The Comparative Enzymology of Lactic Dehydrogenases

IV. FUNCTION OF SULFIHYDRYL GROUPS IN LACTIC DEHYDROGENASES AND THE SEQUENCE AROUND THE ESSENTIAL GROUP*

THOMAS P. FONDY,† JOHANNES EVERSE, GERALDINE A. DRISCOLL, FRED CASTILLO, FRANCIS E. STOLZENBACH, AND NATHAN O. KAPLAN

From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154

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H₄ lactic dehydrogenases crystallized from heart and M₄ lactic dehydrogenases crystallized from muscle tissues of various vertebrates have four functionally essential thiol groups per molecule of enzyme (1). This value suggests that there is one active site thiol group for each of the four subunits of these lactic dehydrogenases. Attempts to introduce a noninterchangeable thiol reagent specifically at the active site of any of these enzymes in the native configuration have failed. Gold and Segal (2) have succeeded in introducing 4 eq of N,N-(dimethylamino-3,5-dinitrophenyl)maleimide into bovine H₄ lactic dehydrogenase by freezing and thawing the enzyme and the reagent in 0.25 M sodium chloride. The enzyme so labeled showed an 80% reduction in activity. The extent of this inactivation was reduced in the presence of NADH.

The other papers in this series (3, 7) outline the preparation of crystalline lactic dehydrogenases from a variety of mammals, birds, reptiles, fishes, an amphibian, a cyclostome, and a crustacean. We have determined the total thiol content of these various H₄ and M₄ lactic dehydrogenases by titration with p-hydroxymercuribenzoate in urea. In addition, we have determined the effect of p-hydroxymercuribenzoate-binding on enzymatic activity as well as the influence of coenzyme on the p-hydroxymercuribenzoate-induced inactivation. This survey has led to the finding that the M₄ lactic dehydrogenase from bullfrog (Rana catesbeiana) has only two other thiol groups in addition to the four essential ones. The nonessential thiols have been blocked by iodoacetate in the native enzyme, thus permitting identification of the essential thiols by alkylation with radioactive iodoacetate in urea. Preliminary reports of this work have been delivered (8, 9).

The four labeled essential thiol groups in the frog M₄ enzyme appeared as a single radioactive peptide in the peptide maps of a tryptic digest. It is reasonable to assume that there are identical active site thiol regions in each of the four subunits of the amphibian lactic dehydrogenase. This same dodecapeptide sequence has been identified essentially unchanged in tryptic digests of mammalian and avian lactic dehydrogenases and in the digest of the enzyme from a primitive fish, the dogfish.

The portion of the primary sequence containing the essential thiol group in lactic dehydrogenases bears varying degrees of relationship to the amino acid sequences around the essential thiol groups in some other pyridine nucleotide dehydrogenases (10-13).

EXPERIMENTAL PROCEDURE

Materials

The crystalline lactic dehydrogenases used in this study were prepared according to procedures presented elsewhere in this series (7). HMB was obtained from Sigma and NADH from Calbiochem. Trypsin was a twice recrystallized, salt-free product of Worthington (Lot 6134). Salt-free α-chymotrypsin (Lot 2065) was purchased from Nutritional Biochemicals. Disopropylfluoridate-treated carboxypeptidase A was batch 0131 from Worthington. Leucine aminopeptidase (batch 5085) containing MgCl₂ was obtained from Worthington. Nagarse was a product of Teikoku Chemical Industry Company, Osaka, Japan. Iodoacetate-1-¹⁴C acid was obtained from New England Nuclear. The acetylpyridine analogue of NADH (AcPyADH) was prepared as described by Kaplan and Stolzenbach (14).

Methods

Lactic Dehydrogenase Assay—Lactic dehydrogenase activity was determined by the method outlined in the first paper of this series (7).

Reaction of Enzyme Thiols with HMB and with Alkylating Agents—The method of Boyer (15), as applied to lactic dehydrogenase by Di Sabato and Kaplan (1) and Di Sabato, Pesce, and Kaplan (16), was employed for HMB reaction with thiol groups in both native and urea-denatured enzymes. Alkylation

* The abbreviations used are: HMB, p-hydroxymercuribenzoate; DDFM, N,N-(dimethylamino-3,5-dinitrophenyl)maleimide; DNS, dimethylaminonaphthalene-5-sulfonyle; PTH, phenylthiohydantoin; CMT, S-carboxymethylcysteine.

† Postdoctoral Fellow on Training Grant 5 T1 NB 5211-16 from the National Institute of Neurological Diseases and Blindness, National Institutes of Health. Present address, Department of Zoology, Syracuse University, Syracuse, New York.

‡ For discussion of the nomenclature of lactic dehydrogenases see Paper II in this series (3) and also Cahn et al. (4). For a general review of lactic dehydrogenase structure and function see Kaplan (5) and Dawson, Goodfriend, and Kaplan (6).

of thiol groups with iodoacetate or iodoacetamide in native enzymes was done at pH 8.4 unless otherwise noted, with a molar excess over thiol ranging from 5:1 to 000:1. Extent of alklylation in native lactic dehydrogenases was determined by HMB titration of available thiols. For the 14C-carboxymethylatation of thiols in urea-denatured lactic dehydrogenases, the molar ratio of labeled alkylating reagent to thiol was generally 5:1. The course of the 14C-carboxymethyl incorporation was followed by titration with HMB.

Specificity of alklylation was determined by paper electrophoresis of the total acid hydrolysate of the labeled protein, followed by scanning for radioactivity with a paper strip scanner. Quantitative amino acid analysis with the Beckman/Spinco analyzer before and after carboxymethylation was done on the frog M4 enzyme.

Extinction coefficients at 280 µM were assumed to be 1.8 × 104 for chicken H4 lactic dehydrogenase (7, 16) and 2.0 × 104 for the other lactic dehydrogenases (16). A value of 7.6 × 102 was used for the extinction coefficient at 250 µM of the mercaptide formed by HMB reaction with thiols in 0.1 M Tris buffer (15). The extinction coefficient for the mercaptide in 8 M urea was determined by using glutathione as standard.

The microcomplement fixation technique of Wasserman and Levine (17) was used to measure the extent of reaction of HMB-bound lactic dehydrogenases with the antibody directed to the native enzyme.

Cysteic acid analysis was performed on the performic acid oxidized enzymes (18, 19) by the method of Moore, Spackman, and Stein (20).

Trypsin and Trypsin-Chymotrypsin Digestions—Digestions with trypsin or with trypsin followed by chymotrypsin were performed at 37° either in 0.1 M ammonium bicarbonate or in distilled water with an automatic titrator to maintain the pH at 8.0. When trypsin-chymotrypsin digests were to be made, a fraction of the trypsin digest was removed for separate study before addition of α-chymotrypsin to the remainder. A weight ratio of 1:50 was used for proteolytic digestions.

Peptide Mapping of Trypsin and of Trypsin-Chymotrypsin Digests—The method of Baglioni (21) as applied to lactic dehydrogenases by Pondy et al. (3) was used in peptide mapping and specific staining of digests of 14C-carboxymethylated lactic dehydrogenases. In most cases the 14C-labeled peptides were located by autoradiography and removed before staining the nonradioactive peptides. About 0.012 µmole (2 µg) of peptide was digested in the applied to the origin of each peptide map.

Paper Electrophoresis of Peptides at pH 3.5—Peptides eluted from peptide maps or obtained by chromatography on a Dowex column were purified on Whatman No. 3MM paper by electrophoresis in a Varsol-cooled tank. The pH 3.5 electrolyte was pyridine-acetic acid-water, 1:10:299 (v/v/v). Column Chromatography of Tryptic Digest of 14C-Carbomethylated Chicken H4 Lactic Dehydrogenase—Isolation of peptides from a tryptic digest of 70 µg (0.5 µmole) of 14C-carboxymethylated chicken H4 lactic dehydrogenase was achieved by initial separation on a Sephadex G-25 column (1 × 50 cm) with distilled water as eluant. The elution pattern was monitored by optical density readings at 219 and 280 µM, by ninhydrin reaction with alkaline hydrolysates and by determination of radioactivity. The fractions containing the labeled peptides were lyophilized and chromatographed on a column of Dowex 50 X2 (1 × 50 cm), prepared and developed by the procedure of Margoliash and Smith (22) and Matsubara and Smith (23). The positions of nonradioactive peptides in the eluant from the Dowex column were determined by alkaline hydrolysis and reaction with ninhydrin according to the procedures of Hirs, Moore, and Stein (24). The radioactivity of the eluant was monitored with a Packard scintillation counter and the 14C-labeled peptides were isolated, lyophilized, and purified by paper electrophoresis at pH 3.5 as already described.

Total Acid Hydrolysis of Peptides—Peptides purified by paper electrophoresis at pH 3.5 were eluted into hydrolysates with 10% pyridine in water. Generally between 0.05 and 0.1 µmole of peptide was eluted and dried under vacuum. The peptides were dissolved in 0.3 ml of 6 N hydrochloric acid, and the hydrolysis was performed at 110° for 20 hours in evacuated, sealed tubes.

Amino Acid Analysis with Beckman/Spinco Analyzer —The procedure of Spackman, Stein, and Moore (25) was followed for quantitative amino acid analysis on ion exchange resins with the Beckman/Spinco automatic amino acid analyzer. Reliable results were obtained with quantities as low as 0.025 µmole per column.

Amino Acid Analysis by Paper Electrophoresis—The procedure of Atfield and Morris (26) for paper electrophoresis of amino acids on a cooled plate at pH 1.8 was modified for use with a Varsol-cooled tank. Two Whatman No. 3MM paper strips (27 × 200 cm), each bearing eight separate amino acid analyses, were subjected to electrophoresis in parallel. The application of a potential of 45 volts per cm for 6 hours permitted the resolution of all the amino acids except of serine from isoleucine.

Partial Acid Hydrolysis of Common Thiol Peptide—Two procedures were employed for partial acid hydrolysis of the labeled thiol peptide common to the various enzymes. Partial acid hydrolysis In 6 N hydrochloric acid at 37° for 24 hours in an evacuated, sealed tube was used to give a large number of small fragments for sequence comparison among the peptides. Hydrolysis in 0.25 M acetic acid at 110° for 24 hours in an evacuated, sealed tube was employed to produce more specific cleavage for use in sequence determination of a peptide. The partial acid hydrolysates were separated into the component peptides by paper electrophoresis at pH 3.5.

Nagarse Digestion of Common Thiol Peptide—Nagarse digestion of 1.0 µmole of the common thiol peptide as isolated from chicken H4 lactic dehydrogenase was carried out in 1 ml of 0.1 M pyridine acetate buffer, pH 7.5, with 0.02 mg of enzyme at 37° for 3 hours. The digestion mixture was applied directly to Whatman No. 3MM paper for paper separation of peptides by electrophoresis at pH 3.5.

Edman Degradation—The method of Schroeder et al. (27) for sequential Edman degradation on strips of Whatman No. 1 paper was employed. In some cases the method was applied to sequence determination of peptides directly on Whatman No. 3MM paper after electrophoresis of the peptides at pH 3.5. Identification of the PTH derivatives of amino acids proved satisfactory in only a few cases. Reliance was placed either on analysis of the residual peptide, or on identification of the free amino acid regenerated from the PTH derivative by the method of Stark and Smyth (28).

DNS-chloride End Group Analysis—DNS-chloride was used to label the amino-terminial group in one peptide following the
method developed by Gray and Hartley (29). DNS-amino acid standards were prepared as described by Dawid, French, and Buchanan (30).

Carboxypeptidase A Digestion of Peptides—For the determination of sequences at the COOH-terminal end of peptides, 0.2 pmole of peptide was dissolved in 0.2 ml of 0.1 M ammonium bicarbonate. Diisopropylfluoridate-treated carboxypeptidase A was added (0.002 pmole), and the mixture was incubated at 37°. Three aliquots were withdrawn over a 4-hour period, and the amino acids released were determined by paper electrophoresis or by the Beckman/Spinco analyzer. The activity of the carboxypeptidase A was tested with carbobenzyglycyl-n-phenylalanine according to the method of Snoke and Neurath (31).

Leucine Aminopeptidase Digestion of Peptides—Enzymatic digestion of a peptide for asparagine determination was performed with the use of a mole ratio of 1:100 leucine aminopeptidase to peptide. The digestion was carried out in 0.1 M Tris-chloride, pH 8.4, at 37° for 24 hours. The amino acids released were determined by paper electrophoresis.

RESULTS

Cysteine Content of Various Lactic Dehydrogenases—The total thiol content of a series of crystalline lactic dehydrogenases as determined by HMB titration in 8 M urea is listed in Table I. Data previously presented by Di Sabato, Pesce, and Kaplan (16) are included in Table I for comparison and completeness.

Effect of HMB on Essential and Nonessential Thiols—The effect on HMB binding on the enzymatic activity of beef and chicken H4 lactic dehydrogenases has been reported by Di Sabato and Kaplan (1). These workers showed that there is a linear relationship between moles of HMB bound and decrease in enzymatic activity, with activity approaching zero when 4 moles of HMB are bound per mole of enzyme.

In extending these studies to many other crystalline lactic dehydrogenases, we have observed that all these enzymes may be divided into two major classes on the basis of the reactivity of their thiol groups with HMB. One group of lactic dehydrogenases fitted the pattern established by the beef and chicken H4 enzymes in that the HMB binding required a period of minutes or hours and in all cases but one, the essential thiol groups appeared to be the first to be attacked by HMB. In addition to the H4 enzymes from chicken and beef this group included the M4 enzymes from chicken, ostrich, pheasant, turkey, duck, tuna, and halibut, and the H4 lactic dehydrogenase from tuna. The effect of HMB binding on the activity of this group of enzymes is shown in Fig. 1. The halibut M4 enzyme differed from the others in that excess HMB failed to affect the activity even after 24 hours. During that period 10 moles of HMB were bound, after which the enzyme began to precipitate.

The second group of lactic dehydrogenases was characterized by a varying number of nonessential thiol groups which bound HMB almost instantaneously. These lactic dehydrogenases were almost all M4 type, and were crystallized from lobster, frog, caiman, and dogfish. One H4 lactic dehydrogenase, that from ostrich heart, had those “rapidly reacting,” nonessential thiol groups. The effect of HMB treatment on these enzymes is presented in Fig. 2. In the lobster enzyme, both the nonessential and the active site thiol groups reacted almost instantaneously with HMB. In order to follow the course of mercurial binding in this case, we found it necessary to perform the reaction in steps.

<table>
<thead>
<tr>
<th>Lactic dehydrogenase*</th>
<th>HMB bound in 8 M urea</th>
</tr>
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<tbody>
<tr>
<td>Chicken H4</td>
<td>25.0</td>
</tr>
<tr>
<td>Chicken M4</td>
<td>24.0</td>
</tr>
<tr>
<td>Ostrich H4</td>
<td>32.2</td>
</tr>
<tr>
<td>Ostrich M4</td>
<td>17.7</td>
</tr>
<tr>
<td>Pheasant M4</td>
<td>26.2</td>
</tr>
<tr>
<td>Turkey M4</td>
<td>24.0</td>
</tr>
<tr>
<td>Duck M4</td>
<td>24.0</td>
</tr>
<tr>
<td>Beef H4</td>
<td>16.0†</td>
</tr>
<tr>
<td>Beef M1</td>
<td>25.0†</td>
</tr>
<tr>
<td>Rabbit M1</td>
<td>16.7†</td>
</tr>
<tr>
<td>Frog M4</td>
<td>5.0</td>
</tr>
<tr>
<td>Alligator M1</td>
<td>36.8</td>
</tr>
<tr>
<td>Caiman M4</td>
<td>30.0</td>
</tr>
<tr>
<td>Halibut M4</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>13.1†</td>
</tr>
<tr>
<td>Tuna H4</td>
<td>18.5</td>
</tr>
<tr>
<td>Tuna M4</td>
<td>16.8</td>
</tr>
<tr>
<td>Dogfish M4</td>
<td>23.5</td>
</tr>
<tr>
<td>Lamprey M4</td>
<td>20.0</td>
</tr>
<tr>
<td>Lobster M4</td>
<td>12.5</td>
</tr>
</tbody>
</table>

* Molar extinction coefficients are assumed to be $1.8 \times 10^5$ for the chicken H4 enzyme (7, 16); $2.0 \times 10^5$ for the others (7).
† Values from Di Sabato, Pesce, and Kaplan (16). Extinction coefficients for chicken M4 enzyme assumed to be $2.2 \times 10^5$ by Di Sabato et al.
‡ See second footnote in Table III.

![Figure 1](http://www.jbc.org/)
by several additions of a limited quantity of HMB. By this technique, the lobster enzyme showed eight rapidly reacting, nonessential thiol groups which bound HMB at rates faster than did the four essential thiols.

Caiman M₄ lactic dehydrogenases bound 12 moles of HMB within 30 sec with no loss in enzymatic activity. Further incorporation of the mercurial led initially to a linear drop in activity, after which the enzyme began to precipitate.

The frog M₄, dogfish M₄, and ostrich H₄ enzyme had varying numbers of nonessential thiol groups which bound HMB almost instantaneously. Further treatment of these enzymes with HMB over a period of minutes and hours produced a decrease in enzymatic activity which had a linear relationship to moles of HMB bound. In all cases, the number of active site thiols approached 4 moles per mole of enzyme. The optical rotatory dispersion curve of the frog M₄ enzyme which had been 50% inactivated by the binding of 4 moles of HMB per mole of enzyme was identical at all wave lengths from 250 to 450 nm to the curve produced by the native enzyme. Similarly, the dogfish M₄ enzyme, completely inactivated by the binding of 8 moles of HMB per mole of enzyme had an optical rotatory dispersion curve identical with that of the untreated enzyme. Both HMB-bound enzymes had a 100% cross-reaction with the antibodies directed toward their respective native enzymes, as measured by microcomplement fixation. Therefore, the inactivation of these lactic dehydrogenases produced by the binding of HMB was not caused by a conformational change significant enough to be measured by optical rotatory dispersion or immunological cross-reaction.

Di Sabato and Kaplan (1) have shown that HMB-bound chicken H₄ and beef H₄ lactic dehydrogenases have sedimentation coefficients identical with those for the native enzymes. We have determined that the same is true for the HMB-bound frog M₄ enzyme. Thus, the inactivating effect of HMB treatment is not due to dissociation of active tetramer into inactive subunits.

Alkylation of Nonessential Thiols—The effect of iodoacetate and iodoacetamide on the thiol groups in several lactic dehydrogenases was examined. A 600-fold molar excess of iodoacetamide to enzyme was reacted with M₄ lactic dehydrogenases from frog and dogfish at room temperature and pH 8.9. No effect on enzymatic activity was observed even after 5 hours. However, HMB titration of the enzymes after removal of the excess iodoacetamide showed that the two rapidly reacting, nonessential thiols in the frog enzyme had been alkylated as had two of the four similar thiol groups in the dogfish enzyme.

This experiment was repeated with a 60-fold molar excess of iodoacetate to enzyme again at 25°C and pH 8.9. No effect on enzymatic activity was observed even after 5 hours. However, HMB titration of the enzymes after removal of the excess iodoacetamide showed that the two rapidly reacting, nonessential thiols in the frog enzyme had been alkylated as had two of the four similar thiol groups in the dogfish enzyme.

This experiment was repeated with a 60-fold molar excess of iodoacetate to enzyme again at 25°C and pH 8.9. This milder alkylation was terminated after only 1 hour with results identical with those obtained with the iodoacetamide-treated enzymes (Fig. 3A). Presumably, iodoacetamide would have been equally effective with the iodoacetate treatment.

Fig. 2. HMB-induced inactivation of lactic dehydrogenases with rapidly reacting, nonessential thiol groups that bind HMB before the essential thiols bind. Conditions in all cases were the same as for Fig. 1 except with the lobster M₄ enzyme. HMB was added to 2.5 μM lobster enzyme in five equal aliquots until a final HMB concentration of 5 × 10⁻⁵ M was reached. Each aliquot was added after the HMB binding produced by the previous addition had ceased.

Fig. 3. HMB-induced inactivation of frog, dogfish, and lobster M₄ lactic dehydrogenases before and after treatment with iodoacetate. Enzyme concentration for iodoacetate treatment was 2.25 μM for the lobster enzyme (B) and a 20-fold molar excess of iodoacetate to enzyme was used. For the frog and dogfish enzymes (A), the concentration was 4.5 μM and a 60-fold molar excess of iodoacetate was used. In all three cases, the buffer was 0.1 M Tris-chloride, pH 8.9. HMB reaction with both untreated and carboxymethylated enzymes was carried out as in Fig. 2.
effective in alkylating the nonessential thiol groups under the milder conditions used for iodoacetate treatment.

A 20-fold molar excess of iodoacetate to enzyme was sufficient to alkylate the eight nonessential thiol groups in lobster M₄ lactic dehydrogenase at 25°C and pH 8.9. This alkylation was complete after 1 hour as determined by HMB titration (Fig. 3B). Iodoacetate treatment failed to affect the essential thiol groups even in an overnight reaction.

Chicken H₄ lactic dehydrogenase was unaffected by a 250-fold molar excess of iodoacetamide to enzyme when the two were mixed at pH 7.6 and kept at room temperature for 5 hours. It should be noted that in spite of the fact that the rate of HMB inactivation of the various lactic dehydrogenases showed a wide variation (Table II), in no case were the essential thio groups reactive enough to bind iodoacetate in the native enzymes.

Effect of Reduced Coenzymes on HMB Binding—The presence of 1 mole of NADH per mole of essential thiol groups had a marked protective effect against HMB-induced inactivation of the frog and dogfish M₄ enzymes. The acetylpyrididine analogue of NADH (AcPyADH) under the same conditions had an equal or greater protective effect on these enzymes (Table II). No protection by reduced coenzyme or its analogue against HMB binding of the rapidly reacting, nonessential thiol groups was observed.

Labeling of Essential Thiol Groups in Frog M₄ Lactic Dehydrogenase—The two rapidly reacting, nonessential thiol groups in the frog M₄ enzyme were alkylated with a 36-fold molar excess of unlabeled iodoacetate under the conditions outlined in an earlier section. After 1 hour, HMB titration showed that the nonessential thiol groups were blocked while 96% of the original activity remained.

The alkylated enzyme was treated with an 11-fold molar excess of iodoacetate-1⁴C in a nitrogen atmosphere. The essential thiol groups were made available for alkylation by addition of recrystallized, metal-free urea to a concentration of 8 M. HMB titration showed that the alkylation was complete after 4 hours. The unbound iodoacetate-1⁴C was removed by exhaustive dialysis after which a measure of the bound radioactivity showed that 3.4 moles of iodoacetate had been incorporated per mole of enzyme.

Amino acid analysis of the total acid hydrolysate of the labeled frog enzyme was carried out both by paper electrophoresis and with the Beckman/Spinco analyzer. Scanning for radioactivity after paper electrophoretic analysis indicated that all the activity was associated with ¹⁴C-carboxymethylcysteine. Quantitative amino acid analysis by the column method showed that the compositions before and after carboxymethylation were identical within the range of error except for cysteine, which was not determined quantitatively by this procedure.

Labeling of All Thiol Groups in Other Lactic Dehydrogenases—H₄ lactic dehydrogenases from chicken, beef, and tuna, and M₄ enzymes from chicken, dogfish, and halibut were labeled with iodoacetate-1⁴C in 8 M urea. The reactions were terminated when alkylation appeared to have ceased as indicated by HMB titration. Table III lists the extent of incorporation of ¹⁴C-carboxymethyl groups into the various enzymes. The alkylation of the halibut M₄ enzyme was repeated for a longer period of time to obtain more complete labeling of available thiols.

The total acid hydrolysate of alkylated chicken H₄ lactic dehydrogenase was examined for specificity of labeling by scanning of an electrophoretogram of the constituent amino acids. All the radioactivity was incorporated into carboxymethylcysteine.

Peptide Mapping and Autoradiography of Digests of ¹⁴C-Carboxymethyl-Lactic Dehydrogenases—The labeled frog M₄ and chicken H₄ lactic dehydrogenases were digested with trypsin and the course of the digestions were followed by an automatic titrator. Both trypsin digests were complete after 6 hours. A portion of each trypsin digest was treated with a-chymotrypsin and the digestion again followed by use of a pH-stat. The chymotrypsin digestions were complete after 6 hours.

The other labeled lactic dehydrogenases (chicken M₄, beef H₄, dogfish M₄, tuna H₄, and two halibut M₄ preparations) were digested with trypsin and with trypsin-chymotrypsin in 0.1 M NH₄HCO₃ buffer. The digestion times were 7 to 9 hours. Digestion of the labeled chicken H₄ enzyme carried out under these conditions was shown by peptide mapping and autoradiography to be identical with the digestion performed with the pH-stat.

Peptide maps of these various digests were made, and the position of the ¹⁴C-labeled peptides was determined by autoradiography. Fig. 4 shows the autoradiographs obtained from pep-
FIG. 4 (upper two rows). Autoradiographs of tryptic peptide maps of $^{14}$C-carboxymethyl lactic dehydrogenases. The essential thiol peptide in the frog M4 enzyme is designated T-1. The common peptide in the chicken, beef, and dogfish enzymes is also designated T-1 in those peptide maps. A labeled peptide containing arginine appears in a similar position in the tuna H4 autoradiograph but has not been further characterized to determine its relationship to the T-1 peptides in the other species. The halibut M4 peptide maps do not show a peptide that can be immediately related to peptide T-1 from other species.

tide maps of tryptic digests. The single intense spot in the autoradiograph of the frog muscle enzyme contains 70% of the total counts incorporated and represents the essential thiol peptide in frog M4 lactic dehydrogenase. A labeled peptide appears in a comparable position in the autoradiographs of peptide maps of all the other lactic dehydrogenases except the halibut enzyme. Some other labeled peptides may share a similar position in the peptide maps of two lactic dehydrogenases, but no other peptide is shared by more than two enzymes. The possible common peptide is indicated in Fig. 4 by an arrow.

Fig. 5 (lower two rows). Autoradiographs of trypsin-chymotrypsin peptide maps of $^{14}$C-carboxymethyl lactic dehydrogenases. The peptide designated TC-1 in the various autoradiographs is identical with the T-1 peptides in Fig. 4. The tuna H4 autoradiograph shows a labeled peptide containing arginine that may be homologous with the TC-1 peptides in the other species. The halibut M4 patterns do not contain a peptide that can be immediately related to the TC-1 peptides in the other autoradiographs.

LDH, lactic dehydrogenase.

Fig. 5 represents the autoradiographs of trypsin-chymotrypsin digests of these $^{14}$C-carboxymethylated lactic dehydrogenases. The autoradiograph of the frog M4 digest shows the presence of a second peptide of apparent intensity comparable to the active site tryptic peptide. However, isolation and quantitative analysis of the two trypsin-chymotrypsin peptides showed that this second peptide contained less than 25% of the radioactivity contained in the proposed active site peptide. The amino acid composition of this second peptide suggests that it may be a fragment of the active site peptide.
The peptide common to the lactic dehydrogenases in the tryptic digests appears essentially unaffected by the action of chymotrypsin. The possible common peptide is indicated in Fig. 5 with an arrow. Table IV summarizes the results obtained in these peptide maps of trypsin and trypsin-chymotrypsin digests of \(^{14}\text{C}-\text{carboxymethylated lactic dehydrogenases.}\)

### TABLE IV

<table>
<thead>
<tr>
<th>14C-Carboxymethylated enzyme</th>
<th>Labeled peptides in digest of trypsin</th>
<th>Labeled peptides in trypsin-chymotrypsin digest</th>
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<tr>
<td>Chicken H₄</td>
<td>6-7</td>
<td>6-7</td>
</tr>
<tr>
<td>Chicken M₄</td>
<td>6-7</td>
<td>6</td>
</tr>
<tr>
<td>Beef H₄</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Dogfish M₄</td>
<td>5-6</td>
<td>5</td>
</tr>
<tr>
<td>Tuna H₄</td>
<td>4-5</td>
<td>4</td>
</tr>
<tr>
<td>Frog M₄</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Halibut M₄-10-14C</td>
<td>3-5</td>
<td>3-5</td>
</tr>
<tr>
<td>Halibut M₄-18-14C</td>
<td>3-5</td>
<td>3-5</td>
</tr>
</tbody>
</table>

* Based on the values given in Table I divided by the four subunits of lactic dehydrogenase.

Amino Acid Compositions of Possible Common Peptide—The \(^{14}\text{C}-\text{carboxymethylated peptide containing the essential sulphydryl group in frog M₄ lactic dehydrogenase was eluted from peptide maps of a tryptic digest and purified by paper electrophoresis at pH 3.5. The possible common peptide present in tryptic digests of chicken H₄ and M₄, beef H₄, and dogfish M₄ lactic dehydrogenases was eluted from peptide maps of each of these digests. The peptide from the tuna H₄ enzyme has been only recently identified and was not included in this study. The active site peptide from frog M₄ lactic dehydrogenase, and the possible common peptide from the other enzymes will be referred to as peptide T-1. A second peptide (T-2), which migrated to a region close to peptide T-1 in the peptide maps of chicken M₄ lactic dehydrogenase, was also eluted and purified along with peptide T-1. Fig. 6 shows the autoradiograph of peptide T-1 from the various lactic dehydrogenases and peptide T-2 from the chicken M₄ enzyme after paper electrophoresis. The T-1 peptides that appeared to migrate to identical positions in the various tryptic peptide maps also migrated identically during electrophoresis at pH 3.5. Peptide T-2 from chicken M₄ lactic dehydrogenase was easily distinguished from the others.

The peptides were eluted from the pH 3.5 electrophoretogram, hydrolyzed, and subjected to amino acid analysis in the Beck

![Fig. 6. Autoradiograph of paper electrophoretic analysis of the T-1 peptides from Fig. 4 and of peptide T-2 from chicken M₄ lactic dehydrogenase.](http://www.jbc.org/)}
tic digests of the various lactic dehydrogenases was in each case H4 lactic dehydrogenase. The appearance of these acidic peptides isolated from the tuna Hb enzyme in Fig. 5 indicates that the peptides were unchanged in autoradiographs of trypsin-chymotrypsin digestion. Analysis has been performed on these acidic peptides from tuna Hb enzyme. No comparison of peptide T-A with peptide T-l will be required to determine the exact relationship between them.

The two extremely acidic peptides which dominate the autoradiograph of the halibut M4 peptide map in Fig. 4 were also purified, hydrolyzed, and analyzed. These analyses are listed in Table V. The more intense of the peptides is designated T-A, the other T-B. None of the other peptides in the halibut M4 digest were present in significant quantities. The compositions of peptides T-A and T-B do not distinguish them from one another, but they do indicate that these peptides from halibut M4 lactic dehydrogenase are not identical with peptide T-l common to the other lactic dehydrogenases. A full sequential comparison of peptide T-A with peptide T-1 will be required to determine the exact relationship between them.

It should be pointed out in Fig. 4 that peptides analogous to the halibut peptides T-A and T-B also appear in the autoradiograph of the tryptic peptide map of the tuna H4 enzyme. No analysis has been performed on these acidic peptides from tuna H4 lactic dehydrogenase. The appearance of these acidic peptides unchanged in autoradiographs of trypsin-chymotrypsin digests of these fish enzymes (Fig. 5) indicates that the peptides are not susceptible to chymotryptic digestion.

Peptide T-l isolated from peptide maps of tryptic-chymotryptic digests of the various lactic dehydrogenases was in each case found to be identical in amino acid composition to the corresponding peptide T-1 from peptide maps of a tryptic digest.

Sequence Comparison of T-1 Peptides by Partial Acid Hydrolysis—The common thiol peptides (T-1) isolated from peptide maps of tryptic digests of the various lactic dehydrogenases and purified by paper electrophoresis were examined for identity of sequence by comparison of partial acid hydrolysates. For this purpose, hydrolysis in 6 N hydrochloric acid for 24 hours at 37°C was the method chosen since this procedure gave the largest number of fragments among the various methods tested. The partial acid hydrolysates were streaked contiguously and resolved into their component peptides by paper electrophoresis at pH 3.5. Fig. 7 is the autoradiograph of the hydrolysates after electrophoresis. The nonradioactive, ninhydrin-positive bands have been indicated. The partial acid hydrolysis patterns obtained for each of the T-1 peptides were almost entirely identical. The hydrolysate of the frog M4 peptide appeared weaker because the amount of peptide available was considerably less than for T-1 peptides isolated from the other species. The ninhydrin-positive fragment absent from the basic region of the chicken H4 peptide hydrolysate is consistent with the fact that a sequence difference was later found near the arginine residue in that peptide.

**Isolation of Peptide T-1 from 14C-Carboxymethylated Chicken H4 Lactic Dehydrogenase by Column Chromatography**—It was found that approximately 0.025 µmole of peptide could be isolated and purified from a single peptide map. The quantity of a given peptide obtained by elution from several peptide maps was adequate for determination of amino acid composition, but to establish amino acid sequence, a larger amount of peptide was required. Gel filtration on a column of Sephadex G-25 followed by ion exchange chromatography on a Dowex 50-X2 column and a final purification by paper electrophoresis yielded 0.4 µmole of peptide T-1 from 0.5 µmole of a tryptic digest of 14C-carboxymethylated chicken H4 lactic dehydrogenase.

Gel filtration of the enzyme digest produced a single broad radioactive peak. The eluate containing the radioactivity was pooled and lyophilized, in preparation for further purification by chromatography on Dowex resin. The nonradioactive eluant analyzed by Spinco analyser. Approximately 0.03 µmole of peptide hydrolysate was used for each column. The results of these analyses are presented in Table V. The composition of peptide T-1 from each of the five species of lactic dehydrogenase is seen to be identical with the exception of the substitution of a threonine for a serine residue in the chicken H4 peptide, and the deletion of valine from the dogfish M4 enzyme. The values for valine and isoleucine were consistently low in all the T-1 peptides (except that from the dogfish M4 enzyme) suggesting the presence of a valine-isoleucine bond that was resistant to acid hydrolysis.

Peptide T-2 from the chicken M4 lactic dehydrogenase digest was shown to be totally different from the peptide common to the lactic dehydrogenases.

The amino acid compositions of tryptic peptides from various lactic dehydrogenases are listed in Table V. The composition of peptide T-l from each of the five species of lactic dehydrogenase is seen to be identical with the exception of the substitution of a threonine for a serine residue in the chicken H4 peptide, and the deletion of valine from the dogfish M4 enzyme.

### Table V

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Beef H4 T-1</th>
<th>Dogfish M4 T-1</th>
<th>Frog M4 T-1</th>
<th>Chicken H4 T-1</th>
<th>Chicken M4 T-1</th>
<th>Halibut M4 T-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>*</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.2</td>
<td>2.0</td>
</tr>
<tr>
<td>14C-Carboxymethylecysteine</td>
<td>0.7</td>
<td>0.6</td>
<td>1.0</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.1</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Serine</td>
<td>0.7</td>
<td>0.6</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.4</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Proline</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.6</td>
<td>0.4</td>
<td>1.0</td>
<td>0.7</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>(0.3)</td>
<td>(0.3)</td>
<td>(0.3)</td>
<td>(0.3)</td>
<td>(0.3)</td>
<td>(0.3)</td>
</tr>
</tbody>
</table>

* Analysis for basic amino acids was not performed.
FIG. 7. Autoradiograph of paper electrophoretic analysis of partial acid hydrolysates of T-1 peptides in Fig. 6. Nonradioactive, ninhydrin-positive fragments are indicated.

FIG. 8. Sephadex G-25 column analysis of 0.5 μmole of a tryptic digest of 14C-carboxymethylated chicken H4 lactic dehydrogenase. Tubes containing the radioactive peptides were pooled and conserved, while the remaining tubes were subjected to optical density readings and ninhydrin analysis of alkaline hydrolysates.

from the Sephadex column was monitored by optical density readings at 219 and 280 μm, and by ninhydrin analysis of alkaline hydrolysates. Fig. 8 shows the elution pattern from the Sephadex column. Resolution of the radioactive peak into the various 14C-carboxymethyl cysteine peptides of which it was composed would require a column larger than the 1 x 50-cm one employed in this initial separation.

After lyophilization, the radioactive peak from the Sephadex column was rechromatographed on Dowex 50-X2. Fig. 9 shows the positions of the peptides in the eluate as determined by monitoring the radioactivity and by the ninhydrin procedure. Two major radioactive peaks were observed (at tubes 156 and 340), along with four peaks of lesser intensity (at tubes 34, 88, 201, and 316). Each of these peptides was purified by paper electrophoresis at pH 3.5. The peak at tube 156 was resolved into three components in the estimated ratio of 10:2:1. The most anionic of these three peptides was also the major component and had an electrophoretic mobility identical with peptide T-1 isolated from peptide maps of the various lactic dehydrogenases. No other labeled peptide from the Dowex eluate migrated to this position. The amino acid composition of the major component of Peak 156 was identical with that of peptide T-1 from peptide maps of 14C-carboxymethylated chicken H4 lactic dehydrogenase. The amino acid compositions of the lesser components of Peak 156 were not related to peptide T-1.

The peptide separation shown in Fig. 9 was sufficient to isolate
peptide T-1 from the enzyme digest. A satisfactory resolution of all the peptides in the digest would require a column larger than 1 × 50 cm.

Sequence Determination of Peptide T-1 from 14C-Carboxymethylated Chicken H4 Lactic Dehydrogenase—The sequence of peptide T-1 as isolated from the chicken H4 enzyme was established by analysis of partial acid hydrolysis fragments and Nagarse peptides. Peptides obtained by partial acid hydrolysis in acetic acid are designated with a subscript A and numbered according to their positions on the pH 3.5 electrophoretogram, beginning with the most anionic as peptide 1A. Similarly, peptides obtained by partial acid hydrolysis in hydrochloric acid and eluted from the electrophoretogram presented in Fig. 7 are designated with a subscript H, beginning with peptide 1H as the most anionic. The products of Nagarse digestion, after electrophoresis at pH 3.5, are designated with a subscript N.

Table VI lists the amino acid compositions of the various fragments of peptide T-1 that were obtained in quantities sufficient for analysis. The analyses of these peptides, along with additional structural information presented below, permitted the reconstruction of the sequence of peptide T-1 as shown in Table VI.

Since peptides 2A and 7A' had identical compositions but different electrophoretic mobilities, the aspartic acid residue identified in the acid hydrolysate of peptide 7A' must have been present as asparagine. This conclusion was confirmed by Edman degradation of peptide 9N. The PTH-amino acid released by Edman degradation of peptide 9N was identified as PTH-asparagine. Amino acid analysis of the residual peptide (9N-) showed that one of the aspartic acid residues present in the acid hydrolysate of peptide 9N had been removed by Edman degradation.

Peptide 9N-1 was digested with leucine aminopeptidase to establish the presence or absence of an amide group on the second aspartic acid residue in peptide T-1. Amino acid analysis by paper electrophoresis showed the presence of aspartic acid but not of asparagine.

Peptide 8A was examined by sequential Edman degradation. The PTH-amino acid derivative obtained after the first Edman cycle was hydrolyzed in sodium hydroxide, and the amino acid regenerated was identified as valine. After the second Edman cycle, the residual peptide (8A-) contained no valine and 0.6 residue each of isoleucine and serine. All the other amino acids were unaffected by the Edman reaction. Since the quantity of both serine and isoleucine decreased after the second Edman cycle, it was not possible to establish unequivocally their order in sequence of peptide 8A. However, it is likely that isoleucine was the amino acid removed in the second Edman degradation, since serine may be destroyed during acid hydrolysis, whereas isoleucine is not so affected. This surmise is confirmed by the amino acid compositions of peptides 10H, 16H, and 18H, which contain serine linked to glycine.

Peptide 11A was subjected to a single Edman degradation and the residual peptide analyzed. The analysis showed that all the threonine had been removed and only alanine and arginine remained. Since it may be assumed that arginine is the COOH-terminal residue, then the sequence of peptide 11A is Thr-Ala-Arg.

Peptide 4N was not present in enough quantity to perform...
Table VI

Determination of amino acid sequence of peptide T-1 from 14C-carboxymethylated chicken H4 lactic dehydrogenase by analysis and partial sequencing of peptide fragments

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Composition and known portions of sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-1</td>
<td>Val - Ile - Ser - Gly - 14CMC - Asn - Leu - Asp - Thr - Ala - Arg</td>
</tr>
<tr>
<td>2A</td>
<td>Gly, Gly, 14CMC, Asp, Leu</td>
</tr>
<tr>
<td>3A</td>
<td>Asp, Asp</td>
</tr>
<tr>
<td>4A</td>
<td>14CMC, Asp, Leu</td>
</tr>
<tr>
<td>6A</td>
<td>Gly, Gly, 14CMC, Leu</td>
</tr>
<tr>
<td>7A'</td>
<td>Gly, Gly, 14CMC, Asn, Leu</td>
</tr>
<tr>
<td>7A''</td>
<td>Thr, Ala</td>
</tr>
<tr>
<td>8A</td>
<td>Val - (Ile, Ser, Gly, Gly, 14CMC, Asp, Leu)</td>
</tr>
<tr>
<td>9A</td>
<td>Leu</td>
</tr>
<tr>
<td>11A</td>
<td>Thr - (Ala, Arg)</td>
</tr>
<tr>
<td>12A</td>
<td>Arg</td>
</tr>
<tr>
<td>10N</td>
<td>Ser, Gly, Gly, 14CMC, Asp, Leu, Asp</td>
</tr>
<tr>
<td>16N</td>
<td>Ser, Gly</td>
</tr>
<tr>
<td>18N</td>
<td>Ser, Gly, Gly</td>
</tr>
<tr>
<td>19N</td>
<td>Val, Ile, Ser, Gly, Gly</td>
</tr>
<tr>
<td>4N</td>
<td>(Val, Ile, Ser, Gly, Gly)-14CMC</td>
</tr>
<tr>
<td>9N</td>
<td>Val, Ile, Ser, Gly, Gly</td>
</tr>
<tr>
<td>9N</td>
<td>14CMC, Asp, Leu, Asp, Thr, Ala, Arg</td>
</tr>
<tr>
<td>9N</td>
<td>Val, Ile, Ser, Gly, Gly</td>
</tr>
</tbody>
</table>

* Subscript A refers to peptides formed by partial acid hydrolysis in 0.25 M acetic acid, at 110°, for 24 hours. Subscript H denotes peptides from partial acid hydrolysis in 6 N hydrochloric acid, at 37°, for 24 hours. Subscript N refers to Nagarse fragments. For details of sequence determination of peptides 7A', 8A, 11A, 4N, and 9N, see text. Amino acid compositions of the various peptides are listed as determined after total acid hydrolysis. Therefore, the asparagine residue appears as aspartic acid in this table unless the presence of the acid amide could be definitely established.

sequential Edman degradation, so the more sensitive DNS-chloride method of Gray and Hartley (29) was used. The NH2-terminal residue was removed by Edman degradation and determined to be nonradioactive. Since the radioactivity remained in the residual peptide, the NH2-terminal residue of peptide 4N was glycine. The residual peptide composed of glycine and 14C-carboxymethylcysteine was purified by paper electrophoresis. The DNS-chloride label was attached to the new NH2 terminus by the method of Dawid, French, and Buchanan (30), and the fluorescent-labeled peptide was hydrolyzed in 6 N hydrochloric acid for 6 hours. Paper electrophoresis was performed on a cooled plate at pH 4.4 (50 volts per cm). The electrophoretogram showed that the fluorescent label had been incorporated into the nonradioactive amino acid, and that the 14C-carboxymethylcysteine had been protected from DNS-chloride labeling. The sequence of peptide 4N thus was established to be glycyl-glycyl-14C-carboxymethylcysteine.

COOH-terminal analysis performed on peptide 6A with active carboxypeptidase A showed that no COOH-terminal amino acid was released. This suggested the presence of terminal 14C-carboxymethylcysteine which resisted the action of carboxypeptidase A.

The foregoing results permitted the complete elucidation of the amino acid sequence of peptide T-1 as isolated from chicken H4 lactic dehydrogenase. This sequence is presented in Table VI.

The comparison of partial acid hydrolysis fragments presented in Fig. 7 strongly indicates an identity of sequence for the common thiol peptide (T-1) isolated from various 14C-carboxymethylated lactic dehydrogenases. This identity of sequence was further substantiated by Edman degradation studies done on an equimolar mixture of T-1 peptides from 14C-carboxymethylated chicken M4 and beef H4 lactic dehydrogenases. Table VII presents the results of four sequential Edman degradations performed on this mixture. The sequence in the NH-terminal region of T-1 peptides from 14C-carboxymethylated chicken M4 and beef H4 lactic dehydrogenases was concluded to be: Val-Ile-Ser-Gly. This sequence was identical with that determined for peptide T-1 isolated from 14C-carboxymethylated chicken H4 lactic dehydrogenase.

Comparison of Ninhydrin-positive Peptides in Tryptic Peptide Maps of 14C-Carboxymethylated Lactic Dehydrogenases—A region of the primary sequence of various lactic dehydrogenases (T-1 peptides) is retained over a wide range of evolution. However, the precise significance of this retention cannot be evaluated unless some knowledge of the relationship among other regions of the primary sequences is available as well. The structural similarities and differences among various lactic dehydrogenases has been the subject of a recent review (32). Additional data on the relationship among the primary sequences of various lactic dehydrogenases is presented in Fig. 10. The 14C-carboxymethyl-labeled peptides originally shown in the autoradiographs of peptide maps in Fig. 4 are indicated by the shaded areas in Fig. 10. As already pointed out, only one of these labeled peptides is
TABLE VII
Amino acid compositions of residual peptides after Edman degrada-
tions of mixed T-1 peptides from 14C-carboxymethylated chicken M4 and beef H4 lactic dehydrogenases

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Initial cycle</th>
<th>First cycle</th>
<th>Second cycle</th>
<th>Third cycle</th>
<th>Fourth cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>14C Carboxymethylerythritol</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Aspartic acid*</td>
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<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>1.9</td>
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<td>2.0</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.2</td>
<td>2.2</td>
<td>2.1</td>
<td>2.1</td>
<td>1.6</td>
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<td>Alanine</td>
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<td>1.0</td>
<td>1.1</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Valine</td>
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<td>0.0</td>
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<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.8</td>
<td>0.9</td>
<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.0</td>
<td>0.9</td>
<td>1.0</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.0</td>
<td>†</td>
<td>†</td>
<td>†</td>
<td>†</td>
</tr>
</tbody>
</table>

* Value for aspartic acid taken as 2.0.
† No analysis of basic amino acids done. Arginine assumed to be COOH-terminal.

Comparison of the alkylated chicken M4 peptide maps with the beef H4 patterns in Fig. 10 shows very few similarities. Likewise, the peptide map of the alkylated dogfish M4 enzyme is quite different from any of the other three lactic dehydrogenases shown in Fig. 10. These peptide map comparisons do not present definitive data on how many regions of the primary sequences of these various lactic dehydrogenases have been retained functionally intact during evolution. However, these peptide maps do contain evidence that the primary sequence of lactic dehydrogenase has not generally been conserved during evolution.

Semiquantitative amino acid analysis was performed by paper electrophoresis on each peptide eluted from peptide maps of alkylated H4 and M4 lactic dehydrogenases of chicken. Since the analyses were carried out without further purification of the peptides, the number of identities observed is to be taken as a minimum figure. Closely related peptides from each digest located in crowded regions of their respective peptide maps might escape detection by this crude technique. However, even among peptides that migrated to isolated regions of the peptide maps of each digest, only three were shown to have identical amino acid compositions in both chicken enzymes. Two of these were dipeptides in the basic region of each peptide map, phenylalanyl-arginine, and leucyllysine. The third ninhydrin-positive peptide identical in both enzymes was located at the lowest point in the positive region of each peptide map (directly below peptide T-2 in the chicken M4 pattern in Fig. 10). The composition by crude paper techniques was determined to be: glutamic (2), serine, glycine, arginine. While this composition may require correction, it seems certain that this ninhydrin-positive fragment was shared by both chicken enzymes. A similarly positioned ninhydrin-positive peptide was present also in the beef H4 and dogfish M4 patterns given in Fig. 10. If this tryptic peptide is indeed shared by other lactic dehydrogenases it, like peptide T-1, may represent a functionally important region in the primary sequences of these enzymes.

DISCUSSION

Di Sabato and Kaplan (1) in a previous study have established that four sulfhydryl groups per molecule are essential for the catalytic operation of beef H4, chicken H4, and chicken M4 lactic dehydrogenases. Moreover, they have shown that these essential sulfhydryl groups are involved almost certainly in coenzyme binding since both oxidized and reduced coenzymes and their respective acetylpyridine analogues protect these thioles against HMB binding, while neither pyruvate nor lactate has any such protective effect.

We have extended the study to include a variety of other crystalline lactic dehydrogenases, all of which showed the presence of essential thiol groups (except the halibut M4 enzyme, which bound HMB to 10 moles of nonessential thiol before precipitating). Where the HMB-treated enzymes did not precipitate immediately on binding the first mole of mercurial, it was possible to show a linear decrease in activity with HMB-binding, the activity approaching zero when 4 moles of HMB were bound.

The essential thiol groups in frog M4 and dogfish M4 lactic dehydrogenases were protected from HMB inactivation by reduced coenzyme and by the acetylpyridine analogue of reduced coenzyme in the same manner as described for the chicken and beef enzymes by Di Sabato and Kaplan (1). The inactivation produced by HMB binding did not involve a major conformational change such as would have been observed by change in optical
rotatory dispersion or change in reactivity toward antibody directed to the native enzymes. Moreover, HMB treatment did not produce dissociation of these enzymes into subunits. Thus, the conclusions of Di Sabato and Kaplan (1) can be applied also to the dogfish M4 and frog M4 lactic dehydrogenases, and presumably to the other lactic dehydrogenases as well.

The essential thiol groups of all these lactic dehydrogenases bound HMB at widely different rates. With the lobster and lamprey enzymes, the reaction was complete almost instantaneously, while the frog M4 and chicken H4 enzymes required several hours for complete inactivation by HMB. Any essential thiol groups in halibut M4 lactic dehydrogenase resisted HMB binding altogether. This observation concerning differences in rates of HMB-induced inactivation among lactic dehydrogenases has already been pointed out for the chicken and beef H4 and M4 enzymes by Di Sabato and Kaplan (1). In spite of this wide variation in susceptibility to the mercurial, none of the essential thiols in any of the enzymes discussed in this paper was sufficiently reactive to bind iodoacetate or iodoacetamide in the native enzymes.

Among the fishes, birds, and mammals listed in Table I, the total thiol content of the various lactic dehydrogenases varied from 16 to 26 per mole of enzyme, with the exception of the ostrich H4 enzyme. Considering the relatively stable thiol content among these H4 and M4 enzymes, ranging from four to seven per subunit over this wide range of evolution, the enormous variation between the amphibian and reptilian M4 enzymes is noteworthy. While the frog enzyme has one or two thiol groups per subunit, the lowest of any lactic dehydrogenase, the alligator and caiman M4 enzymes have eight or nine per subunit, the highest thiol values so far observed.

The total thiol content of the various lactic dehydrogenases listed in Table I do not in all cases give values which are integral multiples of 4. However, most of these nonintegral multiples do not cast serious doubt on the tetrameric nature of the lactic dehydrogenases. A variation of less than 10% between the actual molar extinction coefficient of a given enzyme and the assumed value of 2.0 × 10^4 is sufficient to account for the nonintegral multiples of 4 in all cases but one. In that one case, the number of free thiol groups in the frog M4 enzyme is very likely six per mole, and cannot be explained by error in the extinction coefficient. Three possibilities present themselves. (a) M4 lactic dehydrogenase isolated from R. catesbeiana may be composed of two types of subunits, governed by separate genes, and instead of being M4 is really (M4)_2(M4)_2. This phenomenon has already been documented for some species of mice (33, 34). If the frog enzyme is indeed composed of two types of M subunits, both appear to have the essential thiol peptide (T-1), but one of the M types has an additional nonessential thiol which the other lacks. (b) The frog M4 lactic dehydrogenase preparation was made from

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**Fig. 10. Sketch of ninhydrin-stained peptide map patterns of various C14-carboxymethylated lactic dehydrogenases (LDH).** Peptides containing tryptophan are indicated by T. Major radioactive peptides from Fig. 4 are shown as shaded areas.
many frogs. The six thiol groups observed per mole may be due to a variation within the species among individual frogs, some having four thiol groups per mole, others having eight. (c) The six thiol groups per mole may be an artifact of isolation. The two rapidly reacting, nonessential thiols may be all that survived oxidation from among four or eight highly reactive, nonessential thiols present in the enzyme in vivo.

The alkylation of the two rapidly reacting, nonessential thiol groups in native frog M4 lactic dehydrogenase produced a fully active enzyme which had only four free thiol groups per molecule. Labeling of these four essential thiols with iodoacetate-1-14C was straightforward and unambiguous. The presence of a single radioactive peptide in the tryptic digest of the 14C-carboxymethylated frog enzyme (Fig. 4) accounting for 70% of the radioactivity added establishes this peptide as the one containing the active site thiol group in each subunit. No significant additional labeled peptide appeared in the tryptic-chymotryptic digest of the enzyme (Fig. 5), indicating that no other labeled peptide lay unidentified at the origin of the tryptic peptide maps. Thus, the peptide designated T-1 in tryptic digest of 14C-carboxymethylated frog M4 lactic dehydrogenase (Table V) is the active site thiol peptide of that enzyme.

A 14C-carboxymethylated peptide with electrophoretic and chromatographic properties identical with the active site peptide (T-1) from frog M4 lactic dehydrogenase can be isolated from tryptic peptide maps of 14C-carboxymethylated lactic dehydrogenases from a mammal, a heart, from both the muscle and heart of a bird, and from the muscle of a primitive fish. These four peptides, all designated T-1, have virtually identical amino acid compositions (Table V) consisting of 12 residues, or in the case of the peptide from dogfish, 11. Each peptide T-1 gives a complex partial acid hydrolysis pattern consisting of 20 fragments, each of which has an identical counterpart in the electrophoretic pattern of the hydrolysate from all the other peptides designated T-1 (Fig. 7). Thus, the peptides designated T-1 in tryptic digests of four widely different alkylated lactic dehydrogenases must be virtually identical in sequence to the active site thiol peptide from the frog M4 enzyme. Moreover, peptide T-1 is the only thiol peptide that is retained by more than two different lactic dehydrogenases. The evolutionary stability of peptide T-1 is all the more significant when compared to the evolutionary change that has occurred in other areas of the primary sequences of these enzymes. The papers of Wilson et al. (35) and of Fondy and Kaplan (32) present evidence for the sequential relationship among these various lactic dehydrogenases. These papers, along with the data presented in Fig. 10 and discussed in connection with it, all point to the same conclusion, that the primary sequences of these various lactic dehydrogenases have not generally been conserved during evolution. The essential thiol regions in triosephosphate dehydrogenase (10, 11) and in alcohol dehydrogenase (12, 13) have been retained over the course of evolution from yeast to mammals. If the thiol region that has been conserved during the evolution of the lactic dehydrogenases may likewise be assumed to be the essential thiol region, then peptide T-1 represents the essential thiol peptide in the lactic dehydrogenase. The fact that peptide T-1 has been definitely established to be the essential thiol peptide in one lactic dehydrogenase makes the foregoing assumption practically certain.6

Further evidence for the functional significance of this thiol peptide common to the various lactic dehydrogenases is available from two sources. (a) As mentioned in the introduction to this paper, Gold and Segal (2) have presented a preliminary report on the specific labeling of essential thiol groups in beef H4 lactic dehydrogenase. This labeling has been achieved by freezing and thawing the enzyme in 0.25 M sodium chloride in the presence of DDPM. Since this labeling reaction could be prevented by the addition of coenzyme, it is possible that these workers have specifically labeled the coenzyme-binding site. However, the protective effect of coenzyme in labeling reactions performed by freezing and thawing the enzyme may not be caused by specific active site effects. Chilson, Costello, and Kaplan (36) have observed that coenzyme has a general stabilizing effect on tertiary and quaternary structure of lactic dehydrogenases during freezing and thawing. Di Sabato and Kaplan (37) have observed the same general stabilization by added coenzyme during sodium dodecyl sulfate dissociation of the lactic dehydrogenase tetramer. Thus, coenzyme may protect against DDPM-labeling of the beef H4 enzyme during freezing and thawing by a general stabilizing effect on tertiary structure rather than by specific active site protection.

While the freeze-thaw labeling data of Gold and Segal (2) cannot definitely be attributed to the reaction of DDPM at the coenzyme-binding site, the extent of inactivation is directly proportional to the amount of DDPM incorporated. Thus, Gold and Segal have specifically labeled some essential thiol groups in the beef H4 enzyme. It is likely, although not certain, that these labeled thiols are associated with the coenzyme-binding site. If this is indeed the case, then the composition and sequence of the tryptic peptide containing the DDPM-labeled thiol groups when presented by these workers should correspond closely to peptide T-1 in our study.

(b) The second source available for confirmation of the functional significance of peptide T-1 is lobster M4 lactic dehydrogenase. As mentioned earlier, the lobster M4 enzyme has 12 thiol groups per molecule, 8 of which are nonessential and also bind iodoacetate very rapidly (within 1 hour) in the native enzyme. The four essential thiol groups, on the other hand, do not bind iodoacetate even after standing overnight in the presence of the alkylating reagent. Thus, the specific labeling of the essential thiol groups in the lobster M4 enzyme should be a straightforward procedure. The peptide so labeled should correspond directly to peptide T-1 in the higher species, or be functionally related to it.

The inability to locate a peptide in the digest of halibut M4 lactic dehydrogenase that is comparable to peptide T-1 in other lactic dehydrogenases does not cast serious doubt on the functional significance of peptide T-1. A careful study of the halibut M4 enzyme may yet uncover a region which is functionally homologous with peptide T-1 in other species. The initial indication of such a homologous region by the peptide mapping technique requires that no amino acid substitution has taken place which alters the electrophoretic or chromatographic properties of the peptide or changes the point of trypsin attack. Thus, it is possible for a region homologous with peptide T-1 to exist in the halibut enzyme and go unrecognized until a sequential analysis is available. Allison and Harris7 have observed just such a minor variation in the essential thiol peptide of triosphos-

6 See "Note Added in Proof," p. 4234.
7 Personal communication.
Fondy, Everse, Driscoll, Castillo, Stolzenbach, and Kaplan

November 1965

Table VIII

Amino acid sequences around essential thiol groups in four pyridine nucleotide dehydrogenases

<table>
<thead>
<tr>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg/Lys-Val-Ile-Ser-Gly-Gly-CMC-Asn-Leu-Asp-Thr-Ala-Arg-Lys</td>
</tr>
<tr>
<td>Met-Val-Ala-Thr-Gly-Ile-CMC-Arg-Ser-Asp-Asp-His-Val-Thr-Ser-Gly-Leu-Lys</td>
</tr>
<tr>
<td>-Tyr-Ser-Gly-Val-CMC-His-Thr-Asp-Leu-His-Ala-Try-His-Gly-Asp</td>
</tr>
<tr>
<td>Lys-Ile-Val-Ser-Asn-Ala-Ser-CMC-Thr-Thr-Asn-Cys-Leu-Ala-Pro-Leu-Ala-Lys</td>
</tr>
<tr>
<td>COOH-Lys-Ala-Leu-Pro-Ala-Leu-Cys-Asn-Thr-CMC-Ser-Ala-Asn-Ser-Val-Ile-NH₂</td>
</tr>
</tbody>
</table>

TABLE VIII

Amino acid sequences around essential thiol groups in four pyridine nucleotide dehydrogenases

<table>
<thead>
<tr>
<th>Chicken H₄ lactic dehydrogenase</th>
<th>Horse liver alcohol dehydrogenase</th>
<th>Yeast alcohol dehydrogenase</th>
<th>Rabbit and pig muscle and yeast triose-P dehydrogenase</th>
<th>Rabbit and pig muscle and yeast triose-P dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Tyr-Ser-Gly-Val-CMC-His-Thr-Asp-Leu-His-Ala-Try-His-Gly-Asp</td>
<td>Lys-Ile-Val-Ser-Asn-Ala-Ser-CMC-Thr-Thr-Asn-Cys-Leu-Ala-Pro-Leu-Ala-Lys</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COOH-Lys-Ala-Leu-Pro-Ala-Leu-Cys-Asn-Thr-CMC-Ser-Ala-Asn-Ser-Val-Ile-NH₂</td>
<td></td>
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</tr>
</tbody>
</table>

Table VIII represents the comparison among the essential thiol peptides of various pyridine nucleotide dehydrogenases. For this reason, the essential thiol peptide of triosephosphate dehydrogenase is presented a second time in Table VIII with the nonalkylated cysteine matched with the labeled cysteine residues in the lactic and alcohol dehydrogenases. The sequence of the triosephosphate dehydrogenase peptide is reversed in this comparison since this arrangement shows that a functional similarity may exist among the coenzyme-binding regions of the lactic, alcohol, and triosephosphate dehydrogenases.

The presence of similar amino acid sequences in the active site regions of functionally similar enzymes has been established for ficin and papain (38, 39) and for two separate functionally essential regions in trypsin and chymotrypsin (40, 41). The identical pentapeptide sequence containing the reactive serine residue in both trypsin and chymotrypsin is in addition quite similar in sequence to the pentapeptides containing the reactive serine residues in Escherichia coli alkaline phosphatase (42), pseudocholinesterase (43), and liver aliesterase (44). Moreover, many of the pyridine nucleotide dehydrogenases have been shown to possess functionally essential thiol groups (45). In addition to binding the identical coenzyme and functioning by means of essential thiol groups, many of these dehydrogenases show measurable cross-specificity for one another's primary substrates (46-53).

Thus, the presence of functionally related essential thiol peptides in both alcohol and lactic dehydrogenases is not altogether unexpected. It is hoped that the similarities as well as the differences among the essential thiol peptides of various pyridine nucleotide dehydrogenases will provide some clues to the mechanism of coenzyme binding and of substrate specificity. A comparison of the coenzyme-binding regions of these various enzymes may also be useful in following the course of evolution of the pyridine nucleotide dehydrogenases.

SUMMARY

We treated 19 species of crystalline lactic dehydrogenases with p-hydroxymercuribenzoate in 8 M urea. The number of thiol groups that bound the mercurial varied from 16 to 26 for most of the enzymes but numbered only 6 in frog M₄ lactic dehydrogenase. Four thiol groups per molecule were essential for the catalytic operation of the various lactic dehydrogenase tetramers, suggesting the presence of one active site thiol group per subunit. The native enzymes bound p-hydroxymercuribenzoate to the essential thiols at different rates with no measurable change in
conformation or molecular weight. None of the lactic dehydrogenases bound iodoacetate or iodoacetamide to the essential thiol groups in the native conformation.

The essential thiol groups in frog M₄ lactic dehydrogenase were specifically labeled with iodoacetate-¹³C in 8 M urea. The thiol-containing peptides of each subunit were isolated and characterized. This same dodecapeptide sequence was found in four out of five other lactic dehydrogenases examined: the H₄ enzymes of beef and chicken and the M₄ enzyme of chicken and dogfish. The over-all primary sequences of these lactic dehydrogenases have not been generally conserved during evolution.

The sequence of the essential thiol peptide as isolated from chicken H₄ lactic dehydrogenase was determined. This active site thiol peptide from the lactic dehydrogenases may be functionally related to the active site thiol region of the alcohol dehydrogenases.

Acknowledgments—We wish to give recognition to Miss Regina Ziegler for technical assistance. We are grateful to the New England Enzyme Center for making available large quantities of crystalline enzymes.

Note Added in Proof—While this manuscript was in press, a preliminary report by Holbrook and Pfeiderer (54) appeared on specific labeling of the essential thiol groups in pig H₄ lactate dehydrogenase. These workers employed N-[(N-acetyl-4-³⁵S-sulfamoylphenyl)maleimide to achieve inhibition of enzymatic activity. This inhibition was completely prevented by the presence of reduced coenzyme.

The labeled thiol peptide composition was identical with that reported by us for the active site thiol peptides from beef H₄, chicken M₄, and frog M₄ lactic dehydrogenases. Thus, the work of Holbrook and Pfeiderer and our own work are complementary. The thiol peptide specifically labeled in frog M₄ and pig H₄ lactic dehydrogenases and retained virtually unchanged in several other species is confirmed as the active site thiol region.

REFERENCES

CORRECTION

In the paper by Thomas P. Fondy, Johannes Everse, Geraldine A. Driscoll, Fred Castillo, Francis E. Stolzenbach, and Nathan O. Kaplan (Vol. 240, No. 11, November 1965, page 4219), Table VIII, page 4233, the "-Lys" should be deleted from the right-hand ends of the sequences for chicken H, lactic dehydrogenase and horse liver alcohol dehydrogenase.
The Comparative Enzymology of Lactic Dehydrogenases: IV. FUNCTION OF SULFHYDRYL GROUPS IN LACTIC DEHYDROGENASES AND THE SEQUENCE AROUND THE ESSENTIAL GROUP

Thomas P. Fondy, Johannes Everse, Geraldine A. Driscoll, Fred Castillo, Francis E. Stolzenbach and Nathan O. Kaplan