Peptide Inhibitors of Lactic Dehydrogenase (LDH). II. Isolation and Characterization of Peptides I and II*

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ABSTRACT: Two peptides which inhibit lactic dehydrogenase have been isolated from human urine by solvent extraction, ion-exchange chromatography, and gel filtration. Peptide I has been shown to have a molecular weight of 1760. Peptide II as yet not completely characterized has a molecular weight of about 2500. The intact peptide structure is required for inhibition since enzymatic hydrolysis with Nagarse, carboxypeptidase, and trypsin abolishes the inhibition by both peptides. Peptide I does not contain an aromatic amino acid and is not affected by chymotrypsin; peptide II contains tyrosine and is destroyed by chymotrypsin.

Control of metabolic processes by inhibition of enzymatic activity is now well known to occur in bacteria (Umbarger, 1956; Yates and Pardee, 1956; Jacob and Monod, 1961) and has been postulated for mammalian systems (Kaplan and Goodfriend, 1964). From our previous studies, lactic dehydrogenase appeared to provide a suitable means for establishing in mammalian systems the existence of such enzymatic control processes. In developing a method for the measurement of lactic dehydrogenase (LDH) activity, dialyzable inhibitors of LDH were found to be present in all human urines (Wacker and Dorfman, 1962). The inhibition could not be accounted for by the concentration of known inhibitors of LDH activity such as oxalate or urea in urine. Further experiments indicated, moreover, that they were low molecular weight dialyzable organic compounds destroyed by ashing (W. E. C. Wacker and L. E. Dorfman, unpublished data). Systematic efforts to isolate and determine the chemical nature of the inhibitors have resulted in the isolation from human urine of two low molecular weight peptides which inhibit lactic dehydrogenase activity. Hydrolysis with specific proteolytic enzymes, either in urine or when highly purified, abolishes the inhibitor activities of these peptides. Such peptide inhibitors of lactic dehydrogenase or, indeed, any pyridine nucleotide dependent dehydrogenase had not been known previously. The present communication reports the method of isolation of the inhibitors and their amino acid composition.

Materials and Methods

The reagents for the initial extraction procedure, including 1-butanol and ether, were reagent grade and used without further purification. The organic bases pyridine, 2,6-lutidine, 2,4,6-trimethylpyridine, and triethylamine used as the buffer systems for column chromatography were redistilled before use (Gero and Markham, 1951).

Ion-Exchange Resins. All resins and gels were washed several times with distilled water before use to remove fine particles. Amberlite IR-120 (Rohm and Haas, Co.) was converted to the Na^+ form by treating with 2.0 N NaOH for 2 hr followed by a washing with distilled, deionized water. This treatment was repeated three times. Amberlite IR-400 (Rohm and Haas, Co.) was converted to the OH^- form by treating for 4 hr in NaOH. Dowex 50-X4 (Bio-Rad) was converted to the NH_4^+ form with 2.0 M ammonium hydroxide. Dowex 1 (Dow Chemical) was converted to the acetate form (Rudloff and Braunitzer, 1961).

Molecular Sieves. Sephadex G-25 Fine (Pharmacia, Uppsala, Sweden) excluded molecular weight >3600. Bio-Gel P_2, 100–200 mesh, polyacrylamide-Gel (Bio-Rad), excluded molecular weight >1600.

Columns. For Amberlite IR-120 and IRA-400, 100 × 3 cm columns were used. For Dowex 50-X4, a 50 × 2 cm column was employed. For Dowex 1, a 150 × 2 cm jacketed column was utilized and maintained at 35° by circulating water. Gel filtration with Sephadex G-25 and Bio-Gel P_2 was carried out in 30 × 1 cm columns. Constant flow rates were maintained by use of a peristaltic pump. The Dowex 1 and the gel-filtration columns were coated with dimethyldichlorosilane applied as a 1% solution in benzene at 60°. The benzene was then removed by evaporation at 104°. All effluent fractions were collected on a GME fraction collector.

The presence of peptides in the effluent fractions was determined with ninhydrin. A 0.1-ml aliquot of each fraction was added to 1.0 ml of ninhydrin solution

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and color developed by heating at 100°. After cooling, the samples were diluted with 8.0 ml of propanol–water (1:1), and the absorbance at 575 mμ was measured in a Beckman DU spectrophotometer (Moore and Stein, 1948, 1954).

High-voltage electrophoresis (75 ma, 2000 v, 1.5 hr) on Whatman 3MM paper was used to assess purity of the peptides during the isolation procedure. Electrophoresis was carried out both at pH 1.9 (4% formic acid) and at pH 6.5 (pyridine acetate) (Michl, 1951).

Quantitative amino acid analyses were performed with a Spinco Model 120B automatic amino acid analyzer according to the method of Spackman *et al.* (1958). The samples were hydrolyzed with 6 N HCl at 105° for 24 hr in sealed evacuated tubes.

LDH (rabbit muscle) was obtained from Boehringer, Mannheim, W. Germany; DPN^+ and DPNH were obtained from the Sigma Chemical Corp. Lactic acid was obtained from Fisher Scientific Corp.

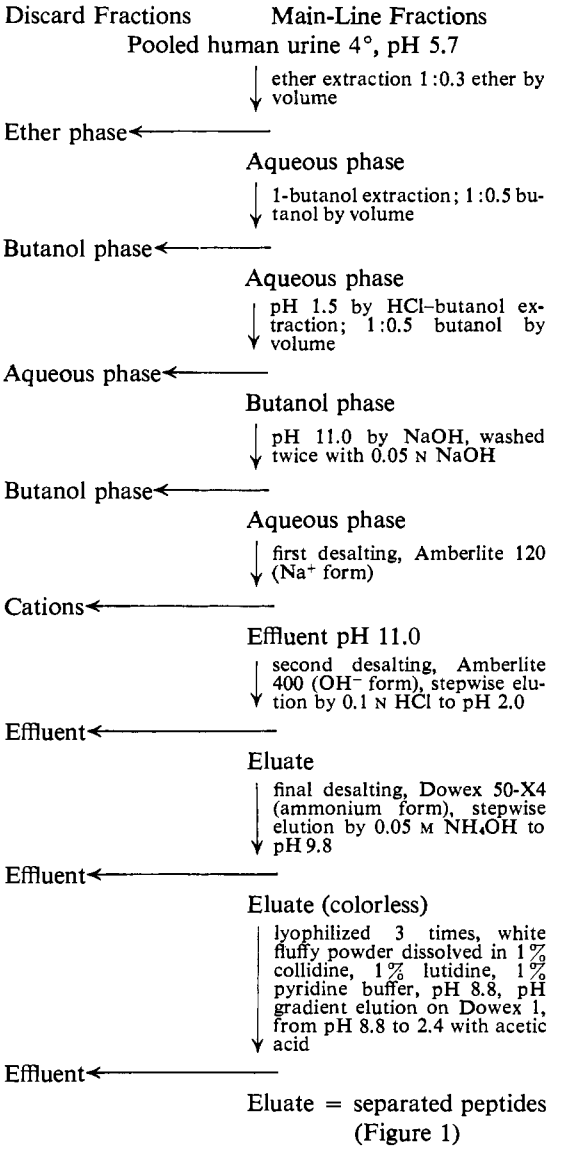
LDH activities were measured using the forward spectrophotometric assay (Wacker *et al.*, 1956) in a Beckman DU spectrophotometer equipped with a Gilford automatic cell changer and recording attachment. The cell compartment was maintained at 25 ± 0.1° by a circulating water bath. Quartz cells of 1-cm path length were used throughout.

Results

Preliminary efforts to purify, by ion-exchange chromatography, the substances in urine which inhibit LDH indicated that they were amphoteric and suggested further that they were peptides. The development of the following purification procedure was based on this hypothesis.

A schematic diagram of the purification is shown in Scheme I. Pooled human urine which had been stored at 4° was extracted with ether to remove chromogenic substances, and the ether phase was discarded. The aqueous phase, containing the inhibitor, was extracted with 1-butanol at an apparent pH of 5 to 6 to remove other noninhibiting substances and the butanol phase was discarded. The remaining aqueous phase was brought to pH 1.5 with HCl, and under these conditions the inhibitors were extracted into butanol. The aqueous phase was discarded. The apparent pH of the butanol phase was then raised to 11.0 with sodium hydroxide and washed twice with 100 ml of 0.05 M sodium hydroxide to transfer the inhibitor into the aqueous phase. The aqueous solution (pH 11.0) was passed through an Amberlite 120 (Na^+ form) column and most of the remaining colored substances were thus removed. The effluent (pH 11.0) was transferred to an Amberlite 400 (OH^- form) column. The column was washed with 0.05 M NaOH until the effluent was completely colorless. The inhibitors were eluted by the addition of 0.1 M HCl until the pH of the effluent reached 3.4. Remaining salts were removed from the eluate by adjusting its pH to 1.5 with HCl and passing it through a column of Dowex 50 × 4 (NH^+ form) previously washed with 1 void volume of 0.1 M sodium citrate, pH 3.3 (Haugaard and Haugaard, 1955). At pH 1.5 the inhibitors were bound to the resin. The column was washed with 0.1 M acetic acid until the effluent reached pH 3.5. The inhibitors were eluted by bringing the pH of the effluent to pH 9.8 with 0.05 M NH_4OH. The resultant water-clear solution was lyophilized three times to remove ammonium acetate, yielding a white fluffy powder.

SCHEME I: Isolation of Inhibitor Peptides from Human Urine.



Further separation and purification was achieved by gradient elution from Dowex 1 (Rudloff and Braunitzer, 1961). The column was coated with dimethyldichlorosilane before packing to provide homogeneous flow characteristics. The sample was dissolved in a buffer containing 3% organic base (1% collidine, 1% lutidine, and 1% pyridine) at pH 9.4. The same buffer adjusted to pH 8.8 with acetic acid was used as the starting buffer. Gradient elution was achieved by addition of 0.1 and 2.0 N acetic acid covering a pH range from 8.8 to 2.4.

Figure 1 shows the pattern of ninhydrin-positive ma-

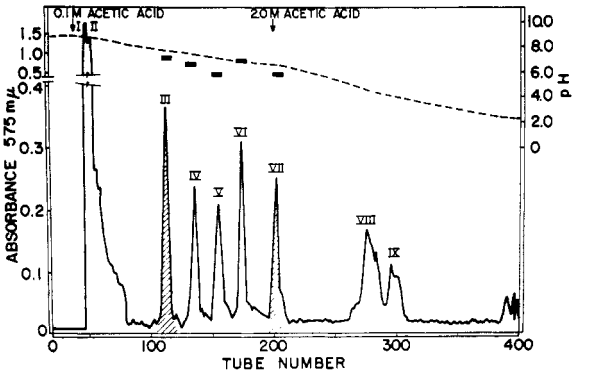


FIGURE 1: Chromatography of urinary peptides on Dowex 1. ———, ninhydrin absorbance; - - - -, pH. Starting buffer: 1% collidine, 1% lutidine, 1% pyridine, pH 8.8; pH gradient from 8.8 to 2.4 with 0.1 and 2.0 N acetic acid. ■, absorbance of ninhydrin after alkaline hydrolysis of the respective peaks. The shaded areas under peaks III and VII indicate the fractions which inhibit LDH.

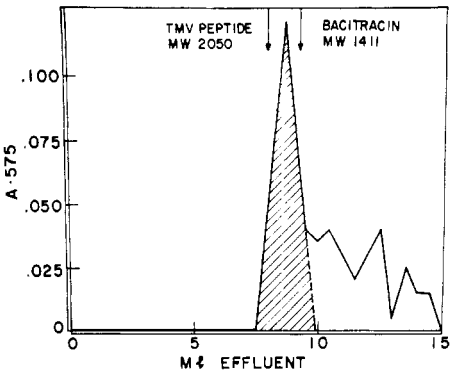


FIGURE 2: Gel filtration of peak III on polyacrylamide gel (Bio-Gel P_2). ———, ninhydrin absorbance. The shaded area under the major peak indicates the fractions which inhibit LDH. The arrows indicate the positions where TMV peptide and bacitracin emerge from the column under these conditions.

terial eluted from the column. The major peaks are numbered 1 to 9. High-voltage paper electrophoresis at pH 6.5 showed that the substances in peaks I and II are basic, those in III through VII are neutral, while those in VIII and IX are acidic peptides. The bars above peaks II through VII in Figure 1 show the increase in ninhydrin absorbance on alkaline hydrolysis, indicating the presence of peptides. Electrophoresis at pH 1.9, however, demonstrated that in addition to the major peptides peaks, III–VII contained small amounts of faster migrating ninhydrin-positive materials. Since these compounds are eluted in the same position but move faster in the electrical field they were judged to be contaminating amino acids or small peptides.

The materials in peaks II–VII were lyophilized three times yielding fluffy white powder. Inhibitory capacity was measured by adding aliquots of the dry powder to the enzyme assay mixture. Materials from peaks III and VII inhibited LDH but those from the remaining peaks were devoid of inhibitory capacity.

The inhibitor peptide in peak III was purified further by passage over Sephadex G-25 in 3% triethylamine, pH 10.8. Ninhydrin-positive material is not excluded under these conditions while the inhibitor is retained, indicating its molecular weight to be less than 3600. To remove lower molecular weight materials, identified by high-voltage electrophoresis, the eluate from Sephadex was passed over polyacrylamide gel (Bio-Gel P_2, 100–200 mesh). The result of the polyacrylamide gel filtration of peak III is shown in Figure 2. The position of peak III is compared with the R_F values of peptides of known molecular weight (bacitracin, molecular weight 1415, and TMV peptide, molecular weight 2050), suggesting an approximate molecular weight of 1750. The peak containing the inhibitor is symmetrical, indicating a high degree of purity. Small peaks in the region of low molecular weight compounds represent contaminating amino acids. Following gel filtration and repeated lyophilization peak III moved as a single band on electrophoresis at both pH 6.5 and 1.9.

Amino acid analysis of the peptide obtained from peak III designated inhibitor peptide I indicates that it contains lysine, histidine, serine, threonine, glycine, alanine, leucine, isoleucine, aspartic acid, and glutamic acid. Gel filtration of peak VII on polyacrylamide gel (Bio-Gel P_4) indicates that it has a molecular weight of approximately 2500. Amino acid analysis of inhibitor peptide II, the peptide obtained from peak VII, as yet not completely purified, shows it to contain lysine, histidine, serine, threonine, glycine, alanine, leucine, isoleucine, aspartic and glutamic acids, tyrosine, and cystine. In addition this peptide contains a ninhydrin-positive material which emerges just after glutamic acid on the amino acid analyzer. The nature of this material is being investigated.

Incubation of the peptides with trypsin, Nagarse, or carboxypeptidase for 1 hr completely abolishes their inhibitory activity (Table I). The presence of a tyrosyl residue in peptide II permits it to be inactivated specifically by digestion with chymotrypsin under the same conditions. Inhibitor peptide I does not contain an aromatic amino acid residue and hence the inhibition is not abolished by chymotrypsin.

Discussion

Previous studies indicated the presence of naturally occurring inhibitors of LDH in human urine (Wacker and Dorfman, 1962). The present data demonstrate that the inhibition is due to the presence in urine of two polypeptides.

Peptide I has a molecular weight of about 1750 as estimated by gel filtration. The amino acid composition is not unusual and the spectrum does not exhibit absorption at 280 mμ, consistent with a lack of aromatic amino acids. Inhibitor peptide II has been purified sub-

TABLE I: Effect of Proteolytic Digestion on the Activity of Inhibitor Peptides I and II.[a]

Proteolytic Enzyme	Inhibitor Peptide	Time of Digestion, V_i/V_c 0 Min	60 Min
Trypsin	I	0.64	1.0
	II	0.66	1.0
Nagarse	I	0.62	1.0
	II	0.66	1.0
Carboxypeptidase A	I	0.63	1.0
	II	0.60	1.0
Chymotrypsin	I	0.62	0.61
	II	0.64	1.00

[a] Each proteolytic enzyme (1 mg) was added to 10 ml of a solution of inhibitor. Aliquots were removed at 0 and 60 min, boiled to destroy the proteolytic enzyme, and then assayed for inhibitory activity.

stantially but has not been characterized completely; its amino acid composition differs from that of inhibitor peptide I and its molecular weight appears to be closer to 2500. In keeping with the presence of tyrosine this peptide has an absorption maximum at 280 mμ at neutral pH which shifts to 293 mμ at pH 11. In addition peptide II contains an as yet unidentified ninhydrin-positive material which may represent an unusual amino acid. The precise chemical nature of this material is currently being studied.

The inhibition brought about by both peptides is clearly related to their intact peptide structure: Nagarse, trypsin, and carboxypeptidase completely destroy their inhibitory activity. As expected chymotrypsin does not abolish the inhibition due to inhibitor peptide I which lacks an aromatic amino acid. Conversely, chymotrypsin destroys the inhibition due to inhibitor peptide II which does contain tyrosine.

The presence of a number of peptides in human urine has been reported (Buchanan *et al.*, 1962; Skarzynski and Sarnecka-Keller, 1962) though their biological function has not been identified. In addition to the two peptides found to inhibit LDH, a number of other peptides have also been isolated from human urine in this study, but none had any inhibitory capacity for LDH (Figure 1). The potential biological activities of the remaining peptides have not been examined as yet. It is, however, possible that some of them may be inhibitors of other enzymes whose activity in urine has been masked by their presence (Amador *et al.*, 1963).

The present studies while clearly demonstrating the presence of peptide inhibitors of LDH in human urine of themselves do not indicate their source. Preliminary experiments in this laboratory, however, suggest the presence of similar peptide inhibitors in homogenates of human liver, cardiac and skeletal muscle, and in human serum (W. E. C. Wacker and G. A. Schoenenberger, unpublished data). Support for the contention that these inhibitors are present in tissues may be gained from previous studies. Detailed examination of the isozyme patterns of normal tissues revealed that the LDH activity of an homogenate was often considerably less than the sum of the LDH activity of the various constituent isozymes after separation on DEAE-Sephadex (Richterich *et al.*, 1964). These results may be interpreted to indicate that the separation of the isozymes may also have resulted in dissociation of inhibitors from the enzyme. Substances inhibiting LDH activity have been obtained from human serum by dialysis, although the possible peptide nature of the inhibiting substances was not investigated (Morgan *et al.*, 1963; Emerson *et al.*, 1965).

Although the present observations represent the first indication that peptides inhibit lactic dehydrogenase, or indeed any pyridine nucleotide dependent dehydrogenase, many peptides are known to have striking biological activity. Peptide hormones and vasoactive peptides produced by limited proteolysis of serum proteins may be cited as examples. Moreover, peptides released by limited proteolysis during the thrombin-catalyzed conversion of fibrinogen to fibrin are active in potentiating the contraction of smooth muscle (Osbahr *et al.*, 1964). Thus, from a biological point of view it is not too surprising that the LDH inhibitors have proved to be peptides. Since little information is available concerning the precise chemical and physical modes of action of peptide hormones or vasoactive peptides, it is conceivable that the biological activity of some of them may be exerted through a similar inhibitory mechanism.

The inhibition caused by these peptides is remarkably specific. Thus peptide I inhibits LDH-M_4 isozyme but not LDH-H_4 isozyme while peptide II inhibits LDH-H_4 but is inactive against LDH-M_4. The details of this specific inhibition is the subject of a separate report (Wacker and Schoenenberger, 1966).

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The Sulfatase of Ox Liver. X. Some Observations on the Intermolecular Bonding in Sulfatase A*

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ABSTRACT: Several independent items of evidence strongly indicate that hydrophobic bonding significantly contributes to the formation of the tetramer (mol wt 411,000) of sulfatase A. Notable in this respect is the increase in weight-average sedimentation coefficient with increasing temperature at pH 5.0. Electrophoretic mobility data are used to show that the isoelectric point of the protein is 3.4 ± 0.05 in sodium chloride–sodium formate–formic acid buffer at ionic strength 0.10. Weight-average sedimentation coefficient results obtained in the pH range 3.47–5.00 are interpreted in terms of an expansion of the tetramer structure with increasing pH, caused by electrostatic repulsive forces acting in opposition to the postulated hydrophobic bonding. As the pH is further increased the increased net negative charge results ultimately in dissociation to the monomer (mol wt 107,000) at pH 6.6. A dissociation is also brought about by the addition of dioxane to a solution of the tetramer. The effect of sodium dodecyl sulfate on the system has been investigated at pH values of 7.5, 6.7, and 5.0. The approach of the anionic detergent has been shown to be governed by the net charge on the molecule. Results obtained at pH 5.0, where the action was most pronounced, have been chosen for detailed study; in particular, the slow sedimenting material (s 3.6) evident in the presence of detergent has been shown to be essentially homogeneous with respect to sedimentation coefficient and has been characterized by estimating its partial specific volume, its sedimentation coefficient to diffusion coefficient ratio, and the amount of bound detergent. The results show that detergent dissociates the tetramer into subunits of approximately equal size (mol wt 24,000) and the several assumptions required for the latter estimation are discussed. The effect of removing the detergent by dialysis under various conditions has been examined.

Aryl sulfatase A (an arylsulfate sulfohydrolase, EC 3.1.6.1.) from ox liver exists in 0.10 ionic strength solutions as a monomer (mol wt 107,000) at pH 7.5 and as a tetramer (mol wt 411,000) at pH 5.0 (Nichol and Roy, 1964, 1965). The latter work showed that an increase in ionic strength resulted in more extensive polymerization at both pH 7.5 and pH 5.0, suggesting that the net charge at the former pH is sufficiently large to prevent polymerization in 0.10 ionic strength buffers, but that it can be masked either by lowering the pH or by increasing the ionic strength. In this study electrophoretic mobility data are presented to support the hypothesis. The data also show that the isoelectric point of the enzyme is surprisingly low and this has necessitated the determination of weight-average sedimentation coefficients, $\bar{s}$, and enzymatic activities at pH values lower than previously studied (Nichol and Roy, 1964, 1965). It is found that $\bar{s}$ values increase as the pH is lowered toward the isoelectric point.

An increase of $\bar{s}$ with temperature at pH 5.0 is also reported, suggesting that the tetramer structure becomes more compact as the temperature is increased. This finding [together with the failure to detect electrostatic bonds or the more common covalent linkages (Nichol and Roy, 1964, and unpublished observations)] suggests that hydrophobic interaction (Kauzmann, 1959; Scheraga, 1963) may contribute toward polymer

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