

AN ABSTRACT OF THE THESIS OF

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Title: LACTATE DEHYDROGENASE FROM HEXANCHUS GRISEUS:
ISOLATION AND CHARACTERIZATION

Abstract approved: Signature redacted for privacy.
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The M_4 isozyme of l(+)-lactate dehydrogenase (LDH) has been isolated from the skeletal muscle of the sixgill shark Hexanchus griseus using a procedure derived from the purifications of dogfish and halibut M_4 LDH. This isolation procedure is distinctive in relying upon a heat treatment in which LDH activity is conserved through stabilization with added NADH and sodium sulfate. Through electrophoresis and coenzyme analogue kinetics, the LDH isozyme system of Hexanchus has been shown to be quite similar to that of dogfish; the M_4 isozyme can be isolated from the skeletal muscle with the exclusion of other isozymes and is the major LDH component in such tissue. With a purification of 23-fold from the water extract, a preparation quite resistant to further purification results; one kilogram of muscle tissue contains about two grams of such protein, of which 24-48% may be thus isolated. The results of polyacrylamide gel electrophoresis, gel filtration, and ultracentrifugation generally

show conformity of the Hexanchus M_4 preparations with the tetrameric molecular structure of 140,000 daltons expected for LDHs of vertebrates, despite some microheterogeneous deviations from the beef M_4 molecular weight-structure profile. However, the coenzyme binding capacity of these preparations predictably falls short of the ideal 4.0 moles per 140,000 grams by a very significant, though variable, margin. The coenzyme-binding deficiency of the Hexanchus samples apparently results both from the obstruction of available coenzyme binding sites by dialysis-resistant residual coenzyme fragments and the inclusion of varying amounts of deactivated enzyme material in a quasi-equilibrium relationship with the active enzyme. The inclusion of residual coenzyme fragments by Hexanchus M_4 can be verified by chemical analyses for nicotinamide, pentose, phosphate, and adenine; though levels of such components are substoichiometric and show much experimental variation, it appears certain that Hexanchus M_4 preparations contain very significant amounts of cyanide-reactive pyridinium-nicotinamide, of which beef M_4 and H_4 appear nearly devoid. The release of coenzyme fragments from Hexanchus M_4 preparations occurs in conjunction with massive losses of enzyme activity, through dissociation, formation of an insoluble precipitate, or both; proportions of coenzyme components thus observed are seldom compatible with NAD^+ . Hexanchus M_4 could thus exemplify a mode of structure-function stabilization in LDH dependent upon the

covalent cleavage of the coenzyme and the covalent attachment of the resulting fragments to the enzyme. A few residue-values of the amino acid analysis of Hexanchus M₄ deviate conspicuously from those of dogfish M₄ (and similarly from those of LDHs in higher vertebrates); these same deviations have been found (to a greater degree) in the amino acid analysis of lobster tail muscle LDH.

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Isolation and Characterization

by

Sylvester Goodrich Spencer

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ABBREVIATIONS

NAD ⁺ :	nicotinamide-adenine dinucleotide
NADH:	reduced NAD ⁺
APD ⁺ :	3-acetylpyridine-adenine dinucleotide (analogue of NAD ⁺ , Ref. 34, p. 3)
APDH:	reduced APD ⁺
TAD ⁺ :	thionicotinamide-adenine dinucleotide (analogue of NAD ⁺ , Ref. 34, p. 5)
NBT:	nitro blue tetrazolium
PMS:	phenazine methosulfate
CMC:	carboxymethyl cellulose
DEAE:	diethylaminoethyl (cellulose)
LDH:	lactate dehydrogenase
M ₄ :	LDH isozyme characteristic of skeletal muscle
M:	moles per liter (when following a number)
Tris:	2-amino-2(hydroxymethyl)-1, 3-propanediol

LACTATE DEHYDROGENASE FROM HEXANCHUS GRISEUS: ISOLATION AND CHARACTERIZATION

I. INTRODUCTION

A. General Background: LDH in Vertebrates

L-lactate dehydrogenase (LDH), systematically classified L(+)-lactate/ NAD^+ oxidoreductase (EC 1.1.1.27) (1, 2), reversibly catalyzes the overall conversion of one equivalent each of L-lactate and NAD^+ to one equivalent each of pyruvate, NADH, and hydrogen ion. The LDH reaction to produce L-lactate from pyruvate has long held the position of being the final step of anaerobic glycolysis in animal tissues (3). The relatively conspicuous fluctuations of lactate levels in vivo have rendered the LDH reaction a rather high-priority target for physiological study, and many studies of other enzymes have relied upon this reaction in coupled reaction systems (4, 5). In the study of the molecule itself, the problem of catalysis shows an experimental accessibility shared by few other dehydrogenases. Complications such as metal ions, linked phosphorylation, or ammonia uptake are absent (2). Its relative stability in a variety of simple buffer systems has contributed considerably to its practical significance in clinical laboratory work (6).

Markert and Møller (7) in 1959 published collected evidence that LDH is a tetrameric molecule of molecular weight 140,000 which dissociates into monomers under denaturing conditions. In conjunction with this proposed structure for LDHs, the phenomenon of multiple molecular forms of LDH occurring within an individual organism was extensively explored. Using various electrophoresis methods (8), differential kinetic studies (9), and immunological studies (10), two distinct subunit types of molecular weight 35,000 could generally be discerned in each organism. The proportions of these subunits are tissue-specific: in heart muscle tissue, a polypeptide recognizable as the H-subunit predominates over the M-subunit, which is typical of skeletal muscle tissue. With appropriate separation techniques (11), a homotetrameric LDH-H₄ may be isolated from heart tissue, and a homotetrameric LDH-M₄ may be obtained in good yield from skeletal muscle. These two "parent" homotetramers H₄ and M₄, whose constituent polypeptide chains arise from the expression of two distinct genetic loci, form the three "hybrid" isozymes, H₃M, H₂M₂, and HM₃, under subunit-interchange conditions in vivo and in vitro (12).

The starch gel electrophoresis of LDH samples has been widely used with both tissue extracts and purified LDHs for characterization in terms of isozyme composition (13). Under conditions in which most isozymes migrate in an anodic direction, LDH components are thus

separated on starch gel slabs, and resulting isozyme locations are defined with an activity stain. Usually, the H_4 isozyme is the most anionic of the LDHs in a given animal, and may be assigned a relative mobility (r. m.) of +1.0. It follows that the M_4 isozyme, least anionic within the same species, may be assigned a r. m. of zero. Hybrid isozymes usually occupy intermediate locations with r. m.'s of +0.25, +0.50, and +0.75. In an ideal hybridization experiment, such that equal activities of the two parent homotetramers survive the subunit-interchange conditions, the spot intensities of the five isozymes would show the binomial expansion-derived proportions of 1:4:6:4:1. Methods of quantitating the spots using densitometer scanning have shown that near-Gaussian distributions of staining may be obtained, if staining conditions are properly controlled; a systematic calculation of relative total H- and M- derived activity in "zymograms" has thus been developed (14). The kinetic properties of the intermediate isozymes range between those of the homotetramers; kinetic measurements on pure samples of hybrids approximate the same data taken with proportionate mixtures of the two homotetramers (15).

The functional significance of the LDH isozymes in vivo has been discussed extensively, but few interpretations have gained universal acceptance or have taken into account the many behavioral criteria by which the M_4 and H_4 molecules may be distinguished. The M_4 LDH predominates over other isozymes in primarily anaerobic tissues

(such as skeletal muscle), whereas the H_4 isozyme occurs more in aerobic tissues, such as heart muscle. Some investigators have cited differences in the kinetics, especially in sensitivity to substrate inhibition (9, 16), claiming that the M_4 is more fit for the anaerobic media than the H_4 , because the lesser substrate inhibition of M_4 may allow further glycolysis with lactate buildup when cellular energy requirements exceed the capacities for aerobic pyruvate metabolism. The kinetic theories involving substrate inhibition by pyruvate have been challenged by Vesell (17) on grounds that cellular accumulations of pyruvate are meager compared to the concentration used in vitro to demonstrate substantial differences in substrate inhibition for the isozymes.

The control of pyruvate utilization as an outcome of the particular isozyme composition also says nothing about a wide variety of catalytic alternatives of which the LDH molecule is capable. LDHs catalyze the reduction of superoxide radical-anion by NADH to produce hydrogen peroxide and radical NAD^{\cdot} , involving an enzyme- NAD^{\cdot} complex (18). Any LDH, if supplied with the proper balance of NAD^+ and NADH near pH 7, may catalyze the disproportionation of two moles of glyoxalate into glycolate and oxalate with no net turnover of coenzyme (19). Actually, many 2-oxo-carboxylic acids other than pyruvate may be reduced by LDH-NADH at rates falling short of the pyruvate rate only to the degree that the radical attached to the

2-carbon of the acid exceeds the size of an ethyl group (20). In view of such alternatives, the formulation of a comprehensive and entirely distinctive catalytic profile for any isozyme appears to be a most formidable task, especially if extrapolation into intrinsically variable physiological conditions is to be considered.

Certain investigators have approached the question of isozyme function by correlating changes in the amounts of H_4 and M_4 (and their hybrids) in tissue cultures in response to imposed conditions. According to Hellung-Larsen and Andersen (14), the phytohaemagglutinin-stimulated growth of human lymphocytes in the presence of increasing oxygen tension allows consistently less of the M_4 LDH to be synthesized, while H-type subunits increasingly predominate in the zymograms of the homogenized cells. Though normal human lymphocytes inherently have the isozyme composition of anaerobic cells, some 48 hours' growth under 70% O_2 produces a population of highly aerobic cells. Conversely, beginning a culture with transformed "aerobic" lymphocytes produces a consistent increase in M_4 LDH as growth under a 5% O_2 atmosphere progresses. These responses to O_2 persist despite culturing the cells in high pyruvate or lactate concentrations, or frequently changing the media so that lactate buildup is disallowed. These studies support the designation of M_4 -producing cells as being fundamentally anaerobic, but also suggest that lactate or pyruvate may have little direct relationship to the isozyme

composition as effectors of genetic expression.

The search for the different and essential functions that distinguish H_4 from M_4 LDH and the basis of their normal tissue-specific expression has led to the isolation of the isozymes from a variety of animal species (21). Certain aspects of the amino acid sequence of LDH remain strictly conserved despite isozymic or interspecific variation. The sequence homology between H- and M-type subunits is likely to be 75% within a species (22), and an active site peptide centered about an essential cysteine residue is virtually identical in H and M within a species, and largely conserved through interspecific variation (23, 77). Much of the LDH structure must remain highly conserved to maintain the tetrameric-globular structure of 140,000 molecular weight and to allow the same active site structure of such high stability in so many species. However, considerable progress has been made in distinguishing M_4 from H_4 on an interspecific basis. Experiments with subunit interchange in interspecific mixtures of isozymes have implied that M_4 molecules from different species are much more like each other than they are to the H_4 molecules of the same species (2, 25), a conclusion supported by chemical and immunological results (10).

The isolation of M_4 and H_4 LDHs from a wide variety of species has culminated in discovering a method of preparing especially large crystals of dogfish M_4 (21). The consequent X-ray crystallography

of M_4 from dogfish (Squalus acanthias) in conjunction with the amino acid sequence determination has allowed Adams, Rossmann, and others (25) to construct a three-dimensional model of a LDH molecule. Not only are the proximities of the amino acids known for a crystalline state of the apoenzyme to a nominal resolution of 2.0 \AA , but the corresponding structure of the LDH- NAD^+ -pyruvate complex has been determined from a separate crystallography using the same amino acid sequence data and assuming the same subunit chain length (some 331 residues) (2). Thus, the crystallographic model derived for dogfish M_4 has considerable dynamic character: a very substantial displacement accompanies the binding of coenzyme. Besides elucidating the general geometry of coenzyme binding, the binding of coenzyme fragments (26) and the structural consolidation associated with functional anion binding sites (other than coenzyme) has been explored (27).

The crystallography of dogfish M_4 confirms much about the structure-function relationship of LDH catalysis that has been deduced from less explicit structural studies on other LDHs: that the catalysis has a compulsory-ordered mechanism depending first on the binding of the adenine moiety concurrent with the "unfolding" of the coenzyme molecule, then the binding of the nicotinamide moiety, followed by positioning of the substrate molecule near the 4-position of the nicotinamide ring and also near essential residues of the active site. The crystallography of dogfish M_4 quite exactly measures the unfolding

of the coenzyme and contraction of the subunits about their active sites upon forming the ternary complex. These structural transformations would apply similarly to most any coenzyme-LDH complexing, although the specific changes and their actual extent would depend upon the species, isozyme, and the chemical states of coenzymes and substrates involved.

Because of the wide taxonomic divergence between dogfish and the other species in which LDH has been extensively studied, there is considerable uncertainty in comparisons of the coenzyme binding and structure-consolidating behavior that might make use of the dogfish model. Also, the comparison of in vivo geometries of H_4 and M_4 within a species could never rely wholly upon the X-ray, since only certain crystalline states of the enzyme may be characterized in this manner. Methods of wider applicability and intrinsically more inclusive of hydrodynamic influences are especially needed to make use of the dogfish model in multiple interspecies comparisons that would better define the consistent differences between M_4 and H_4 . Fortunately, considerable structure-function data has been collected for dogfish M_4 preparations from which the "model" crystals may be obtained. The studies involving interactions with coenzymes and their relationship to enzyme structure in aqueous solution have been applied quite extensively to other species, particularly to beef, pig, and chicken. Apparently, a most desirable addition to the present

body of information would be the extension of the M_4 structure-function studies to species of elasmobranchs other than dogfish. Such comparative studies would be likely to elucidate some interspecies variability among the elasmobranchs which would have bearing on the general applicability of the dogfish M_4 model to LDH studies in all vertebrates.

The particular elasmobranch LDH of interest in this study is the M_4 isozyme isolable from the soluble cytoplasm of skeletal muscle of the sixgill shark Hexanchus griseus. As well as to provide additional information on the variability of the M_4 isozyme among elasmobranchs, the sixgill shark is particularly interesting as an enzyme source since it may be regarded as a "living fossil." This primitive vertebrate appears to have diverged from the main evolutionary line of sharks in late Paleozoic times, now a surviving remnant of elasmobranch stock representing a different stage of development than does the dogfish. The phylogeny of the sixgill shark designates the genus Hexanchus as the sole genus of the family Hexanchidae, diverging from a transitional phase in the evolution of elasmobranchs, apparently before the batoids (such as skates and rays) diverged from the rest of modern elasmobranchs (including dogfish) (28). The characterization of Hexanchus M_4 offers to bring considerable additional insight into the evolution of LDH; no isozyme of LDH from Hexanchus has been isolated and characterized to date.

B. Specific Goals of Hexanchus M₄ Studies

The central object of this study was the completion of a coherent comparative characterization of Hexanchus M₄ LDH. At the outset of these studies, this glycolytic enzyme, specific for the L-isomer of lactate and NAD⁺-dependent, appeared to be much the same in Hexanchus as in the corresponding tissues of other vertebrates. However, detailed characterization shows this LDH to have some unique traits, whose appearance not only reflects the biochemical uniqueness of the shark species itself, but also reflects the general intrinsic variability of the M₄ LDH of any vertebrate. The specific procedures described herein have been selected both in view of precedent and the emerging character of the enzyme under investigation; these fall into four general categories of characterization:

Section III: behavior through purification;

Section IV: general molecular size, shape, and subunit composition;

Section V: interactions with natural coenzyme (NADH) and analogues;

Section VI: chemical composition (to include analyses for coenzyme fragments).

Most of the experimental procedures applied to the isolated Hexanchus M₄ were previously (or concurrently) applied to known

samples of beef or chicken LDHs.

Recently, most isolations of LDHs have been based upon ion-exchange chromatography in conjunction with ammonium sulfate fractionations (2), and the Hexanchus M_4 isolation in Section III is basically in accord with this precedent. Using the dogfish M_4 purification of Pesce and others (21) as a guideline, the behavior of Hexanchus M_4 during purification is explored. Numerous deviations from dogfish purification behavior have been observed with the Hexanchus, and adaptations from other enzyme purifications have been incorporated, including some sodium sulfate procedures taken from a recent glutamate dehydrogenase purification (29). The design of the Hexanchus M_4 isolation embodies certain expectations about the isozyme composition of the skeletal muscle tissue. Initially, it was expected that electrophoretically distinct M_4 and H_4 components occurred in Hexanchus with tissue specificity comparable to the dogfish, and that the M_4 could be isolated from the skeletal muscle with the exclusion of all other isozymes. To verify these assumptions, and to ascertain the degree isozymic separation actually achieved in purification procedures, a purification of a small amount of the H_4 isozyme (from Hexanchus heart) is followed concurrently.

Some anomalous interactions of Hexanchus M_4 with coenzymes (or analogues) and the relationship of these interactions to enzyme structure (and even intrinsic chemical composition) are the major

concerns in Sections IV-VI. Section IV mostly is a critical examination of the degree of purification achieved in the isolation, but also explores the relationship of tetramer-monomer dissociation to the apparent degree of homogeneity, and provides some preliminary data on interactions with coenzymes. In Section V, extensions of the fluorescence-titration procedures of Anderson and Weber (30) are applied to explore the anomalous coenzyme binding of Hexanchus M_4 ; the limitation on binding capacity due to partial obstruction of active sites by coenzyme fragments is suggested. Section VI presents a comparative amino acid analysis, then provides information supporting the above coenzyme fragment hypothesis. Enzyme fractionation experiments involving chemical determinations for coenzyme fragments suggest that the obstructive moieties in otherwise purified enzyme samples probably represent a functional mode of coenzyme binding distinct from that usually monitored in the fluorescence titrations of active sites. Atypical modes of coenzyme binding have been reported for lobster muscle LDH (Kaloustian and others) (31), and for pig H_4 (Wieland and others) (32), and the Hexanchus results are compared to these precedents.

The final objective in summarizing these observations is to provide a useful resolution to the question of whether Hexanchus M_4 is simply a uniquely difficult enzyme to purify, or whether its anomalous coenzyme binding reflects an aspect of LDH-coenzyme interaction

more generally influential. If the latter statement is upheld, then Hexanchus M_4 provides a uniquely prominent example of a mode of structure stabilization by the coenzyme hitherto not extensively explored.

II. GENERAL METHODS AND MATERIALS

The following materials and procedures apply to enzyme preparations specifically (Section III), and to all other sections generally.

A. Enzyme Sources

Hexanchus skeletal muscle LDH preparations were taken from a flank muscle sample weighing 10 Kg. stored at -10°C in this laboratory. The sample was obtained early in 1972 through Dr. David Crawford of the Oregon State University Seafoods Laboratory, Astoria, Oregon. Specimens taken from a single individual six-gill shark weighing some 2000 lb. were vacuum sealed and quickly chilled to -30°C and shipped in frozen condition to this laboratory.

One Hexanchus heart weighing some 100 gm., from the same source, was also available for an enzyme source at the same time.

Beef Muscle LDH: Highly purified M_4 homotetramer prepared in July 1971 in this laboratory was stored under refrigeration. Crystals were harvested from ammonium sulfate suspension by centrifugation for experiments.

Beef Heart LDH: Two significantly different sources were employed in these studies, both of which were ammonium sulfate suspensions stored under refrigeration: (1) H_4 homotetramer purified August 1971 in this laboratory, and (2) samples from Miles-Seravac, lot 36-301, 79A.

Chicken Heart LDH: Highly purified H_4 homotetramer previously prepared in this laboratory was available as an ammonium sulfate suspension stored under refrigeration.

B. Enzyme Assay Methods

Generally, these were modifications of the LDH kinetic procedures of Schwert and others (33). The rates of utilization of pyridine nucleotide coenzyme in buffered substrate solutions were monitored spectrophotometrically after initiating reactions by adding micro-aliquots of enzyme solutions of interest. Assay mixtures were prepared by pipetting reagents and buffers into cuvettes from stock solutions, giving final volumes from 1.0 to 3.0 ml. and pH values from 7.00 to 9.00. For the forward reaction, initial oxidized coenzyme concentrations were near 10^{-4} M, and L(+)-lactate ranged from .01 to 0.1 M. For the reverse reaction, initial reduced coenzyme concentration was 1.27×10^{-4} M and pyruvate concentration was 0.1 M. Principal uses of kinetic procedures were (1) to monitor the progress of enzyme purifications, (2) to determine stability of enzyme samples through various experimental procedures, and (3) qualitative characterizations of Hexanchus LDHs based on comparison with beef isozymes H_4 and M_4 , involving coenzyme analogues.

C. Coenzymes and Other Reagents

The pyridine nucleotide coenzyme samples described below were used in procedures without further purification.

NAD^+ : For earlier portions of this work (Section III), Sigma grade III, lot 71C-7290 was used. For later procedures, Calbiochem #481911, lot 200476, labeled as the tetrahydrate of the free acid, was used. Solutions of these crystalline preparations in either distilled water or buffers near pH 7 generally showed good agreement between molarity calculated from weight concentration and that calculated from UV absorbance. However, solutions of these samples in 1M NaCN showed that only 90% of the molarity deduced from weight concentration was capable of cyanide adduct formation, based on absorbance at 325 nm. (34).

NADH : For earlier procedures (mostly Section III), P. -L. Biochemicals #2200, lot 22002 was used. For later procedures, Calbiochem #481913, lot 201044 was used. As with NAD^+ , solutions of these crystalline preparations in buffer (usually pH 9 Tris-HCl) showed good agreement between molarities calculated from measured weight concentration and UV absorbance at 260 nm., though only 91% of the weight-concentration molarity could be found in the characteristic absorbance at 340 nm. (34).

APD^+ : P. -L. Biochemicals #2700, lot 2708.

APDH: Reduced coenzyme analogue prepared from P. -L. Biochemicals #2700, crystallized as the barium salt. Stock solutions were prepared by dissolving the crystals in .01 M Tris sulfate, pH 8, and removing the BaSO_4 residue by centrifugation. Concentration was determined from absorbance at 363 nm. (34).

TAD⁺: P. -L. Biochemicals #4600, lot 200476

DL-Lactic Acid: Sigma grade DL-III, 86% total solids, containing approximately equal amounts of D and L isomers, with 20% as anhydride, lot 39B-1020. Stock solutions were prepared by heating the acid, dissolved with the estimated equivalent amount of NaOH or KOH in H_2O , at 90-100°C for 3 hours, then neutralizing and diluting the solution to the desired concentration (1.0-2.0 M).

Sodium Pyruvate: Sigma, dimer-free, 37B 2200

Bovine Serum Albumin: Pentex, crystalline

H_2O : Glass-distilled, deionized water was used to prepare all solutions and rinse containers for all procedures described in this work.

Ammonium Sulfate: Schwarz/Mann Ultra-Pure, lot W3375

Sodium Sulfate: Mallinckrodt analytical, anhydrous, lot VJK

Sodium Chloride: Mallinckrodt rgt. 7581

Sodium Phosphate (dibasic): B&A rgt. lot B107

Sodium Phosphate (monobasic): Baker rgt. 3818

Sodium Hydroxide: Mallinckrodt rgt. 7708

Sodium Carbonate: Mallinckrodt rgt. 7516

Sodium Bicarbonate: Mallinckrodt rgt. 7412

Potassium Hydroxide: Mallinckrodt rgt. WVBM

Potassium Phosphate (monobasic): Baker rgt. 3246, lot 41426, and Mallinckrodt rgt. Lot XEN

Potassium Phosphate (dibasic): Fisher rgt. P-288

Phosphoric Acid (syrup): Mallinckrodt rgt. lot ZXB, and B&A rgt. ACS code 1149

Sulfuric Acid: Dupont rgt. lot 31123 I

Nitric Acid: Dupont rgt. lot 91027 I

Hydrochloric Acid: Baker rgt. lot 504640

Tris: Sigma "Trizma Base," rgt. lot 63C-5310

EDTA: Matheson, Coleman, & Bell EX 545, practical

Sodium Cyanide: Mallinckrodt rgt. 7616 lot XCH

Guanidine Hydrochloride: Mann R. L. lot U-1484

Magnesium Sulfate: Mallinckrodt rgt. 6066

Mercaptoethanol: Calbiochem

D. Chromatography and Electrophoresis

CM-Cellulose used in Section III was Whatman CM22, recycled after previous use in this laboratory. Prior to use, this material was soaked at room temperature in 0.1 M NaOH, 0.1 M HCl, and 0.1 M Phosphate buffer, and then was rinsed with several volumes of the

final buffer solution. Soak treatments were one hour apiece; solutions were drained by aspiration in a Buchner funnel between treatments. Packing of column was accomplished by continuous flow of a dilute slurry over a period of 2-3 hours.

DEAE-cellulose used was Whatman DE52, whose preparation and handling was essentially the same as for the CM-cellulose, though Tris rather than phosphate was the final buffer ion generally used.

Gel filtration media were from Bio-Rad (polyacrylamide), prepared and handled according to the supplier's directions, except as specifically described in the text:

Bio Gel P-200, 100-200 mesh, cont. no. 79783

Bio Gel P-20, 50-150 mesh, cont. no. 2812

Bio Gel P-2, 50-150 mesh, cont. no. 56742

For affinity chromatography, 1,6-hexanediamine was bound to Sepharose 4B (Pharmacia, Uppsala, Sweden, lots 3861 and 5979) using the CNBr method of Cuatrecasas (35). The terminal amino groups of the derivatized Sepharose were then condensed with oxalate to form the oxamate derivative according to the method of O'Carra and Barry (36). Sources of additional reagents for this procedure were as follows:

Cyanogen Bromide: Apache Chemicals, 99%

Potassium Oxalate: B&A rgt. lot 2106

1,6-Hexanediamine: Matheson, Coleman, & Bell HX315

Water-soluble carbodiimide: 1-ethyl 3(3-dimethylaminopropyl)-carbodiimide hydrochloride; Calbiochem lot 300066

Aquacide II, lot 900091, Calbiochem; this was used to concentrate small, dilute enzyme samples after dilution during affinity chromatography.

The starch gel electrophoresis methods of Costello, Kaplan, and others (13) were applied to monitor the isozyme composition of preparations and to demonstrate the LDH isozyme system of Hexanchus. Zymograms were developed using the LDH activity stain derived from the procedure of Dewey and Conklin (37), whereby localizations of beef LDH isozymes on the gel slabs comparable to those presented by Markert (38) were visually observed. Additional materials useful in these procedures were:

Starch, hydrolyzed for electrophoresis, Sigma lot 109B-0170

Nitro Blue Tetrazolium: Calbiochem lot 000159

Phenazine Methosulfate: Sigma #P 9625

Additional chromatographic and electrophoretic procedures and materials are described in Sections IV, V, and VII, which involve disc gel electrophoresis, amino acid chromatography, and various applications of gel filtration methods.

E. Spectroscopy

The following spectrophotometers were used in their ordinary

modes of operation to measure absorbance or fluorescence, and were maintained in accord with the manufacturer's directions in conjunction with such use:

(1) Cary 15 UV-VIS spectrophotometer. For kinetics, 0-0.1 OD scale was used to follow coenzyme reduction, using glass cuvettes and the tungsten lamp down to 340 nm. Otherwise, (for scanning especially), the change from the tungsten to hydrogen lamp was made at 350 nm., and Pyrocell cuvettes were used.

(2) Beckman Model B single beam spectrometer, equipped with red-sensitive phototube, for simple colorimetric determinations.

(3) Varian Techtron UV-VIS spectrometer, model 635, with chart recorder. Use similar to Cary 15.

(4) Perkin-Elmer Fluorescence Spectrometer MPF-2A, equipped with Hitachi chart recorder QPD33 and Perkin-Elmer 150 Xenon Power Supply (high-pressure xenon lamp). 1 cm. square cuvettes were used, placed in the thermostatted 4-place cell block, generally using only the #1 position. Temperatures were maintained with a Forma circulating bath, and actual cuvette temperatures were determined with a YSI telethermometer (thermocouple probe). Sample volumes required were generally around 2 ml.

Cuvettes used for UV-VIS absorbance spectroscopy were sets from Pyrocell (Westwood, N. J.), codes S22-260 and S18-260, having path lengths 0.1, 0.2, 0.5, and 1.0 cm. All cuvettes exposed to

protein or coenzyme (or otherwise soiled) were cleaned by soaking in nitric acid between uses, and thoroughly rinsed with glass-distilled water (and drained) immediately before use. In accord with the precautions given by Anderson and Weber (30) the square cuvettes used in fluorescence titrations were silicone-treated after soaking in nitric acid, except as otherwise described in the text.

F. Centrifugation

For all routine sedimentations in preparing or handling enzyme samples, a Sorvall Superspeed RC2-B refrigerated centrifuge was used. Either the GSA or SS34 rotor was used, depending on sample sizes. Unless otherwise described in the text, separations were carried out near 0°C.

Use of the Beckman Model E Analytical Ultracentrifuge is described in Section IV.

G. Glassware and Miscellaneous

Volumetric measurement was routinely accomplished with graduated cylinders for volumes over 10 ml. and with various pipettes for volumes below 10 ml. Repeatability of concentration values resulting from volumetric dilutions was generally within 1-2% under these conditions. Micro-volume measurements and transfers were carried out with Hamilton syringes, Eppendorff pipettes, and the

smaller sizes of Bellco graduated serological pipettes. Between use periods, all Bellco pipettes were soaked in potassium dichromate- H_2SO_4 cleaning solution, Eppendorff pipette tips were soaked in 1% Na_2CO_3 , and Hamilton syringes were thoroughly rinsed in nitric acid. All containers, volumetric or otherwise, were rinsed thoroughly with distilled water after any cleaning procedures and were inspected for cleanliness before use.

While containers of plastic (usually Nalgene) were generally selected in preference to glass when contact with enzyme solutions was involved, no consistent difference in sample stabilities could be delineated under the experimental conditions generally maintained in this work. Enzyme solutions were generally stored at 0-4°C in glass or plastic containers during experiments, capped with Parafilm, unless as otherwise described in the text.

Large dialysis tubing used in enzyme isolations was from Union Carbide, ca. 1" diam., and was prepared by heating with dilute acetic acid and soaking with 10^{-3} M EDTA; prolonged storage of prepared tubing was at 0-4°C in EDTA solution. Smaller dialysis tubing used in later procedures was from Will Scientific, 5/8", 25225-226, prepared by soaking for 1 hour in 50% ethanol, then heating for 1 hour each in solutions of 1% NaHCO_3 and 10^{-3} M EDTA. Tubing was thoroughly rinsed with glass-distilled water immediately before use, and inspected for leakage.

Additional materials and methods, as well as further descriptions of some of those set forth above, are described in the sections where they are specifically of interest.

III. ISOLATION OF HEXANCHUS ENZYMES

A. Introduction

An isolation procedure for Hexanchus M_4 LDH was derived by selecting procedural steps from the purification formats for dogfish and halibut M_4 LDHs presented by Pesce and others (21). The efficacy of each step in separating unwanted components from the enzyme population, while simultaneously conserving most of the total activity, was tested with small batches, and modifications of the selected steps were made accordingly. The various steps were eventually integrated with the overall objective of reaching the limits of purification with a number of steps and final percent yield comparable to that obtained for large batches of LDH types previously characterized. The resulting purification format is described below and summarized in Table 1.

Isolation of Hexanchus H_4 LDH made use of some preliminary steps from the M_4 procedure, but being performed with very small batch sizes, provided the opportunity to use the affinity chromatography procedure of O'Carra and Barry (36) to complete purification. The H_4 isolation is set forth below as a subroutine of the M_4 procedure; the small amount of H_4 isozyme prepared was used to compare its own behavior to that of the Hexanchus M_4 isozyme in starch gel zymograms and in coenzyme analogue kinetic studies (39). The final

Table 1. Isolation of Hexanchus M₄.

	Volume (ml.)	Total Protein (mg.) ^{***}	Total Activity (units) ^{**}	Specific Activity	Yield (%)
1. Crude Extract	4000	46,400	4360	.094	100
2. Supernatant from 45% (NH ₄) ₂ SO ₄	4430	14,450	3785	.262	87
3. Precipitate from 70% (NH ₄) ₂ SO ₄	487	10,320	3210	.321	74
4. Filtrate from heat step (56.5°C for 15 min.)	800	2705	3000	1.11	69
5. Redissolved precipitate from saturated Na ₂ SO ₄	105 [*]	1627	2275	1.40	52
6. Dialysis and charcoal	211	1288	2115	1.64	49
7. DEAE chromatography, pH7 in .005 M Tris-HCl	937 [*]	609	1247	2.05	27
8. Recrystallization with 70% (NH ₄) ₂ SO ₄	61 [*]	490	1025	2.09	24

* The above data represent minimum yields obtained from one kilogram of shark muscle. By increasing the amount of material recovered at these steps, yields up to 48% were obtained.

** 10⁻³ units of enzyme catalyzes a change in OD₃₄₀ of 10⁻³/sec. at or around .01 OD and 10 seconds' reaction time. Assay medium: 0.02 M L-lactate, 1.5 x 10⁻⁴ M NAD⁺, 0.02 M Tris-HCl, pH 9, 25°C, Volume: 3.0 ml.

*** Protein concentration estimated using 1.55 OD₂₈₀ -.76 OD₂₆₀ (Warburg and Christian) (82).

objective in preparing Hexanchus H₄, therefore, was to establish the isozymic purity and identity of the final M₄ preparations, as well as to confirm and clarify the existence of an LDH isozyme system in Hexanchus comparable to that generally observed in other vertebrates (40).

B. Skeletal Muscle Enzyme (Hexanchus M₄)

(1) Extract

One kilogram of tissue was sliced in thin sections from a large slab of frozen flank muscle of six-gill shark; the sections then thawed at 4°C. By far, most of the mass was fibrous material or frozen fluids, so no attempt was made to divest the sample of the small amounts of fatty or connective tissue included. The slices were then minced with glass-distilled water at 4°C in a Waring Blendor, occasionally stopping the motor to free the blades of adhering fibers. The resulting slurry was strained through four layers of cheesecloth to give a milky, rose-tinted extract. The solid residues were then reprocessed in the blendor with more water and likewise strained until 4 liters total of water extracts were collected; overall, the solid residue was exposed to water for at least 2 hours during the extraction.

(2) Ammonium Sulfate Supernatant

To the 4 liters of water extract, solid $(\text{NH}_4)_2\text{SO}_4$ was added gradually over a 15-minute period with stirring at 4°C until 45% of room-temperature saturation was reached. The slurry was allowed to stand 2 hours at 4°C , and was then filtered through fluted Whatman #1 paper and glass wool overnight. The fatty, odorous precipitate was then discarded. Around 87% of the extract activity was consistently recovered in the filtrate.

(3) Ammonium Sulfate Precipitate

The filtrate was brought to 70% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ over a 15-minute period with stirring at 4°C . The resulting slurry stood for 2 hours at 4°C and was then centrifuged with the GSA rotor at 10,000 RPM, 15 minutes per batch, and all supernatant was eventually discarded. Precipitates were resuspended and pooled in about 400 ml of glass-distilled water at 4°C .; 85-92% of the filtrate activity was recovered in the resuspended precipitates.

(4) Heat Step

To every 470 ml. protein solution resulting from the above, 71 mg. solid NADH, 94 ml. 1.0 M potassium phosphate (equal molarities mono- and dibasic), and 141 ml. 1.0 M sodium or potassium

DL-lactate were added at room temperature. When the original volume of the protein solution differed from 470 ml., these amounts of additives were adjusted proportionately. To the resulting solution (700 ml. or more), 5 grams of anhydrous sodium sulfate were added for every 100 ml. solution, with stirring at room temperature. A water bath in excess of 10 liters at 56-57°C was prepared, and a U-tube was improvised by joining two 3 x 50 cm. glass columns together by a Tygon tube. The protein solution with its additives was placed in this U-tube, and by plunging the tube into the water bath, the solution was brought quickly to 56.5°C and was held at this temperature for 15 minutes. The mixture was occasionally stirred with a long glass rod as coagulation took place. After this, the tube was placed in an ice bath, holding the mixture near 0°C for 10 minutes. The pasty suspension was then filtered through fluted Whatman #1 paper until maximal drainage was accomplished (more than 1 hour). The voluminous precipitate was discarded, and in the filtrate, 93-97% of the heated mixture's previous activity was recovered. If either the sodium sulfate or NADH were not included in the mixture, 50% of the activity would be lost upon heating; if both components were omitted, nearly 100% loss would occur.

(5) Sodium Sulfate Precipitate

Soon after filtration, an additional 16 grams of Na_2SO_4 were

added for every 100 ml. protein solution. This was accomplished gradually, with stirring, at room temperature, while the solubility limit of the solute was closely approached. A milky white, or slightly yellow, suspension was produced, which was stirred at room temperature for at least 1 hour. The precipitate was then collected by centrifugation, using the GSA rotor at 25°C, 10,000 RPM, requiring 20-40 minutes for adequate packing of sediment. After carefully decanting, all supernatant was discarded.

(6) Dialysis and Charcoal Treatment

The Na₂SO₄ precipitate was collected and resuspended in about 100 ml. glass-distilled water, and dialysed against three changes of .005 M Tris-HCl buffer, pH 7.0 (800 ml. and 12 hours each at 4°C). To the turbid dialysate, 1 mg. activated charcoal (Norit A) was added for every ml. protein solution, and the resulting slurry was stirred for 2 hours at 4°C. The gelatinous, carbon-cruusted precipitate was discarded, and some 95% of the activity was retained in the supernatant.

(7) DEAE-Cellulose Chromatography

DEAE-cellulose was equilibrated with .005 M Tris-HCl buffer, pH 7.0, packed in a 3.0 x 50 cm. column and chilled to 4°C. About 200 ml. protein solution (charcoal-treated dialysate) was applied, and

this sample was eluted over a period of six hours with two liters of the same buffer without an ionic strength gradient and without collecting individual small fractions. About 60% of the applied activity was found in the first liter of eluate, and an additional 14% was in the second; the specific activities of these fractions were equal, and no increases in specific activity beyond this point were observed using any further purification procedures shown in Section III. Additional fractions could be eluted from the DEAE with buffers of greater ionic strength, but none of these constituted a significant proportion of the yield or possessed maximal specific activity. Here, the remaining yellow color was mostly lost.

(8) Ammonium Sulfate Suspension

Major fractions eluted from DEAE-cellulose were pooled and brought to 70% saturation with $(\text{NH}_4)_2\text{SO}_4$ and were allowed to settle at 4° C. Excess supernatant was discarded and material was harvested by centrifugation for further study.

C. Heart Muscle Enzyme (Hexanchus H₄)

(1) Extract

Eighteen grams of dark red muscle tissue was sliced from the ventricle of a frozen Hexanchus heart with a scalpel. This material

was placed in a mortar and pestle and ground with broken glass and distilled water at 4°C, and allowed to drain through four layers of cheesecloth. After about 2 hours' mincing and drainage, 37 ml. of extract eluted from the tissue mash.

(2) Heat Step

To the extract, NADH, Na₂SO₄, DL-lactate, and phosphate buffer were added in the same proportions as for the heat step of the M₄ procedure, giving a final volume of 53 ml. The sample was brought quickly to 56.5°C and held at this temperature for 15 minutes, after which, the sample was placed in an ice bath. The precipitate was removed by centrifugation with SS34 rotor, 15,000 RPM, for 30 minutes. During the heat step and removal of precipitate, the sample underwent a 7-fold increase in specific activity, while 100% of the initial activity was recovered in the supernatant. The sample was concentrated by dialysis against 80% sat. ammonium sulfate followed by centrifugation and removal of supernatant. The sediment was resuspended in a buffer of 0.5 M NaCl and 0.02 M phosphate, pH 6.8, in preparation for affinity chromatography at room temperature.

(3) Affinity Chromatography

Following the method of O'Carra and Barry (36), a column of Sepharose 4B-oxamate was packed and equilibrated with 0.5 M NaCl,

0.02 M sodium phosphate buffer, pH 6.8. To a column of dimensions 2 x 20 cm., 30 ml. 10^{-4} M APDH (in the same buffer) was applied, followed by 7 ml. protein sample (also 10^{-4} M with APDH with the same buffer). As the sample was washed in and eluted with APDH-buffer, fractions of 7 ml. each were collected for analysis. After 60 ml. of APDH-buffer had passed through, elution was concluded with the same buffer in the absence of APDH. The resulting separation of the purified H_4 -LDH from the contaminating proteins is shown in Figure 1; nearly 100% of the applied activity was recovered in the active fractions, and a 16-fold purification was obtained, resulting in a final specific activity comparable to that of beef H_4 preparations. Neglecting losses occurring in reconcentration and transfer of samples between steps, nearly 100% yield and an overall purification of 112-fold for the Hexanchus heart LDH was observed. Active fractions were pooled and reconcentrated with Aquacide (41) for further study.

D. Hexanchus LDH Isozyme System

Using starch gel electrophoresis (13), composite zymograms of the type presented by Markert (38) were produced, with which the electrophoretic mobilities of the Hexanchus heart and skeletal muscle LDHs were displayed along with those of the corresponding beef enzymes. On these gel slabs, samples of Hexanchus M_4 from the final step of the above isolation procedure showed localizations

Figure 1. Affinity Chromatography of Hexanchus H₄ LDH. Method of O'Carra and Barry; preliminary sample chromatogram. Buffer: pH 6.8 phosphate, .02 M, NaCl, 0.5 M.

- A. Unwanted protein components, mg. /ml.
(Lowry method) (54).
- B. Coenzyme concentration, APDH, as OD₃₇₅^{1 cm.}
- C. LDH activity, units of Table 1 x 10. Dashed line ends with 100% recovery of activity.

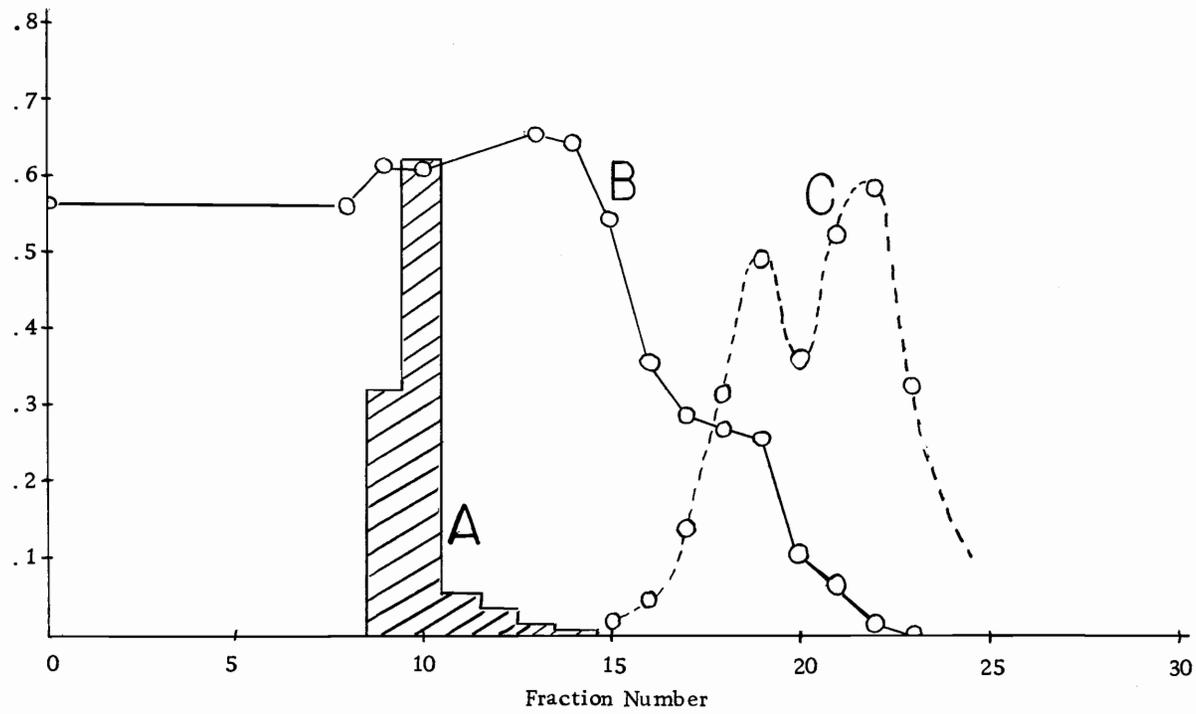


Figure 1.

of activity comparable to those of the beef M_4 of known purity (slight displacement toward anode under these conditions). Under the same conditions, the Hexanchus H_4 from the final step of its procedure showed a single localization of activity falling short of that for beef H_4 , equal (or nearly so) to the H_3M isozyme of beef. Since only single localizations of activity for both M_4 and H_4 Hexanchus preparations were found (even when zymograms were purposely overstained), these preparations were judged to be homogeneous with respect to isozyme composition. The Hexanchus M_4 isozyme thus appeared to be clearly distinguished from the H_4 isozyme, and the two components gave evidence of an isozyme system in Hexanchus comparable to that found in beef.

Zymograms produced with impure preparations of Hexanchus LDHs showed isozymes having mobility intermediate between the pure M_4 and H_4 localizations. Like the well-known hybrid isozymes, these localizations (three) were confined to the space between the extremes shown with the pure M_4 and H_4 preparations. But unlike the typical distribution of LDH hybrids (38), spot intensities and locations were not analyzable as unbiased groupings of H and M monomers into tetramers from various sizes of H and M populations. As examples: (1) all stages of purification of Hexanchus M_4 previous to heat treatment showed an isozyme which had a relative mobility (r. m.) of +0.44, whose intensity was about 10% of that for the major M_4

band (r. m. zero), occurring in the complete absence of any bands with r. m. values near +0.25 or +0.75. Upon heat treatment, all evidence of this isozyme in the preparation vanished, though 93-97% of the activity was apparently conserved (discussed further in part F).

(2) Despite nearly 100% retention of activity through the affinity chromatography of Hexanchus H_4 (r. m. = +1.0), bands of r. m. +0, +0.78, and +0.56 were removed; even though the sum of the magnitudes of these contaminating isozymes was much in excess of the H_4 before their removal, the total activity of the preparation was conserved. Identifying bands with r. m. 's of +0.78 and +0.56 as hybrids H_3M and H_2M_2 was uncertain, since their placement deviated significantly from the r. m. 's +0.75 and +0.50 expected for hybrids, and no bands near r. m. +0.25 were seen, despite having larger magnitudes of r. m. +0.56 and +0.0 adjacent to the r. m. +0.25 neighborhood.

The anomalous isozyme compositions described above resemble the results of such experiments with various coral reef fish presented by Rosenberg (42), who discussed anomalous preponderances of LDH hybrids in conjunction with epigenetic regulation. These results are quite different from the usual outcome of LDH hybridization experiments (38) in which random recombination of H and M subunits occurs, and from the isozyme distribution phenomena described by Leberz (43) in which isozymes are proportioned by intercellular differences in synthesis rates of parent monomers. Generally speaking, impure

LDH preparations of Hexanchus give electrophoresis zymograms in which isozymes localized between the parent homotetramers are anomalous in proportion and position compared to those of mammals.

Additional support was provided for the distinction of the M_4 preparation from the heart muscle extracts by use of coenzyme analogue kinetic studies after Ciotti, Kaplan, and others (39). Table 2 shows activities of Hexanchus heart, liver, and skeletal muscle preparations with analogues APD^+ and TAD^+ relative to activity with NAD^+ under the same assay conditions. Corresponding activity ratios for beef M_4 and H_4 are set forth for comparison. Approximately the same alteration in magnitude of activity ratios occurs in going from the Hexanchus H_4 to the M_4 as in going from the H_4 of beef to its M_4 complement. Yet also, as an interspecies variation, activity ratios of APD^+ to either TAD^+ or NAD^+ are higher in Hexanchus than in beef, whether either the heart or skeletal muscle enzyme is compared to its counterpart in the other species. A very similar result for interspecies comparison has been set forth for dogfish LDH (44).

In summary, Hexanchus M_4 LDH, of central importance in this writing, can be readily distinguished from its H_4 complement by both coenzyme analogue kinetics and electrophoresis. The observed distinction is closely paralleled in comparing beef M_4 to H_4 , despite considerable interspecies variation which has been described. By the isolation procedures portrayed (Table 1), the Hexanchus M_4 may

Table 2. Hexanchus isozyme system: coenzyme analogue kinetics (41).

Sample	NAD ⁺	APD ⁺	TAD ⁺
A. Specific activity relative to assay with NAD ⁺ ; Coenzyme concentrations 1.5×10^{-4} M			
Beef M ₄	1.0	.041	.300
Beef H ₄	1.0	.016	.560
<u>Hexanchus</u> M ₄	1.0	.164	.204
<u>Hexanchus</u> heart (extract)	1.0	.075	.450
B. Coenzyme concentrations 1.0×10^{-4} M			
Beef M ₄	1.0	.054	.334
Beef H ₄	1.0	.020	.528
<u>Hexanchus</u> M ₄	1.0	.235	.274
<u>Hexanchus</u> liver (extract)	1.0	.180	.290
C. Relative activities with NAD ⁺ :			
Beef M ₄		100	
Beef H ₄		50	
<u>Hexanchus</u> M ₄		79	

be prepared with the complete exclusion of H_4 and all intermediary isozymes.

E. Hexanchus M_4 LDH: Criteria of Purity

The final step of the isolation of Hexanchus M_4 produced a population of protein molecules whose homogeneity compared very favorably to that of beef M_4 samples of known purity by several criteria. Electrophoresis on cellulose acetate membranes, using the same buffers as with starch gel procedure and Ponceau S stain (with a dialysed-protein concentration estimated at 10 mg/ml.), gave evidence of a single component with very little mobility. The distribution produced with disc gel electrophoresis with Amido Black stain compared very favorably with that of beef M_4 under the same conditions, and comparisons of gel filtration elution profiles showed comparable homogeneity. Ultracentrifugation also upheld the designation of homogeneity for these preparations and indicated sedimentation properties compatible with other LDHs. The foregoing criteria of purity are given more detailed examination in Section IV.

Despite giving the above evidence of being homogeneous relative to the beef M_4 samples, Hexanchus M_4 displayed other results showing anomalous limits to the apparent degree of purification which could be attained by the methods described in this section. While a highly purified LDH would hypothetically be expected to bind four

molecules of reduced coenzyme per 140,000-dalton tetramer if an adequate excess of coenzyme is present in solution (45) binding stoichiometries for Hexanchus M_4 preparations fell consistently below 3.0 per tetramer despite use of a variety of conditions and methods of determination. Further attempts at purification did not increase the binding ratio much, and the specific activity was not increased to give a purification beyond 23-fold (in Section III). The preparations ranging from 24 to 48% yield from the extract were typical of most LDH isolations for similar batch sizes (21), while Hexanchus M_4 showed a specific activity consistently midway between those of beef M_4 and H_4 , within a few percent of the geometric mean of the two when all three enzymes were assayed under the same conditions. Thus, despite the apparent limit on the stoichiometry of coenzyme binding, the final specific activity was not inordinately low for a highly purified M_4 LDH. In summary, the observed limits in the stoichiometry of binding appeared to be a highly stable intrinsic characteristic of the enzymes populations isolated, in contrast to certain other LDHs already well-known. The characterization of Hexanchus M_4 continued with special consideration of this discrepancy.

F. Further Attempts at Purification

To further illustrate the limit of purification attainable by the

methods described, and to provide additional information about this molecular population suggestive of causes of this limit, some further purification attempts and their results may be considered.

(1) CM-cellulose Chromatography

The resuspended ammonium sulfate precipitate from step 3 was prepared for fractionation by dialysis against three changes of .005 M pH 6.5 potassium phosphate buffer, 10 volumes each, over a 72-hour period at 4°C. A 3 x 50 cm. column was packed and equilibrated with the same buffer; 200 ml. dialysed enzyme sample was applied and washed in, and 800 ml. of elutions were collected, as four fractions of 200 ml. each. Of the four fractions collected, the second and third contained 50% of the applied activity, and showed a specific activity 50-100% in excess of the applied sample. The fourth fraction showed only negligible additional activity, indicating the cessation of elution of unbound protein. The remainder of the sample was eluted with a linear ionic strength gradient, 0-0.1 M NaCl, while individual small fractions were collected. The resulting elution profile is shown in Figure 2.

In the ionic strength gradient elution, the pooled active fractions amounted to only 17% of the original sample activity. Repetition of this procedure at pH 6.0 with application of a smaller sample gave basically the same result, except that percent yields of corresponding

Figure 2. CM-Cellulose Chromatography of Ammonium Sulfate Fraction from Step 3 of Isolation format (Table 1). Broken line, activity, same units and conditions as Table 1; Solid line, protein concentration, as $OD_{280}^{1\text{cm}}$. Buffer: .01 M potassium phosphate, pH 6.5; ionic strength gradient 0-0.1 M NaCl.

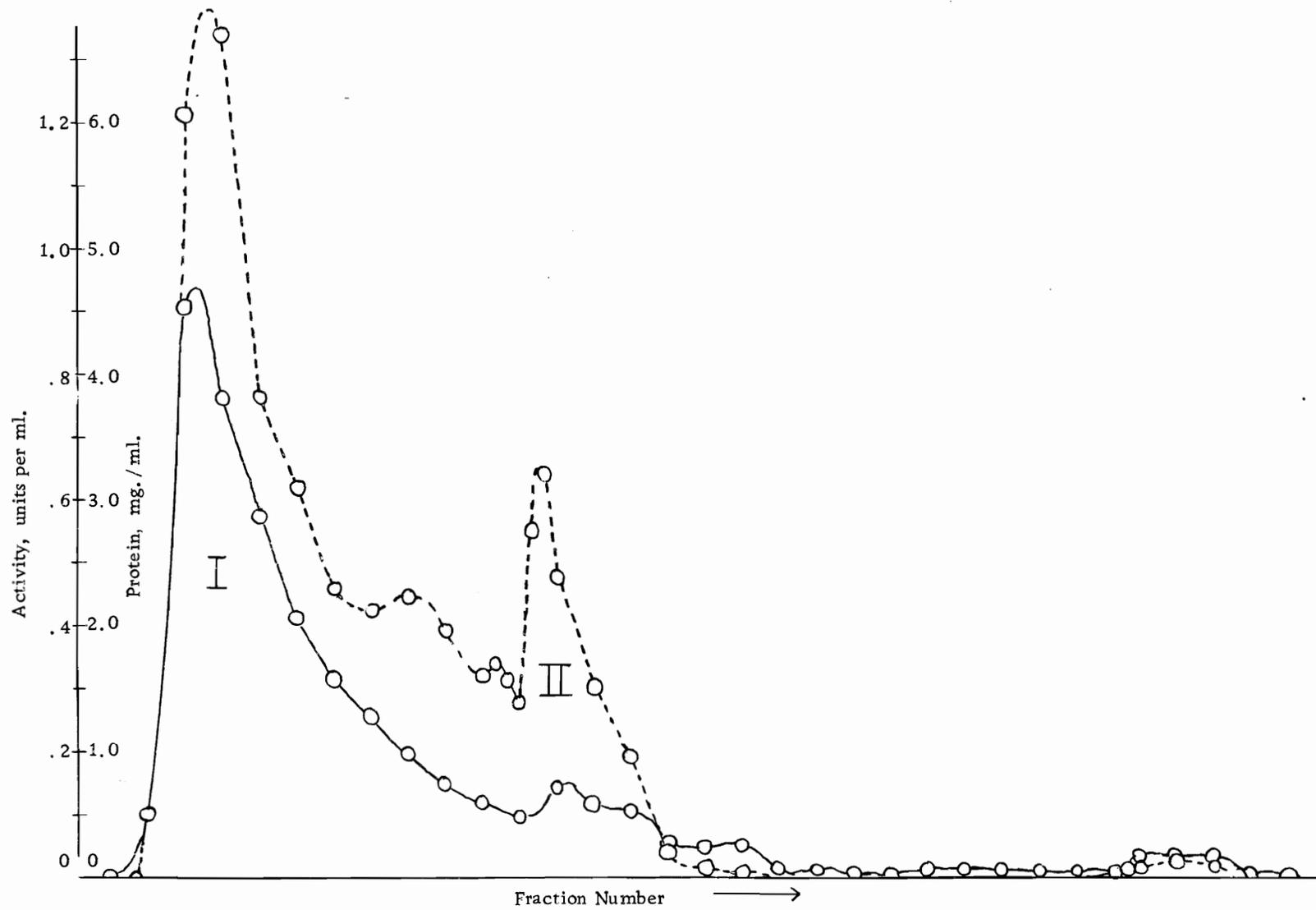


Figure 2.

active fractions were somewhat lower than with pH 6.5. Additional washings from the column with 0.5 M NaCl returned no further contributions to total activity of any size, and the average specific activity of bound protein requiring ionic strength gradient elution was very low compared to the average specific activity of the initial sample washings. Thus, binding to the column appeared to represent both activity loss and distribution into multiple fractions, and that sample integrity and the combination of percent yield and specific activity gain were best found in the initial sample washings, that is, with less interaction with the column.

Neither the washings or gradient elution fractions were homogeneous with respect to isozyme composition when tested with starch gel electrophoresis. Though all fractions tested contained both the M_4 spot and the anomalous isozyme already described (this section, part D), peak II of the gradient elution exhibited a larger proportion of the anomalous isozyme than the rest of the fractions. The removal of similar isozyme contaminants from dogfish M_4 by DEAE-cellulose was reported (21); with Hexanchus, however, the CMC separation of the isozyme from the main population was not very efficient. In contrast, the heat step, which was adapted in preference to CMC fractionation at this stage of the purification, removed all evidence of isozymic contamination while retaining 93-97% of the total batch activity.

The use of CMC at this stage of the purification corresponded to the use of DEAE in the dogfish M_4 isolation procedure of Pesce and others (21). The response of the Hexanchus samples to ion-exchange media was very different from that of dogfish, however, offering considerably less efficient steps of purification. The CMC fractions most enhanced in specific activity, the initial washings which contained only 50% of the applied activity to begin with, responded inefficiently to further ammonium sulfate fractionation (by methods similar to steps after DEAE treatment in the dogfish format): for very slight increases in specific activity, large proportions of total activity were lost. The heat step, a modification of the halibut M_4 heat step of Pesce's group (21) gave 3.5-fold purification with at least 93% activity recovery while using a much simpler and less time-consuming procedure; as a replacement for the CMC procedure, the heat step appeared to be a most desirable adaptation. When using the heat step, additional fractionations gave considerable further specific activity increase, in contrast to ammonium sulfate fractionation of CMC samples.

(2) DEAE-cellulose Chromatography

In addition to the DEAE fractionation already described as part of the isolation procedure, DEAE-cellulose was used to seek further purification of the final samples. The procedures were basically the

same as before, except that small samples were applied and individual small fractions were collected to examine distribution and resolution. Whether pH 7.2 .005 M Tris or .01 M phosphate was used, elution without gradient at room temperature gave a single component centered on the void volume of the column. Distributions of protein concentration gave sharp, but slightly skewed, peaks; the trailing edges of the distributions appeared to represent minimal interaction with the column. Major fractions had specific activities equal to that of the applied sample, and samples in or near the trailing edges showed losses in specific activity. Similar to the DEAE step in the isolation format, 70-80% of the applied activity was recovered, the remainder strongly bound to the column, partially deactivated and dispersed into multiple fractions. This step offered some evidence of homogeneity for the sample, not providing further purification, but instead, suggesting that the limits of purification were already reached. Further exposure to ion-exchange media would only cause further losses of activity.

(3) Ammonium Sulfate Fractionation

Fractional crystallizations of the type described in the dogfish M_4 isolation procedure (21) were attempted with Hexanchus samples both from the heat step supernatant and from the final stage of purification. As with DEAE-cellulose, major fractions showed no increases

in specific activity over the starting batches, and minor fractions showed substantial losses in specific activity. These results showed substantial losses in total activity. These results were very different from the response of dogfish M_4 preparations to crystallization attempts with ammonium sulfate, suggesting resistance to further purification.

A variation of the ordinary ammonium sulfate fractionations used in LDH isolation was attempted, whereby a precipitate formed in 70% sat. ammonium sulfate was extracted with a series of ammonium sulfate washings of successively lower concentration. When the procedure was executed with the precipitated dialysed heat step supernatant (from step 6), the major fractions again did not surpass the previously established limit of purification, nor was the orange-yellow colored material removed from the major fractions. As before, minor fractions at the extremes of solubility showed losses of specific activity and represented a substantial loss in total batch activity. The major fractions were again pooled and reconcentrated in 60% sat. ammonium sulfate, and another series of extractions yielded the same result, further exhibiting the same resistance to purification.

(4) Miscellaneous

Treatment of Hexanchus M_4 samples from steps 6 and 8 by

various other purification methods gave results similar to ammonium sulfate fractionation. Heat treatments of these samples under conditions similar to the heat step in the purification format gave massive losses of activity, and no variations in proportions of additives tested produced improvements (although low enzyme concentration was not tested). Partial denaturation by prolonged dialysis at room temperature (pH 8 Tris, .005 M), affinity chromatography with NADH (this section, part C-3), and gel filtration (Section IV), all produced major fractions with specific activity equal to or less than the maximum value reached in the purification format, and the recrystallization of these major fractions with ammonium sulfate only further demonstrated the same limit. Inclusion of 10^{-3} M 2-mercaptoethanol or 10^{-4} M EDTA, or both, in sample buffers caused no changes in specific activities of samples or the results of ammonium sulfate fractionations. Though no further possibilities for increasing the specific activity of the preparations were revealed in these tests, much about the stability of the purified samples through storage, dialysis, and reconcentration with ammonium sulfate could be deduced.

(5) Exposure to Coenzyme (NADH)

Three purification procedures involving addition of NADH to Hexanchus M_4 samples were of interest in probing the limits of

Figure 3. Hexanchus M₄ + NADH: Absorbance spectra of 30-fold dilutions of ammonium sulfate supernatant at four stages of fractionation:

- A. Before ammonium sulfate precipitate, pH 8.3
- B. After ammonium sulfate addition to 47% saturation concentration
- C. 55% saturation
- D. 63% saturation

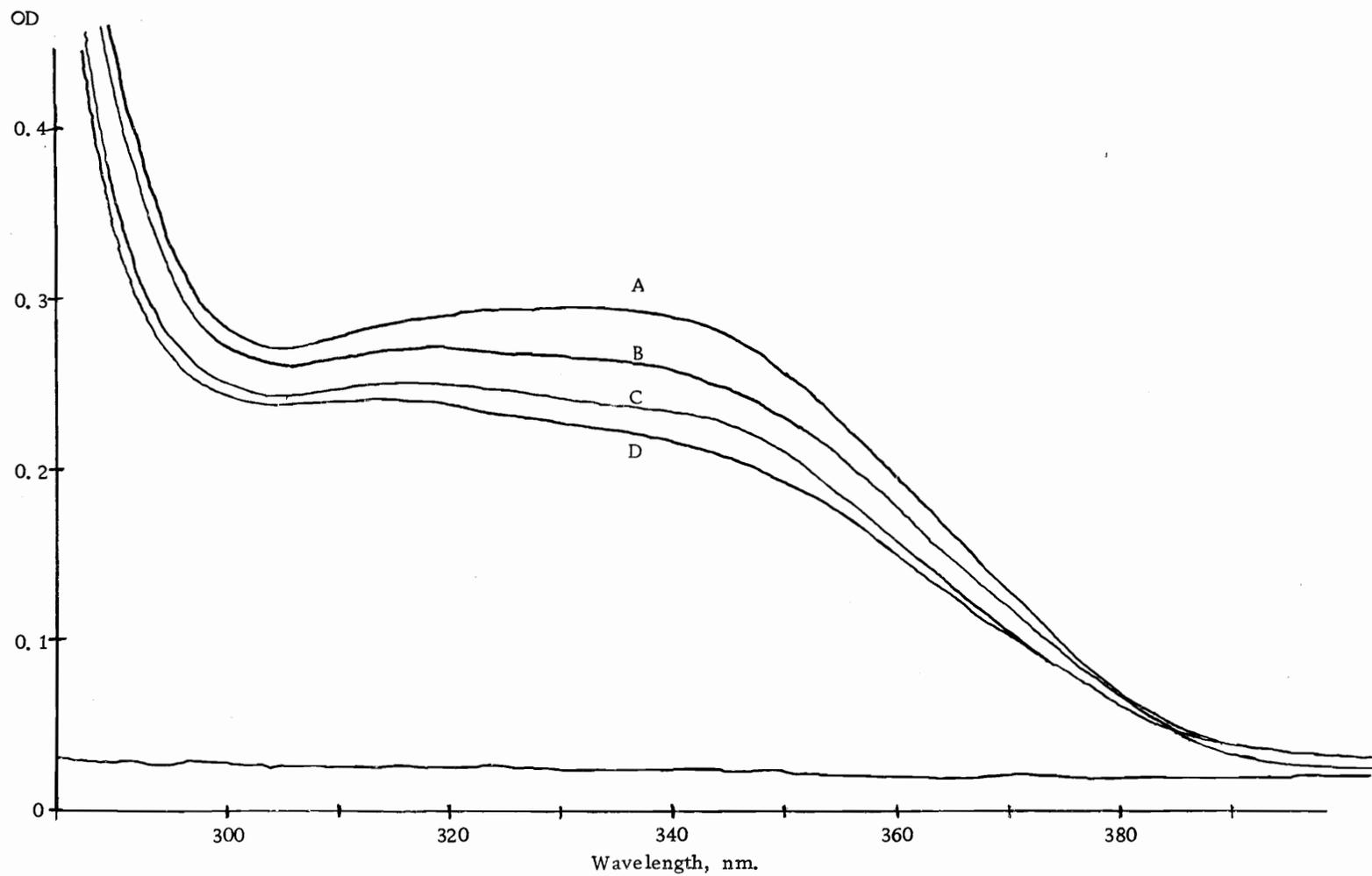


Figure 3.

purification. Two of these, the affinity chromatography and the coenzyme-stabilized heat step, have already been described. In addition, crystallization with ammonium sulfate in the presence of NADH was attempted. Though for increasing specific activity, this procedure was no more effective than the other ammonium sulfate methods described above, nevertheless the nature of the interaction of NADH and the enzyme during fractionation was of interest.

Beginning with 5 ml. of sample eluted from DEAE-cellulose (major fraction, $OD_{270}^{1\text{ cm.}} = 7.27$), 2 ml. potassium DL-lactate (1 M), .142 grams Na_2HPO_4 , and 8.3 mg. NADH, these materials were mixed to give a solution of pH 8.3. This solution was brought to 47% sat. with ammonium sulfate and allowed to crystallize overnight at $4^{\circ}C$; the precipitate was harvested by centrifugation. By addition of ammonium sulfate, the pH was reduced to 6.0. From the remaining supernatant, two more precipitates were similarly harvested by increasing the ammonium sulfate first to 55%, then 63% of saturating concentration. The harvested precipitates were each resuspended in 3 ml. 0.1 M Tris-HCl, pH 7.0, and 0.1 ml. aliquots of the supernatant were withdrawn at each of the four stages of precipitation and diluted to 3 ml. in the same buffer. Spectra of the resulting sample solutions from 240-450 nm. were recorded, and their enzymic activity was measured.

Figure 3 shows the spectra in the neighborhood of 340 nm. of

the four stages of ammonium sulfate fractionation described above, in which the supernatant is diluted 30-fold. As NADH is removed by coprecipitation with the enzyme, a new absorbance peak in the neighborhood of 313 nm. emerges, whose relative magnitude is similar to the loss in absorbance at 340 nm. In contrast, the re-suspended precipitates showed absorbance spectra devoid of such peaks at 313 nm.; their spectra were quite close to being simple sums of the spectra of NADH and LDH. On the average, 4.0 moles of NADH coprecipitated with every mole LDH, the first fraction (minor) with 7.0, the second (major) with 4.0, and the third (minor, much lower specific activity) with 3.0. Because extraneous NADH may be trapped in precipitates, this result was not considered a determination of specific coenzyme binding stoichiometry. The emergence of an altered, non-binding, UV-absorbant component in conjunction with the NADH-LDH coprecipitation was suggestive of some disruption in the covalent structures of LDH and NADH under the experimental conditions.

IV. MOLECULAR WEIGHT AND HOMOGENEITY

A. Introduction

Hexanchus M_4 LDH may be shown to have a molecular weight comparable to the accepted value for most other LDHs of vertebrates (21) (140,000 daltons) and accordingly appears to be a tetramer dissociable into monomers of about 35,000 daltons under certain conditions of interest. With the electrophoresis, ultracentrifuge, and gel filtration methods used, the Hexanchus M_4 preparations from the final stage of the purification format (Table 1) showed behavioral profiles indicating a degree of homogeneity similar to that of the beef M_4 sample, to a first approximation. As stated previously (Section III, part E), several criteria of purity may indicate a homogeneity-molecular size profile comparable to that of the previously characterized beef M_4 sample; however, a detailed examination of sample distributions produced with gel filtration and disc-gel electrophoresis showed some finite departures from the above generalizations. Both the Hexanchus and beef LDH showed latent microheterogeneity, which is here explored.

B. Materials and Methods

(1) Disc-Gel Electrophoresis

The objectives in the design of disc-gel electrophoresis

experiments included evaluation of the homogeneity and electrophoretic behavior of the isolated Hexanchus M_4 relative to previously characterized beef M_4 , verifications of its ability to dissociate into subunits (7), evaluation of influence of reduced coenzyme (NADH) on electrophoretic behavior, and analysis of activity-protein concentration relationship in disc electrophoresis distributions.

Crystals of beef M_4 and Hexanchus M_4 were harvested from their ammonium sulfate suspensions, each sample redissolved in 2.5 ml. glass distilled water, and each dialysed in its own container against three changes of buffer, 100 ml. apiece. The dialysis buffer was .01 M Tris-HCl, room temperature pH 7.6 (Tris from Canalco, no. 210, FW 121.14). Dialysis was carried out at 4° C, the first two changes for 3 hours apiece, and the third for 16 hours. Concentrations of enzyme in the resulting stock solutions were estimated from the following absorbance data:

	OD_{280}	OD_{260}	$\text{OD}_{340}^{1/}$	mg/ml. x 50 $^{2/}$
Beef	.149	.084	.008	5.45
<u>Hex.</u>	.221	.135	.008	8.26

$^{1/}$ -value subtracted from OD_{280} as turbidity correction.

$^{2/}$ -absorbance data taken with 50-fold dilution of samples in dialysis buffer; concentrations estimated with extinction coefficient $E_{1\text{ cm}}^{1\%} = 12.9$ at 280 nm (11).

For the above data, the Hexanchus sample was centrifuged at 5000 RPM, 10 min. with SS34 rotor, immediately before reading, to remove a small amount of precipitate formed during dialysis. A small amount of opalescence remained with the Hexanchus sample despite further attempts to remove it by centrifugation. Solutions were stored at 4°C until ready for further use.

For preparation of electrophoresis samples, the following sample buffer was prepared:

Sucrose (Canalco, #165773),	2.0 gm.
0.5 M Tris-HCl, pH 6.8,	1.0 ml.
0.1% Bromophenol Blue (Canalco, #165237),	0.2 ml.
Water, glass-distilled, to	5.0 ml.

Using this buffer, the following samples were prepared:

(a) Beef M_4 , 5.45 mg. /ml. ,	1.0 ml.
Sample buffer,	1.0 ml.
Final concentration, 2.73 mg. /ml.	
(b) Same as above, plus 1.0 mg. NADH dissolved.	
(c) <u>Hexanchus</u> M_4 , 8.26 mg. /ml. ,	0.5 ml.
Water, glass-distilled,	0.5 ml.
Sample buffer,	1.0 ml.
Final concentration, 2.07 mg. /ml.	
(d) Same as above, plus 1.0 mg. NADH dissolved.	

As a test of sample stabilities, and to enhance possible reactions with

NADH, the above samples were heated in a water bath at 45°C for 10 min., and placed in an ice bath immediately thereafter. After heat treatment (45°) the Hexanchus samples increased slightly in opalescence, the NADH sample less so than the other. In comparison, the beef samples remained perfectly clear. The specific activity of the samples was not altered by the 45° incubation; after the treatment plus two weeks storage at 4°C, the specific activities held constant within experimental error, equal to those of samples freshly harvested from ammonium sulfate. Prior to the electrophoretic separations described here, 3.9 mg. of crystalline BSA was added to sample (b) to display the relative mobilities of the major fractions of NADH, BSA, and beef M₄ LDH in the polyacrylamide media.

Separations were carried out in gel tubes 7.5 cm. in length, I. D. .488 cm. (tube volumes determined gravimetrically with H₂O). Between runs, tubes were cleaned by boiling in nitric acid, rinsing, soaking in "Fotoflow" (Kodak, 0.1% soln.), rinsing, and oven drying. Polyacrylamide gels of 5.0 and 7.5% were prepared using the following reagents from Canalco (Rockville, MD 20852), basically in accord with the procedures of Davis and others (46). Acrylamide, #165055 (99%); BIS, #165545; TEMED, #165818; and ammonium persulfate, #165135. The buffer of the 7.5% gel was Tris-HCl, pH 8.8, final concentration in gel mixture, .372 M; the 5.0% gel had phosphate adjusted to pH 6.8 with solid Tris, final concentration 0.1 M

phosphate. Samples were placed on both 5.0% and 7.5% gels without the use of any "stacking gel." The 7.5% samples were set up by first pipetting 1.2 ml. gel solution into the tubes, allowing them to polymerize in the preparation rack. After discarding excess fluid, the samples were placed on top of the gel columns, to be in direct contact with both the separating gel and the running buffer. The 5.0% samples were set up by first pipetting 0.7 ml. gel solution into the tubes, allowing polymerization and discarding excess fluid, then pipetting samples (0.055-.06 ml.) onto the gel columns. Then, taking care not to disturb the sample zones, 0.5 ml. additional gel solution was placed above the sample in each tube to allow samples to be simultaneously tested for cationic migrants. Anionic migration was of primary importance in both types of gel, however. Samples were uniformly 0.05 ml. in volume for the 7.5% gel runs, and contained no components other than those already described. The 5.0% runs each had .005 ml. 1% 2-mercaptoethanol added to the samples after placement on the gels; the mercaptoethanol was expected to cause considerable modification in observable localizations, likely to "equalize" the behavior of the two samples (ref. 38, p. 121).

Separations were carried out with a Buchler Polyanalyst disc electrophoresis chamber, using up to 12 of its 18 positions at once. Reservoir buffers were pH 6.8 Tris, 0.1 M phosphate for the 5.0% gel, and 2% solution Canalco #165875 Tris-glycine buffer, pH 8.3 for

the 7.5% gel. During operation, the apparatus was cooled with circulating tap water which held a very nearly constant temperature of 17.2°C during the period in which these separations were performed. A Bio-Rad Model 400 power supply was employed, operated in the constant current mode, generally at 2.5 ma. per tube. The voltage, which varied with time during the runs, was recorded at various times, and the average potential applicable to each run was estimated by graphic analysis.

Tubes were removed from the apparatus at various intervals, and the current setting on the power supply was adjusted accordingly to maintain the same current density in the remaining tubes. The removed tubes were kept at 0-4°C until all samples had been removed from the apparatus, at which time all gels were removed from their tubes with a blunt syringe and distilled water, as described by Davis (46). Gels stained for activity were placed in small test tubes, in which they were incubated with the following solution (modification of the activity stain of Dewey and Conklin used with starch gel zymograms (37).

NAD ⁺ ,	50 mg.
NBT,	30 mg.
PMS,	2 mg.
2.0 M Sodium DL-lactate,	5 ml.
pH 9.0 Tris-HCl, .022 M,	50 ml.

Each gel was exposed to 5.0 ml. of the above for 10 min. at 37°C before washing the staining solution away and fixing the gels in 7% acetic acid. Activity-stained gels did not change in distribution or intensity of bands upon immersion into 7% acetic acid, as judged by visual inspection.

Protein distribution in gels was determined by Amido Black staining (47), in which gels were soaked in 1% Amido Black 10B (Canalco #165113) in 7% acetic acid for 1 hour before electrolytic destaining. Destaining was accomplished with the Buchler Polyanalyst equipped with destaining tubes, using 7% acetic acid as solvent-electrolyte and operating at 200 volts. Under these conditions, the progress of migration of excess stain out of the gels was easily observed. (Prior to destaining, some excess stain was removed by washing with 7% acetic acid). Gels stained for activity, for protein, or for both, were stored in small test tubes under 7% acetic acid for further inspection. (Acetic acid, glacial, from B&A, code 1019).

After visual inspection, comparison, and gross physical measurement of some band positions on gels, certain gels were selected for graphic representation. For this purpose, a Beckman DU model 2400 spectrometer equipped with a Gilford gel scanner (model 2520) and OD converter was used, recording scans with a Moseley Autograf model 2D-2AM X-Y recorder. Where appropriate, various scans were superimposed, as in Figures 4-9. For scanning,

gels were placed in a 1 x 10 cm. rectangular cuvette with some 7% acetic acid, and were scanned at 540 and/or 600 nm. through their entire length. No evidence of distortion of gels could be found by comparing distance measurements before removal from tubes and after fixation in 7% acetic acid.

(2) Gel Filtration

Columns of Bio-Gel P200 were used to determine the average molecular size and degree of homogeneity of various Hexanchus M₄ preparations. These values were sought for the isolated Hexanchus samples relative to the known beef M₄ sample. Also, the relationship of activity (both kinetic and coenzyme binding capacity) to protein concentration in molecular sieve distributions was of interest both as a probe to the limits of purification and as a measure of latent micro-heterogeneity.

Bio-Gel P200 was packed into columns having settled bed dimensions of 2.0 x 33.5 cm. and 5.73 x 62.0 cm. (cylindrical). The material was prepared and handled according to the published specifications from Bio-Rad, involving hydration in 0.1 M potassium phosphate buffer and aspiration before packing. Elution of the columns was accomplished at 4°C with the same buffer with a flow rate of 1.42 ml. /hr. per cm.² cross section for the shorter column and about 0.8 ml. /hr. per cm.² for the larger column. Fractions of

about 3.5 ml. each were collected to define the distribution, and for the larger column, fractions of about 15 ml. were collected. To analyze the distribution, exact elution volumes were determined by weighing individual sample tubes before and after collection of fractions. The cumulative elution volume appears in the abscissa in Figures 10 and 11, and was the basis of graphic analysis.

Protein concentration of individual fractions was deduced from $OD_{280}^{1\text{ cm.}}$ and from intrinsic protein fluorescence (48) at the extremes of low concentration in the edges of distributions. LDH activity was measured kinetically under the conditions shown for the isolation format (Table 1), and coenzyme binding capacity was measured by the fluorescence enhancement of analogue APDH (49). For the APDH-binding assay, 2.0-2.5 ml. of APDH solution in 0.1 M potassium oxalate, 0.1 M potassium phosphate buffer, pH 7, was kept in the fluorometer cuvette at 15°C; small aliquots of samples to be tested were added to this solution, which contained a vast excess of APDH over enzyme. With APDH around 10^{-4} M, the fluorescence of the unbound ligand was nearly insensitive to dilution, which facilitated simple multiple calculations of $F/F_0 - 1$, where F_0 is the fluorescence of the unbound ligand, and F is the total fluorescence of the sample and ligand in solution. The fraction of total ligand bound was assumed to be $(F/F_0 - 1)/230.5$, where 230.5 is the enhancement factor applicable to binding of 1 mole of APDH to 1 mole (140,000 gm./mole) of

Hexanchus M₄ under the given conditions, as discussed in Section V, part C. The distributions of protein concentration, activity, and binding capacity thus determined were recorded and analyzed as a function of elution volume by graphic methods (Figures 10, 11).

The fractionations here described apply to protein solutions in 0.1 M potassium phosphate applied to the gel columns in volumes around 1% of the settled bed volume.

(3) Ultracentrifuge

The objectives of sedimentation studies were to provide an absolute measurement of molecular size readily comparable to published values for other LDHs, to provide additional information on the degree of homogeneity of the isolated Hexanchus M₄ (final stage of purification format), and to provide some representation of the concentration-dependency and repeatability of the S-values.

Sedimentation velocity runs were carried out using a Beckman model E analytical ultracentrifuge equipped with an absorbance scanning optical system (50). Samples were run at temperatures of 14-28°C at speeds of 48,000-52,000 RPM for periods of around one hour to obtain the desired data; 0.4 ml. aliquots of protein solutions of OD₂₈₀^{1 cm.} 0.5-1.5 were placed in 12 mm. cells in the rotor (AN-F) and were scanned at 8-minute intervals during sedimentation. The progress of the midpoint of the boundary on the recorded scans was

used to calculate the s-value (51), and some scans were traced and superimposed (Figure 12) to allow a careful survey of homogeneity and to allow some comparison of diffusion properties.

(4) Miscellaneous

Preliminary electrophoresis experiments involved use of 15% polyacrylamide gels (as described in this section, part B-1) and cellulose acetate membranes. Additional materials useful in these procedures were a gel preparation rack (Parafilm, American Can Co., #1812), Ponceau S stain (Millipore Filter Corp., #M 000000 2B), Gelman electrophoresis tray and power supply, and cellulose acetate membranes from Millipore. Membranes stained with Ponceau S were fixed and destained with 5% acetic acid prior to visual inspection.

C. Results

(1) Disc Electrophoresis

The electrophoretic migrations of representative samples and the conditions of their measurement are summarized in Table 3. Under the two general sets of conditions represented by the 5.0% and 7.5% gel preparations, both the beef and Hexanchus M₄ samples migrated into the gels (in the anodic direction) as two components, viewing the results of Amido Black staining (Figures 4-7). The

Table 3. Polyacrylamide Gel Electrophoresis Summary.

Sample	Gel***	Component	Figure	Average Potential (volts)	Time (hours)	Anodic* Migration (cm.)	Mobility, ** cm ² / volt-sec
(b)	7.5%	NADH	N. S.	119.0	1.13	6.05	8.01 x 10 ⁻⁵
(b)	7.5%	BSA	N. S.	119.0	1.13	3.81	5.04 x 10 ⁻⁵
(b)	5.0%	NADH	N. S.	9.70	3.70	6.41	31.8 x 10 ⁻⁵
(b)	5.0%	BSA	N. S.	9.70	3.82	0.83	3.97 x 10 ⁻⁵
<u>Hexanchus M₄</u> :							
(c)	5.0%	Peak I	4	32.52	24.05	0.74	0.17 x 10 ⁻⁵
(c)	5.0%	Peak II	4	32.52	24.05	2.83	0.64 x 10 ⁻⁵
(c)	5.0%	Activity	4	32.52	24.05	1.19	0.27 x 10 ⁻⁵
(c)	7.5%	Peak I	7	145.9	2.425	1.10 ± .02	0.55 x 10 ⁻⁵
(c)	7.5%	Peak II	7	145.9	2.425	0.61 ± .05	0.31 x 10 ⁻⁵
<u>Beef M₄</u> :							
(a)	5.0%	Peak I	6	32.52	24.05	0.88	0.20 x 10 ⁻⁵
(a)	5.0%	Peak II	6	32.52	24.05	3.32	0.76 x 10 ⁻⁵
(a)	5.0%	Activity	6	32.52	24.05	1.48	0.34 x 10 ⁻⁵
(a)	7.5%	Peak I	8,9	145.9	2.425	0.0	0.0
(a)	7.5%	Peak II	8,9	145.9	2.425	0.81 ± .05	0.41 x 10 ⁻⁵

* measured from gel-sample zone boundary

** Calculated using average gel length of 6.41 cm.

*** 7.5% gel: pH 8.8; 5.0% gel: pH 6.8 (Initial pH-values of gel buffers)

Figure 4. Hexanchus M₄ LDH: Electrophoretic distribution of protein concentration and enzymic activity. Solid line: protein, Amido Black stain; broken line: activity stain, NBT/PMS. Scale in cm. represents migration distance from sample zone boundary toward anode. Cationic slice (beyond 5 cm.), not aligned with scale, is included for baseline values. 5% polyacrylamide, 24.05 hours, 32.52 volts, 17°C. Gel buffer: pH 6.8

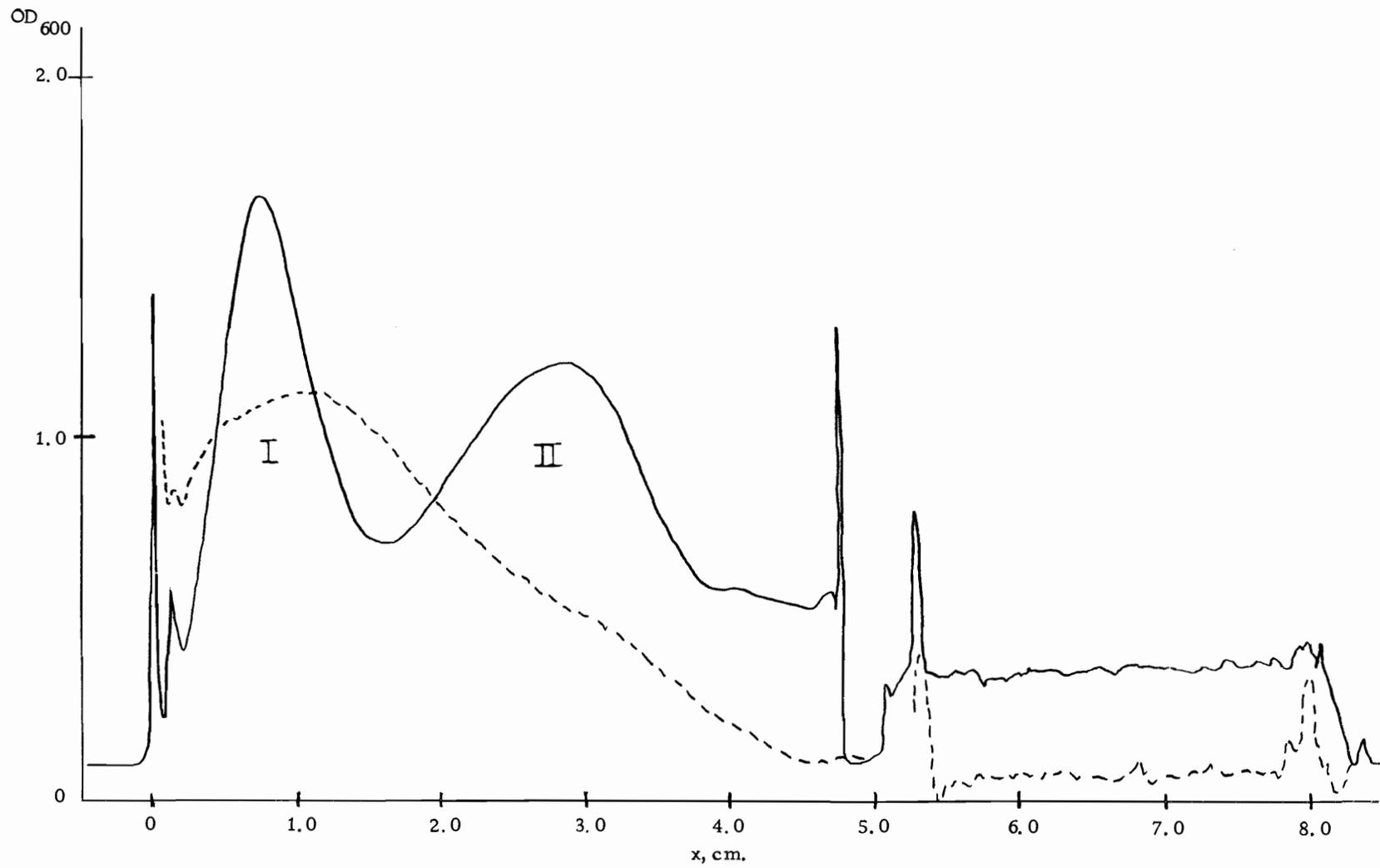


Figure 4.

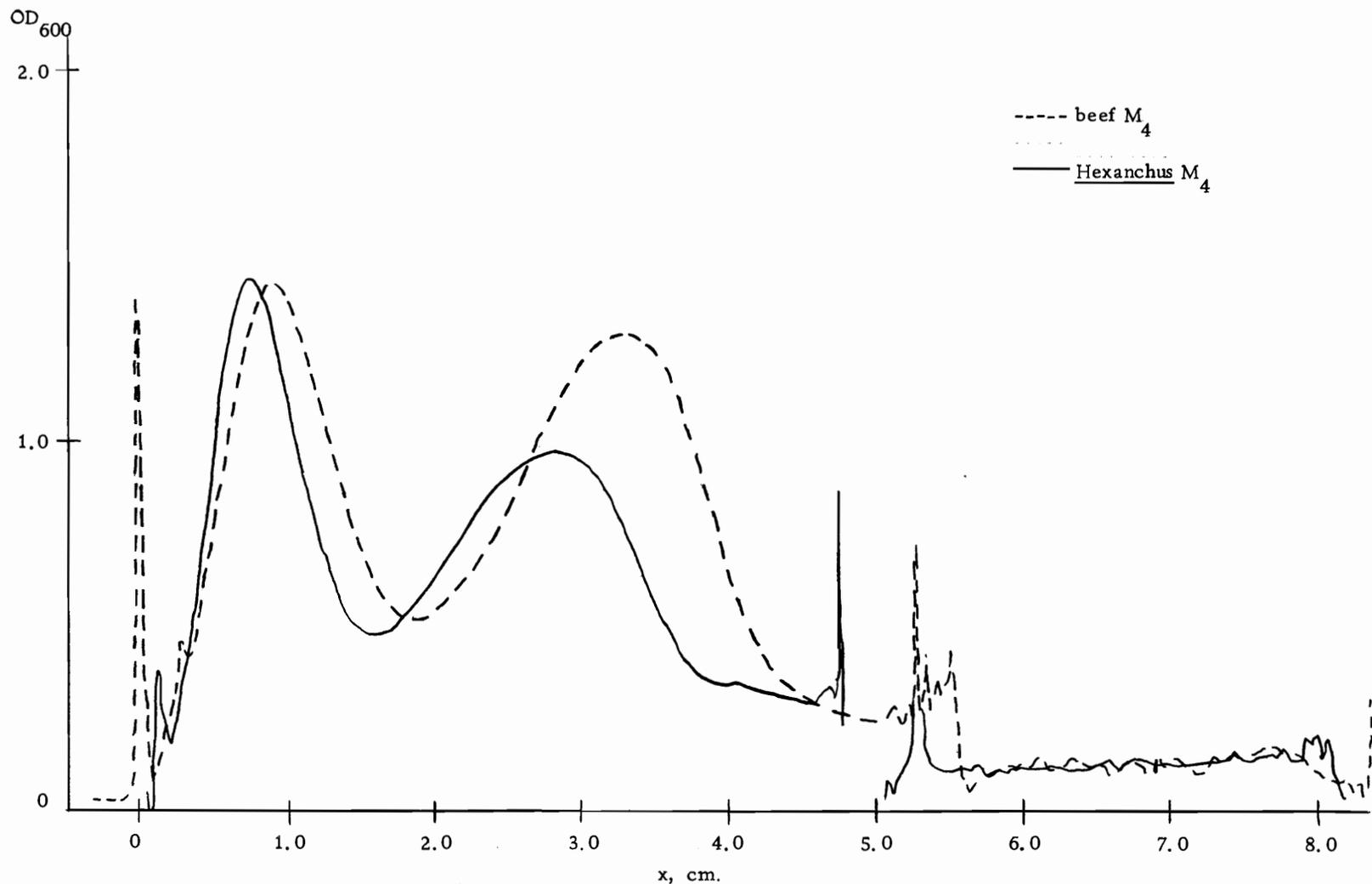


Figure 5. Hexanchus and beef M₄ LDH, 5% gel scans, Amido Black stain only.

Figure 6. Beef M₄ LDH: Electrophoretic distribution of protein concentration and enzymic activity. Solid line: protein, Amido Black stain; broken line: activity stain, NBT/PMS. Scale in cm. represents migration distance from sample zone boundary toward anode. Cationic slice (beyond 5 cm.), not aligned with scale, is included for baseline values. 5% polyacrylamide, 24.05 hours, 32.52 volts, 17°C. Gel buffer: pH 6.8

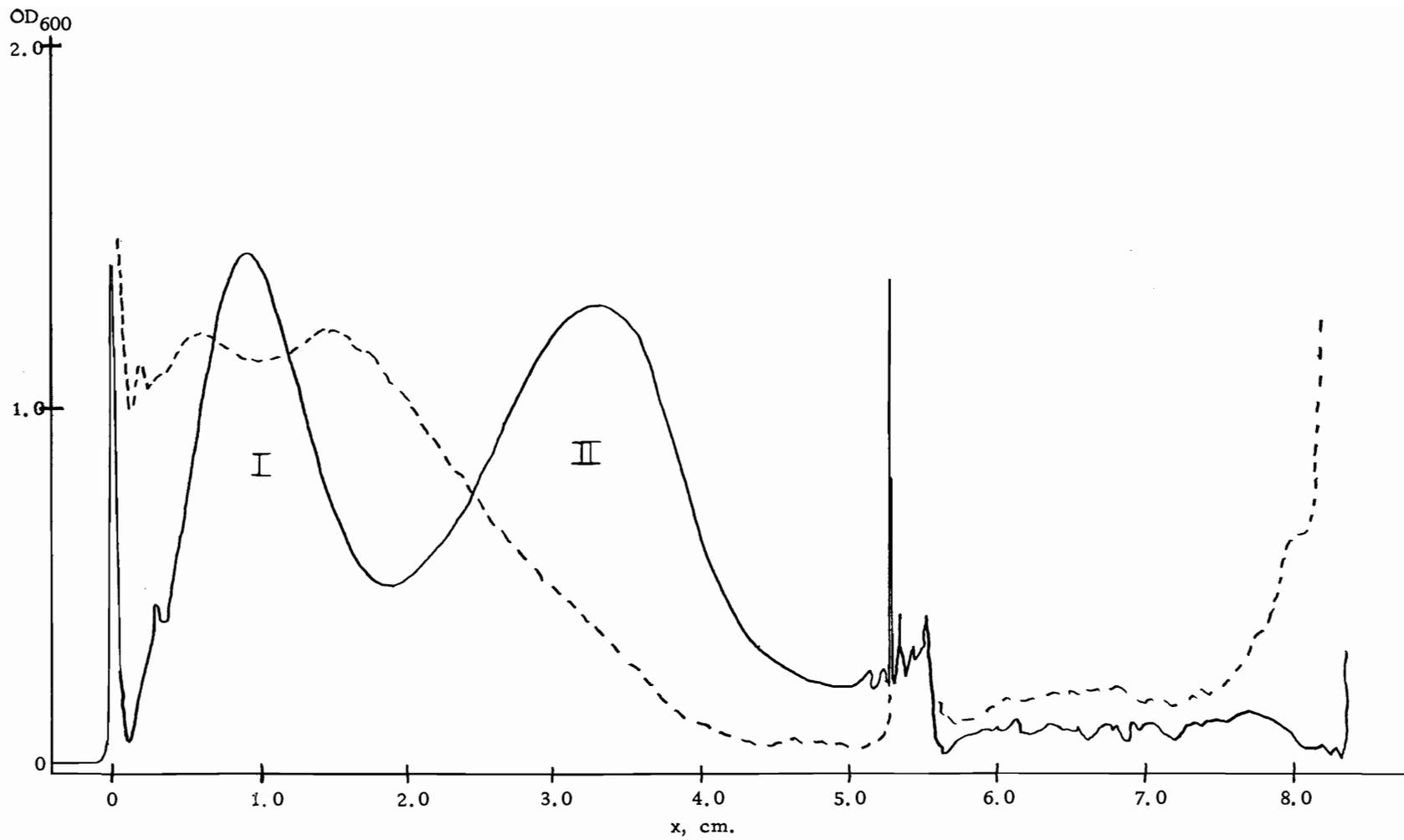


Figure 6.

Figure 7. Hexanchus M₄ LDH: electrophoretic distribution of protein concentration, Amido Black stain only. Solid line: Hexanchus M₄ only; broken line: Hexanchus M₄ with NADH. 7.5% polyacrylamide, 2.43 hours, 145.9 volts, 17°C. Gel buffer: pH 8.8

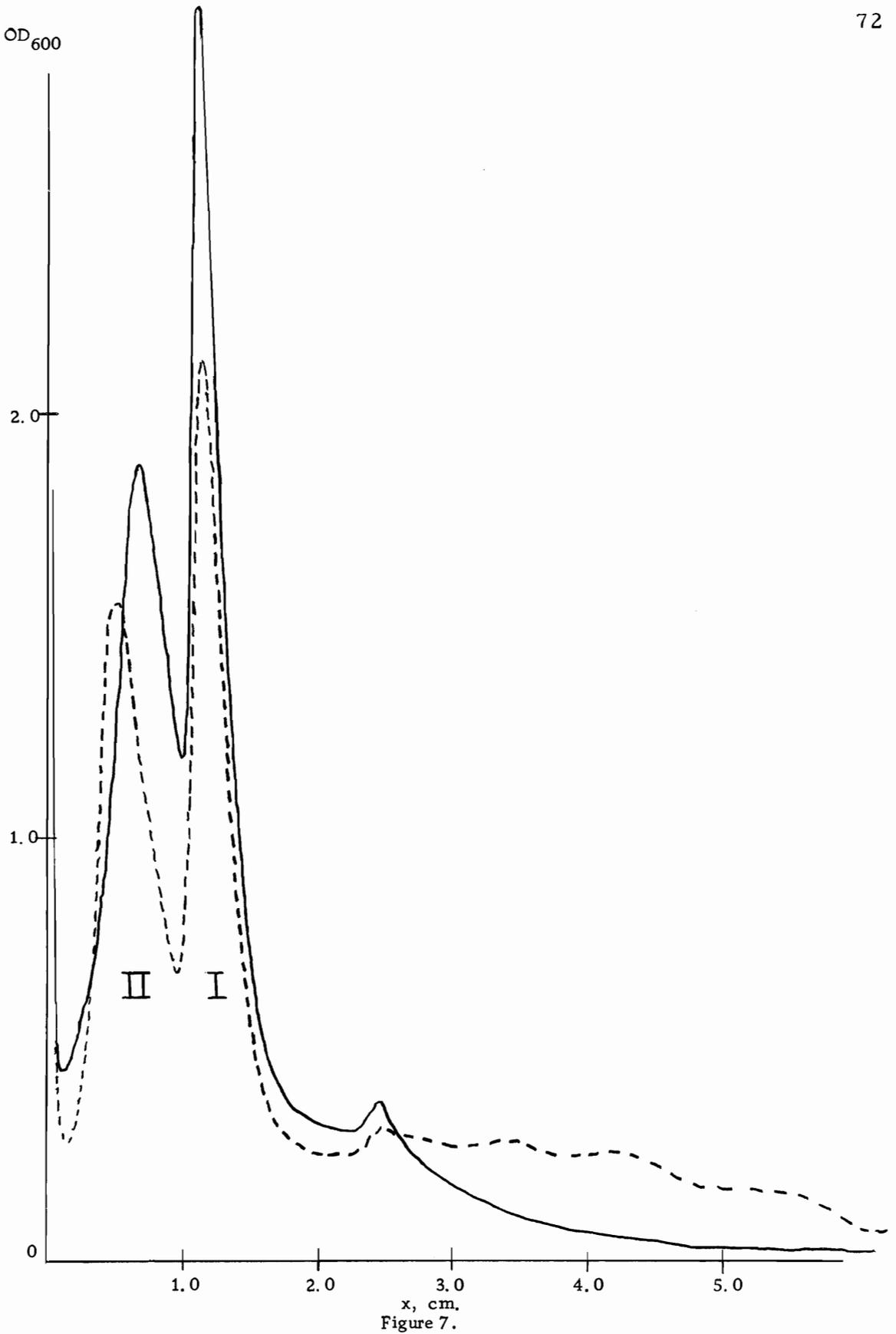


Figure 7.

Figure 8. Beef M₄ LDH: electrophoretic distribution of protein concentration, Amido Black stain only. Solid lines: Beef M₄ only, Sample (a); broken line: Beef M₄, BSA, and NADH, Sample (b). 7.5% polyacrylamide, 2.43 hours, 145.9 volts, 17°C. Gel buffer: pH 8.8

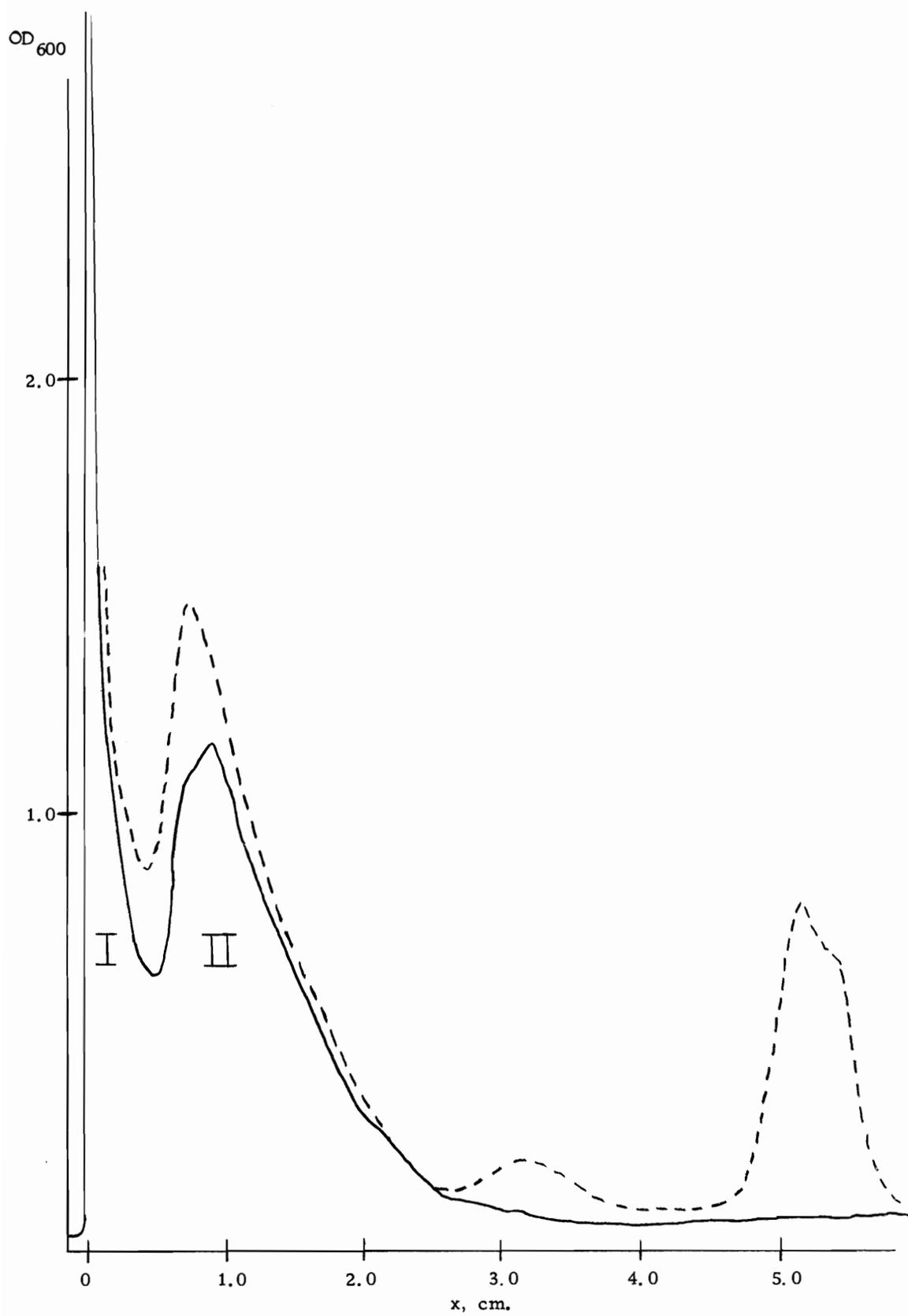


Figure 8.

Figure 9. Beef M₄ LDH: electrophoretic distribution, combined Amido Black and NBT/PMS activity stain. Solid line: gel scanned at 600 nm. ; broken line: gel scanned at 540 nm. 7.5% polyacrylamide, 2.43 hours, 145.9 volts, 17°C. Gel buffer: pH 8.8

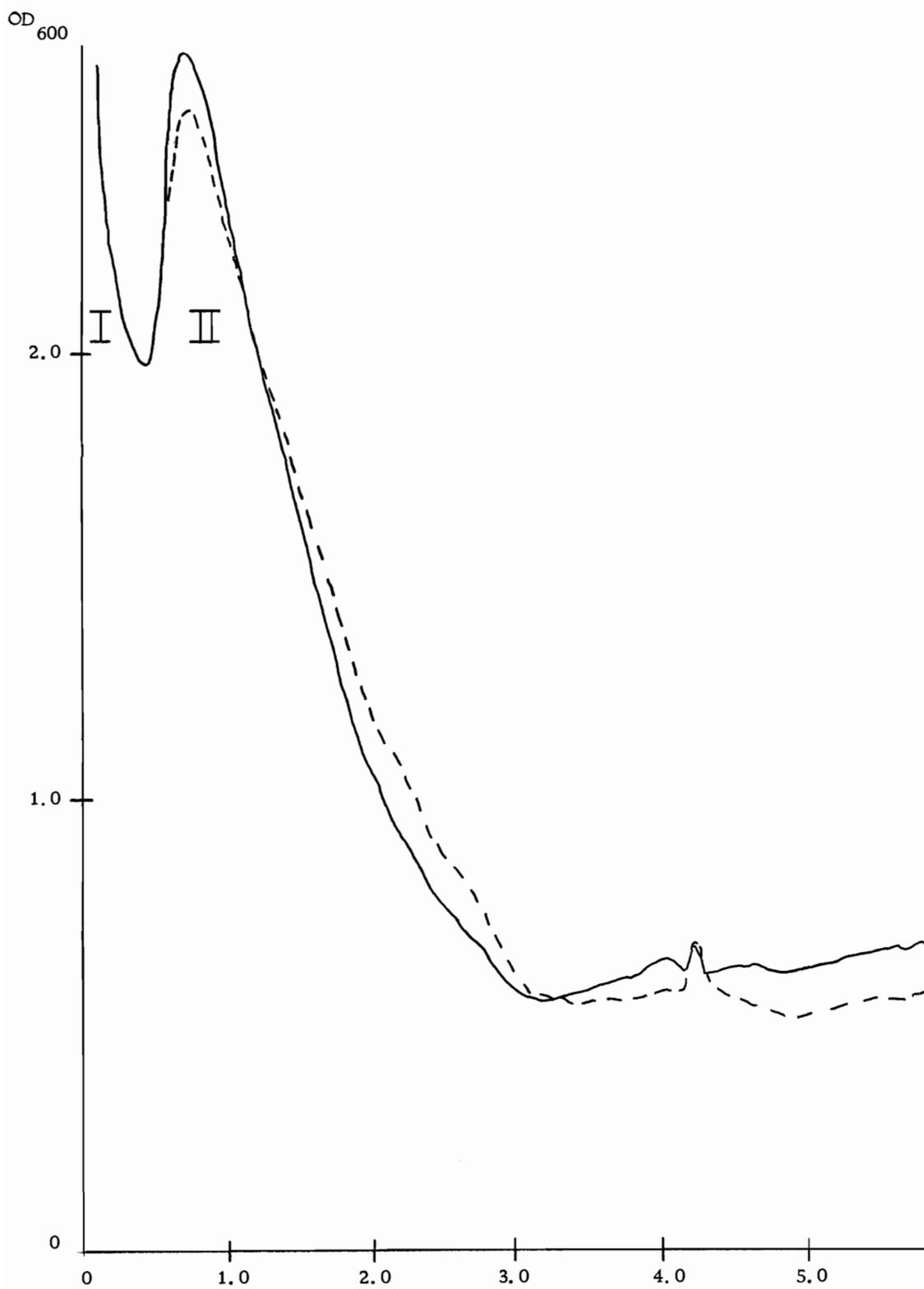


Figure 9.

components of the LDHs designated as Peaks I and II in Table 3 are labeled as such in Figures 4-9. The components designated as "activity" in the table are NBT/PMS stain maxima occurring closest to the minima between Peaks I and II. The latent ability for the molecular populations of beef and Hexanchus to migrate in these gels as two components persists despite considerable difference in the conditions of separation found in the 7.5% and 5.0% gel runs. Assuming that Peaks I and II can be consistently identified as such by their area/height ratios despite their potential ability to trade places with pH change, one also finds their relative proportions to total protein well conserved. The degree to which this latent divisibility is manifested, in terms of mobility units (52) of separation, is similar in all cases here illustrated, though the Hexanchus components trade places in going from the pH 6.8 system to the 8.8, while the beef components maintain their original order.

In the 5.0% gels, represented in Figures 4-6, the 24-hour period of separation and the greater porosity (unlike 7.5% gel) combine to give quite favorable conditions for comparing the diffusion properties of the Hexanchus and beef components both inter- and intraspecifically. Peak II of both Hexanchus and beef (Figures 4-6) has a width at half-height about twice that of Peak I, a relationship which holds constant despite considerable variation in the ratio of total area of Peak I to that of Peak II. The simplicity, symmetry,

and consistency of interrelationship ascribable to these peaks prompts an explanation involving the dissociation of tetrameric LDH into monomers. Apparently Peak II could represent a population of monomers being sloughed off from a Peak I comprised mostly of tetramers under the ion-exchanging conditions involved in the electrophoresis. Because the half-height widths of Peaks I and II in Hexanchus correspond well to those widths in beef, one can readily propose that the two components have the same average molecular weights in both species, though their proportions to total protein in the separation differ considerably between species.

The distribution of enzymic activity in the gels and its relation to protein distribution belie the simplicity of the above tetramer-dissociation interpretation, however. In Figures 4-6, a simple overview shows that Peak I is certainly associated with most of the remaining activity, but variation of activity level within individual peaks suggests that Peak I and II themselves are far from being homogeneous. Because both an activity peak and the epicenter of activity distribution appear consistently near the minimum point between Peaks I and II, it would appear that the activity-staining zones are really heterogeneous ensembles of sub-active enzyme fractions which incorporate both Peak I and Peak II protein forms for their integrity. The non-linearity of the stain-intensity-activity relationship only partially explains this lack of simple coincidence.

Figure 7 shows a small Amido Black component in Hexanchus more anionic than either major Peak. Beef M_4 requires a combination of activity and Amido Black staining (in that order) to reveal the location of a similar peak (Figure 9). The lack of coincidence of activity and Amido Black stain also occurs in the 7.5% gels.

Other than the components described thus far, no contamination by other molecular species is in evidence; examination of cationic slices, removal and staining of gels at earlier times during runs, and visual inspection of gel-samples duplicating the scanned gels of Figures 4-9 all uphold the degree of homogeneity hereto described, and ascribe considerable reproducibility to the measurements.

Though its components tend to be of differing proportions, and its activity distribution somewhat less extensive, the Hexanchus M_4 preparation possesses heterogeneity of the same character as the beef M_4 sample displayed for comparison.

Figure 7 displays the striking sensitivity of Hexanchus M_4 electrophoretic distribution to pretreatment and separation in the presence of NADH. It appears that some "isozyme" material has been created by the exposure, whose mobility exceeds that of the major bands already described, and depletes these bands accordingly. Not only has protein been removed from Peaks I and II, but Peak II has also undergone considerable morphological change. The new satellites observed are broad bands, suggestive of either reduced

molecular weight (monomers or below), or extensive microheterogeneity, or both. Observation of the progress of the NADH bands in such samples with a UV hand lamp gave no evidence of NADH fluorescence being detained by binding to LDH during electrophoretic migration; in fact, NADH migrated with the bromophenol blue marker band without evidence of retardation or diminution. In contrast, beef M_4 showed no diminution or obvious changes in average mobility of Peaks I and II with the NADH exposure, as shown in Figure 8. The additional peaks appearing in Figure 8 seem to arise from the BSA; NADH contributes some additional staining in the Peak I and Peak II region.

(2) Gel Filtration

Figure 10 shows the distribution of protein concentration, kinetic activity, and binding avidity (APDH) of individual fractions of 3.5 ml. each eluted from the 33.5 cm. (107 ml.) P200 column. The abscissa-values for the points are the midpoints in the increments of elution volume (cumulative) for the individual samples, and the logarithmic ordinate values are extrapolated from points approaching the maximum (graphically) to obtain the modal elution volume, shown as the intersection of two curves to form a cusp. The values obtained for the modal elution volumes of protein concentration, activity, and APDH binding avidity of Hexanchus M_4 are compared to the corresponding value obtained for beef M_4 protein concentration on the same

- Figure 10. Gel Filtration of Hexanchus M₄. Bio-Gel P200, column height 33.5 cm., volume 107 ml. 0-4°C, 1.42 ml./hr. per cm.²; sample volume applied: 1.0 ml. Buffer: 0.1 M PO₄, pH 7
- A. Protein Concentration, log₁₀ of Relative Fluorescence Yield (RFY), excitation and emission wavelengths 280 and 340 nm., respectively. 115,700 RFY = 1.0 OD₂₈₀. RFY measured at high dilution to assure proportionality of RFY and OD₂₈₀.
 - B. APDH binding, assuming a constant fluorescence enhancement factor, log₁₀ of moles per liter capacity.
 - C. Apparent binding stoichiometry, ratio of B to A, using $F_{1\text{ cm.}}^{1\%} = 13.0$ and Mol. Wt. = 140,000; non-logarithmic.

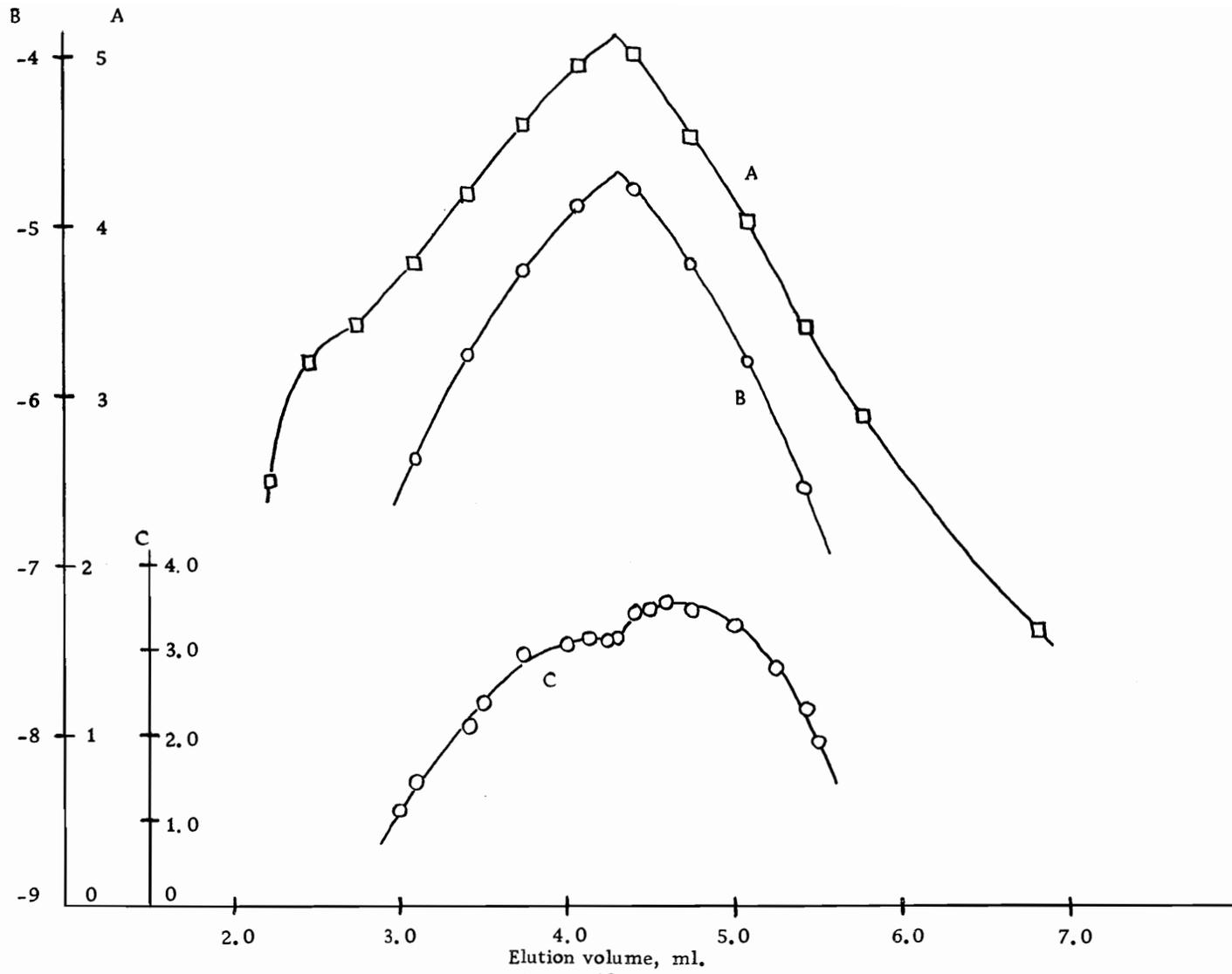
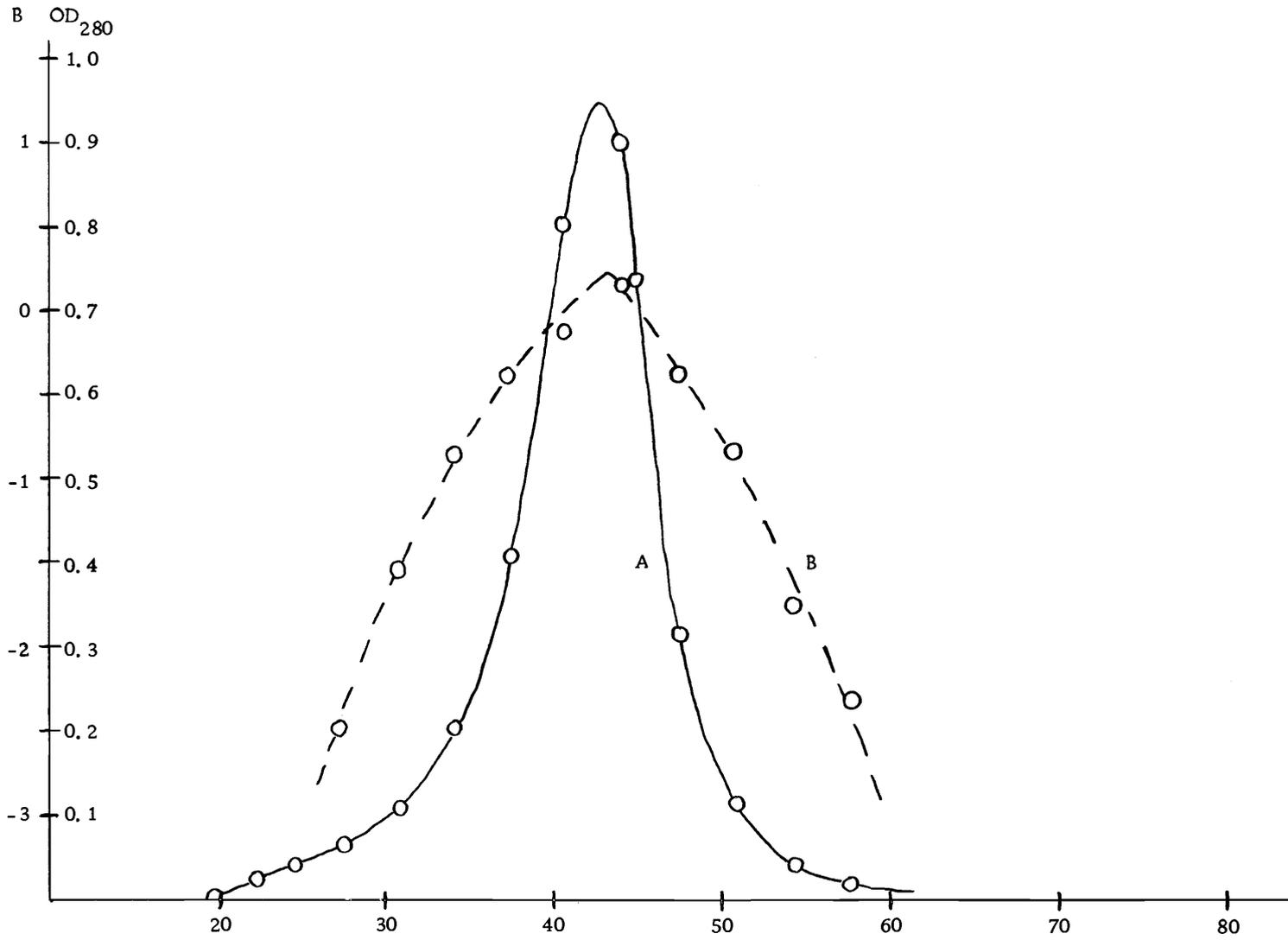


Figure 10.

Figure 11. Gel Filtration of Hexanchus M₄. Bio-Gel P200, column height 33.5 cm., volume 107 ml., 0-4°C, 1.42 ml./hr. per cm.²; Sample volume applied: 1.0 ml. Buffer: 0.1 M PO₄, pH 7.

A. Protein Concentration, as OD₂₈₀

B. Activity, log₁₀ of units/ml.; same units and conditions as assay in Table 1.



Elution volume, ml.
Figure 11.

column (beef enzyme elution not shown). These values, and the corresponding molecular weight estimates, may be compared as follows:

	<u>Parameter</u>	<u>V_e (modal)</u>	<u>$(V_e - V_o)^{-1.5}$</u>	<u>Mol. Wt.</u>
Beef M_4 standard:				
	OD ₂₈₀	42.35 ml.	.00886	140,000
<u>Hexanchus</u> M_4 :				
	OD ₂₈₀	42.90 ml.	.00856	135,200
	APDH bound	43.20 ml.	.00840	132,700
	RFY _{280,340}	43.10 ml.	.00845	133,600
	Activity	43.40 ml.	.00830	131,200
	OD ₂₈₀ ($\frac{1}{2}H$)	42.23 ml.	.00893	141,200

In the above comparison, the proportionality between estimated molecular weight and $(V_e - V_o)^{-1.5}$ is based on an approximation of a theoretical relationship between average molecular radius and relative elution volume proposed by Laurent and Killander (53). (The value $V_o = 19.0$ ml. is used in the calculations. This is the abscissa-intercept of the leading edge of the OD₂₈₀ distribution shown in Figure 11, and also the leading edge shown with a preliminary elution of partially purified glutamic dehydrogenase. The use of $(V_e - 19.0)^{-1.5}$ to estimate and scatter of the molecular weight comparisons is also somewhat vindicated by calculating a molecular weight of 19,100,

using the beef M_4 standard, to correspond to the total column bed volume of 107 ml. Thus, a V_e of 107 ml. would designate a molecular weight comparable to the "total inclusion" molecular weight for P200 designated by the supplier.). The parameter $RFY_{280,340}$ is the relative fluorescence yield due to intrinsic protein fluorescence (as in Figure 10), and the parameter $OD_{280}(1/2H)$ designates an elution volume determined by averaging the two half-height V_e values taken from the OD_{280} function in Figure 11.

Comparison of the distributions of protein concentration for Hexanchus (Figure 11) and beef (not shown) shows an unmistakable skewing of the Hexanchus distribution not observed with the beef sample. Some material of higher molecular weight appears, at first glance, to be eluting ahead of the major fraction, with molecular weight in the 200,000-400,000 range. No other "contaminants" appear in the elution profile for either beef or Hexanchus M_4 samples of final purity. But inspection of the corresponding distributions of activity and APDH binding capacity shows a corresponding skewing into this zone of high molecular weight, showing again, as with gel electrophoresis, the capability of the population to be displayed as a heterogeneous ensemble of active forms. This microheterogeneity differs from that observed with the electrophoresis, in that promotion of molecules to states of high molecular weight seems to be involved in the process more than any dissociation into subunits. Other than

this skewing, no indication of Hexanchus being less homogeneous than the beef sample comes to light: the beef elution profile showed a symmetrical height-width relationship, matching the lower molecular weight phase of the Hexanchus distribution (Figure 11).

Examining the distribution of APDH binding stoichiometry (Figure 10, curve C) shows the expected loss of avidity upon approaching either extreme of molecular weight, but the interrelationship of the skewed protein concentration and binding (and activity) curves gives rise to a double-phase maximization of apparent stoichiometry and of specific activity. This occurrence of maxima in off-center locations relative to protein concentration maxima is highly similar to the occurrence of activity peaks in similar locations in gel electrophoresis distributions. As in that case, the rather simple, near-Gaussian distribution of protein concentration is shown to be quite heterogeneous within itself by activity measurements.

Figures 10 and 11 represent a dialysate of Hexanchus M_4 against the running buffer (3 changes of 50 volumes, 6 hours apiece at 0-4°C). For the larger (1600 ml.) column, crystals were simply harvested from an ammonium sulfate suspension, redissolved in the 0.1 M phosphate running buffer, and the sample was promptly applied to, and eluted from the column. In this case, no skewing into the high molecular weight region of the elution profile was observed, but similar heterogeneity within the protein concentration peak was

maintained (Table 4).

The aspect of heterogeneity that holds true for all gel filtration of Hexanchus M_4 is the variation of binding stoichiometry (of coenzyme) and specific activity within the protein concentration peak. In Figures 10 and 11, binding stoichiometry of APDH (apparent) and specific activity are proportional and reach a maximum not coinciding with the protein concentration maximum. The maximum binding ratio observed (some 3.57 moles per 140,000 grams in Figure 10) still falls short of the hypothetical maximum of 4.0, but represents a purification over the applied sample. While this result shows that further purification with gel filtration is physically possible, the changes in concentration, elution rate, and sample preparation represented in Table 4 produce a lower maximum binding ratio. As an additional purification step, P200 seems to lack repeatability to a significant degree. Also, a substantial fraction of the total sample activity must be discarded if optimization of binding capacity is the objective of purification, and samples are selected accordingly from distributions.

The use of P200 with impure Hexanchus M_4 preparations (such as from step 5, Table 1) resulted in distributions with at best obvious losses of binding capacity (and specific activity) compared to the applied sample. However, good yields of protein in the 140,000-dalton region of elution could be reliably predicted, with peak symmetries

Table 4. Gel filtration, Hexanchus M₄, Bio-Gel P200, 1600 ml. Column (5).

Fraction ⁽¹⁾ number	OD ₂₈₀	APDH bound, ⁽²⁾ M x 10 ⁵	Binding ⁽³⁾ ratio	Protein ⁽⁴⁾ mg/ml.	E ^{1%}
32	.545	.51	1.76	.445	12.2
33	1.430	1.77	2.32	1.040	13.8
34	2.295	3.65	2.98	1.700	13.5
35	2.280	3.35	2.76	1.660	13.7
36	1.700	2.39	2.64	1.300	13.1
37	1.050	1.44	2.57	.806	13.0
38	.610	.54	1.66	.441	13.8
39	.308	.22	1.34	.218	14.1
Chicken H ₄	1.200	2.40	3.95	.883	13.6

- (1) Increasing numbers correspond to fractions of decreasing molecular weight.
- (2) Enhancement factors: Hexanchus M₄, 230.5; Chicken H₄, 245. Calculated as described (this section, part B-2).
- (3) Assures $E_{1\text{ cm}}^{1\%} = 13.4$. Purification shown in Figures 10-11 not repeated with 1600 ml. column.
- (4) Lowry method (54), using Chicken H₄ standard, assuming $E_{1\text{ cm}}^{1\%} = 13.6$ (55).
- (5) Diameter 5.7 cm.; height 62 cm.

and proportions rivalling those obtained for highly purified samples. The 140,000-dalton fractions were well-separated from the peaks of high molecular weight contaminants eluting near void volume.

(3) Ultracentrifuge

Table 5 summarizes the results of sedimentation velocity runs with samples prepared from the Hexanchus M_4 of Table 1, final sample. The uncorrected and corrected s -values are listed along with conditions influential in the corrections. The density, viscosity, and temperature correction factors applicable to the determinations were deduced from interpolation of Handbook (56) values for the component salts of the buffers used, proportioned for pH using the Henderson-Hasselbalch equation. The \bar{v} value used in density corrections was 0.74 ml./gm., a value often used by previous investigators of LDH (11). The format of calculation of composite correction factors was as given by Van Holde (51).

The values near 7.2S calculated for the sedimentation coefficient of Hexanchus M_4 are in reasonable agreement with the values listed for other LDHs (57). The traced and superimposed scans used in a determination are compared in Figure 12. Here, the appearance of homogeneity is well-conserved during the progress of sedimentation. The concentration-dependence of the s -value appears to be less than the margin of experimental error under the conditions of

Table 5. Ultracentrifuge Summary.

Sample	OD ₂₈₀ ^{1 cm.}	Buffer	T ^o C	Protein, mg/ml.	S _{T, b} ^(b)	S _{20, w} ^(b)
<u>Hexanchus</u> M ₄	0.76	(a)	22.7	.567	7.07	7.18
" "	1.52	(a)	"	1.13	7.04	7.15
" "	0.0	-	-	0.0	-	7.21
Beef heart ^(c)	-	-	-	0.0	-	7.06 ^(f)
" "	-	-	-	9.0	-	6.48
Beef H ₄ ^(d)	-	-	18-23	0.0	-	7.45
" "	-	-	" "	1.13	-	7.38 ^(e)
Beef M ₄ ^(d)	-	-	" "	0.0	-	7.32
" "	-	-	" "	1.13	-	7.28 ^(e)

(a) 0.1 M sodium phosphate, pH 7.0

(b) s-values in Svedberg units (S₁).

(c) Meister (1950). J. Biol. Chem. 184, 117.

(d) Pesce and others (1964). J. Biol. Chem. 239, 1753.

(e) Values calculated to compare to Hexanchus M₄, based on average concentration dependencies, (d).

(f) Extrapolation from 9.0 mg./ml. using H₄ concentration-dependence from (d).

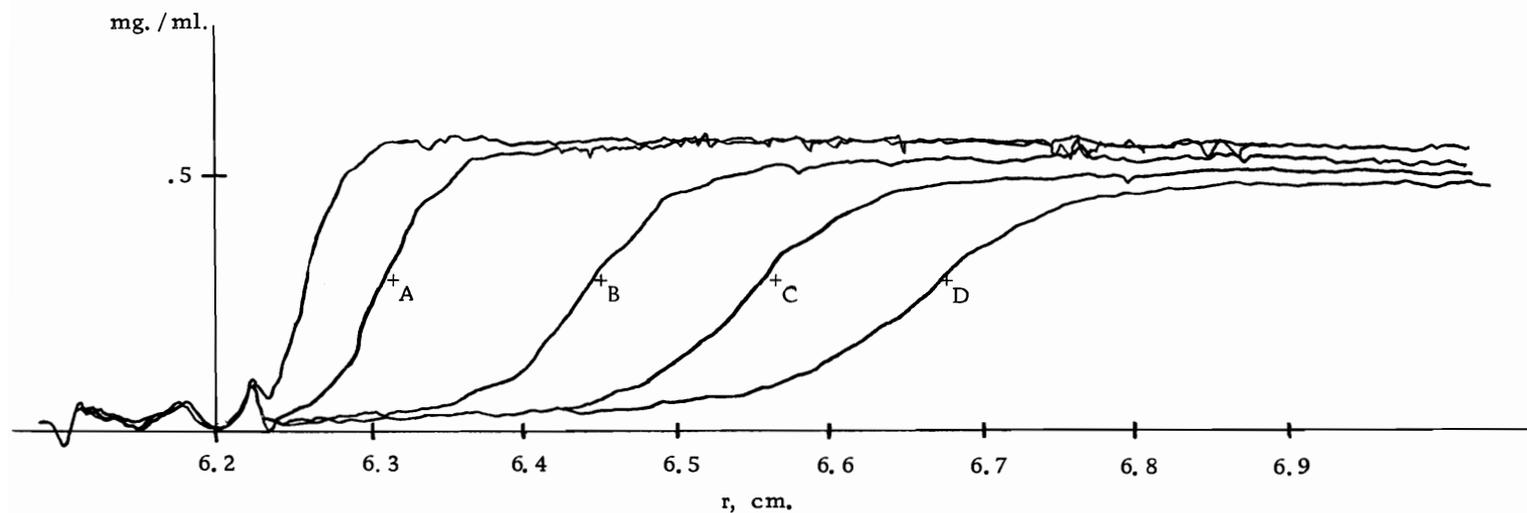


Figure 12. Ultracentrifugation: Hexanchus M₄; Absorbance Optics, Superimposed Scans. Wavelength 290 nm., path length 12 mm.; 22.7°C, 0.1 M sodium phosphate, pH 7.0; calculated rotor speed 48,013 RPM. Point A, 110.9 min., B, 138.3 min., C, 159.5 min., D, 183.6 min. Calculated (corrected) s-value = 7.18 S.

measurement; sedimentation properties are compatible with a homogeneous preparation of tetrameric LDH. This result is particularly important for the Hexanchus samples, since a limited stoichiometry of coenzyme binding and a low factor of purification have been established for the preparations, and other homogeneity probes have given extensive evidence of latent microheterogeneity (largely held in common with the beef M_4 preparations).

(4) Polyacrylamide Electrophoresis:
Elution of Samples from Gel

Further comparison of the electrophoretic behavior of Hexanchus and beef M_4 uses the 5.0% gel procedure set forth in part B-1, but with fewer modifications from the work of Dietz and Lubrano (1967), who have investigated the polyacrylamide resolution of LDH isozymes quite extensively (58). No stacking gel has been used in this work; Dietz and Lubrano showed that use of the stacking gel with LDH isozymes results in failure to resolve the M_4 and HM_3 isozymes in human serum samples. For the separations displayed in Figure 13, no 45° heat treatment is used, and no mercaptoethanol is added to the sucrose buffer sample; these modifications are expected to give less denaturation and consequent separation of components than shown in Figures 4-6. With the sectioning of the gels and allowing the segments to equilibrate with 1.0 ml. pH 7.0 phosphate buffer, while

Figure 13. 5% Gel electrophoresis: Elution of samples from Gel.
pH 6.8, 11.3° C, 24 hours, 28.1 volts.

Curves:

- a. Cumulative elution of activity, ordinate: % of total applied activity.
- b. Cumulative elution of protein fluorescence, ordinate: % of total applied F_{280} (emission 340 nm.). F readings corrected to linear dependence on sample concentration in pH 7.0 phosphate buffer, 0.1 M.
- c. Coomassie Blue stain of intact gel, ordinate:
100% = 3.2 OD_{550}

Abscissa: separation (cm) from sample zone-gel interface

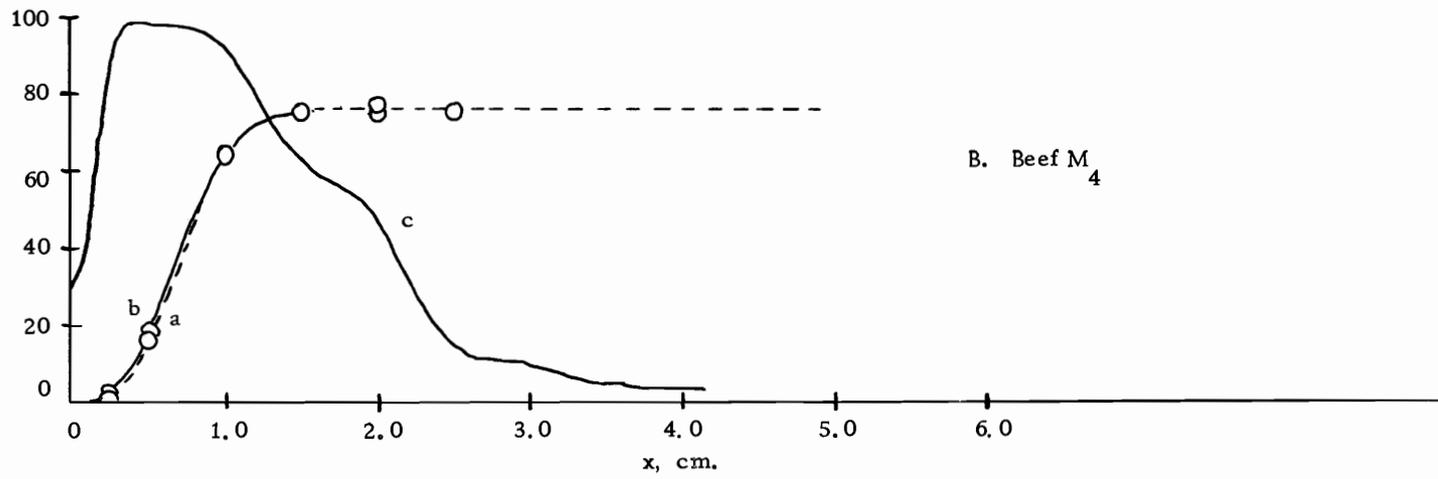
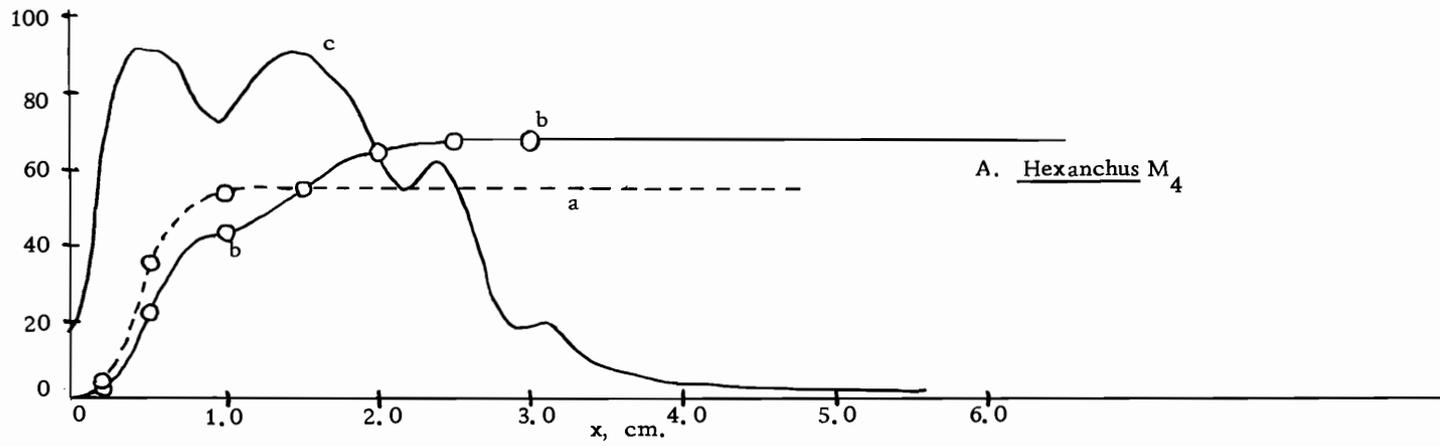


Figure 13.

staining the intact gels with Coomassie Blue (59), one obtains a comparative profile of the enzymes emphasizing differences in the nature of the heterogeneity rather than the sameness implied in Figures 4-6. The lower separation temperature (11.3° C) and potential (28.1 volts) for the 24-hour period only partly explain the lesser migration from the origin and the concurrent greater fusion of component peaks. Also, the lack of coincidence of the activity maxima and the major active protein component (Peak I) is not repeated under the conditions for Figure 13.

In beef M_4 , 75% of the applied activity can be accounted for in its cumulative elution profile, which shows near-perfect coincidence with the cumulative recovery of protein fluorescence; this represents complete conservation of specific activity, if only the cumulative elution results are considered. The superimposed profile of the Coomassie stain allows quite different interpretations of these results to be considered, however. In comparison, the Hexanchus cumulative elution profiles appear to offer a contrasting example of heterogeneity, with an actual purification taking place in the active fraction, with inactive contaminants sloughed off into what appears to be the Peak II region (as in Figure 4) of relative mobility. However, though an actual increase in specific activity is confirmed for the active fraction (Peak I, perhaps), only 55% of total activity and 68% of applied protein fluorescence can be recovered for the

Hexanchus, showing an overall loss in specific activity and a greater structural dispersal than observed for beef M_4 . Much of the apparent lack of purity in Hexanchus seems to be due to instability; the separations in Figures 4-6 show that with nearly equal separations of Peak I and II components (through partial denaturation), the activity-protein distribution relationships for the two enzymes become very much more equalized. Apparently, the separations of the components in Figure 13 are not great enough for any lack of coincidence of the activity maximum and Peak I to be readily observable.

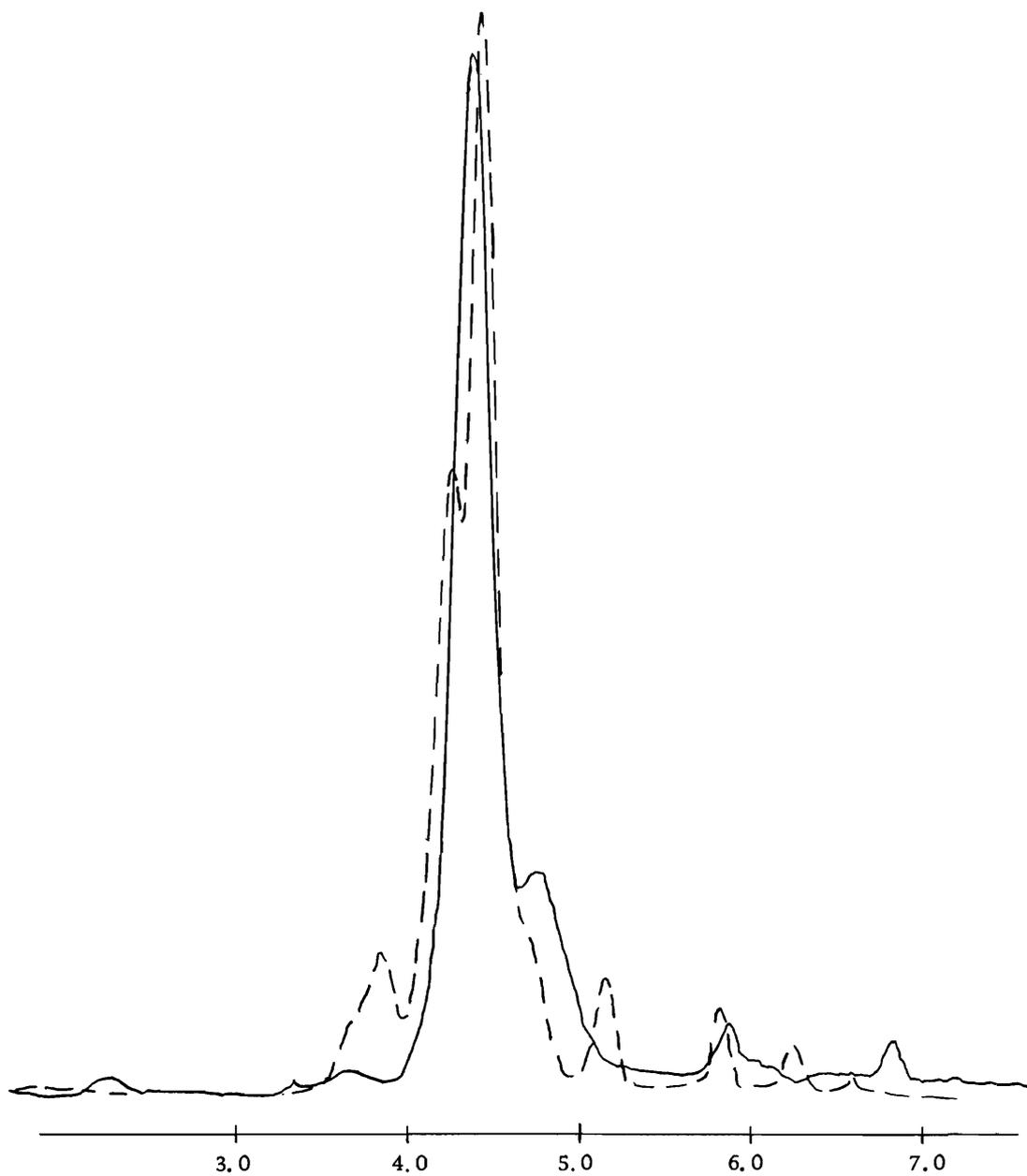
While from outward appearance, the protein fluorescence and distributions show greater homogeneity for beef M_4 than for Hexanchus, close inspection of these activity bands shows that that of beef is considerably wider. The broad activity band coincides with an anomalously broadened Coomassie peak, which could be described as two peaks (as with Hexanchus) nearly merged into one. If indeed the major peak (Coomassie) of beef is really two interacting components (upon whose interaction the stabilization of the active enzyme depends) which tend to resist separation (more than in Hexanchus), then the two enzymes have the same number of components under these separation conditions. In Hexanchus, their resolution is more pronounced, with sub-active material already present in the sample before electrophoresis (hence, the possibility of a purification).

(5) SDS Gels: Subunit Homogeneity

Using the pH 6.8 buffer system (part B-1), 7.5% acrylamide gels, with sodium dodecyl sulfate added to samples, buffers, and gel preparation to a final concentration of 1.0%, separations were carried out at 3.7 mamp./tube for one hour. After removing the gels from the tubes and staining with Coomassie Blue (64), and destaining, gels are scanned as for Figure 4-9; results for typical scans of Hexanchus and beef M_4 (same sample stocks as for Figure 13) are shown in Figure 14. (generally a standard procedure of this laboratory).

Though the subunit molecular weight homogeneities of Hexanchus and beef M_4 are being compared in Figure 14, the hypothetical dissociation of both enzymes into populations of 35,000 daltons is not straightforwardly represented in either case. Instead, many of the heterogeneity-related discrepancies in the structures of the two enzymes shown in Figure 13 are conserved through resolution with SDS: where a single major peak is localized for beef M_4 , designated as 35,000 daltons, the major component of Hexanchus splits into two bands, the more intense of the two at 33,000 daltons and the lesser with 37,000. While the single major band of beef may nominally signify greater homogeneity, close inspection shows that this band is much broader (greater width to height ratio) than the more intense

Figure 14. Subunit homogeneity: SDS Gel electrophoresis.
pH 6.8, 1.0% Sodium Dodecyl Sulfate, 7.5% gel,
one hour separation time.
-----Hexanchus M₄, 7.2 micrograms
—————Beef M₄, 4.8 micrograms
Stain: Coomassie Blue, absorbance at 550 nm.



x, cm.
Figure 14.

major Hexanchus band, and therefore microheterogeneous (as with Figure 13, most likely two peaks nearly merged into one). Other than the major bands described above, minor components are more obviously displayed in Hexanchus than in beef, though (again) close inspection can show that aggregate amounts of stray components (including shoulders imperfectly resolved from major bands) are present in similar proportions in the two preparations.

(6) UV Spectroscopics, Extinction Coefficient, and Protein Concentration

For the remainder of this study, concentrations of Hexanchus M_4 are estimated from a turbidity-corrected value of OD_{280} , using $E_{1\%}^{1\text{cm.}} = 13.4$, with 140,000 daltons to calculate molar concentrations of LDH tetramers. The turbidity correction $OD_{280} - OD_{340}$ is often used; the ratio of OD_{340} to OD_{280} may vary widely between preparations, with OD_{340} sometimes virtually zero, with no consistent correlation with solution conditions being discernible.

Often, the spectrum of Hexanchus M_4 in neutral buffers would show a considerable increase (greater than first-power) in OD with wavelength loss at 340 nm., although the simple subtraction (as above) gave reasonably consistent results when OD_{340}/OD_{280} would change when samples were diluted or reconstituted. Solutions with obvious turbidity were clarified by centrifugation before OD_{280}

measurement and further use. Figure 15 shows a typical absorbance spectrum of Hexanchus M_4 compared to that of beef M_4 . The above descriptions of spectroscopic methods apply only to simple solutions of the enzyme in neutral buffer, with no inclusion of coenzymes or other components. The relatively high ratio of OD_{260} to OD_{280} distinguishes the Hexanchus samples from the beef and chicken, and prompts further speculation about the nucleotide content and attainable degree of purification of the preparations.

Table 6 summarizes extinction coefficient determinations for Hexanchus M_4 employing the above spectroscopic techniques; beef M_4 and chicken H_4 have been used as standards. Part A of this table uses the Amido Black-stained polyacrylamide gel scans of Figures 4 and 6 (Section IV) to determine relative amounts of Hexanchus and beef M_4 applied to the gels. Height \times width calculations are used to represent areas of individual peaks, with areas of Peaks I and II summed to represent total protein on the gel. Heights are measured from the average baseline-value (cationic zone), with 1.12 mm. as the unit of distance measurement represented in Table 6, part A. Beef M_4 with $E_{1\text{ cm}}^{1\%} = 12.9$ is used as a standard to calculate $E_{1\text{ cm}}^{1\%}$ for Hexanchus M_4 in part A. Part B shows $E_{1\text{ cm}}^{1\%}$ calculated independently for both beef and Hexanchus M_4 , using the summed microgram quantities of amino acids (Section VI, hydrolysis-corrected); fair agreement with the values for both in part A is shown.

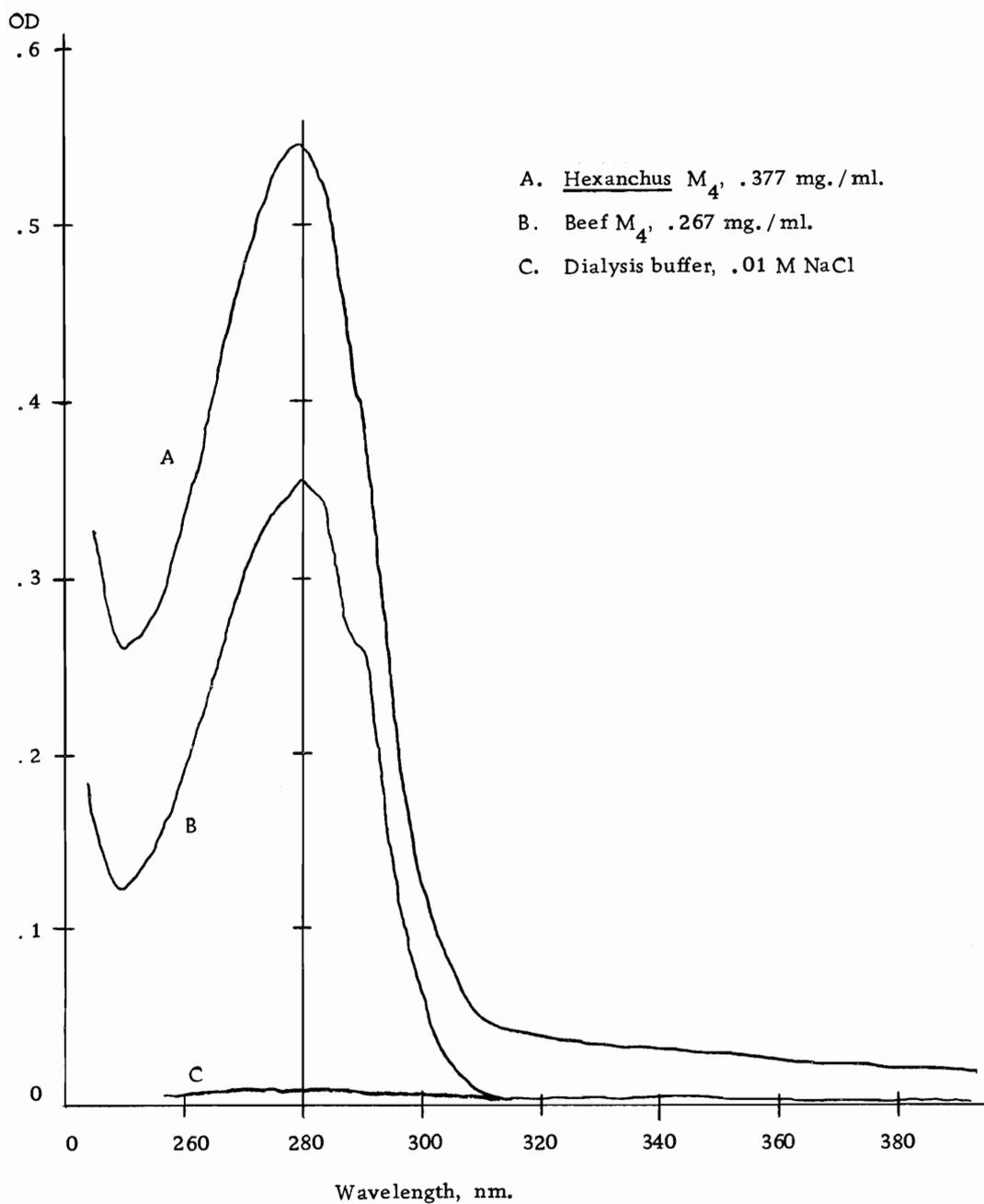


Figure 15. Absorbance spectra.

Table 6. Extinction coefficient: Hexanchus M.

A. From 5% Polyacrylamide Gel Electrophoresis, Amido Black Stain, Figures 4, 6						
	H	W	H X W	Sum I + II	OD ₂₈₀ ^{1 cm.}	E ₂₈₀ ^{1%}
<u>Beef</u> M ₄						
peak I	47.1	13.7	645.3	1624	3.52	12.9 (assumed) (11)
peak II	42.0	23.3	978.6			
<u>Hexanchus</u> M ₄						
peak I	46.5	10.6	492.9	1208	2.67	13.2 (calculated)
peak II	29.8	24.0	715.2			
B. From Amino Acid Analysis, Section VI						
<u>Beef</u> M ₄	650.2	.100	8.25	.650	12.7 (calculated)	
<u>Hexanchus</u> M ₄	445.3	.050	12.12	.891	13.6 (calculated)	
C. Lowry method (54), Samples from Gel Filtration, Table 4						
<u>Chicken</u> H ₄	E ₂₈₀ ^{1%} = 13.6 (assumed) (55)					
<u>Hexanchus</u> M ₄	E ₂₈₀ ^{1%} = 13.4 ± .36 standard deviation of individual fractions (calculated)					

Part C of Table 6 states the average $E_{1\text{ cm}}^{1\%}$ and the standard deviation (60) of individual samples determined by the method of Lowry and others (54), using samples from a gel filtration of Hexanchus M_4 (Table 4, Section IV). (Reagents employed: Folin-Ciocalteu, Uni-Tech, cat. no. CC4-445-4; cupric sulfate, B&A rgt. code 1651; sodium tartrate, B&A rgt. code 2305.). Chicken H_4 was used as a standard, and because of non-linear and irregular dependencies of Lowry OD_{750} upon protein concentration often observed, the Hexanchus sample sizes were so adjusted as to produce OD_{750} outputs within a few percent of a consistent chicken H_4 standard; calculations of total sample protein involved only small proportionate adjustments, and the result for each fraction repeated itself well. The average value of $E_{1\text{ cm}}^{1\%} = 13.4$ from parts A and B of Table 6 is confirmed in part C and Table 4. Of most interest in Table 4 is the relative independence of $E_{1\text{ cm}}^{1\%}$ and the apparent ADPH binding capacity; inspection of the table shows a maximum variation over 50%. The fluctuations of these variables also do not consistently correspond. Thus, despite large variation of kinetics and coenzyme binding through perturbations of enzyme structure involving large changes in average molecular weight, a reliable estimate of protein concentration via OD_{280} is nevertheless conserved.

D. Discussion

The Hexanchus M_4 isolation, as portrayed in Table 1, reaches apparent limits to purification at 23-fold, when the methods of Section III are used. The 23-fold purification is low compared to other LDH isolations reported (dogfish M_4 is among the lowest at 40-fold) (11, 21). The limited stoichiometry of coenzyme binding (Section V) would suggest that some 40-50% additional protein material must be removed to bring the binding ratio up to 4.0 and the purification, correspondingly, up to 40-fold or more.

The above designation of an incomplete purification does not allow for intrinsic behavioral characteristics of the enzyme that may limit its stability to purification, that may give rise to some ambiguities in the applicable criteria of purity, or both. And the results described in this section suggest that such characteristics apply to both Hexanchus and beef M_4 . Both enzymes may be displayed as microheterogeneous ensembles of sub-active forms with gel filtration and electrophoresis (Hexanchus more so than beef), though the sedimentation results portray the isolated Hexanchus samples to be homogeneous tetrameric LDH. If indeed a non-LDH protein species is contaminating the characterized Hexanchus M_4 preparations, then its molecular size, charge, and tertiary structure must be so close to that of the M_4 LDH that reliable separation of the enzymes in good

yield is very difficult.

A purification factor of 23-fold from the water extract represents a high yield of LDH from the tissue. Since 4.3% of the protein in the extract is apparently LDH of the sort characterized in this writing, the tissue must contain close to two grams LDH per kilogram (if kinetic activity is used as the guideline), of which 24-48% is isolable as described in Table 1. The gel filtration of the material suggests that purification may be thereby further increased, but to do this consistently, additional specific sample preparation techniques must be applied, and concurrent losses in activity yield would be introduced. These losses appear to be sufficient to lower the kinetic activity yield to a proportion far lower than that generally reported for other LDH characterizations (21). With such lower yields, though highly refined coenzyme binding data in conjunction with proof of high purity could be obtained, the representation of the active enzyme population actually extracted from the tissue would be very incomplete. Because of this rationale, the final step from Table 1 is designated as the material subject to characterization; much of Sections V and VI investigates the nature of the observed limitation in purification.

V. INTERACTIONS WITH COENZYME

A. Introduction

Preliminary results with Hexanchus M_4 LDH indicated that the isolated enzyme samples (resulting from following the format of Table 1) had a stoichiometry of coenzyme binding falling short of the ideal 4.0 moles per 140,000 grams by at least 1.0 mole. This binding capacity measurement refers to the mode of coenzyme binding described by Anderson and Weber (30) in the study of beef M_4 and H_4 and their hybrids; its ideal stoichiometry is verifiable by a variety of methods and its applicability to many LDHs has been proven (2). The primary objective of this section is to measure the capacity of Hexanchus M_4 to bind NADH and analogue APDH under varying conditions using fluorescence titration, ultracentrifuge, and equilibrium dialysis methods. The limit of binding stoichiometry and subsidiary parameters related to it are to be determined and their reliability and mutability explored.

Another objective of this section is the extension of the coenzyme binding study, using fluorometric methods, to investigate the phenomenon of residual (dialysis-resistant) fluorescent moieties in Hexanchus M_4 . These extensions are pursuant to the concluding statements of Section I, regarding additional modes of coenzyme binding, their relation to enzyme structure, and the unique behavior

of the Hexanchus samples relative to these phenomena. Both beef M_4 and H_4 , as well as the Hexanchus, showed evidence of trace amounts of dialysis-resistant fluorophores resembling NADH; this section provides some measurements of these residual fluorescences, allowing some comparisons of the various preparations.

B. Materials and Methods

(1) Fluorescence-monitored Binding of APDH

These procedures make use of the very large fluorescence enhancements predictably obtained upon reaction between LDH and reduced coenzymes in the presence of oxalate in previous studies (49, 61).

Samples of Hexanchus M_4 and chicken H_4 were harvested from ammonium sulfate suspensions, washed with excess 80% sat. ammonium sulfate (using centrifugation), and dissolved in 0.1 M potassium phosphate buffer, pH 7.0, final volume of 3 ml. each. Concentrations of these stock solutions were determined by $OD_{280}^{1\text{ cm.}}$ using $E^{1\%} = 13.6$ for chicken H_4 (55) and 13.4 for Hexanchus M_4 ; these concentrations were 1.986 and 3.33 mg/ml., respectively. A stock solution of APDH in .01 M Tris- H_2SO_4 , pH 8.0, 2.48×10^{-3} M (determined from OD_{363}), was prepared also. Solutions were stored at 0-4° C until ready for use.

Aliquots of the above enzyme solutions, phosphate buffer, and buffered potassium oxalate solution, were transferred into fluorometer cuvettes (Section II) to give a final volume of 2.0 ml. with pH 7.0, and 0.1 M oxalate. The cuvettes were maintained at 15.2° C in the sample compartment of the instrument, while up to .150 ml. APDH solution was added in small increments. Between additions of APDH, fluorescence intensity was recorded, using excitation and emission wavelengths of 360 and 430 nm., and slit widths of 4 and 10 nm., respectively. Samples and APDH solution were mixed by manual stirring with teflon wands, and time spans of 20-30 minutes were employed to complete titration of samples. To determine the fluorescence-concentration relationship for the unbound APDH, a sample of the oxalate-buffer was titrated in the absence of any enzyme. The wavelengths selected for fluorescence measurement were near the maxima of the fluorescence excitation and emission bands of interest. Binding of APDH by LDHs in the presence of oxalate at pH 7 predictably enhances its fluorescence 200-250 times.

Calculation of APDH bound at each step of the titration of an enzyme sample uses the quantity $(F/F_o - 1)$ (as in the studies of Anderson and Weber, 30), where F is the total fluorescence intensity of an enzyme sample at some step of its titration with APDH, and F_o is the intensity for the same concentration of ligand in the absence of enzyme. The above F -values are corrected for both protein and

buffer-blank values before use in calculations. Values of F_o applicable to particular F-values are obtained from a standard curve of F_o plotted against ligand concentration; such curves generally show maxima in F_o around 10^{-4} M. The objective of such calculations is to obtain a plot of Y (ordinate) against x_o/P_o (abscissa) where

$$Y = (F/F_o - 1) (x_o/P_o) (d. f.)$$

where x_o/P_o is the accrued ratio of total ligand to total enzyme in the titrated sample (in moles per 140,000 grams) and (d. f.) is a dilution factor correcting to constant volume and constant protein concentration. One expects plots of Y vs. x_o/P_o (Figure 16) to show a pronounced titration endpoint (ideally at $x_o/P_o = 4.0$) if enzyme concentrations are high enough to permit strong binding of the ligand.

(2) Equilibrium Dialysis, *Hexanchus* M₄ vs. APDH

A dialysate of *Hexanchus* M₄ against 0.1 M potassium phosphate, pH 7.0 was diluted with the same buffer to a final concentration of 1.13 mg/ml, or 8.1×10^{-6} M in LDH tetramers; protein concentration was determined using $OD_{280}^{1 \text{ cm}}$. A stock solution of APDH at 5.09×10^{-4} M in .01 M Tris-H₂SO₄, pH 8.0 (concentration from $OD_{363}^{1 \text{ cm}}$) was also prepared. The APDH stock was diluted with the pH 7.0 buffer to provide sample solutions having concentrations approximately 4, 8, and 13 times the concentration of tetramers in the LDH

stock. Dialysis cells from La Pine Scientific Co., size A, # 10403, 1.0 ml. for each chamber-half, were prepared by placing a square of moist, prepared dialysis tubing wall between the two chambers, dividing them equally as possible, securing the cell-halves with bolts. Aliquots of the LDH stock (1.0 ml. each) were placed in one chamber of each prepared cell, while 1.0 ml. of an APDH sample was pipetted into each opposing chamber. After closing the cell ports, the cells were secured in a shaker and allowed to equilibrate some 20 hours at 20° C with agitation (10 strokes/sec., 2-3 cm., reciprocation parallel to plane of membrane). Before and after use, dialysis cells were soaked for at least 24 hours in 2% sodium carbonate, and thoroughly rinsed with glass-distilled water.

After equilibration, samples were withdrawn from the chambers, and their APDH concentrations determined spectrometrically. LDH-containing chambers showed considerable turbidity after the prolonged agitation; protein precipitates (small) were removed by centrifugation before measuring $OD_{363}^{1 \text{ cm}}$ of these samples. The amount of LDH bound in each cell was determined by both the loss of APDH from the non-enzyme chamber, and from the apparent excess of ligand in the protein chamber over the non-protein chamber. The latter estimate of binding incorporates a correction to isobestic conditions; complexing of APDH with LDH is known to shift its absorbance maximum, lowering the molar absorbancy at 363 nm. by 5% (62).

(3) Ultracentrifuge

Dialysates of Hexanchus M_4 were diluted with neutral buffers to final concentrations of 3.29, 7.24, and 8.01 mg/ml. These enzyme samples included added coenzyme (either APDH or NADH) in proportion of 2.88 to 7.5 moles per 140,000 grams LDH. For each LDH-coenzyme sample, a control sample containing an equal concentration of coenzyme was prepared. One aliquot each of an enzyme sample and its corresponding coenzyme control (ca. 0.4 ml. each) were placed in the two compartments of a 12 mm. cell in the rotor (AN-F), opposed to samples of the corresponding buffer in the other cell. Separations were carried out with a Beckman Model E ultracentrifuge, employing speeds around 48,000 RPM, temperatures 18.9-22.2° C, and separation times up to one hour. As with sedimentation velocity runs, cells were scanned at 8-minute intervals using the absorbance optical system. The wavelength used was selected to monitor only the coenzyme absorbance; in the resulting print-out, sedimenting and non-sedimenting coenzyme could be clearly distinguished.

The binding of coenzyme under ultracentrifuge conditions was calculated by measuring the proportion of non-sedimenting optical density of the corresponding control. For such calculations, only scans having very pronounced separations of the sedimenting boundary from the meniscus were selected. Successive scans after separation

of the boundary showed very good agreement in measured percent non-sedimenting coenzyme (Table 8).

(4) Fluorescence Titrations with NADH

Dialysates and redissolved ammonium sulfate sediments of Hexanchus M_4 and beef heart LDH were diluted to 2.0 ml. in fluorometer cuvettes with .01 M sodium phosphate, pH 7.0. Using the same procedure as for the APDH titrations (represented in Figure 16), the samples were titrated with an NADH stock solution (using Hamilton syringes) from 2.0 up to 2.1 ml. total volume. From the resulting plots of Y vs. x_o/P_o , enhancement factors $(F/F_o)_{1.0} = (Y_{1.0} + 1)$ were obtained by interpolating the Y -values at $x_o/P_o = 1.0$ (which gives the cumulative average F/F_o from $x_o/P_o = 0.0$ to 1.0). Using these (F/F_o) values, the values $\bar{n}_{4.0}$, or average number of NADH molecules bound to each tetramer at $x_o/P_o = 4.0$, were calculated as $Y_{4.0}/Y_{1.0}$ with the interpolated Y -values occurring at $x_o/P_o = 4.0$. Table 9 summarizes the $(F/F_o)_{1.0}$, $\bar{n}_{4.0}$, and $\bar{n}_{6.0}$ values thus obtained for various LDH preparations. The above \bar{n} calculations assume that only two interacting components are present, and that all added coenzyme is completely bound through $x_o/P_o = 1.0$.

(5) Equilibria with Fluorescent Residuals

Because of the prominent residual blank-value fluorescence in

NADH titrations of Hexanchus M₄ samples, further information was desired which would help clarify its source and significance. Since almost all known proteins show such fluorescence (63), three questions were asked, whose answers were sought through extension of the fluorescence titration methods:

a) Can these residual moieties, possibly including strongly bound coenzyme in mixed oxidation and covalently altered states, be further reduced by lactate, such that blank-value fluorescence is increased thereby (similar to NADH)?

b) Does reduction (by lactate) of the residuals affect the fluorescence enhancement relationship to NADH binding in the samples of interest?

c) To what extent are the fluorescence-enhancing properties of Hexanchus M₄ in NADH-lactate mixtures comparable to those of beef M₄ and H₄, or to those observed in the absence of lactate?

Samples of Hexanchus M₄, beef M₄, and beef H₄ were harvested from ammonium sulfate suspensions and dialyzed against 0.1 M sodium phosphate buffer, pH 7.00, three changes of 30 volumes for eight hours each, 0-4° C, with all three samples in separate containers. In fluorometer cuvettes, dialysates were diluted to 2.0 ml. with the same buffer, to final concentrations at which the three enzymes showed comparable blank-value fluorescence. For Hexanchus M₄, beef M₄, and beef H₄, these concentrations proved to be 1.56, 3.66,

and 2.95×10^{-6} M in LDH tetramer, respectively. To these samples, aliquots from stock solutions of 2.0 M sodium DL-lactate, 1.0 M sodium carbonate, 1.17×10^{-3} M NADH, and 1.31×10^{-3} M NAD^+ were added using Hamilton syringes at 20-21° C over 30-minute periods. As with other fixed-wavelength work with NADH in this section, the excitation and emission wavelengths used were 340 and 430 nm., respectively, while the corresponding slit widths were 4.0 and 7.0 nm. Over the course of titrations and reagent additions, sample volumes were increased from 2.0 to 2.29 ml. maximum. Sodium carbonate was used for pH adjustments; maximum pH used was 7.54.

The lactate-NADH titrations were represented by plotting F_{obs} (corrected only for estimated light scattering; not for blank-value fluorescence) vs. added coenzyme concentration. From F_{obs} plots, values of interest were the fluorescence intensities of samples occurring when $x_o/P_o = 1.0$ and 0.0 (that is, F_{bk}). A plot of F_o vs. x_o for NADH in the lactate buffer (pH 7.54) was prepared, and found to be no different from that of NADH in pH 7.0 phosphate buffer without lactate. Fluorescence excitation spectra of enzyme samples before and after addition of reagents were taken; with all samples, blank-value fluorescence bands appeared to be largely compatible with NADH. These data are represented in Table 10 and Figure 17.

C. RESULTS

(1) Binding Capacity, Reduced Coenzyme

Figure 16 shows titrations of Hexanchus M_4 and chicken H_4 with APDH in 0.1 M oxalate. Both as a standard and a quality control for the reagents and procedure, the chicken H_4 LDH shows a sharp titration endpoint at $x_o/P_o = 4.0$, or within 2% of that value. The regression of Y with x_o/P_o (logarithmic) shows good linearity with a dependency slightly in excess of first power; an enhancement factor (E. F.)_{1.0} of 256 is observable as the Y-value at $x_o/P_o = 1.0$. In contrast, the Hexanchus samples show an approach to an endpoint near $x_o/P_o = 3.0$, dependent on concentration in a complex manner. The regression of Y with x_o/P_o is generally less than first-power; its irregular form shown with the most concentrated Hexanchus sample produces Y-values in excess of those of chicken H_4 at low x_o/P_o , suggesting partial applicability of an enhancement factor greater than 256, though the accumulated average of Y at $x_o/P_o = 1.0$ is only 208. The medial value of 230.5 has been used in the "apparent binding capacity" measurements of Section IV, performed under the same conditions as for Figure 16, but restricted to single points with vast excesses of coenzyme and relying on the calculation $\bar{n} = Y/(E. F.)$. Such simple \bar{n} calculations assume only two interacting components are present, and that (E. F.) represents a condition in

Figure 16. Fluorescence titrations with APDH

Conditions: 0.1 M potassium phosphate, 0.1 M potassium oxalate, pH 7.0; 15.2° C; maximum dilution from 2.0 to 2.15 ml.

$$Y = (X_o/P_o)(F/F_o - 1) (V/V_o)$$

- A. Chicken H₄, .993 mg. /ml. (upper solid line)
- B. Hexanchus M₄, .584 mg. /ml. (upper broken line; triangular points)
- C. Hexanchus M₄, .133 mg. /ml. (lower broken line)
- D. Hexanchus M₄, .067 mg. /ml. (lower solid line)

Wavelengths: excitation, 360 nm.; emission, 430 nm.

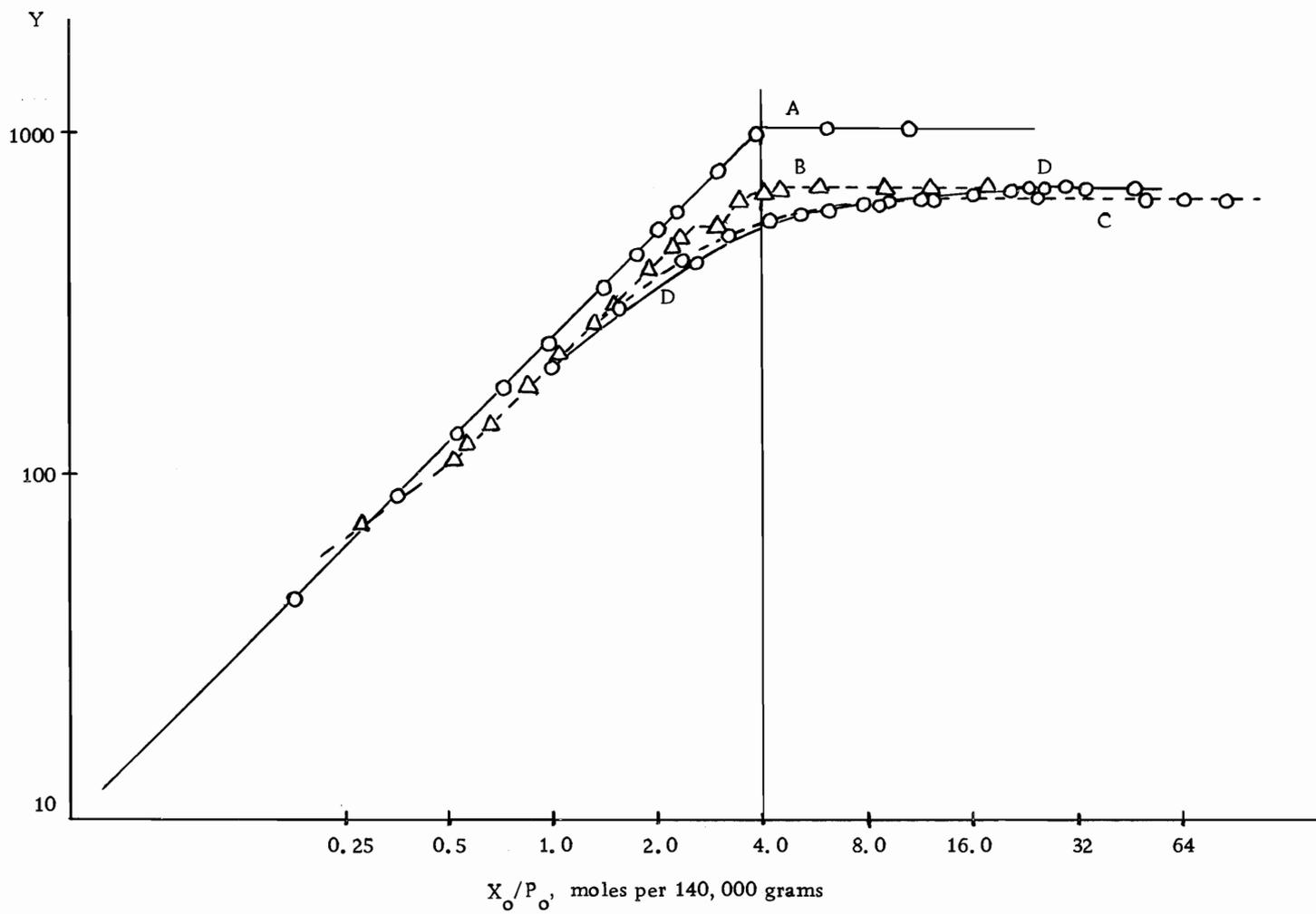


Figure 16. Fluorescence titrations with APDH.

which all ligand is totally bound to the enzyme. The ambiguity of the \bar{n} values is apparent from Figure 16: use of $(E. F.) = 256$ and the maximum Y observed with Hexanchus gives $\bar{n} = 2.71$, while $(E. F.) = 208$ gives $\bar{n} = 3.34$. The irregularities shown with the Hexanchus curves (especially in the endpoint region) prompt questions about homogeneity of the binding site population and the applicability of the $(E. F.)$ values at various points in titration. As for the gel filtration distributions in Section IV, what appears to increase \bar{n} may well also be increasing $(E. F.)$, so that an apparent purification beyond $\bar{n} = 3.0$ may not be verifiable through repetition at differing concentrations (Figures 10, 11; Table 4).

Repetition of the above procedures, substituting NADH for APDH, or omitting the oxalate from the reaction mixture, showed similar limits to \bar{n} , though the enhancement factors were lower in these cases, intermediate between APDH-oxalate and NADH without oxalate (Table 9). Initial fluorescence titration experiments were performed using silicone-treated cuvettes (Section II), though repetition of the procedures later showed that the same performance of the samples could be demonstrated without silicone-cuvettes at the enzyme concentrations of interest in this writing. (The data of Tables 9 and 10 are non-silicone.) A similar limit in binding capacity (Figure 16) was found for preparations ranging from 24-48% yield (Table 1), showing the equivalence of the various experimental

Table 7. Equilibrium dialysis: Hexanchus M₄ with APDH.

Sample ^(a)	Initial OD ₃₆₃	Final ^(b) OD ₃₆₃	Protein ^(c) OD ₃₆₃	Protein ^(d) $\frac{\text{Protein}}{n}$	Non-Protein ^(e) $\frac{\text{Non-Protein}}{n}$
A. 4.48	.330	.086	.201	1.65	1.80
B. 4.48	.329	.078	.201	1.77	1.98
C. 8.33	.614	.203	.401	2.74	2.73
D. 13.30	.980	.359	.533	2.50	2.59

(a) Designation refers to ratio of total molar amounts of coenzyme analogue APDH and tetrameric LDH added to dialysis chambers to begin the equilibration.

(b) Designates amount of coenzyme remaining in the non-protein chamber after 20 hrs. agitation at 20°C.

(c) Designates amount of coenzyme passing through membrane into the protein chamber during the 20 hrs. equilibration. A small protein precipitate generated during agitation was removed by centrifugation before measuring OD₃₄₀.

(d) Ratio of total coenzyme excess in protein chamber (over the non-protein chamber) to total protein. Calculation uses $a_m = 9100 \text{ cm}^{-1} \text{ M}^{-1}$ for APDH; $E_{280}^{1\%} = 13.4$, and MW = 140,000 for LDH. Correction to isosbesticity, + 5.5%.

(e) Same calculation, theoretically, though the amount of excess coenzyme transferred (corrected for coenzyme destruction) is estimated from the depletion of the coenzyme in the non-protein chamber.

General: Conditions for all was 0.1 M potassium phosphate buffer, pH 7.0. All calculations assumed constant solution volumes of 1.0 ml, for all chambers during equilibration. Protein concentration was held constant at 8.1×10^{-6} M in sample preparation and in calculations, while APDH was variable.

Table 8. Ultracentrifuge: coenzyme binding, Hexanchus M₄.

Ligand	LDH conc., mg. / ml.	Total ligand, M X 10 ⁶	Non-sedimenting ligand, %	\bar{n}	T, °C
1. APDH	3.29	142.3	73.5	1.60	18.9
			73.3	1.62	
			73.0	1.63	
2. NADH	7.24	149.0	39.7	1.74	22.2
			39.6	1.74	
			38.5	1.77	
3. APDH	8.01	429.0	75.1	1.87	20.0
			75.1	1.87	
			75.1	1.87	

Sample Preparations: (1) Enzyme stock, 13.47 mg. / ml. dialysate vs. .01 M Tris-HCl, pH 6.8, 500X, 26 hrs., 0-4°C; APDH buffer, .01 M Tris-H₂SO₄, pH 8; sample, .244 ml. LDH stock + .16 ml. APDH stock + .5 ml. 0.1 M pH 7.0 sodium phosphate + .096 ml. H₂O. (2) LDH stock, 1 hr. dialysate, 0-4°C, 500X, vs. .05 M sodium phosphate, 7.0; sample, 0.5 ml. LDH stock + 0.13 ml. NADH stock + 0.37 ml. H₂O, final pH 6.92. (3) LDH stock, as in #2; no H₂O used; final pH 7.20.

samples.

The results of equilibrium dialysis and ultracentrifuge-measured coenzyme binding offer non-fluorometric confirmation of the limited stoichiometry described above. The dialysis results show evidence of some complications possibly arising from protein precipitation and coenzyme destruction occurring during equilibration (Table 7), but suggest a maximum $\bar{n} = 2.7$, approximately. Despite various ratios of ligand to protein and substitution of NADH for APDH, the binding under ultracentrifuge conditions holds quite persistently to \bar{n} -values below 2.0 (Table 8). From these results, the limits to binding capacity observed in fluorometric comparisons of Hexanchus M_4 to other LDHs may be considered physically actual and not merely apparent due to changes in fluorescence quantum yields during titrations.

Table 9 shows the binding of NADH to Hexanchus M_4 compared to beef H_4 and a commercial beef heart LDH preparation. The summary of the beef H_4 titration curve agrees well with previous observations of its behavior (30). The approach to saturation of beef H_4 with added NADH is characterized by $\bar{n} = 3.47$ observed with $x_o/P_o = 4.0$, and the attainment of $\bar{n} = 4.0$ near $x_o/P_o = 6.0$. The Hexanchus M_4 , by contrast, shows leveling off with $\bar{n} = 2.0$, reaching complete saturation of two sites at $x_o/P_o = 4.0$, with almost no additional binding produced in proceeding to $x_o/P_o = 6.0$.

Table 9. Fluorescence titrations: NADH binding in various LDH preparations.

Sample (a)	$(F/F_{0.1})_{1.0}$ (b)	conc., mg. / ml.	$\bar{n}_{4.0}$ (b)	$\bar{n}_{6.0}$ (b)
1. Beef H ₄	6.02	.301	3.47	4.02
2. Beef heart LDH (comm.)	5.14	.405	3.08	3.50
3. <u>Hexanchus</u> M ₄	3.65	1.47	1.92	2.07
4. " "	3.17	.760	2.15	2.29
5. " "	2.37	.880	1.76	1.90
6. " "	3.35	.365	1.74	1.87

(a) Sample preparations: All samples diluted to 2 ml. in cuvette with .01 M sodium phosphate, pH 7.0; titration temperature 22.4°C; all samples titrated incrementally from 2.0 to 2.1 ml. with 0.1 ml. 1.2×10^{-3} M NADH over an average period of 20 minutes.

1. Beef H₄, .601 mg./ml. dialysate (1000X) vs. Tris-HCl, .01 M, pH 6.8, diluted 2X for titration.
2. Beef heart LDH, commercial, 1.62 mg./ml. dialysate (10,000X) vs. 1.0 M NaCl + .0001 M Tris-HCl, pH 6.56, diluted 4X for titrations.
3. Hex. M₄, 14.7 mg./ml. dialysate (500 X, 1 hr. only) vs sodium phosphate, .05 M, pH7.0, diluted 10X.
4. Hex. M₄, 7.6 mg./ml. dialysate (10,000X) vs. 1.0 M NaCl + .001 M Tris-HCl, pH 6.56, diluted 10X.
5. Hex. M₄, ammonium sulfate sediment resuspended in H₂O to 3.52 mg./ml., diluted 4X for titration. Clear preparation becomes turbid upon dilution into phosphate buffer.
6. Hex. M₄, dialysate vs. .05 M phosphate (500X) followed by treatment with excess APDH and 10 mg./ml. charcoal. Concentration after charcoal, 1.46 mg./ml.; diluted 4X.

(b) $(F/F_{0.1})_{1.0}$ is the average enhancement factor accrued in titration of a sample from zero to 1.0 moles per 140,000 grams protein. \bar{n} -values have units of moles/140,000 grams.

Large variation of the enhancement factor, $(F/F_0)_{1.0}$, is observed with various sample preparation methods; appreciable, though irregular, co-variation occurs in the accompanying \bar{n} values. The obstruction of binding sites could give cancellation of the fluorescence of added coenzyme; it would appear that some bound material in the preparation is oxidizing the added NADH. While some cases of $(F/F_0)_{1.0}$ loss are due to high OD or turbidity (sample 5), the Table 10 information gives evidence of a specific quenching reaction being latently present in the Hexanchus samples.

(2) Residual Fluorescence and its Effects

With the relatively low enhancements of fluorescence observed with NADH-LDH binding (compared to APDH or NADH with oxalate), residual (blank-value) fluorescence, F_{bk} , becomes conspicuous relative to that caused by adding coenzyme to LDH samples. Moreover, the blank-value excitation spectrum is comparable to that of NADH. Figure 17 shows an excitation spectrum of Hexanchus M_4 before adding coenzyme, its response to reduction by lactate, and with bound coenzyme. The blank-value spectra of beef M_4 and H_4 are very similar to the Hexanchus, and their proportion-wise increases in magnitude at 340 nm. upon addition of lactate are almost identical. Thus, the residual fluorescence appears to partially represent some distribution of endogenous nicotinamide adducts, whose preponderance

Table 10. Influence of lactate on $F_{bk}/F_{1.0}^{obs}$.

Sample ^(a)	pH	L-Lactate M	NADH conc. ^(b) M X 10 ⁶	(F/F _o) _{1.0} ^(c)	F _{bk} /F _{1.0} ^{obs} ^(d)
1. Beef M ₄	7.0	0.0	3.66	2.76	.052
2. " "	7.0	.091	3.32	1.53	.121
3. " "	7.54	.088	3.26	2.08	.095
4. Beef H ₄	7.0	0.0	2.86	6.02	.026
5. " "	7.54	.088	2.63	4.88	.044
6. <u>Hexanchus</u> M ₄	7.0	0.0	7.96	3.41	.140
7. " "	7.0	.091	1.40	.19	.712

(a) Sample preparations: Dialysates vs. 0.1 M sodium phosphate, pH 7.0, diluted to 2.0 ml. with same buffer, titrated to $X/P = 1.0$ with NADH after lactate and/or carbonate additions. Exceptions: sample 6 is average of samples 3 and 4 in Table 9; samples 4 and 8 are taken directly from Table 9.

(b) Molar concentration both of NADH and LDH tetramers ($X_o/P_o = 1.0$).

(c) Enhancement factor observed at $X_o/P_o = 1.0$. F-values here corrected for blank-value fluorescence.

(d) F_{bk} is blank-value fluorescence, corrected only for light scattering estimate. F_{1.0}^{obs} is total fluorescence (includes F_{bk}), corrected as above, observed at $X_o/P_o = 1.0$.

of reduced quaternary nicotinamide may be increased by reduction with lactate.

The measurement of F_{bk} from excitation scans is accomplished by recording the difference in apparent fluorescence at 340 nm. (usually at the band maximum) from that at 400 nm. (at a minimum point, where NADH-related fluorescence wanes and light scattering takes over). While this representation quite likely underestimates F_{bk} , an adequate deduction for light scattering is ensured. The strong F_{bk} of Hexanchus M_4 has an excitation spectrum maximum near 340 nm., somewhat obscured by protein fluorescence, yet morphologically similar to bound added NADH (Figure 17). Thus, the "quenching reaction" of Hexanchus M_4 may actually be a redox equilibration of the added NADH with some covalent adducts of quaternary nicotinamide moieties (other than residual reduced forms). The presence of oxidizing adducts could well result in attenuation both of observed enhancement factors and binding, and could endow LDHs from differing source (and modes of preparation and handling) with many finitely distinctive traits.

Table 10 provides support for the hypothesis of endogenous (residual) coenzyme material attenuating the fluorogenic binding of added coenzyme (even to the point of limiting or obstructing total number of sites available). While changes in F_{bk} upon lactate addition to the three LDHs are of similar proportions to the F_{bk} values themselves, they appear very different if expressed in proportion to

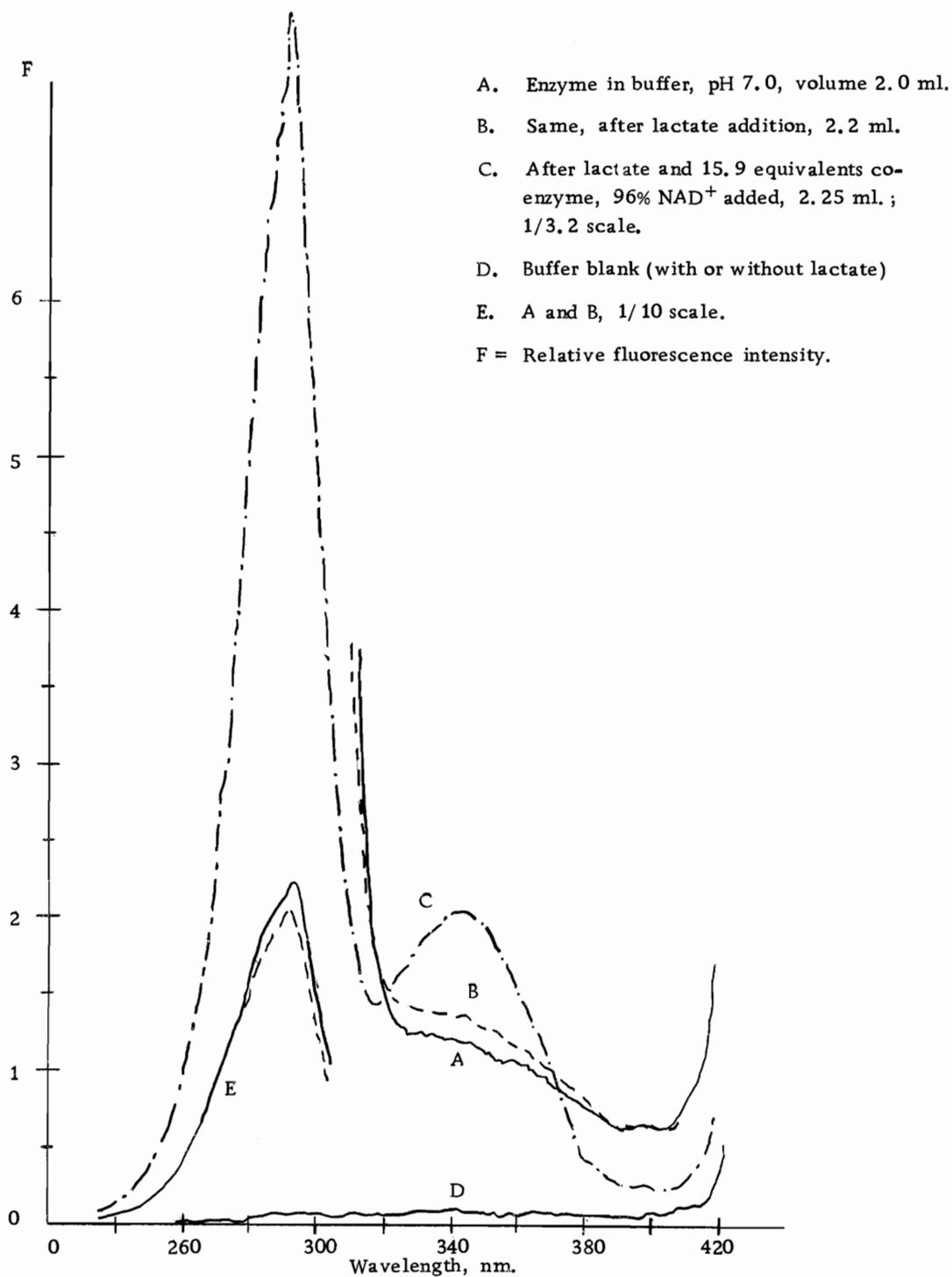


Figure 17. Fluorescence excitation spectra, Hexanchus M₄. Emission wavelength, 430 nm.

total observed fluorescence upon adding NADH up to $x_o/P_o = 1.0$.

Probably due to bound oxidizing coenzymic residuals, the native

Hexanchus M_4 in lactate shows cancellation of fluorescence of added

coenzyme, which goes along with an increase of $F_{bk}/F_{1.0}^{obs}$ to .712

(much higher an increase than for beef isozymes). Under these condi-

tions of dynamic equilibration of substrates, coenzymes, and residual

adducts, the increases in the ratio of F_{bk} to $F_{1.0}^{obs}$ upon reduction in

lactate buffer coincide consistently with losses in enhancement factor

$(F/F_o)_{1.0}$.

Table 10 unambiguously places the three enzymes in the order

Hexanchus, beef M_4 , and beef H_4 , in terms of their reducible residual

coenzyme content, an order which also describes the degree they tend

to fall short of complete saturation of 4.0 binding sites at $x_o/P_o = 4.0$,

given uniform concentrations of components in the comparison. Not

only do individual enzyme samples (regardless of the source) show

losses in F/F_o upon increasing $F_{bk}/F_{1.0}^{obs}$ by lactate addition, but

some inherent differences in NADH binding between sources and

species of LDH seem ascribable to the particular distribution of

residual coenzymic materials represented by F_{bk} . These residual

fluorescent moieties do very likely represent a potential obstruction

of further coenzyme binding, whose influence is not confined to

Hexanchus M_4 , where such obstruction is singularly obvious.

D. Discussion

The above results have been presented in light of a certain hypothesis: that coenzyme binding of LDH is partly regulated by another mode of interaction with coenzyme, which may be viewed as residual, or dialysis-resistant, coenzyme fluorescence. This mode, demonstrated in Figure 17, has such influence on the well-known fluorogenic coenzyme binding that introducing a mere 0.5 moles per 140,000 grams (apparent residual NADH content) would cancel the ability of Hexanchus M₄ to bind any additional NADH with fluorescence enhancement. Because all enzyme samples employed in the procedures of this section showed comparable amounts of blank-value NADH-related fluorescence (including chicken H₄), residual co-enzymic moieties may well be considered universally present influences in the establishment and maintenance of LDH structure and activity. Hexanchus M₄ exhibits a singular prominence in its reliance on coenzymic residuals, reflected in its "limited" titration capacity, its large increase in $F_{bk}/F_{1.0}^{obs}$ upon lactate addition, its sensitivity to structural modification with NADH (Section IV), and its reliance upon (and chemical reaction with) NADH in its behavior through purification (Section III).

Using the binding capacity results to develop the above hypothesis results requires a cautious overview of the binding process and supplementation by chemical analyses (Section VI). The loss in

F/F_0 would include losses of saturation due to binding-constant relationships; no corrections were made taking the protein concentration and ligand dissociation into account. Doubtlessly, this influence contributes substantial uncertainty to the interpretation of particular F/F_0 values, but does not begin to challenge the overall correlation: protein concentration showed no correlation with the overall trend. Previous studies, however, show considerable inconsistency in the observable binding behavior of beef M_4 and H_4 (64); probably much of the observed variation in classical binding constant determinations may be related to coenzymic residuals and their alteration.

VI. CHEMICAL ANALYSES

A. Introduction

Through the preceding section, a hypothesis regarding residual (non-dialysable) coenzyme-derived moieties inhabiting purified LDH samples in variable, substoichiometric quantities was brought out. The mode of coenzyme interaction thus defined appeared to be very influential in LDH structure-function relationships, and of multispecific applicability. Hexanchus M_4 appeared to offer an outstanding example of this occurrence of coenzyme residuals in LDH. With reports of similar residuals in LDH from pig (32) and lobster (31) on record, the Hexanchus residuals could hardly be considered a novel or necessarily unexpected occurrence. A high-priority objective of chemical analyses of Hexanchus M_4 is therefore investigation of the presence and nature of the apparently obstructive residuals.

With the amino acid analysis of Hexanchus M_4 , not only is routine characterization of interest (with concurrent handling of a beef M_4 preparation as a quality control), but also a search for distinctive features related to the above hypothesis. In view of some suggestions of impurity at the outset of this study, a primary goal is demonstrating an amino acid composition compatible with LDHs of the M_4 type. Thenceforth, the information so obtained is used to compare Hexanchus M_4 with other enzymes (including dogfish M_4), and to correlate

perturbations in amino acid composition with coenzyme fragment enrichments occurring during fractionation procedures.

Specific chemical methods are applied to demonstrate the presence of adenine, phosphate, pentose, and quaternary nicotinamide in Hexanchus M₄. The extent to which the residuals may be dialysis-resistant, the consistency of their proportions, and the possibility for their removal from the enzyme population is investigated. Some of the tests are concurrently run on beef M₄ and H₄.

With enzymic fractionations in concert with chemical methods, knowledge of any specific changes in coenzyme fragments (uptake, release, or alteration) covariant with denaturation is sought. The results set forth support the hypothesis of LDH structure-function integrity being influenced by coenzyme residuals, and preliminary data showing how the universality of the coenzyme residual effects might be more extensively investigated is provided. Extensive information on the influence of exogenously supplied coenzyme on the thermal stability of various LDHs is already available in literature (65).

B. Materials and Methods

(1) Amino Acid Analyses

Amino acid analyses of purified Hexanchus and beef M₄ were

performed on a modified Beckman model 120B amino acid analyser using the two-column procedures described by Spackman and others (66). Samples of Hexanchus and beef M₄ were dialysed against three changes of .01 M NaCl in separate containers, 0-4° C, over a 24 hr. period. Spectra of 24-fold dilutions of these samples are shown in Section IV, Figure 15; 0.1 ml. of beef M₄ and .05 ml. of Hexanchus were diluted to 0.5 ml. with distilled H₂O, thence to 1.0 ml. with 12 N HCl (6N final conc.) for hydrolysis; portions each of Hexanchus and beef M₄ were hydrolyzed for 20 and 70 hrs. at 110° C. Valine and isoleucine contents were determined from the 70 hr. results, and serine and threonine contents were corrected to zero hydrolysis time according to tables in the handbook (67). Cysteine contents apparent at 20 and 70 hrs. hydrolysis for the two enzymes are shown in Tables 11 and 12.

A low molecular weight fraction of Hexanchus M₄ was eluted from a gel filtration column; a 0.5 ml. portion was diluted to 1.0 ml. with 12 N HCl for a hydrolysis of 20 hrs. Amino acid analysis was performed as above, except that a single-column system using Hamilton HP-AN 90 resin was adapted. Residues were eluted with the following buffers:

pH 3.49 0.2 M citrate

pH 4.30 0.2 M citrate

pH 6.20 1.8 M Na⁺, .066 M citrate

An overall elution time of four hrs. was employed.

(2) Detection of Adenine

Because of its characteristic strong absorbance at 260 nm. and its affinity for charcoal in aqueous solution, the release of adenine moieties in fractionations could be indicated by difference spectroscopy. Spectra were compared before and after treatment with Norit A (68).

(3) Nicotinamide, Quaternary

NAD^+ is known to form a cyanide adduct in solutions 0.5-1.0 M in cyanide ion, pH near 11, which (similar to NADH) has an absorbance maximum at 327 nm. (34). The apparent NAD^+ content of LDH dialysates was measured fluorometrically, using NAD^+ as a standard, assuming no fluorescence enhancement occurred upon interaction of the adduct and LDH at pH 11. The wavelength and slit widths used were the same as for NADH work (Section V). Blank-value samples of LDH in pH 10.25 sodium carbonate were also prepared.

With long-term observation of the cyanide samples (LDH and NAD^+ standards), the initially formed adduct fluorescence was decreased markedly within 24 hours; the polymerization of cyanide which ensued, as described by Oro and Kimball (69), caused all samples to develop a fluorescent component with a spectrum very similar to

NADH, which replaced and overwhelmed any evidence of the initially formed adduct in ten day's storage time (20-22° C.). The graphical correction to zero time was therefore necessary to determine initially formed cyanide adduct in samples measured more than one hour after cyanide addition. Prolonged exposure to cyanide or pH 10.25 carbonate resulted in complete loss of activity in Hexanchus and beef M₄; beef H₄, however, retained 20% of its activity at pH 10.25 and 5% in cyanide after 500 hours.

(4) Phosphate

Modifications of the original Fiske-SubbaRow method were adapted for Method I (71, 72). A typical procedure is as follows:

(a) Mix 0.1 ml. sample (or more, when taken to dryness and ashed) with 0.1 ml. 14.4 N H₂SO₄, 10% NaClO₄ in a Pyrex tube (ashing at this point, if needed); dilute samples to 0.9 ml. with H₂O; hydrolyze samples 1 hr. at 100° C. (for pyrophosphate breakdown).

(b) Add 0.1 ml. 6.6% ammonium molybdate (Mallk. Rgt. 3420); mix and record OD₈₀₀ as a turbidity baseline (if necessary), then add 0.1 ml. 5% ascorbic acid (Mallk. lot ASU).

(c) After about 30 minutes at room temperature, record OD₈₀₀ and estimate PO₄ relative to standards and blank (also carried through ashing procedure).

Method II used the malachite green method of Itaya and Michio

(72) with the magnesium nitrate ashing method of Ames (73), scaled proportionately to a final sample volume of 1.0 ml. Special reagents: Malachite Green, Matheson, Coleman, and Bell, B329, MX95 (94%); Tween 20; magnesium nitrate, Baker 547 7075. Considerable time-dependence (inconsistent, both positive and negative) was observed in OD_{660} readings; values for samples, standards, and blanks were recorded graphically, and all PO_4 estimates were based upon values occurring at 60 minutes.

(5) Pentose

Modifications of the photometric method of Brin (74) were adapted using Bial's Reagent (75), employing two parts reagent mixed with one part sample:

Orcinol (5-methyl resorcinol, MC&B MX1411)	0.15 gm.
Concentrated HCl	50.0 ml.
Ferric chloride (10%, Mallk. Rgt. 5092)	0.10 ml.

The above was prepared fresh immediately before use, and discarded after several hours. Standards used were d-ribose (H. -LaRoche lot 112 610312), adenosine (Calbiochem lot 30284), nicotinamide mononucleotide (Sigma lot 73C-75-00), and NAD^+ (Section II). Identical responses for all pentose standards were obtained under conditions of interest; the orcinol condensation product arising from LDH residuals showed the same absorbance spectrum (maximum near 670 nm. ;

shoulder at 600) as the pentose standards. Hexose samples, by contrast, showed no such color production in Bial's Reagent. Pentose was quantitated only in samples producing spectra in close agreement with the pentose standards. Samples 0.5 to 1.0×10^{-6} M (lower limit for useful data) showed 16% expected error for individual determinations. Samples near lower limits of detection were quantitated using measurements of OD_{800} and OD_{670} , where OD_{800} was used to correct the baseline values (taking very slight turbidity differences into account).

(6) Enzyme Fractionation

The release or alteration of coenzyme fragments in conjunction with enzyme denaturation was investigated relying mostly upon induced formation and removal of a protein precipitate. Denatured supernatants were prepared by the following methods:

Method I: nine parts sample and one part 14.4 N H_2SO_4 + 10% $NaClO_4$ were mixed and allowed to stand 15-30 minutes at room temperature; precipitates were removed by centrifugation; supernatants were neutralized with 0.13 gm. $NaHCO_3$ per ml. and stored at 0-4° C.

Method II: Samples were diluted to various concentrations in neutral buffers and heated to 57° C for 15 minutes in a water bath, then abruptly cooled in an ice bath. Precipitates were removed by

centrifugation and supernatants stored at 0-4° C.

Other methods: Samples of Hexanchus M₄ and commercial beef heart LDH were treated with trypsin (Worthington TRL 33N633, lyophilized) for 24 hours at room temperature in pH 7.55 Tris-HCl buffer; proportion of trypsin to LDH was 1/200 w/w. Higher molecular weight materials were removed by heat treatment (Method II above), and supernatants were stored 0-4° C.

Ten parts chicken H₄ (resuspended ammonium sulfate precipitate in pH 7.55 Tris-HCl buffer, .01 M) were mixed with one part concentrated ammonium hydroxide, heated gently over a bunsen burner until bubbles first appeared, then cooled abruptly in an ice bath. Precipitate was removed by centrifugation, and supernatant was stored at 0-4° C.

Parameters of interest in preparation of denatured supernatants were conditions (buffers) of dialysis, concentrations of enzyme upon inducing denaturation, and the apparent enzyme concentrations in supernatants. OD₂₈₀ was used for these estimates; the ratio OD₂₆₀/OD₂₈₀ was also of some interest in denatured supernatants. Molar enzyme concentration estimates were calculated using $E_{1\text{ cm.}}^{1\%} = 13.4$ for Hexanchus M₄ (Section V) and a molecular weight of 140,000 was assumed for all LDHs (Section IV). Such calculations were performed knowing that such a molecular weight and extinction coefficient in actuality were not rigorously accurate for the supernatants; the

apparent molecular concentrations were of primary interest.

Bio-Gel P2 was hydrated and equilibrated with .01 M Tris-HCl, pH 7.55, and packed into a column of .95 cm. diameter with final bed volume of 26 ml. (36.7 cm. high). A funnel was affixed to the column, through which samples of 0.5 ml. were applied and washed in, and in which elution buffer was placed to allow continuous flow with 50-55 cm. hydrostatic pressure at about 50 ml./hr. at room temperature. A standard mixture of BSA, NAD^+ , and tryptophan was applied and eluted with .01 M Tris, and fractions of 1.0 ml. were collected. Elution was monitored using OD_{220} , selected because of high sensitivity to passage of many types of components, and elution profiles were analyzed graphically. The molecular weight estimation using $V^{-1.5}$ (as in Section IV) allowed the very accurate value 662 to be established for the modal elution volume of NAD^+ , using tryptophan as a standard (mol. wt. 204). Upon reducing the buffer concentration and pH (.0005 M, pH 6.7), NAD^+ appeared to undergo self-association (as well as exhibiting the expected heterogeneity (76), reaching molecular weights (modal) as high as 3500, while the elution volume of tryptophan and the relative symmetry of its distribution remained unchanged. (BSA was not included in samples showing the reversible association of NAD^+).

Bio-Gel P20 was hydrated (.2 M NaCl, 90-100° C, 2 hrs.), then thoroughly rinsed and packed with glass-distilled H_2O . The gel

was (rather loosely) packed in a Buchner funnel to a final bed volume of 26 ml. with a diameter of 3.0 cm., height, 3.67 cm.; a diaphragm of Whatman #1 filter paper (2 layers) was secured over the bed surface with a retaining ring improvised from Tygon tubing to prevent gel disruption through sample and buffer applications. The bed surface was flattened so that a sample of 0.5 ml. would wet the entire bed surface when applied; a useful buffer reservoir space of 21 ml. remained in the funnel over the gel. Flow was controlled by capping the funnel orifice with a heat-sealed piece of large Tygon tubing; removal of the cap allowed dropwise hand-collection of fractions during which the eluate flowed in a single fine stream from the sintered glass disc edge to the orifice region of the outlet tube. Capping the orifice halted the drainage without filling the outlet tube; thus, the gel was continuously kept under a head of fluid. An average flow rate of 150 ml./hr. was easily maintained. OD_{280} was used to monitor elution, and distributions resulting were analyzed graphically. (Between runs: 1 M NaCl wash).

C. Results

Tables 11 and 12 show the amino acid analyses of Hexanchus and beef M_4 LDH, giving the micromolar amounts of each residue measured at 20 and 70 hours' hydrolysis. Trace amounts of similar unidentified components are found in the initial elution period of the

Table 11. Amino acids of Hexanchus M₄ LDH.

	micromoles amino acid			Micrograms	Residues ^e	% Deviation
	20 hrs	70 hrs	Composite			
Lys	. 3306	. 3126	. 3216	46.95	102.2	2.8
His	. 1266	. 1542	. 1404	21.76	44.6	9.8
NH ₃ ^d	. 3324	. 3758	. 3214	5.46	102.2	--
Arg	. 1302	. 1284	. 1293	22.50	41.1	0.7
Asp	. 4230	. 4386	. 4308	57.30	136.9	1.8
Thr ^c	. 2268	. 2058	. 2279	27.12	72.4	0.9
Ser ^c	. 2322	. 1794	. 2372	24.91	75.4	3.1
Glu	. 2958	. 2952	. 2955	43.44	93.9	0.1
Pro	. 1476	. 1524	. 1500	17.25	47.7	1.6
Gly	. 3300	. 3342	. 3321	24.91	105.6	0.6
Ala ^b	. 3642	. 3654	. 3648	32.47	116.0	0.2
Cys ^a	. 0396	. 0198	. 0396	4.75	12.6	--
Val ^a	. 3642	. 3864	. 3864	45.21	122.8	--
Met ^a	. 0870	. 0870	. 0870	12.96	27.7	0.0
Ilu ^a	. 2346	. 2520	. 2520	33.01	80.1	--
Leu	. 3156	. 3222	. 3189	41.78	101.4	1.0
Tyr	. 1032	. 0900	. 0966	17.48	30.7	6.8
Phe	. 1290	. 1320	. 1305	21.53	41.5	1.1
U ^{b, f}	. 0414	. 0132	. 0414	4.89	13.2	--
U ₁	. 0114	. 0096	. 0105	1.24	3.3	--
U ₂						
SUM	4.2654	4.2522	4.3139	506.92	1371	Avg. 2.2%
SUM ^g			4.402	524.52		
SUM (hydrolysis-corrected)				445.3		

a) 70 hr. value used to estimate complete release.

b) 20 hr. value used in estimate.

c) correction to t=0 according to Handbook, 2nd Ed., CRC, p. C-281 (67).

d) empirical logarithmic extrapolation to t=0.

e) moles per 140000 grams protein, assuming 440.4 mcgm. total protein in hydrolyzate

f) unidentified peaks quantitated using Asp conversion constant in H X W calculation. U₂ is probably cysteic acid, or mostly so.

g) Includes tryptophan estimate of 2.0 mole %.

Table 12. Amino acids of Beef M₄ LDH.

	micromoles amino acid			micrograms	Residues ^d	% Deviation
	20 hrs	70 hrs	Composite			
Lys	.4888	.4806	.4847	70.77	104.4	0.8
His	.1632	.1422	.1527	23.67	32.9	6.9
NH ₃ ^b	.5380	.6690	.5380	9.15	115.8	--
Arg	.2092	.2046	.2069	36.00	44.6	1.1
Asp	.5696	.5682	.5689	75.66	122.5	0.1
Thr ^c	.2404	.2238	.2446	29.11	52.7	0.4
Ser ^c	.3428	.2616	.3480	36.54	74.9	3.7
Glu	.5716	.5700	.5708	83.91	122.9	0.1
Pro	.2396	.2424	.2410	27.72	51.9	0.6
Gly	.4748	.4764	.4756	35.67	102.4	0.2
Ala ^b	.4364	.4272	.4318	38.43	93.0	1.1
Cys ^a	.0916	.0660	.0916	10.99	19.7	--
Val ^a	.5064	.5592	.5592	65.43	120.4	--
Met ^a	.1156	.0918	.1037	15.45	22.3	11.5
Ilu ^a	.3732	.4266	.4266	55.88	91.9	--
Leu	.6140	.6222	.6181	80.97	133.1	0.7
Tyr	.1296	.1188	.1242	22.48	26.7	4.3
Phe	.1292	.1314	.1303	21.50	28.1	0.8
U ^e	.0236	.0210	.0223	2.63	4.8	5.8
U ₁ ^a	.0012	.0072	.0072	.85	1.6	--
U ₂						
SUM	6.2588	6.3102	6.3462	742.81	1367	Avg. 2.5
SUM ^f			6.462	766.5		
SUM (hydrolysis-corrected)				650.2		

a) 70 hr. value used to estimate complete release.

b) 20 hr. value used in estimate.

c) Correction to t=0 according to Handbook, 2nd Ed., CRC, p. c-281 (74).

d) Moles per 140000 grams protein, assuming 650.19 mcgm. protein in hydrolysate.

e) Unidentified peaks quantitated using Asp conversion constant in H X W calculation. U₂ is probably cysteic acid, or mostly so.

f) Includes tryptophan estimate of 2.0 mole %.

Table 13. Amino acids: comparative profile, moles per 140,000 grams.

	Hexanchus ^c	Dogfish ^a	Dogfish ^b	Beef ^a	Beef ^c	Pig ^d			Hexanchus ^f
	M ₄	M ₄	M ₄	M ₄	M ₄	GPD-M	M ₄ ^e	H ₄ ^e	M ₄
Lys	102.2	119.7	115.1	103.0	104.4	104	105.2	98	102.2
His	44.6	42.3	41.6	33.0	32.9	44	38	26	44.6
Arg	41.1	29.6	34.2	42.0	44.6	40	39.2	32	41.1
Asp	136.9	132.6	131.1	127.0	122.5	152	121.2	135.2	136.9
Thr	72.4	55.9	50.6	48.0	52.7	88	48	51.2	65.9
Ser	75.4	100.9	101.9	87.0	74.9	76	88	89.2	87.5
Glu	93.9	97.0	101.1	121.0	122.9	72	119.2	126.0	93.9
Pro	47.7	39.8	46.7	51.0	51.9	48	44	46	47.7
Gly	105.6	94.1	96.8	100.0	102.4	128	103.2	92	105.6
Ala	116.0	76.4	86.6	78.0	93.0	128	82	82	97.3
Val	122.8	124.2	128.7	115.0	120.4	136	129.2	135.2	117.3
Met	27.7	41.4	40.8	32.0	22.3	36	33.2	33.2	39.7
Ilu	80.1	78.5	73.9	91.0	91.9	84	86	88	80.1
Leu	101.4	130.3	128.7	136.0	133.1	72	139.2	136	103.6
Tyr	30.7	25.7	27.6	29.0	26.7	36	28	27.2	33.3
Phe	41.5	25.7	25.7	29.0	28.1	56	28	22	42.8
Error	± 2.2%	± 2.3%		± 3.3%	± 2.5%				
SUM	1240	1214	1221	1222	1225	1300	1232	1219	1240

a) Pesce et al., *J. Biol. Chem.* **242**, p. 2159, 1967 (21).

b) Taylor et al., *Proc. Nat. Acad. Sci. USA*, vol. 70, no. 6, p. 1790-1794, 1973 (77).

c) This laboratory

d) Glyceraldehyde-3-phosphate dehydrogenase, pig muscle; rabbit muscle is very similar (78, 79).

e) Mammalian-average lactate dehydrogenases, Goldberg, 1972 (80).

f) Hexanchus M₄, residue values normalized according to proportion of beef M₄ values of Pesce (above) to those of this laboratory.

longer column (acidics and neutrals) for both beef and Hexanchus, indicated U_1 and U_2 . Hexanchus M_4 appears to have 2.5 times as many "U" residues as beef M_4 , a ratio similar to the relative pentose contents of their unfractionated dialysates. The average errors of individual residue values for both enzymes (based on deviation of 20 and 70 hour values from average of residues not expected to change) are similar and are significantly less than the expected deviation reported in literature for beef M_4 (Table 13).

The multiple comparisons made possible in Table 13 (putting cysteine, tryptophan, and ammonia aside) show that Hexanchus M_4 , as isolated according to Table 1, bears a considerable resemblance to glyceraldehyde-3-phosphate dehydrogenase (GPD) of the skeletal muscle variety. The ratio of threonine to serine, the quantities of non-tryptophan aromatics, and total non-aromatic hydrophobics all point to an enzyme population intermediate in character between GPD-M and M_4 LDH; the observed behavior in interaction with co-enzymes (Section V), especially that suggesting the retention of some form of oxidized coenzyme through purification, supports this observation.

The absorption spectrum of Hexanchus M_4 typically shows a ratio OD_{260}/OD_{280} .59 to .62, while similar dialysates of beef M_4 and H_4 will give the value .52. Taking the difference between these ratios, it appears that Hexanchus M_4 has enough additional OD_{260} to

account for 1.0 mole adenine per 140,000 grams protein (using $E_{1\text{ cm}}^{1\%} = 13.4$ for LDH and $a_m = 15,400$ for adenine (81). Attempts to remove the alleged ligand with charcoal treatments (68) do not decrease the spectrometric ratio; evidently, Hexanchus M_4 does not readily shed intact coenzyme while remaining mostly in soluble, tetrameric form. Exposure of dialysed Hexanchus M_4 to high charcoal concentrations will cause a large proportion of the soluble enzyme to be adsorbed, while leaving the remaining enzyme with an increased ratio OD_{260}/OD_{280} . (In Table 14, samples #4 and 5 show substantial increases in pentose levels, showing that pentose and OD_{260}/OD_{280} increase together upon fractionation with charcoal.)

However, in the preparation of certain denatured fractions, adenine appears to be released in a form that may preferentially bind to charcoal. Difference spectra, "before" minus "after charcoal," show the loss of components sometimes giving well-defined maxima near 260 nm.

Hexanchus M_4 , unlike the beef enzymes, had an absorption spectrum suggestive of containing $\bar{n} = 1.0$ moles adenine. To agree with this finding, a cyanide adduct determination might show 1.0 moles NAD^+ (apparent) per 140,000 grams protein (apparent from OD_{280}) with a fluorescence spectrum compatible with NAD^+ -cyanide. Cyanide studies with dialysates of intact Hexanchus M_4 (pH 7.0, 0.1 M sodium phosphate, 8000 X, 24 hours, 0-4° C) showed only

0.38 moles NAD^+ (apparent) per 140,000 grams, however. Dialysates of beef M_4 and H_4 (same conditions; separate containers) showed that M_4 had a trace of NAD^+ (only .05 moles apparent NAD^+ per 140,000 grams, spectrum not well defined), while H_4 gave no evidence of adduct formation. Thus, in terms of immediate NAD^+ -cyanide adduct formation, Hexanchus M_4 , beef M_4 , and beef H_4 maintain the same order as seen with proportions of residual coenzyme fluorescence in Section V, with Hexanchus M_4 showing a similar perturbation from the value set for the beef enzymes (Tables 9 and 10).

Table 14 shows the resistance of pentose and phosphate to removal by dialysis in the three enzymes mentioned above. Pentose and phosphate in Hexanchus M_4 dialysates may be lowered to levels comparable to the apparent NAD^+ content deduced from initial cyanide adduct formation ($\bar{n} = 0.38$), but the apparent adenine content and the amount of quaternary nicotinamide apparent from lactate reduction are in excess of these values (Section V).

Beef H_4 , despite appearing to be relatively free of cozymic obstruction in Section V, now appears to equal or exceed Hexanchus M_4 in its residual pentose and phosphate. Generally speaking, all LDHs here considered contain residual coenzyme components, but the mutual ratios of detectable pentose, phosphate, adenine, and nicotinamide in stable dialysates are seldom compatible with NAD^+ .

Table 15 shows pentose release into supernatants of LDH

Table 14. Dialysis-resistant pentose and phosphate under various conditions.

Sample	Dialysis (a)	Phosphate:		Pentose:
		Method	\bar{n} (b)	\bar{n} (b)
<u>Hexanchus</u> M ₄	1	II	.60	--
Beef H-LDH (comm.)	1(d)	II	1.20	--
<u>Hexanchus</u> M ₄	2	II	.55	.58
Beef H-LDH (comm.)	2(d)	II	.90	.52
<u>Hexanchus</u> M ₄	3	II	.82	.74
Beef M ₄	3	II	.55	.16
Beef H ₄	3	II	.84	.32
<u>Hexanchus</u> M ₄	4	--	--	1.03
" "	5	--	--	.96
G3PD ^(c) , inactive	6	I	1.52	1.99
<u>Hexanchus</u> M ₄	6	I	.84	.40
Beef M ₄	6	I	.33	.13
Chicken H ₄	6	I	.17	neg./trace
G3PD ^(c)	Ammonium sulfate susp.			8.12
<u>Hexanchus</u> M ₄	"	"	"	.73±.30
Beef H-LDH (comm.)	"	"	"	1.71

- (a) Dialysis conditions: Generally 0-4°C, with slight agitation in neutral aqueous buffer, using dialysis tubing (Section II).
- 1 M NaCl with .001 M Tris-HCl, pH 6.56; 10,000X; separate containers. (Fluorescence titration, Table 9, Section V.)
 - Same solutions as above, with additional buffer change (330,000X); exterior phosphate decreases 5X to 9.2×10^{-6} M.
 - Dialysed in same container, .01 M Tris-HCl, pH 6.8; exterior phosphate, 27.4×10^{-6} M; exterior pentose 0.64×10^{-6} M. (330X, rel. to total sample volume.)
 - Dialysate, .05 M sodium phosphate, pH 7.2; enzyme remaining after adsorbing more than 50% with 20 mg./ml. charcoal.
 - Same as #4, except #4 contained four equivalents of APDH prior to charcoal treatment.
 - Exhaustive dialysis vs. .01 M NaCl; same as for Figure 13 (Section V) and for amino acid analysis.
- (b) Calculations of \bar{n} values: the differences of the sample concentrations of pentose and phosphate over those of the exterior buffer are expressed in moles per 140,000 grams protein for all samples.
- (c) G3PD: rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Sigma, ammonium sulfate suspension, 10.7 mg./ml.).
- (d) Commercial beef heart LDH, same as in Table 9, Section V.

Table 15. Pentose release upon precipitate formation.

Sample (a)	"Before" OD ₂₈₀	"After": (b)	Ratio (c)	Pentose, M X 10 ⁶	\bar{n}_{su} (d)	\bar{n}_t (e)
<u>Hexanchus M₄</u>						
1	8.54	.268	.959	3.06	2.14	.067
2	7.08	1.06	.75	14.69	2.60	.39
3	1.59	.272	1.11	4.83	3.33	.57
4	1.39	.310	1.06	6.00	3.63	.81
5	.903	.247	.64	2.88	2.19	.60
6	1.85	.383		7.1	3.48	.72
7	1.94	.450		3.4	1.42	.33
8	14.95	3.14		75.28	4.49	.94
<u>Hexanchus M₄; denatured supernatant</u>						
9	8.14	3.39	.99	84.5	4.67	1.94
10	8.14	5.32	.91	139.8	4.93	3.22
<u>Beef H₄</u>						
11	4.65	.938	.73	11.8	2.64	.53
12	1.91	.733	.78	15.4	4.41	1.69
13	.950	.808	.58	1.44	.37	.31
<u>Beef M₄</u>						
14	.788	.638	.56	1.44	.41	.33
<u>Trypsin +</u>						
<u>Hexanchus M₄</u>	4.88	1.86		19.9	2.01	.77
<u>Beef H₄</u>	3.86	2.09		20.7	2.08	1.13

(a) Sample preparations: 1, 3, 11, 12: Exhaustive dialysates of Hexanchus M₄ and commercial beef heart LDH, pH 7.6, .01 M Tris, precip. method II (heat treatment). 2, 4: Method I (ClO₄⁻). 6, 7: Same as samples 4 and 5, Table 14, method II. 8: Ppt. with .12 N HCl. 9, 10: Method I, except #10 had H₂SO₄ added before ClO₄⁻. 5, 13, 14: Same as samples with dialysis #3, Table 14, method II. Trypsin samples: ammonium sulfate suspensions, comm. beef heart LDH. 11, 12: also comm. beef heart LDH.

Table 15. (Continued)

- (b) OD_{280} of samples measured before and after precipitate formation and removal. Values given are corrected approximately for turbidity as described in Section V, part B-6. Values given proportioned to 1 cm. path. OD_{280} "before" represents protein concentration at which precipitation actually was induced.
- (c) "Ratio" refers to OD_{260}/OD_{280} , where both OD measurements are corrected as above. The ratio .52 for the beef isozymes and .59 for Hexanchus may be considered typical of neutral dialysates of the unaltered, pure enzymes.
- (d) \bar{n} is the apparent stoichiometric ratio of pentose to protein in the supernatant, where protein concentration is calculated simply from the OD_{280}^{su} "after", using $E_{1\%}^{1\text{cm}} = 13.4$ for Hexanchus M_4 , 140,000 grams per mole. This is an apparent pentose ratio, since much of the measured OD_{280} in the supernatant cannot be due to LDH protein.
- (e) \bar{n}_t is stoichiometric ratio of pentose (measured after precipitation) based on total original protein concentration (before inducing precipitation).

samples denatured by precipitation. In some preparations (Sample #8 , especially), total pentose apparently extracted, \bar{n}_t , significantly exceeds the original \bar{n} of pentose in the starting material (given in Table 14). Concurrently, there is also a tendency for detectable pentose to coprecipitate with the aggregated enzyme, and produce a loss in \bar{n}_t (as in sample #1). These processes combine in the dynamics of denaturation (and probably during the course of isolation and preparation of active enzyme samples as well) to produce a complex, implicitly defined relationship between \bar{n}_t of supernatant pentose and fraction of OD_{280} lost in precipitation. This mode of pentose binding is such that maintaining the tetrameric structure causes interference with pentose detection when the sample dissolves in Bial's Reagent (9N HCl), while the previous precipitate removal completely removes some bound pentose from the reaction mixture. Yet, dissociation and cleavage of enzymes can release bound pentose into supernatants in an easily detectable condition. Hexanchus M_4 and the beef isozymes all follow the same general pattern of behavior, showing in all the latent ability of pentose to be "created" coinciding with the dismantling of tetramers. Apparently, both Hexanchus M_4 and beef H_4 contain no less than 2.0 moles pentose per 140,000 grams protein, bound to the enzyme in a covalently altered state.

Further investigating pentose enrichments in denatured supernatants, some of these preparations were further fractionated on the

Bio-Gel P2 column. Perchlorate and ammonia supernatants of chicken H₄ (same dilution of sample) each showed an OD₂₂₀-average molecular weight of around 720; this appeared as a single peak (predominantly) with the ammonia preparation, and with the same amount of material split into peaks (primarily) of 1000 and 520 molecular weight with the perchlorate. This amount of material thus eluted would represent 15 moles NAD⁺ per 140,000 gm., using only OD₂₂₀ and an NAD⁺ standard run for interpretation. However, the dialysates of chicken H₄ tested minimally for both pentose and phosphorus (Table 14), and no release of pentose was detected in the P2 elution. Nevertheless, some F_{bk} was observed for chicken H₄ samples in amounts comparable to the beef enzymes (Section V, Table 10). By comparison, Hexanchus M₄ showed an OD₂₂₀-average molecular weight of 900 for the perchlorate supernatant.

Using cyanide fluorometry to monitor the P2 elution of the Hexanchus M₄ perchlorate supernatant, a considerable proportion of the nicotinamide in the supernatant was distributed into multiple fractions with consequent dilution below the limits of reliable detection. However, a barely discernible localization with an approximate molecular weight of 260 was obtained with a relatively concentrated sample; this could be a nicotinamide nucleoside moiety.

Supernatants from heat and trypsin treatment samples showed no distinct localizations of low molecular weight components or high

proportions of the coenzyme fragments. In such samples, materials of higher molecular weight predominated.

Hexanchus M_4 dissociates extensively upon gel filtration with Bio-Gel P20 (also with P2, but not as obviously due to closeness of the fractions of interest to the void volume and their consequent crowding/reassociation). Hexanchus M_4 , already in a partly destabilized condition (Section IV), partitions readily under P20 fractionation. While ion-exchange effects doubtlessly contribute to the retardation here observed, protein cleavage also occurs. In Figure 18, the cumulative elution plots (monitoring the elution with OD_{280} , pentose, and kinetic activity) show that gross deactivation accompanies retardation by the gel, with less than 1% of the applied activity concentrated near the void volume. If the narrow zone of activity elution represents a small amount of LDH remaining in active, tetrameric form, then the OD_{280} -average and pentose-average elutions of Hexanchus M_4 both show an apparent molecular weight of 12,000 daltons.

The pentose distribution does not correspond to the OD_{280} curve in the P20 profile (Figure 18), but shows large enrichment of pentose content relative to OD_{280} at 12,000 apparent molecular weight for Hexanchus M_4 (Fraction #10). The accumulated elutions of pentose and OD_{280} give an apparent stoichiometry of 2.0 moles per 140,000 grams for the whole sample, verifying the latent stoichiometry

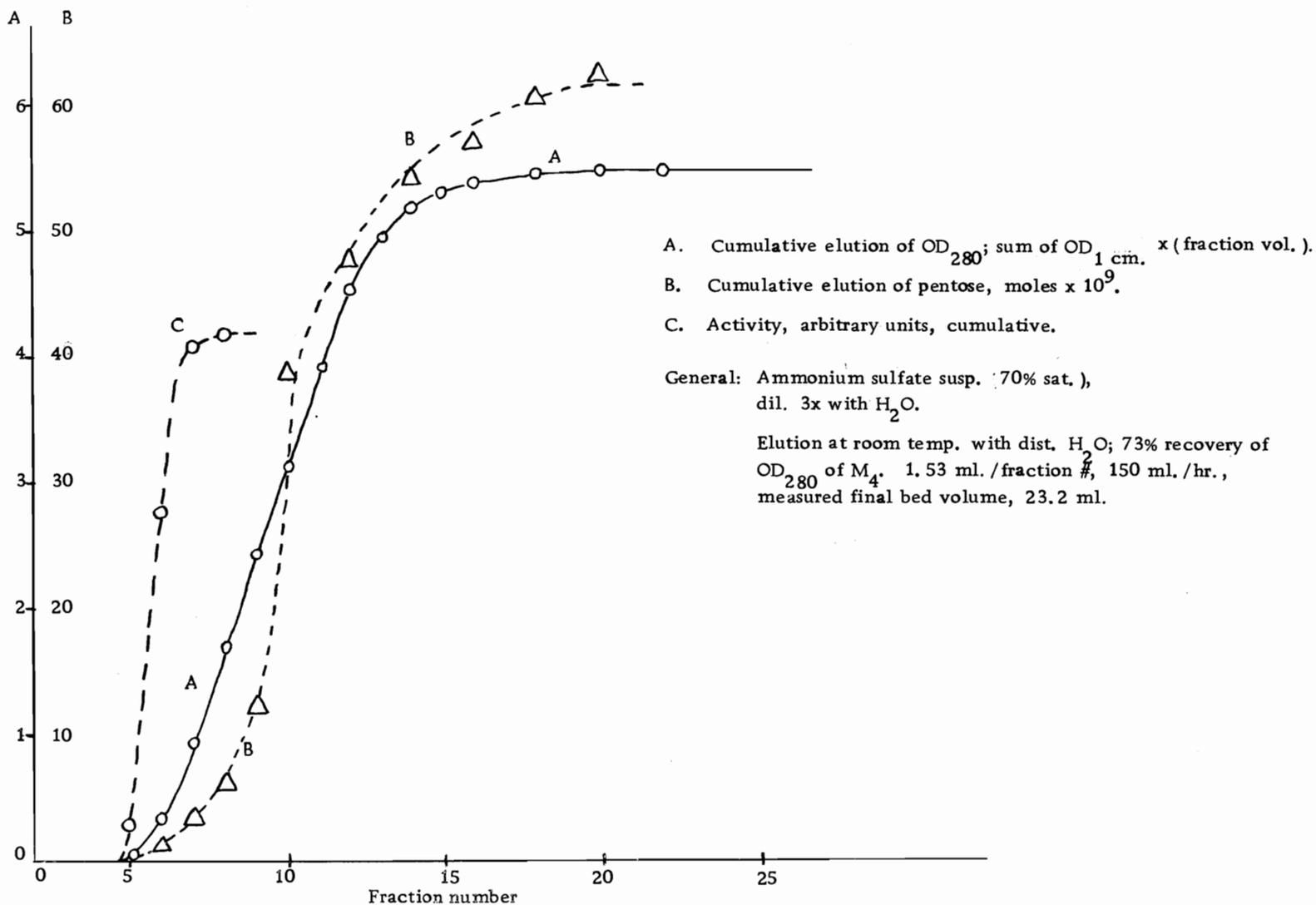


Figure 18. Hexanchus M_4 fractionation: Bio-Gel P20.

deduced from Table 15.

The observed enrichments of pentose (with certain amino acids) occurring at intermediary elution volumes suggests that indeed its incorporation is affecting protein structure, in that breakdown into extreme low molecular weight fractions gives a loss of elevated stoichiometry. Yet approaching the void volume (return to native tetrameric structure) shows a loss of detectible pentose apparently contingent upon establishing the active M_4 . This result repeats the multiphasic covariance of denaturation with covalent alteration and release of coenzymic satellites implied in Table 15, though a different means of denaturation is involved.

Table 16 shows the amino acid analysis of Fraction #10 (1.53 ml.) taken from the M_4 elution (Figure 18). The enrichment of pentose relative to total peptide mass in this fraction is much greater than that apparent from elution of OD_{280} ; tryptophan is concentrated here along with pentose. The pentose proportion to peptide is sufficient to indicate two pentose molecules bound to each 12,000-dalton fragment, on the average. Phosphate distribution did not correspond to the observed pentose enrichment in Fraction #10; phosphate appears to maintain a nearly constant proportion to OD_{280} in the distribution, the apparent stoichiometry of 0.52 moles per 140,000 grams. Thus, as stated previously, the proportions of coenzymic components are not likely to be compatible with NAD^+ ; since some disruption or reintegration of coenzyme structure (such as breaking or forming a

Table 16. Amino acids of 12,000-dalton fraction, P20 elution of Hexanchus M₄^(c)

Residue	micromoles	Mole %	% difference ^(a)
Lys	.0408	8.49	+0.04
His	.0120	2.50	-0.77
NH ₃	(15.39) (ammonium sulfate sample)		
Arg	.0168	3.50	+0.14
Asp	.0620	12.90	+1.98
Thr	.0353	7.34	+1.49
Ser	.0300	6.24	+0.25
Glu	.0268	5.58	-2.05
Pro	.0176	3.66	-0.15
Gly	.0448	9.32	+0.80
Ala	.0560	11.65	+2.25
Cys	neg		
Val	.0431	8.97	-0.43
Met	.0036	0.75	-1.36
Ilu	.0280	5.47	-0.58
Leu	.0364	7.57	-0.57
Tyr	.0112	2.33	-0.33
Phe	.0180	3.74	+0.41
SUM	.4807	100.0	
Cy-SO ₃ ^(b)	.0048	1.00	+0.71
Pentose	.00951	1.95	+1.88

Pentose ratio to total peptide mass: 27.3 moles per 140,000 grams.

(a) Deviation of "mole%" above from mole % of each residue in 20 hr. amino acid analysis of intact enzyme, Table 11.

(b) Identification tentative, from elution location.

(c) 12,000 daltons is an apparent molecular weight.

ribose-phosphate bond) may be involved in the process, intact NAD^+ , or even a near-unity pentose/phosphate ratio, may not be observed upon denaturation of the tetrameric enzyme.

Further experiments with Bio-Gel P20 showed that pentose stoichiometry of LDH dialysates could be increased simply by prolonged exposure at room temperature. Dialysates of beef and Hexanchus M_4 (0.1 M sodium phosphate, pH 7.0) were allowed to stand in tubes with 20% by volume (hydrated) Bio-Gel P20 for 48 hours without significant agitation. The pentose \bar{n} of Hexanchus increased from 0.73 to 1.08 and that of beef from 0.24 to 0.57; the increases of the two were really the same, and showed a similar latent pentose inclusion. The P-20-catalyzed pentose-release of LDH therefore similarly applies to species other than Hexanchus. The effects of P20 exposure must also persist through considerable variation of buffer composition, though the process may be influenced greatly by ionic strength and sample movement relative to the gel.

Pentose-enriched supernatant fractions appeared to be potent effectors of enzyme structure-function as well as arising from dismantling of LDH structure in the manner described in this section. Addition of such material in sub-stoichiometric proportions (with respect to pentose) caused LDH-coenzyme-substrate equilibrium mixtures (as shown in Table 10, Section V) to show a time-dependent turbidity increase. Thus, the relationship of the pentose residuals

to structure-function integrity can be demonstrated from either the standpoint of endogenous content or exogenous supply.

D. Discussion

The implicitly demonstrated correlation of pentose release with extent of denaturation in Table 15 displays a large experimental scatter; along with the inconsistent proportions of pentose and phosphate in Table 14, a general picture of substoichiometric amounts with a large experimental error is conveyed. However, the results of Section V show that such small amounts of coenzyme residuals may exercise very substantial effects on enzyme function. The exact nature and extent of covalent disruption of the residual coenzyme (in concert with covalent binding to peptide functionalities) seems to be significant: the average coenzymic oxidation state involved in a particular enzyme-substrate-coenzyme encounter can be influenced by equilibration with coenzyme residuals.

The distributions of pentose encountered in Hexanchus M₄ fractions suggest that some multiple covalent attachment of pentose may partially assist in stabilizing the enzyme's structure. The enrichment of tryptophan (or OD₂₈₀) along with pentose during fractionation complicates the use of OD₂₈₀ to estimate protein concentration in denatured fractions (Table 15); this is made clear by the amino acid analyses of pentose-enriched fractions. The bonding of pentose

involved must be something other than the bonds found in NAD^+ , which do not obstruct the reactivity of ribose in Bial's Reagent.

In Table 16, threonine shows an enrichment (in mole % deviation from the intact enzyme mole % threonine) corresponding quite closely to pentose enrichment compared to other amino acids. Threonine is also one of the more conspicuous residues of Hexanchus M_4 that is elevated in the analysis relative to other LDHs shown for comparison; a similar elevation of threonine is found in the amino acid analysis of lobster tail muscle LDH (31).

VII. GENERAL DISCUSSION

Hexanchus M_4 LDH shows a behavior through purification in many ways quite distinct from that of dogfish M_4 (21). Though Hexanchus LDH seems to have an isozyme system virtually identical to that of dogfish in terms of tissue-specific predominances of discrete H and M subunit types, the zymograms of Hexanchus tissue extracts show deviations in expected occurrences of intermediary isozymes and in the type of procedures required to isolate M_4 from other isozymes. The heat step (#4 of Table 1) is the principal procedure through which isozymic purity is achieved; also, it appears conspicuously more effective in advancing the progress of isolation than ion-exchange methods at that stage of purification. While this substrate-stabilized heat step has been previously used on halibut M_4 (21), the additional reliances upon sodium sulfate and NADH in Hexanchus are of interest. Likewise of interest is the appearance of considerable amounts of discrete active LDH fractions in CMC chromatograms of the sample immediately before heat treatment, yet 93-97% of the activity is conserved in producing the isozymically pure supernatant.

The isolation of Hexanchus M_4 through the steps of Table 1 results in a preparation quite resistant to further purification, though showing an overall purification factor of only 23-fold and a coenzyme

binding capacity (30) predictably falling short of the ideal 4.0 moles per 140,000 grams by a very significant margin. The yield of this enzyme from tissue is high; some 4.3% of the protein in the water extract is LDH of this sort, with one kilogram of tissue containing about two grams of the enzyme. Polyacrylamide gel filtration and disc electrophoresis show greater heterogeneity for the Hexanchus M_4 than for beef M_4 in some comparative determinations, with a latent possibility for further purification of the Hexanchus samples demonstrated. However, the heterogeneity exhibited by Hexanchus M_4 appears in a similar fashion in beef M_4 , except not as extensively manifested under ordinary conditions of sample preparation. Despite the microheterogeneous deviations from the beef M_4 molecular weight profile, the Hexanchus M_4 generally shows conformity with the tetrameric molecular structure of 140,000 daltons expected for LDH of vertebrates.

The latent heterogeneity which Hexanchus and beef M_4 share is most openly displayed in Figures 4-7, Section IV, where both enzymes, given the same destabilizing pretreatment, separate very similarly into Peak I and II components in gel electrophoresis. While the Peak I components of these enzymes are associated with most of the remaining activity, the sub-active Peak II components appear to be of lower average molecular weight, predominantly in a dissociated condition.

The high incidence of coenzyme residuals in Hexanchus M₄ further distinguishes this LDH from the beef homotetramers. The fluorescence partly attributable to dialysis-resistant coenzyme-derived moieties is more prominent in Hexanchus M₄ than in the beef LDH samples, and is spectroscopically quite compatible with reduced quaternary nicotinamide. The residual fluorescence data (Table 10) in conjunction with cyanide adduct tests (Section VI, NAD⁺ standard) show that Hexanchus M₄ contains at least 0.3 molecules per tetramer of a tightly bound, reducible quaternary nicotinamide in excess of whatever of this moiety the beef isozymes contain.

Wieland and others (32) have encountered a similar occurrence of tightly bound coenzyme material in pig H₄. The coenzyme residuals of pig H₄ may occupy one binding site per H₄ tetramer, which obstruction may be overcome by titrating the enzyme with NAD⁺ in the presence of excess sulfite ion, displacing the covalent adduct to form the strongly associated NAD⁺-SO₃-LDH ternary complex.

The release of coenzyme fragments from Hexanchus M₄ (as followed by chemical analyses for pentose, phosphate, adenine, and cyanide-reactive nicotinamide in Section VI) occurs only in conjunction with massive losses of enzyme activity; these losses take place through dissociation, formation of an insoluble precipitate, or both. Though the possibility of removing a substantial proportion of the residuals as intact coenzyme while maintaining most of the enzyme

activity has not been absolutely ruled out, these findings strongly imply that structure-function integrity in Hexanchus M_4 partially depends upon incorporation of coenzyme fragments. The above could also be described as a mode of coenzyme binding in which covalent attachment of parts of the coenzyme to the enzyme is contingent upon coenzyme cleavages and consequent alteration of the distribution of available oxidation states. The evidence for a covalent disruption of NADH during coprecipitation with Hexanchus M_4 (Figure 3) and the reliance upon NADH in isolating this enzyme (Table 1) place further emphasis upon this aspect of coenzyme residual content.

Evidence for such involvement of coenzyme residuals in LDH structure is not confined to Hexanchus M_4 ; some differing, but comparable, findings with the beef isozymes in this study and a study of lobster M_4 LDH by Kaloustian and others (31) provide further examples. In isolating lobster M_4 , a discrepancy between the percent yield determined by assay of pyruvate reduction by NADH and that determined by lactate oxidation arises; this deviation increases steadily as purification of the enzyme progresses. The "pure" lobster M_4 proves to be totally incapable of lactate oxidation with NAD^+ , yet represents a high yield of the total reverse reaction activity. Apparently, some form of altered coenzyme residual, derived from NADH, is involved in this discrepancy; during dialysis and other purification procedures, such material is lost or altered and requires

restoration by supplying reduced coenzyme in order to regain enzyme activity. In concert with this anomalous progress of purification is the appearance of discrete, sub-active minor enzyme fractions in ion-exchange chromatography. This combination of behavioral traits is quite comparable to that encountered in isolating Hexanchus M_4 , with its NADH-dependence in the heat step, the ambiguities in applicable criteria of purity, and the separation of components in the electrophoresis.

Interactions with coenzymes, amino acid composition, crystalline structure, and residual coenzyme moieties are all categories of characterization in which very substantial differences between the Hexanchus M_4 preparations of this study and dogfish M_4 (25) can be seen. In encountering this apparently large interspecific variation, considerable evidence of extensive intrinsic variability in the M_4 -LDH molecular population itself has been gained.

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