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AN ONTOGENETIC STUDY OF LACTATE DEHYDROGENASE IN POROCEPHALUS CROTALI (PENTASTOMIDA)

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CHAPTER I

INTRODUCTION

The detailed characterization of enzymes involved in metabolic regulation of multistep reaction sequences serves as a foundation for current investigations of the regulation of metabolic processes in living organisms. Particular interest has been generated with regard to regulatory enzymes which initiate reaction sequences and dictate the rate of these sequences. Examination of a number of regulatory enzymes has revealed structural and kinetic characteristics that are generally not typical of other enzymes: e.g., these regulatory enzymes are characterized as follows: (1) they are composed of subunits, (2) the substrate saturation curves are usually sigmoidal, and (3) they undergo conformational changes when exposed to "effectors" (Changeux, 1963). The effectors, which may be activators or inhibitors, are bound to these enzymes at sites distinctly different from the substrate sites. This type of alteration has been termed an allosteric transition (Monod et al., 1965).

Recently the application of electrophoretic and, to a lesser

extent, chromatographic techniques has led to the isolation and characterization of multiple forms of enzymatically active proteins. These enzymes, which are chemically distinct species of protein molecules occurring in the same species and catalyzing the same biochemical reaction(s) are referred to as isoenzymes. Lactate dehydrogenase (LDH), malic dehydrogenase (MDH), aldolase, and hexokinase are examples of isoenzymic systems which have been described from animal tissues (Vessel and Baron, 1968; Markert and Ursprung, 1962; Lindsay, 1963; and Thorne, 1963).

The hypothesis that regulation of metabolic reaction sequences could be brought about by allosteric transitions, differential activation, or inhibition of isoenzymes is based on the occurrence of isoenzymes and enzymes which undergo allosteric transitions. Metabolic regulation of this type was demonstrated by Wolin (1964), who showed that LDH from Streptococcus bovis specifically requires fructose-1,6-diphosphate for activity and only phosphate and fructose-1,6-diphosphate prevented inactivation of the dehydrogenase.

Tricarboxylic acid cycle (T.C.A.) intermediates have been reported as being activators of LDH_5 (slowest migrating band towards the anode at pH 7.6) in rabbit muscle (Fritz, 1966), and it has been suggested that lactate accumulation in tissue may be due in part to increased reduced nicotinamide adenine dinucleotide (NADH) from glycolysis and, in part, to activation of LDH_5 by the T.C.A. intermediates. Markert and Ursprung, (1962) had previously suggested that the fundamental role of LDH is regulation of the NAD^+/NADH ratio.

Activation would result when more NAD^+ is available from other metabolic pathways (Fritz, 1966).

Although LDH, MDH, and other enzymes have been characterized in a number of helminths by histochemical, as well as spectrophotometric techniques, few of these studies have concerned the ontogenetic, physiological, and adaptive aspects of the enzyme.

Rhodes et al., (1964), using DEAE-cellulose ion exchange columns and working with extracts of Ascaris suum and swine heart muscle, found that extracts from both tissues contained three isoenzymes of MDH. These isoenzymes differed in: (1) relative migration rate in starch-gel electrophoresis, (2) conditions for adsorption and elution from DEAE-cellulose ion exchanges columns, (3) stability at 55 C, (4) inhibition by zinc ion, and (5) relative activity at different pH values. Dungan and de Luge (1966) demonstrated that multiple molecular species of LDH and MDH exist in Macracanthorhynchus hirudinaceus. Conde-Del Pino et al., (1966, 1968) discovered that LDH and MDH isoenzymes are present in extracts of adults and cercariae of Schistosoma mansoni. Bayne et al., (1968) demonstrated that whole organism extracts of the frog lung trematode Hematoloechus medioplexus contain four isoenzyme fractions, two of which correspond to the two isoenzymes of frog lung tissue. Coles (1970) revealed that S. mansoni and S. haematobium isoenzyme patterns of LDH, MDH, and glucose-6-phosphate dehydrogenase vary according to the sex, species and developmental stages.

Rhodes et al., (1970), found seven fractions of MDH in soluble

extracts from adult male and female Haemonchus contortus. The isoelectric points of these seven fractions were found to be between pH 5.9 and 9.0 by using the technique of isoelectric focusing. Isoelectric focusing in polyacrylamide and starch-gel electrophoresis failed to give discrete bands of MDH activity. The pH optima, maximal velocity (V_{max}), Michaelis-Menten constant (K_m), and the affinity constant (K_a or $1/K_m$) were different for H. contortus vs. sheep serum MDH.

Epigenetic adaptations should be particularly important in animals with complex life cycles (Fairbairn, 1970). The eggs of Porocephalus crotali are able to survive free for a limited time. Immediate hatching of the eggs and development of primary larvae is inducted by the quite different conditions encountered in the intestine of the intermediate host. The nymph transforms to a sexually mature adult in the lung of crotalid hosts. Clearly, there must be great differences in the physiological conditions under which LDH functions in the various life cycle stages.

Thus the objectives of this research were to investigate and demonstrate in the egg, sixth nymphal stage, and adults of P. crotali the following: (1) LDH activity; (2) multiple molecular forms (isoenzymes) of this enzyme; (3) kinetic differences as shown by isoelectric focusing separations; (4) changes in the protein, lipoprotein and LDH patterns; and (5) subunit structure of LDH.

CHAPTER II

METHODS AND MATERIALS

Eggs were obtained from the distal uterus of gravid P. crotali females, taken from rattlesnakes (Crotalus atrox). Eggs were placed in a phosphate buffered reptile Ringer's solution (pH 7.2), and 25 to 40 eggs were given per os to a strain of mice inbred for 35 years. Development was allowed to continue in vivo to the infective sixth nymph. The remainder of the egg suspension, and the non-gravid adults with empty guts, were washed twice with Tris-glycine buffer (pH 8.2, ionic strength 0.05), and frozen at -15 to -20 C in fresh Tris-glycine buffer.

For all experiments involving nymphal stages, only those with (1) empty guts and (2) either moving free or encysted in the intestinal mesentery were selected. They were cut free of host tissue and washed with mammalian Ringer's solution (pH 7.6), allowed to equilibrate for 30 minutes in Tris-glycine buffer (pH 8.8, ionic strength 0.05) at 5 to 7 C, and resuspended in two ml of fresh Tris-glycine buffer. Subsequently they were homogenized in an all glass Potter-Elvehjem homogenizer at 4 C. The previously frozen eggs and adults were allowed to thaw and then homogenized as described above.

All homogenates were centrifuged at 5 C in a high speed refrigerated centrifuge at 30,000 x g for 45 minutes. The supernatant was decanted and used for the separations of the fractions by polyacrylamide-gel electrophoresis, fractionation of the enzyme by isoelectric focusing, spectrophotometric assays for LDH and determination of protein.

The methods of Orstein and Davis (1962) were used for polyacrylamide-gel electrophoresis. The anodal system with a Tris-glycine upper buffer (pH 9.2) and a Tris-HCl lower buffer (pH 8.3) was used. Egg, nymph, and adult protein samples (200 µl) samples of mouse and snake serum (50 µl) were added to separate gel columns. A current of 1.5 ma/gel column was applied until a band of tracking dye, (bromophenol blue) migrated to the bottom of the gel columns.

To stain LDH isoenzymes, gel columns were removed from the glass tubes and immediately placed in the following staining medium for 30 minutes at 37 C: 10 ml 0.3 M Tris-glycine buffer, pH 8.4; 5 ml 0 12 M lithium lactate; 12 mg NAD^+ (3.76×10^{-4} M); 25 mg phenazine methosulfate (1.63×10^{-4} M); 13 mg nitro BT (2,2'-di-p-nitrophenyl-3,3'(3,3'-dimethoxy-4,4'-biphenylene ditetrazolium); 5 mg potassium cyanide (3.09×10^{-3} M); and 0.56 M hydrazine, pH 8.4.

Gels were then fixed in 5% acetic acid. Visualization of lipoprotein was achieved by staining gels in 0.5% Ponceau S for 24 hours, of protein by staining gels in 5% amino Schwartz (naphthol blue-black) for 30 minutes. Destaining for both was in 5% acetic acid.

Gels were scanned with a model 2400 Gilford spectrophotometer with linear transport attachment at the following wavelengths:

LDH, 620 mμ; protein, 570 mμ; lipoprotein, 400 mμ. Relative electrophoretic flow rates (E_f) for LDH, protein and lipoprotein were determined by averaging the values obtained from the scanning of six individual gels.

Photographic records of gels were made with a Pentax Spotmatic SLR camera using Kodachrome II film and transmitted light.

Isoelectric focusing was by the method of Vesterbery and Svennson (1966) on an LKB 440 ml isoelectric focusing column. A wide range ampholine carrier (pH 3-10) was used, and the protein sample electrofocused for 48 hours using a potential of 800 volts (40-45 ma). After electrofocusing, the contents of the focusing column were fractionated with an automatic fraction collector into 120 equal portions. The pH of all fractions was determined with a semi-micro combination electrode. Fractions were assayed for LDH (L-lactate: NAD oxidoreductase, EC 1.1.1.27.) activity by measuring the reduction of NAD^+ in the presence of L-lithium lactate (Kornberg, 1955). This same assay method was also used for determining LDH activity in the original protein supernatants. Enzyme assays were conducted using saturating substrate concentration (1.2×10^{-2} M) and varying the cofactor concentration (NAD^+). At least four assays were performed at each substrate and cofactor concentration. In all enzyme assays 0.25 mg protein was used. A Gilford model 2000 spectrophotometer with a Beckman DU monochromator was used for recording the progress of all enzyme assays. The results of these assays were evaluated graphically by plotting reaction velocities against substrate concentrations.

For most enzymes this yields a hyperbolic saturation curve. However, for many regulatory enzymes similar plots produce sigmoid curves. Such curves indicate the presence of two or more dependent binding sites on the enzyme molecule. Similar interactions frequently occur in the binding of the allosteric effector substances. This suggests that some regulatory enzymes contain more than one binding site. Furthermore, from this type of graph, one can extrapolate the maximal velocity (V_m), Michaelis-Menten constant (K_m), and the affinity constant (K_a or $1/K_m$).

Data were also represented graphically by a Lineweaver-Burke plot (1/v vs. 1/s), since this type of plot allows interpretation of V_m, and K_m without extrapolation. Derivations of equations are from Dixon and Webb (1964) and Walter (1965).

When a sigmoidal curve was obtained, indicating an allosteric mechanism in the enzyme, a Hill plot was used to determine K_m, V_m, and the minimum number of interacting sites (n).

Using the Michaelis-Menten assumption of equilibrium kinetics:

$$v_o = \frac{V_m (S)^n}{K_m + (S)^n}$$

where v_o is initial velocity, (S) is substrate concentration, and n is the number of interacting binding sites, and by taking log form of the above equation and arranging the log expression for the equation to fit a straight line ($y = mx + b$), one obtains the following equation:

$$\log (v_o/V_m - v_o) = n \log (S) - \log K_m$$

If the data are plotted using the coordinates log $(v_o/V_m - v_o)$ vs. log (S), a Hill plot results where n equals the minimum number of interacting binding sites. The entire saturation range from 15 to 85% V_m was employed.

In order to determine the pH optima of the enzyme, assays under saturation conditions were carried out in a pH range of 5.0 to 10.0 using the following three buffers: (1) 0.01 M Phosphate (2) 0.01 M Tris-glycine; (3) 0.01 M Glycylglycine.

The assay mixtures were also subjected to graded series of temperatures from 10 to 60 C for 30 minutes in order to determine optimum temperatures. These assays were performed at saturation (1.2×10^{-2} M L-Lithium lactate).

Total protein of the supernatant fluid was determined by the method of Lowry et al., (1951), using crystalline bovine serum albumin as a standard and by determining a 280/260 mμ wavelength ratio and referring to a nomograph (Warburg and Christian, 1942).

Dissociation, recombination and hybridization experiments were conducted according to the methods of Markert and Massaro (1968), modified to provide partial staining of the gels for approximately five minutes. The gel bands from the egg, nymph, and adult stages were excised with a razor blade and immediately placed in chilled 1M NaCl solution and frozen at -15 C for 24 hours. Samples were thawed, homogenized, and dialyzed against distilled water for two hours. Samples were then used in various recombination and hybridization experiments.

CHAPTER III

RESULTS

Since the nymphal and adult stages of P. crotali consume host blood, LDH assays were performed on these two parasitic stages in order to determine whether their gut contents (mostly blood) would exhibit an elevated enzymatic rate. Graphs 1 and 2 show the results of the LDH assays on the nymph and adult (equal weight ± 0.27 mg) protein homogenates with and without gut contents. Very little increase in velocities or difference in K_m's were detected.

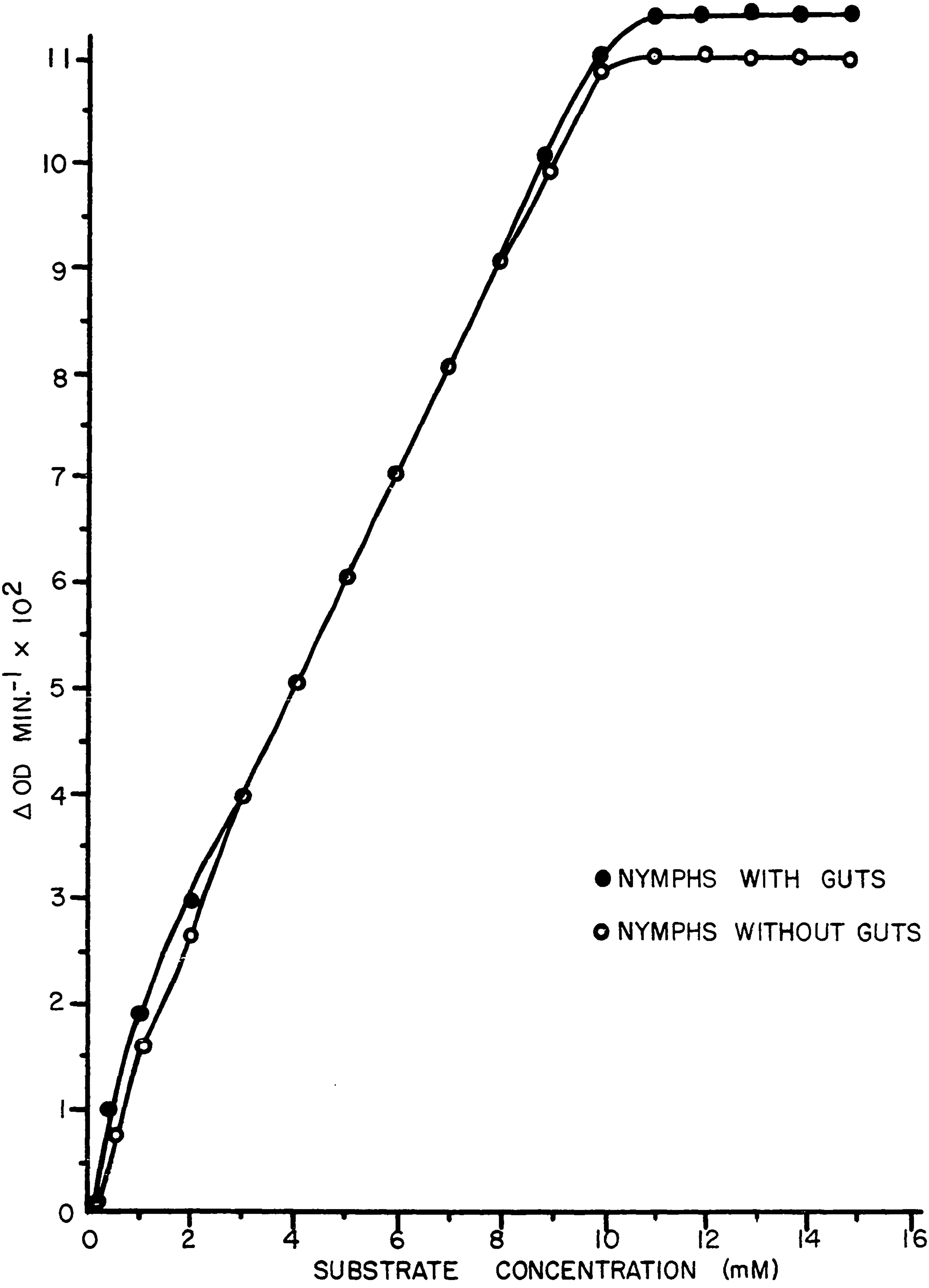
Assays performed using whole tissue homogenates and various substrate concentrations (L-Lithium lactate) for lactate dehydrogenase on the nymph and adult stages display rectangular hyperbolic kinetics, while the egg stage displays sigmoidal kinetics. The latter is characteristic of interacting binding sites (Graph 3). The Michaelis-Menten constants, (K_m's) were calculated for the egg, nymph, and adult stages and found to be 6.9×10^{-3} M, 4.1×10^{-3} M, and 4.4×10^{-3} M, respectively (Table I).

A Hill plot of the data from Graph 3 revealed that the egg possessed a minimum of two interacting binding sites, and the nymph and adult one non-interacting binding site (Graph 4).

Assaying LDH activity at various cofactor concentrations (NAD^+),

GRAPH 1

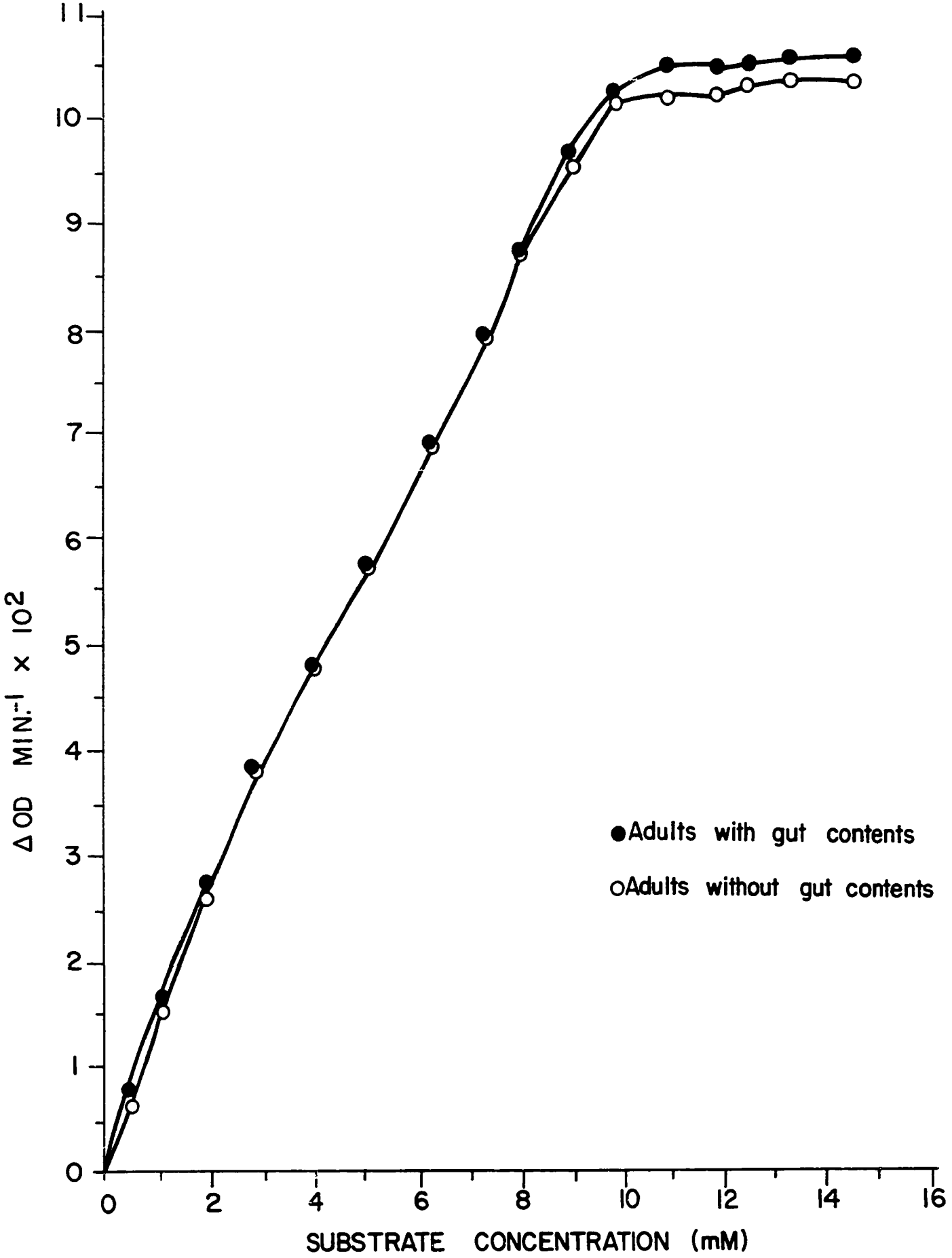
Effect of substrate concentration on LDH activity of whole tissue homogenates of P. crotali nymphs with and without gut contents. Each point represents a mean of four values.



ΔOD MIN.$^{-1}$ × 10^2
0
1
2
3
4
5
6
7
8
9
10
11
0
2
4
6
8
10
12
14
16
SUBSTRATE CONCENTRATION (mM)
NYMPHS WITH GUTS
NYMPHS WITHOUT GUTS

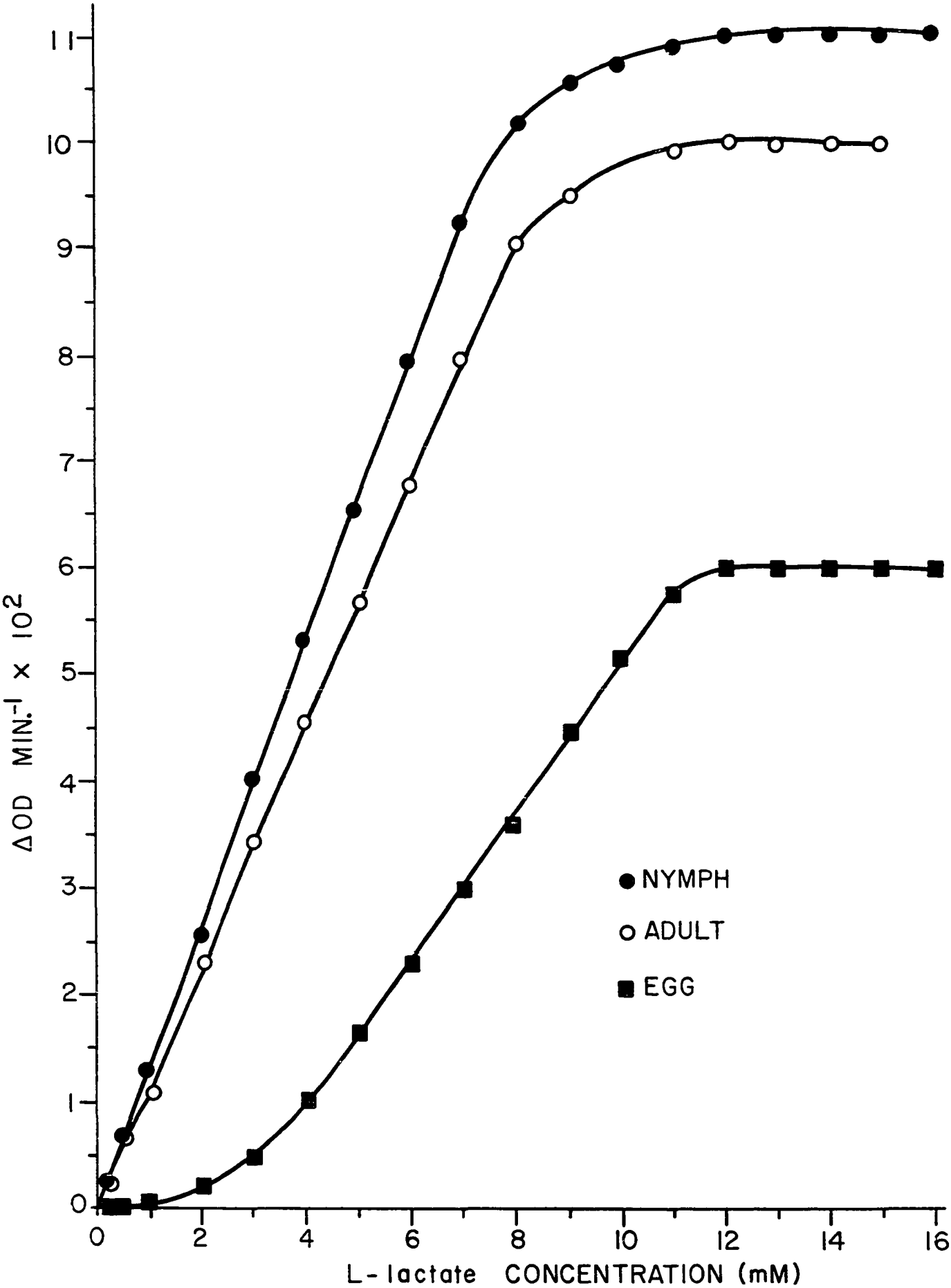
GRAPH 2

Effect of substrate concentration on LDH activity on whole tissue homogenates of P. crotali adults with and without gut contents. Each point represents a mean of four values.


11
10
9
8
7
6
5
4
3
2
1
0
ΔOD MIN.-1 x 10²
0
2
4
6
8
10
12
14
16
SUBSTRATE CONCENTRATION (mM)
●Adults with gut contents
○Adults without gut contents

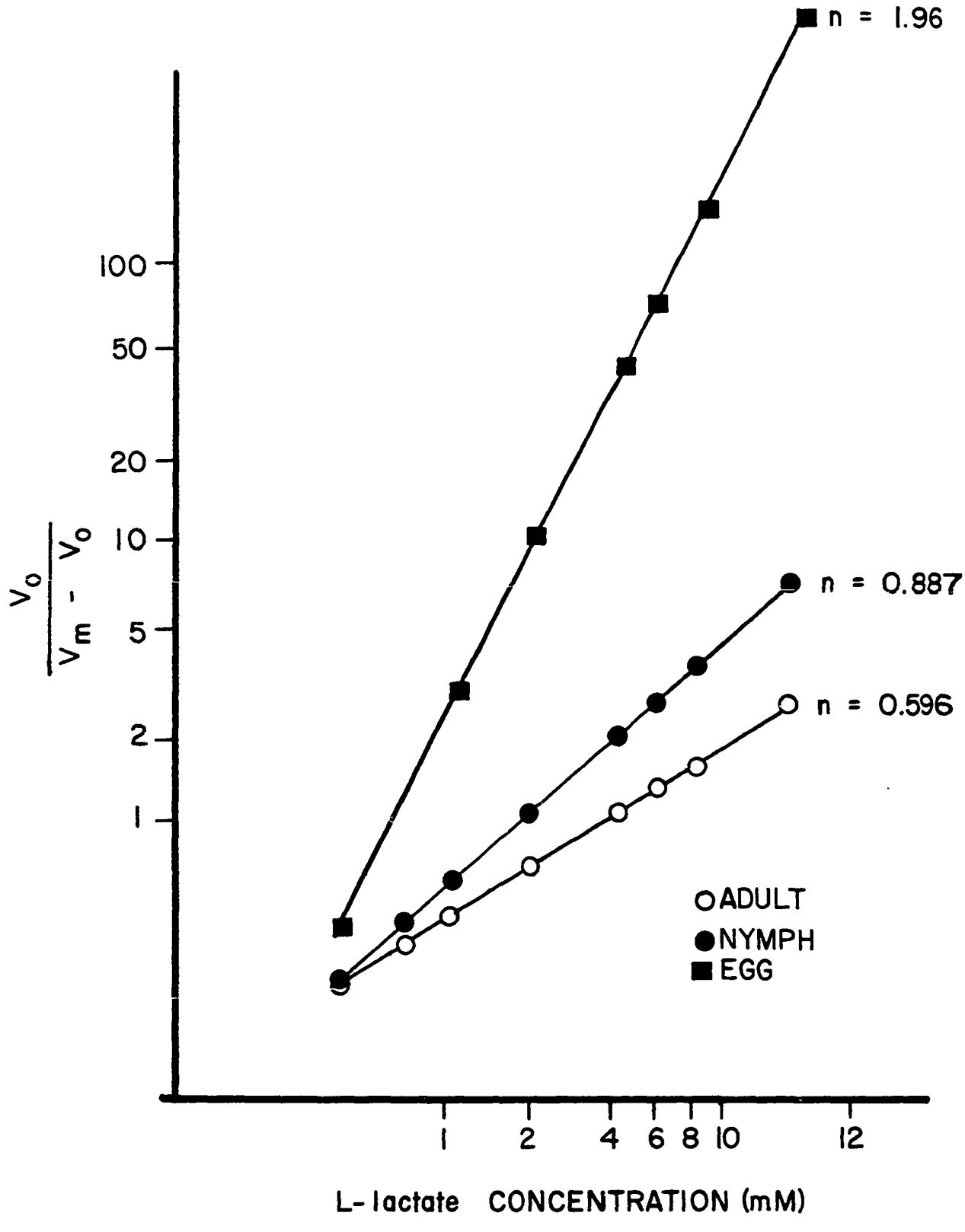
GRAPH 3

Effect of substrate concentration on LDH activity of P. crotali egg, nymph, and adult whole tissue homogenates. Each point represents a mean of five values.


ΔOD MIN.-1 x 10-2
0
1
2
3
4
5
6
7
8
9
10
11
0
2
4
6
8
10
12
14
16
L-lactate CONCENTRATION (mM)
NYMPH
ADULT
EGG

GRAPH 4

Empirical Hill plot for LDH assayed at various substrate concentrations.


n = 1.96
n = 0.887
n = 0.596
100
50
20
10
5
2
1
$\frac{V_o}{V_E - V_o}$
ADULT
NYMPH
EGG
1
2
4
6
8
10
12
L-lactate CONCENTRATION (mM)

while keeping the substrate at saturation (1.3×10^{-2} M), showed that the nymph and adult display apparent rectangular hyperbolic kinetics, while the egg displays sigmoidal kinetics (Graph 5).

A Hill plot of these data revealed that there was an interaction at more than one site in all three homogenates assayed (Graph 6).

The pH profile for the enzyme in all three developmental stages was obtained by plotting the maximum enzyme activities as a function of buffer pH. The egg homogenate showed optimal activity at pH 7.9 in all three buffer systems, and activity fell sharply on both sides of pH 7.9. Highest amount of activity was obtained in a phosphate buffer (Graph 7). The nymphal stage showed highest activity when buffered with Tris-glycine buffer at pH 7.8 (Graph 8). Activity again dropped sharply on both sides of pH 7.8. Results of the pH profile of the adult stages are shown in Graph 9. Highest activity was detected when using a phosphate buffer at a pH of 8.1.

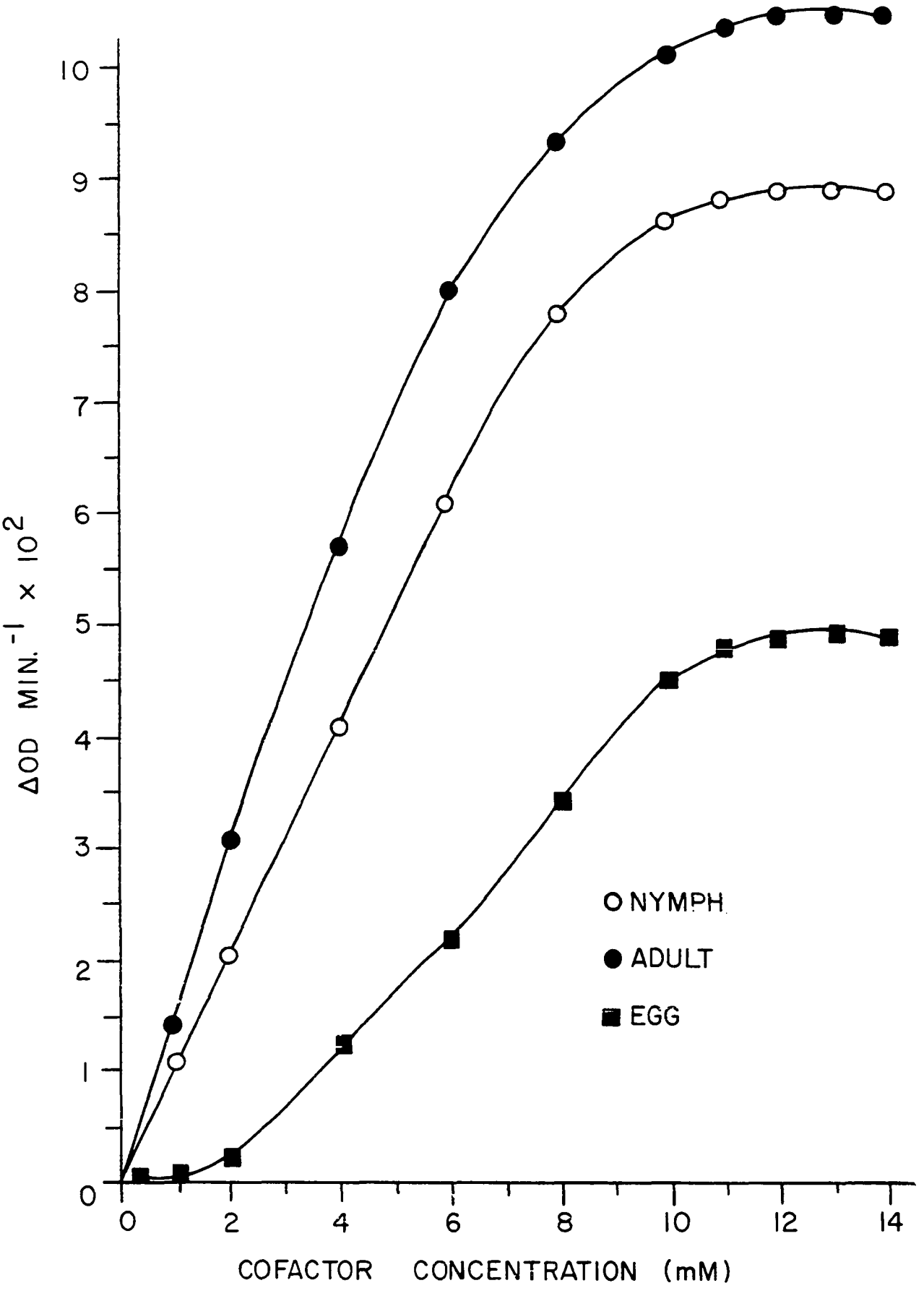
Thermal stability of the enzyme was determined in a temperature range of 10 to 60 C. All three developmental stages exhibited optimal stability at 41 C (Graph 10, Table I).

The K_m of the total LDH activity from egg, nymph and adult stages varied with temperture in a characteristic way (Graph 11). At each stage of the life cycle the K_m was minimal (and by implication the affinity of the enzyme for its substrate was maximal) at a temperature near 37 C.

Graph 12 represents the results of substrate stereospecificity experiments. Assays were performed on nymph whole tissue homogenates

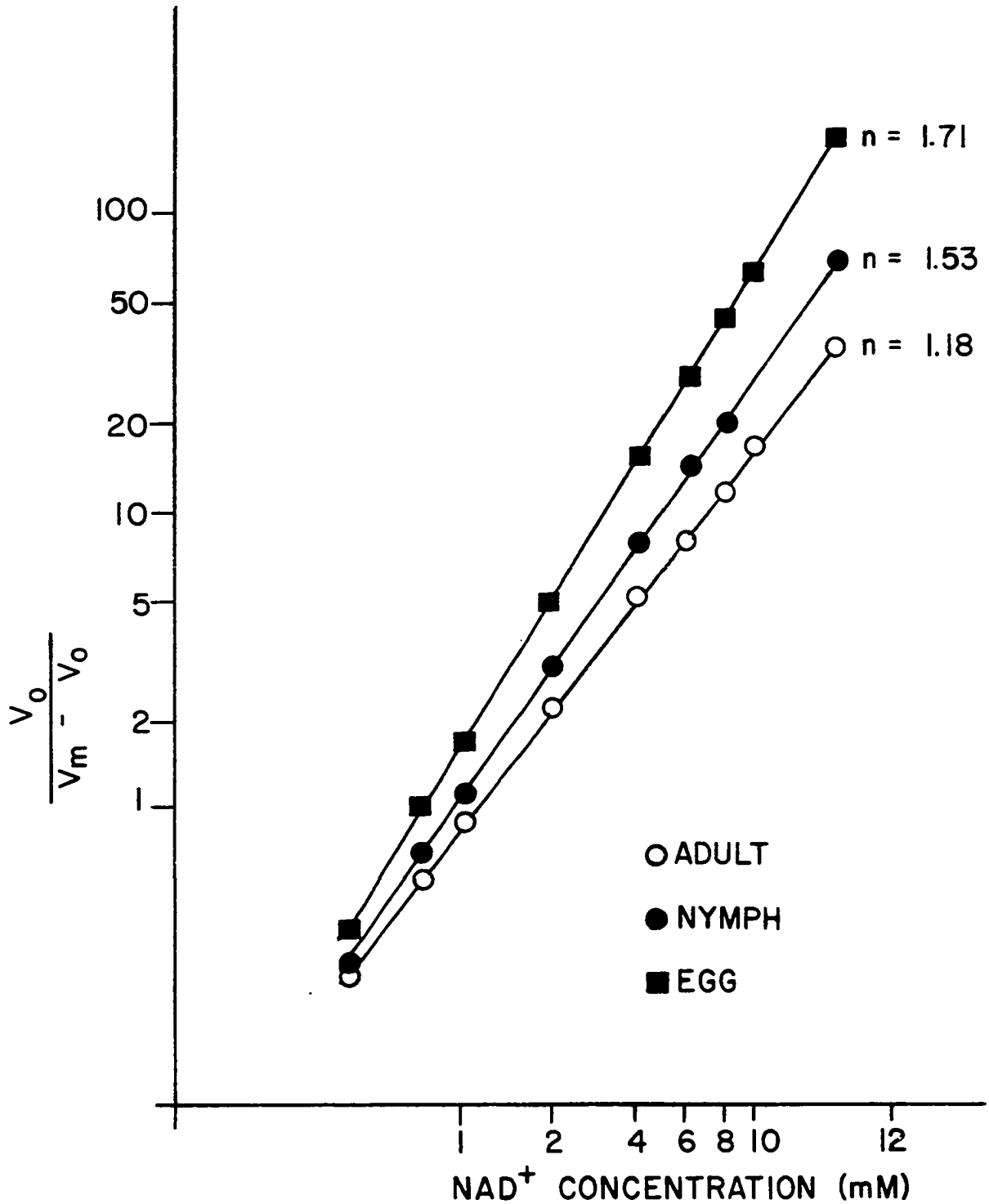
GRAPH 5

Effect of cofactor concentration on LDH activity of P. crotali egg, nymph and adult whole tissue homogenates. Each point represents a mean of five values.


ΔOD MIN.$^{-1}$ x 10^2
0
1
2
3
4
5
6
7
8
9
10
0
2
4
6
8
10
12
14
COFACTOR CONCENTRATION (mM)
NYMPH
ADULT
EGG

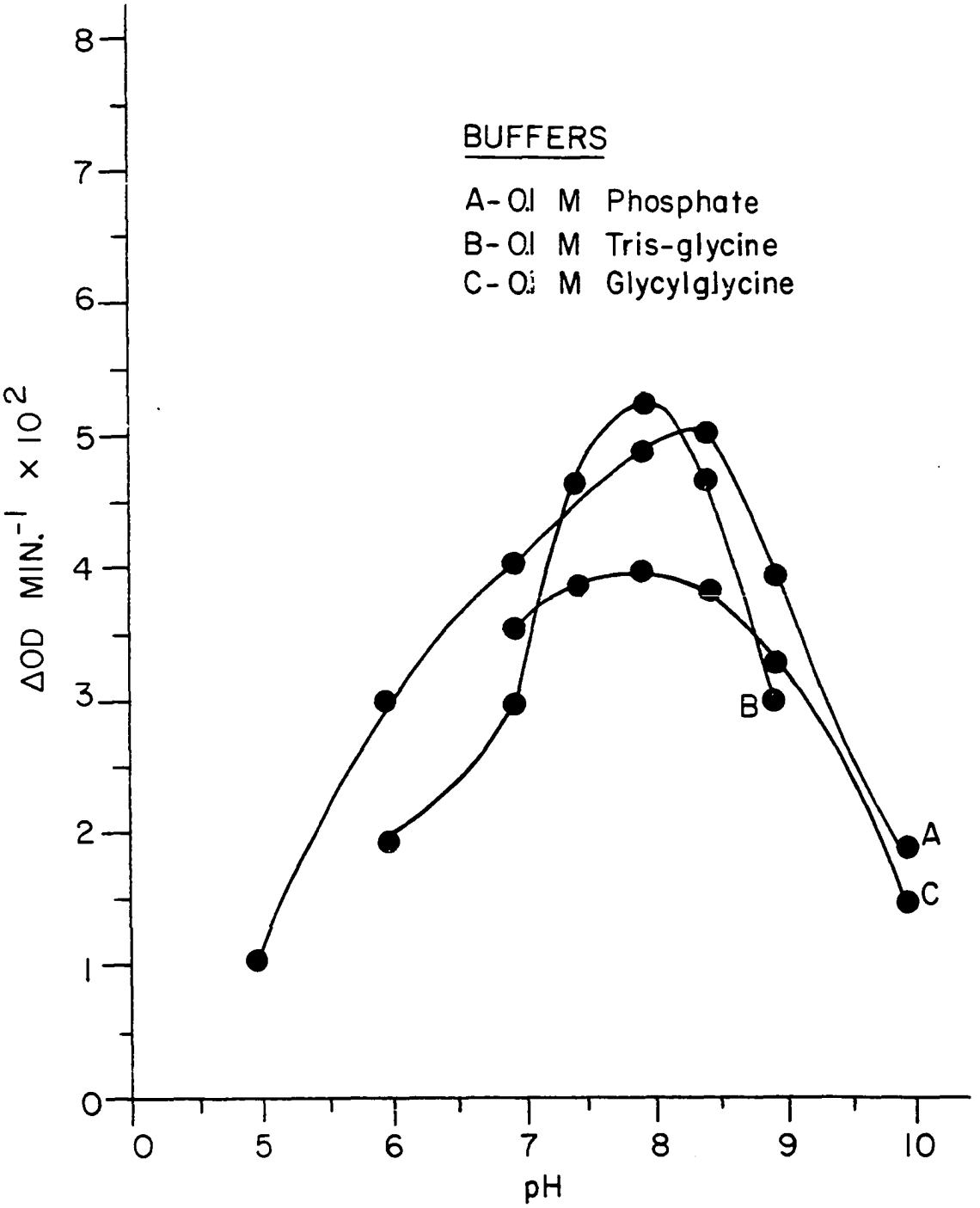
GRAPH 6

Empirical Hill plot for LDH assayed at various cofactor concentrations while keeping substrate at saturating levels.


n = 1.71
n = 1.53
n = 1.18
100
50
20
10
5
2
1
$\frac{V_o}{V_m - V_o}$
ADULT
NYMPH
EGG
1
2
4
6
8
10
12
NAD+ CONCENTRATION (mM)

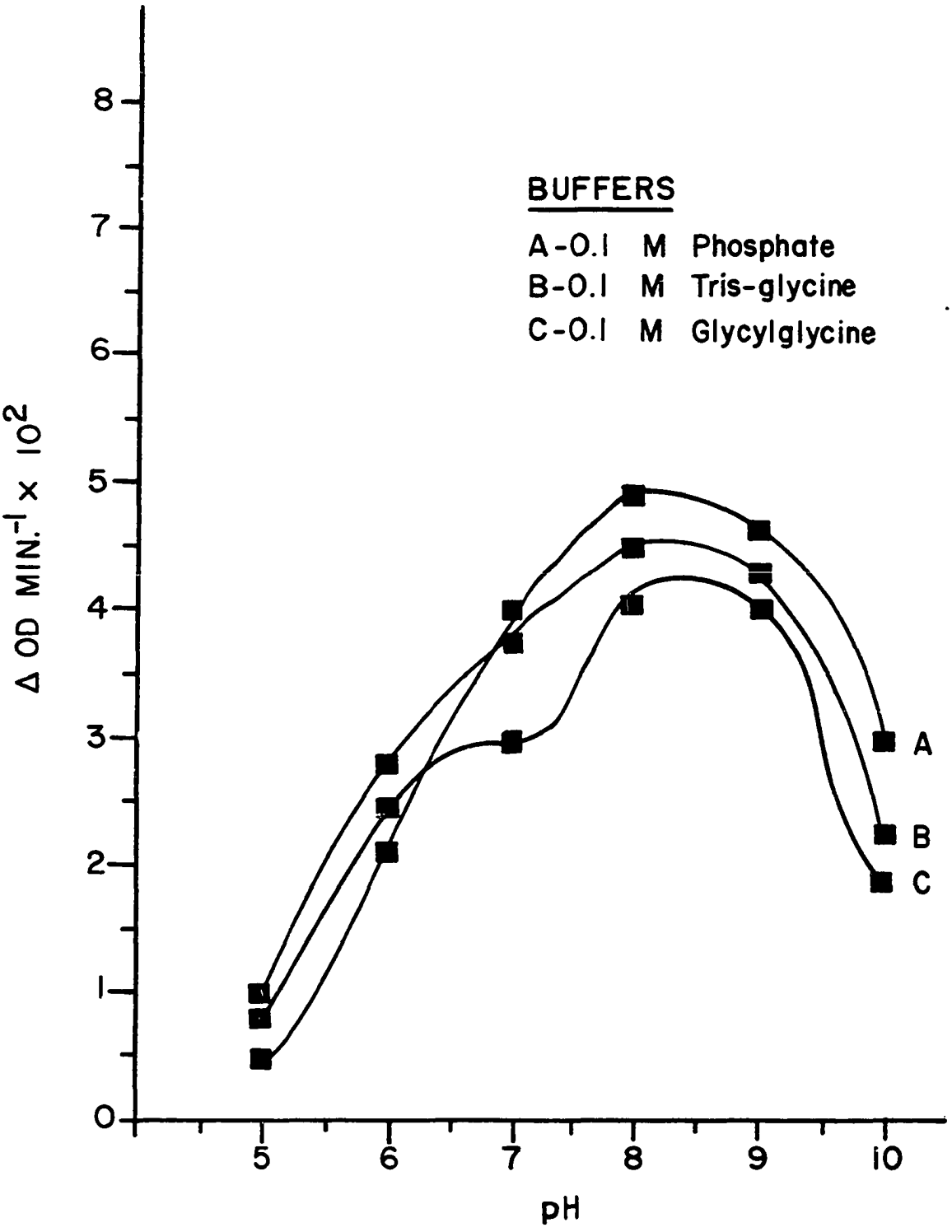
GRAPH 7

Effect of pH on LDH activity from whole tissue homogenates of P. crotali eggs. Assays were performed using three buffers listed and substrate at saturating concentrations. All points represent a mean of five values.


BUFFERS
A- 0.1 M Phosphate
B- 0.1 M Tris-glycine
C- 0.1 M Glycylglycine
ΔOD MIN.⁻¹ x 10²
pH
A
B
C

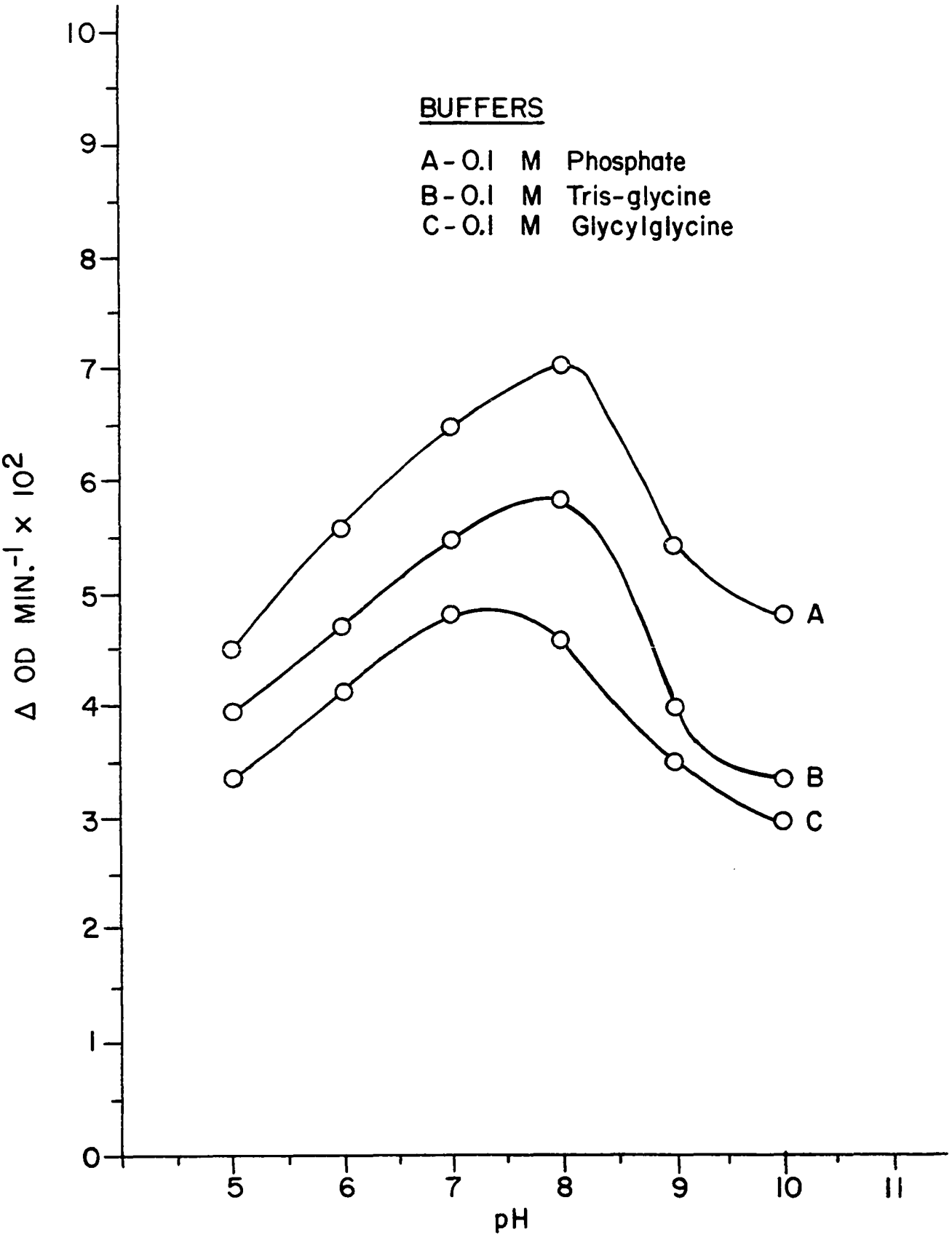
GRAPH 8

Effect of pH on LDH activity from whole tissue homogenates of P. crotali nymphs. Assays were performed using three buffers listed and substrate at saturating concentrations. Each point represents a mean of four values.


BUFFERS
A-0.1 M Phosphate
B-0.1 M Tris-glycine
C-0.1 M Glycylglycine
Δ OD MIN.$^{-1}$ x 10^2
pH
A
B
C

GRAPH 9

Effect of pH on LDH activity from whole tissue homogenates of P. crotali adults. Assays were performed using the three buffers listed and substrate at saturating concentrations. Each point represents a mean of five values.


BUFFERS
A - 0.1 M Phosphate
B - 0.1 M Tris-glycine
C - 0.1 M Glycylglycine
Δ OD MIN.$^{-1}$ x 10^2
pH
A
B
C
0
1
2
3
4
5
6
7
8
9
10
5
6
7
8
9
10
11

GRAPH 10

Effect of incubation at various temperatures on LDH activity from whole tissue homogenates of P. crotali eggs, nymphs, and adults. Each point represents a mean of five values.

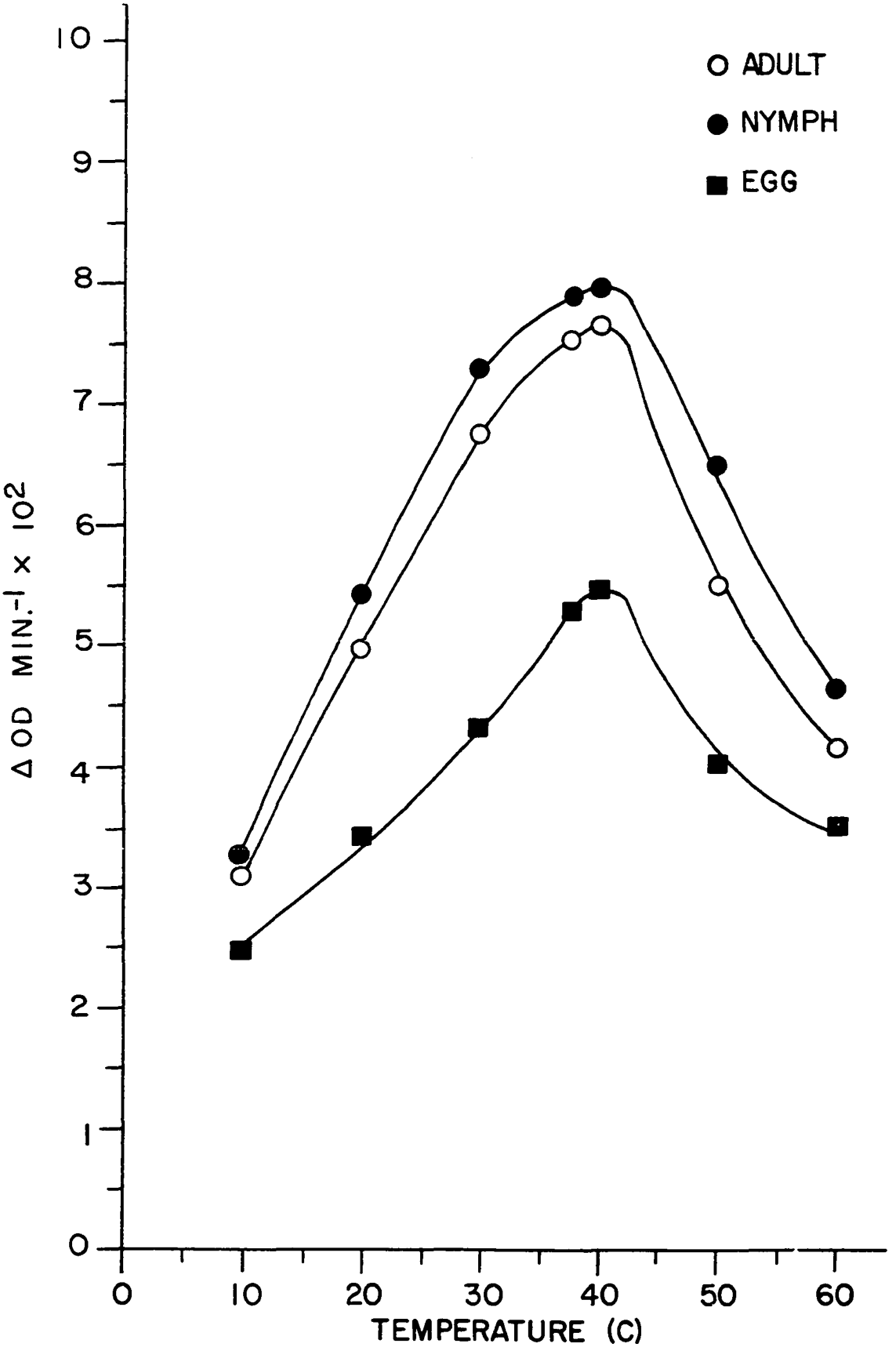

O ADULT
● NYMPH
■ EGG
Δ OD MIN.$^{-1}$ x 10^2
0
1
2
3
4
5
6
7
8
9
10
0
10
20
30
40
50
60
TEMPERATURE (C)

TABLE I. Comparisons of specific activity, optimal temperature stability, optimal pH, and K_m from whole tissue homogenates of P. crotali egg, nymph and adult stages.

ENZYME SOURCE	*SP. ACT.	OPTM. TEMP. STAB.	OPT. pH	K_m **
EGG	(3.6±.1)	41	7.9±.1	(6.9×10^{-3})
NYMPH	13.5±.09	41	7.8±.1	4.1×10^{-3}
ADULT	12.8±.08	41	8.1±.1	4.4×10^{-3}

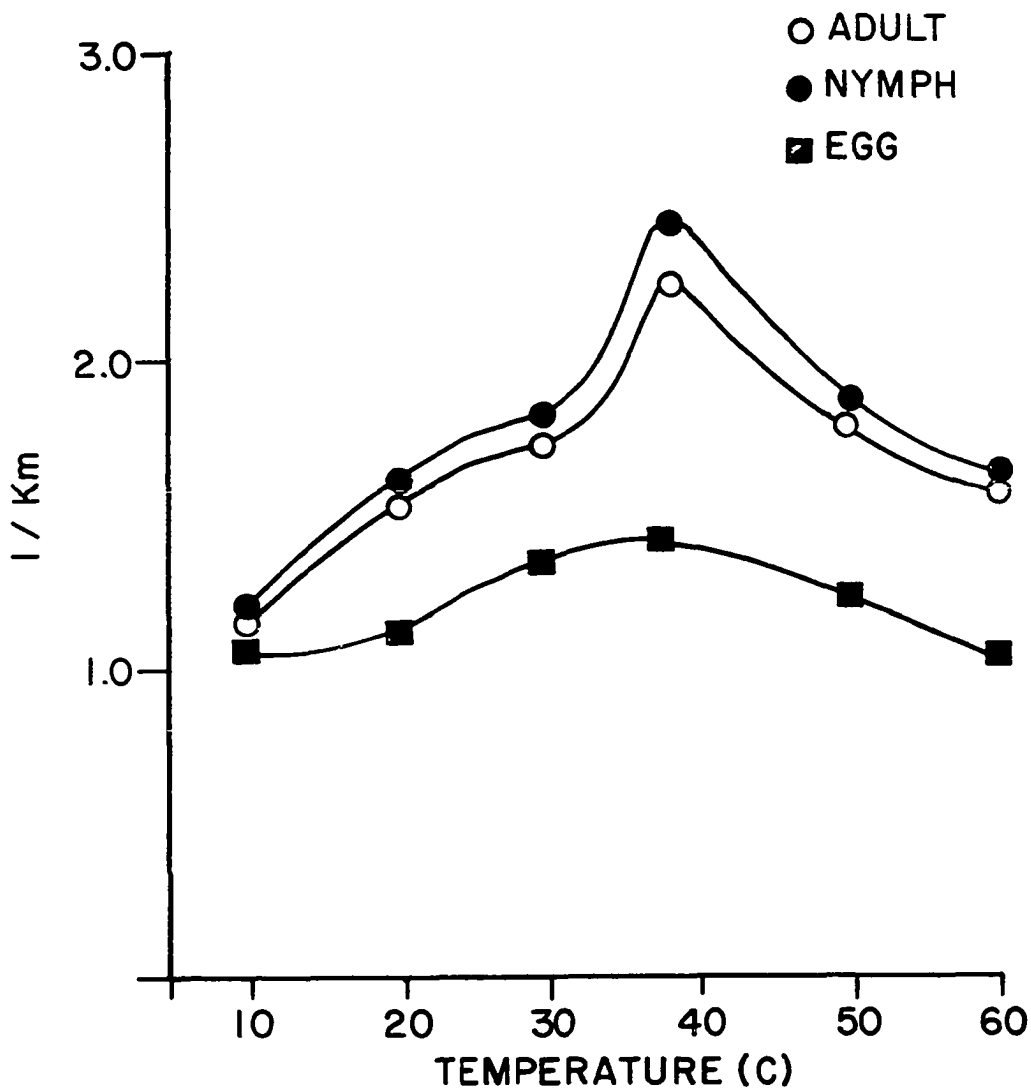
* Specific activity (SP. ACT.) = units/mg protein, where one unit = 10^{-3} O.D. units/minute at optimal pH and temperature.

Values in parentheses are significantly different from other values in same column ($p<0.05$) when compared by the Student's t test.

** Michaelis-Menten constant (K_m) = molar substrate concentration which gives 1/2 maximal velocity (V_m).

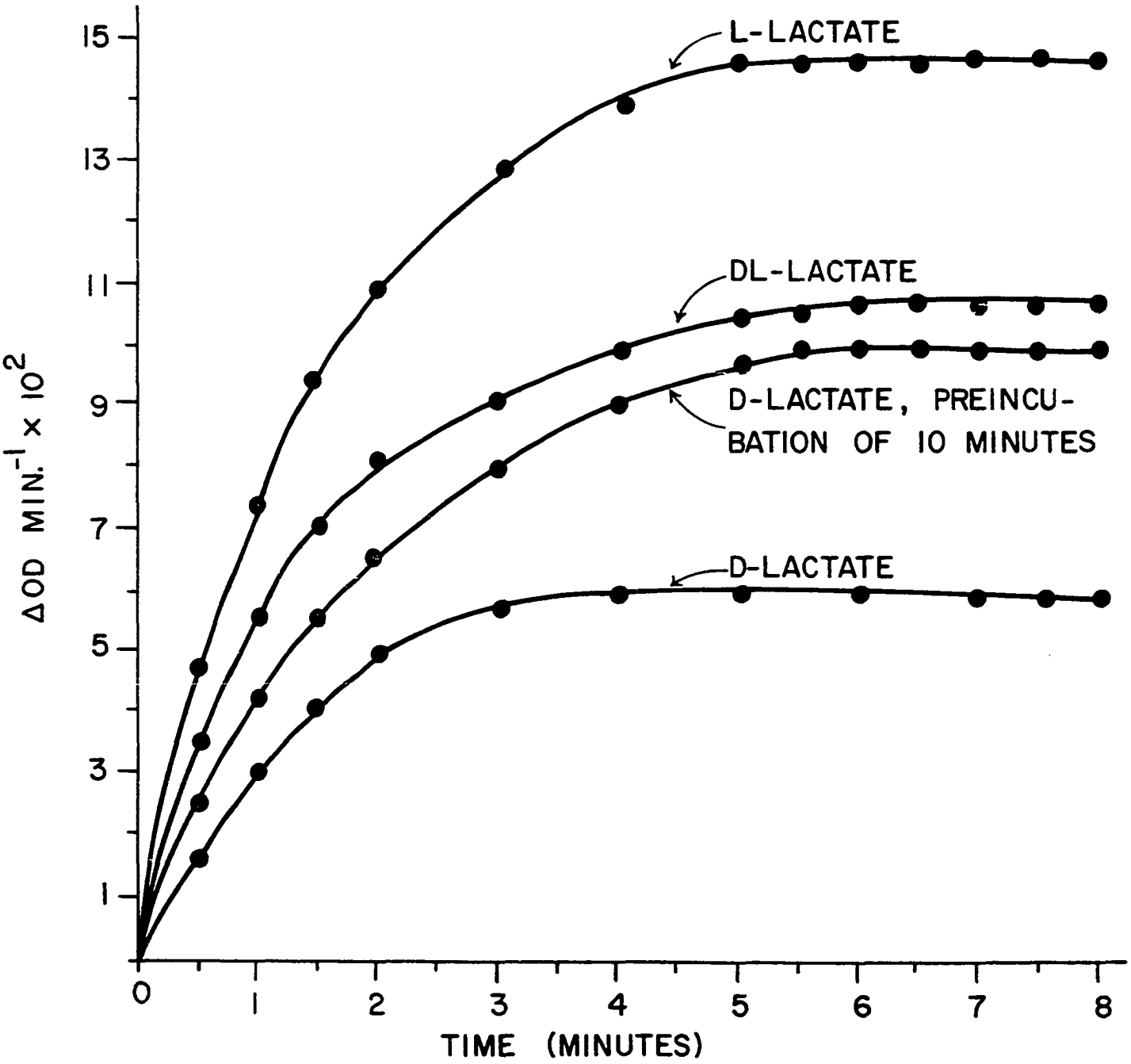
GRAPH 11

Effect of temperature on the apparent K_m of the total LDH activity from the three developmental stages of P. crotali. Each point is a mean of five values.


O ADULT
● NYMPH
EGG
3.0
2.0
1.0
1 / Km
10
20
30
40
50
60
TEMPERATURE (C)

GRAPH 12

Reaction rate of LDH from whole tissue homogenates of P. crotali nymphs. Each point represents a mean of three values.


L-LACTATE
DL-LACTATE
D-LACTATE, PREINCU-
BATION OF 10 MINUTES
D-LACTATE
ΔOD MIN.$^{-1}$ x 10^2
TIME (MINUTES)
15
13
11
9
7
5
3
1
0
1
2
3
4
5
6
7
8

using D-, D,L-, and L-lactate, at 37 C, pH 8.0, while the substrate (1.2×10^{-2} M and 1.3×10^{-2} M, respectively). A similar assay was performed on a reaction mixture which contained D-Lithium lactate and that was pre-incubated for 10 minutes. L-lactate gave the highest velocity after five minutes incubation. Low activity was obtained when using D-lactate. D,L-lactate (racemic mixture) gave an intermediate velocity after five minutes incubation. Pre-incubation of the protein supernatant plus D-lactate for 10 minutes gave results which closely resemble the curve for the D,L-lactate assays. Polarimetric assays were of limited success. Results of polyacrylamide-gel electrophoresis of nymphal protein using D- and L-lactate separately as a substrate gave the same pattern (Plate 1, Figure 1).

The ontogenetic patterns of protein, lipoprotein, and LDH were examined in the eggs, nymphs, and adults (Plate 1, Figures 2, 3, and 4). A comparison of the electrophoretic flow rate (E_f) and relative optical densities is represented in Figure 15. Only one band each of protein, lipoprotein, and LDH was found to exist in the free egg stage. The relative optical density in all three cases ranged from 21% to 26%, where 100% equaled 1.5 optical density. The parasitic sixth nymphal and adult stages showed five LDH isoenzyme bands. The most prominent band was LDH_4 with a relative optical density of 57% and 62% for nymph and adult respectively. The least prominent band was LDH_1; and had relative optical densities of 21% and 19% respectively, where 100% equals 1.5 optical density. Analysis of the protein and lipoprotein of the parasitic nymph and

PLATE 1

Figure 1. LDH isoenzyme pattern of P. crotali nymph whole tissue homogenates using D-lactate (left) and L-lactate (right).

Figure 2. Ontogenetic protein pattern of P. crotali egg, nymph, and adult whole tissue homogenates (left to right).

Figure 3. Ontogenetic lipoprotein pattern of P. crotali adult nymph and egg whole tissue homogenates (left to right).

Figure 4. Ontogenetic LDH pattern of P. crotali adult, nymph, and egg whole tissue homogenates (left to right).

Figure 5. Comparison of mouse serum protein pattern with P. crotali nymph protein and lipoprotein patterns.

Figure 6. Comparison of mouse serum LDH pattern with P. crotali nymph LDH pattern.

Figure 7. Comparison of infected rattlesnake serum LDH pattern with P. crotali adult protein pattern.

Figure 8. Comparison of infected rattlesnake serum protein pattern and P. crotali adult protein pattern.

Figure 9. Electrophoretic comparison of LDH fractions from P. crotali nymph whole tissue homogenates isolated by isoelectric focusing.

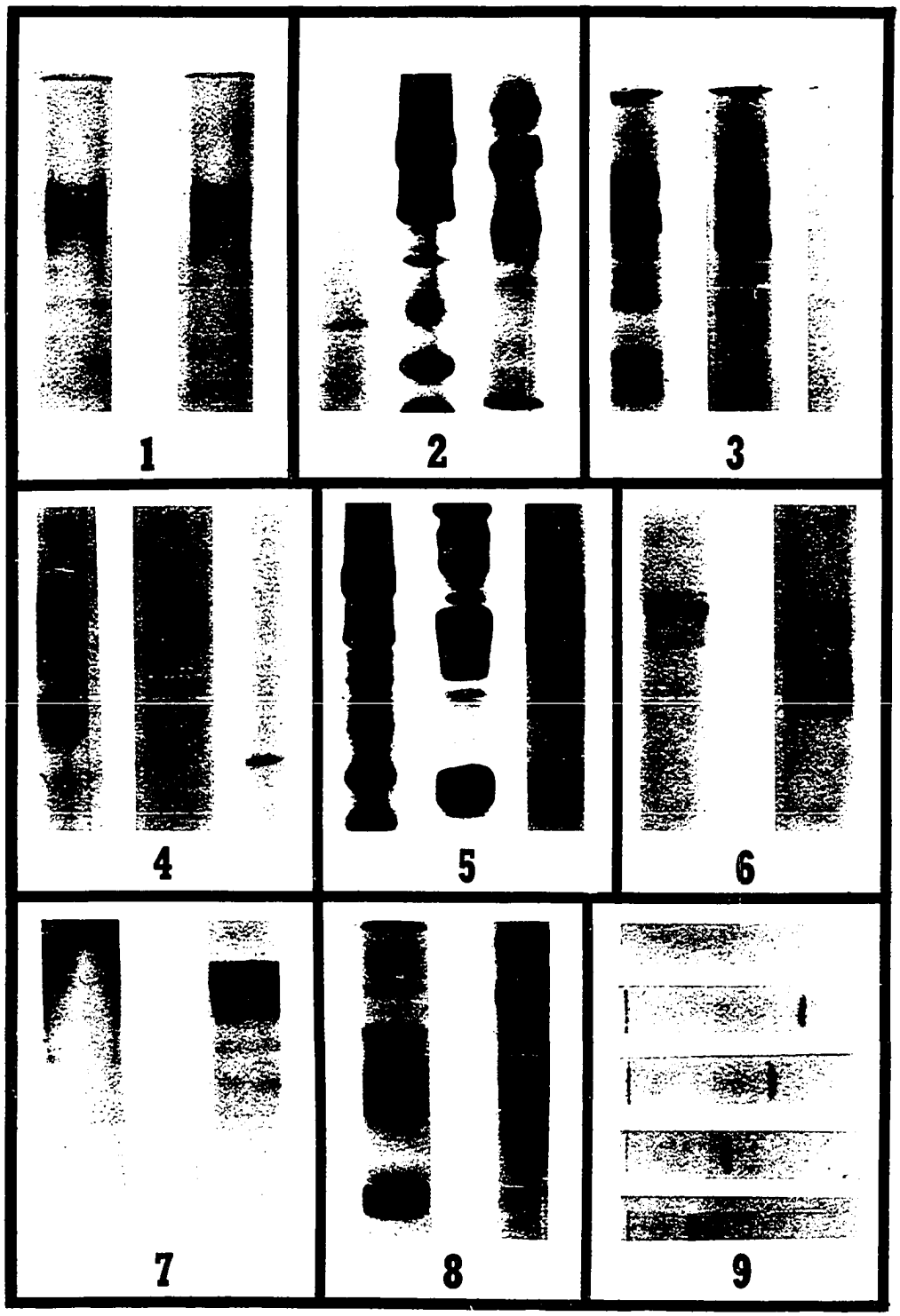

1
2
3
4
5
6
7
8
9

PLATE 2

Figure 10. Hybridization of LDH_1 from P. crotali eggs and nymphs.

Figure 11. Hybridization of LDH_5 from P. crotali nymphs and adults.

Figure 12. Dissociation and recombination of LDH_1 and LDH_5 from P. crotali nymphs.

Figure 13. Electrophoretic comparison of LDH fractions isolated by simultaneous isoelectric focusing of P. crotali egg, nymph, and adult whole tissue homogenates.

Figure 14. Hybridization of LDH_1 from P. crotali nymphs and adults.

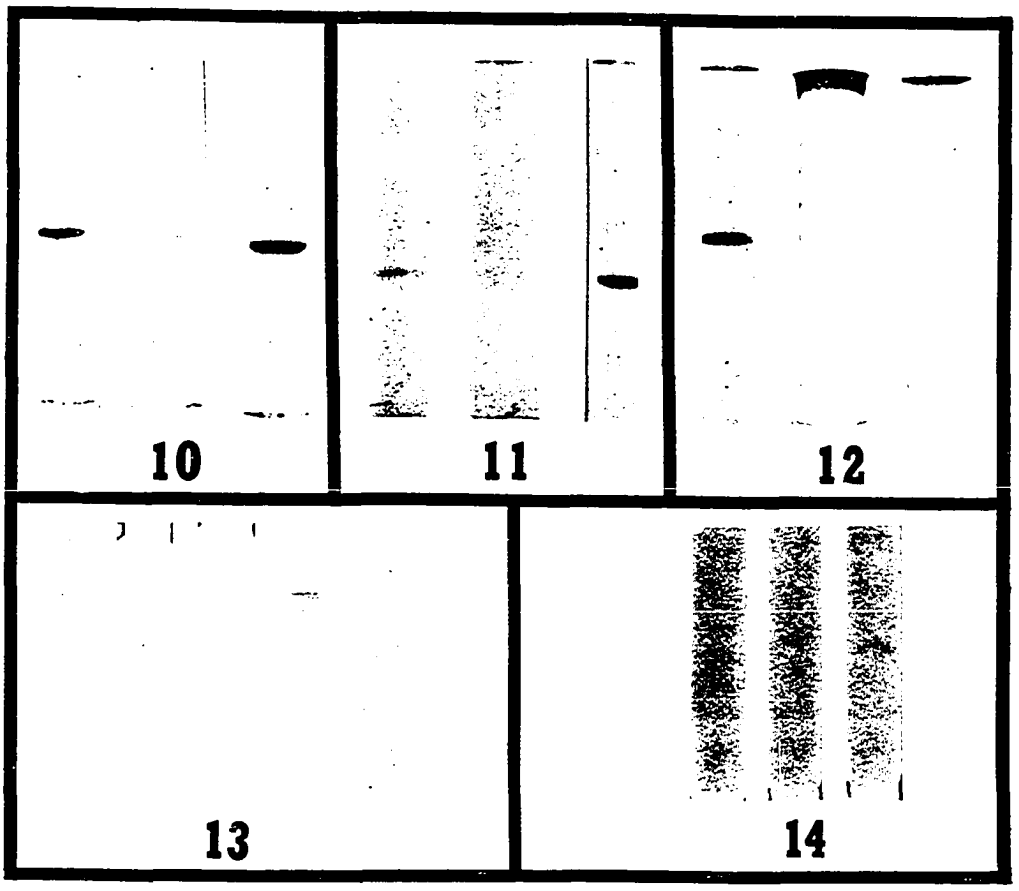
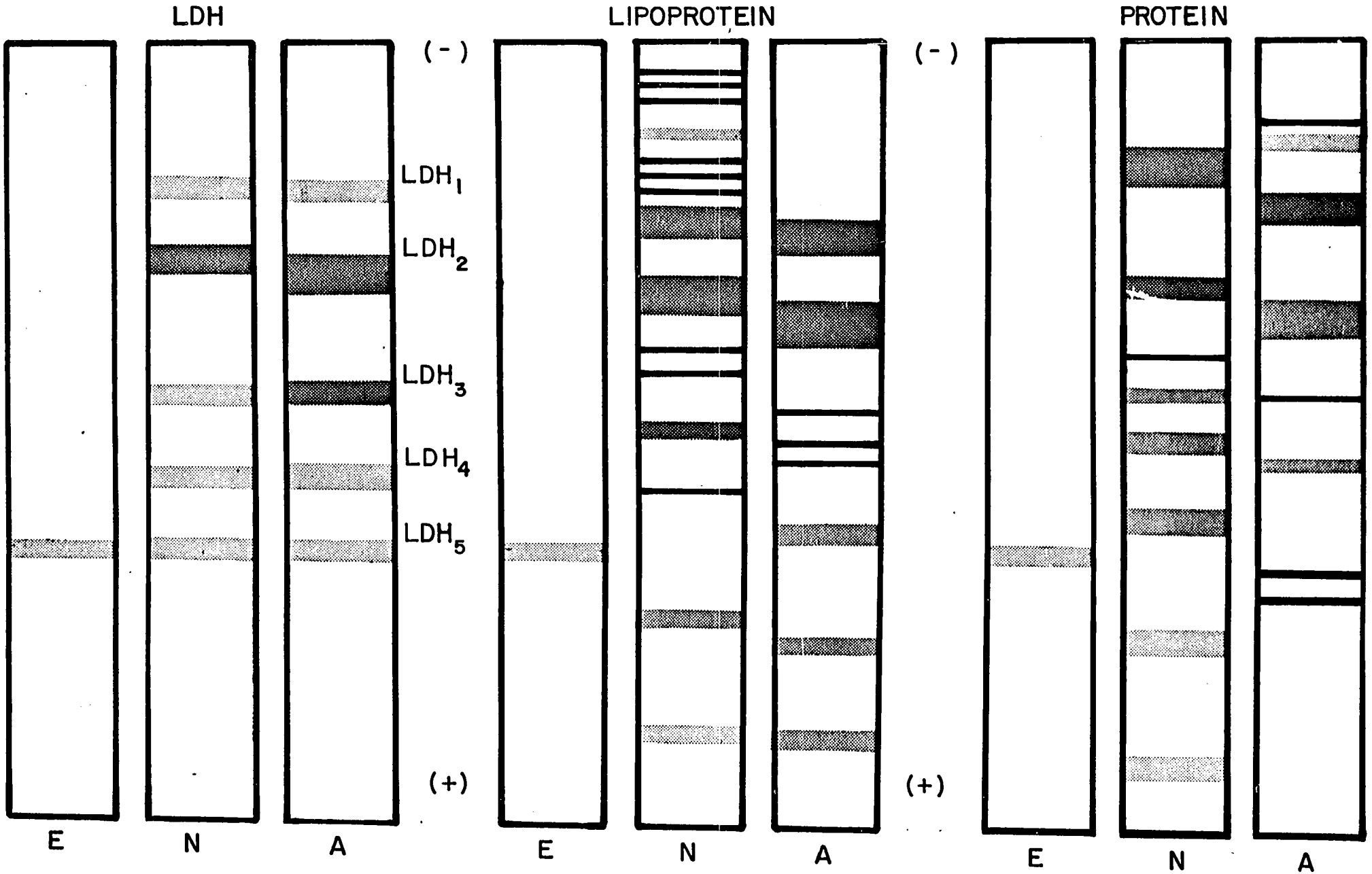

10
11
12
13
14

FIGURE 15

Zymogram showing comparisons of lactate dehydrogenase (LDH), lipoprotein, and protein patterns from P. crotali eggs, nymphs, and adults. Each band is an average of the electrophoretic flow rate (E_f) and relative optical density (O.D.), which were determined by ten densitometer scans.


LDH
LIPOPROTEIN
PROTEIN
(-)
(+)
LDH1
LDH2
LDH3
LDH4
LDH5
E
N
A
E
N
A
E
N
A

adult stages showed a significant increase in bands which were not present in the free-living egg.

The nymphal stage of P. crotali is a mammalian tissue dweller and blood consumer, and the adult stage feeds on blood in the rattlesnake lung. Therefore, comparisons of protein and LDH fractions were performed between the respective hosts and parasites (Plate 1, Figures 5, 6, 7, and 8).

No differences in the isoenzyme patterns were detected with relation to sex and tissue of adult stages.

Control experiments were performed in order to demonstrate the amount of substrate, cofactor, and staining dye specificity. Results of the omission of one factor from the LDH staining media, in an attempt to detect the so-called "nothing dehydrogenase" (Shaw and Koen, 1965) were all negative. Alternate substrates (glyoxylate, alpha-keto-butyrate, alpha-keto-isovalerate, oxaloacetate, and alpha-keto-glutarate) were substituted for lactate in the incubation media in an attempt to determine substrate specificity. All results were negative.

The technique of isoelectric focusing demonstrated only one molecular form of LDH in egg homogenates with an isoelectric point (pI) of 4.2 (Graph 13, Table II). Five molecular forms of LDH were detected in the nymph and adult homogenates. The pI's of the five fractions in both stages were determined and found to exist between 4.0 and 8.6 (Graph 13, Table II). These results further substantiated that the egg possesses only one LDH form, while the nymph and adult

GRAPH 13

Fractionation of whole tissue homogenate of P. crotali egg, nymph and adult stages by isoelectric focusing. Each point represents a mean of five values. A pH gradient of 3 to 10 was established in all experiments, as indicated by the straight line and ordinate on the right.

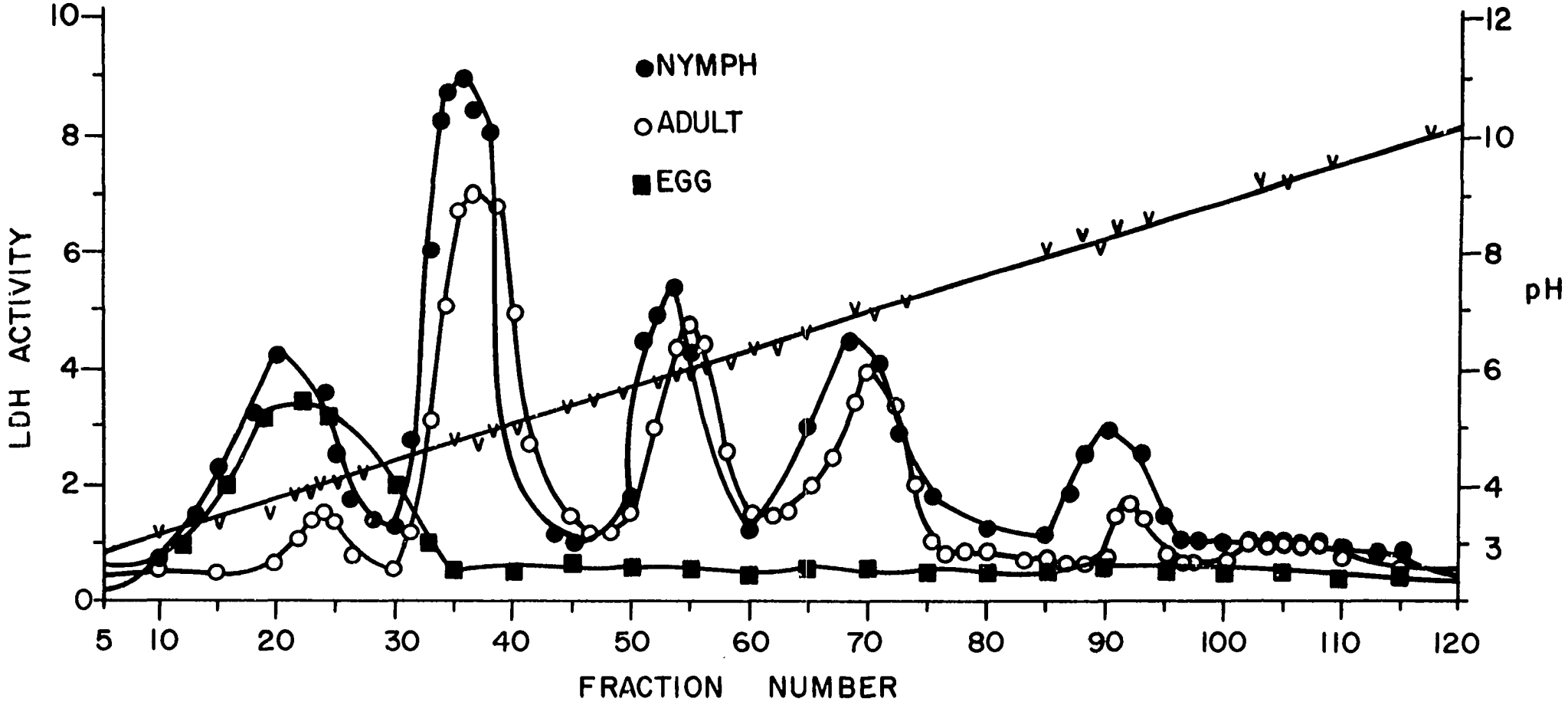

LDH ACTIVITY
pH
FRACTION NUMBER
NYMPH
ADULT
EGG
0
2
4
6
8
10
12
3
5
10
20
30
40
50
60
70
80
90
100
110
120

TABLE II. Comparisons of specific activity, isoelectric points (pI), and K_m from P. crotali egg, nymph and adult stages after isoelectric focusing.

ENZYME SOURCE	FRACTION	SP. ACT.	pI	K_m
EGG	LDH_1	3.6	4.2	5.01×10^{-3}
NYMPH	LDH_1	2.1	4.0	5.53×10^{-3}
	LDH_2	2.5	4.9	4.02×10^{-3}
	LDH_3	2.9	6.0	4.61×10^{-3}
	LDH_4	4.2	7.0	4.70×10^{-3}
	LDH_5	1.8	8.5	4.93×10^{-3}
ADULT	LDH_1	2.3	4.3	4.21×10^{-3}
	LDH_2	2.4	5.0	4.25×10^{-3}
	LDH_3	2.1	6.1	4.12×10^{-3}
	LDH_4	4.1	7.2	4.01×10^{-3}
	LDH_5	1.9	8.6	3.51×10^{-3}

possess five different LDH forms.

In order to determine whether each molecular form was indeed isolated by this technique, electrophoretic separations were performed on fractions showing maximal activity. Results of these experiments showed only one molecular form of LDH was present (Plate 1, Figure 9). Results of assays on the LDH fractions isolated by isoelectric focusing from the three developmental stages executed at various substrate concentrations are shown in Graphs 14 and 15. All LDH fractions from nymphal and adult stages displayed apparent rectangular hyperbolic kinetics.

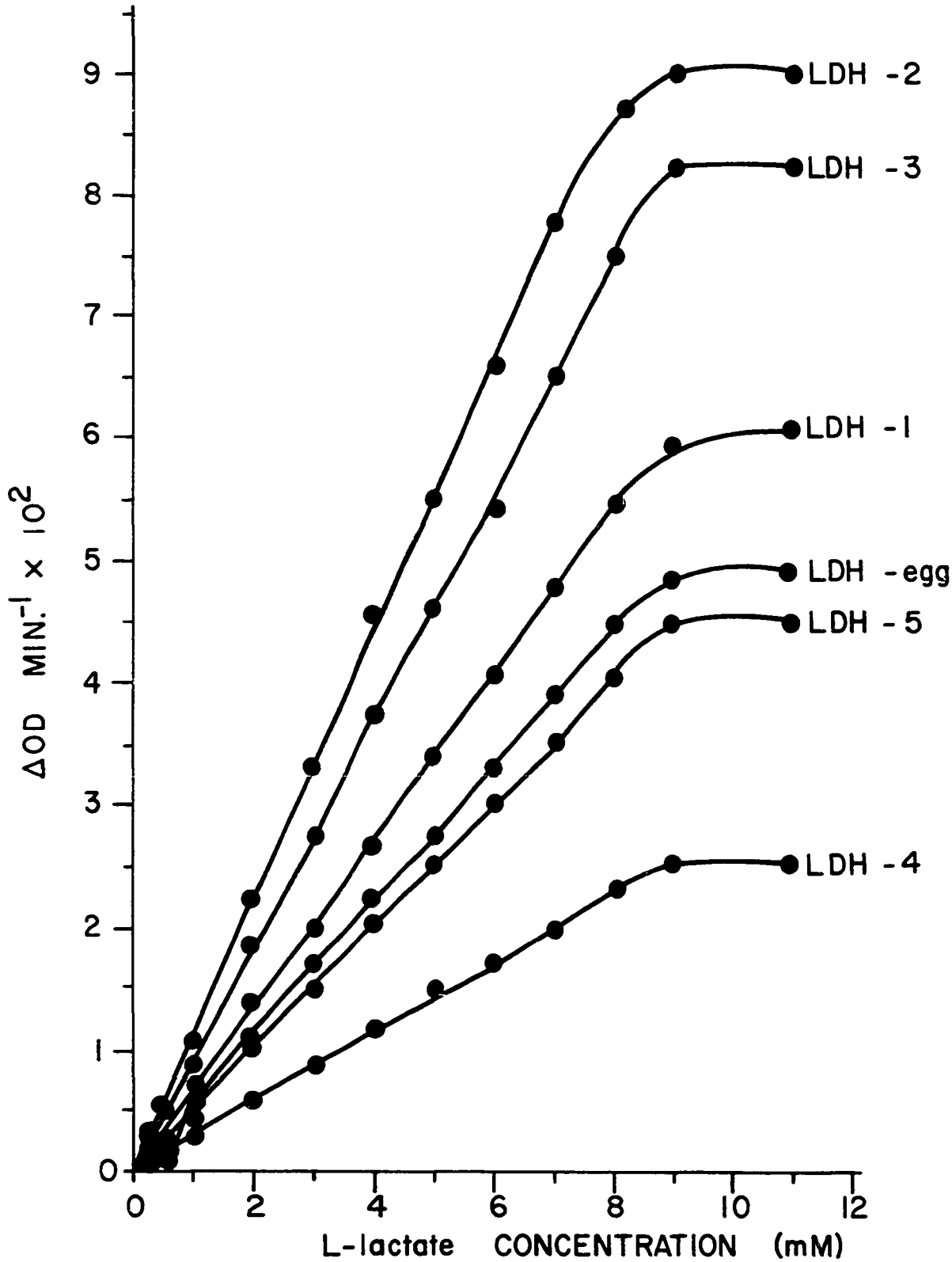
Graph 16 shows isoelectric focusing of the combined egg, nymph, and adult protein supernatants. Five fractions were isolated in this experiment and electrophoresed in order to determine whether one or more different molecular forms were present in the various fractions. In no instance did electrophoresis of these various fractions reveal more than one band (Plate 2, Figure 13), suggesting that the LDH's produced by each developmental stage are the same.

Results of hybridization experiments performed on LDH_1 fractions of eggs, nymphs, and adults, and LDH_5 on nymphs and adults are shown in Plate 2, Figures 11, 12 and 14. They revealed only one band with almost identical electrophoretic flow rate (E_f) after electrophoresis indicating that the LDH forms are the same in each developmental stage.

Efforts to elucidate information on the protein structure involved the reassembling of functional LDH molecules after dissociation in urea, acetone, chloroform, guanidine or at acid pH (2.1). These

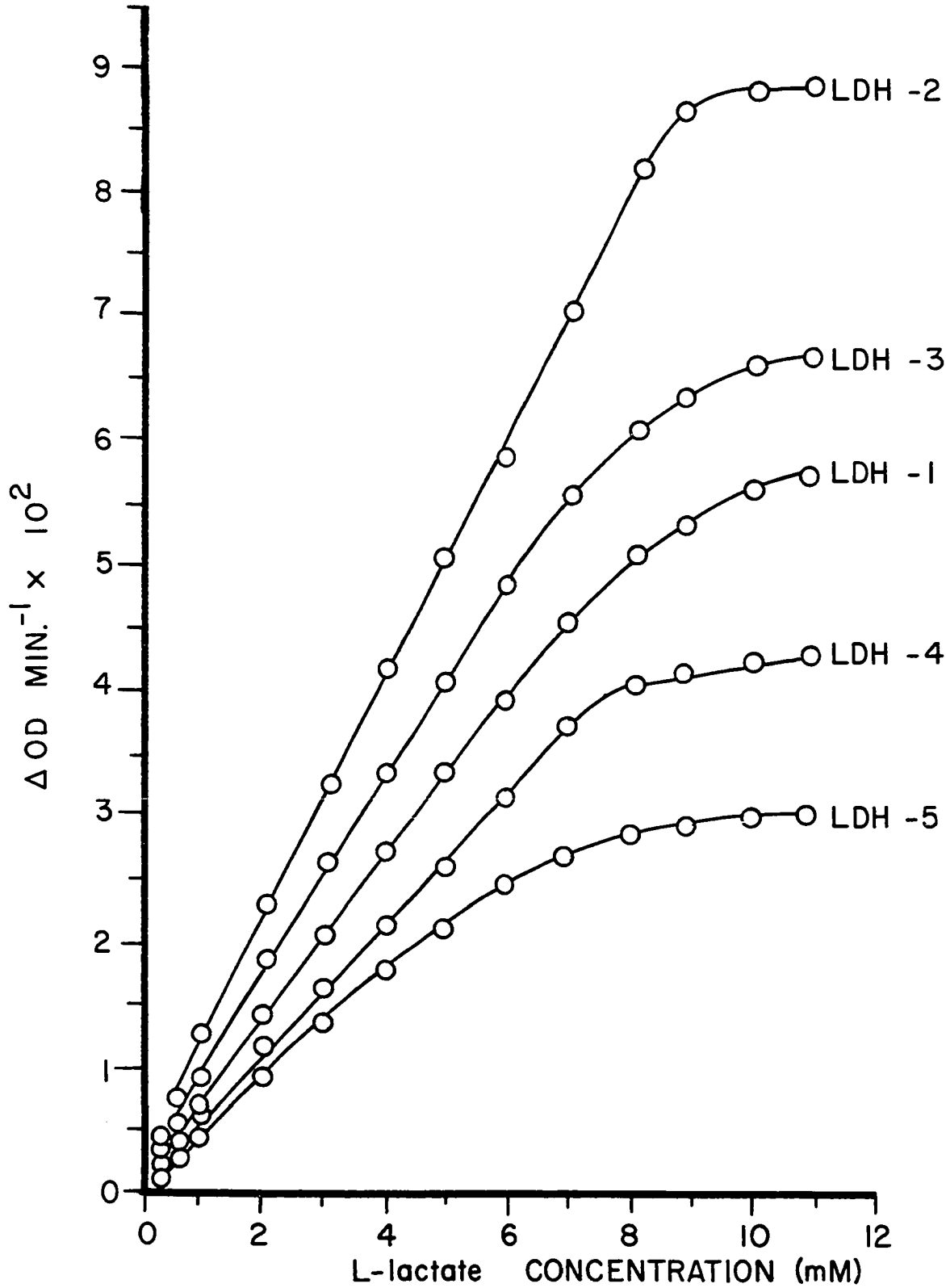
GRAPH 14

Effect of substrate concentration of P. crotali egg and nymph lactate dehydrogenase fractions after separation by isoelectric focusing. Each point is a mean of four values.


LDH -2
LDH -3
LDH -1
LDH -egg
LDH -5
LDH -4
ΔOD MIN.$^{-1}$ x 10^2
0
1
2
3
4
5
6
7
8
9
0
2
4
6
8
10
12
L-lactate CONCENTRATION (mM)

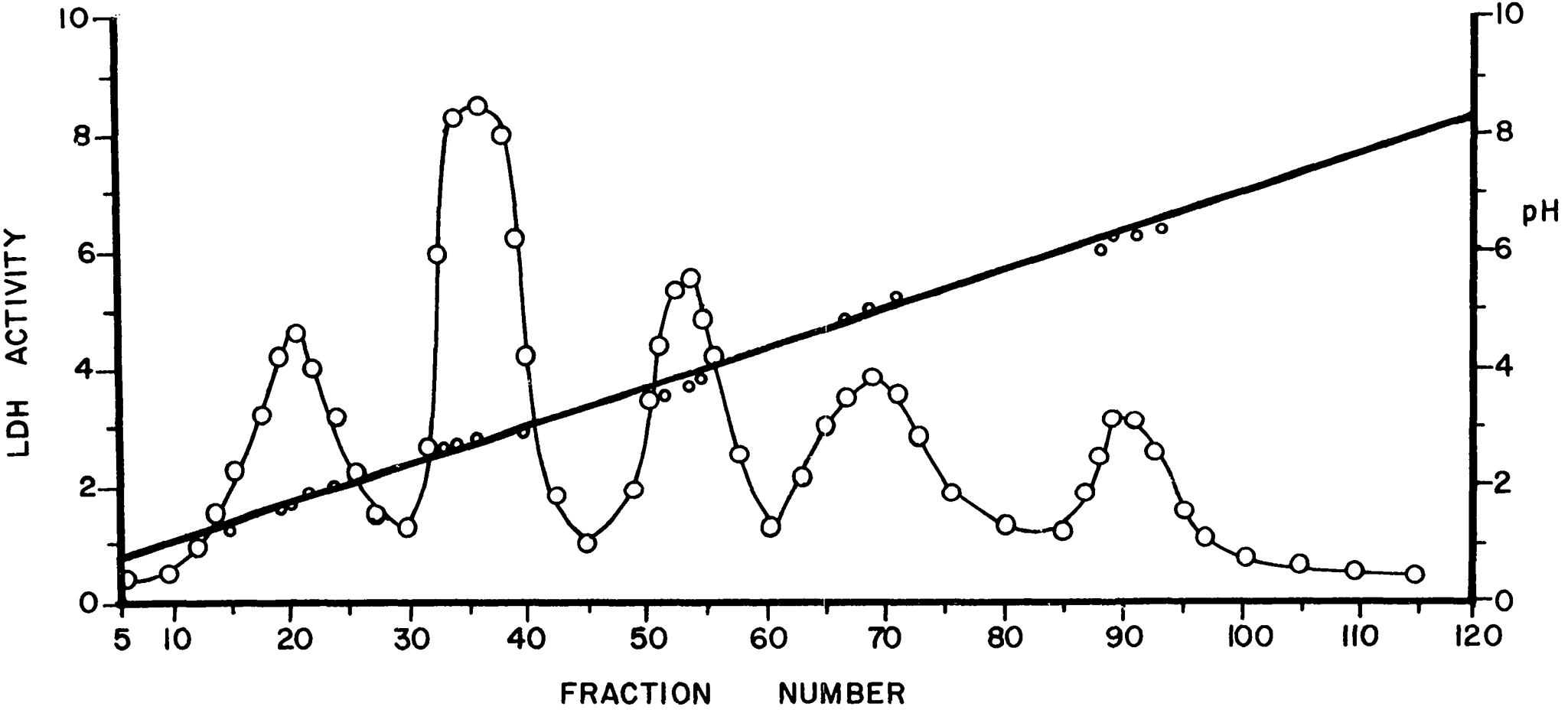
GRAPH 15

Effect of substrate concentration on P. crotali adult lactate dehydrogenase fractions after separation by isoelectric focusing. Each point is a mean of four values.


LDH -2
LDH -3
LDH -1
LDH -4
LDH -5
ΔOD MIN.$^{-1}$ x 10^2
0
1
2
3
4
5
6
7
8
9
0
2
4
6
8
10
12
L-lactate CONCENTRATION (mM)

GRAPH 16

Simultaneous isoelectric focusing of P. crotali egg, nymph and adult whole tissue homogenates. A pH gradient of 3 to 10 was established in all experiments, as indicated by the straight line and ordinate on the right.


LDH ACTIVITY
pH
FRACTION NUMBER
0
2
4
6
8
10
5
10
20
30
40
50
60
70
80
90
100
110
120

experiments were of limited success. Enzyme activity was not greatly reduced by treatment with 1M NaCl. Plate 2, Figure 12 shows the formation of enzymatically active tetramers by random association of nymph LDH_1 and LDH_5 monomers after dissociation by freezing in 1M NaCl.

CHAPTER IV

DISCUSSION

Although LDH isoenzymes have been extensively studied, and in some respects are well understood, explanations of their epigenetic adaptive values with respect to their physiological function, are still incomplete. The LDH isoenzymes of P. crotali provided an opportunity to investigate the properties of a total enzyme, as well as those of individual isoenzymes, the production of which is epigenetically controlled.

Analysis of substrate and cofactor saturation curves for LDH by use of a Hill plot indicate that all three developmental stages have at least two interacting binding sites for NAD^+. This type of enzymatic mechanism, involving at least two binding sites, may play some physiological role in controlling the NAD^+/NADH ratio in the parasite.

Results of LDH assays from whole tissue homogenates of nymphs using L-, D,L-, and D-lactate indicate that a racemase may be present. The actual physiological significance of a racemase in this developmental stage is unknown. A possible explanation is that the presence of a racemase may lower the reaction rate of

lactate to pyruvate under certain physiological conditions and therefore in an indirect way regulate the $NAD^+/NADH$ ratio. The rate increase which resulted when a ten minute pre-incubation of D-lactate + protein supernatant was employed before the LDH assay was carried out is consistent with this explanation.

Specific activity, optimal pH, thermal stability, and Michaelis-Menten constants were the only parameters studied in this investigation. Thus, no correlation with physiological parameters is provided. No modulators of LDH activity in P. crotali are known. There is, however, evidence that certain modulators affect the isoenzymes fructose diphosphatase (Berisch, 1969) and pyruvate kinase (Somero and Hochachka, 1968) in certain fish. Zee and Zinkham (1968) and Barrett and Fairbairn (1971) have shown that the K_m's of the three malate dehydrogenases from Ascaris lumbricoides are closely correlated with temperature. Therefore modulation of similar type in LDH from the three developmental stages of P. crotali is possible. The maximum difference in K_m between the three developmental stages was less than a factor of two. Although relatively small, such differences may nevertheless be significant at the low substrate concentrations occurring in most cells (Atkinson, 1966). The changes in K_m for the three developmental stages are approximately the same as those reported for LDH and acetycholine esterase in trout (Hochachka and Somero, 1968; Baldwin and Hochachka, 1970) and LDH and pyruvate kinase in Paralithodes camtochatica, the Alaskan king crab, during acclimation to temperature changes (Somero and Hochachka, 1969). These changes in

K_m are also larger than those of LDH and pyruvate kinase in the peripheral tissues of Phoca vitulina, the harbor seal (Somero and Johanson, 1970). Although none of the mentioned investigators included the examination of individual isoenzymes, electrophoretic changes in isoenzyme patterns of fish during temperature acclimation have been described (Hochachka, 1967; Baldwin and Hochachka, 1970). Thus the possibility of changes in isoenzyme pattern could be a possible mode of enzyme modulation.

Optimal pH and temperature stability for LDH in the three developmental stages were found to exist between pH 7.8 and 8.1 and 41 C respectively. It should be pointed out that the results of pH and thermostability studies on whole tissue homogenates of P. crotali must, however, be interpreted cautiously since the rate of thermal and pH denaturation is greatly affected by interaction with other proteins. In any case, it appears that the correlations between thermostability and pH and the environmental temperature and pH have been found in several structural proteins (Alexandrov, 1969) and in aldolase of antarctic fishes (Komatsu and Feeney, 1970) and could possibly exist in P. crotali.

Ontogenetic analysis of protein, lipoprotein and LDH patterns in P. crotali by polyacrylamide-gel electrophoresis demonstrated a change in the synthesis of protein and lipoprotein during the life cycle of the parasite. A dramatic change in the LDH isoenzyme pattern of this parasite is shown, going from one to five different molecular forms. Isoelectric focusing of the three protein super-

natants from the three developmental stages further substantiates that the egg contains only one LDH form, while the nymph and adult possess five different forms. Although the actual physiological function of LDH in P. crotali is uncertain, the shift from one to five isoenzymes may be an epigenetic adaptation in this parasite. The changes in protein and lipoprotein may be indicative of regulatory processes of ontogeny, cellular differentiation and morphogenesis which are manifested during the growth and development of the parasite. Moreover, this type of regulation would involve the synthesis of proteins, lipoproteins, and enzymes.

The predominant LDH band found in the nymphal and adult stages was LDH_4. Pfleider and Wachsmuth (1961) demonstrated that LDH_5 and LDH_4 are predominant in anaerobically metabolizing tissues such as human liver and skeletal muscle. Wilson et al., (1963), have shown a correlation between the flying habits of various birds and the breast muscle LDH composition. Therefore, it may be that P. crotali nymph and adult stages are anaerobic but can function satisfactorily in an aerobic environment such as the lung of a snake.

Evidence that the isoenzymes are five different proteins, and not five different conformations of the same protein caused by intracellular chemical reactions (such as deamination or decarboxylation) was derived by dissociation and recombination experiments. When LDH_1 and LDH_5 of the nymph were dissociated and recombined, five bands were detected, but the classical 1:4:6:4:1 ratio (according to Markert and Massaro, 1968), was not obtained. This was probably

due to incomplete dissociation. These results can be most easily interpreted by assuming that the synthesis of the cytoplasmic LDH isoenzymes is controlled by two genetic loci, A and B. According to this hypothesis the five isoenzymes are tetramers arising from aggregation of the two different subunits into five possible combinations, LDH_1 = AAAA; LDH_2 = AAAB; LDH_3 = AABB; LDH_4 = ABBB; and LDH_5 = BBBB (Markert and Massaro, 1968). Results from dissociation and reassociation of LDH_1 and LDH_5 respectively, indicate that they are each composed of four identical subunits.

The presence of five isoenzymes in P. crotali are indicative of only two possible conformations for the tetrameric protein. Klotz et al., (1970) discuss the possibility of only two different types of quaternary structure for a tetramer such as LDH. Cyclic symmetry is the simplest and resembles a square with no free ends, such as in a linear bead arrangement. The second type of arrangement would be dihedral symmetry. Dihedral symmetry would be present only if the two fold axes exist at right angles to any single fold axis. The simplest arrangement of dihedral symmetry would involve a tetramer and would resemble a triangular arrangement of three subunits with the fourth subunit located on the top or bottom of the triangle. If neither of these two conformations of the tetrameric LDH protein existed, then there would be a possibility of more than five molecular forms present in the cytoplasm of P. crotali.

Hybridization results on LDH_1 fractions from the different developmental stages indicate that this enzyme is a homologous tetramer

and the same specific gene product. The same is true for LDH_5. These conclusions are further substantiated by the similarities in electrophoretic flow rates and isoelectric points of the three protein supernatants from the three developmental stages.

Failure to reassemble functional LDH molecules after dissociation in urea, guanidine, or at acid pH (2.1) can be explained by assuming that dissociation procedures rupture hydrogen bonds and destroy the tertiary and secondary structure of the monomers. Such denatured monomers apparently do not recombine into tetramers nor do they exhibit enzymatic activity. Since the monomers that were dissociated in NaCl behave differently, they must have retained much of their original structure.

Enzyme activity was not greatly reduced by treatment with 1M NaCl, and this suggests that the monomers retain enzyme activity. Thus, the overall structure of the monomers remained essentially intact. LDH with a molecular weight of 72,000 has been reported as enzymatically active, which indicates that the dimer is functional (Markert and Massaro, 1968).

The formation of enzymatically active tetramers by random aggregation of monomers demonstrates that the functional requirements for the quaternary structure of LDH are readily available in the structure of the monomers. However, after the disruption of the secondary and tertiary structure of the monomers (as with urea, acetone and chloroform) they apparently lose their ability to become functional LDH molecules again even though the primary structure probably remains

intact. Therefore the physico-chemical environment in the cell at the site of protein synthesis, at least in the case of LDH_1 can be of decisive importance in determining the higher states of molecular configuration.

It is evident that studies of LDH offer biologists from different areas great opportunities for further research. The existence of the multiple forms of LDH furnishes for geneticists a simple system to follow in higher animals, and for those interested in developmental biology, a simple tool for following specific biological changes in ontogeny. The physiological implications of the multiple forms of LDH studied in this research hopefully provide a new approach to problems in multicellular organisms, and interesting structural characteristics of LDH are a challenge to the enzymologist and protein chemist.

CHAPTER V

SUMMARY

The ontogenetic study of P. crotali was undertaken using enzyme assays, isoelectric focusing and polyacrylamide-gel electrophoresis. The purposes of this research were to investigate and demonstrate in the egg, sixth nymphal stage, and adults of P. crotali the following: (1) LDH activity; (2) multiple molecular forms of LDH; (3) kinetic differences after separation by isoelectric focusing; (4) changes in the protein, lipoprotein, and LDH patterns, and (5) subunit structure of LDH.

LDH assays on nymph and adult whole tissue homogenates displayed rectangular hyperbolic kinetics, while the egg homogenate displayed S-shaped kinetics, the latter is characteristic of interacting binding sites.

Hill plot revealed that the egg enzyme possessed a minimum of two interacting substrate binding sites, and the nymph and adult a maximum of one non-interacting binding site.

LDH activity assayed at various NAD^+ concentrations revealed by use of a Hill plot that all three developmental stages possessed a minimum of two interacting cofactor binding sites.

Optimal pH for the egg, nymph and adult was found to be 7.9,

7.8, and 8.1 respectively. All three stages exhibited optimal thermal stability at 41 C.

Nymphal homogenates were found to possess a racemase-like enzyme that could convert D-lactate to L-lactate. Polyacrylamide-gel electrophoresis demonstrated a tremendous increase in the synthesis of protein and lipoprotein during development of the egg to the adult stage. LDH was found to shift from one molecular form in the egg to five different molecular forms in the nymph and adult stages. Fractionation by isoelectric focusing substantiated that only one molecular form was present in the egg, while the nymph and adult possess five different forms.

Evidence for tetrameric structure was obtained by dissociation and recombination experiments. Hybridization of the LDH_1 fraction from the three developmental stages indicates that they are products of the same gene. The same is true for the LDH_5 fraction in the nymph and adult.

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