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GEOGRAPHIC VARIATION OF MUSCLE-TYPE LACTATE DEHYDROGENASE IN THE FROG, *Rana pipiens*

by

PAUL LEVY

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1972

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

May 24, 1972
Date

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Special thanks are due too to Dr. Aaron Lukton, for his helpful advice and criticism of the kinetic studies. His frequent role of Devil's Advocate allowed crystallization of many experimental approaches used here. I also thank him for his generosity in supplying both equipment and facilities whenever needed. His excellent humor and humanity helped me through some difficult moments.

The greatest amount of thanks are due Dr. Stanley Salthe. Dr. Salthe is largely responsible for instilling in me a keen interest in molecular evolution. His quick mind and active imagination leave me with the greatest respect for him. His profound philosophical musings, although frequently at variance with my own ideas and prejudices, were always valuable because they never failed to stimulate questions, both scientific and otherwise.

For this reason alone, he must be considered a great teacher.

I would also like to acknowledge Mrs. Jean Needleman, without whose help and support I probably would not have entered the graduate program. Her genuine concern for the doctoral students was a breath of fresh air in an otherwise polluted beaurocracy. Her ability to cut through red tape and her willingness to do so for us on innumerable occasions made it a constant joy to deal with her.

One extremely unfortunate note must be sounded here. During the course of an otherwise peaceful demonstration on the Brooklyn College Campus, Dr. Salthe's entire frog collection (which formed the basis for the studies reported here) was destroyed. This senseless act of vandalism deprived him and others of an irreplaceable collection which took many years to build. It is with a sense of shame that I make note of this extremely juvenile act on the part of supposedly mature college students.

As a final happy thought, I acknowledge my wife, Maxine, whose unswerving faith in me allowed me to continue through some rather grim days. Her wit and humor encouraged me; her sensitivity preserved my sanity. In short, I don't think this thesis would have been possible, were it not for Maxine.

ABSTRACT

Variability in muscle-type lactate dehydrogenases (M-LDH) in the frog, Rana pipiens, was studied. Of 589 animals from 81 populations, only 4 animals exhibited electrophoretic variants, and no polymorphisms were observed. Twenty five populations were tested for immunological distinctness from the typical (Wisconsin) allotype by the micro-complement fixation test, and no differences were noted. Pooled samples from four populations were examined for differences in M-LDH thermostability, and on this basis the Guasave allotype was distinguished from the others, since it was labile at lower temperatures.

Detailed kinetic comparisons were made between Wisconsin and Guasave M-LDHs. No differences could be discerned for K_ms over a range of temperatures, nor could any be found for energies of activation, suggesting that these allotypes might be the result of neutral allelic replacement.

The reliability of various screening methods is discussed. It is concluded that electrophoretic estimation of variability may be the most useful method for those cases in which the degree of variability is low, providing that corrections are made to reflect that proportion of variability resulting from interchanges which do not involve differences in charge.

While the data fit both a selectionist and neutral allele hypothesis, it is likely that the latter is the most probable explanation for the pattern of variability observed for LDHs in R. pipiens.

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INTRODUCTION

Since the initial demonstration of widespread genetic variability at the molecular level (Harris, 1966; Hubby and Lewontin, 1966; Johnson et al., 1966; Lewontin and Hubby, 1966), it has become evident that a large proportion of enzyme loci are polymorphic in a wide variety of organisms, including man. Some examples of the estimates of the percentage of polymorphic loci in natural populations, based upon random samplings of proteins are 36% in Limulus polyphemus (Selander et al., 1970), 39% in Drosophila pseudoobscura (Lewontin and Hubby, 1966), 44% in the European house mouse (Selander et al., 1969), and 54% in Avena fatua (Marshall and Allard, 1970). It has frequently been assumed that balancing selection of some description is maintaining this large array of polymorphisms (Kojima and Yarbrough, 1967; Johnson et al., 1969; Koehn, 1969; Prakash, 1969; Prakash et al., 1969; Burns and Johnson, 1971). However, such balancing selection, operant upon large numbers of polymorphisms would impose impossibly large genetic loads (i.e., genetic deaths required to maintain polymorphisms) on populations if genetic loads were calculated according to traditional models of selection (i.e., multiplicative models), even if very small coefficients of selection are assumed (Lewontin and Hubby, 1966; Maynard-Smith, 1968; Kimura and Crow, 1969; O'Donald, 1969).

In addition to the genetic load due to balanced polymorphisms, the amount of genetic death associated with speciation is considerable (Haldane, 1957; Haldane, 1960; Crow, 1970). For example, it is estimated that only 34% of all alleles are shared between the sibling species pair D. melanogaster and D. simulans (Berger, 1970), implying intense directional selection during speciation. Thus, the genetic load due to the combined effects of directional selection and stabilizing selection seems to be too high to allow the survival of the organism.

This apparent impasse could be resolved in several ways. If our conception of how natural selection operates is revised, so that a culling or threshold mechanism is considered (King, 1967; Milkman, 1967; Sved et al., 1967; Wills et al., 1970), then heterosis could be capable of maintaining large numbers of polymorphic loci without burdening the population with an unbearable genetic load. Such models are extreme forms of epistatic and linkage disequi;ibrium models (Lewontin, 1964; Mukai et al., 1971), by which the genetic load is thought to be reduced through non-random association of alleles within linkage groups. A similar model has been proposed for directional selection in haploids (Maynard-Smith, 1968).

Alternatively, some authors consider that the evolution of primary gene products proceeds through gradual, random accumulation of selectively neutral alleles through

a combined process of continual mutation and genetic drift leading to allele fixation (Kimura, 1968a,b; Arnheim and Taylor, 1969; King and Jukes, 1969). If alleles are selectively neutral, then only the effective population size and the neutral allele mutation rate determine the number of alleles of a particular gene (Kimura and Crow, 1964). Polymorphisms are thought to be transient events in the continuum of allelic replacement (Kimura and Ohta, 1971). This formulation does not consider the various allotypes as nonadaptive, but rather as equally adaptive.

Vertebrate lactate dehydrogenase, LDH (L-lactate: NAD oxidoreductase, E.C. 1.1.1.27) is a tetrameric enzyme (Appella and Markert, 1961; Allison et al., 1969) drawn from two different types of subunits in the frog, Rana pipiens (Salthe et al., 1965; Wright and Moyer, 1966; Goldberg and Wuntch, 1967). These subunits have been designated as heart-type LDH (H-LDH) or B, and muscle-type LDH (M-LDH) or A (Markert, 1963; Kaplan, 1964). It has been demonstrated that the different subunits are under the control of separate structural genes (Shaw and Barto, 1963; Boyer et al., 1963; Nance et al., 1963), which have been designated LDh and LDm respectively (Wright and Moyer, 1966). Additional subunits are known in other vertebrates (Zinkham et al., 1963; Markert and Faulhaber, 1965; Whitt, 1969). Homotetramers composed of the H subunit exhibit substrate inhibition at lower concentrations of substrate

than do homopolymers of the M (Salthe, 1965; Levy and Salthe, 1971). Similar observations in other species have led to the hypothesis that the five isoenzymes generated from the two subunits may operate in different physiological contexts (Wilson et al., 1963; Lindsay, 1963; Salthe, 1965; Nemchinskaya et al., 1968; Everse et al., 1970; Levy and Salthe, 1971; Levy et al., 1971).

In an extensive investigation of the geographic variation of H-LDH in R. pipiens, 11 variants were reported, all of which were involved in local polymorphisms (Salthe, 1969). Although it was initially assumed that these alleles were maintained by balancing selection, a subsequent investigation revealed no evidence to indicate differences in the kinetic properties of some of these H-LDH variants (Levy and Salthe, 1971). The purpose of the present investigation was to determine the extent of variability of the M subunit. To this end, 589 animals from 81 populations, representing the entire range of R. pipiens were examined for M-LDH variability. Since no single experimental approach would be sufficient to adequately describe protein variability (Salthe, 1969), three methods were utilized: electrophoretic, kinetic and immunological. The evidence to be presented will indicate that variability of M-LDH in natural populations of R. pipiens is minimal, and that this is probably the result of intense selection. This notion will be shown to be consistent with the neutral allele hypothesis.

MATERIALS AND METHODS

(a) Animals

The Rana pipiens used in this study were principally those used by Salthe (1969) for his study of H-LDH variability, which were generously made available to me. The Panamanian frogs were the gift of Dr. Richard G. Zweifel of the American Museum of Natural History. The frogs from Guasave, Sinaloa, were purchased from Hermosa Reptiles, Inc., and the frogs designated as Wisconsin were obtained from Lemburger, Inc., and were the ones used for the purification of M-LDH. Table 1 is a summary of the localities from which the animals used in this study were derived. All of those used, with the exception of those from Guasave, were determined to be members of the R. pipiens complex by S.N. Salthe.

Male and female frogs and tadpoles at several developmental levels were used. However, developmental stage has no influence upon LDH variability (Levy and Salthe, unpublished), although the relative amounts of isoenzymes do change ontogenetically (Salthe et al., 1965; Wright and Moyer, 1966; Wright and Moyer, 1968; Moyer et al., 1968).

Table 1. Geographic Distribution of R. pipiens Populations Studied. Locality refers to the closest recognized geographical landmark to where the animals were captured. These animals were for the most part identical to those studied by Salthe (1969).

Table 1. Geographic distribution of R. pipiens populations studied.

State	Locality	Number
Alabama	Franklin County	9
Arizona	Portal	2
	Tuscon	2
Florida	Fort Lauderdale	8
	Jacksonville	1
	Tarpon Springs	24
Georgia	Fairburn	31
	Green Hill Region	7
Louisiana	La Place	4
Maryland	College Park	10
Massachusetts	Wayland	1
Mississippi	Jackson	3
New Jersey	Green Village	2
	Jenkins	7
	Lakewood	5
	North Bergen	3
	Penn State Forest	3
	Springdale	4
New Mexico	Arroyo Blanco	5
	Carlsbad	2
	Delaware Creek	1
	Tucumcari	3

Table 1 (con't.) Geographic distribution of *R. pipiens* Populations Studied.

State	Locality	Number
North Carolina	Nags Head	1
Oklahoma	Tulsa	4
South Carolina	Florence	1
	Jasper County	1
South Dakota	Tyndall	2
Tennessee	Ashland City	61
	Burns	3
	Dickson	4
	Donelson	8
	Erin	1
	Goodletsville	4
	Kingston Springs	1
	Lebanon	2
	Linton	2
	Nashville	9
	Petway	1
	Smithland	6
	White Bluff	1
Texas	Alpine	9
	Austin	5
	Balmorhea	25
	Bastrop	3
	Brownwood	5

Table 1 (con't.) Geographic distribution of R. pipiens populations studied.

State	Locality	Number
Texas (con't.)	Buffalo	1
	Davis Mountain	9
	Fort Davis	9
	Kerrville	21
	Lulling (Palmetto Park)	42
	Midlands	3
	Ottine	9
	Pfluggerville	32
	Sheffield	3
	Silvertone	1
	Sinton	1
	Uvalde	7
	Wichita Falls	6
Utah	Logan	4
Vermont	Alberg	16
West Virginia	Williamstown	6
Coahuilla, Mexico	San Juan de Sabinas	3
Morelos	Cuautla	8
	Cuernavaca	7
Oaxaca	Ayutla	3
	Ejutla	1
	Guelatao	13
	Huajuapan de Leon	3

Table 1 (con't.) Geographic distribution of *R. pipiens* populations studied.

State	Locality	Number
Oaxaca (con't.)	Ixtlan de Juarez	1
	Mitla	2
	Oaxaca	9
	Ocotlan	1
	Tehuantapec	11
Sinaloa	Guasave	22
Tabasco	Villa Hermosa	1
Taumalipas	Matamoros	1
Alberta, Canada	Calgary	6
Ontario	Pine Portage	1
Costa Rica	San Rafael	9
Nicaragua	Bluefields	3
Panama		2
Totals	81	589

(b) Preparation of Homogenates

Homogenates were prepared from the gastrocnemius muscle of adult frogs, or in the case of small or immature animals, the muscles of one or both legs. With tadpoles, the tails were used. Muscle homogenates were made in a Sorvall Omnimixer equipped with a micro-attachment by grinding excised tissue at top speed for about 1 minute with sufficient ice-cold 0.25 M sucrose to yield a 25% homogenate. Crude homogenates were clarified by centrifugation at 15,000 x g for 30 min. at 5°. Homogenates thus prepared were stored frozen at -25° with no apparent loss of enzymatic activity or change in immunological properties. Because some subunit rearrangement of electrophoretic bands has been noted upon prolonged storage (Salthe, 1969), electrophoresis was carried out within a week of homogenization.

(c) Electrophoresis

Horizontal zone electrophoresis was carried out in 14% starch gels using electrostarch (Electrostarch Inc., Madison, Wisc.) in 0.2 M citrate-phosphate buffer, pH 7.0. To make an origin, a slit was cut across the width of the gel, into which were inserted small squares of filter paper (Whatman #2) containing absorbed crude homogenate. Electrophoresis was carried out for 8 hrs. at a constant current of 50 ma, yielding about 20V/cm. across the gel.

Following electrophoresis, the gels were sliced and stained for LDH activity by the tetrazolium method of Fine and Costello (1963). Variant LDH patterns were always rerun for confirmation of the pattern.

(d) LDH Assay

LDH was assayed as previously described (Levy and Salthe, 1971; Levy et al., 1971). The reaction was initiated by the addition of 0.02 ml of a suitable diluted enzyme preparation to the reaction mixture consisting of 1.0 mM NADH, 1.0 mM Na pyruvate in 0.1 M phosphate buffer, pH 7.2. The final volume of the reaction mixture was 3.0 ml. The initial reaction rate was determined by following the enzymatic oxidation of NADH spectrophotometrically at 340 nm in a Zeiss PMQ II spectrophotometer. Absorbance readings were made every 15 sec., for 75 sec., and the initial rates were expressed in arbitrary enzyme units defined as the change in optical density per minute at 25°. All determinations were made in triplicate and the mean value reported.

Km and V_{max} were determined by the method of Lineweaver and Burk (1934) using a minimum of six experimental points. The inhibition constant, K_i, was determined by plotting the reciprocal of initial rate against substrate concentration, and determining the substrate value at which the extrapolated straight lines intersect the abscissa (Dixon and Webb, 1964; Stambaugh and Post, 1966).

Since the degree of substrate inhibition was found to vary from one lot of coenzyme to another (Salthe, 1965), only a single lot was used throughout a series of experiments. Enzyme concentrations were adjusted to yield a maximum change in O.D. of 0.150 optical density units/min., since concentrations in excess of this value tended to give non-linear velocities over the period of measurement.

Temperature studies on LDH required the use of a specially modified cuvette holder allowing the circulation of thermostatically controlled water through the block. Temperature was monitored in a blank cuvette.

(e) Purification of M-LDH

Wisconsin R. pipiens legs were skinned and homogenized in ice-cold buffer (0.1 M K_2HPO_4 and 0.001 M EDTA, pH 6.5) with a Waring blender. All subsequent steps, with the exception of column chromatography, were carried out in the cold. Solid ammonium sulfate was slowly added to the homogenate until it was 40% saturated, and the precipitate was allowed to form overnight. The precipitated protein was discarded, and the addition of ammonium sulfate continued until 65% saturation had been achieved in the supernatant. The precipitate was centrifuged and redissolved in a minimal quantity of water. The pH was then adjusted to 5.0 by the dropwise addition with stirring, of 0.1 N acetic acid. The precipitate which formed overnight was discarded. In order to take advantage of the

fact that the presence of substrate is capable of protecting enzymes during thermal denaturation (Vessell and Yeilding, 1966; Sudi, 1970), a ten-fold excess of NAD and Na lactate was added, assuming that four moles of substrate are bound per mole of LDH (Takenaka and Schwert, 1956), and that there are about 2.3×10^{11} units per mole of LDH, if the molecular weight is taken as 145,000 (Pesce et al., 1964), and the specific activity as 1500 (see below). Thermal denaturation also has the advantage of preferentially destroying H-LDH owing to its greater thermolability (Wuntch and Goldberg, 1968). The preparation was then heated to 60° in a water bath for 7 min., quickly chilled, and the coagulated protein discarded. The supernatant was brought to 40% saturation with ammonium sulfate, the precipitate discarded, and the LDH precipitated by the addition of ammonium sulfate to 57% saturation. The partially purified LDH was redissolved in a minimal quantity of 0.03 M Na_2HPO_4 and 0.04% (v/v) mercaptoethanol, pH 7.8, and dialyzed against two changes of the same buffer for five hours each. The dialysate was applied to a column of DEAE-Sephadex, A-50 (Pharmacia), equilibrated with 0.03 M Na_2HPO_4, pH 7.8, and eluted from the column with the same buffer. Column chromatography could be performed at room temperature, owing to the general stability of LDH. Under these conditions, the LDH does not bind to the column, and appears in the eluant just

Table 2. Purification of M-LDH from Wisconsin R. pipiens. An enzyme unit was defined as that quantity of enzyme yielding an initial rate of 1.000 O.D./ml./min under the conditions of assay stated in the text. Protein was measured by the micro-biuret.

Table 2

Procedure	Volume (ml.)	Enzyme Conc. (E.U./ml.)	Specific Activity (E.U./mg Protein)	Purification X	Yield %
Homogenate	4950	327	17	1	100
40% - 65% Ammonium sulfate	1750	925	62	3.5	100
pH 5.0	1650	930	116	7	90
60°	1550	1640	357	21	138
40% - 57% Ammonium sulfate	270	4000	434	26	67
After A-50 chromatography	129	6750	1500	88	54

after the void volume. The LDH was then concentrated by the addition of 55% ammonium sulfate, and the precipitate was allowed to form slowly over a period of several weeks (the LDH was not crystallized by this method). M-LDH thus prepared has a specific activity of about 1500 U/mg. protein. Table 2 summarizes the steps involved in the purification procedure.

This method is not applicable to the purification of M-LDH from the Guasave frogs owing to the apparent thermal lability of that enzyme (see below). Consequently, only partially purified material was available for the kinetic studies I report. However, no differences in kinetic properties between crude homogenates and purified enzyme could be discerned in Wisconsin enzyme preparations (Salthe, 1965; Levy and Salthe, 1971).

(f) Criteria of Purity

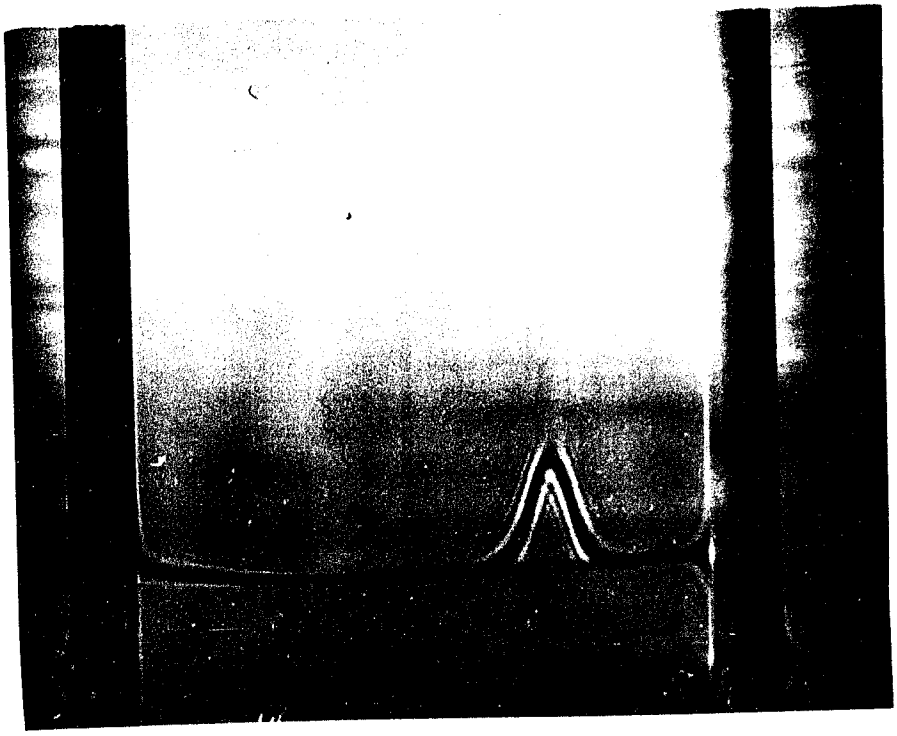
The criteria of purity used were (1) only a single band was formed with cellulose acetate electrophoresis when the strips were stained for total protein (2) a single protein band corresponding to the position of M-LDH was formed with starch-gel electrophoresis at pH 5.6 and 7.0 (3) only a single protein band was formed upon polyacrylamide gel electrophoresis, and (4) a single peak appeared when the enzyme was run in the analytical ultracentrifuge, equipped with Schlieren optics. Fig. 1a

Fig. 1. Criteria of Purity of M-LDH-Wisconsin.

a. Disc gel electrophoresis at pH 4.5 (Reisfeld et al., 1963).

b. Ultracentrifugal pattern of M-LDH, 4.5 mg/ml at 105,000xg for 30 min.

1 (a)



1 (b)

shows the result of disc gel electrophoresis, and 1b, the ultracentrifugal pattern obtained.

(g) Antiserums

The antiserums used in this study were prepared by immunization of each of four young male New Zealand white rabbits with purified M-LDH. Each rabbit received an initial injection containing 3.0 mg of purified Wisconsin M-LDH (3.0 mg of enzyme in 1.5 ml of 0.03 M phosphate buffer, pH 7.5, homogenized with an equal volume of Freund's complete adjuvant) distributed over several intradermal back sites. A booster, consisting of 5.0 mg of enzyme in 1.0 ml of 0.03 M phosphate buffer, pH 7.5, was injected intravenously in a peripheral ear vein at 8 weeks after the initial immunization. Bleedings were made from the peripheral ear vein at 4 weeks, and again at 9 weeks.

The antiserums were obtained by allowing the blood to clot, and the clot to retract overnight at 5°. The serum fraction was separated from the clot by gentle centrifugation at 1500 rpm at 5° for about 10 min.; antiserums were stored frozen at -25° with no noticeable loss of antibody titer. For routine use, they were diluted 1:100 in isosatris buffer (see below) and stored frozen.

The antiserums are designated by two digits: the first indicates the animal, and the second the bleeding. A total of 8 antiserums were obtained.

(h) Micro-complement Fixation

Quantitative micro-complement fixation determinations (Wasserman and Levine, 1961) using rabbit antibodies directed against purified Wisconsin M-LDH, were performed as modified by Sarich and Wilson (1966) in a final volume of 7.0 ml, except that all dilutions were made with isosatris buffer (Rose and Wilson, 1966) (0.14 M NaCl, 0.01 M tris hydrochloride, 5×10^{-4} M $MgSO_4$, 1.5×10^{-4} M $CaCl_2$, and 0.1% bovine serum albumin, fraction V, pH 7.2 - 7.4). All experiments were controlled for both pro- and anticomplementarity by including a series of antigen and antibody controls within each experiment. Table 3 summarizes the experimental protocol.

The method depends upon the fact that there is a direct relationship between the concentration of antigen-antibody complex and the amount of complement bound by the complex. The relative concentration of unbound complement may be estimated by determining the relative degree to which it is capable of lysing hemolysin-sensitized erythrocytes.

Sensitized erythrocytes were prepared by washing week-old sheep red blood cells (Connecticut Biological

Table 3. Experimental Protocol for Micro-complement Fixation. Numbers refer to milliliters of the appropriate solution.

Table 3

Experimental protocol for micro-complement fixation

	Experimental	Antigen Cont.	Antibody Cont.	Comp. Cont.	Comp. Cell Cont.	Cell C
Isosatris	3.0	4.0	4.0	5.0	4.0	6.0
Antibody	1.0	-	1.0	-	-	-
Complement	1.0	1.0	1.0	1.0	2.0	-
Antigen	1.0	1.0	-	-	-	-
Total	6.0	6.0	6.0	6.0	6.0	6.0

Laboratories, Inc.) for 15 min. at 37° in a water bath, and the mixture was then rapidly diluted with 9 volumes of ice-cold isosatris buffer.

After the experimental tubes were allowed to equilibrate at about 4° for 18 hrs., 1.0 ml of freshly prepared, sensitized erythrocytes was dispensed into each tube, and the tubes were incubated at 37° until about 80% of the cells had lysed in the complement control tube. All of the tubes were then rapidly chilled in an ice-water mixture, and the unlysed erythrocytes were gently pelleted by centrifugation at 5°. The concentration of free hemoglobin in each tube was determined spectrophotometrically at 413 nm.

Fig. 2 shows the results of a typical antibody titration experiment. Antibody titers are expressed as those concentrations of antibody which will fix 50% of the available guinea pig complement. Figure 3 demonstrates the relationship between antibody concentration and the percent complement fixed at equivalence. It should be noted that a straight line is obtained when the logarithm of the antibody dilution is plotted against the percent complement fixed, in agreement with the data published by other workers (Sarich and Wilson, 1966; Arnheim and Wilson, 1967). Because antibody titer will vary to some extent with the lot of complement, only a single lot of complement was used for all of the experiments with any

Fig. 2. Typical Antibody Titration Experiment. Antibody 1-2 was titered against purified M-LDH. See text for details on the method. Antibody titers -- X = 1:4500; ▲ 1:5500; O 1:6500; ● 1:7500; ◻ 1:8500.

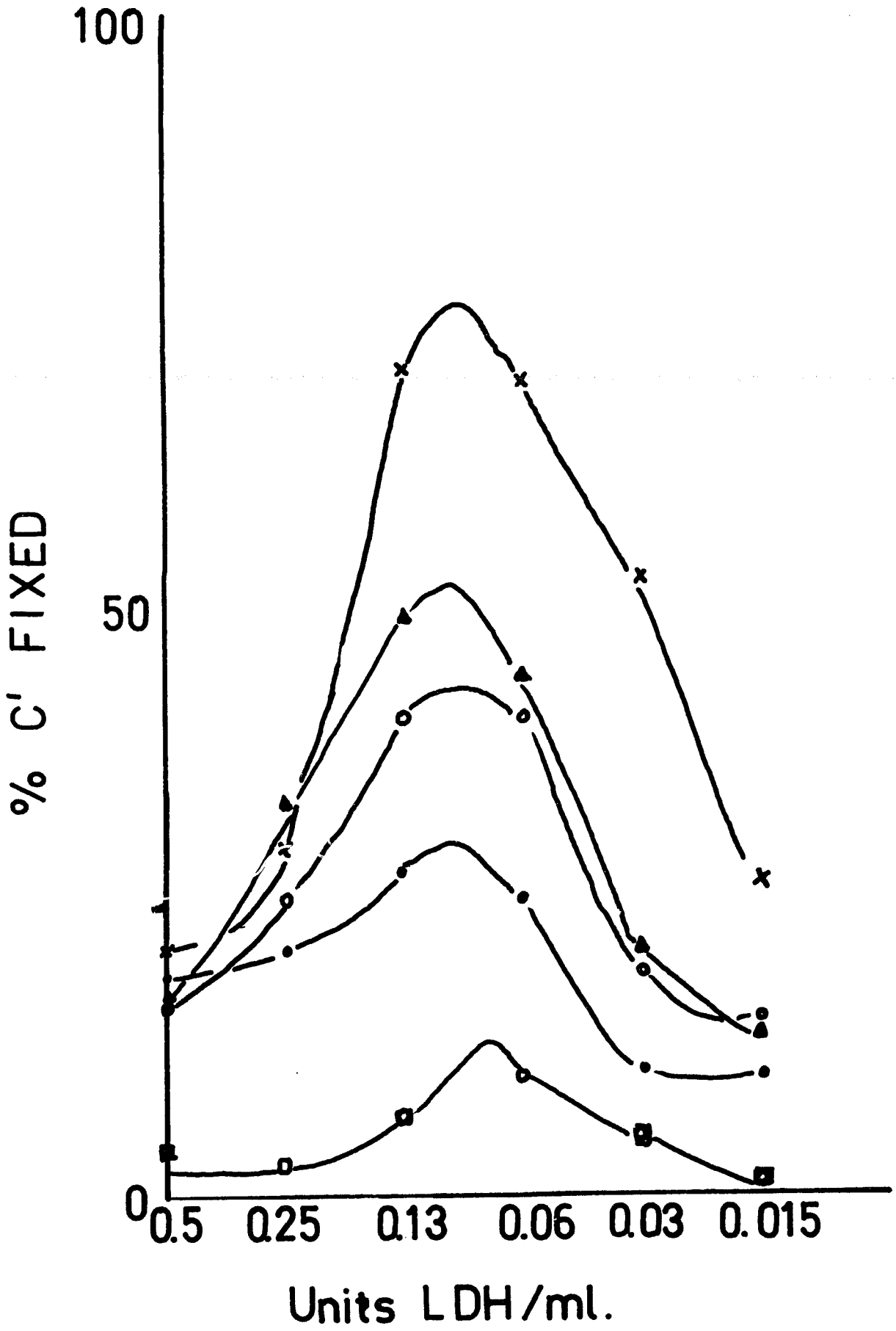
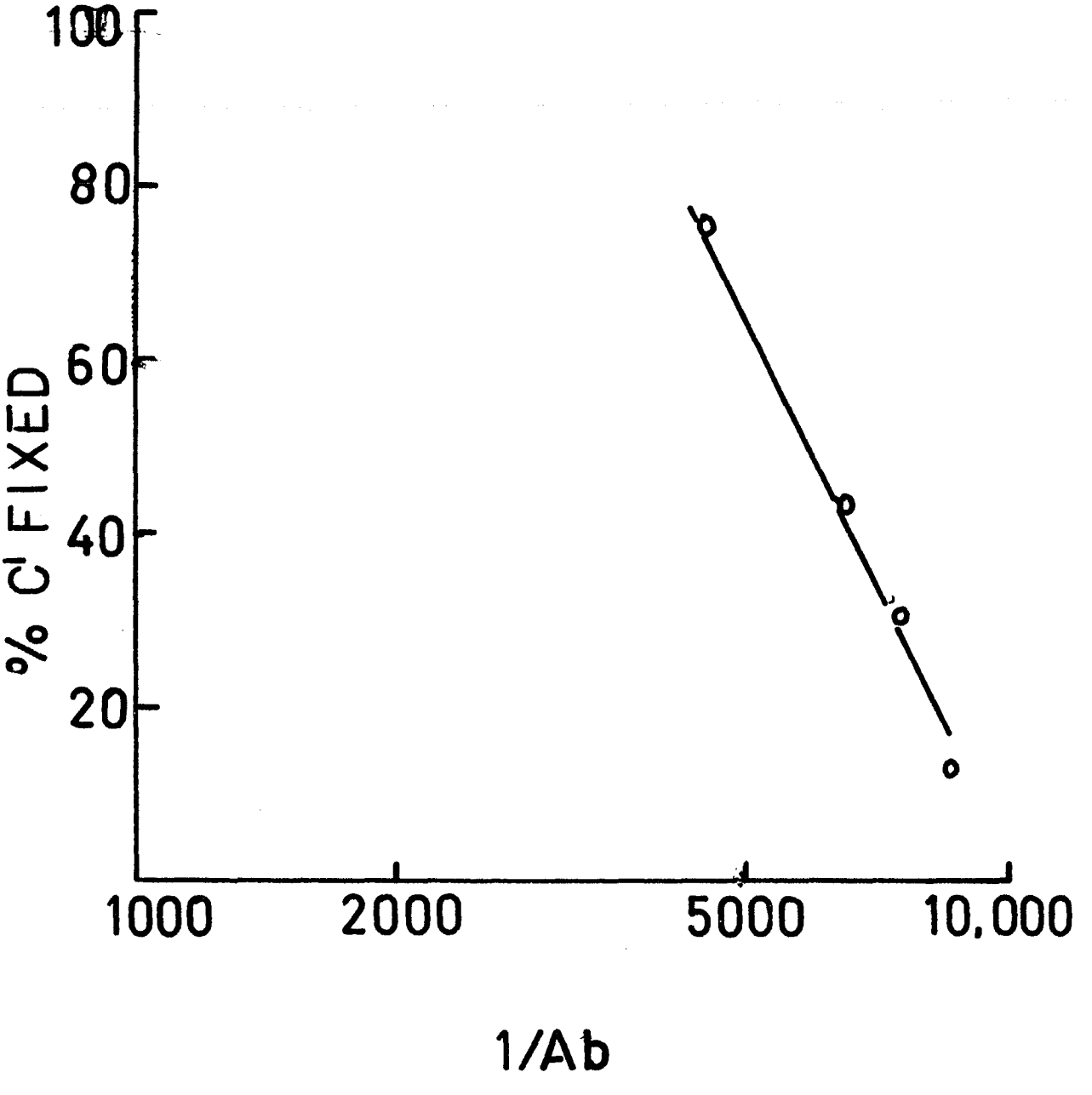

100
50
0
% C' FIXED
0.5
0.25
0.13
0.06
0.03
0.015
Units LDH/ml.

Fig. 3. Relationship Between Antibody Concentration and Percent Complement (C') Fixed at Equivalence. The data were redrawn from Fig. 2.


100
80
60
40
20
% C' FIXED
1000
2000
5000
10,000
1/Ab

particular antibody.

The degree of heterologous cross reaction has been variously expressed as either the complement fixation difference (Cocks and Wilson, 1969), or the index of dissimilarity, ID_{50} (Wilson et al., 1964; Sarich and Wilson, 1966). The latter measure, used here, was defined as the ratio of heterologous to homologous titer. The ID_{50} of the homologous reference antigen is defined as 1.00, while ID of a heterologous reaction is greater than 1.00. The experimental error is less than ± 2% for distantly related antigens (Sarich and Wilson, 1966), and ± 11% for closely related antigens (Salthe and Kaplan, 1966; Salthe, 1969).

RESULTS

(a) Electrophoresis

The primary objective of this study was to assess the extent of M-LDH variability within and between the populations collected. In order to accomplish this, electrophoresis was carried out on starch gel as noted above. In order to control for the presence of "nothing dehydrogenase"(Beutler, 1967; Cuatrecasas and Segal, 1967; Shaw and Koen, 1967), or for enzymes in the crude homogenate capable of reducing tetrazolium in the absence of exogenous lactic acid, duplicate zymograms were developed, one of which contained no lactate in the staining solution. In all cases, no significant staining of the gels was detected in the absence of lactic acid.

If the gels were allowed to incubate for long periods of time in the staining solution in the presence of fluorescent light, they became suffused with a blue color. There were, however, distinct colorless areas on the gels which corresponded to the placement of the homogenates. These colorless bands probably represent either an enzyme capable of oxidizing tetrazolium salts, or the presence of some metabolite preventing tetrazolium reduction. In any case, these "oxidase" bands migrated considerably faster than the LDH bands, and thus, presented no difficulty for the interpretation of LDH isoenzyme

patterns. No attempt was made to study the "oxidase".

Since crude homogenates were used for the electrophoretic studies, it was essential to distinguish between (1) isoenzymes containing H subunits, (2) conformers of M_4, and (3) isoenzymes composed exclusively of allotypic variants (alloenzymes) of M. Hybridization studies _in vitro_ and _in vivo_ conclusively indicate that when two allotypic forms are present, they hybridize to form five isoenzymes, and that the concentration of each alloenzyme is consistant with the relative concentrations expected from a binomial expansion (Salthe et al., 1965). Fig. 4 shows the expected molecular species and their relative concentrations, resulting from the hybridization of two electrophoretically distinguishable allelomorphs of M-LDH.

Zymograms, however, are incapable of detecting enzyme concentrations in crude homogenates. They are useful only insofar as the activity stains used to visualize them are capable of detecting catalytic activity. It is therefore necessary to consider the consequences of isoenzyme formations from two subunits with dissimilar catalytic activities. Table 4 summarizes the distribution of enzyme activity expected for LDH composed of subunits with dissimilar K_{cat}s, assuming equal subunit concentrations. Of special interest is the fact that if a homogenate contains one subunit which is totally devoid of enzymatic activity,

Fig. 4. Theoretical Subunit Composition and Relative Concentration of Alloenzymes of M-LDH. M^a and M^b refer to monomeric allotypes of M-LDH.

species	M^aM^a M^aM^a	M^aM^a M^aM^b	M^aM^a M^bM^b	M^aM^b M^bM^b	M^bM^b M^bM^b
distri- bution	.063	.250	.375	.250	.063

Table 4. The Distribution of Relative Activities of Isoenzymes of M-LDH Heterozygotes. The numbers were calculated by assuming different relative K_{cat}s for the two allotypic subunits; activity was computed by multiplying the expected statistical means of the tetramers by distribution factors generated by the binomial theorum.

Table 4

Species	$M^a{}_4$	$M^a{}_3M^b$	$M^a{}_2M^b{}_2$	$M^aM^b{}_3$	$M^b{}_4$
Relative Vmax M^b					
1.00	.063	.250	.375	.250	.063
0.75	.072	.287	.372	.238	.054
0.50	.084	.292	.374	.208	.042
0.25	.100	.325	.375	.175	.025
0	.125	.376	.376	.125	.000

its presence will still be detected on zymograms, provided that the electrophoretic mobilities of the two allelic subunits are sufficiently different.

For the reasons considered above, electrophoretic patterns were presumed to be indicative of heterozygous allotypes if, and only if, binomially distributed activity was obtained on zymograms, with either four or five bands. Fig. 5 shows some typical zymograms clearly demonstrating the expected distribution of M-LDH activity anticipated for heterozygous individuals. In no case was an electrophoretic pattern obtained which could be interpreted as indicative of a homozygous variant animal.

A total of 589 animals from 81 different localities were screened (see Table 1), and only four variants with altered electrophoretic mobility were detected. Table 5 summarizes the geographical distribution of electrophoretically detected allotypic variants. Although exact comparisons of the activity of variant M_4s were not made, in no case was a zymogram with only four activity bands observed.

These data indicate that electrophoretic variants of M-LDH are extremely rare, and are widely scattered through the R. pipiens complex range. It may be tentatively assumed, owing to the small number of variants detected and the complete absence of homozygotes, that normalizing selection pressures are great at the Ldm locus

Fig. 5 Sketch of Typical Starch Gel Zymograms. H_4 refers to the Wisconsin allotype. Place names refer to specific variant allotypes. See text for details of preparation.

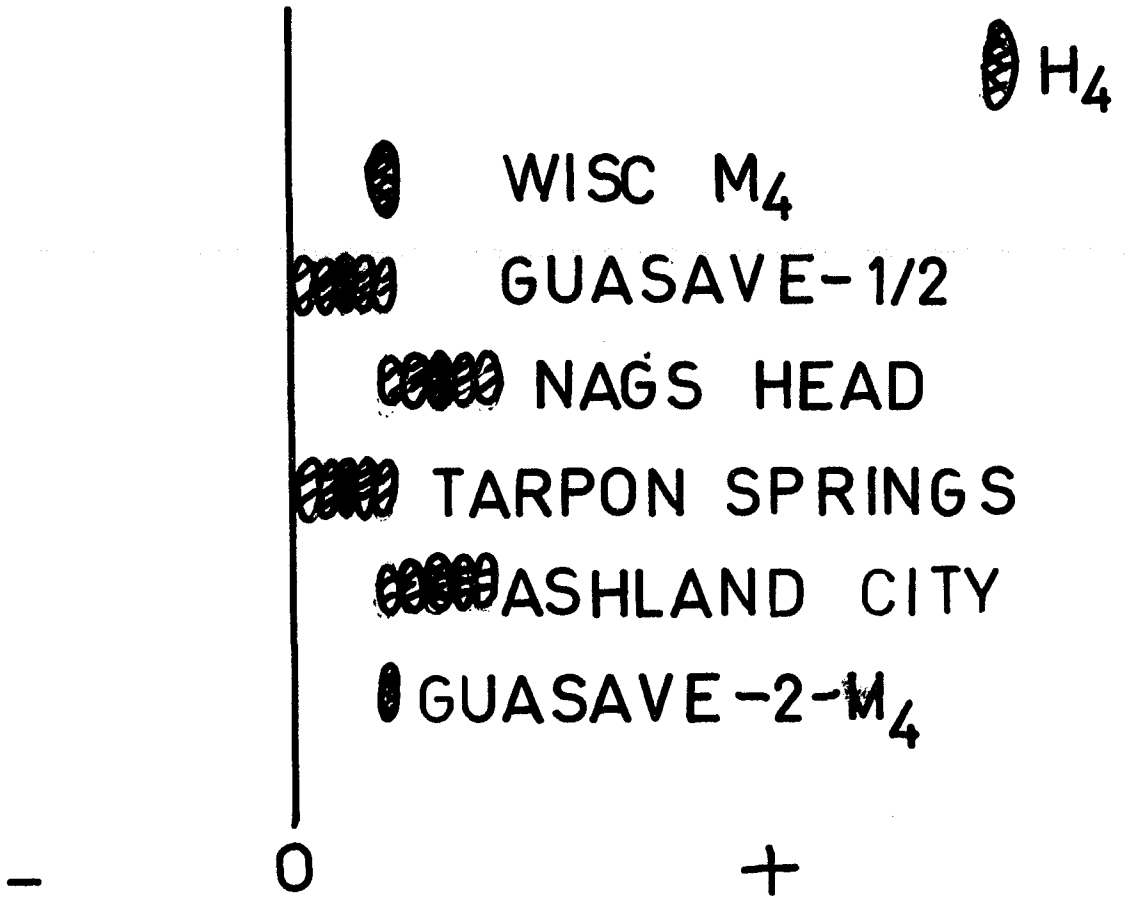

H_4
WISC M_4
GUASAVE-1/2
NAGS HEAD
TARPON SPRINGS
ASHLAND CITY
GUASAVE-2-M_4
−
0
+

Table 5. Geographical Distribution of Electrophoretically Identifiable Allotypes of M-LDH.

Table 5

Homogenate number	Mutant designation	Collection site
34	M-LDH- Tarpon Springs	Tarpon Springs, Fla.
319	M-LDH-Nags Head	Nags Head, No. Caro.
361	M-LDH-Guasave-1	Guasave, Sinaloa
464	M-LDH-Ashland City	Ashland City, Tenn.

in frogs.

However, before such a conclusion may be reached, it is essential that some estimate be obtained of the proportion of electrophoretically detectable mutants in the total mutant population. There are several approaches to this question, the simplest being a consideration of the consequences of all of the possible amino acid interchanges, assuming that all such interchanges are equally likely, and proteins are composed of all 20 amino acids in equal quantities. For the purpose of this, and subsequent analyses, I assumed that the basic amino acids are lysine, arginine and histidine, and that the acidic ones are glutamic acid and aspartic acid. It was further assumed that, at neutral pH, no electrophoretic distinctions could be made within the acidic or basic groups; the only electrophoretic distinctions that could be made are between acidic, neutral and basic residues. The estimate resulting from this calculation indicates that only about 35% of all of the possible amino acid interchanges will result in electrophoretically altered proteins.

However, it is clear that some of the above assumptions are incorrect. For example, if the amount of variability within a species is minimal, as seems to be true in the case of M-LDH in R. pipiens, it is extremely unlikely that the amino acid interchanges that do occur

are the result of more than a single nucleotide change within any codon. Thus, the calculation must be corrected to reflect only altered charge resulting from single step mutations excluding mutations to synonymous codons. Codon assignments were taken from Crick (1966). The second assumption, namely that all amino acids occur with equal frequencies in enzymes is also incorrect. Table 7 summarizes the information contained in Table 6. It is clear from these calculations that interchanged amino acid residues with altered charge characteristics are more likely to result from mutations of codons specifying charged amino acids than from mutations of codons coding for neutral residues. Thus, it is apparent that the frequency of electrophoretically detectable mutations will depend entirely upon the amino acid sequence of the "typical" allotype of the protein under consideration.

Table 8 compares the amino acid composition of R. pipiens M-LDH (A. Pesce in Salthe, 1969) to that of the idealized protein of King and Jukes (1969). A recalculation of the proportion of electrophoretically detectable allotypes of M-LDH, taking the above considerations into account, yields an estimate of 34.1% detectability, whereas the idealized protein would be expected to have 39.4% of its variants detected by electrophoretic methods. Thus, it is expected that, all other factors being equal, the actual number of variants in the populations examined

Table 6. The Amino Acid Interchanges Resulting from a Single Nucleotide Change in the Codon. Codon assignments listed below the amino acid were those used by Crick (1968). The denominator indicates the total number of codons that will result in changed amino acids. The numerator indicates the total number of interchanges that would be expected to give rise to a change in charge of the protein at neutral pH. Underlined residues are those which would be expected to alter the charge of the protein.

Table 6

PHE	Ser	Tyr	Cys	Leu	Leu	Leu	Ile	Val	0/8
UUU	UCU	UAU	UGU	UUA	UUG	CUU	AUU	GUU	
PHE	Ser	Tyr	Cys	Leu	Leu	Leu	Ile	Val	0/8
UUC	UCC	UAC	UGC	UUA	UUG	CUC	AUC	GUC	
LEU	Ser	Phe	Phe	Ile	Val				0/5
UUA	UCA	UUU	UUC	AUA	GUA				
LEU	Ser	Trp	Phe	Phe	Met	Val			0/6
UUG	UCG	UGG	UUU	UUC	AUG	GUG			
LEU	Pro	His	Arg	Phe	Ile	Val			2/6
CUU	CCU	CAU	CGU	UUU	AUU	GUU			
LEU	Pro	His	Arg	Phe	Ile	Val			2/6
CUC	CCC	CAC	CGC	UUC	AUC	GUC			
LEU	Pro	Gln	Arg	Ile	Val				1/5
CUA	CCA	CAA	CGA	AUA	GUA				
LEU	Pro	Gln	Arg	Met	Val				1/5
CUG	CCG	CAG	CGG	AUG	GUG				
ILE	Thr	Asn	Ser	Phe	Leu	Met	Val		0/7
AUU	ACU	AAU	AGU	UUU	CUU	AUG	GUU		
ILE	Thr	Asn	Ser	Phe	Leu	Met	Val		0/7
AUC	ACC	AAC	AGC	UUC	CUC	AUG	GUC		
ILE	Thr	Lys	Arg	Leu	Leu	Met	Val		2/7
AUA	ACA	AAA	AGA	UUA	CUA	AUG	GUA		
MET	Thr	Lys	Arg	Leu	Leu	Ile	Ile	Ile Val	1/9
AUG	ACG	AAG	AGG	UUG	CUG	AUU	AUC	AUA GUG	
VAL	Ala	Asp	Gly	Phe	Leu	Ile			1/6
GUU	GCU	GAU	GGU	UUU	CUU	AUU			
VAL	Ala	Asp	Gly	Phe	Leu	Ile			1/6
GUC	GCC	GAC	GGC	UUC	CUC	AUC			
VAL	Ala	Glu	Gly	Leu	Leu	Ile			1/6
GUA	GCA	GAA	GGA	UUA	CUA	AUA			
VAL	Ala	Glu	Gly	Leu	Leu	Met			1/6
GUG	GCG	GAG	GGG	UUG	CUG	AUG			

Table 6 (con't.)

SER	Phe	Tyr	Cys	Pro	Thr	Ala	0/6
UCU	UUU	UAU	UGU	CCU	ACU	GCU	
SER	Phe	Tyr	Cys	Pro	Thr	Ala	0/6
UCC	UUC	UAC	UGC	CCC	ACC	GCC	
SER	Leu	Pro	Thr	Ala			0/4
UCA	UUA	CCA	ACA	GCA			
SER	Leu	Trp	Pro	Thr	Ala		0/5
UCG	UUG	UGG	CCG	ACG	GCG		
PRO	Leu	His	Arg	Ser	Thr	Ala	2/6
CCU	CUU	CAU	CGU	UCU	ACU	GCU	
PRO	Leu	His	Arg	Ser	Thr	Ala	2/6
CCC	CUC	CAC	CGC	UCC	ACC	GCC	
PRO	Leu	Gln	Arg	Ser	Thr	Ala	1/6
CCA	CUA	CAA	CGA	UCA	ACA	GCA	
PRO	Leu	Gln	Arg	Ser	Thr	Ala	1/6
CCG	UUG	CAG	CGG	UCG	ACG	GCG	
THR	Ile	Asn	Ser	Ser	Pro	Ala	0/6
ACU	AUU	AAU	AGU	UCU	CCU	GCU	
THR	Ile	Asn	Ser	Ser	Pro	Ala	0/6
ACC	AUC	AAC	AGC	UCC	CCC	GCC	
THR	Ile	Lys	Arg	Ser	Pro	Ala	2/6
ACA	AUA	AAA	AGA	UCA	CCA	GCA	
THR	Met	Lys	Arg	Ser	Pro	Ala	2/6
ACG	AUG	AAG	AGG	UCG	CCG	GCG	
ALA	Val	Asp	Gly	Ser	Pro	Thr	1/6
GCU	GUU	GAU	GGU	UCU	CCU	ACU	
ALA	Val	Asp	Gly	Ser	Pro	Thr	1/6
GCC	GUC	GAC	GGC	UCC	CCC	ACC	
ALA	Val	Glu	Gly	Ser	Pro	Thr	1/6
GCA	GUA	GAA	GGA	UCA	CCA	ACA	
ALA	Val	Glu	Gly	Ser	Pro	Thr	1/6
GCG	GUG	GAG	GGG	UCG	CCG	ACG	

Table 6 (con't.)

TYR	Phe	Ser	Cys	His	Asn	Asp			2/6
UAU	UUU	UCU	UGU	CAU	AAU	GAU			
TYR	Phe	Ser	Cys	His	Asn	Asp			2/6
UAC	UUC	UCC	UGC	CAC	AAU	GAC			
HIS	Leu	Pro	Arg	Tyr	Gln	Gln	Asn	Asp	7/8
CAU	CUU	CCU	CGU	UAU	CAA	CAG	AAU	GAU	
HIS	Leu	Pro	Arg	Tyr	Gln	Gln	Asn	Asp	7/8
CAC	CUC	CCC	CGC	UAC	CAA	CAG	AAC	GAC	
GLN	Leu	Pro	Arg	His	His	Lys	Glu		5/7
CAA	CUA	CCA	CGA	CAU	CAC	AAA	GAA		
GLN	Leu	Pro	Arg	His	His	Lys	Glu		5/7
CAG	CUG	CCG	CGG	CAU	CAC	AAG	GAG		
ASN	Ile	Thr	Ser	Tyr	His	Lys	Lys	Asp	4/8
AAU	AUU	ACU	AGU	UAU	CAU	AAA	AAG	GAU	
ASN	Ile	Thr	Ser	Tyr	His	Lys	Lys	Asp	4/8
AAC	AUC	ACC	AGC	UAC	CAC	AAA	AAG	GAC	
LYS	Ile	Thr	Arg	Gln	Asn	Asn	Glu		6/7
AAA	AUA	ACA	AGA	CAA	AAU	AAC	GAA		
LYS	Met	Thr	Arg	Gln	Asn	Asn	Glu		6/7
AAG	AUG	ACG	AGG	CAG	AAU	AAC	GAG		
ASP	Val	Ala	Gly	Tyr	His	Asn	Glu	Glu	6/8
GAU	GUU	GCU	GGU	UAU	CAU	AAU	GAA	GAG	
ASP	Val	Ala	Gly	Tyr	His	Asn	Glu	Glu	6/8
GAC	GUC	GCC	GGC	UAC	CAC	AAC	GAA	GAG	
GLU	Val	Ala	Gly	Gln	Lys	Asp	Asp		5/7
GAA	GUA	GCA	GGA	CAA	AAA	GAU	GAC		
GLU	Val	Ala	Gly	Gln	Lys	Asp	Asp		5/7
GAG	GUG	GCG	GGG	CAG	AAG	GAU	GAC		
CYS	Phe	Ser	Tyr	Trp	Arg	Ser	Gly		1/7
UGU	UUU	UCU	UAU	UGG	CGU	AGU	GGU		
CYS	Phe	Ser	Tyr	Trp	Arg	Ser	Gly		1/7
UGC	UUC	UCC	UAC	UGG	CGC	AGC	GGC		

Table 6 (con't.)

TRP	Leu	Ser	Cys	Cys	Arg	Arg	Gly		2/7
UGG	UUG	UCG	UGU	UGC	CGG	AGG	GGG		
ARG	Leu	Pro	His	Cys	Ser	Gly			5/6
CGU	CUU	CCU	CAU	UGU	AGU	GGU			
ARG	Leu	Pro	His	Cys	Ser	Gly			5/6
CGC	CUC	CCC	CAC	UGC	AGC	GGC			
ARG	Leu	Pro	Gln	Gly					4/4
CGA	CUA	CCA	CAA	GGA					
ARG	Leu	Pro	Gln	Trp	Gly				5/5
CGG	CUG	CCG	CAG	UGG	GGG				
SER	Ile	Thr	Asn	Cys	Arg	Arg	Arg	Gly	3/8
AGU	AUU	ACU	AAU	UGU	CGU	AGA	AGG	GGU	
SER	Ile	Thr	Asn	Cys	Arg	Arg	Arg	Gly	3/8
AGC	AUC	ACC	AAC	UGC	CGC	AGA	AGG	GGC	
ARG	Ile	Thr	Lys	Ser	Ser	Gly			5/6
AGA	AUA	ACA	AAA	AGU	AGC	GGA			
ARG	Met	Thr	Lys	Trp	Ser	Ser	Gly		6/7
AGG	AUG	ACG	AAG	UGG	AGU	AGC	GGG		
GLY	Val	Ala	Asp	Cys	Arg	Ser			2/6
GGU	GUU	GCU	GAU	UGU	CGU	AGU			
GLY	Val	Ala	Asp	Cys	Arg	Ser			2/6
GGC	GUC	GCC	GAC	UGC	CGC	AGC			
GLY	Val	Ala	Glu	Arg	Arg				3/5
GGA	GUA	GCA	GAA	CGA	AGA				
GLY	Val	Ala	Glu	Trp	Arg	Arg			3/6
GGG	GUG	GCG	GAG	UGG	CGG	AGG			

Table 7. The Frequency of Charge Change Resulting from Single Site Mutations. The data presented have been summarized from the data listed in Table 6.

Table 7

Amino Acid	Number of possible single step mutations	Frequence of Charge Change
Alanine	24	0.25
Arginine	34	0.88
Asparagine	16	0.50
Aspartic acid	16	0.75
Cysteine	14	0.14
Glutamic acid	14	0.71
Glutamine	14	0.71
Glycine	23	0.44
Histidine	16	0.88
Isoleucine	21	0.10
Leucine	33	0.26
Lysine	14	0.86
Methionine	9	0.11
Phenylalanine	16	0.0
Proline	24	0.25
Serine	32	0.19
Threonine	24	0.25
Tryptophan	7	0.29
Tyrosine	12	0.33
Valine	24	0.25

Table 8. The Amino Acid Composition of M-LDH Compared to that of an Idealized Protein. The composition of the idealized protein is listed under the heading "Expected frequency" and has been drawn from King and Jukes (1969). These data have been recalculated in order to compare them to those available for M-LDH, since the number of aspartic acid, asparigine, glutamic acid, glutamine, cysteine and tryptophan residues were not reported for M-LDH.

Table 8

Amino Acid	Expected frequency*	Observed frequency in LDH**
Serine	9.0	9.7
Leucine	8.0	10.3
Arginine	11.2	2.9
Glycine	7.5	7.5
Alanine	6.3	5.7
Valine	6.4	10.6
Threonine	7.2	4.6
Proline	5.2	3.7
Isoleucine	5.5	7.2
Lysine	5.8	9.1
Glx[+]	9.0	8.4
Asx[++]	8.2	11.0
Phenylalanine	2.3	2.2
Tryosine	3.3	2.3
Histidine	3.1	2.2
Methionine	1.9	2.8

*Calculated from the data of King and Jukes (1969) excluding Cysteine and Tryptophan.

**Calculated from the data of A. Pesce in Salthe (1969).

[+]Glx = Glutamic Acid + Glutamine

[++]Asx = Aspartic acid + Asparagine

is about 12. The allele frequency of Ldm-Wisc can then be calculated to be 0.990, which is only slightly less than 0.997, the value which is calculated from the number of electrophoretic variants.

(b) Micro-complement Fixation

From the foregoing section, it is clear that electrophoresis is capable of detecting only a small proportion of all of the potential variability of M-LDH. It would, therefore, be of interest to estimate the degree of variability with some parameter independent of charge density. Since it has been shown that micro-complement fixation is capable of distinguishing between electrophoretically identical H-LDHs in R. pipiens (Salthe, 1969), the technique would appear to be ideal for the purposes of this study.

In order to test the sensitivity of the method with respect to its ability to distinguish between known electrophoretically variant allotypes, all previously detected heterozygotes were tested against the antibodies designated as 1-2, 2-2 and 4-2. The results of these experiments are presented in Table 9. Under the conditions used for determining ID_{50}, the experimental error was found to be ± 13%. Based upon general experience in handling closely related antigens, an antigen yielding an I.D. in excess of 1.20 was considered immunologically

Table 9. Indices of Dissimilarity of Electrophoretic Heterozygotes of M-LDH. Only a single determination could be made because of the extremely limited quantities of homogenates.

Table 9

Antibody	1-2	2-2	4-2
Location			
Tarpon Springs	1.03	1.07	0.98
Nags Head	1.04	1.00	1.06
Guasave	1.08	1.09	0.99
Ashland City	1.33	1.04	1.07

different (Salthe, personal communication). However, since no statistical analysis of the data were performed, it's possible that some of the higher $I.D._{50}$'s reflect immunological differences (see Table 10; e.g., Brownsville and LaPlace.) An examination of the data indicates that only antiserum 1-2 was capable of detecting any differences between the test antigens and purified Wisconsin M-LDH, which was used as the reference antigen throughout these studies. Attempts to use the four week bleedings (antiserums 1-1, 2-1, 3-1 and 4-1) were unsuccessful, owing to the fact that sharp complement fixation peaks could not be obtained, resulting in very high experimental errors. Antiserum 3-2 was not used, since it yielded double complement fixation peaks.

It is of interest to note that only a single heterozygous allelomorph (Ashland City) was presumed to be immunologically detectable, in marked contrast to the findings of Salthe (1969). There is, however, one important difference between our approaches. Owing to the polymorphic nature of the Ldh locus, Salthe was able to select homozygous allelomorphs for all of his comparisons. Clearly, such an approach was impossible here.

Because of its ability to immunologically distinguish between the Ashland City heterozygous variant and purified Wisconsin M-LDH, antiserum 1-2 was selected to be the sole test antiserum. In order to determine whether there were

geographical polymorphisms which might be immunologically differentiated, homogenates were pooled from animals derived from the same collection sites. A total of 25 population pools were examined, and the results of this survey are summarized in Table 10. These data suggest that no population appears to be immunologically differentiated from the Wisconsin standard antigen if an arbitrary choice of IDs of 1.2 or greater is considered to be significant. Thus, the general conclusion derived from these studies is that the typical M-LDH structure in R. pipiens would appear to be constant throughout its range.

(c) Thermal Denaturation

During the course of enzyme purification, it had been noted that M-LDH from the Guasave population was considerably more thermolabile than enzyme derived from the Wisconsin animals. It was therefore deemed of interest to study the rate of thermal denaturation of M-LDHs derived from several different populations of R. pipiens.

Since the enzymes used in this phase of the study were not purified, it was necessary to compare the rate of thermal denaturation of M-LDH in crude homogenates to that of purified enzyme isolated from the same source. In order to minimize the effects of other proteins in the homogenates, which could lead to enhanced thermal stability,

Table 10. Indices of Dissimilarity for Selected Populations. All $I.D._{50}$ values are the means of two determinations using antibody 1-2.

Table 10

Location	Number in Pool	$I.D._{50}$
Alberg, Vermont	16	1.06
Ashland City, Tennessee	13	1.02
Balmorhea, Texas	5	1.01
Bluefields, Nicaragua	3	1.07
Brownsville, Mexico	1	1.15
Calgary, Alberta	6	0.98
College Part, Maryland	10	1.06
Cuernavaca, Mexico	7	1.00
Fairburn, Georgia	31	1.07
Guasave, Mexico*	24	1.09
Guelatao, Mexico	12	1.04
Lakewood, New Jersey	5	1.02
La Place, Louisiana	4	1.13
Logan, Utah	4	0.97
Lulling, Texas	7	1.02
Pine Portage, Ontario	1	1.02
Portal, Arizona	2	0.97
Panama**	2	1.00
San Juan de Sabinas, Mexico	3	1.03
San Rafael, Costa Rica	9	0.98
Tarpon Springs, Florida	23	1.06
Tucumcari, New Mexico	3	0.99

Table 10 (con't.)

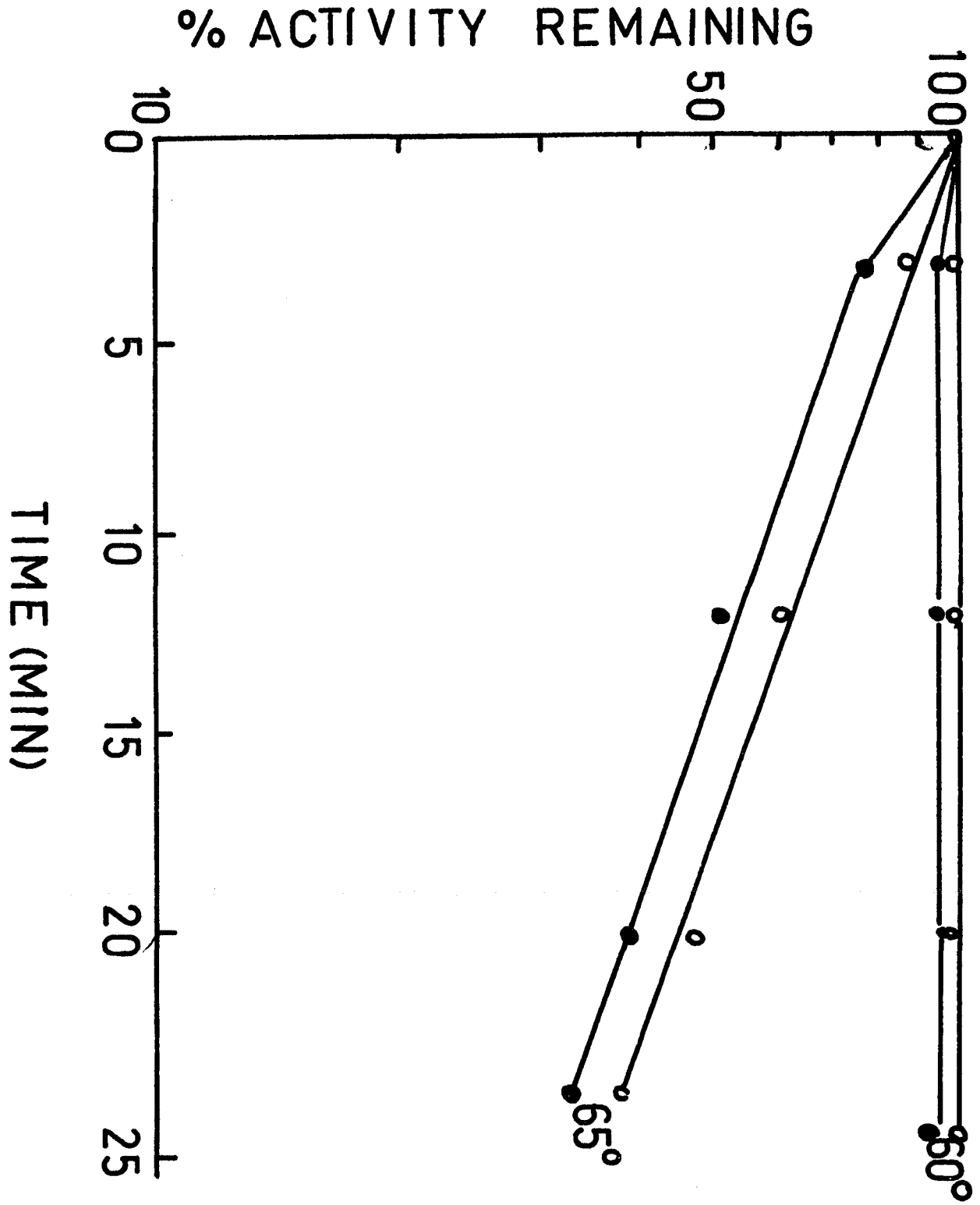
Location	Number in Pool	$I.D._{50}$
Uvalde, Texas	7	0.97
Villa Hermosa, Mexico	1	1.07
Wichita Falls, Texas	6	0.97

*Antigen was partially purified through the thermal denaturation step. See Materials and Methods for details on preparation.

**Collection site unknown.

Fig. 6. Comparison of the Rate of Thermal Denaturation of Purified and Crude M-LDH-Wisconsin at 60^{o} and at 65^{o}.

● = crude; o = purified.

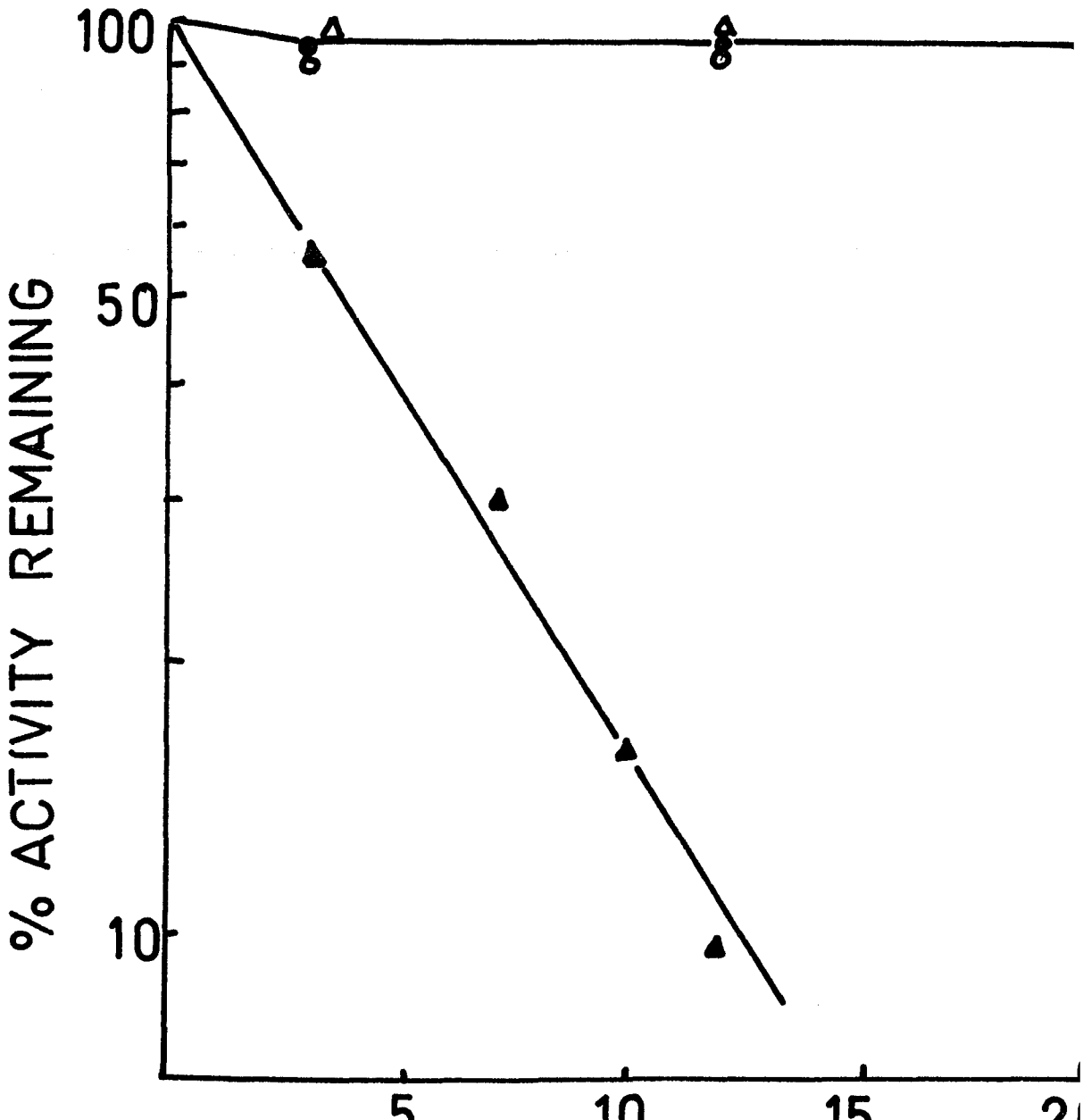

% ACTIVITY REMAINING
100
50
10
0
5
10
15
20
25
TIME (MIN)
65°
60°

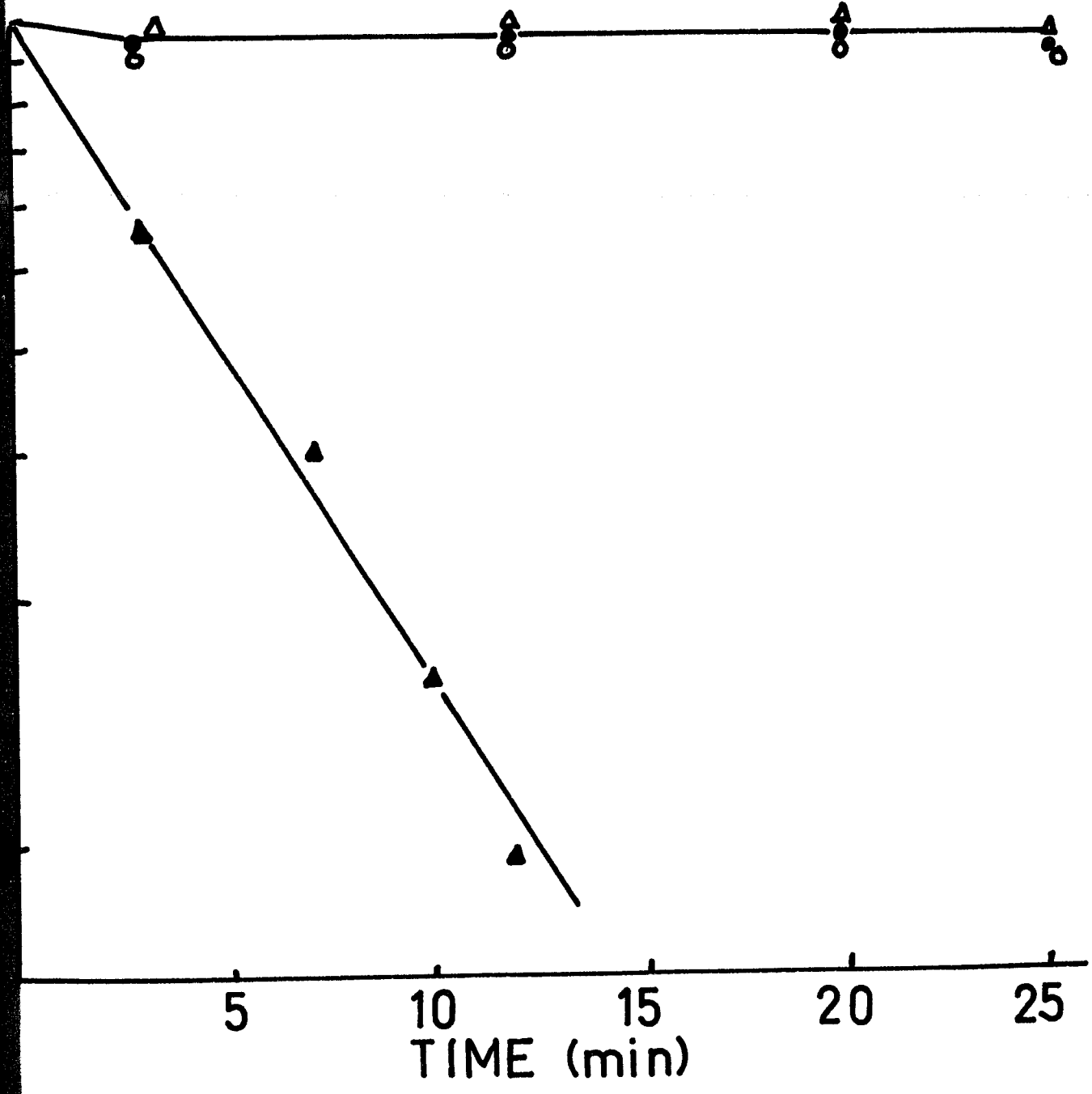
1:50 dilutions were made into isosatrous buffer prior to denaturation (Salthe, 1969). At the end of the specified time of heating, experimental tubes were plunged into an ice-water bath, and assayed immediately. Fig. 6 is a comparison of the rate of thermal denaturation of crude Wisconsin enzyme with purified enzyme from the same source. At 56°, there was no loss of enzyme activity in either case. At 60°, however, there was a slight loss of activity in the crude material during the first three minutes, and the enzyme activity was stable thereafter. On the other hand, there was no loss in activity detected in the purified enzyme preparation under these conditions. It is probable that the loss of activity found in the crude material was due to the denaturation of the small amount of H subunits found in muscle tissue. At 65°, both the crude and the purified preparations denatured at the same rate.

The pooled extracts used in these experiments have been described previously in Table 10. The population pools studied were Alberg, Vermont; Cuernavaca, Mexico; Cuasave,Mexico; and Tarpon Springs, Florida. These populations were selected on the basis of their geographical distance from one another. Figs. 7 and 8 depict the data that were derived from these experiments at 60° and at 65°. Only the Guasave population exhibited thermal denaturation properties different from those of Wisconsin

Fig. 7. Thermal Denaturation of M-LDH Allotypes at 60°.

O = Alberg; ● = Cuernavaca; ▲ = Guasave; Δ = Tarpon Springs.


100
50
10
% ACTIVITY REMAINING
5
10
15
TIME (min)


5
10
15
20
25
TIME (min)

M-LDH. Wisconsin, Alberg, Cuernavaca, and Tarpon Springs homogenates denatured at identical rates. Thus, these results suggest that the Guasave population contains an M-LDH different from that of the other R. pipiens populations, and hence, was designated as M-LDH-Guasave-2.

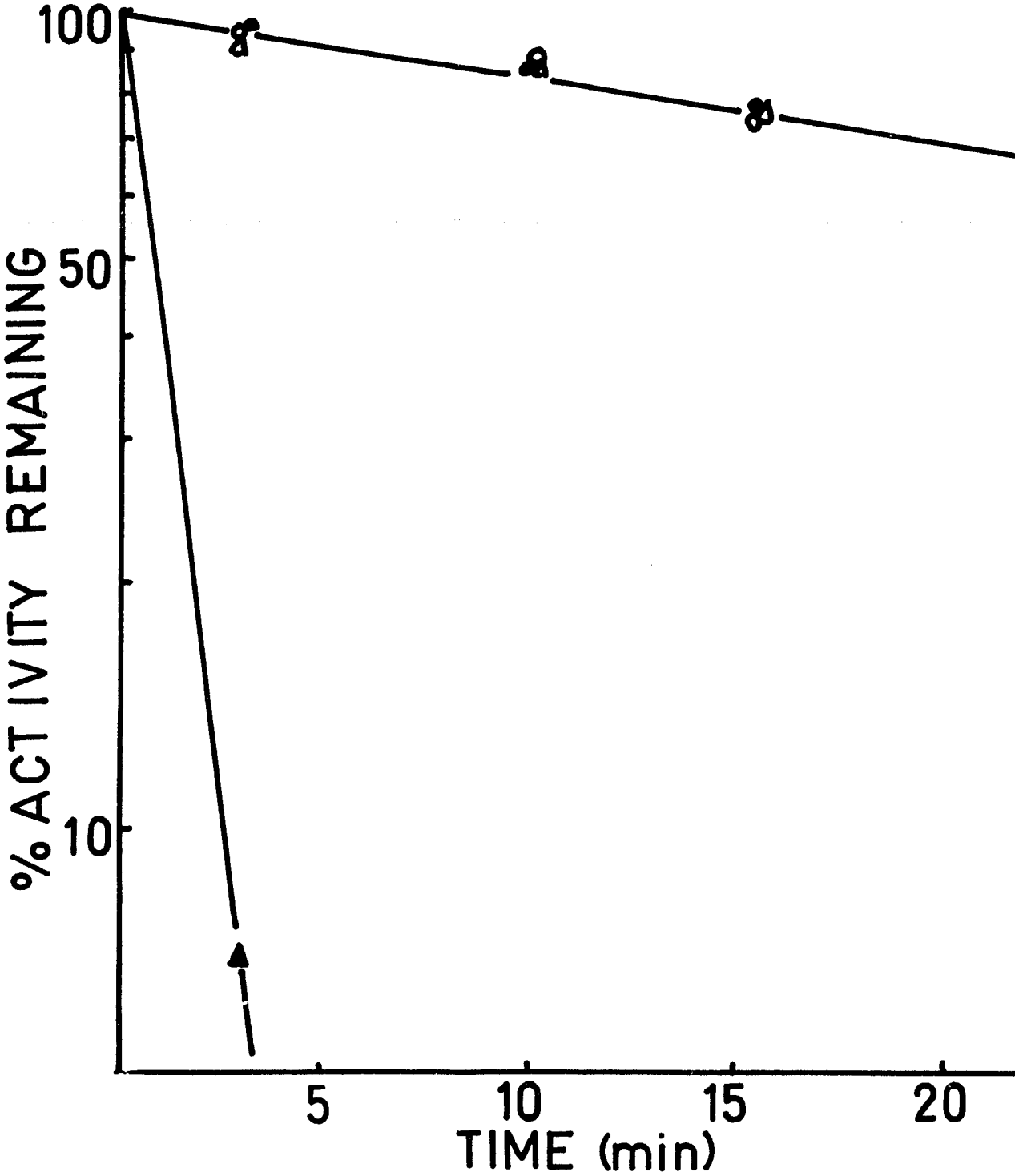
(d) Kinetic Studies

Evolutionary arguments, regardless of whether or not they are concerned with organisms per se, distinct morphological features of organisms, or primary gene products, must be placed in physiological contexts in order to have meaning. It was therefore essential to study in detail some of the presumed functional properties of M-LDHs from the R. pipiens complex. Towards this end, substrate inhibition, thermal adaptations, and thermodynamic properties of variant enzymes were investigated, largely with respect to the conversion of pyruvic acid, although some aspects of the reverse reaction were considered.

In sharp contrast to the situation of H-LDH in R. pipiens, where a large number of allotypes were present in sufficient quantities for kinetic investigations (Levy and Salthe, 1971), only a single M-LDH variant, M-LDH-Guasave-2, was available for similar study.

As is the case with H-LDH, M-LDH is apparently inhibited by high concentrations of pyruvate or lactate.

Fig. 8. Thermal Denaturation of M-LDH Allotypes at 65°. Symbols are the same as in Fig. 7.


100
50
10
% ACTIVITY REMAINING
5
10
15
20
TIME (min)

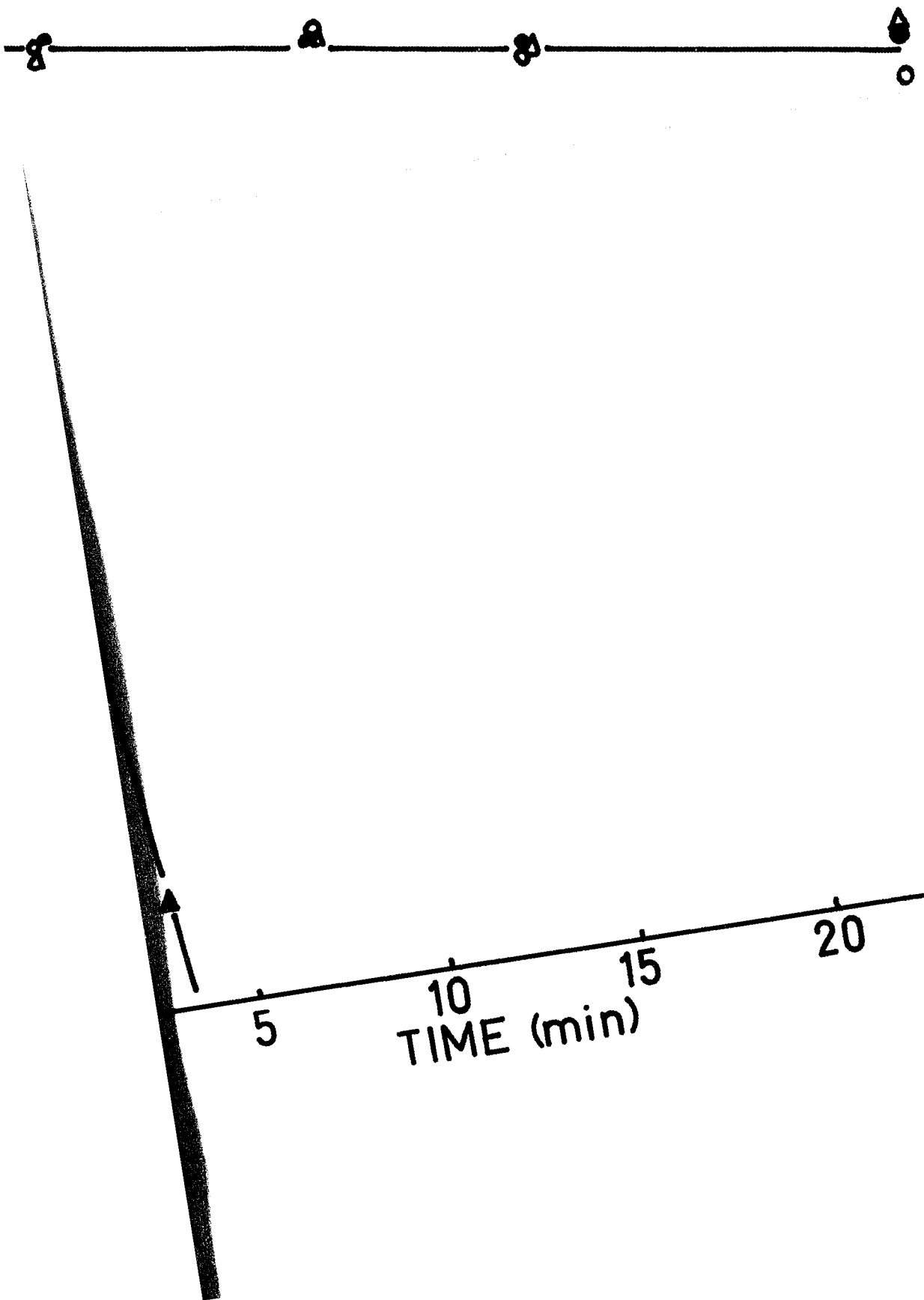
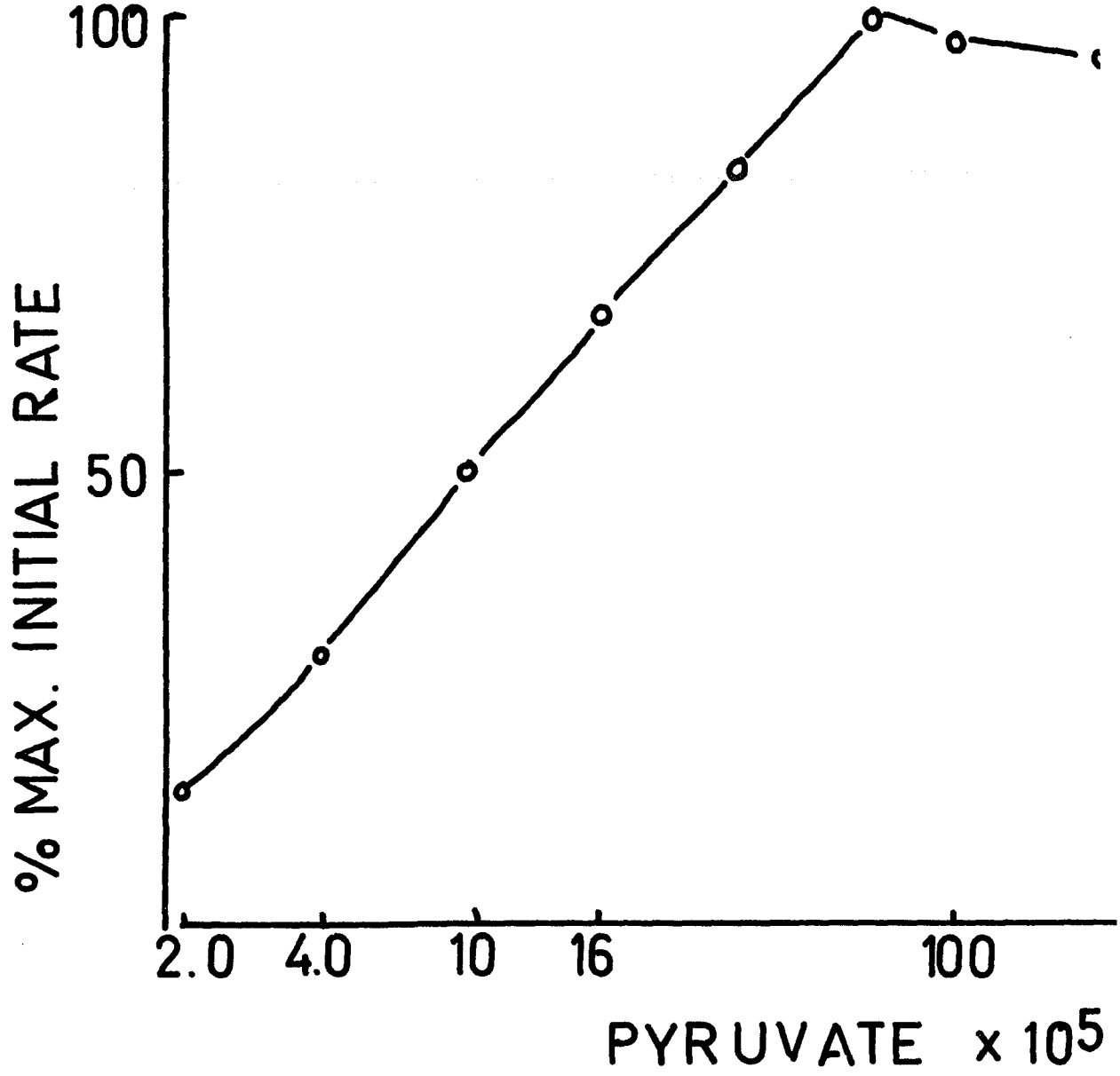

5
10
15
20
25
TIME (min)

Fig. 9 shows a typical substrate velocity curve for purified Wisconsin-LDH at 25° and pH 7.2 using pyruvate as the substrate. The reaction velocity increases with increasing concentrations of pyruvate, but only up to 6.6×10^{-4} M, after which increases in the concentration of substrate lead to progressively lowered velocities. Similar data may be obtained using lactate as the substrate as shown in Fig. 10. It is not clear, from such data, which molecular species is the primary cause of the inhibition; certainly substrate or product inhibition are reasonable alternative explanations for these data. In order to distinguish between these two alternative hypotheses, 0.01 M hydroxylamine was added to the reaction mixture, and the reaction was allowed to proceed using DL-Lactate as the substrate. The initial velocity was determined by following the rate of enzymatic reduction of NAD^+ at 340 nm for no longer than 2 minutes. Hydroxylamine would be expected to react with the carbonyl group of pyruvic acid to form an oxime derivative (Roberts and Caserio, 1965), thus trapping product as it formed. Thus, if the previously observed inhibition were due to non-productive binding with pyruvate, hydroxylamine would be expected to relieve it. If, on the other hand, the inhibition were due to lactate, the presence of hydroxylamine in the reaction mixture would not be expected to have any effect on the substrate velocity curve. The

Fig. 9. Substrate-velocity Relationship for Purified M-LDH-Wisconsin. Conditions were pH 7.2 and 25°. See Materials and Methods for details on the assay method.


100
50
% MAX. INITIAL RATE
2.0
4.0
10
16
100
PYRUVATE x 10^5

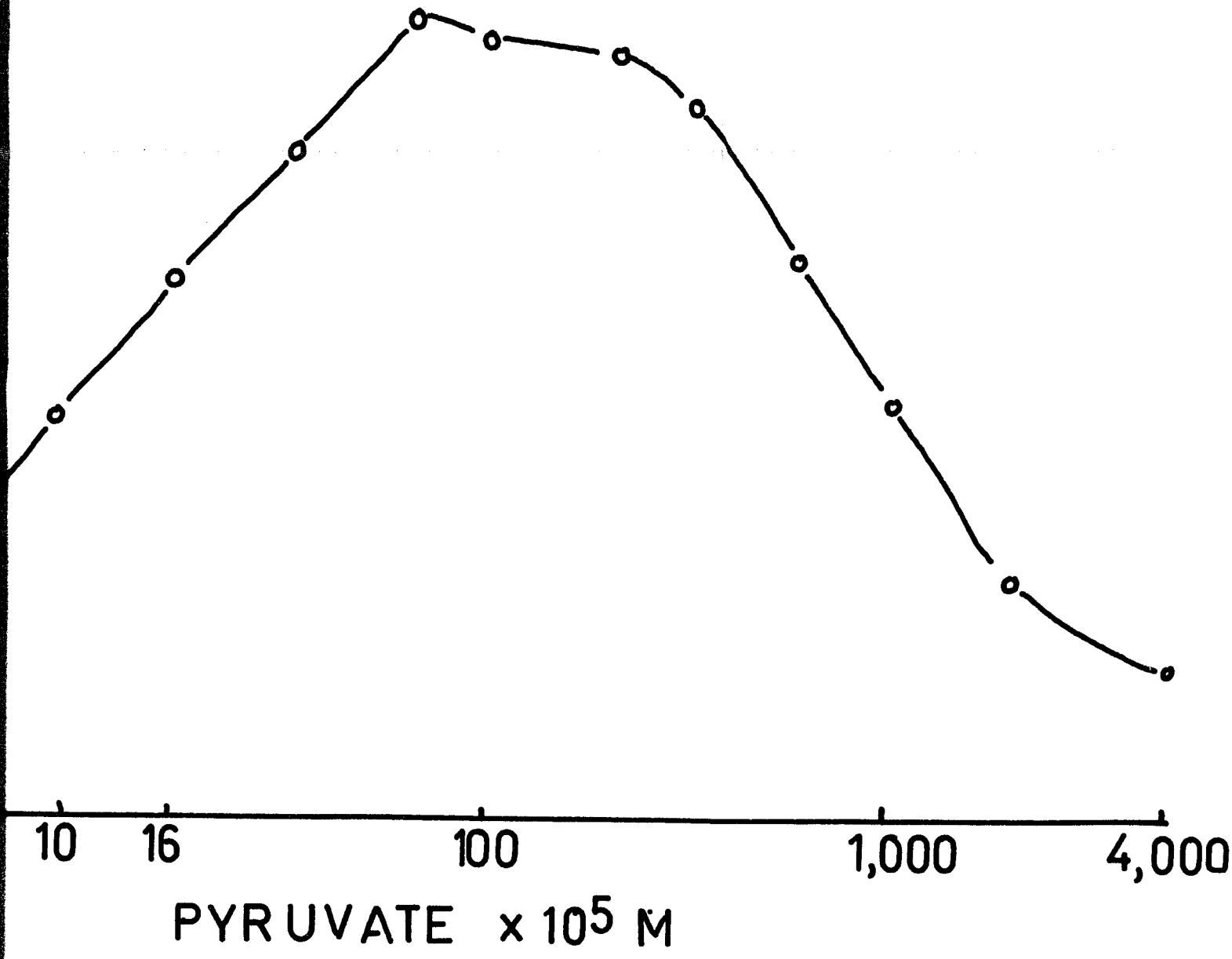
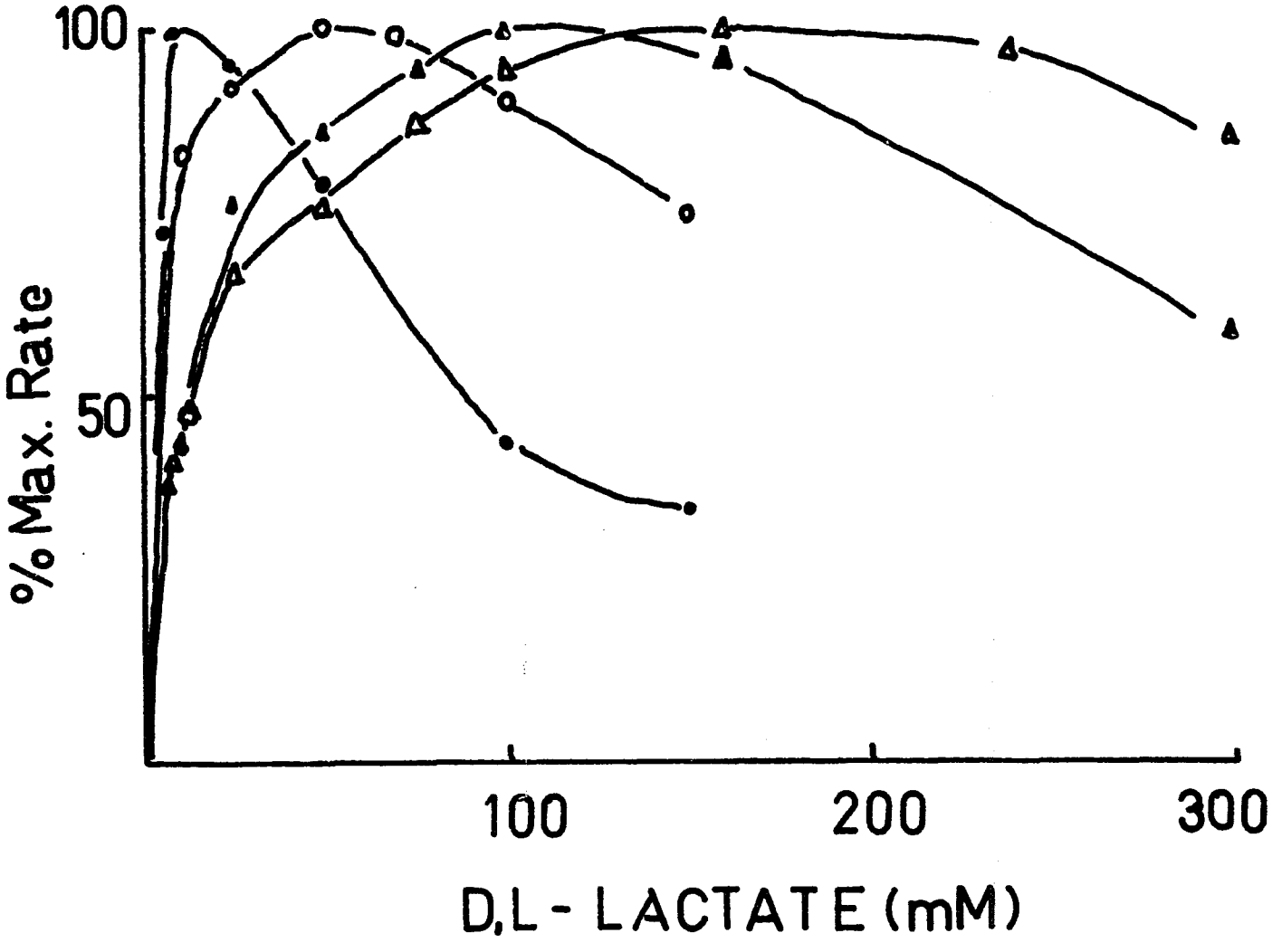

10
16
100
1,000
4,000
PYRUVATE x 10⁵ M

Fig. 10. Effect of Hydroxylamine on Substrate-velocity Curves. O = crude heart extract; ● = heart extract + 0.01 M hydroxylamine; Δ = purified M-LDH-Wisconsin; ▲ = purified M-LDH-Wisconsin + 0.01 M hydroxylamine.


100
50
% Max. Rate
100
200
300
D,L - LACTATE (mM)

results of these experiments are shown in Fig. 10. They confirm that the inhibiting molecular species is pyruvate.

It has often been asserted that enzymes exhibit thermal adaptations to temperatures which closely approximate those under which the species in question is best adapted (Somero et al., 1968; Somero, 1969). Of special interest in this context, is the demonstration that varying temperatures differentially affect developmental rates in R. pipiens, and that such differences in rate are roughly correlated with the altitudinal and latitudinal collection site of the animal (Moore, 1949; 1950). It was thought, therefore, that different thermal adaptations might be found in enzymes purified from Wisconsin frogs, which are presumably cold-adapted, as compared to enzymes isolated from Guasave frogs, which would be considered warm-adapted.

It should be emphasized that, while the thermal denaturation data reported above suggest that the enzymes may be structurally different, differences in the rate of thermal denaturation do not necessarily imply differences in thermal adaptations, since such denaturation occurs at temperatures considerably higher than those at which R. pipiens would be expected to survive.

Thermal adaptations were studied in three ways: (1) The behavior of K_m at various temperatures was investigated to determine the inflection point (Somero, 1969), (2) The energy of activation (E_a) was measured

from an Arrhenius plot by the method of Dixon and Webb (1964). The behavior of the overall reaction was studied as a function of temperature.

V and K_m were estimated at varying temperatures by the method of Lineweaver and Burk (1934), and a typical plot is shown in Fig. 11. Since E_a is determined from the slope of the Arrhenius plot, it is unnecessary to know V_{max} for purified enzyme preparations. All V determinations were made on fixed volumes of enzyme solution, are expressed in arbitrary units.

Fig. 12 shows the relationship of K_m with temperature for both enzymes. The data clearly indicate that the K_ms are very nearly the same for the two enzymes at any given temperature. Furthermore, the data indicate that the K_ms for both enzymes appear to be at a minimum at about 14°, the K_m rising with either an increase or a decrease in temperature. Thus, M-LDH would appear to be a cold-adapted enzyme (for a discussion, see Somero, 1969), but not so cold-adapted as H-LDH, which has a temperature-K_m minimum at about 5° (Levy and Salthe, 1971). Moreover, in neither enzyme was a temperature adaptation noted which could be positively correlated in some way to the collection sites of the animals.

Fig. 13 shows the relationship between log V with $1/T_K$, where T_K is the absolute temperature. There is a discontinuity at about 18°. Below this temperature,

Fig. 11. Lineweaver Burk Plot. Data for M-LDH-Wisconsin is shown at pH 7.2 and 25°.

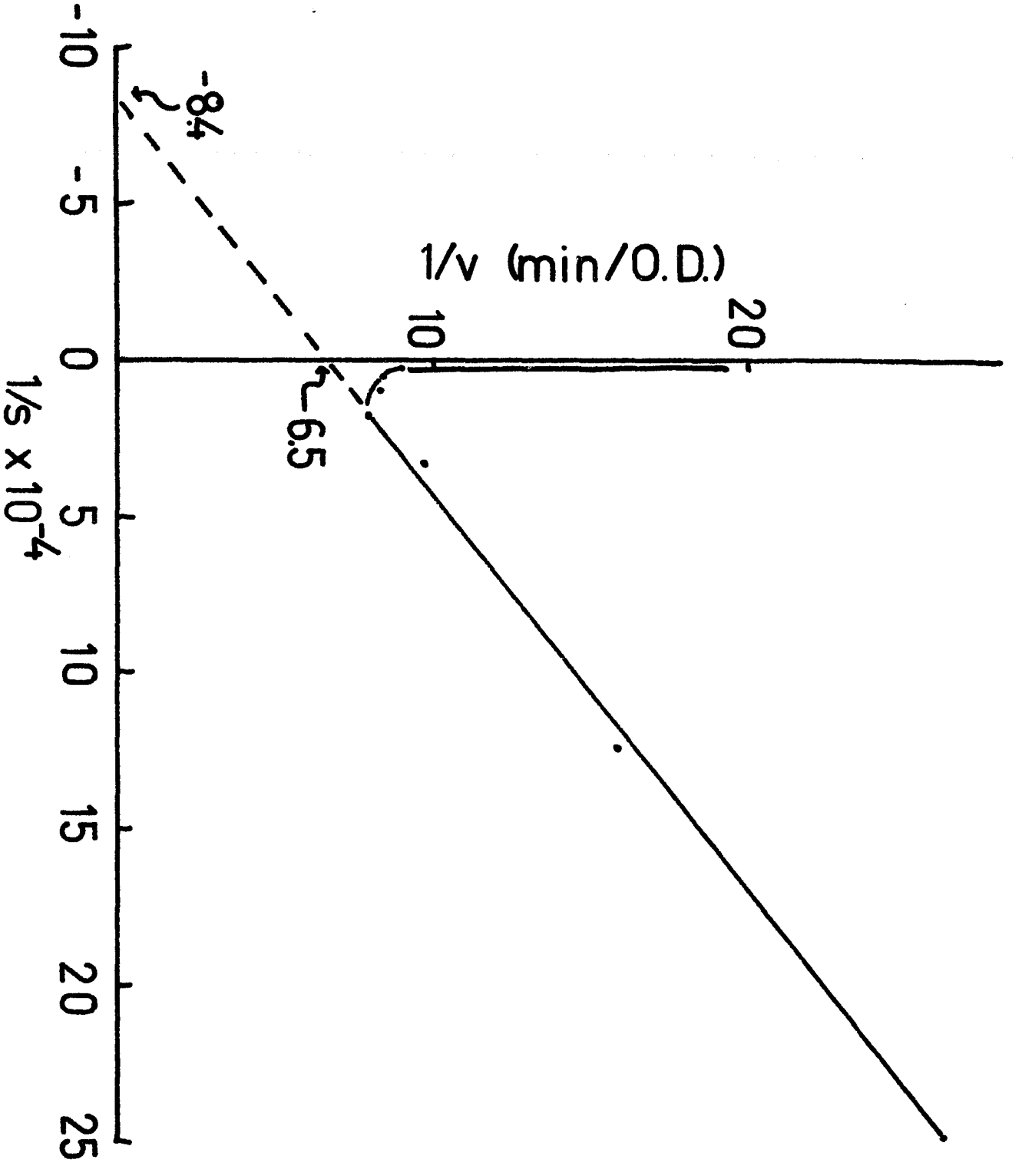

-10
-5
0
5
10
15
20
25
1/s x 10^-4
-8.4
-6.5
10
20
1/v (min/O.D.)

Fig. 12. Relationship of K_m with Temperature. ● = M-LDH-Wisconsin; O = M-LDH-Guasave-2. Determinations were made at pH 7.2.

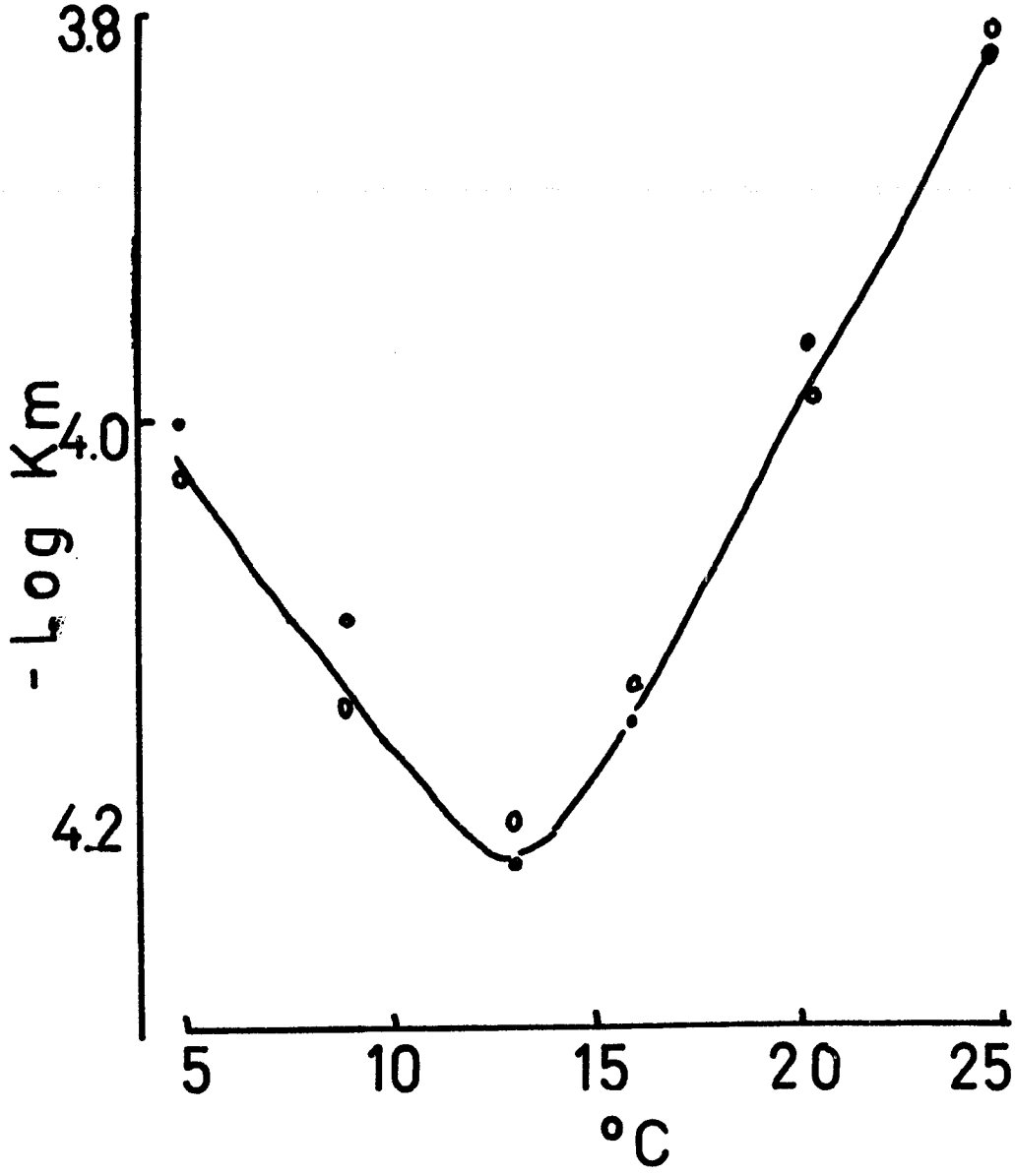
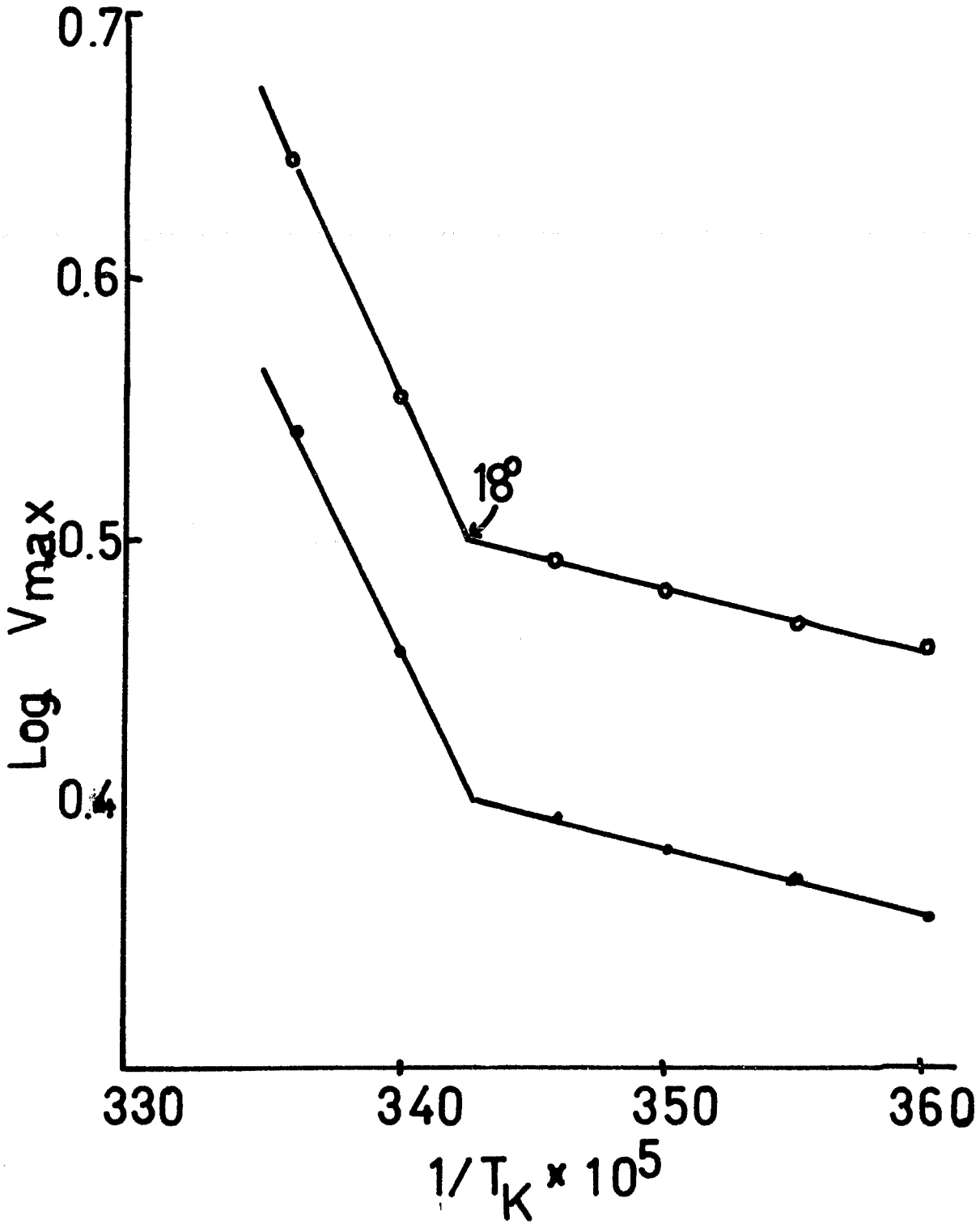

3.8
4.0
4.2
- Log Km
5
10
15
20
25
°C

Fig. 13. Relationship of log V with $1/T_K$. Symbols and conditions are the same as in Fig. 12.

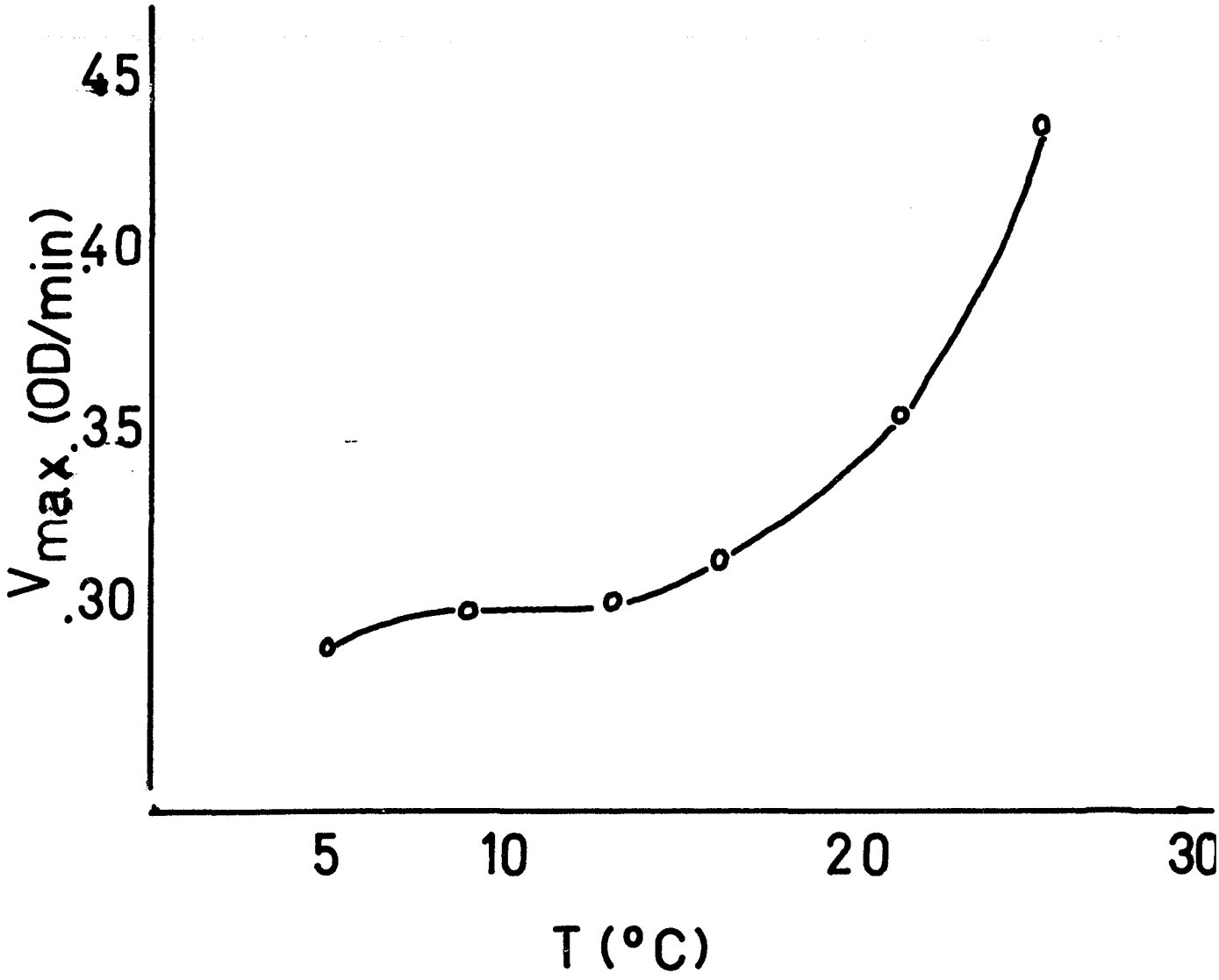

0.7
0.6
0.5
0.4
Log Vmax
18°
330
340
350
360
1/T_K x 10^5

the E_a is considerably higher: 38,000 cal/mole. These data also indicate that the enzyme may be cold adapted. Once again, M-LDH-Wisconsin and M-LDH-Guasave-2 behave very similarly, and could not be distinguished by this method.

If, however, one examines the behavior of the enzymes with varying temperatures at any concentration of substrate, a somewhat different picture emerges. Under all conditions examined, increasing temperatures result in increasing initial reaction rates. Fig. 14 shows the relationship between V and temperature for M-LDH-Guasave. Exactly the same curve can be generated from the data for M-LDH-Wisconsin, except for the fact that the values for V would be different for the reasons noted above.

Since overall enzyme performance, rather than our mathematically defined enzyme constants is of critical importance to the organism, changes in enzyme constants with temperature may be largely irrelevant from a physiological-ecological point of view. However, regardless of whether or not these constants are physiologically meaningful, M-LDH-Guasave-2 and M-LDH-Wisconsin could not be differentiated from one another by any kinetic method utilized here.

Fig. 14. Relationship of V with Temperature. The data shown are for M-LDH-Wisconsin and were drawn from the same data shown in Fig. 13.


45
.40
.35
.30
$V_{max.}$ (OD/min)
5
10
20
30
T (°C)

DISCUSSION

The experiments that I have reported consistently indicate that there are no polymorphisms and only a minimal amount of variability at the Ldm locus throughout the R. pipiens complex range. This conclusion is based upon electrophoretic mobility studies which yielded an estimated Ldm-Wisc allele frequency of 0.990, and immunological studies which are suggestive of no hidden polymorphisms. Thermal stability data suggest that the Guasave population may have an allele different from Ldm-Wisc, and that Ldm-Guasave-2 is fixed within the Guasave population. A critical examination of the criteria used to arrive at these conclusions is therefore warranted.

Many of the animals used in this study were the identical ones used by Salthe (1969), who was able to show widespread polymorphisms for Ldh. Furthermore, Salthe (1969) demonstrated that when populations were polymorphic, genotypes were present in approximately Hardy-Weinberg proportions. These considerations render sampling errors an unlikely cause for the lack of variability found in the present study.

Electrophoretic mobility differences are probably the most easily accessible property of proteins that may be exploited for estimating the extent of polymorphisms and variability within a population. In addition, these determinations are extremely rapid, so large numbers of

samples can be run in a minimum period of time. Zone electrophoresis, however, suffers from one serious drawback: it consistently underestimates the number of variants in a population. Correction factors can be calculated if the amino acid composition of the typical allotype is known. For example, I have calculated that electrophoretic methods are maximally capable of distinguishing only about 35% of the single site mutants possible for Ldm. However, since it is possible that this correction factor can vary significantly from one protein to another (e.g., proteins with a high content of charged residues would be expected to have a smaller correction factor than those rich in uncharged residues), extreme care must be taken when comparing the frequencies of variants of two or more different proteins. Futhermore, even corrected estimates of variability may be too small, since the calculations presuppose that the substitution of any differently charged amino acid will lead to altered electrophoretic mobility in every case. This assumption is not always correct. For example, in hemoglobin New York there is no increase in electrophoretic mobility despite the presence of an additional charge due to the substitution of glutamic acid for valine at 113 (Ranney et al., 1967).

A second, and much more difficult factor to assess is the nature of the interchanged residues; i.e., are there

truly random charge interchanges, or do the interchanged residues tend to be conservative with respect to charge? While it is true that the rigorous data necessary to provide a satisfying answer to this question are not yet available for a wide variety of proteins, the data compiled by Jukes (1971) seem to indicate that there are no serious discrepancies between frequencies known for amino acid interchanges in globins and exp-ctancies for random charge interchanges (however, see Zuckerkandl and Pauling, 1965). Apparent random charge interchange may be true only on an overall statistical level, but incorrect at the level of the amino acid position (Uzell and Corbin, 1971). Such considerations should not, however, diminish the potential utility of electrophoretic screening methods. Electrophoretic mobility, then, appears to be a bias-free estimator of the frequency of protein variants if, and only if, corrections are made to include the probability of altering the charge of the particular protein, given that a single step mutational event occurred.

The foregoing discussion assumes that the electrophoretic variation observed was allotypic. However, it is possible that the presumed allotypes are in fact conformers (Kitto et al., 1966). Ideally, one would like to demonstrate both normal Mendelian genetics and differences in primary structure of the variants in order to prove their allotypic nature. In any case, even if

the variants are really conformers, this in no way detracts from the conclusion that electrophoretic variability of M-LDH is minimal.

The usefulness of immunological data is somewhat more difficult to assess. While it is true that microcomplement fixation is capable of detecting single amino acid interchanges (Reichlin et al., 1964; Wilson et al., 1964; Reichlin et al., 1966; Margoliash et al., 1967; Phillips et al., 1968; Cocks and Wilson, 1969), it is also true that this technique is incapable of detecting every single amino acid interchange (Cocks and Wilson, 1969; Margoliash et al., 1970). In general, two explanations may be advanced to account for this observation: (1) an interchange did not occur within the antigenic mosaic on the surface of the molecule, i.e., it did not occur within the neighborhood of an antigenic determinant, and (2) an interchange resulted in a negligible conformational change in the antigen. Of particular interest in this context are the findings of Arnheim et al., (1971) showing that, in the case of lysozyme, the interior of the enzyme is not normally antigenic, i.e., the enzyme does not denature when it is used to elicit antibodies. However, when the enzyme is first reduced and carboxymethylated, the core becomes antigenic. Thus any interchanges occuring within the interior of the molecule would not be normally detected, unless steps were taken to totally denature

the antigen. Such procedures usually require milligram quantities of purified protein (Hirs, 1967) thus rendering this approach generally impractical for measuring intra-specific variability.

Since LDH is a large molecule, it would be expected to be an excellent antigen (Margoliash et al., 1970); indeed, this expectancy is borne out since the titers of the second bleeding antibodies used for this study ranged from 1:4,900 to 1:11,000. The data presented here indicate that only one of the four variants is immunologically detectable. It is unlikely that three of the four interchanged positions would be outside of an antigenic mosaic. Furthermore, it has been shown that the vast majority of amino acid replacements in man which do not lead to perceptible hemoglobinopathies are located on the surface of the hemoglobin molecule (Perutz and Lehmann, 1968); on the other hand, alterations within the hydrophobic core tend to have rather drastic functional effects. There is no reason to believe that other enzymatic proteins, including M-LDH, should have a different pattern of amino acid replacements. One would logically expect, then, that the sites of amino acid replacements in M-LDH (if they result in unaltered phenotypic properties) would tend to be on the surface of the enzyme, and hence, near an antigenic determinant. Arnheim et al., (1971) confirmed this pattern of replace-

ments in the case of lysozymes.

Since I.D.s for single amino acid replacements tend to be rather small for large antigens (for discussions of this point, see Arnheim et al., 1969; Margoliash et al., 1970; Prager and Wilson, 1971), small I.D.s would be expected in the case of single amino acid interchanges for LDH. Furthermore, each of the Ldm variants examined (with the exception of M-LDH-Guasave-2) were in heterozygous combination with a second allelomorph (M-LDH-Tarpon Springs, M-LDH-Nags Head and M-LDH-Ashland City were heterozygotes with M-LDH-Wisconsin; M-LDH-Guasave-1 was in heterozygous combination with M-LDH-Guasave-2). Thus, it would seem that if small antigenic differences existed between pure variant antigen and homologous antigen, these differences would be further diminished by the presence of the presumed homologous antigen in the homogenates. This interpretation is indirectly supported by Kaplan and White (1963), and by Fondy et al. (1964) who showed that intermediate isozymes have intermediate I.D.s when subjected to micro-complement fixation using an antibody directed against only one of the subunits. If this interpretation is correct, then it is conceivable that M-LDH-Guasave-2 represents either the lack of an interchange within the immunological mosaic or an interchange resulting in a negligable conformational change.

In spite of the fact that quantitative micro-complement fixation is potentially a very powerful technique for the investigation of allotypic variability, the above considerations warrant the conclusion that, practically, its utility is severly diminished in diploid populations where variants are present only as heterozygotes. However, when variability is great, as is the case with R. pipiens H-LDH, micro-complement fixation may provide an extremely useful adjunct to electrophoresis (Salthe, 1969).

On the other hand, kinetic analysis of enzymes is not a reasonable adjunct to screening methods such as electrophoresis or differences in antigenicity for several reasons. First, while one can measure Km and Vmax in crude preparations, providing that intrinsic modifying factors such as small molecule effects and natural inhibitors are adequately controlled, the determination of other kinetic constants, such as K_{cat}, require the use of highly purified preparations. Clearly, the effort involved in large numbers of purifications would be enormous. Furthermore, where independently determined allotypic variability is low, techniques must be used to purify material from single animals, and the resulting preparation must be further purified to isolate the two allotypes. Fortunately, the use of affinity chromatography may potentially overcome some of these obstacles (Cuatrecasas and Anfinsen, 1971), provided that the amount

of enzyme contained within the organism is sufficient.

However, a second and more fundamental objection to kinetic screening is that not all allotypes must, of necessity, possess differences in their kinetic properties. While it is true that, in some systems, such differences have been found, e.g., hemoglobin (for a summary, see Perutz and Lehmann, 1968) and esterases (Koehn, 1969), similar approaches in other systems have yielded opposite results, e.g., transferrins (Turnbull and Giblett, 1961) and H-LDH (Levy and Salthe, 1971). Indeed, the data presented here indicates that M-LDH-Guasave-2 cannot be kinetically distinguished from the Wisconsin allotype in spite of the fact that these allotypes are apparently discerned by differences in their thermostability (see below).

The thermal stability of enzymes can vary significantly from one allotype to the next, providing a reasonable, independent test of allotypic variability (Salthe, 1969). It has the advantage of theoretically being capable of distinguishing allotypes in heterozygous combination since the thermal denaturation would be expected to yield a curve resulting from several simultaneous first-order denaturation rates. Thermal stability difference was the only criterion which justified the distinction between M-LDH-Wisconsin and M-LDH-Guasave-2. The fact that an allotype of M-LDH was found with increased thermolability

without associated kinetic alterations is not unprecedented. For example, in the case of red blood cell glucose-6-phosphate dehydrogenase, the Chicago allotype is electrophoretically and kinetically normal, but exhibits greatly reduced thermostability (Kirkman, 1968). In all probability, differences in thermolability may be attributable to alterations in the hydrogen bonding characteristics of the molecule. Unfortunately, the general lack of information in the literature does not permit a more meaningful discussion of this phenomenon.

Despite the technical limitations discussed above, all of the available data indicate that, in R. pipiens, M-LDH is a relatively non-variable, monomorphic enzyme compared to H-LDH. This remarkable difference in variability may be explained in two ways: (1) by traditional models of selection, or (2) by the effects of genetic drift on neutral alleles and the neutral allele mutation rate.

The selectionist hypothesis would most probably have to include intense balancing selection, where balanced polymorphisms would be presumed to be responsible for maintaining the large array of Ldh alleles. The different polymorphisms could be interpreted as adaptations to either extrinsic factors, or intrinsically determined physiological differences, i.e., those which are genetically determined. On the other hand, M-LDH would not be

affected by normal environmental or physiological differences to any great extent. In other words, it would be adequately adapted for all normal contingencies. Rare variants of M-LDH would most probably be interpreted as deleterious mutants which are being eliminated from the gene pool. The Guasave population might be the only one containing an adaptive variant; most certainly its adaptive nature must have nothing to do with increased thermolability. Most probably, the heightened thermolability is a secondary effect of some adaptive change in the structure of the molecule.

It is known that R. pipiens is an extensively subspeciated grouping of morphologically variable animals found in a wide range of climatic conditions (Moore, 1944; Wright and Wright, 1949), suggesting that differences in climate might provide a basis for adaptive differences between the H-LDH allotypes. Such correlations have been found in esterases and malate dehydrogenases in natural populations of Pogonomyrmex barbatus (Johnson et al., 1969). Differences in the temperature tolerances of R. pipiens embryos from various populations (Moore, 1964) suggest that temperature adaptations may be a very important consideration in the genetic differentiation of the complex. However, Levy and Salthe (1971) demonstrated that there were no differences among temperature adaptations of several allotypes of Ldh, thus ruling out the most probable

extrinsic factor which could influence Ldh variability. Furthermore, these investigators found no K_m minimum as a function of temperature. These results were confirmed by Hochachka and Lewis (1971) who demonstrated no K_m minima in the case of fish LDHs. The fact that a minimum was seen in the present study could be interpreted as a pH artifact, since pH varies with temperature. The salient point here is that Guasave-2 and Wisconsin are indistinguishable by this yardstick. On the other hand, E_as tend to reflect temperature adaptations of organisms (McNaughton, 1972), and the thermodynamic data presented here indicate that M-LDH may be cold-adapted. On the other hand, the data of Levy and Salthe (1971) may be interpreted as evidence for warm adaptation of the H subunits in this regard.

While it has been assumed by most investigators that LDH isoenzyme patterns in various tissues are the result of differential rates of synthesis of the two subunits followed by random combination at the site of synthesis to form five isoenzymes in binomial proportions (Appella and Markert, 1961; Cahn et al., 1962), alternative explanations are feasible. For example, Vesell and Yielding (1966) have suggested that differential rates of attack by proteolytic enzymes may provide an important control mechanism regulating the amounts of different enzymes in various tissues. Thus, Fritz et al. (1969)

consider that the major determining factors in tissue distribution of LDH isoenzymes are the differential rates of proteolytic degradation of the various isoenzymes. This might result in adaptive differences between a number of allotypes which are more or less resistant to proteolytic attack.

Since LDH is a metabolic enzyme, other potential intrinsic differences between allotypes might be expected to manifest themselves either as altered affinity for membranes, or as altered forms of some hypothetical supermolecular aggregate of enzymes similar to those reviewed by Ginsberg and Stadtman (1970). Unfortunately, no evidence is available on these hypotheses. However, if these latter hypotheses are true, one must further speculate that H-LDH and M-LDH have different spatial arrangements on membranes (if altered membrane structure is responsible for the presumed adaptations). Otherwise, one would expect a corresponding pattern of variability for the two subunits. Clearly, further work needs to be done to establish the relationships between the LDH isoenzymes and membranes, as well as LDH and other metabolic enzymes.

Alternatively, it may be argued that the pattern of H-LDH variability is determined partially by the neutral allele mutation rate of Ldh, and partially by the rate of allele fixation. The time required to fix newly arisen alleles in a population may be significantly increased if

the mutated cistron is closely linked to another cistron upon which selection is acting to maintain a balanced polymorphism (Ohta and Kimura, 1971). This would have the effect of increasing the amount of neutral variability within the population without altering the expected rate of protein evolution (amino acid replacement rate). In spite of this effect, the extreme difference in the patterns of H and M subunit variability in the frog leads to the prediction that Ldh is evolving considerably faster than Ldm.

If the neutral allele hypothesis is true, a second prediction can be made. The degree of variability of any protein should be directly proportional to that percentage of the molecule not responsible for catalytic properties, as well as well as that percentage not necessary for such ancillary functions as sites of membrane attachments, etc. Thus, it is predicted that a greater proportion of the primary structure of M-LDH is necessary for its function than the proportion of primary structure required for the function of H-LDH.

All of the available evidence seems to support the theory that LDH variability is primarily controlled by its neutral allele mutation rate. Differences in enzyme variability have been shown to be generally dependent upon their metabolic role (Gillespie and Kojima, 1968). What is remarkable here is that two subunits having identical

biochemical roles differ so markedly in their patterns of variability. Physiologically, it has been shown that the subunits behave as if they operate in different contexts. (Wilson et al., 1963; Dawson et al., 1964; Salthe, 1965; Everse et al., 1970; Levy et al., 1971), which could allow a sufficiently different number of intermolecular contacts to render the proportion of immutable positions of M-LDH extremely large compared to H-LDH. The data do not permit an evaluation of whether or not M-LDH variability is neutral; clearly, however, it does not seem to be adaptive. It is to be hoped that amino acid sequencing will finally resolve the question of whether Ldh is evolving more rapidly than Ldm, as predicted by the neutral allele hypothesis.

Unfortunately, even if the rate of evolution of Ldh can be shown to be much greater than the rate of evolution of Ldm, this datum cannot be used as a satisfying test to distinguish between the two evolutionary hypotheses, since it can be argued that the accelerated rate of Ldh evolution is the result of adaptations to environmental flux, and is independent of its variability. Probably the only way that these hypotheses may be distinguished from one another, in a definitive manner, would be by an experimental population approach, where one can speak in terms of measurable coefficients of selection in controlled environments.

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