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1 UNPUBLISHED PRELIMINARY DATA

Evolution of Dehydrogenases

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In recent years studies have clearly shown that enzymes from different species, which catalyze the same reaction, differ in their catalytic, physical, and immunological properties as well as in their amino acid compositions. Margoliash and Smith have elucidated differences in amino acid sequences for cytochrome c, which have been summarized in the present symposium. Comparative enzymological investigations have led to the suggestion that the evolution of enzymes may be related to morphological evolution and ^{of} considerable significance in natural selection. Furthermore, the comparative enzymological approach has added a new parameter to studies in systematics and phylogeny. It is the purpose of this paper to describe methods whereby the relationship among the same enzymes from different organisms can be compared; in addition, the significance of changes in enzyme structure during evolution is discussed. The present review deals largely with the DPN-linked dehydrogenases.

Coenzyme-Enzyme Relationships

Comparative biochemical studies have demonstrated that the coenzymes are ubiquitous in all living systems, and that there has been little or no evolutionary change in the structure of coenzymes. The proteins appear to have been evolving and not the small molecules. It is worthwhile to consider briefly the implications of such observations. We have found that a number of pyridine bases can be incorporated into the DPN instead of nicotinamide to form analogs of the coenzyme (20). One such base is 3-acetylpyridine (3-AcPy) (18,19); the structure of this compound is given in Fig. 1. The question may be asked why 3-AcPy cannot replace nicotinamide as a growth factor. Table I shows the relative rate of the 3-AcPy analog

Fig. 1

Table I

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of DPN with a number of dehydrogenases obtained from the rabbit. An enzyme such as liver alcohol dehydrogenase has a higher turnover rate with the DPN analog than with the natural coenzyme. If it is assumed that an increased $V_{\max}$ with an enzyme is beneficial to the organism, then 3-AcPy would be more valuable to the rabbit as a vitamin than nicotinamide, particularly with respect to ethanol oxidation. The dinucleotide analog can also effectively replace DPN in liver glutamic dehydrogenase and in mitochondrial malic dehydrogenases. However, enzymes such as the β -hydroxybutyrate dehydrogenase and the α -glycerophosphate dehydrogenase show little or no activity with the analog. There may be more than several hundred different pyridine nucleotide-linked enzymes in one organism. Hence, if there was a change in the nature of the pyridine base during evolution, a change in many proteins would consequently have been required to accommodate the modified coenzyme resulting from the alteration in the structure of the vitamin. Such changes might have no effect on some dehydrogenases, as indicated in Table I, but the chances that the modified coenzyme could replace DPN in all DPN-linked proteins are very slim. Therefore, it seems reasonable to predict that a change in DPN structure would not occur, because of the large number of enzymes that would be affected. One might expect the chances for evolutionary change in vitamins to be related to the number of different types of enzymes of which the particular vitamin is a constituent.

In contrast to DPN, the structure of vitamin B_{12} has undergone changes without significant effect on the organism (4). This may have been due to the fact that the B_{12} coenzyme is a cofactor for only a few enzymes. Minor alterations of carotene or ubiquinone structures may also have been possible, because these cofactors are

associated with only a relatively small number of proteins. This is evident in the existence of both vitamin A₁ and vitamin A₂. Evolution of enzymes requiring particular coenzymes, therefore, must be restricted to the extent that the enzyme can interact with the co-factor. In view of the above facts, one must conclude that enzymes have undergone changes in structure as a result of primary sequence changes; whereas the various vitamins and coenzymes have experienced little evolution.

There are two types of pyridine nucleotides - DPN and TPN - that occur in nature. The two coenzymes are ubiquitously distributed. They also appear to have different functions. Reduced DPN seems primarily to be used as a potential source of high energy phosphate in the form of ATP after oxidation. Reduced TPN, on the other hand, appears to have a primary role as a reducing agent. Aspects of the functional roles of the two nicotinamides containing coenzymes have been recently considered elsewhere (25), and will not be discussed in detail here.

A large number of enzymes are known that are either DPN- or TPN-linked enzymes. TPN specific dehydrogenases are usually very specific, and will not react with DPN. Some of the DPN-linked dehydrogenases show slight activity with TPN.

It is interesting that a large number of enzymes can react with a specific coenzyme such as DPN. This would imply that there might be common structural features of the various DPN dehydrogenases that are essential for the binding of the pyridine coenzyme. At present there are a number of DPN-linked dehydrogenases that have been purified and their properties studied in some detail. These investigations permit at least a preliminary approach to comparative

studies. Knowledge of the properties of TPN specific enzymes is relatively limited and there is now available little information from which a comparison of properties of the TPN- and DPN-linked dehydrogenases can be made. In this paper, only the DPN-linked proteins are considered.

Size of the Dehydrogenases

During the past several years, we have crystallized a large number of DPN-linked specific lactic dehydrogenases (LDH's) and have studied their properties in some detail. In most animals there are essentially two main types of LDH. One type we have referred to as the H form, since it is found largely in heart muscle; and the second type we have termed the M type LDH, because it is usually present in skeletal muscle. The M type LDH and the H type LDH appear to be under the control of separate genes; each has quite different physical, catalytic and immunological properties. Differences in amino acid compositions of the two types are easily detectable, as illustrated in Table II, for the chicken and for the beef enzymes. The chicken M catalyst has an unusually high number of histidine residues. This high histidine content is characteristic of avian M type LDH's (Table III). Tables III and IV, which have selective lists only for a number of vertebrate H LDH's and M LDH's and a few selected amino acids, show that closely related species appear to have relatively similar amino acid compositions. Certain amino acids appear to be more variable than others. For example, in the M type, the number of histidine units appears to have changed considerably during evolution, whereas the phenylalanine content seems to have varied little. Some changes can be detected in arginine and

isoleucine residues of the H type, whereas the values for lysine appear to be quite constant.

Examination of the tryptic fingerprint patterns of the chicken H LDH and M LDH indicated that approximately one-half of the peptides was different (Table V) (15). A comparison of the patterns of the chicken M and dogfish M enzymes showed that there were considerable differences in the primary structures of these two related LDH's. H LDH's from different species were also different. As indicated in the table, less tryptic peptides were found when equal mixtures of chicken H and turkey H LDH were chromatographed than when equal mixtures of chicken H and beef H were run together. These results indicate that there are less differences between the chicken and the turkey enzyme than between the chicken H LDH and the beef H LDH. This is what would be expected. Differences in M type LDH's are also shown by this method of "fingerprint" comparison. It seems that one can make a preliminary index of differences between two enzymes by fingerprinting the enzymes together and then counting the number of peptides. The increase in number of peptides when the enzymes are fingerprinted together over the quantity observed when they are fingerprinted separately gives this index of difference. From the analysis of fingerprint patterns it is evident that there has been considerable change in the primary structures of the LDH's during evolution.

Although the LDH's vary considerably in many of their properties, they all appear to have about the same molecular weights (Table VI). The L specific LDH from Lactobacillus arabinosus is approximately the same size as the animal enzymes. A preliminary estimate, by Miss E. Tarmy of our laboratory, of the molecular weight

of Escherichia coli L specific LDH gave a weight about the same as that of the Lactobacillus enzyme. In contrast, the D specific LDH's from Leuconostoc mesenteroides and Lactobacillus arabinosus have somewhat lower molecular weights than the L specific dehydrogenases, with values of about 85,000. The L specific LDH's all appear to have the same shape, as indicated by their similar sedimentation constants (Table VII).

Table
VII

The L specific LDH's consist of four subunits of identical ^{about} weights of/35,000. In the pure H or M enzymes, the four subunits are identical. However, in cells that synthesize both types of LDH polypeptides, hybrids containing units of the M form and the H form can be detected.

Most DPN-linked dehydrogenases apparently consist of subunits (Table VIII); the mannitol-1-phosphate dehydrogenase of Aerobacter aerogenes is an exception that appears to consist of a single polypeptide chain. As indicated in Table VIII, the size of the subunits of the various dehydrogenases is approximately the same. These data suggest the possibility that all DPN-linked dehydrogenases arose from a common ancestral dehydrogenase, and that the size of the genes responsible for synthesis of dehydrogenases has not been altered appreciably during duplication and evolution.

Table
VIII

In general we, as well as other investigators, have found that the molecular weights of like enzymes from different species are quite similar. Recently we have used Sephadex columns to estimate molecular weights. We have found an unusually good correlation between values obtained by sedimentation methods as compared to the molecular weights determined by the Sephadex procedure. Mr. B. Kitto and Mr. J. Everse of our laboratory have recently surveyed the

weights of malic dehydrogenases from many sources. Several crystalline malic dehydrogenases have been found by the Ehrenberg method (12) to be 67,000 (16,36): an almost identical value was obtained by the use of a Sephadex G-100 column. The malic dehydrogenases in crude extracts can be eluted from the Sephadex column in the identical way that they can with the purified proteins. With the Sephadex column it is possible to estimate the molecular weights of the malic dehydrogenases directly in crude extracts without purification. As shown Table IX, in Tables IX^{and IXA}, nearly all malic dehydrogenases examined gave values of 67,000. Only a few of the Bacillus species (those principally related to B. subtilis) had the higher value of 97,000. The higher molecular weight obtained for the B. subtilis enzyme is easily reproducible on the Sephadex/^{column}. The value obtained for the Bacillus malic dehydrogenase does not appear to be due to an artifact. Dr. W. Murphey of our laboratory has recently obtained the B. subtilis malic dehydrogenase in pure form; this enzyme retained the molecular weight of 97,000 on the Sephadex G-100 column. A molecular weight of 100,000 was obtained by the Ehrenberg ultracentrifugal method; there seems to be no doubt that the molecular weight of the subtilis malic dehydrogenase is larger than that found in most organisms. It will be worthwhile to determine whether the difference in size observed with the subtilis enzyme is due to the fact that this enzyme contains three subunits instead of the usual two.

Active Site of Dehydrogenases

Most DPN-linked dehydrogenases seem to contain essential sulfhydryl (-SH) groups that can react with alkylating agents or with mercurials. These essential groups appear to be involved largely

Fig. 2 in the coenzyme binding site. Figure 2 shows the inactivation produced by iodoacetate with crystalline horse liver alcohol dehydrogenase (HL-ADH). Ethanol exerts some protection against this inactivation; the reduced DPN, however, gives complete protection. It has been the usual observation that the substrate offers little protection against -SH reagents. Lactate and pyruvate are ineffective with LDH (11). The protection of ethanol, however, on the HL-ADH is reproducible.

It has been found that p-mercuribenzoate can inactivate chicken H and beef H LDH. The extent of inactivation of these enzymes depends on the number of moles of mercurial bound (11). For example, when one mole of mercurial is bound, approximately 25% of the enzymatic activity is lost. These titrations suggest that there is one active -SH group per subunit of the LDH. After p-mercuribenzoate binding, the activity can be restored by the addition of cysteine. DPNH and AcPyDPNH can protect against the binding of the mercurial and thereby prevent inactivation; the oxidized coenzymes have somewhat less of a protective action (see Fig. 3). The rate of reaction of p-mercuribenzoate with the beef H LDH and chicken H LDH is quite slow. It should be pointed out that only slightly more than four -SH groups react per mole of enzyme. As indicated in Table X, the total -SH groups of different LDH's vary.

The unusually low number of only six cysteines in the frog M LDH led us to investigate this enzyme in detail. The finding of only six cysteines is somewhat disturbing, since this would give one-and-a-half -SH groups per subunit. Eight cysteic acid residues, however, were found after performic acid oxidation of the frog M LDH. We believe that during the purification of the enzyme two of the -SH

groups might have been oxidized. The stoichiometry of binding of p-mercuribenzoate to the loss of enzymatic activity of a number of LDH's is compared in Fig. 4. The frog M enzyme is quite different from the mammalian or avian LDH's in that only after two moles of p-mercuribenzoate are bound is any loss of catalytic properties observed. However, the additional four cysteines react to give a stoichiometry of inhibition identical to that described for the beef H LDH and the chicken H LDH. Dr. T. Fondy of our laboratory has found that the two fast reacting -SH groups of the frog LDH will also react with iodoacetate. None of the -SH groups in the native avian and mammalian LDH's will react with the alkylating reagent. The first two -SH groups per subunit of dogfish M LDH appear not to be essential for enzymatic activity, whereas the third and slower reacting group (cysteine) is essential. In the halibut enzyme, there appear to be three -SH moieties per subunit, which react faster than the essential -SH groups. It should be emphasized that reduced DPN and its analogs interfere with the binding of p-mercuribenzoate to only the essential -SH groups and not with the non-essential -SH's.

Dr. Fondy has been able to obtain a peptide from the frog M LDH that contains the apparent essential -SH group involved in the binding of the pyridine coenzyme. This was achieved by reacting the native enzyme with "cold" iodoacetate, then removing the excess of the reagent by dialysis. Only the two non-essential -SH groups of the frog dehydrogenase were alkylated. The four essential -SH's were alkylated by the use of C^{14} iodoacetate in 6 M urea. After tryptic digestion, the peptides were fingerprinted by high voltage electrophoresis, and all the radioactivity was found essentially in one peptide. This peptide was extensively purified and its composition

determined. Since the position after migration of the peptide was known, treatment of five other LDH's with C^{14} iodoacetate in urea led to the finding that the same peptide is present in LDH's other than the frog M enzyme. These results therefore suggest that an active site peptide containing an -SH group and involved in coenzyme binding is common to all LDH's. The peptide has been found both in the H and M type of LDH of the same species.

Fig. 5 The amino acid sequence of the "active site" has now almost unequivocally been established and is shown in Fig. 5. Also shown in the figure are active site peptides for triosephosphate dehydrogenase (TPD), obtained by Perham and Harris (30), as well as those for yeast alcohol dehydrogenase (Y-ADH) and HL-ADH recently reported by Harris (17). The sequence for the HL-ADH is relatively similar to that recently determined by Li and Vallee (28). With respect to TPD, the sequence is identical with both the yeast and the rabbit enzymes. Although there are some obvious differences, the similarities among the various dehydrogenases suggest that their sequences are related. Of particular interest are the close similarities between the two alcohol dehydrogenases even though the two enzymes show great differences in their specificities as well as in other catalytic properties. The LDH sequence seems to be somewhat closer to the sequence of the two alcohol dehydrogenases than to that of the TPD peptide. We are at present attempting to elucidate the sequence of "active site" peptides from glutamic and malic dehydrogenases.

Amino Acid Compositions of Dehydrogenases

The ideal method for elucidating evolutionary changes in enzymes would, of course, be to show the number of changes in the amino acid

sequence. This approach has been carried out by Margoliash and Smith for cytochrome c. However, "sequencing" is such a time-consuming endeavor that it seems impractical to consider such an approach as a useful adjunct in evolutionary studies.

Some index of relationships can be determined by comparisons of the amino acid composition. As discussed on p. 5, with respect to the LDH's, enzymes from closely related species generally can be recognized by their amino acid compositions; the extent of changes in amino acid composition varies with different dehydrogenases.

Table XI Table XI gives the amino acid compositions of 11 TPD's from different sources (2). Although there are some definite differences among them, the changes are not quite as dramatic as those observed with the LDH's. It is clearly indicated, however, that the TPD's isolated from closely related species have similarities in their patterns of amino acid distribution. Within the limitations of the method, the beef and rabbit enzymes appear to be identical in amino acid composition. The human TPD differs significantly, in some respects, from the beef and rabbit proteins; it has fewer residues of basic amino acids, a higher number of aspartic acids, a higher number of serine moieties, and fewer valine residues. It is noteworthy that catalytic differences also distinguish the human catalyst from the rabbit and beef dehydrogenases. The three galliform bird enzymes seem to have almost identical compositions. On the other hand, the compositions of the two bony fishes are somewhat different; these differences are also suggested by immunological experiments. When enzymes from taxonomically distinct sources are analyzed, considerable differences in certain amino acid residues are observed. For example, the lobster dehydrogenase has a considerably lower basic

amino acid (histidine and arginine) content than does the vertebrate enzymes. The coli and yeast enzymes have a number of differences that distinguish them from each other as well as from the animal proteins. A feature that appears to be common to all the TPD's is the constantly high proportion of residues with aliphatic hydrocarbon side chains. A further feature is the relatively constant number of amino acid residues (1083 to 1117) in all of the enzymes; this fact suggests that the size of the gene has not been appreciably altered during evolution.

Use of Catalytic Characterizations in Evolutionary Studies

Close analyses of the catalytic properties of the same enzyme from a variant species have indicated that differences exist in this enzyme that may be valuable in phylogenic and taxonomic studies. These differences are reflected by affinities for substrate or coenzyme, relative rates of reaction with several different substrates or coenzyme analogs, as well as by the inhibition of excess substrate and various types of inhibitors. In our comparative studies, we have extensively used DPN and TPN analogs to show differences in pyridine nucleotide requiring enzymes. Here we shall give only a few examples of this use.

Table XII Table XII shows the difference of DPN analogs with Y-ADH and HL-ADH (23). The Y-ADH is much more specific than the HL-ADH and reacts to only a slight extent with most of the coenzyme analogs. Several of the analogs give higher maximum rates of reaction with the HL-ADH than with the natural coenzyme.

A comparison of relative rates of reaction with several coenzyme analogs has been made for LDH for a large number of different

Table
XIII

animals. Table XIII summarizes this study briefly for both the H type LDH and the M type LDH (40). By such a comparison the H LDH's and the M LDH's of a single species can easily be distinguished. As shown in the table, the H type enzymes from different mammals are quite similar; the M enzyme shows somewhat more variation in the mammals. Differences in ratios among the various classes of vertebrates are also observed.

Table
XIV

In our early studies with the coenzyme analogs, we were amazed by the differences found in the phylum Arthropoda (21). It was found that the crustacean muscle LDH could be characterized by a much higher reactivity with the AcPyDPN than with DPN itself (see Table XIV). In contrast, limulus (horseshoe crab) possesses an enzyme that gives a higher rate of reaction with the natural coenzyme than with the analog. Furthermore, the thionicotinamide analog (TNDPN) reacted with the crustacean enzymes but not with the limulus LDH. Other arachnids reacted similarly with the two DPN analogs as did the limulus LDH. From such catalytic data, it can easily be seen that the LDH's of the Arthropoda fall into two main groups, and that the division of the LDH's is certainly in line with the known classification of this phylum. The insects, by the LDH criteria, appear to be more closely related to the crustaceans than to the arachnids. This agrees with the consensus of views on classification of the Arthropoda.

There appear to be two types of LDH in invertebrates as well as in vertebrates. However, differences in catalytic, chemical and physical properties of LDH's make it almost impossible at present to ascertain whether there is an ancestral enzyme of either the vertebrate H or M in the invertebrates. It will be interesting to

determine if such relationships exist or whether the gene duplication into H and M occurred as an independent evolutionary event in both invertebrates and vertebrates.

Table XV

Table XV shows another example of the use of the catalytic method in studies of classification. It is well known, as illustrated in the table, that there is considerable variation in the % of G+C content of DNA of members of the Bacillus group. The catalytic properties with the malic dehydrogenases from the various Bacilli correlate with the DNA composition of the enzymes. This correlation reaffirms that closely related species have like enzymes as indicated by their catalytic characteristics. Listed in Table XV is an organism (megaterium cereus) supposedly an intermediate between the two types of Bacillus, which appears to be very closely related to cereus and not to megaterium. The relationship among the Bacilli is not limited to the malic dehydrogenases but the same relationships are shown in analog reactions rates with the mannitol-1-phosphate dehydrogenase (Table XV).

Table
XVI

Relationships of enzymes also can be ascertained by the use of inhibitors. A number of years ago, we studied the inhibitory effect of a number of pyridine bases on the enzyme DPNase which cleaves DPN at the nicotinamide riboside bond (42). It was found, as shown in Table XVI, that isonicotinic acid hydrazide strongly inhibited the DPNase from a number of ruminants but not the enzyme from other animals (42). These results are particularly interesting since they demonstrate that a particular characteristic (isonicotinic acid hydrazide sensitivity) is limited to one group of mammals, and that this particular characteristic is a feature of all ruminant DPNases. By such inhibition studies it may be possible to detect subtle

evolutionary changes in a given enzyme.

Table XVII Table XVII summarizes data on the inhibition of a number of TPD's by the pyridine-3-aldehyde analog of DPN (Py3AlDPN) and shows that differences among the various crystalline dehydrogenases can be detected. The human enzyme has a somewhat different inhibition pattern as compared to the beef and rabbit proteins. This difference is also reflected in some physical properties. Data such as given in Table XVII are useful for showing relationships as well as for indicating subtle differences.

Immunological Characterizations

Immunological methods have been used to show that similar proteins in different species vary in structure (27,5), and it has been possible in this way to show the relationship of enzymes in various species (6,7,41,1,3,40,39).

In our immunological comparisons we have used primarily the micro complement (C') fixation method of Wasserman and Levine (37). For comparative purposes only the enzyme to be used as the immunizing antigen must be pure. If the purity of the immunizing antigen is definitively established, then it is possible to carry out cross reactions with crude tissue extracts. It should be emphasized that this method gives the same fixation curves whether the antigen is in the pure form or whether it is present in crude extracts. The micro C' fixation method is advantageous because the procedure requires a hundred to a thousand times less enzyme or antibody than is necessary for other quantitative immunological methods.

Another advantage of the micro C' fixation method is that this method is considerably more sensitive to changes in structure than

the quantitative precipitin or the usual micro C' fixation method; this fact allows for the recognition of differences among homologous proteins of different species that is not possible by the other methods (see Table XVIII). The relatively large differences given by the homologous and ^{the} heterologous reaction with the diverse types of proteins strongly indicate that the micro C' fixation procedure is a desirable method for taxonomic and evolutionary studies.

The micro C' fixation method can detect differences in which a change of only one amino acid is involved, as indicated by a comparison of the reaction of human hemoglobins S and C with an antibody to hemoglobin A (see Table XIX) (33). The data in this table suggest that there is a correlation between the number of sequence differences and the cross reaction by C' fixation. This possibility is now under intensive study in our laboratory as well as in other laboratories.

The index of dissimilarity between the fixation reactions of a homologous and a heterologous antigen has been defined as the ratio of antiserum required to give 50% fixation with the homologous antigen, as compared to the amount of antiserum to give 50% fixation with the heterologous antigen. A value of 10 would mean that for a heterologous enzyme 10 times more antibody is required than for the homologous enzyme to give 50% C' fixation.

Table XX contains data showing the dissimilarity of several enzymes and hemoglobins from different species, as compared to the chicken. The turkey proteins are quite close to the chicken proteins; the two turkey LDH's can be distinguished from the corresponding enzymes in the chicken, whereas with the other proteins listed, no differences can be detected by the C' fixation method.

Table
XVIII

Table
XIX

Table XX

The indexes of dissimilarity increase from the turkey to fish in relatively the same way with each immune system in a manner that might be expected from the evolutionary positions of the various species. This fact suggests that parallel rates of change in enzymes might have occurred during evolution. The rates are by no means identical, but the order of magnitude is roughly the same.

Table XXI Table XXI shows that changes in LDH and TPD have also occurred in fish. The indexes of dissimilarities are given for various fish, as compared to the halibut TPD and LDH. The fish are arranged in an approximate evolutionary series. Close relatives of the halibut are listed near the top; the more distant relatives are at the bottom of the table. It is noteworthy that closely related fish have relatively similar indexes of dissimilarities as compared to the halibut.

Table XXII Table XXII presents data with an antibody to lobster TPD, with respect to indexes of dissimilarity to the dehydrogenase from other Arthropods. The evolutionary relationships are again quite apparent.

Table XXIII The use of the immunological structure of an enzyme as a means of determining taxonomic distance is shown for the Enterobacteriaceae in Table XXIII. An antibody to Escherichia coli phosphatase can react with the enzyme from E. freundii to the same extent as the homologous enzyme. However, the enzyme does not react as well with the Aerobacter aerogenes phosphatase, reacts less so with Serratia marcescens, and very poorly with the Proteus species. The order of cross reaction correlates with the taxonomic relationship of the organism.

Table XXIV Dr. Allan Wilson has made a detailed comparison of the relationships of enzymes from halibut, chicken, and the sturgeon (Table XXIV).

The table also includes some results with two other higher teleosts, the tuna and the mackerel. With the exception of the H_4 , it is evident that the chicken enzymes are more closely related to the sturgeon proteins than to those of the higher teleosts. Conversely, it has been found that antibodies to halibut enzymes cross react to a greater extent with the sturgeon enzymes than with the corresponding chicken catalysts. As might be expected, antibodies to sturgeon enzymes react comparatively well with both the chicken and higher teleost proteins. The evolutionary relationships between the sturgeon, chicken, and halibut are given in Fig. 6. The point of divergence of the fish line from the chicken time is some 400 million years. The data suggest that both the chicken and halibut enzymes have retained some of the characteristics of the primitive sturgeon ancestor. The morphological characteristics of the sturgeon, based on fossil data, apparently have changed little. The enzyme data appear to substantiate this finding, since it seems reasonable to assume that the sturgeon enzymes as a whole have also undergone little change in structure. Further details of this study will be presented elsewhere.

The above examples certainly suggest that the immunological approach can be a useful adjunct in taxonomic and evolutionary studies. It should be emphasized that in order to elucidate relationships between closely related species, antibodies to enzymes of one of the members of the group must be obtained. For example, if one hopes to establish relationships within the reptilian class, it is preferable to use an antibody to a reptile rather than an antibody to a bird or amphibian enzyme.

Temperature Stability and Electrophoresis

Certain physical criteria, such as temperature stability and electrophoretic migration, can be used as parameters for showing relationships of enzymes from different species. An intensive study of the electrophoretic properties and stability of the H₄ LDH of vertebrates has been made in our laboratory and is summarized in Table XXV. These measurements can be made in crude extracts, if carefully controlled conditions are maintained.

In general, it has been found that the H₄ LDH of closely related species has similar migratory and thermal stability properties. Small but significant differences can be observed with some closely related species. In the bullfrog H, 50% of the enzymatic activity is lost after 20 minutes at 52°; the temperature must be raised to 56° to obtain the same degree of inactivation in 20 minutes for the leopard frog H LDH. These small differences are quite reproducible and represent subtle differences in the LDH structure. We have had adequate experience to state that these differences in heat lability can be used as an index of species relationships.

Table XXV indicates that a relatively large increase in the heat stability of the H type LDH occurred at one point during vertebrate evolution. As the table shows, all the lower invertebrates examined, which include a diverse number of fish and amphibia, have a half-inactivation temperature of roughly 60°. This is also true of the lower reptiles and the mammals. In contrast, the higher reptiles have temperatures of inactivation of close to 80°. Apparently this high temperature stability occurred at a time when the higher reptiles were evolving from the lower reptiles. Birds

Table
XXV

generally have the high temperature stable H LDH characteristic of the higher reptiles. There are some exceptions with regard to thermostability in a few higher birds; it is possible that those species with relatively unstable H type LDH may have originated comparatively recently from the stable "primitive" higher reptile or bird enzyme. It is of interest that the thermal stability of the mammalian H type is similar to that of the lower invertebrate, indicating that at least with respect to this physical property the mammalian H LDH has undergone little change.

Closely related species usually have rather similar electrophoretic migrations for their H type LDH's (8,40) (see Table XXV). There are some closely related species, such as the bullfrog and leopard frog, which have significantly different mobilities: the mammals examined all have a very rapidly migrating H type LDH (12 to 15 CM). Most fish, amphibia, and reptiles have enzymes that migrate considerably slowly (5 to 10 CM). The bird H LDH's as a whole are exceptionally slow-moving. The paleognathous birds (ostrich, rhea, and tinamou), however, have somewhat faster migrating enzymes and are more like the higher reptiles in this respect. Among birds, on the basis of anatomical data, this group has long been considered to be primitive and appears to have evolved from reptiles before the modern birds had diversified. It is interesting that both the heat stability and electrophoretic properties of the primitive birds are similar to those of the higher reptiles. Figure 7 summarizes the evolutionary changes of LDH in vertebrates; these results show good agreement with what is known about the general direction of vertebrate evolution.

Significance of Evolutionary Changes in Enzymes

The question can be posed as to whether change in enzyme structure during evolution has any functional or survival significance. At present, this is a difficult question to answer; however, our growing understanding of subtle differences in catalytic and physical properties of like enzymes from different species suggests that these changes may have been important in natural selection.

We have previously advanced the premise that the two types of LDH have functionally different roles (22,24). This premise was based on observations that there was a marked difference in the degree of inhibition of the two types by excess pyruvate, as illustrated in Fig. 8 for the beef LDH's. The H type is maximally active at low levels of pyruvate and is inhibited by higher concentrations of the keto acid. In contrast, the M type shows little substrate inhibition. These findings may be related to the metabolic differences of heart and skeletal muscles. In the heart a steady supply of energy is required and is maintained by a complete oxidation of pyruvate in the mitochondria; hence, inhibition of the LDH by excess pyruvate would favor the oxidation of the keto acid. In voluntary muscles, the need usually is for sudden energy and is associated with a rapid glycolytic formation of pyruvate. To maintain glycolysis it is essential continuously to oxidize the DPNH by LDH and pyruvate. Therefore, it appears reasonable that an M type enzyme that can operate in the presence of temporarily high concentrations of pyruvate should be found in tissues that have a sudden demand for energy. The M type enzyme appears to be geared for reduction of pyruvate, whereas the H type LDH may be more directed toward lactate oxidation.

Fig. 8

Investigations in our laboratory have supported the view of the different roles of the two types. In studies on various striated muscles it has consistently been found that those muscles that contract tonically or rhythmically have considerably more H subunits, as contrasted to muscles without contractions (26,9,38). A detailed examination of the breast muscle composition of a number of different birds showed that those birds that are sustained flyers have a large percentage of H subunits, whereas birds that have only a sudden need for use of their breast muscle have largely the M form. There is an excellent correlation between the flying habits of the birds and the LDH composition of the breast muscle (38).

Dr. Salthe of our laboratory has recently made a survey of the substrate (pyruvate) inhibition characteristics of a number of different amphibians. The terrestrial species have higher indexes of inhibition, as compared to the aquatic amphibians (see Table XXVI). There appears to be a correlation between the environmental oxygen tension and the LDH^{type}. The more anaerobic the environment, as in the case of the aquatic animals, the greater the relative amount of M type LDH, as aquatic and terrestrial amphibians also have been found to differ in their hemoglobins (29); the aquatic animals have hemoglobins with considerably greater affinity for oxygen, as compared to the hemoglobins of the terrestrial amphibians. It is noteworthy that such relatively anaerobic vertebrates as the flat fish (halibut, flounder, sole) and the lamprey have only the M type LDH in all their tissues.

Fig. 9 Figure 9 shows that there have been evolutionary changes in the mammalian M type LDH; this is indicated by the differences in sensitivity to high pyruvate concentration; there are other criteria

for distinguishing the various mammalian LDH's. From the point of view of natural selection, the differences in the curves of Fig. 9 may be of some interest. The rabbit is an animal that produces a great deal of lactic acid during activity, and the evolution of a strongly anaerobic M type LDH may have been advantageous to the survival of this mammal. In contrast, the human, whose dependence on sudden and extensive activity for survival is considerably less, has developed an enzyme that is somewhat more sensitive to high concentrations of pyruvate.

The LDH's also differ in their turnover numbers with respect to both pyruvate and lactate. Table XXVII lists some turnover numbers with pyruvate of several LDH's. The M type LDH's have a higher turnover number than the H catalyst does; the fish M types have significantly higher turnover numbers than the corresponding enzymes in avian and mammalian species do. The Lactobacillus LDH appears to have a considerably lower turnover number than the animal enzymes studied. An E. coli enzyme, purified by Miss Tarmy, also appears to have a relatively low turnover number.

Similar enzymes operate under varying environmental conditions in different species. Organisms live in a manifold of temperatures and hence one might expect that enzyme evolution has permitted adaptation to particular temperatures. Table XXVIII compares the rate of LDH activity at 45° to that at 15° using a low level of pyruvate. It is of interest that for the warm-blooded animals there is an increase in rate of reaction when the temperature is raised. However, the lobster and the halibut enzyme, whose habitat is a low temperature one, show a decrease in activity as the temperature is increased. These data indicate that the affinity for low amounts of

Table
XXVII

Table
XXVIII

pyruvate in the warm-blooded animal increases with temperature and that for the LDH's of the cold-blooded animals generally decreases. We have found that the halibut M enzyme functions at temperatures of 8 to 10° like the chicken M LDH does at 30°. Apparently, cold-blooded animals have evolved enzymes with structures capable of functioning at low temperatures.

There are two types of malic dehydrogenases in animals. One usually is localized in the mitochondria; the second is found in the soluble part of the cell (10,13,34,35). The mitochondrial enzyme appears to be geared toward malate oxidation, whereas the soluble malic dehydrogenase appears to function in the direction of reduction of oxaloacetate (10,25).

The above examples certainly suggest that changes in enzyme structure may be important factors in natural selection. The rapid development of methodology for evaluating the chemical, catalytic, and physical properties of enzymes promises to add new understanding to the physiological significance of specific molecular changes in enzymes.

It should be emphasized that the use of the new biochemical procedures will not supercede the classical phylogenic and taxonomic studies but will add quantitative parameters for use together with the classical approach. Specific problems that the classical methods cannot resolve may be solved by the comparative enzyme techniques outlined in this paper. Perhaps, in years to come, investigators utilizing both classical and molecular biology approaches will be able to clarify the intimate mechanisms involved in evolutionary change and in the origin of new species.

Acknowledgments

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Footnote

- ¹ Publication No. 329.

TABLE I

Rates of Reaction of the 3-Acetylpyridine Analog of DPN with
Different Rabbit Dehydrogenases

<u>Dehydrogenase</u>	<u>% Rate of AcPyDPN Compared to DPN</u>
Liver alcohol	450
Liver glutamic	150
Heart mitochondrial malic	125
Muscle lactic	22
Muscle triosephosphate	10
Heart lactic	4
Liver β -hydroxybutyrate	<1
Muscle α -glycerophosphate	0

TABLE II

Amino Acid Compositions of Heart and Muscle Type Lactic
Dehydrogenases From Chicken and Beef^a

Amino acid	Chicken		Beef	
	<u>H₄</u>	<u>M₄</u>	<u>H₄</u>	<u>M₄</u>
Lys	99	112	96	103
His	30	63	26	33
Arg	35	35	34	42
Asp	129	125	132	127
Thr	75	51	56	48
Ser	107	110	97	87
Glu	122	102	129	121
Pro	38	44	46	51
Gly	96	104	98	100
Ala	88	81	80	78
Val	125	121	138	115
Met	25	31	36	32
Ileu	66	85	85	91
Leu	149	121	143	136
Tyr	31	19	29	29
Phe	19	27	21	29
Try	22	24	22	24
Cys	26	26	17	26

^a From Pesce et al. (32).

TABLE III

Amino Acid Composition of H₄ Lactic Dehydrogenase^a

	Arginine	Isoleucine	Lysine
	<u>(residues/mole)</u>		
Chicken	35	66	99
Pheasant	38	67	97
Turkey	34	69	87
Cow	34	85	96
Rabbit	33	84	97
Man	30	88	96
Frog	21	65	95

^a Data from A. Pesce (31).

TABLE IV

Amino Acid Composition of M₄ Lactic Dehydrogenase ^a

	<u>Histidine Phenylalanine</u> <u>(residues/mole)</u>	
Chicken	63	27
Pheasant	61	29
Turkey	73	24
Duck	57	27
Caiman	46	26
Cow	33	29
Rabbit	41	26
Leopard frog	26	28
Bullfrog	29	26
Halibut	49	29
Dogfish	42	26
Lamprey	41	24

^a Data from A. Pesce (31).

TABLE V
Fingerprint Patterns of H Type Lactic Dehydrogenase
and M Type Lactic Dehydrogenase^a

	<u>No. of ninhydrin positive spots</u>
Chicken	34-38
Chicken M	35-38
Turkey H	33-35
Beef H	33-35
Turkey M	34-37
Dogfish M	34-37
Chicken H + chicken M	50-53
Chicken H + turkey H	35-40
Chicken H + beef H	45-47
Chicken M + dogfish M	52-56
Chicken M + turkey M	36-38

^a Data largely from Dr. T. P. Fondy.

TABLE VI

Molecular Weights of Various Lactic Dehydrogenases^a

<u>Species</u>	<u>H₄</u>	<u>M₄</u>
Beef	131	153
Human	146	-
Rabbit	-	149
Chicken	151	140
Turkey	147	144
Pheasant	-	148
Bullfrog		154
Dogfish	-	141
Halibut	-	148
Lobster		152
<u>Lactobacillus arabinosus</u>		153

^a Data were obtained by the Ehrenberg method of approach to sedimentation equilibria, mainly by Dr. A. Pesce and Mr. J. Everse of our laboratory.

TABLE VII
Sedimentation Constants for H_4 and M_4 Lactic
Dehydrogenases and for Lactic Dehydrogenase
from Lactobacillus arabinosus ()

<u>Species</u>	$S_{20,w}$	
	H_4	M_4
Beef	7.45	7.32
Human	7.46	-
Chicken	7.31	7.33
Turkey	7.49	7.52
Bullfrog		7.56
Dogfish		7.56
Lobster		7.40
<u>Lactobacillus arabinosus</u>	7.50	

TABLE VIII

Molecular Weight and Number of Subunits of Different Dehydrogenases

<u>Dehydrogenase</u>	<u>M.W.</u>	<u>No. of subunits</u>
Lactic	145,000	4
Triosephosphate	150,000	4
Yeast alcohol	140,000	4
Liver alcohol	68,000	2
Malic	67,000	2
Glutamic	1,000,000	30 ^a

^a Approximate.

TABLE IX

Malic Dehydrogenases Molecular Weight Determination by
Sephadex Gel Filtration

The following samples all have malic dehydrogenases of 67,000 molecular weight by gel filtration. All the crude extracts, except for the bacterial, were examined by starch gel electrophoresis and found to contain multiple forms of malic dehydrogenase.

CRYSTALLINE ENZYMES

Chick heart (mitochondrial)

Pig heart (mitochondrial)

Ostrich heart (supernatant)

Propionibacterium shermanii

PURIFIED ENZYMES AND CRUDE EXTRACTS

Pig heart (supernatant)

Chick heart

Beef liver

Bullfrog heart (Rana catesbiana)

Garter snake heart (Thamnophis sirtalis sirtalis)

Tuna heart

Dogfish heart

Horseshoe crab heart (Limulus)

Oak silkworm (Antheraea pernyi)

Potato

Slime mold (Polysphondylium pallidum)

Neurospora crassa

Euglena gracilis

TABLE IX (continued)

BACTERIAL EXTRACTS

<u>Pseudomonadaceae:</u>	<u>Rhodopseudomonas palustris</u>
	<u>Xanthomonas pruni</u>
	<u>Spirillum serpens</u>
<u>Rhizobiaceae:</u>	<u>Chromobacterium violaceum</u>
<u>Achromobacteraceae:</u>	<u>Alcaligenes faecalis</u>
	<u>Achromobacter parvulus</u>
<u>Enterobacteriaceae:</u>	<u>Escherichia coli B</u>
	<u>Citrobacter freundii</u>
	<u>Aerobacter aerogenes</u>
	<u>Erwinia caratovora</u>
	<u>Proteus vulgaris</u>
<u>Micrococcaceae:</u>	<u>Micrococcus lysodeikticus</u>
<u>Actinomycetaceae:</u>	<u>Streptomyces coelicolor</u>

TABLE IXA

Malic Dehydrogenases of Bacillus Species Molecular Weight
Determination by Sephadex Gel Filtration

	<u>M.W.</u> <u>97,000</u>	<u>M.W.</u> <u>67,000</u>
<u>B. subtilus</u> 168 W	+	
<u>B. brevis</u>	+	
<u>B. licheniformis</u>	+	
<u>B. macerans</u>	+	
<u>B. stearothermophilus</u>	+	
<u>B. coagulans</u>	+	
<u>B. megaterium</u>	+	
<u>B. cereus</u>	+	
<u>B. subtilus</u> var. <u>niger</u>	+	
<u>B. pasteurii</u>		+
<u>B. sphaericus</u>		+
<u>B. circulans</u>		+
<u>B. laterosporus</u>		+
<u>B. thuringiensis</u>		+
<u>B. lentus</u>		+
<u>B. natto</u>	+	+

TABLE X

Cysteine Sulfhydryl Content of Different Lactic Dehydrogenases^a

<u>LDH</u>	No. of cysteine -SH <u>residues</u>
Pheasant H	26
Chicken H	25
Beef H	17
Turkey M	24
Chicken M	24
Frog M	6
Beef M	26
Dogfish M	24
Halibut M	14
Lamprey M	20
Lobster M	20
Caiman	30

^a Data of T. P. Fondy and J. Everse (14).

TABLE XI

Amino Acid Compositions of 11 Triosephosphate Dehydrogenases^a

Amino acid	Rabbit	Beef	Human	Chicken	Turkey	Pheasant	Hallibut	Sturgeon	Lobster	<u>E. coli</u>	Yeast
Lys	90	90	84	84	87	87	87	97	94	87	91
His	34	34	31	30	31	32	42	20	15	19	27
Arg	34	35	31	34	35	36	35	37	28	36	36
Asp	131	131	139	138	137	134	110	130	118	148	130
Thr	70	71	70	68	68	66	59	68	65	92	77
Ser	58	57	68	61	62	59	72	58	76	50	88
Glu	71	74	74	64	63	62	77	65	84	76	72
Pro	43	43	42	42	42	42	41	42	38	31	41
Gly	113	112	117	115	115	112	94	97	108	106	90
Ala	114	111	108	121	120	117	106	111	114	124	108
Val	105	110	95	116	117	110	105	118	123	105	105
Met	31	30	31	30	31	31	28	29	34	24	21
Ileu	66	63	65	58	58	56	71	67	65	58	68
Leu	62	64	65	64	65	67	61	64	61	69	73
Tyr	30	31	31	31	31	31	32	39	30	28	38
Phe	48	48	46	44	45	46	50	46	50	37	35
Try	12	13	13	12	13	13	13	12	13	28	12
Total	1112	1117	1110	1112	1120	1098	1083	1110	1116	1118	1112

^a From Allison and Kaplan (2).

TABLE XII

Comparison of Rates of Reaction of Some Analogs of DPN with
Yeast Alcohol Dehydrogenase and Horse Liver Alcohol Dehydrogenase^a

Ethanol (0.1 M) was used in all reaction mixtures.

<u>Coenzyme</u>	<u>Y-ADH</u>	<u>HL-ADH</u>
DPN	1	1
Deamino DPN	0.12	1.1
3-Acetylpyridine DPN	0.05	6.0
Pyridine-3-aldehyde DPN	<0.02	0.95
3-Thionicotinamide DPN	0.16	3.5
3-Benzolpyridine DPN	0	0.31
Propyl pyridyl ketone DPN	<0.01	4.8
Uracil DPN	0.02	0.75

^a From Kaplan and Ciotti (23).

TABLE XIII

Catalytic Properties of Vertebrate Lactic Dehydrogenases^a

<u>Species</u>	<u>AcPyDPN/DPN</u>	
	<u>H₄</u>	<u>M₄</u>
Chicken	0.15	1.1
Turkey	0.17	2.0
Duck	0.18	0.8
Pigeon	0.11	-
Caiman	0.07	0.7
Turtle	-	0.6
Bullfrog	0.18	0.3
Sturgeon	0.08	0.2
Halibut	-	1.0
Mackerel	0.12	0.7
Dogfish	0.26	0.9
Lamprey	-	0.2
Cow	0.06	0.3
Man	0.04	0.2
Rabbit	0.05	-

^a Data from Wilson et al. (40).

TABLE XIV

Muscle Lactic Dehydrogenase of the Phylum Arthropoda^a

<u>Subphylum</u>	<u>AcPyDPN</u> <u>DPN</u>	<u>Detectable Reaction</u> <u>with TNDPN</u>
Mandibulata		
Hermit crab	6	+
Fiddler crab	40	+
Green crab	18	+
Edible crab	22	+
Crayfish	15	+
Lobster	17	+
Chelicerata		
Limulus	0.08	-
Tarantula	0.12	-
Wolf spider	0.06	-
Scorpion	0.16	-

^a Data from Kaplan et al. (21).

TABLE XV

Malic Dehydrogenase, Mannitol Phosphate Dehydrogenase, and DNA
Base Composition of Species in the Genus Bacillus^a

<u>Species</u>	Malic ^b <u>Dehydrogenase</u>	Mannitol Phosphate <u>Dehydrogenase</u> ^c	G + C ^d (%)
<u>B. subtilis</u>	16.0	0.70	43
<u>B. natto</u>	17.4	0.68	43
<u>B. subtilis</u>	22.5	0.75	42.5
var. <u>aterrimus</u>			
<u>B. subtilis</u>	17.9	0.75	43
var. <u>niger</u>			
<u>B. niger</u>	14.8	0.73	43
<u>B. polymyxa</u>	13.6	0.72	44
<u>B. licheniformis</u>	10.5	0.55	46
<u>B. pumilus</u>	6.4	0.56	39
<u>B. macerans</u>	0.8	0.53	50.5
<u>B. circulans</u>	0.6	0.31	35
<u>B. megatherium</u>	1.1	0.22	37
<u>B. cereus</u>	>100	-	33
<u>B. alvei</u>	>100	-	33
<u>B. cereus-</u>	>100		
<u>megatherium</u>			

^a From Wilson and Kaplan (39).

^b Rate with DPN relative to rate with the ethyl pyridyl ketone analog; measurements made at 25° in the presence of 5×10^{-2} M L-malate at pH 8.9.

^c Rate with the 6-hydroxyethylamino purine analog relative to the rate with DPN.

^d DNA from species above the dotted line can transform B. subtilis.

TABLE XVI

The Effect of Isonicotinic Acid Hydrazide (INH) on the
Diphosphopyridine Nucleotidases of Various Animals

The action of all the "sensitive" enzymes was inhibited to an extent greater than 65% by 7.5×10^{-4} M INH; the action of the "insensitive" enzymes was not significantly inhibited by 7.5×10^{-4} M INH.

INH-"sensitive"

Goat
Beef
Lamb
Deer
Buffalo

INH-"insensitive"

Pig
Horse
Mouse
Guinea pig
Rat
Rabbit
Frog
Man

TABLE XVII

Inhibition of the Purified Triosephosphate Dehydrogenases by
Pyridine-3-Aldehyde Diphosphopyridine Nucleotide^a

<u>Enzyme</u>	Inhibition at pyridine-3-aldehyde-DPN concentration of				
	1 μ g	2 μ g	5 μ g	10 μ g	100 μ g
	%	%	%	%	%
Rabbit	3	13	25	38	80
Beef	4	16	25	37	79
Human	0	0	8	20	60
Chicken	12	14	26	47	83
Turkey	10	15	28	41	81
Pheasant	6	18	28	39	83
Halibut	8	18	31	41	75
Sturgeon	6	14	20	22	56
Lobster	7	12	34	65	89
<u>E. coli</u>	0	0	0	3	40
Yeast	39	54		89	95

^a From Allison and Kaplan (2).

TABLE XVIII

Comparison of Sensitivity of Immunological Methods^a

Antiserum	Heterologous antigen	Cross Reaction ^b		
		Micro C' fixation	Macro C' fixation	Quantitative precipitin reaction
Anti-human hemoglobin A ₁	Human hemoglobin S	41	86	100
Anti-human serum albumin	Chimpanzee serum albumin	52	97	89
Anti-chicken ovalbumin	Turkey ovalbumin	3	89	96
Anti-chicken H ₄ LDH	Turkey H ₄ LDH	32	112	91

^a From Wilson et al. (40).

^b Heterologous reaction is expressed as a percentage of the homologous reaction.

TABLE XIX

Micro Complement Fixation by Antiserum to Hemoglobin A₁^a

<u>Hemoglobin</u>	<u>No. of amino acid substitutions</u>	<u>Anti-serum concentration for 50% C' fixation</u>
A ₁	0	1.0
S	1	1.3
C	1	1.3
A ₂	7-8	2.0
F	40	> 5

^a From Reichlin et al. (33).

TABLE XX

Micro Complement Fixation with Antisera to Pure Chicken Proteins^aAntiserum concentration required for 50% C' fixation^b

Species	H ₄ LDH	M ₄ LDH	TPD ^c	GDH ^d	Aldo-lase	Hemo-globin
Chicken	1.0	1.0	1.0	1.0	1.0	1.0
Turkey	1.4	1.2	1.0	1.0	1.0	1.0
Duck	1.5	4.3	1.2	1.2		2.2
Pigeon	2.3	2.0	1.3	1.3		3.6
Ostrich	1.9	3.1	1.3	1.4	5.0	
Caiman	3.3	4.2	3.8	4.0	6.5	
Painted turtle	4.0	5.2	4.2	4.0		6.5
Bullfrog	14	40	30	19	18	
Sturgeon	80	20	12	25		
Halibut	c	> 200	> 50		>100	
Dogfish	>100	> 200	> 50			
Lamprey	c	30	> 50			
Hagfish	>100	>100				

^a From Wilson *et al.* (40). ^b Data presented are based on the use of several anti-chicken H₄ LDH sera, two anti-chicken M₄ LDH sera and one each of the other anti-chicken protein sera. ^c Halibut and lamprey tissues contain no detectable H₄ LDH.

^c Triosephosphate dehydrogenase.

^d Glutamic dehydrogenase.

TABLE XXI

Micro Complement Fixation by Antibodies to Halibut Enzymes^a

<u>Species</u>	Relative Ab conc. for 50% C' fixation at equivalence	
	TPD	M ₄ LDH
BONY FISHES (OSTEICHTHYES)		
<u>Teleostei</u>		
Heterosomata		
Pacific halibut, <u>Hippoglossus stenolepis</u>	1.0	1.0
Petrale sole, <u>Eopsetta jordani</u>	1.3	1.1
Commercial flounder (Boston)	1.3	1.1
Commercial sole (Boston)	1.3	1.1
Perciformes		
Scorpion fish, <u>Scorpaenopsis gibbosa</u>	2.0	1.2
Mackerel, <u>Scomber scombrus</u>	1.8	1.5
Beryciformes		
Squirrel fish, <u>Holocentrus ensifer</u>	1.8	1.3
Ostariophysi		
Carp, <u>Cyprinus carpio</u>	16	1.6
Brown bullhead catfish, <u>Ictalurus nebulosus</u>	18	1.7
Apodes		
Conger eel, <u>Conger marginatus</u>	17	2.0
Moray eel, <u>Gymnothorax hepaticus</u>	20	4.0
Moray eel, <u>Gymnothorax flavimarginatus</u>	20	4.2
Isospondyli		
Brook trout, <u>Salvelinus fontinalis</u>	6.0	1.6
Commercial salmon (Boston)	6.0	1.6
Commercial smelt, <u>Osmerus mordax</u>	4.1	3.2
Chain pickerel, <u>Esox niger</u>	11	6.0
Atlantic herring, <u>Clupea herengus</u>	13	35

TABLE XXI (continued)

<u>Holostei</u>		
Gar pike, <u>Lepisosteus spatula</u>	17	20
<u>Palaeoniscoidei</u>		
Sturgeon, <u>Acipenser transmontanus</u>	67	35
<u>Dipnoi</u>		
African lungfish, <u>Protopterus</u> sp.	NC ^b	100
CARTILAGINOUS FISHES (CHONDRICHTHYES)		
Spiny dogfish, <u>Squalus acanthias</u>	NC ^b	NC ^b
JAWLESS FISHES (AGNATHA)		
Lamprey, <u>Petromyzon marinus</u>	NC ^b	NC ^b

^a Data of A. C. Wilson and W. S. Allison.

^b NC indicates that no cross reaction was detected at the highest antibody concentration tested.

TABLE XXII

Cross Reactions of Various Arthropod Triosephosphate Dehydrogenases
with Antibody to Lobster Triosephosphate Dehydrogenase^a

	Relative antibody concentration for 50% C' fixation at equal enzyme concentration
Lobster	1.0
Crayfish	1.1
South African lobster	1.3
Centipede	7.2
Honey bee	11.0
Limulus	30.0

^a Data taken from Allison and Kaplan (1).

TABLE XXIII

Complement Fixation by Antiserum Against Alkaline

Phosphatase of Escherichia coli K12^a

<u>Species</u>	<u>Strain and source</u>	Antiserum amount ^b required for <u>50% O' fixation</u>
<u>Escherichia coli</u>	K12 (C. Levinthal)	1.0
	T ⁻ A ⁻ U ⁻ (S. S. Cohen)	1.0
	O	1.0
	W	1.0
	I (S. Zamenhof)	1.0
	Crookes	1.0
<u>Escherichia freundii</u>	17 (H. Blechman)	1.0
<u>Aerobacter aerogenes</u>	1088 (Harvard Medical School)	3.0
<u>Serratia marcescens</u>	SR11 Parental (L. Baron)	10
<u>Proteus mirabilis</u>	35 (H. Blechman)	~70

^a See Wilson and Kaplan (39).

^b Relative units: One ml of an approximately 1/70,000 dilution of antiserum gave 50% O' fixation with the Escherichia enzymes. Micro O' fixation data from Drs. S. Cordes and L. Levine (unpublished) using a rabbit antiserum against pure alkaline phosphatase from E. coli K12. The enzyme was purified by Dr. C. Levinthal. The enzymes of other strains and species were studied in sonic extracts of cells grown in inducing medium.

TABLE XXIV

Immunological Index of Dissimilarity of Some Vertebrates^a

	<u>H₄ LDH</u>	<u>M₄ LDH</u>	<u>TPD</u>	<u>Aldolase</u>	<u>Mitochondrial MDH</u>
Chicken	1.0	1.0	1.0	1.0	1.0
Sturgeon	80	20	12	25	20
Mackerel	-	140	-	-	-
Tuna	80	>200	-	>100	>100
Halibut	no H ₄	>200	>50	>100	-

^a Data of Dr. A. Wilson and Miss Natalie Grimes.

TABLE XXV

Temperature Stability and Electrophoretic Mobility of H₄
Lactic Dehydrogenase^a

<u>Taxonomic group</u>	<u>Inactivation temperature^b</u>	<u>Electrophoretic mobility^c</u>
Neognathous birds^d		
Passeriformes (6/67)	74	1.8
Piciformes (3/7)	79	2.8
Coraciiformes (2/9)	80	2.2
Trogoniformes (1/1)	68	0.2
Apodiformes (1/3)	77	2.2
Caprimulgiformes (1/5)	>63	0.2
Strigiformes (1/2)	68	2.1
Cuculiformes (2/2)	80	2.4
Psittaciformes (1/1)	67	2.9
Columbiformes (2/2)	76	2.2
Charadriiformes (6/15)	77	1.5
Gruiformes (2/12)	63	2.2
Galliformes (1/3)	77	2.8
Falconiformes (3/5)	78	2.1
Anseriformes (1/2)	76	2.1
Ciconiiformes (1/7)	79	2.1
Pelecaniformes (3/5)	80	1.7
Procellariiformes (2/4)	76	1.5
Podicipediformes (1/1)	79	1.5
Gaviiformes (1/1)	80	1.8
Sphenisciformes (1/1)	79	2.0
Paleognathous Birds		
Tinamiformes (1/1)	80	6.6
Rheiformes (1/1) (Rhea)	79	6.8
Struthioniformes (1/1) (ostrich)	80	6.6

TABLE XXV (continued)

Higher Reptiles

Caiman, <u>Caiman crocodilus</u>	76	5.1
Lizard, <u>Iguana iguana</u>	82	5.6
Lizard, <u>Varanus flavicens</u>	85	2.5
Snake, <u>Natrix</u> species	80	6.3
Snake, <u>Crotalus atrox</u>	80	6.3
Snake, <u>Constrictor constrictor</u>	77	5.3

Lower Reptiles

Snapping turtle, <u>Chelydra serpentina</u>	58	6.1
Painted turtle, <u>Chrysemys picta</u>	52	5.7
Cooter turtle, <u>Pseudemys scripta</u>		6.3
Soft-shell turtle, <u>Trionyx ferox</u>	<60	5.2

Mammals

Man	65	15
Domestic cow	61	12
Domestic pig	61	16
Laboratory rabbit	65	12
Laboratory mouse	60	15
Laboratory rat	60	15
Squirrel, <u>Sciurus carolinensis</u>	68	15
European hedgehog, <u>Erinaceus europaeus</u>	65	13
Short-tail shrew, <u>Blarina brevicauda</u>	66	7.7
Opossum, <u>Didelphis virginiana</u>	60	15
Kangaroo, <u>Macropus robustus</u>	69	15

Amphibians

Bullfrog, <u>Rana catesbiana</u>	52	10
Leopard frog, <u>Rana pipiens</u>	56	3.4
Toad, <u>Bufo marinus</u>	65	7.4
Congo eel, <u>Amphiuma tridactylum</u>	68	11

TABLE XXV (continued)

Bony fish

Sturgeon, <u>Acipenser transmontanus</u>	62	6.9
Haddock, <u>Melanogrammus aeglefinus</u>	63	7.8
Mackerel, <u>Scomber scombrus</u>	60	6.1

Cartilaginous fish

Seven-gill shark, <u>Notorhynchus maculatum</u>	68	5.0
Spiny dogfish, <u>Squalus acanthias</u>	64	4.1
Chimaera, <u>Hydrolagus collei</u>	<65	7.0

Cyclostomes

Lamprey, <u>Petromyzon marinus</u>	e	e
Hagfish, <u>Eptatretus stouti</u>	<65	4.8

^a From Wilson et al. (40). ^b Temperature (°C) required for 50% inactivation in 20 min. ^c Distance (cm) moved during horizontal starch-gel electrophoresis at pH 7 for 16 hr, at 10 v/cm. ^d So many birds have been studied that we have averaged the data for each order. The neognathous orders are arranged in a sequence, those near the bottom of the list being considered by ornithological authorities to be more primitive than those near the top of the list. In parentheses, we record the number of families examined as a fraction of the total number of families in the order. ^e The lamprey has no H₄ LDH in the heart; all lamprey tissues examined contain only M₄ LDH, as judged by immunological, electrophoretic, and catalytic criteria.

TABLE XXVI

Correlation Between Environmental Oxygen Availability and Substrate
Inhibition of Lactic Dehydrogenase in Amphibian Hearts^a

<u>Species</u>	<u>DPNH^b_L</u> <u>DPNH_H</u>
Terrestrial	
Spadefoot toad (<u>Scaphiopus</u>)	2.54
Spring peeper (<u>Hyla</u>)	2.43
Ant-eating toad (<u>Rhynophrynus</u>)	2.32
Spadefoot toad (<u>Pelobates</u>)	2.31
Green toad (<u>Bufo</u>)	2.30
American toad (<u>Bufo</u>)	2.18
Wood frog (<u>Rana</u>)	2.08
Marine toad (<u>Bufo</u>)	2.01
Pacific tree toad (<u>Hyla</u>)	1.94
Fire salamander (<u>Salamandra</u>)	1.92
European tree frog (<u>Hyla</u>)	1.62
Aquatic--cold, running water	
Pacific giant salamander larva (<u>Dicamptodon</u>)	2.25
Bell toad (<u>Ascaphus</u>)	1.66
Aquatic--standing water	
Painted frog (<u>Discoglossus</u>)	1.70
Fire-bellied toad (<u>Bombina</u>)	1.66
Axolotl (<u>Ambystoma</u>)	1.64
Surinam toad (<u>Pipa</u>)	1.46
Mudpuppy (<u>Necturus</u>)	1.45
Congo eel (<u>Amphiuma</u>)	1.43

TABLE XXVI (continued)

Dwarf siren (<u>Pseudobranchus</u>)	1.37
African clawed water frog (<u>Xenopus</u>)	1.32
Greater siren (<u>Siren</u>)	0.86
Lesser siren (<u>Siren</u>)	0.76

^a From Salthe and Kaplan, in preparation. ^b Indicates ratio of reaction of low to high pyruvate (see refs. 9,38).

TABLE XXVII

Analog Ratios and Turnover Numbers of Various Heart
and Muscle Type Lactic Dehydrogenases^a

<u>Species</u>	Turnover Numbers ^b	
	<u>H₄</u>	<u>M₄</u>
Beef	49,400	80,200
Chicken	45,500	93,400
Frog	-	86,000
Dogfish	-	109,000
Rabbit	41,500	-
Haddock		146,000
Tuna		165,000
Halibut		153,000

^a Data mainly from A. Pesce (31,32). ^b Represents moles DPNH oxidized per mole of enzyme per minute at 25°, pH 7.5, with pyruvate at V_{max}.

TABLE XXVIII

Ratio of Activity of $\frac{45^\circ}{15^\circ}$ of Different Lactic Dehydrogenases at
 Low Pyruvate Concentration (2×10^{-4} M)^a

Chicken H	4.6	Frog M	0.97
Beef H	3.8	Sturgeon M	1.10
Chicken M	1.7	Dogfish M	0.80
Turkey M	1.7	Lamprey M	0.80
Duck M	1.9	Halibut M	0.64
Beef M	2.0	Lobster	0.45

^a N. O. Kaplan and S. White, unpublished experiments.

Figure Legends

Fig. 1. Structure of 3-acetylpyridine and nicotinamide.

Fig. 2. Effects of addition of DPNH and ethanol on inactivation of HL-ADH by iodoacetate. Control contained only 10^{-4} M iodoacetate; DPNH added at 10^{-4} M.

Fig. 3. Effect of DPNH + AcPyDPNH on p-mercuribenzoate binding of beef H LDH (from Di Sabato and Kaplan (11)).

Fig. 4. p-Mercuribenzoate binding of active site -SH groups in various LDH's. Data of T. P. Fondy and J. Everse.

Fig. 5. Active site peptides of various LDH's.

^a Presence or absence of amide not yet established.

^b From Harris (17).

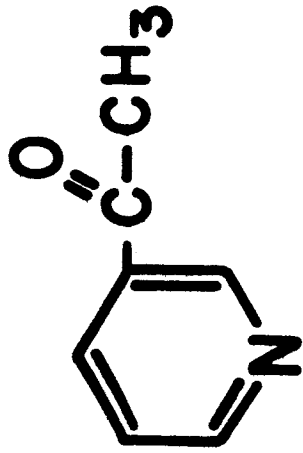
^c From Perham and Harris (30).

Fig. 6. Divergence of three evolutionary lines.

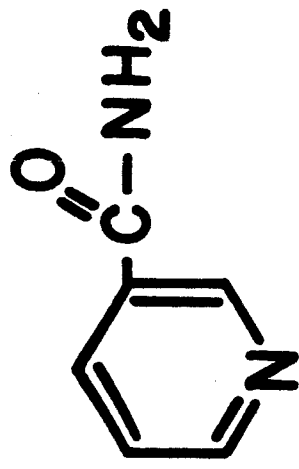
Fig. 7. Evolution of vertebrate LDH.

Fig. 8. Effect of pyruvate concentration on rate of beef M and chicken H LDH's (22).

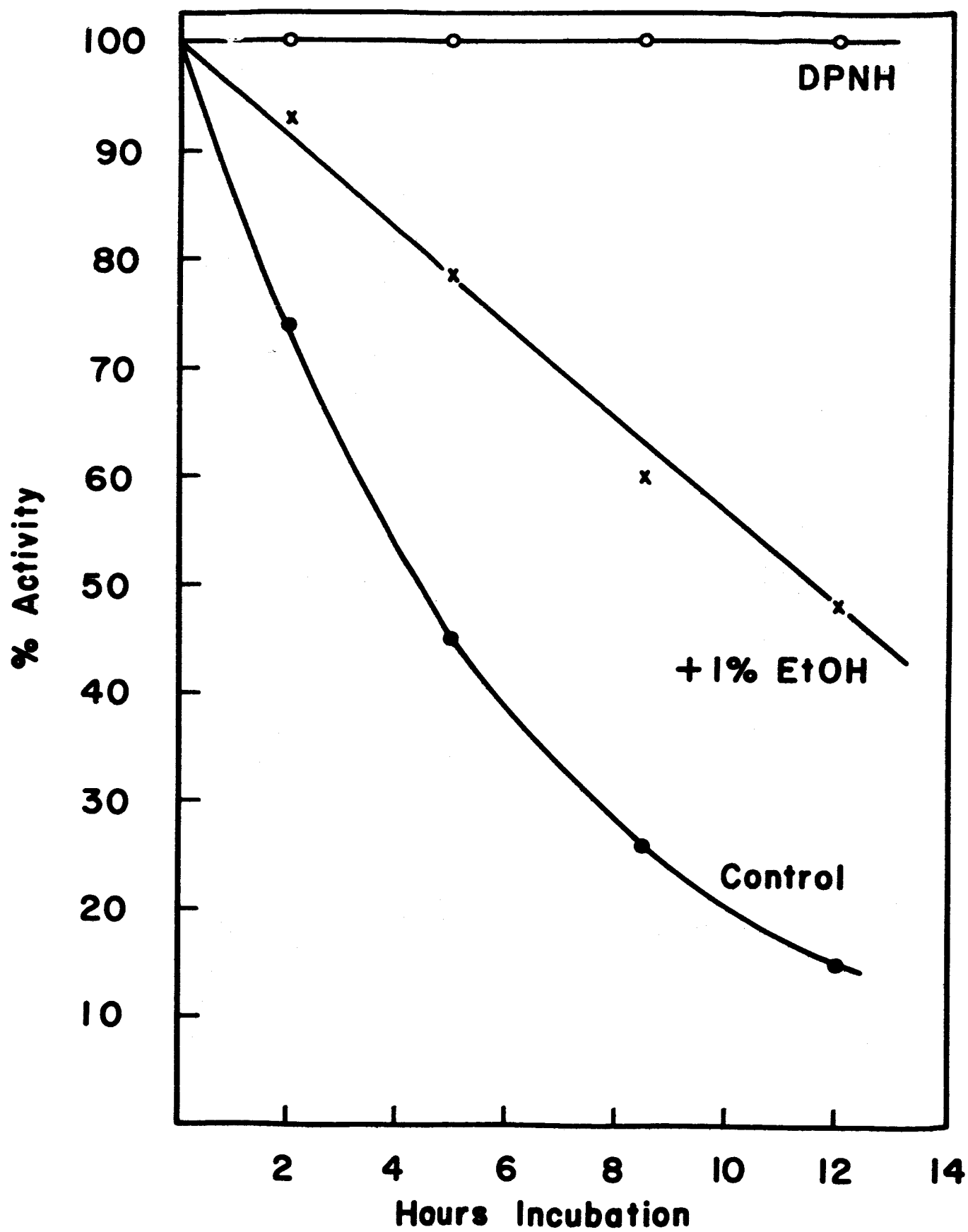
Fig. 9. Substrate inhibition of various mammalian M LDH's (M. M. Ciotti and N. O. Kaplan, unpublished results).

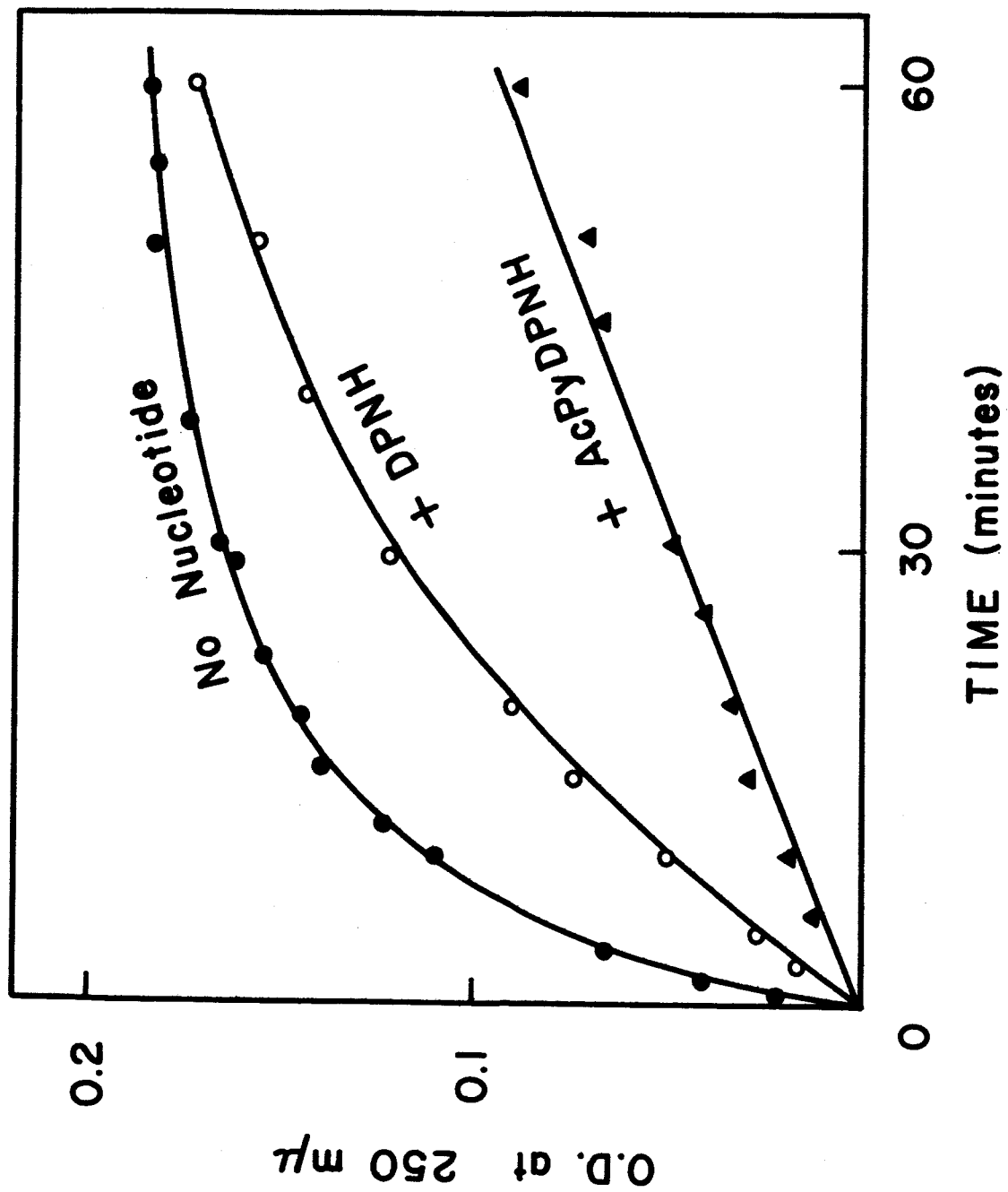


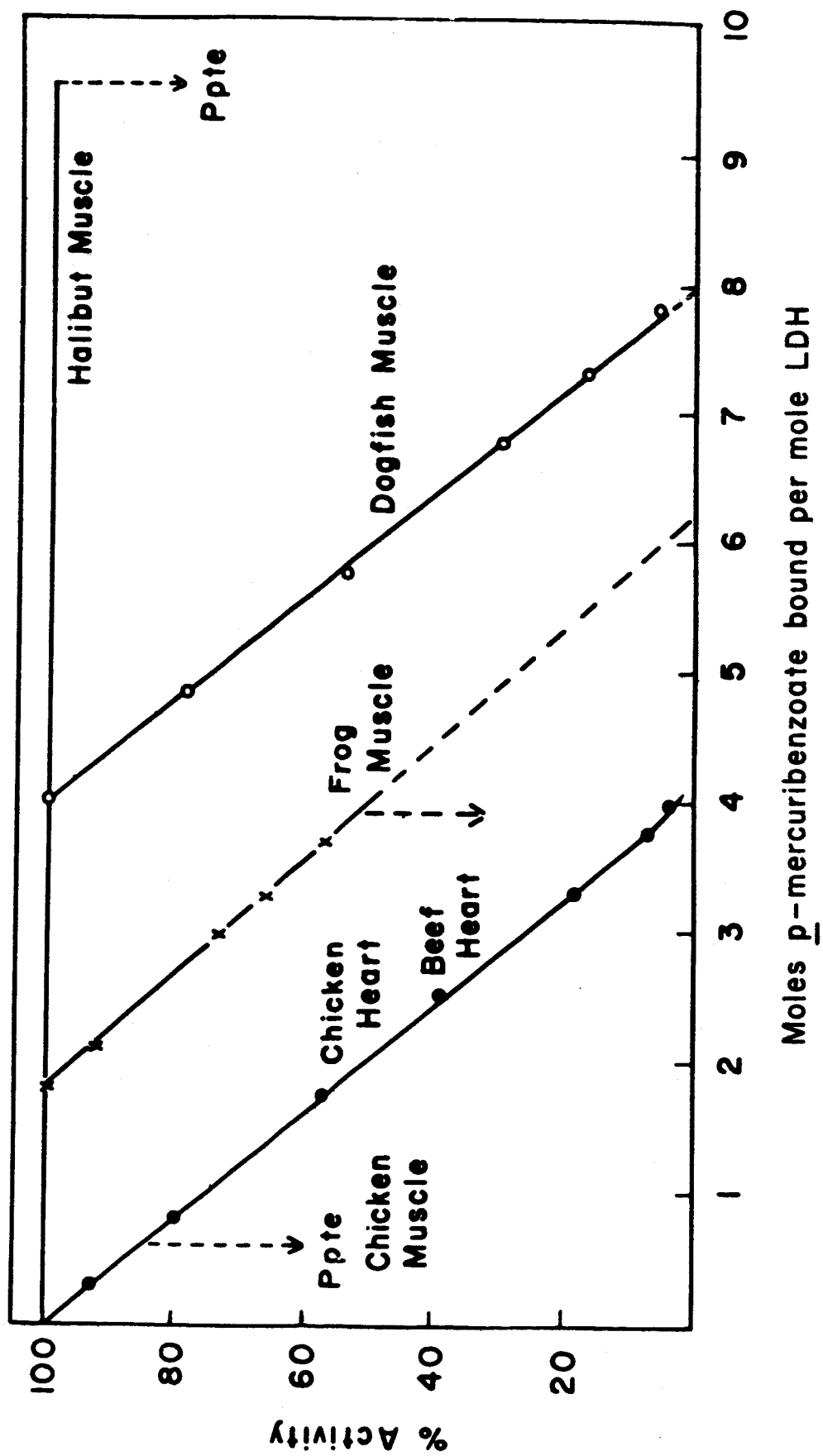
3-ACETYL PYRIDINE



NICOTINAMIDE







LDH : - Val - Ileu - Ser - Gly -(Gly, CMCys) - Asn - Leu - Asp^a - Thr - Ala - Arg -
 HL-ADH^b: - Val - Ala - Thr - Gly -Ileu -CMCys - Arg - Ser - Asp - Asp - His - Val -
 Y-ADH^c: - Tyr - Ser - Gly - Val -CMCys - His - Thr - Asp - Leu - His - Ala -
 TPD : - Val - Ser - Asn - Ala - Ser -CMCys - Thr - Thr - Asn - Cys - Leu - Ala -

Fig. 5

Chicken

Sturgeon

Halibut

Common Ancestor

- 0

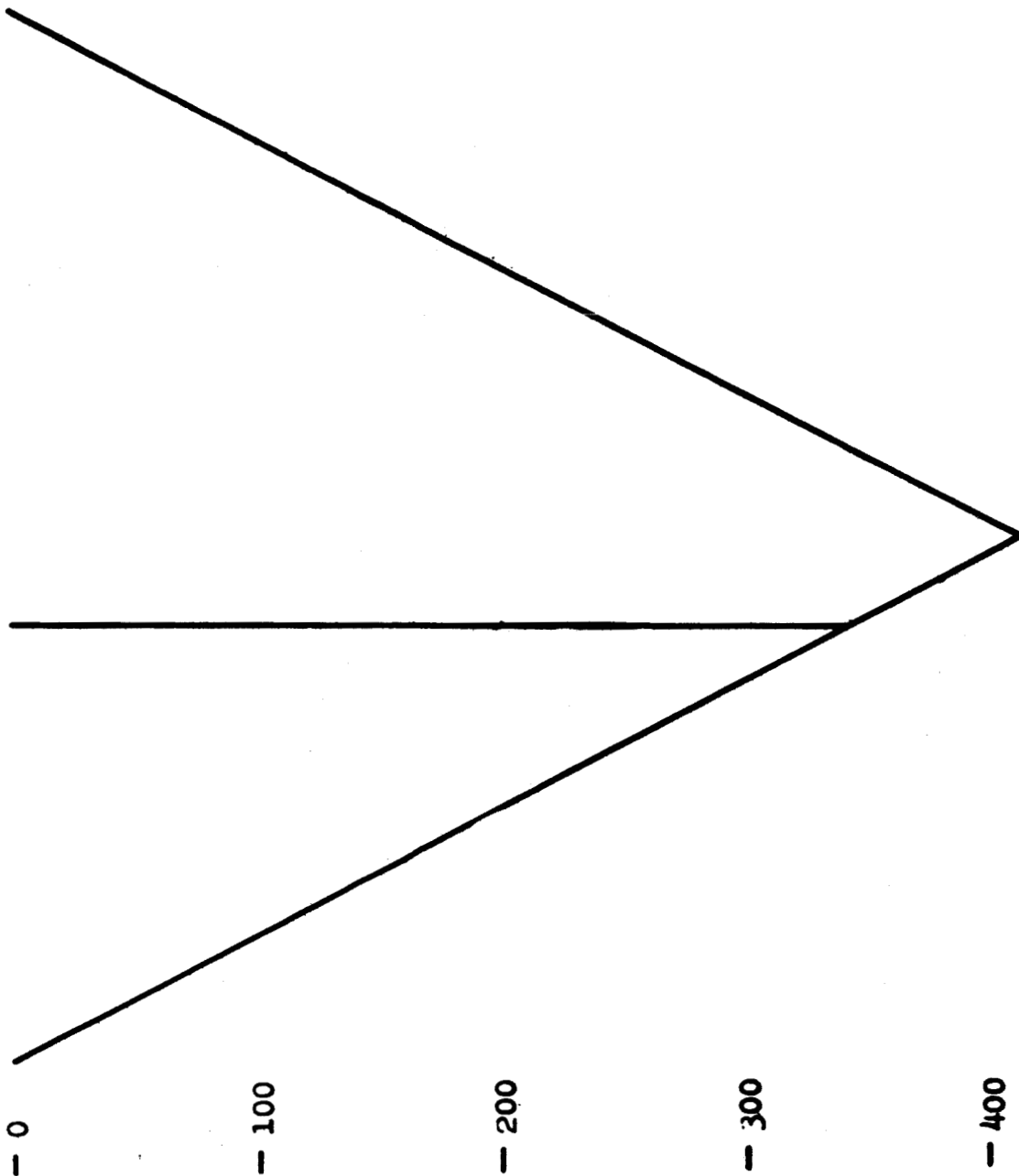
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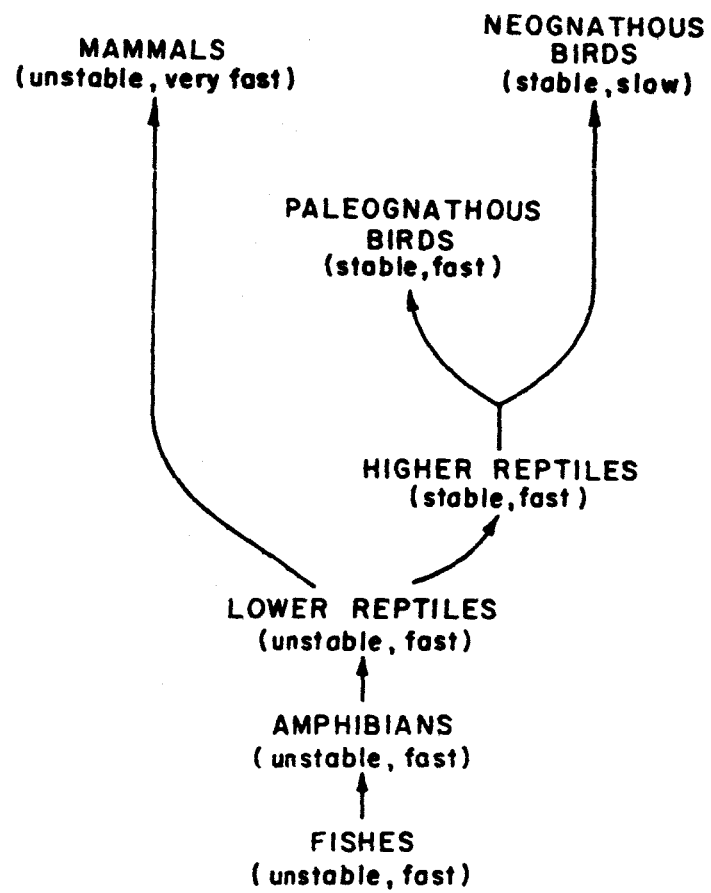
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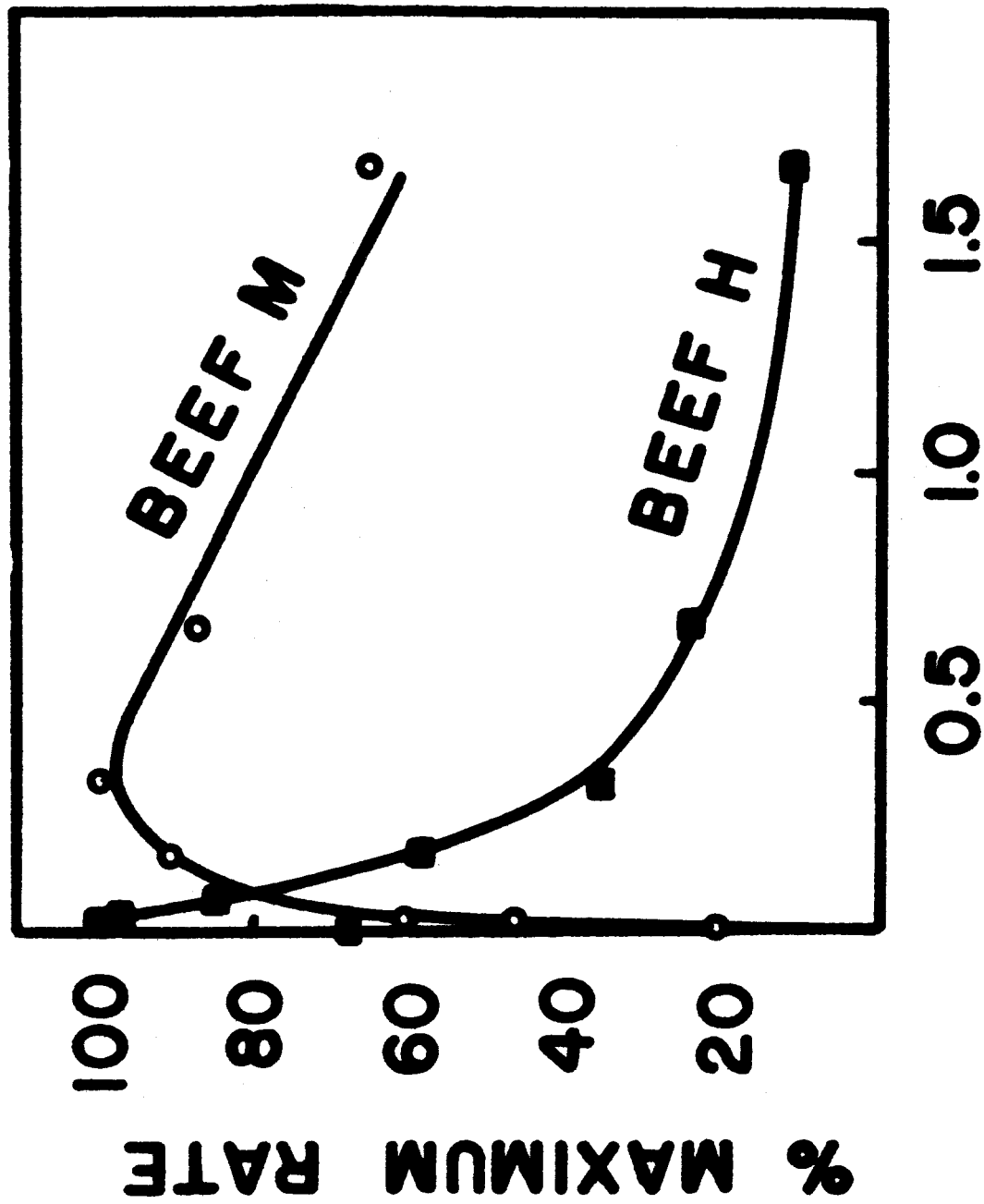
- 300

- 400

Millions of Years







MOLARITY OF PYRUVATE (x10²)

