Glyceraldehyde-3-Phosphate Dehydrogenase Variants in Phyletically Diverse Organisms

Abstract. *Electrophoretically distinct forms of glyceraldehyde-3-phosphate dehydrogenase (TDH) have been detected in turtle, perch, trout, spinach, and yeast. Multiple forms were not detected in rat, rabbit, chicken, frog, honey bee,* Euglena, *or* Escherichia coli. *The combination of two different subunits into tetramers is a probable explanation for the five-membered sets usually detected in extracts exhibiting TDH multiplicity.*

Many enzymes have been shown to exist in multiple molecular forms. Of the glycolytic sequence, distinct molecular variants of hexokinase (*1*), phosphoglucomutase (*2*), aldolase (*3*), enolase (*4*), pyruvate kinase (*5*), and lactic dehydrogenase (*6*) have been described. In this paper we report the presence of electrophoretically distinct forms of glyceraldehyde-3-PO_4 dehydrogenase (TDH) in several vertebrates as well as in yeast and spinach. Five-membered TDH sets were usually apparent in extracts exhibiting TDH multiplicity. By analogy with other systems [for example, aldolase (*3*) and lactic dehydrogenase (*6*)], at least some of the TDH variants detected in the present studies probably result from the formation within cells of different TDH subunits which then combine to produce multiple tetrameric molecules (two different subunits can form five different tetramers, three of which are hybrid molecules). In vertebrates in which multiple forms of TDH were detected, there was considerable variation in the profile of TDH variants in homologous organs; thus, there was no evidence in these studies of an organ-specific requirement for a specific TDH variant or group of variants.

Zone electrophoresis on cellulose acetate strips, using a specific TDH activity stain (see Fig. 1 legend for details), was employed to detect TDH multiplicity. As shown in Fig. 1, single bands of TDH activity were observed in all rat, rabbit, chicken, and frog (*Rana pipiens*) tissues tested. Slight differences in TDH mobility between species were apparent, but no differences in TDH mobility in tissues of the same species were distinguishable.

In an attempt to detect the presence of additional TDH forms, several tissue extracts from a given species (rabbit, chicken) were subjected to electropho-

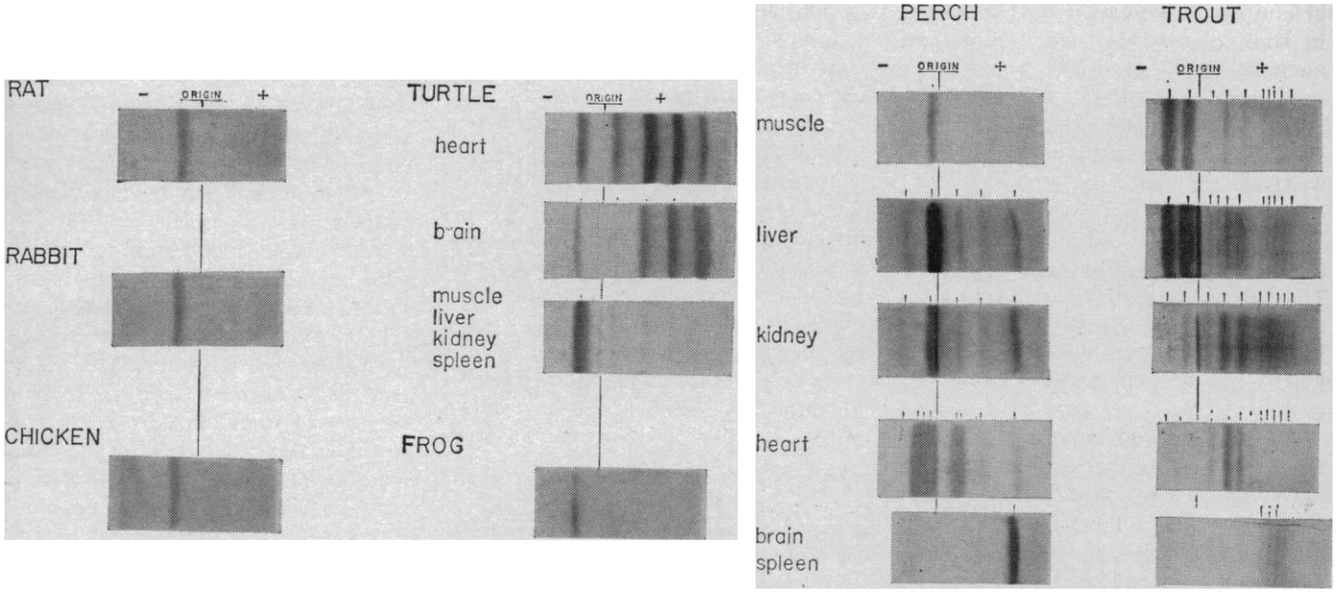

Fig. 1 (left). TISSUE DISTRIBUTION OF TDH IN RAT, RABBIT, CHICKEN, TURTLE, AND FROG. Rat, rabbit, chicken, turtle (*Chrysemys picta*), and frog (*Rana pipiens*) were obtained from commercial sources. Tissues were homogenized, 1 g/ml (approximately), in either 0.01*M* tris, 0.001*M* EDTA, 0.07 percent β-mercaptoethanol (BME) (*p*H 7.5), or 0.05*M* veronal, 0.07 percent BME (*p*H 8.6), and then centrifuged at 100,000*g* for 45 minutes. The resulting supernatants were used for assay. Three to five microliters containing 0.003 to 0.03 unit of TDH activity (*13*) were subjected to electrophoresis in 0.05*M* veronal buffer, 0.07 percent BME (*p*H 8.6), or other buffers as indicated in the text. Electrophoresis was routinely carried out on cellulose polyacetate strips (Gelman Sepraphore III) at 4°C, 250 volts for 90 minutes. Afterwards, the strips were placed on 0.5 percent agar gel prepared in 0.05*M* tris (*p*H 8.3 at 25°C) containing sodium arsenate (0.01*M*), crystalline rabbit muscle aldolase [0.1 mg/ml, specific activity 12 units/mg of protein (*3*)], fructose diphosphate (0.01*M*), phenazine methosulfate (0.024 mg/ml) and *p*-nitroblue tetrazolium (0.4 mg/ml). On incubation at 37°C, the areas containing TDH were recognized by the deep purple bands formed from reduction of the tetrazolium dye. With this system, prolonged incubation (longer than 15 minutes) of strips containing extracts of liver, kidney, and yeast resulted in the formation of an activity band which preliminary experiments indicated was caused by alcohol dehydrogenase. Consequently, for these extracts incubation of the strips with and without fructose diphosphate was carried out so that the bands produced by TDH could be positively identified. The electrophoretic pattern of skeletal muscle from rat, rabbit, chicken, and frog are shown. Brain, spleen, liver, kidney, and heart patterns were indistinguishable from those of muscle. Heart, brain, and muscle patterns are shown for turtle. Spleen, liver, and kidney patterns were indistinguishable from muscle. With higher concentrations, a faint cathodal penultimate band did appear in turtle brain as indicated in the figure. Fig. 2 (right). TISSUE DISTRIBUTION OF TDH IN PERCH AND TROUT. Tissue extracts of rainbow trout (*Salmo gairdneri*) (*17*) and perch (*Embiotoca lateralia*) (*18*) were prepared and subjected to electrophoresis as described in the legend of Fig. 1. Typical electrophoretic profiles of TDH activity for skeletal muscle, liver, kidney, heart, and brain of each species are presented. Spleen patterns were indistinguishable from those of brain. When higher concentrations of trout muscle and heart preparations were assayed, additional bands appeared, as indicated in the patterns.

resis (both separately and mixed), in a number of other buffer systems (*p*H 6.5 to 10.5, at 4° and 37°C, with and without diphosphopyridine nucleotide) (*7*). Although the mobilities of the TDH activity varied widely in the different buffer systems, no additional bands were resolved in mixed extracts. Under a given set of conditions, a single TDH band with the same electrophoretic mobility was observed in all the tissues tested from a given organism. Of course, these negative observations do not eliminate the possibility that multiple forms of TDH exist in these species. Marian Kochman, in this laboratory, has crystallized rabbit brain TDH. This enzyme appears to be identical to rabbit muscle TDH. Papadopoulos and Velick (*8*), on the other hand, have recently reported that crystalline rabbit liver TDH differs from crystalline rabbit muscle TDH in both kinetic properties and in electrophoretic mobility. The isolated proteins therefore must be distinct. We have been unable to resolve these two TDH activities in fresh rabbit tissue extracts by the methods employed.

In contrast with the above observations, five regularly ordered bands of TDH activity (a five-membered set) were clearly demonstrable in extracts of heart and brain of turtle (*Chrysemys picta*) (Fig. 1). Other turtle tissues exhibited a prominent band of TDH activity with an electrophoretic mobility corresponding to that of the most cathodic band; other bands of the five-membered set were weakly detected.

A more complex TDH activity profile was observed in various tissues of the perch (*Embiotoca lateralia*) (Fig. 2). Five-membered sets of TDH activity were detected in perch liver and kidney. The activity of the penultimate cathodic band was especially predominant. Unexpectedly, the mobility of the single muscle TDH did not coincide with that of the terminal cathodic band, but rather with that of the intense penultimate cathodic band of liver and kidney. It seems possible, therefore, that the muscle TDH activity and a portion of the major band of activity in liver and kidney may be contributed by a distinct TDH which has a mobility similar to this penultimate band of the set. The mobility of the single TDH band of brain and spleen coincides with that of the most anodic band of the set. Perch heart exhibits a more complex pattern. Bands of TDH activity corresponding to those of the five-membered set in kidney and liver are present, but several others are also apparent. On prolonged electrophoresis (180 minutes), eleven bands are discernible.

The electrophoretic profiles of TDH activity in trout (*Salmo gairdneri*) tissues were more complex than those described for perch. A strongly anodic five-membered set of TDH activity was observed in brain and spleen and at lower relative concentrations in all other tissues assayed. An additional five-membered set was detected in extracts of muscle, kidney, liver, and heart. The latter two tissues also exhibited additional bands. The first two bands of the five-membered set in muscle and liver tissue are particularly prominent. The observed multiplicity apparently is not the result of an analysis of a hybrid between two trout populations, since four-generation inbred rainbow trout, wild steelhead, and a hybrid between two different rainbow trout populations gave indistinguishable TDH profiles. These trout all belong to the species *S. gairdneri*.

As shown in Fig. 3, a single TDH band was detected in the head, thorax, and abdominal regions of the honey bee (*Apis mellifera*), in *Escherichia coli*, and in *Euglena*. Five activity bands were detected in extracts of baker's yeast (*Saccharomyces cerevisiae*). Krebs, in 1953, reported the resolution by moving boundary electrophoresis of four peaks with TDH activity in yeast (*9*). More recently Davis, in Krebs's laboratory, has chromatographically resolved several peaks of TDH activity from baker's yeast extracts (*10*). Krebs and associates have raised the question whether the multiple TDH activities are present in the yeast cell or are produced during the isolation procedure. Our results demonstrating the presence of a five-membered TDH set in fresh extracts suggest that multiple forms of TDH are present within the yeast cell.

Five bands of TDH activity were also observed in extracts of spinach leaves (Fig. 3). Three different TDH's [diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN), or both, being used as coenzyme] have been reported in plant systems (*11*). In the present studies, however, the same patterns were obtained when ei-

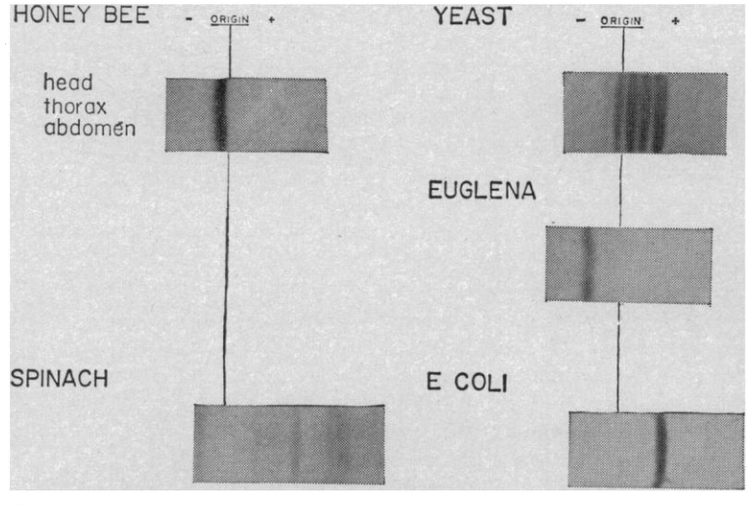

Fig. 3. Distribution of TDH in honey bee, spinach, yeast, *Euglena*, and *E. coli*. Honey bees (*Apis mellifera*) were dissected and extracts prepared in the same manner as vertebrate tissues (Fig. 1 legend). Chopped fresh spinach leaves were washed in cold water. A small amount (1 ml/10 g) of 0.05*M* veronal buffer, 0.001*M* EDTA, 0.1 percent BME (*p*H 8.6) was added, followed by grinding with sand (previously washed in 6*N* HCl and then in 0.05*M* EDTA). The resulting brei was sonicated at 15-second intervals for 1 minute with a Branson Sonifier (9 amp setting) followed by centrifugation at 100,000*g* for 45 minutes. The supernatant was subjected to electrophoresis as described in Fig. 1. *Euglena gracilis* "Z" was grown as outlined by Groves (*19*). The cells were harvested in log phase and isolated by centrifugation at 1465*g* for 5 minutes. The cells were then washed (three times) in cold distilled water and isolated by centrifugation at 1465*g* for 5 minutes. The final pellet was sonicated as described above in 0.1*M* tris, 0.1 percent BME (*p*H 7.5) and centrifuged at 100,000*g* for 45 minutes; then the supernatant was assayed. *Escherichia coli* strain 3000 was grown by R. Weiss by the method of Rushizky *et al.* (*20*) and isolated in log phase. Extracts of *E. coli* were prepared for electrophoresis as described for *Euglena*. Fleischmann's cake yeast (*Saccharomyces cereviseae*) (procured from Standard Brands) was suspended in distilled water (1 : 1 wt/vol) and ground with glass beads (120 μ) in a colloid mill (Gifford-Wood). After centrifugation at 100,000*g*, the supernatant was electrophoresed.

ther DPN or TPN was present in the assay system. The five TDH activities detected did not exhibit the ordered electrophoretic mobilities characteristic of a five-membered set. From the above it seems probable that at least some of the TDH bands detected may have distinct cell localization or metabolic functions, or both.

The present experiments clearly demonstrate the presence of multiple forms of TDH in extracts of certain tissues of the turtle, perch, and trout, as well as in spinach and yeast. With the exception of spinach, all of the organisms exhibiting multiplicity contain at least one five-membered TDH set. Such five-membered sets probably result from the random combination of two homologous, yet distinct, subunits into tetrameric molecules. It is now known that rabbit muscle TDH is a tetrameric molecule composed of identical subunits (*12*). Moreover, the extensive comparative studies of Allison and Kaplan (*13*), as well as the studies of Perham and Harris (*14*), suggest that the TDH molecules from widely divergent sources are closely homologous; hence, it can be inferred that the TDH variants detected here are tetramers. The five-membered sets of lactic dehydrogenase (*15*) and of aldolase (*3*) are the result of random formation of tetramers from subunits which have different amino acid sequences and which are, therefore, coded by separate genes. By analogy, it appears likely that some of the multiple forms detected in the present study will have a similar explanation. Indeed, all of the TDH variants detected could be the result of the synthesis of different TDH subunits, some of which could interact to form hybrids. However, other possibilities for the formation of different subunit types should also be considered. For example, the same polypeptide chain may exist in two or several conformations (*16*); alternatively, a single polypeptide chain may be modified by degradation or by derivative formation. We think it is unlikely that there are separate genes for every TDH variant detected.

The profile of TDH variants in perch and trout tissues are also of interest from a physiological point of view. Although the tissues of both organisms exhibited a characteristic pattern, there is no consistent relationship between the profiles of the same tissues from the two species. Thus, there is no evidence from these studies of an organ-specific distribution and, hence, no apparent specific requirement by an organ for a given variant or set of variants. It will be of considerable interest to determine whether the kinetic properties of the various TDH variants are modulated in accordance with the metabolic activities of the various tissues.

Because of the multiple forms of TDH found in some organisms, and the apparent absence of variants in others, this enzyme may be a useful phylogenetic and physiological probe.

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References and Notes

1. L. Grossbard and R. T. Schimke, *J. Biol. Chem.* **241**, 3546 (1966); H. M. Katzen and R. T. Schimke, *Proc. Nat. Acad. Sci. U.S.* **54**, 1218 (1965); H. M. Katzen, D. D. Soderman, H. M. Nitowsky, *Biochem. Biophys. Res. Commun.* **19**, 377 (1965); C. González, T. Ureta, R. Sánchez, H. Niemeyer, *ibid.* **16**, 347 (1964); C. Sharma, R. Manjeshwar, S. Weinhouse, *J. Biol. Chem.* **238**, 3840 (1963); F. J. Ballard and I. T. Oliver, *Biochem. J.* **90**, 261 (1964); D. G. Walker, *Biochim. Biophys. Acta* **77**, 209 (1963).
2. J. C. Joshi, J. Hooper, T. Kuwaki, T. Sakurada, J. R. Swanson, P. Handler, *Federation Proc.* **26**, 557 (1967).
3. E. Penhoet, T. Rajkumar, W. J. Rutter, *Proc. Nat. Acad. Sci. U.S.* **56**, 1275 (1966); W. J. Rutter, T. Rajkumar, E. Penhoet, M. Kochman, R. Valentine, *Ann. N.Y. Acad. Sci.*, in press.
4. R. P. Cory and F. Wold, *Biochemistry* **5**, 3131 (1966); H. Tsuyuki and F. Wold, *Science* **146**, 535 (1964).
5. T. Tanaka, Y. Harano, H. Morimura, R. Mori, *Biochem. Biophys. Res. Commun.* **21**, 55 (1965); W. Susor, unpublished observation.
6. C. L. Markert and F. Møller, *Proc. Nat. Acad. Sci. U.S.* **45**, 753 (1959); Th. Wieland and G. Pfleiderer, *Ann. N.Y. Acad. Sci.* **94**, 691 (1961); F. Wróblewski and K. F. Gregory, *ibid.* **94**, 912 (1961); J. M. Allen, *ibid.* **94**, 937 (1961); N. O. Kaplan *et al.*, *Science* **131**, 392 (1960).
7. Namely, 0.05*M* imidazole, *p*H 6.5 (90 minutes, 4°C); 0.02*M* tris, *p*H 8.0 (150 minutes, 4°C); 0.02*M* glycine, 0.001*M* ethylenediaminetetraacetate (EDTA), *p*H 10.5 (150 minutes, 4°C); 0.02*M* glycine, 0.001*M* DPN, *p*H 10.5 (150 minutes, 4°C); 0.02*M* phosphate, *p*H 7.5 (90 minutes, 4°C); 0.05*M* veronal, *p*H 8.6 (150 minutes, 37°C); 0.02*M* and 0.05*M* tris, *p*H 8.0 (150 minutes, 37°C). We included 0.07 percent β-mercaptoethanol in each buffer. Tissues were always homogenized in the appropriate buffer; in some cases crystalline horse liver alcohol dehydrogenase was added as a standard for determination of relative mobilities.
8. C. S. Papadopoulos and S. F. Velick, *Federation Proc.* **26**, 557 (1967).
9. E. G. Krebs, *J. Biol. Chem.* **200**, 471 (1953).
10. C. H. Davis, thesis, University of Washington (1962).
11. L. L. Rosenberg and D. I. Arnon, *J. Biol. Chem.* **217**, 361 1955); R. C. Fuller and M. Gibbs, *J. Plant Physiol.* **34**, 324 (1959); R. H. Hageman and D. I. Arnon, *Arch. Biochem. Biophys.* **57**, 421 (1955).
12. J. I. Harris and R. N. Perham, *J. Mol. Biol.* **13**, 876 (1965); H. C. Watson and L. J. Banaszak, *Nature* **204**, 918 (1964); W. F. Harrington and G. M. Karr, *J. Mol. Biol.* **13**, 885 (1965).
13. W. S. Allison and N. O. Kaplan, *J. Biol. Chem.* **239**, 2140 (1964). Enzyme units were expressed as micromoles of DPN reduced per minute, per milliliter of extract.
14. R. N. Perham and J. I. Harris, *J. Mol. Biol.* **7**, 316 (1963).
15. C. L. Markert, *Science* **140**, 1329 (1963).
16. G. B. Kitto, P. M. Wassarman, N. O. Kaplan, *Proc. Nat. Acad. Sci. U.S.* **56**, 578 (1966).
17. Obtained through the courtesy of Dr. Lauren Donaldson, School of Fisheries, University of Washington, and Dr. G. Klontz, Western Fish Disease Lab, Seattle.
18. Obtained through the courtesy of the Seattle Public Aquarium.
19. W. E. Groves, thesis, University of Illinois (1962).
20. G. W. Rushizky, A. E. Greco, D. L. Rogerson Jr., *Biochim. Biophys. Acta* **108**, 142 (1965).
21. Supported in part by PHS grant HD-02126.

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Heterophile Reactive Antigen in Infectious Mononucleosis

Abstract. *Specific heterophile reactive antigen has been localized by means of indirect immunofluorescence in 12 of 13 kidney biopsy specimens obtained during the acute phase of infectious mononucleosis. I feel that this may represent the identification of infectious agent antigen. Evidence is also presented for the possible existence of two different strains of the agent of infectious mononucleosis.*

The etiologic agent of infectious mononucleosis has long eluded detection. Its epidemiology suggests that an infectious agent is responsible and the general suspicion is that the agent is a virus. A rise in titer of heterophile antibody with specificity for beef red blood cells is a curious and specific laboratory finding during the early stages of infectious mononucleosis. Because many viruses have the ability to grow well in tissue cultures of kidney cells (*1*), it appeared feasible to use specific heterophile antibody to search for antigen of the infectious agent in kidney tissue of patients during the acute phase of the illness.

Thirteen patients with typical clinical symptoms and signs of infectious mononucleosis and high serum heterophile titers specific for the antigen of beef red blood cells were investigated. Throat cultures were taken initially and every other day to insure the absence of streptococci. Antistreptolysin O and antistreptococcal F (*2*) titers were also determined initially and at weekly intervals (see Table 1). Urinalyses and urine cultures were made initially and at weekly intervals to help rule out the possibility of any bacterial infections of the genitourinary tract. Double needle biopsies (*3*) of the kidneys were performed on all 13 pa-