LACTIC DEHYDROGENASE ISOZYME VARIATIONS
IN SELECTED MAMMALS

by

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STATEMENT BY AUTHOR

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INTRODUCTION

In 1959, Markert and Moller demonstrated that the enzyme lactic dehydrogenase could be separated by gel electrophoresis into five distinct forms, all of which retained the original enzymatic activity. These separable entities were named isozymes, the term now being applied to all enzymes that have multi-molecular forms. The lactic dehydrogenase (LDH) isozymes in particular are widespread in nature, being found in both vertebrates and invertebrates with little difference in the overall size of the basic molecule. Since 1959 most of the work on the LDH isozymes has attempted to explain the biochemical differences between these forms. By using preparative electrophoresis the individual isozymes have been separated and purified. The comparisons of the separated proteins by amino acid analyses, immunological specificities, and reaction rates with coenzyme analogues have shown the isozymes to be real entities and not artifacts incurred in the separation procedure. These analytical methods also provided enough insight to allow for the development of a phylogenetic relationship between the major classes of animals based on the evolutionary changes in the LDH molecule (Wilson et al. 1964), much like that done with hemoglobin and cytochrome c.

The consistent finding of five LDH isozymes in all mammals investigated so far has been explained by the subunit character of the LDH molecule. The molecule consists of four subunits which combine at random; the subunits, however, are of only two different types. Each different subunit, indicated by the letters M and H, is thought to be the product
of a single gene. The possible combinations of these two subunits into groups of four yield five entities: \( \text{HHHH} \), \( \text{HHHM} \), \( \text{HHMM} \), \( \text{HMMM} \), and \( \text{MMMM} \). Hereafter each of these groups, representing a LDH isozyme, will be referred to as \( \text{H}_4\text{-LDH} \), \( \text{H}_3\text{M}_1\text{-LDH} \), \( \text{H}_2\text{M}_2\text{-LDH} \), \( \text{H}_1\text{M}_3\text{-LDH} \), and \( \text{M}_4\text{-LDH} \). The two genes are not equally effective in all tissue, resulting in different amounts of the individual isozymes appearing in the gel patterns obtained by electrophoretic studies of respective tissues. Any one isozyme from a tissue, however, is identical to the corresponding isozyme found in another tissue from the same animal, even though the amounts of this particular isozyme may be quite different in the two tissues. There appears to be no preferential formation of any one isozyme over another. Markert (1963) demonstrated this by freezing purified \( \text{H}_4 \) and \( \text{M}_4 \) LDH's in 1M NaCl so that the molecules separated into their individual subunits. He allowed known ratios of H and M subunits to combine, resulting in isozyme amounts in the gel patterns identical to those predicted by random association of the subunits.

The significance of the different distribution of LDH isozymes in the organs of an individual animal remained unknown until Cahn et al. (1962) noted the difference in pyruvate inhibition among the five forms, the differences being greatest between \( \text{H}_4\text{-LDH} \) and \( \text{M}_4\text{-LDH} \). The heart requires a steady supply of energy which is maintained by the complete oxidation of pyruvate and lactate. As the heart of all animals contains mainly \( \text{H}_4\text{-LDH} \), which is inhibited by high pyruvate concentrations, the entrance of the products of anaerobic glycolysis into the more efficient Krebs cycle is favored. On the other hand, \( \text{M}_4\text{-LDH} \) still functions to catalyze the conversion of pyruvate to lactate at high pyruvate concentrations, and is found
in greatest quantities in white muscle. This muscle, in contrast to the
ever-active heart muscle, is characterized by sporadic activity, easy
fatigue, and high production of lactic acid when the energy demand exceeds
the oxygen supply. The other isozymes of mixed subunits are intermediate
in their properties. Further physiological relationships to the isozyme
patterns have been noticed (Dawson, Goodfriend, and Kaplan 1964) such as
in the kidney where the more anoxic medullary portions have more M-LDH as
compared to the cortical portions having more of the H-type. That the
skeletal muscle LDH isozyme patterns could shift in response to selection
pressures and correlate with the activity of the muscle was shown using
avian flight muscle (Wilson, Cahn, and Kaplan 1963). These studies dem-
onstrated that those species which were the strongest flyers had more
$H_4$-LDH as compared to the weak flyers which either have all five isozymes
in equal amounts or a preponderance of $H_1M_3$-LDH and $M_4$-LDH.

It was the purpose of this study to further investigate the
isozymes present in situations in which strenuous or unusual metabolic
demands are placed upon an animal. The Order Chiroptera, consisting of
the only truly flying mammals, is thought to be one of the oldest mammalian
orders. Fossil records indicate that bats much like those of today were
flying in mid-Eocene times, over 50 million years ago, if not earlier
(Simpson 1945). During the long course of their evolution radial adapta-
tions have resulted in pronounced differences in food habits, flight habits,
and wing structure. In the present study twelve species of bats including
representatives of the families Phyllostomatidae, Vespertilionidae and
Molossidae were obtained. Although these three families do not show the
extreme variations in flight capabilities seen in birds, they nevertheless
show significant differences in their flight habits. For example, because of ground-based feeding habits the phyllostomatid *Macrotus californicus* does not appear to engage in strenuous flight. The *Macrotus* wing is short and wide, giving it the maneuverability necessary for foraging in the low-lying vegetation. A far different situation exists in the molossid *Taderida braziliensis*, which shows migratory behavior and has long slender wings. This type of wing is not suited for maneuverability at close quarters since it has a higher stalling speed (failure to maintain lift), but by reducing the inefficient drag forces it allows for speed and strong flying ability. The family Vespertilionidae is a diversified group that contains species with varying flight habits. The larger species of this group appear to be stronger flyers than the smaller vespertilionid bats, which often exhibit an erratic, fluttering type of flight. However, none of the vespertilionids used in this study, except *Lasiurus cinereus*, appears to cover any distances approaching that seen for *Taderida* migrations. The vespertilionid wing is intermediate in shape between that of *Macrotus* and *Taderida*.

Along with the observed differences in wing shapes and flight habits, one would expect both morphological and metabolic differences in the flight muscles of the various species. With this in mind, the isozyme patterns in the flight muscle of each of twelve species of bats were determined, and the results were related to what is known of the natural history of each species. Since *Macrotus californicus* and *Taderida braziliensis* showed the widest divergence in isozyme patterns, various histochemical procedures were used to differentiate the muscle fiber types found in the flight muscle of each of these species. In view of the extreme
specialization found for *Taderida* flight muscle, electron micrographs of this tissue were prepared to determine the size of the mitochondria and their relationships to the surrounding muscle fibers. Previous studies have indicated a relationship between mitochondrial size and the activity of a muscle. For a comparison with the flight muscle, the less active leg muscle from *Taderida* was included in the electron microscope study.

A second purpose of this study was to determine whether animals that hibernate have any differences in the isozyme patterns of various tissues as compared to the numerous patterns reported in the literature for the same tissues of non-hibernators. For some time there has been speculation as to the extent of the anaerobic capacities in the metabolism of hibernators. Recently Burlington and Wiebers (1965), using the ground squirrel *Citellus tridecemlineatus*, have shown that anaerobic glycolysis in the cerebrum of this hibernating animal increases under severe hypoxic conditions, whereas anaerobic glycolysis decreases under similar conditions in the cerebrum of a non-hibernator such as the laboratory rat. The extent of anaerobic glycolysis was measured by the production of lactic acid. Since the hibernator was able to produce more lactic acid from pyruvic acid than the non-hibernator (homeotherm), under the same conditions of oxygen lack, one might suspect a greater efficiency for this enzymatic conversion in the hibernator. The brain tissues of all adult homeothermic mammals examined so far show a greater amount of the LDH isozymes that contain a preponderance of H-type subunits (H₄-LDH and H₃-LDH). The hypothesis of the present study was that the nervous tissues (and perhaps other tissues) of hibernators would show the presence of greater amounts of M₄-LDH than occur in non-hibernators. The M₄-LDH, in
contrast to the $H^-\text{LDH}$, shows no substrate inhibition by pyruvic acid and is the best suited of all the isozymes to produce large quantities of lactate. Greater amounts of $M^-\text{LDH}$ in the nervous tissue of hibernators, then, could explain the greater capacity for lactic acid production observed by Burlington and Wiebers for these animals. To test this hypothesis, brain and other tissues from hibernators both in and out of hibernation were examined for their isozyme content. For this purpose, two species of bats were taken out of their natural hibernals and maintained in a state of hibernation until use. Also used for further comparisons were three species of hibernating rodents, a rock squirrel *Citellus variegatus*, a ground squirrel *Citellus teretecaudus*, and a pocket mouse *Perognathus baileyi*. Although the resultant isozyme patterns were compared only with the abundant patterns already published for non-hibernators, the round-tail ground squirrel *Citellus teretecaudus* used in this study afforded the opportunity for an interesting direct comparison. This hibernating animal often exists in close association with a near relative, the non-hibernating Harris ground squirrel *Citellus harrisi*. As the isozyme proteins of closely related forms have been shown to be very similar if not identical (Wilson et al 1964), a simultaneous determination of the isozyme patterns in the Harris and round-tail ground squirrels served to give a sensitive comparison of any possible differences in the isozyme amounts.

In addition, the relative rates of migration on the starch gels for the enzyme proteins of various species of bats were compared to see if the extent of amino acid differences (which affect the migration rate
under electrophoresis) had any consistent relationship to the taxonomic grouping of these species.
MATERIALS AND METHODS

Starch Gel Electrophoresis

The starch gel electrophoresis technique as described in Colowick and Kaplan (1963) was used, with some slight modifications. Gel electrophoresis was used in preference to other possible methods as it does not involve complicated procedures, yields reliable results, and has much better resolution than, for example, paper electrophoresis. Starch electrophoresis has proved to be a sufficiently gentle procedure for proteins so that the danger of denaturation effects are minimized. This is a great advantage in that by using a specific substrate the localization procedure on the gel can be coupled to the enzyme activity alone, and the site and amount of the enzyme is demonstrated even though there are numerous other proteins in the gel. Thus a crude homogenate can be applied to the gel without having to go through purification procedures for the enzymes. After the proteins have been separated by electrophoresis, localization of lactic dehydrogenase activity is accomplished by incubating the gel in a staining solution containing lactic acid, NAD, phenazine methosulfate, and the dye Nitro-blue tetrozolium. The enzyme and its coenzyme NAD remove two hydrogen atoms from each lactic acid molecule. An intermediary hydrogen-transferring agent, phenazine methosulfate, then transfers the hydrogen atoms to the Nitro-BT, which becomes colored upon being hydrogenated. This easily reduced dye yields discrete purple spots on the gel, whose size and color intensity are proportional to enzyme
activity. In the case of the lactic dehydrogenase isozymes, the $H_4$-LDH is fastest moving and migrates towards the anode, whereas the slower moving $M_4$-LDH is attracted to the cathodic end of the gel.

The trays which hold the starch gels are ten inches long; this distance allows for complete resolution of the individual isozymes. The trays can be made from one-quarter inch plexiglass (Figure 1). During electrophoresis the migrating proteins interact with the top and bottom surfaces of the gel, causing artifacts in the localization of the isozymes. These artifacts can be avoided by slicing thin horizontal strips off the top and bottom of each gel slab. This is accomplished most easily by making the gel trays so that a one-sixteenth inch thick strip of plexiglass can serve as a false bottom. After electrophoresis, a probe can be inserted in a hole provided in the bottom of the gel tray, raising the false bottom and the gel to allow for the insertion of another one-sixteenth inch thick plexiglass strip. This raises the top surface of the gel above the sides of the gel tray. A Weck hair stylist's razor blade, which is longer than the standard single-edged blade, is placed so that it straddles both sides of the tray. The blade is kept in contact with the sides of the tray and moved with an even motion so that the amount of gel protruding above the sides is sliced off. This procedure is again followed after more plexiglass strips are inserted under the false bottom so that only a one-sixteenth inch thick portion of the gel is left in the tray. The remaining middle section of gel is used for the enzyme localization. The wire-slicing method proposed by Colowick and Kaplan proved unsatisfactory since it appeared to tear rather than actually slice the gel. Plugging the hole provided for manipulating the
false bottom made the trays an ideal vessel for incubating the gels during the staining procedure.

A plexiglass box, divided into four compartments by three parallel plexiglass strips, served as an electrode apparatus and buffer tank (Figure 1). The electrode buffer consisted of 30 ml of 0.2 citric acid plus 240 ml of 0.2M Na₂HPO₄ and 1230 ml of distilled water at a pH of 7.0. The amounts of buffer in each compartment of the box are kept at the same level, and the inner and outer compartments are connected by strips of eight thicknesses of Whatman No. 1 filter paper. The outer compartments are connected to the gels by six thicknesses of filter paper cut to the width of the gel. The filter paper is soaked in buffer and pressed firmly to the end of the gel. Since undue heating of the gel due to the current flow should be avoided, the apparatus was used in a cold room at 4°C. Good separation of the isozymes was given by electrophoresis carried out for 10-14 hours at a constant current of 25 milliamps and a voltage of 10-14 volts/cm. of gel.

The starch gel itself is prepared by adding twenty-one grams of Connaught hydrolyzed starch to a 500 ml Pyrex vacuum filtration flask containing 150 ml of pH 7.0 buffer. The buffer consisted of 7.0 ml of 0.2M citric acid plus 43 ml of 0.2M Na₂HPO₄ and 950 ml of water. The contents of the flask are thoroughly mixed by swirling while heating over a Bunsen burner. As soon as boiling starts and the mixture changes in consistency and becomes translucent, the flask is connected to an aspirator and the contents are allowed to boil under reduced pressure for one minute in order to eliminate trapped air bubbles. The liquid starch suspension is then poured into the gel trays so that overfilling occurs. After the gel
sets, the overfilled part of the gel can be sliced off, giving it a uniform thickness. To prevent drying, each tray and gel is wrapped in Saran Wrap and stored in a cold room at least six hours before use. After electrophoresis and slicing of the gels, the Nitro-BT staining solution used by Colowick and Kaplan (1963) was used to localize the enzyme activity. The time of incubation of the gels at 37°C was determined by inspecting the amount of dye formed, usually taking from 20-45 minutes for adequate localization. The stained gels can be conveniently kept in two per cent formalin, using large diameter test tubes for storage.

**Preparation of Tissue Homogenates**

In order to avoid loss of enzyme activity, tissues used for determination of isozyme patterns were from freshly killed animals only. The tissues were dissected out into cold 0.9% NaCl, then washed and blotted several times to remove as much adherent blood as possible. The avoidance of adherent blood and the careful trimming of fat and connective tissue insures that these contaminating substances do not alter the subsequent isozyme determinations in the desired tissue under study. Since lactic dehydrogenase is a soluble cytoplasmic enzyme, nearly the full enzymatic activity present in the cell can be obtained by simply disrupting the cell structure. This was accomplished in a motor-driven all-glass homogenizer. The various tissues are homogenized in a ratio of one part by volume of tissue to two parts of 0.07M Tris buffer. This concentration of Tris buffer was used instead of distilled water since it appeared to insure the stability and subsequent localization of the more labile Mg-LDH isozyme (Vesell and Brody 1964). With this procedure a single piece of
No. 1 filter paper (5mm, square) soaked in the homogenate sufficed for adequate enzyme localization, except for brain and adipose tissue, which required two and four thicknesses, respectively. The squares of filter paper, having any excess fluid drawn off so that they exhibited only a slight sheen, were inserted by using the tip of a micro-spatula to make a vertical slice in the gel. Since the H$_6$-LDH isozyme moves fastest towards the anode, the insertions are placed in a line approximately one-third the length of the gel away from the cathodal end. This allows for maximum separation of the isozymes and insures that they do not migrate off the end of the gel during a maximum 14 hour electrophoresis run. The width of the gel trays (Figure 1) allows for two insertions per tray.

**Animals Used**

**Flight Muscle Studies**

The bats used for comparing the flight muscle LDH isozymes were the phyllostomatids *Macrotus californicus* and *Pteronodus*, the molossid *Taderida braziliensis mexicana*, and the vespertilionid bats *Myotis velifer*, *M. californicus*, *M. thysanodes*, *M. volans*, *Lasiurus cinereus*, *Eptesicus fuscus*, *Plecotus townsendi*, *Pippistrellus hesperus*, and *Antrozous pallidus*. Identifications were provided by Mr. Alfred Gardner, Mr. Robert Baker, and Dr. E. Lendel Cockrum, of the Department of Zoology, University of Arizona at Tucson. Representative specimens have been deposited in the mammology collection of that department and bear catalogue numbers 13402, 13403, 13404, 13405, and 13406. In most instances the isozyme content of the heart as well as that of the flight muscle of each species was determined for comparison.
Hibernation Studies

For the studies on hibernation, *Eptesicus fuscus* and *Plecotus townsendi* were taken in the month of January from a mine tunnel serving as a hibernal in the Patagonia Mountains, Santa Cruz County, Arizona. The animals were kept in the hibernating state before use, and homogenates of liver, kidney, pectoral (flight) muscle, rear leg muscle, heart and brown fat were tested for the pattern of LDH isozyme activity. Similarly, heart, rear leg muscle, liver, kidney, brown fat and brain tissues of a hibernating rock squirrel *Citellus variegatus* were tested. The cerebral cortex, cerebellum, and medulla of a second hibernating rock squirrel were dissected out and each area was tested separately to see if any one region of brain tissue changed its isozyme pattern significantly as compared to the generalized brain tissue patterns previously reported for numerous non-hibernators (Bonavita 1964). The rock squirrels had been maintained in the laboratory for a year but entered into hibernation 10-15 days after being placed in a cold room at 5°C, without food or water. One animal was used after 15 days, and the other after 30 days in hibernation. The animals were judged to be in true hibernation by a number of observations. They assumed the characteristic hibernating posture, being curled up in a tight ball with the tail wrapped around the head and body. While undisturbed in this position there was no observable respiratory movements. Only by moving the animal to a more favorable position for observation could the respiration rate be determined and this was usually three to four respiratory movements per minute. This is slightly higher than that reported for many hibernators, but handling the animals probably increased the minimal rate. The rectal temperature taken
at a depth of two inches gave the normal situation for hibernators, staying constant at 6-7°C, only a few degrees above ambient temperature. The animals responded with uncoordinated swimming type movements only when pinched fairly hard, and never opened their eyes. Lastly, there is the obvious fact of survival at 5°C for 45 days without food or water in the case of one of the animals. The animals did not appear to be emaciated upon autopsy.

Brain tissue was also used from the ground squirrel *Citellus tereticaudus* and the pocket mouse *Perognathus baileyi*, both capable of hibernation (Neal 1964; Bartholomew and Cade 1957), and from the non-hibernating *Citellus harrisi*. These three animals were not in hibernation before use; the main purpose was to compare closely related forms that differ in their ability to hibernate.

**Intra-species Comparisons of the Gel Migration Rates for the LDH Isozymes**

In comparing two proteins by their migration rates on starch gel, it is impossible to use any absolute values for the migration rates since the gels cannot be standardized, i.e., the separation properties vary for each gel made. Thus any comparison between two or more proteins must be made on the same gel. It was found that crude homogenates could be stored at 5°C, for several weeks and still retain sufficient activities for comparing rates of migration, even though there was a substantial loss of activity for the slower moving isozymes. Using the stored homogenates enabled a much wider range of comparisons, since it would be impossible to capture at one time all of the bat species used in this study. The
relative rates of migration of the isozymes were determined for all combinations of the species within each of the three groups: (1) *Macrotus*, *Pteronodus*, and *Leptonycteris*, (2) *Myotis velifer*, *M. thysanodes*, *M. velans*, and *M. californicus* and (3) *M. velifer*, *Antrozous pallidus*, *Eptesicus fuscus*, and *Lasiurus cinereus*. The species in group (1) are in the family Phyllostomatidae; the species in groups (2) and (3) are vespertilionid bats.

**Histochemical Techniques**

Flight muscles from *Tadarida* and *Macrotus* were frozen in liquid nitrogen, embedded on the microtome tissue carrier in a three per cent gelatin solution, and trimmed to the form of a rectangular block (Taylor 1964). In this way cryostat-cut serial sections could be obtained at both 10 micron and 6 micron thicknesses. Histochemical procedures were used that have demonstrated their ability to differentiate muscle cell types, namely, the tests for succinic dehydrogenase and lactic dehydrogenase (Nachmias 1958, Stein and Padykula 1962, Dubowitz and Pearse 1961, Ogata and Mori 1964) and the test for phosphorylase (Dubowitz and Pearse 1961). The method for succinic dehydrogenase uses Nitro-BT (after Nachlas et al., 1957) as an electron acceptor for the enzyme reaction, forming a blue formazan deposit at the sites of activity. Adequate localization was given by a twenty-minute incubation time. The incubation-film method (Fahimi and Amarasingham 1964), using Nitro-BT for staining and phenozine methosulfate as an intermediary electron carrier, was used for demonstrating the DPN-linked lactic dehydrogenase. The improved Takeuchi method (Eranko and Palkama 1961) was used to demonstrate phosphorylase, utilizing
sections air-dried for twenty minutes and kept in the incubation mixture for three hours. In addition, sections were stained for lipids by the Oil Red O, propylene glycol-Sudan Black, and propylene glycol-Fettrot methods (Pearse 1960, pps. 854-855). For these non-enzymatic stains the sections were fixed at the moment of thawing by a acetic acid-ethanol-formalin adhesive fixative (McMannus and Mowry 1960, p. 67).

Electron Microscopy

For comparative purposes both the rear leg muscle and the flight (pectoral) muscle of *Taderida braziliensis* were used. The tissue was fixed in glutaraldehyde and osmium tetroxide (Sabatini et al. 1964) and embedded in Epon (Pease 1964) and Maraglass (Spurlock et al. 1963). Epon sections were double-stained with uranyl acetate and lead citrate (Pease 1964). Maraglass sections were stained with uranyl acetate. Pictures were taken with the Phillips E.M. 100-B and the Phillips E.M. 200 electron microscopes.
RESULTS

Flight Muscle Isozyme Patterns

The gel patterns are summarized in Figure 2, arranged in increasing order of H-type predominance. The results indicate a certain amount of variability between most of the species, with the flight muscle of Taderida, however, being the only one containing solely $H_4$-LDH. The heart of each species was included for comparison, and in many instances it appeared to parallel the degree of isozyme specialization seen in the flight muscle. The Taderida flight muscle is considered to be the most specialized, since all other mammalian skeletal muscles that have been examined show at least some of the isozymes containing $M$ subunits. The different patterns found in the flight muscles of the species examined do not in all cases fit the pattern predicted by the random association of different amounts of $M$ and $H$ subunits (this is more apparent when viewing the actual gels). It is more likely that some of the different patterns reflect varying contributions of different cell types in the flight muscle. Only within each cell type would the random association principle be valid. However, for all the species examined, except Macrotus, the flight muscle patterns are shifted more toward the direction of $H_4$-LDH than in any of the red muscles of the mouse, guinea pig, or rabbit (Blanchaer and van Wijhe 1962). Moreover, the majority of the bat flight muscle isozyme patterns were comparable to the flight muscle patterns exhibited by strong flying birds (Wilson et al. 1963).
Evaluation of Stains

All of the histochemical methods used, including the lipid stains, clearly differentiated the two cell types in *Macrotus* flight muscle. Fahimi and Amarasingham (1964) stated that their incubation-film method for LDH did not give different staining intensities with white and red fibers due to the limited diffusion of the soluble LDH (achieved by using a gel overlay instead of an aqueous incubation medium). In the present study, however, this method demonstrated different staining intensities of the two cell types in pectoralis muscle of *Macrotus*. By using specific inhibitors for the $H_4$ and $M_4$ isozymes, Brody and Engle (1964) claimed to be able to demonstrate the selective intracellular localization of the LDH isozymes, the $M_4$-LDH appearing in the sarcoplasm and the $H_4$-LDH being associated with the mitochondria. However, there is no evidence from ultracentrifuge separation of cellular components (DeDuve 1963) that LDH is in any way associated with mitochondria. There is also some dispute over the validity of localizations obtained using Nitro-BT (Novikoff 1965). Therefore, a description of the LDH intracellular localizations will not be attempted even though this determination for the *Taderida* flight muscle containing only the $H_4$ enzyme would be a worthwhile addition to the present study. In a no-substrate control reaction for LDH, the Nitro-BT still stained the numerous small lipid inclusions that are difficult to distinguish from mitochondria, making impossible any judgment of purely enzymatic staining. The succinic dehydrogenase reaction, however, also using Nitro-BT, appeared to be much stronger than the background stainings, as the
heavy mitochondrial deposition of formazan crystals was seen only when succinate was present.

**Macrotus Flight Muscle**

The existence of two cell types is clearly indicated in Figures 3, 4, 5 and 6. By the criteria of Stein and Padykula (1962) the fibers would appear to be of the B and C types, thought to be different types of red fibers, with the A type representing the classical "white" fiber. As the evolutionary development of the fiber types in the flight muscle of bats is unknown, it becomes somewhat meaningless to label them with terms applied to another animal, particularly when only two instead of three types are evident. However, sections prepared from the flight muscle of a white-winged dove indicated two fiber types with a much greater difference in size and staining intensity than those found in *Macrotus*. By this comparison it appears that the dove flight muscle contains white (A) and red (C) fibers whereas the *Macrotus* muscle contains two types of red fibers. The two types exhibit a reciprocal relationship between phosphorylase activity and oxidative enzyme activity.

**Taderida Flight Muscle**

Figures 7, 8, 9 and 10 indicate only one fiber type present in Taderida flight muscle. This lipid-rich fiber of relatively small diameter undoubtedly corresponds to the C type, found to the greatest extent in red muscles with high oxidative capacity and reliance on anaerobic metabolism. Phosphorylase activity, taken as an indication of glycolytic activity, was shown to be moderate, but appeared greater than the phosphorylase seen in the small fibers of the pigeon pectoralis (Dubowitz and Pearse 1961).
Electron Microscopy

There is a clear distinction between the leg muscle and flight muscle from the same animals, as seen in Figures 11-15. More observations are needed to fully evaluate the ultrastructure of Taderida flight muscle as compared to other vertebrate skeletal muscle. Note that the mitochondria in the leg muscle (Figure 11) are located in a regular array beside the Z lines of each sarcomere. The mitochondria in the flight muscle, however, are not regularly oriented to any of the myofibrillar markings, and are larger and more densely packed than those in the leg muscle. The holes in the tissue would appear to be extracted lipid droplets. This conclusion was reached from the fact that identical preparation of heart tissue from Taderida gave the same type of holes, which corresponded to the location of intact lipid droplets seen in a published photograph of bat heart muscle (Bloom and Fawcett 1962, p. 204). The lipid droplets were probably incompletely fixed by the osmium and the extraction occurred during the alcohol dehydration and propylene oxide treatments of the tissue. Identical treatment of the less lipid-rich leg muscle did not result in such holes appearing. The unusually large number of lipid droplets and abundance of glycogen granules in the flight muscle can be noticed (Figures 13 and 15).

Comparison of Gel Migration Rates

The classification of the bats in Table 1 was taken from Simpson (1945). All species that are joined together in Table 1 have identical migration rates. For example, all four representatives of the genus
Myotis had LDH proteins with identical mobilities, while the Lasiurus and Pteronotus proteins were different from any of the others in their immediate family.

Family Phyllostomatidae

Subfamily Chylonycterinae

Pteronotus

Subfamily Phyllostomatinae

Macrotus

Subfamily Glossophaginae

Leptonycteris

Family Vespertilionidae

Subfamily Vespertilioninae

Myotis velifer

Myotis californicus

Myotis volans

Myotis thysonodes

Lasiurus cinereus

Eptesicus fuscus

Subfamily Nyctophilinae

Antrozous pallidus

Table 1--Grouping of bat species by similarities in the migration rates of the LDH isozymes. Those species that are joined together have isozymes with identical migration rates.

Hibernation

The isozyme patterns of liver, kidney, pectoral, rear leg and heart muscles from the hibernating bats Eptesicus and Plecotus show no significant variation from the previously published patterns obtained for the same tissues in non-hibernators. Similar results were obtained with these tissues from hibernating rock squirrels. There has been no previous analysis of the LDH isozymes of brown fat; however, no interpretation can be made of the occurrence of all five isozymes in the brown fat of a hibernating rock squirrel except to note that the overall LDH activity was much
greater in this thermogenic tissue than in white fat. This reflects the known greater amount of glycolytic activity in brown fat. In the rock squirrel's brain tissue, where a difference in isozymes was thought to be most likely as compared to non-hibernators, the slower moving isozymes exhibited perhaps a slightly greater density than the whole-brain pattern reported by Weiland and Pfleiderer (1961) for the rat, but did not exhibit any retention of the embryonic pattern typified by the mouse and guinea pig (Flexner et al. 1960). Patterns exhibited by the separate areas cerebral cortex, cerebellum, and medulla from a hibernating rock squirrel likewise did not differ significantly from the patterns found in these areas for various non-hibernators (Bonavita, 1964). The same results were obtained for Perognathaus, which appears to enter the hibernating state more readily than other hibernators, regardless of season (Bartholomew and Cade 1957). Moreover, the same pattern as well as identical mobilities were obtained for the LDH isozymes in nervous tissue from the hibernator Citellus tereticaudus and the non-hibernator Citellus harrisi. Thus the demands of hibernation do not appear to result in a greater production of M-type subunits in nervous or other tissue.
DISCUSSION

Flight Muscle

Wilson, Cahn, and Kaplan (1963) demonstrated a good correlation between the isozyme patterns of flight muscles and the flight habits of the 40 species of birds examined. Of these, the majority exhibited a greater preponderance of the slower-moving isozymes (M-types) than any of the 13 species of bats (Figure 2) except Macrotus. The only birds that gave patterns similar to the bats were the hummingbird and those birds that spend most of their life over water. Only the petrel showed the degree of specialization found in Taderida. The isozyme patterns found for bats are thought to be valid, since overloading the gel did not change the basic pattern and the patterns from other bat organs were identical to those obtained for the same organs in other mammals. If the correlation between isozyme activity and flight habits is valid, the results might imply that most bats have the capacity for sustained flight as great as that found in some oceanic birds. A more reasonable explanation would be that although bats are highly maneuverable they have not developed the aerodynamic efficiency of flight to the same extent as birds, thus needing more efficient muscles to keep themselves aloft.

The variation in isozyme patterns in the bats (Figure 2), with Taderida and Macrotus at the extremes, has two possible explanations. One possibility would be that the patterns reflect the contributions of two cell types, with the patterns shifting more towards H4-LDH as the cell
type found in *Taderida* flight muscle becomes more prominent. A second possibility is that the red fiber (C type) can itself evolve different degrees of isozyme specialization. Micro-electrophoresis of an isolated human red fiber showed that all five isozymes were indigenous (Van Wijhe, Blanchaer, and St. George-Stubbs 1964). The *Taderida* red fiber, however, appears unique in that it contains only the $H_4$-LDH. This was shown by overloading the gel so that isozymes other than $H_4$ appear. Since Markert (1963) has shown that there is no preferred formation of any one isozyme over another, the probability of combination for any $M$ subunits produced in the muscle cell would result in the band $M_1 H_3$ appearing before any other $M$-containing isozymes. The very weak uniform appearance of all the isozymes other than $H_4$-LDH on the overloaded gel indicates that they are not indigenous to the muscle cell but arise most likely from adherent blood cells or connective and adipose tissue contaminants. The pattern from the flight muscle of *Pteronodus* (Figure 2) shows a large amount of $H_4$-LDH along with lesser amounts of the other isozymes. In contrast to the *Taderida* muscle, these weaker isozyme bands are thought to be inherent in the muscle tissue and not contaminants, since their presence was very evident without overloading the gel. The *Pteronodus* muscle cannot consist of a homogenous fiber type, however, since if this were the case the amount of $H_3 M_1$-LDH, by the random association principle, would be greater than that observed. The most likely situation is that *Pteronodus*, being in the same family as *Macrotus*, also has two cell types in its flight muscle but with the fiber type seen in *Taderida* flight muscle being much more numerous than the larger-diameter fiber type seen in *Macrotus*. Thus it appears that at least two evolutionary trends, a tendency towards a
uniform muscle fiber type and a further shift in this fiber towards more 
H₄-LDH predominance, have probably occurred in the chiropteran order. A 
more definitive answer as to which explanation of the varied isozyme pat-
terns is most important could be determined by further histochemical 
studies.

The known migratory habits of Taderida braziliensis (Villa and 
Cockrum 1962) and observations of some amazing flight performances fit 
in well with the extreme specializations found for its flight muscle. By 
oberving the mass movements of Taderida, Davis et al. (1962) estimate 
that the bats sometimes flew up to 300 miles in one night. They further 
oberved that Taderida flocks disappear from binocular-aided view at an 
estimated height of 10,000 feet. Dilworth Carter, who has extensively 
followed the movements of Taderida, believes there is a good possibility 
that Taderida found in Texas may be in northern South America for part of 
the year (personal communication). The comparison of the isozyme and 
histochemical results with those of strong flying birds certainly indi-
cates that the Taderida have the capacity for such feats. In other 
mammalian mixed muscle there appears to be a division of labor, with the 
cytoplasmic glycolytic system being predominant in the larger fiber type 
whereas the mitochondria-based fatty acid oxidations occur in the smaller 
fiber. Phosphorylase activity has often been taken as an indication of 
glycolytic capacity, and the usual inverse relationship between the phos-
phorylase activity and mitochondrial oxidative capacity is seen in the 
Macrotus flight muscles (Figures 3, 4, 5 and 6). The Taderida flight 
muscle, however, appears to have developed the capacity for utilizing 
both energy sources in the one cell type present. This is seen in the
substantial amount of phosphorylase activity (Figure 10) existing simultaneously with strong oxidative enzyme activity in a lipid-rich red fiber (Figure 7). The evidence for this situation is further seen in Figures 13 and 14, where an unusually large number of sizable lipid droplets co-exist with substantial amounts of stored glycogen. It would be of interest to follow the amounts of lipid and glycogen in the flight muscle on a seasonal basis to see if the amounts changed before or during the periods of sustained migration.

The characteristic size and orientation of the mitochondria in red muscle fibers of the rat (Padykula and Gauthier 1963) appear very similar to the situation seen in the leg muscle of the bat (Figure 11). The Taderida flight muscle mitochondria are larger and less uniformly arranged, but are comparable in size to those seen in the pigeon (Howatson 1956) and hummingbird (Lasiewski et al. 1965) flight muscles. They do not, however, approach the size and density of packing seen in insect flight muscle (Smith 1961), nor are they as extensive as those in the extremely fast-acting bat cricothyroid muscle (Revel 1962), which controls the high frequency echo-ranging sounds emitted by bats in flight. Even though the gross mitochondrial appearance in Taderida flight muscle does not differ significantly from other flight muscles, a comparison of the fine internal structure of these mitochondria with other typical mitochondria may prove fruitful.

The only other bat examined in this study that shows migratory behavior is Lasiurus cinereus, whose range extends into Canada, the total distance approaching that of Taderida distribution (Findley 1962). The actual extent of its migration is not as well documented as for Taderida.
but the lack of isozyme specialization may indicate that *Lasiurus* proceeds at a more leisurely pace than *Tadarida*. By not having as narrow a wing as *Tadarida*, the *Lasiurus* bats would need a lesser number of wing beats per minute to stay aloft. This in turn would place lesser demands on the flight muscles. Also, by traveling in smaller numbers than *Tadarida*, more roosting spots at closer intervals may be available to the *Lasiurus* bats.

The flight habits of two other bats used in this study, *Myotis velifer* and *Macrotus*, have been extensively described by Vaughan (1957). He states that *M. velifer* appears to be a stronger flyer than other small vespertilionids. The isozyme pattern for *M. velifer* is shifted more towards $H_4$–LDH than all the other small bats except *M. californicus*. As for the *Macrotus*, showing the least isozyme specialization, Vaughan states that the sound of its wing beats are much softer than those resulting from the forcible action of other bat wings. Furthermore, *Macrotus* feeds by flying, only three to four feet above the ground, often stopping to eat the larger ground insects that it catches, with its total feeding period lasting a maximum of one hour and forty-five minutes. This type of activity would be less strenuous than that of the bats which have to pick insects out of the air. Thus, under the hypothesis that stronger flying bats should show a more pronounced shift towards the $H_4$–LDH isozyme in their flight muscles, the results summarized in Figure 2 do indicate a correlation between the isozyme patterns and the flight habits of the various bats. This is similar to the results obtained for birds by Wilson et al. (1963). The only isozyme patterns that appear out of line are those of *M. californicus* and *Antrozous* (Figure 2). *M. californicus* had isozymes shifted more towards $H_4$–LDH than any of the other vespertilionids.
This is somewhat surprising in that Haywood and Davis (1962) stated that this small bat was a weak flyer and appeared to tire more easily than the other bats which they examined. However, the more rapid wing beats seen in a small bat such as *M. californicus* may give rise to the same metabolic specializations as would endurance capabilities. As for *Antrozous*, Orr (1958) noted that this bat has ground-based feeding habits similar to *Macrotus*. One reason that *Antrozous* does not show the same flight muscle isozyme pattern could be a result of different evolutionary histories for the two bats. Before more meaningful correlations of isozyme patterns with flight habits can be attempted, more detailed knowledge of the individual species must be obtained. This would include such items as the amount of time spent feeding, the total distance covered, and the number of wing beats per second during flight. Since the bats are nocturnal animals, much of this information is difficult to obtain.

**Comparison of Gel Migration Rates**

Starch gel electrophoresis is a highly sensitive technique, capable of detecting one amino acid difference between two proteins. Studies of other common proteins (Zuckerkandl 1965; Smith and Margoliash 1964), have shown that certain parts of a molecule, thought to be the critical functional groups, tend to remain constant throughout evolution whereas other parts of the same molecule are highly variable. It seems reasonable that many amino acid substitutions would not affect the critical function of the molecule. For example, cow and sheep ribonuclease differ in some amino acids but have hardly any difference in their enzymatic functions (Anfinsen et al. 1959). Such substitutions could be termed non-adaptive.
characters, since the number of different random, non-critical amino acid substitutions seen in two homologous proteins from two animals would be dependent only on the amount of time that had elapsed since the species had diverged. The rate of change of adaptive phenotypic characters, however, can be varied according to the selection pressures an animal encounters, whereas the rate of non-adaptive amino acid substitutions would not be varied by sudden environmental changes or convergent evolution. Thus a comparison of species by non-functional differences in their proteins would give added insight into the actual extent of divergence among groups of animals. Of course, the stabilization of an amino acid substitution in the population would have to depend on some adaptive mechanism, but one not related to the effect of the new amino acid per se; it could appear simultaneously with a closely-linked highly adaptive gene, or occur within a favorable chromosomal inversion. For taxonomic purposes the principle would be valid probably within an order or class at most, using the average results for a number of proteins for which amino acid differences could be shown to have no observable functional significance. Recognizing that the LDH enzymes have not been shown to have identical functional properties in the bats, and that any conclusions would depend on the results from many proteins, a comparison of the gel migration rates for bat LDH isozymes was made. The results (Figure 16) are what one would expect except for the Eptesicus isozymes. Instead of showing the same migration rates as those from members of the same subfamily, the Eptesicus isozymes have a migration rate identical to those from Antrozous, which is in a different subfamily. It is possible that the Eptesicus and Antrozous
isozymes are actually different but the net balance of charges comes out the same, which could be determined only by more extensive analysis.

Hibernation

Hiestand et al. (1950) has shown that the comparative hypoxic resistance and anoxic survival time is far greater for hibernators (even when not in hibernation) as compared to non-hibernators, except for the newborn of several rodent species. The newborn appear to have smaller energy requirements for the nervous tissue than the adults, as their cerebral glycolysis rate is lower (Himwich et al. 1942), making it possible for them to exist for some time on the energy production of anaerobic glycolysis alone. This is seen from the fact that cyanide, poisoning the terminal cytochrome chain, had little effect on the survival time of hypoxic newborn rats as compared to adult rats, whereas the glycolytic poison iodoacetate profoundly affected the survival time of the infant rats (Himwich, Fazekas and Alexander 1941). This observation eliminates alternative explanations for the hypoxic resistance such as greater myoglobin oxygen stores or more efficient cytochromes. By actually measuring the lactate produced, Burlington and Wiebers (1965) showed that lactate increased upon exposure to hypoxic conditions in both newborn rats and adult hibernators but not in adult rats, where anaerobic glycolysis decreased. Thus their data indicate that an increased capacity for anaerobically produced energy is retained during maturation in the hibernator more so than in the homeotherm. The assumption made in the present study was that the fetal isozyme pattern, rich in the anaerobically-specialized M₄-LDH capable of producing large amounts of lactate, would be retained.
in the brain tissue of an adult hibernator. This assumption appeared even more attractive when it was noticed that the age at which growing rats lost their hypoxic resistance (12-20 days) corresponded exactly with the time at which these animals shift from the fetal to the adult isozyme pattern in their cerebral cortex (Flexner et al. 1962). Even if the exact fetal pattern was not retained, the following observations (Kaplan et al. 1963) could have significance for a seasonal adaptation in a hibernator.

The effect of hormones on the LDH isoforms has usually been to increase the production of both H and M subunits without favoring one over the other. During pregnancy, however, as the uterus approaches term there is a sudden production of M subunits that appears to anticipate the demands of expelling the fetus. Also, incubating chick eggs in 100% oxygen results in an increased production of H subunits. It was thought that the low oxygen tensions during hibernation might result in the opposite effect, i.e., an increased production of M subunits, or that the preparations for hibernation might accomplish the same effect by a hormonally-mediated mechanism. The actual determination, however, using hibernators both in and out of hibernation, showed no shift towards M₄-LDH in brain or any other tissue as compared to the same tissues for non-hibernators.

The amounts of each isozyme formed by the random combination of subunits within a homogeneous tissue are proportional to the coefficients before the individual terms of the binomial expansion \((aH + bM)^4\), where \(a/b\) is the ratio of H to M subunits produced. The form which best fits the brain isozyme patterns observed is given by \((3H + M)^4 = 0.81H^4 + 108H^3M + 54H^2M^2 + 12HM^3 + M^4\). It is seen that the M₄-LDH constitutes only 0.4% of the total LDH activity and thus could hardly bear the brunt of converting
pyruvate to lactate under hypoxic conditions, even though it is best suited for it. Perhaps the amounts and properties of $H_2M_2$ and $HM_3$-LDH's are sufficient to handle the pyruvate load, or pyruvate may never even build up to inhibitory concentrations within the cell. The lack of $M_4$-LDH may mean that anaerobic metabolism is actually not important in hibernation. If anaerobic metabolism were important, one would expect a shift in the isozyme patterns towards $M_4$-LDH, much like the shift towards $H_4$-LDH seen in the isozyme patterns of flight muscle, where aerobic metabolism has gained predominance. The hypoxic resistance of these animals has often been cited as evidence for the possible utilization of anaerobic metabolism, but its existence has never been proven during actual hibernation. Zimney and Tyrone (1957) demonstrated that the absolute amounts of lactate and pyruvate decreased markedly in hibernation but the lactate/pyruvate ratio was within values found for the same animal when it was not hibernating, indicating no profound alteration in enzyme activity at this particular site. Perhaps the greater increase in lactate production in vitro for the hibernator's brain tissue reflects a decreased sensitivity to the normal feedback mechanisms that regulate glycolysis. This would allow anaerobic glycolysis to continue in the presence of abnormal amounts of end products.

There is ample evidence that over a long period of natural selection the isozyme patterns have become correlated with the predominant type of metabolism in a certain tissue. One might ask, however, why $H_4$-LDH should even exist if it cannot effectively catalyze the conversion of its substrate. The pyruvate inhibition is effective, for the heart containing only $H_4$-LDH shows no appreciable pH change under anaerobic conditions.
The reason for substantial amounts of an LDH enzyme in the heart lies not in the need for lactic acid production but in the need for the lactate to pyruvate reaction without the reverse occurring, since the heart uses blood lactate as an energy source (Bing 1954). H-subunit production in other tissues may reflect the need for a moderate, controlled production of acid that would not upset a more delicate homeostasis present in tissue other than muscle. It would be informative to determine whether the Taderida flight muscle, containing only H4-LDH, uses lactate produced elsewhere as an energy source, or whether this adaptation serves only to avoid muscle fatigue.
SUMMARY AND CONCLUSIONS

1. The lactic dehydrogenase isozyme patterns of the flight muscles from 14 species of bats are unlike other mammalian skeletal muscles, showing a shift towards the pyruvate-inhibited $H_4$-LDH normally found in the heart. This is an adaptation for sustained activity, favoring the oxidation of pyruvate through the Krebs cycle, rather than the production of lactic acid. The differences in isozyme patterns for most of the bats correlate with their known differences in flight habits.

2. The least specialized flight muscle, containing the greatest amounts of $H_4$-LDH, is found in *Macrotus californicus*, thought to be the weakest flyer of the bats examined. Its pectoral muscle consists of two cell types.

3. The most specialized flight muscle, containing only $H_4$-LDH, is that of *Taderida*. This is an adaptation for its migratory habits. Its pectoral muscle contains only one red fiber type, probably depending on fat for its major fuel, but exhibiting more glycolytic capacity than red muscle in other mammalian mixed muscle. Electron micrographs of this muscle show many large mitochondria comparable in size to those in bird flight muscle but larger than those seen in most mammalian red muscle. Also evident are an unusually large number of lipid droplets coexisting with substantial glycogen deposits.

4. Of the bats examined, species within a genus have LDH proteins with identical starch gel migration rates, whereas the proteins from different genera within a subfamily migrate at different rates, except for
the identical movements shown for the proteins from *Eptesicus* and *Antrozous*, and for those of *Macrotus* and *Leptonycteris*.

5. The occurrence in hibernators of isozyme patterns identical to those of non-hibernators does not appear to have any rate limiting significance as to the hibernator's greater hypoxic resistance or its abilities to hibernate. Preparation for hibernation or actual hibernation does not result in any greater production of M₄-LDH isozyme.
Fig. 1.--Plexiglass trays for holding the starch gel, and the electrophoresis buffer tank. During electrophoresis the gel trays rest on the compartment dividers of the buffer tank.
Fig. 2. -- Representation of the LDH isozyme patterns of flight and heart muscle from twelve species of bats. Note that the heart appears to parallel the flight muscle patterns.
<table>
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<tr>
<th>LDH isozymes</th>
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The table shows the distribution of LDH isozymes in different tissues of MYOTIS californicus and TADERIDA species.
Fig. 3. — *Macrotus* flight muscle, 200x and 400x. Stain for neutral lipids. Nuclei counterstained with hematoxylin. Note the two cell types present, with the less lipid-rich fibers appearing to be slightly larger than the more heavily stained fibers.
Fig. 4.—Macrotus flight muscle. Succinic dehydrogenase. 200x. The larger diameter fiber is stained more heavily at the periphery whereas the smaller diameter fibers stain more evenly.
Fig. 5.—Macrotus flight muscle. Lactic dehydrogenase. 480x.
The smaller diameter fibers appear to stain more heavily, although this could result from the lipid substantivity of the Nitro-BT dye.
Fig. 6.—Macrotus flight muscle. Phosphorylase. 200x and 400x. The larger diameter fibers have greater phosphorylase activity.
Fig. 7.—*Taderida* flight muscle. Lipid stain. 200x and 480x. Note the even staining of all fibers as compared to the *Macrotus* muscle (Fig. 3).
Fig. 8.--Taderida flight muscle. Succinic dehydrogenase. 640x. All fibers stain evenly for this enzyme.

Fig. 9.--Taderida flight muscle. Lactic dehydrogenase. Again one fiber type is demonstrated. 480x.
Fig. 10.--Taderida flight muscle. Phosphorylase. 480x. Note the even staining of moderate intensity. Compare with Fig. 6.
Fig. 11.--Electron micrograph of Taderida leg muscle. Uranyl-lead stain, 1800x and 3000x. Note the orderly arrangement of paired mitochondria at the level of the Z line. This situation and the size of the mitochondria are typical for mammalian red muscle.
Fig. 12.--Electron micrograph of Taderida flight muscle. Uranyl-lead stain. 1800x. A somewhat oblique longitudinal section, showing the unusual packing of large mitochondria. The holes correspond to extracted lipid droplets.

Fig. 13.--Electron micrograph of Taderida flight muscle. Uranyl-lead stain. 9900x. Note the dense packing of mitochondria and the abundant glycogen granules.
Fig. 14.—Electron micrograph of *Taderida* flight muscle. Uranyl stain. 1800x. Note the unusually large number of lipid droplets, always occurring next to a mitochondrion.

Fig. 15.—Electron micrograph of *Taderida* flight muscle. Uranyl stain. 3000x. Note the abundance of glycogen granules and the dense packing of mitochondria in the lower right of the photograph.
LIST OF REFERENCES


