Evolution of Malate Dehydrogenase in Birds

Abstract. *Heart extracts from over 100 species of birds were subjected to starch-gel electrophoresis at pH 7. The "supernatant" form of malate dehydrogenase, an enzyme present in every extract, was then located on the gels by a specific staining method. The mobility of this enzyme shows very little interspecific variation. Nearly all birds tested have a supernatant malate dehydrogenase that moves as fast as the chicken enzyme. Those species with an enzyme of unusual mobility are of taxonomic interest. For example, hummingbirds and swifts, which are usually considered as two suborders of Apodiformes, are unique among the birds tested in having an enzyme that moves 63 percent as fast as the chicken enzyme. This finding appears to confirm the unity of the Apodiformes, an order whose unity has long been open to question. Similarly all families tested in the shorebird order (Charadriiformes) are unique in having an enzyme that moves 55 percent as fast as the chicken enzyme. The unity of this order was also previously open to question.*

Proteins have often been suggested as sources of taxonomic information (*1*). The usual finding is that the more closely two species are related, according to morphological criteria, the more likely they are to have similar proteins (*1*). For species whose taxonomic relationships are poorly known, careful study of their proteins should lead to an improvement in their classification. The relationships of the higher taxonomic categories (suborders and orders) of birds are still poorly known (*2, 3*). We have compared a single property of a specific protein, malate dehydrogenase, in a great variety of bird species. Our biochemical results appear to confirm the homogeneity of certain orders which have hitherto been regarded as possibly heterogeneous assemblages of families and suborders.

The enzyme malate dehydrogenase (*4*) exists in two major forms in mammals and birds (*5*). One form (M) occurs in mitochondria and may be distinguished by its movement toward the cathode during starch gel electrophoresis at *p*H 7. The other form (S) occurs in the soluble fraction of the cytoplasm and moves toward the anode. The enzymes of the supernatant and of the mitochondria have been isolated in pure form. They are similar in molecular weight (*6–8*), but differ markedly in amino acid composition, two-dimensional peptide patterns, immunological properties, and susceptibility to substrate inhibition (*5, 7, 9, 10*).

We have compared the electrophoretic mobility of the S malate dehydrogenase (S-MDH) from specimens of more than 100 species of birds. The birds were obtained from a variety of sources, many of which have been listed (*11, 12*), and stored in the frozen state until needed for the preparation of extracts of heart and skeletal muscle. Extracts were prepared in glass homogenizers by grinding 1 g of tissue in 5 ml of cold 0.25*M* sucrose. They were clarified by centrifugation in a Spinco ultracentrifuge (model L) at 100,000*g* for 1 hour. The same extracts have been used for taxonomic and evolutionary studies of lactate dehydrogenases (*11, 12*). Samples of all the heart extracts and many of the muscle extracts were subjected to electrophoresis in horizontal starch gels, with phosphate-citrate buffer at *p*H 7.0 (*13*). The general procedure has been described by Fine and Costello (*13*). A voltage gradient of 10 volt/cm was applied for about 16 hours at about 10°C, the average gel temperature. After electrophoresis, the position of the enzyme activity of MDH on the gels was determined with a nitroblue tetrazolium staining mixture containing L-malate as substrate (*13*). Because the extracts were made from frozen tissues, both M and S enzymes were present, the M form being distinguished by its cathodal mobility (Fig. 1). The anodic mobility of S-MDH from different species is recorded as a percentage of the mobility of the chicken enzyme. At least one sample of chicken S-MDH was applied to each gel to serve as a standard. Crystalline and crude preparations of chicken S-MDH moved identically on electrophoresis.

As indicated in Fig. 1, the S and M enzymes of birds both exist in multiple forms. Multiple forms, or sub-bands, are also detected upon electrophoresis of the S and M enzyme from pig tissue (*14*). The sub-bands appear to be slight modifications of the form with the lowest anodic mobility (*15*). The mobility recorded (Tables 1 to 4) is that of the major form of S-MDH present in avian extracts. This form is, with certain exceptions (*16*), the one with the lowest anodic mobility (Fig. 1).

To test for intraspecific variation in mobility of S-MDH, individual heart extracts from 50 wild pigeons (*Columba livia*) were subjected to electrophoresis (*17*). No variation was detected in the mobility or in the spacing and relative intensities of the sub-bands. Similarly, no intraspecific variation of this sort was encountered when 100 domestic chickens of various breeds, or 12 Japanese quail (*Coturnix coturnix*), were compared. In the case of most other species with which we have worked, only one or a few individuals were examined.

There was little interspecific variation in the electrophoretic mobility of S-MDH. Thirty-seven species were ex-

Table 1. Birds (order Galliformes) with S-MDH of mobility 100.*

Cracidae (1/11)†
Ortalis vetula

Numididae (2/5)
Numida meleagris
Acryllium vulturinum

Phasianidae (24/48)
Ammoperdix griseogularis
Alectoris barbara
Alectoris graeca
Francolinus erckeli
Coturnix delagorguei
Coturnix coturnix
Coturnix (Excalfactoria) sinensis
Synoicus ypsilophorus
Perdicula asiatica
Perdicula erythrorhyncha
Rollulus roulroul
Ptilopachus petrosus
Bambusicola thoracica
Galloperdix spadicea
Gallus gallus
Polyplectron chalcurum
Pavo cristatus
Lophophorus impeyanus
Crossoptilon auritum
Lophura edwardsi
Chrysolophus pictus
Chrysolophus amherstae
Syrmaticus reevesi
Phasianus colchicus
Odontophorus capueira
Cyrtonyx montezumae
Colinus virginianus
Lophortyx californica

Meleagridae (1/2)
Meleagris gallopavo

Tetraonidae (4/11)
Bonasa umbellus
Canachites canadensis
Dendragapus obscurus
Tympanuchus cupido

* A single specimen of the ferruginous wood partridge *Caloperdix oculea* yielded S-MDH with mobility 155. † The numbers in parentheses indicate the number of genera examined, as a fraction of the total number of genera in the family.

amined in the Galliformes, which includes pheasants, partridges, quails, chickens, turkeys, and others (Table 1). Only one of these species had an S enzyme that differed detectably in mobility from that of the chicken enzyme. Despite the peculiar mobility (155) of its S-MDH, this species, the ferruginous wood partridge *Caloperdix*, is thought by ornithologists (*18*) to be closely related to the other partridges listed in Table 1 (such as *Rollulus*).

When a broader survey of birds was made, further evidence for restricted variation was obtained. Species representing 62 families and 22 of the 27 orders of birds are listed in Table 2. More species were examined but, to save space, each family is represented in the table by a single species. Nearly every species examined had S-MDH with a mobility of 100, that is, like that of the chicken enzyme. Three exceptional species were found—the cedar waxwing *Bombycilla*, the cuckoo *Coccyzus*, and the partridge *Caloperdix*, with S-MDH mobilities of 75, 123, and 155, respectively. The total number of species in the 22 orders is about 8000 (*19*). It seems likely that nearly all of these species have S-MDH with a mobility like that of the chicken enzyme.

Lower mobilities were encountered in the three other orders of birds—Caprimulgiformes, Apodiformes, and Charadriiformes. The sole representative tested from Caprimulgiformes—the poor-will *Phalaenoptilus nuttalli*—has an S enzyme of mobility 27.

All hummingbirds and swifts tested had an S enzyme of mobility 63 (Table 3). No other birds possess an S enzyme with such a mobility (*20*). The hummingbirds (Trochilidae) and swifts (Apodidae) have traditionally been considered as two separate suborders of Apodiformes (*19, 21*), but many ornithologists have questioned whether there is any special affinity between the two groups and have considered it possible that they should be put in separate orders (*2, 19*). The electrophoretic evidence is in favor of the unity of the Apodiformes.

The Charadriiformes, sometimes known as the shorebird order, includes the gulls, terns, sandpipers, and plovers. The birds of this order are evidently unique in having S-MDH that moves 55 percent as fast as the chicken standard (Table 4). The species tested represent all three suborders and 10 out of the 16 families in this order (*22*). From a taxonomic point of view, Charadriiformes is highly varied; only one other order (Passeriformes) contains more families than this order does. The order is usually thought to be homogeneous, that is, monophyletic, but there have been persistent suggestions that it may be polyphyletic. According to some workers (*2, 19, 23*) certain families presently included in the order may not belong there, such as Jacanidae, Thinocoridae, and Alcidae. It is also considered possible that some families in other orders, for example, Gruiformes (*2, 19, 24*), Columbiformes (*2, 19*), and Gaviiformes (*24, 25*), may be closely related to, or included in, the order Charadriiformes. The electrophoretic data appear to provide evidence that the order Charadriiformes, as usually defined, is homogeneous and distinct from other orders (*22*).

Our results show that the electrophoretic mobility of S-MDH is useful for studies of the relationships of higher

Table 2. Birds (order and species) with S-MDH of mobility 100. The orders are arranged in a traditional sequence, those in the left-hand column being considered by systematic ornithologists to be less primitive than those in the right-hand column (*19, 21*).

Passeriformes* (16/67)†
- *Calyptomena viridis*
- *Pitta brachyura*
- *Pitangus sulphuratus*
- *Chiroxiphia linearis*
- *Iridoprocne bicolor*
- *Corvus brachyrhynchos*
- *Picarhartes gymnocephalus*
- *Hylocichla mustelina*
- *Regulus satrapa*
- *Toxostoma rufum*
- *Sturnus vulgaris*
- *Nectarinia famosa*
- *Dendroica striata*
- *Pipilo erythrophthalmus*
- *Quiscalus quiscula*
- *Passer domesticus*

Piciformes‡ (3/6)
- *Colaptes auratus*
- *Ramphastos toco*
- *Lybius torquatus*

Coraciiformes (4/9)
- *Megaceryle alcyon*
- *Momotus momota*
- *Upupa epops*
- *Coracias caudata*

Trogoniformes (1/1)
- *Pharomacrus mocino*

Strigiformes (1/2)
- *Otus asio*

Cuculiformes (2/2)
- *Tauraco hartlaubi*
- *Coccyzus americanus*§

Psittaciformes (1/1)
- *Ara chloroptera*

Columbiformes (2/2)
- *Pterocles lichtensteini*
- *Columba Livia*

Gruiformes (4/12)
- *Balearica pavonina*
- *Rallus limicola*
- *Podica senegalensis*
- *Eupodotis senegalensis*

Galliformes (5/7)
(See Table 1)

Falconiformes (4/5)
- *Cathartes aura*
- *Buteo lagopus*
- *Pandion haliaetus*
- *Falco sparverius*

Anseriformes (2/2)
- *Anhima cornuta*
- *Anas platyrhynchos*

Ciconiiformes (4/7)
- *Ardea herodias*
- *Ibis leucocephalus*
- *Plegadis falcinellus*
- *Phoenicopterus ruber*

Pelecaniformes (4/5)
- *Phaethon lepturus*
- *Pelecanus occidentalis*
- *Sula sula*
- *Anhinga anhinga*

Procellariiformes (3/4)
- *Diomedea nigripes*
- *Puffinus pacificus*
- *Oceanites oceanicus*

Sphenisciformes (1/1)
- *Spheniscus mendiculus*

Podicipediformes (1/1)
- *Podiceps auritus*

Gaviiformes (1/1)
- *Gavia immer*

Tinamiformes (1/1)
- *Nothura maculosa*

Rheiformes (1/1)
- *Rhea pennata*

Apterygiiformes (1/1)
- *Apteryx australis*

Struthioniformes (1/1)
- *Struthio camelus*

* Three cedar waxwings (*Bombycilla cedrorum*), representative of the passeriform family Bombycillidae, each yielded S-MDH of mobility 75. † The numbers in parentheses indicate the number of families examined, as a fraction of the total number of families in the order. ‡ Birds of the order Piciformes yielded S-MDH with two sub-bands of approximately equal intensity; the mobilities of the sub-bands were 93 and 102 (*16*). § The cuckoo enzyme had a mobility of 123.

Table 3. Birds (order Apodiformes) with S-MDH of mobility 63.

Apodidae (2/8) *
- *Aeronautes saxatalis*
- *Chaetura pelagica*

Trochilidae (8/123)
- *Archilochus colubris*
- *Calypte anna*
- *Selasphorus sasin*
- *Chlorostilbon ricordi*
- *Eulampis jugularis*
- *Chlorestes notatus*
- *Heliomaster longirostris*
- *Phaethornis yaruqui*

* The numbers in parentheses indicate the number of genera examined, as a fraction of the number of genera in the family.

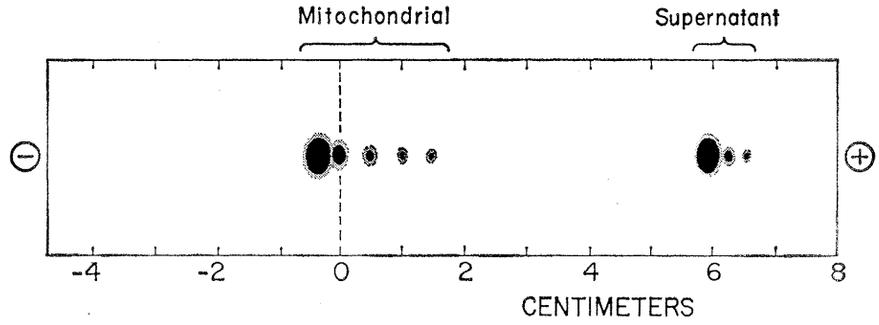

Fig. 1. Starch-gel electrophoresis of the malate dehydrogenases in a chicken heart extract.

Table 4. Birds (order Charadriiformes) with S-MDH of mobility 55.

Jacanidae (4/6)
Jacana spinosa
Metopidius indicus
Actophilornis africana
Hydrophasianus chirurgus

Recurvirostridae (2/4)
Recurvirostra americana
Himantopus himantopus

Charadriidae (3/7)†
Charadrius hiaticula
Charadrius vociferus
Pluvialis dominica
Pluvialis (Squatarola) squatarola
Vanellus (Belonopterus) chilensis
Vanellus (Hoplopterus) spinosus

Scolopacidae (4/21)
Tringa solitaria
Erolia fuscicollis
Philohela minor
Numenius phaeopus

Phalaropodidae (1/3)
Phalaropus fulicarius

Burhinidae (1/3)
Burhinus capensis

Glareolidae (1/5)
Glareola maldivarum

Stercorariidae (1/2)
Catharacta antarctica

Laridae (3/7)
Larus argentatus
Rissa tridactyla
Larosterna inca

Alcidae (6/11)
Uria lomvia
Plautus alle
Fratercula cirrhata
Alca torda
Ptychorhamphus aleutica
Synthliborhamphus antiquum

* The numbers in parentheses indicate the number of genera examined, as a fraction of the total number of genera in the family. † A sample of breast muscle, labeled semipalmated plover (*Charadrius semipalmatus*), was sent to us. It yielded S-MDH of mobility 26. This species is considered by some ornithologists to be a subspecies of the ringed plover (*Charadrius hiaticula*), whose S-MDH mobility is 55. No other tissue samples from this species have been tested.

taxonomic categories, namely families and suborders of birds. It might have been expected, however, that an electrophoretic property would be very susceptible to evolutionary change, and that convergent evolution of similar electrophoretic mobilities could easily occur in unrelated taxonomic groups. Nevertheless, the data strongly suggest that the mobility of S-MDH is generally a very conservative character. In the great majority of bird species, on the basis of our results, the S-MDH mobility appears to be the same. Yet some of these species are products of evolutionary lines that have been separate for about 100 million years (*26*). By contrast, the mitochondrial form of MDH is less conservative than S-MDH in electrophoretic mobility (*27*) and so is the H_4 lactate dehydrogenase of birds (*28*). Some egg-white proteins (*24, 29*) and serum proteins (*30*) appear to be even more variable in this respect. The conservative electrophoretic mobility of S-MDH raises the possibility that other properties of the enzyme, such as immunological properties and primary structure, may also have been conserved during bird evolution.

As the biological significance of the electrophoretic mobility of S-MDH is unknown, the reason for the relative lack of evolutionary variation in this property is also unknown. The fact that in hummingbirds, swifts, and the poorwill the S-MDH mobility is low is of interest because these birds share an unusual physiological capacity—that of lowering their body temperature and becoming torpid (*31*). However, charadriiform birds also possess an S-MDH of low mobility and they are not known to have this capacity.

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