New Creatine Kinase Isoenzymes in Birds. Distribution Among Avian Orders, Tissue-Specific Patterns and Their Appearance in Ontogeny

Creatine kinase (E.C. 2.7.3.2) from vertebrates occurs in multiple molecular varieties. Three isoenzymes are regularly distinguished by electrophoretic separation. They exhibit tissue-specific patterns of distribution[1]. The active enzymes have a dimeric structure[2]. Two different subunits have been recognized and named M and B, according to their predominant occurrence in skeletal muscle and brain. The active dimer is formed by combination of either two identic or two different monomers. MM-type creatine kinases are found in skeletal muscle of adult vertebrates, BB-type enzymes in brain. Heart tissue very often contains the MB-type creatine kinase in addition to either MM or BB, which depends upon the species (Figure 1a, incubations without the specific substrate, indicated as 'blank' in Figure 1a, indicate one band, which is not creatine kinase activity). A fourth creatine kinase isoenzyme has been regularly observed in brain and heart tissues of certain birds[3,4]. Its electrophoretic mobility is clearly distinct from the usual isoenzymes. It is the most anodically migrating enzyme (Figure 1b, a juvenile bird has been chosen to show all four creatine kinases in muscle tissue. Adult birds show only the MM-type creatine kinase in skeletal muscle).

It was noted that this new isoenzyme could only be observed in birds of the orders Passeriformes and Caprimulgiformes[3]. This finding could be explained by the assumption that a mutation – possibly a duplication of a structural gene – which had occurred in the evolution of the avian order Passeriformes, gave rise to this isoenzyme. This hypothesis led to a more detailed investigation of the occurrence of the additional isoenzyme band in birds.

Mitochondria-free supernatant fractions were subjected to electrophoresis on 1.5% agarose-plates (10 × 10 cm) for 1–2 h at a current of 40 mA. In order to obtain optimal electrophoretic separation of the various creatine kinase bands, 2 different buffer systems were used: (a) veronal 0.02 *M*, pH 8.6, (b) citrate-NaOH, 0.01 *M*, pH 6.5 for preparation of the gels. The same buffers at the same pH but 3-fold concentrated served as vessel buffer. Visualization of creatine kinase activity on the gels was achieved by a method described earlier[5]. Most birds were deep-frozen shortly after death and sometimes stored for several weeks. Controls with fresh material showed that the enzyme activity decreased in deep-frozen tissue, leaving the isoenzyme pattern unaffected. Patterns analogous to the ones found in agar gels, were obtained with starch-gel-electrophoresis[3].

Distribution of the new isoenzyme among avian orders. In a first series of experiments with pH 8.6 buffer, 2 isoenzyme bands with fast electrophoretic migration typical for the brain enzymes were regularly found in Passeriformes, but also in some Anseriformes and Columbiformes. There was considerable variation of the absolute and relative rates of isoenzyme migration within different species (Figure 2). In birds from other avian orders 2 brain bands were detectable only occasionally. If 2 brain bands were observed, they always had very similar mobilities. In most cases it could not be decided whether only one single band was present or actually 2 bands with almost identical electrophoretic mobility (Figure 2, pheasant). Therefore a different buffer system was used, since it was suspected that the 2 brain enzymes were always present, but not separable with pH 8.6 buffer because of a relatively small difference in net-charge of these proteins. Using pH 6.5 buffer for electrophoresis the 2 isoenzymes were separated in brain extracts of all birds (Figure 3) except a few Psittaciformes.

Altogether some 250 birds belonging to 15 orders have been examined: (8) Podicipediformes, (11) Ciconiiformes, (12) Anseriformes, (13) Falconiformes, (14) Galliformes, (15) Gruiformes, (16) Charadriiformes, (17) Columbiformes, (18) Psittaciformes, (19) Cuculiformes, (20) Strigiformes, (22) Apodiformes, (25) Coraciiformes, (26) Piciformes, (27) Passeriformes. The numbers preceding the names of avian orders refer to the classification of WETMORE[6], who subdivides living species into 27 orders.

Tissue-specific isoenzyme patterns and their appearance in ontogeny. The new isoenzyme always occurred together with the BB-type enzyme. This association has been detected in supernatant fractions from tissue homogenates of brain, heart, stomach and intestine. The portion of the total activity contributed by the 2 isoenzyme bands varied and was characteristic for each organ. In all species examined the faster moving band was more active in brain-extracts, whereas the slower moving band was more pronounced in heart-extracts (Figure 1b, Figure 2). This observation was confirmed by densitometric estimations of the stained enzyme bands.

The new isoenzyme has also been observed in undifferentiated muscle tissue from bird embryos. Early embryonic skeletal muscle from vertebrates generally shows the BB-enzyme[7]. During muscle differentiation changes in relative activities of the 2 structural genes for creatine kinase lead to a shift in isoenzyme distribution towards the pattern of adult muscle, i.e. a predominance of the MM-enzyme. Concomitantly a hybrid enzyme with a subunit composition MB appears at intermediate stages of differentiation[7].

In various bird embryos (domestic duck, domestic pigeon, and 2 Passeriformes, i.e. *Taeniopygia guttata* and canary) the new isoenzyme could regularly be detected together with the BB-enzyme in early stages of embryonic muscle (Figure 4). During muscle differentiation, when the appearance of an MB-enzyme was expected, rather 2 clearly separated enzyme bands appeared in positions between the isoenzymes of adult organs. This brings the number of creatine kinase isoenzymes in birds to a total of 5. When extracts from adult muscle tissue and brain- or heart tissue were combined and dissociated in 8-M urea[2], these 2 intermediate bands could be formed artificially, when the dimeric enzyme was reconstituted by reactivation upon dialysis against *tris*-buffer.

Furthermore a variety of mammals, reptiles, amphibia and fishes were examined. In agreement with other investigators[8] we found only 3 creatine kinases in these

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[2] D. M. DAWSON, H. M. EPPENBERGER and N. O. KAPLAN, Biochem. biophys. Res. Communs. *27*, 346 (1965).
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vertebrates. The occurrence of a double band in heart-tissue has recently been reported for the chick[9].

Subunit structure of the new creatine kinases. Preliminary experiments on purified brain enzymes from birds have previously been published[4]. They indicate that the 2 brain bands may represent 2 thermodynamically possible conformations of the brain enzyme (BB) or its subunits (B) as already suggested in an earlier publication[3]. Alternatively it could be thought of as a third structural gene, coding for subunits X in addition to the subunits M and B. This seems less likely from the experiments reported here, since in the case of 3 different subunits forming dimeric enzymes one would expect a total of 6 isoenzymes. However, 5 isoenzymes only have been found. It could be argued that the sixth theoretically expected combination of subunits is enzymatically inactive or does not form in the cell. At least the first argument can be excluded from dissociation and reaggregation experiments on purified enzymes[4].

From this investigation it is evident that if a third structural gene for creatine kinase has evolved in birds by gene duplication, this must have occurred early in birds' phylogeny since the new isoenzymes have been found in all birds. Therefore sufficient time has allowed for considerable divergence of the 2 genes by mutations.

[9] B. T. HOOTON, Biochemistry 7, 2063 (1968).

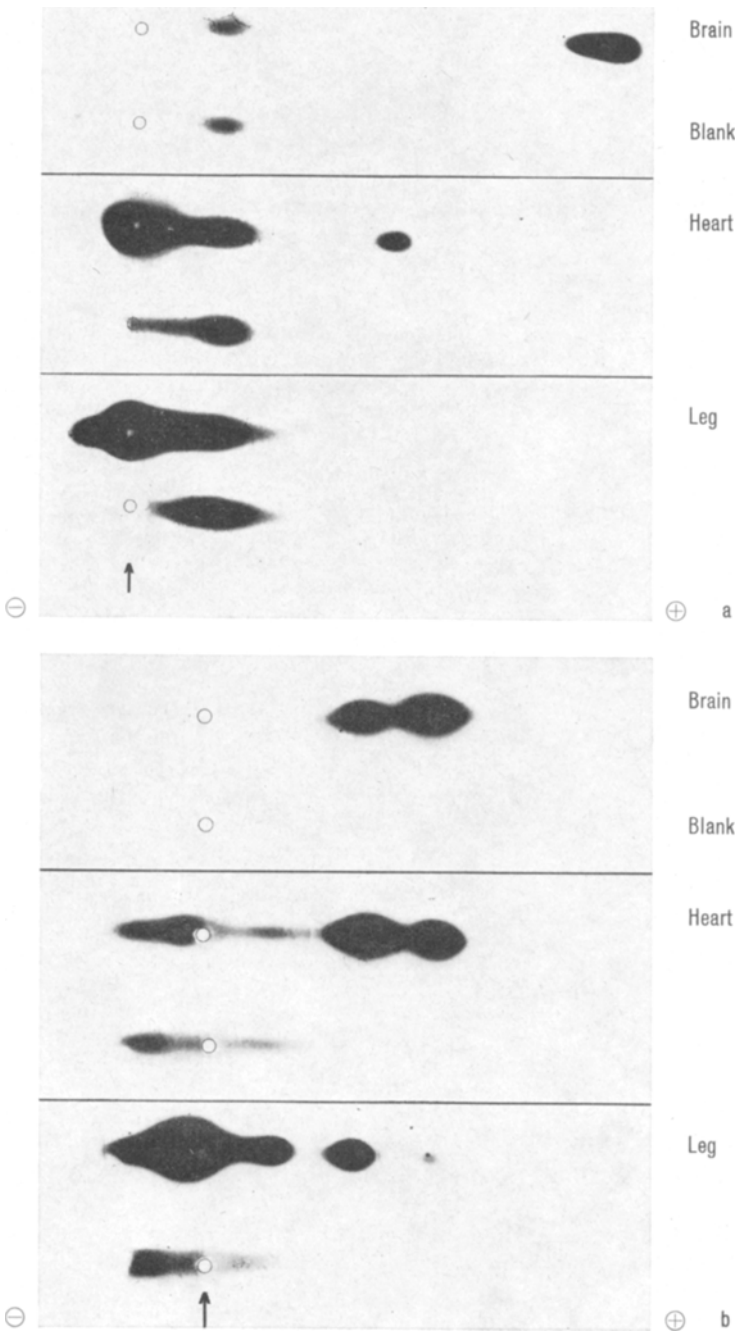

Fig. 1. Electrophoretic patterns of creatine kinases from brain, heart and skeletal muscle from (a) a mammal (laboratory mouse) and (b) a bird (*Taeniopygia guttata*, Passeriformes). Electrophoresis in 1.5% agarose-gels, made up in 0.02 *M* veronalbuffer, pH 8.6 at 40 mA for 60 min. Other explanations see text.

Consequently one might expect differences in primary structure of the creatine kinases. Determinations of amino acid compositions of purified brain enzymes from sparrows however do not indicate significant differences.

It is evident that only experiments on purified enzymes can definitely decide which hypothesis is correct. Such experiments are in progress in our laboratories. If it can be confirmed that the new isoenzymes are different conformations of the brain enzyme or its subunits, it is very likely that this is not a random phenomenon but has physiological implications, since first of all different organs show typical differences in relative amounts of these isoenzymes. Furthermore it is not very probable that a mutation, which only led to a random phenomenon, should persist over a long period of immense morphological and physiological divergence in bird's evolution[10,11].

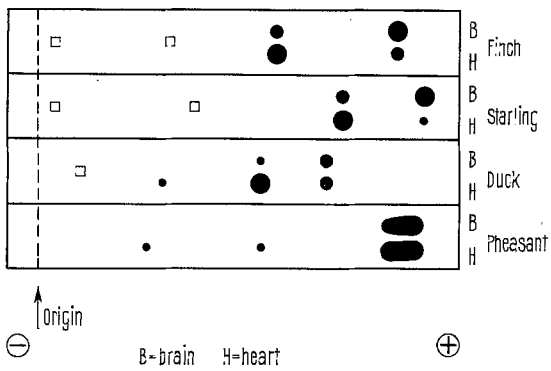

Fig. 2. Tracings of creatine kinase isoenzymes from brain- and heart-extracts of various birds (finch, *Fringilla coelebs*; starling, *Sturnus vulgaris*; duck, *Aythya fuligula*; pheasant, *Chrysolophus pictus*). Conditions of electrophoresis see Figure 1. The relative intensities of isoenzyme bands (compare Figure 1) are indicated by the different size of the spots. The positions of isoenzymes which have been observed in muscle tissue of adult birds are indicated by open squares.

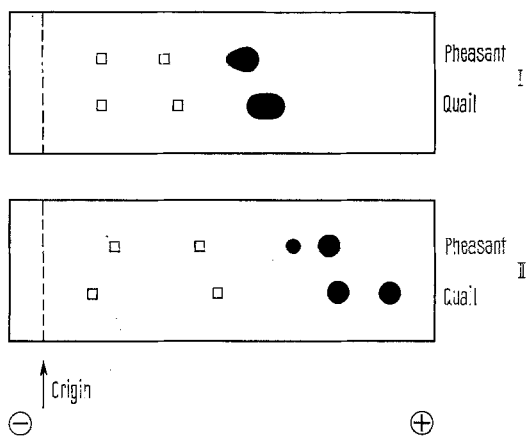

Fig. 3. Electrophoretic separation of creatine kinases in supernatant fractions from brain homogenates in (I) veronalbuffer 0.02 *M*, pH 8.6, 40 mA, 75 min and (II) citrate-NaOH-buffer 0.01 *M*, pH 6.5, 30 mA, 90 min. Pheasant, *Chrysolophus pictus*, quail, *Francolinus francolinus* (Galliformes). The positions of the other isoenzymes (MM/MB) are indicated by squares.

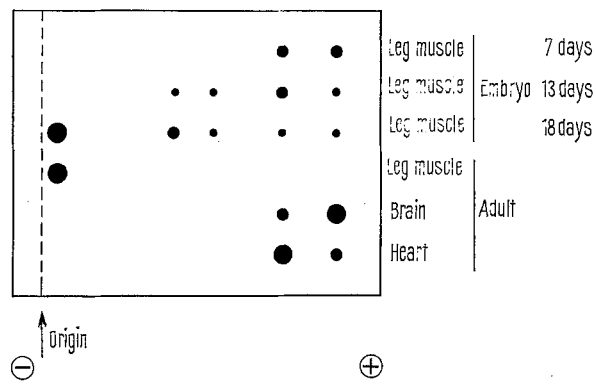

Fig. 4. Ontogenetic changes of isoenzyme patterns in developing leg muscle (domestic duck). The patterns in various organs from the adult duck are added for comparison (lower 3 separations). Conditions as given in Figure 1.

Zusammenfassung. Durch Abwandlung der elektrophoretischen Auftrennungsmethode wird das Vorkommen einer neuen Isoenzymbande der Kreatinkinase, das ursprünglich auf die Vogelordnung Passeriformes beschränkt zu sein schien, allgemein in verschiedenen Geweben von Vögeln nachgewiesen. Eine weitere neue Isoenzymbande wird in bestimmten Entwicklungsstadien der Skelettmuskulatur beobachtet. Es wird diskutiert, dass diese neuen Isoenzyme alternative thermodynamisch mögliche Konformere der Gehirn-Kreatinkinase (BB-Enzym) oder ihrer Monomere (B) sein könnten. Anhand der Untersuchungsergebnisse wird aufgezeigt, dass diesen vermutlich durch Konformationsunterschiede bedingten Isoenzymen physiologische Bedeutung zukommen könnte.

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[11] The technical assistance of Miss Evelyne Mathys is gratefully acknowledged.

Creatine of Mouse Brain: Evidence of Active Uptake from Blood

The existence of a distinct concentration gradient of creatine between plasma and brain in man and animals is known and well documented for some time[1]. Although it was shown more recently that cerebral tissue is capable of synthetizing creatine from suitable precursors[2,3], it is yet to be determined whether intracerebral formation of creatine constitutes the sole source for the brain to replenish its comparatively high content of this compound. To examine the possible contribution of peripheral creatine to the cerebral pool by way of its transfer from blood to brain, we have studied the distribution

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