**References and Notes**

1. G. Kolmodin and C. Skoglund, *Acta Physiol. Scand.* **44**, 11 (1958); R. Granit, J.-O. Kellerth, T. Williams, *J. Physiol. London* **174**, 435 (1964).
2. B. Katz and R. Miledi, *J. Physiol. London* **168**, 389 (1963).
3. J. Eccles, *The Physiology of Synapses* (Springer, Berlin, 1964), pp. 29–36.
4. M. Kuno, *J. Physiol. London* **175**, 81 (1964).
5. R. Granit, *Receptors and Sensory Perception* (Yale Univ. Press, New Haven, 1955), pp. 201–240.
6. J. Eccles, R. Eccles, A. Lundberg, *J. Physiol. London* **137**, 22 (1957).
7. ———, *ibid.* **136**, 527 (1957).
8. V. Wilson and M. Kato, *J. Neurophysiol.* **28**, 545 (1965). This work suggests that the group II muscle spindle afferents may also contribute EPSP's to some extensor motoneurons, but the synaptic length of this pathway is unclear and may well include at least one interneuron.
9. Since this paper was completed, results exactly like those illustrated in Fig. 3 have been obtained from a cell quite definitely identified as a plantaris motoneuron. This cell appeared to be connected monosynaptically with only one spindle in the plantaris muscle, and conduction velocity of the afferent fiber was in the group Ia range.
10. A. Martin, *J. Physiol. London* **130**, 114 (1955).
11. Mnemotron Div., Technical Measurement Corp., CAT 400-B that uses a model 607 peak detector which recognized the large rhythmic synaptic potentials with an accuracy of better than 95 percent.
12. Benson-Lehner Corp., OSCAR model J.



5 November 1965


## Lactate Dehydrogenase of Trout: Hybridization in vivo and in vitro


Abstract. *Speckled trout and lake trout contain five forms of lactate dehydrogenase, but a different electrophoretic distribution of isozymes characterizes each species. The hybrid splake, which is produced artificially by fertilizing lake trout eggs with speckled trout sperm, contains nine isozymes. This complement of isozymes in vivo could be produced in vitro by recombination of subunits from tissues of the parent species. In the splake trout, this complement is the result of heterozygosity at the gene locus responsible for synthesis of LDH-5. Extracts of trout eyes contain at least two additional forms of LDH which could not be demonstrated in other tissues.*


In describing the lactate dehydrogenase isozyme composition of various tissues from the speckled trout, *Salvelinus fontinalis*, I proposed that three active nonallelic genetic loci controlled the synthesis of LDH subunits (*1*). Evidence that requires modification of this hypothesis and provides a firm genetic basis to describe the multiplicity of lactate dehydrogenases in these trout is now presented.

As many as five forms of electrophoretically distinguishable lactate dehydrogenases may be produced in most vertebrates by the random association of two classes of polypeptide subunits (*2*). These subunits are coded by two nonallelic genes (*3*). Polyacrylamide-gel electrophoretograms exhibited nine regularly spaced bands of LDH activity from various tissues of *S. fontinalis* (*1*). In order to fit these data within the framework of the subunit hypothesis it was reasonable to propose that there was active in this species a third cistron coding a third LDH polypeptide subunit. This then would result in the occurrence of three homopolymers of subunit composition, AAAA, BBBB, and CCCC, and of 12 heteropolymers produced by the random combinations of the three polypeptides.

I have now determined that nine LDH isozymes occur in the hybrid (heterozygous) trout, while in the parent (homozygous) species there are the usual five forms of the enzyme. The original data on trout LDH were obtained with fish from the Gaspé Hatchery of Quebec, Canada, and those reported now were obtained with *S. fontinalis*, *S. namaycush* (lake trout), and the hybrid splake from various hatcheries in the United States and Canada.

The lactate dehydrogenase isozymes were separated electrophoretically on polyacrylamide gels. Enzyme activity was localized on the gels as sites of nitro blue tetrazolium formazan deposition. The reaction mixture at *p*H 8.3 contained, in final concentration, 0.1*M* tris (hydroxmethyl) aminomethane, nicotinamide adenine dinucleotide (NAD) (1.3 mg/ml), nitro blue tetrazolium (0.45 mg/ml), 0.05*M* L(+)-lactic acid sodium, and phenazine methosulfate (0.14 mg/ml). The mixture was incubated at 37°C, usually from 5 to 30 minutes, depending upon the activity of the preparation. Isozymes were better separated on a 5-percent polyacrylamide gel rather than on the standard 7½-percent gel.

Organs were removed from freshly killed fish, washed in ice-cold 0.05*M* sodium phosphate buffer at *p*H 7 to remove excess blood, blotted with filter paper, weighed, and then homogenized in an amount of buffer sufficient to make a 10-percent homogenate. Testes were homogenized and then disrupted with high-frequency sound. The extracts were centrifuged at 4°C for

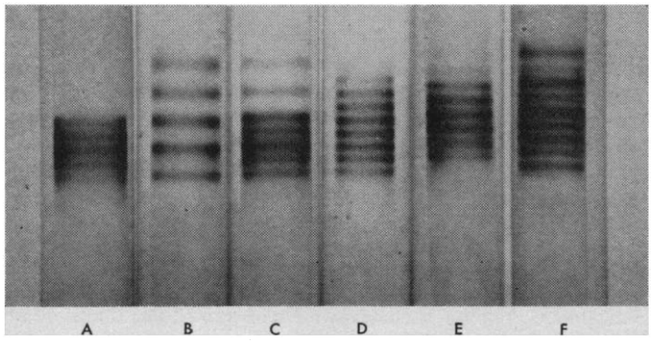


Fig. 1. LDH electrophoretograms from trout. The sample was applied to the top of the gel, and the proteins migrated toward the anode. LDH-1 is the isozyme closest to the anode. Patterns: A, lake trout; B, speckled trout; C, mixture of A and B, demonstrating coincidence of LDH bands; D, splake; E, recombinant isozyme pattern formed by combining A and B and freezing; F, mixture of B and E, demonstrating coincidence of LDH bands.

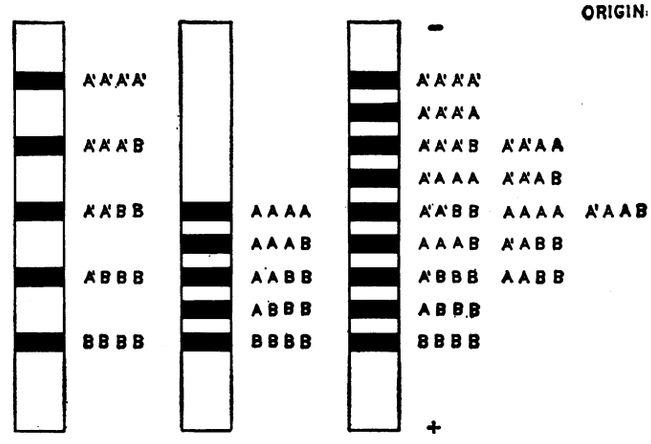


Fig. 2. Diagrammatic representation of LDH isozymes showing proposed subunit composition of (left to right) speckled trout, lake trout, and splake.

20 minutes at 10,000*g*, and the supernatants were used for assay. The LDH activity in the extracts was assayed spectrophotometrically by measuring the oxidation of reduced NAD at 340 m$\mu$ with pyruvate as substrate. Ratios of $NADH_L$ to $NADH_H$ (*4*)—that is, the ratio of LDH activity in the presence of a low concentration of pyruvate to that in a relatively high concentration of this substrate—were determined with $9 \times 10^{-2}M$ and $1.8 \times 10^{-4}M$ pyruvate (final concentrations). This ratio represents a measure of the kinetic characteristics of the tissue isozyme complement and thereby indicates the relative proportion of the different subunits in the preparations. Extracts prepared from heart, muscle, testes, sperm, brain, and eye were assayed spectrophotometrically and electrophoretically. With the exception of the eye, there were no qualitative differences in isozyme patterns of the various tissues. Tissue-specific isozyme patterns were reflected in the differences in relative proportions of the individual isozymes. For example, heart extracts contained more LDH-1 and muscle extracts contained more LDH-5.

The five forms of lactate dehydrogenase from tissues of lake trout (Fig. 1, pattern A) show a different electrophoretic distribution from the five isozymes in speckled trout extracts (Fig. 1, pattern B). In both species, LDH-1, the form that migrates most rapidly to the anode, moves the same distance on the gels. However, wider separation on the gels of the isozymes from the speckled trout suggests that the difference in charge between the A and B subunits is greater in this species than in the lake trout. If the extracts from the two species are combined prior to electrophoresis, it is apparent that LDH-1 of both have the same charge, that speckled trout LDH-2 and lake trout LDH-3 are coincident in migration, and that speckled trout LDH-3 and lake trout LDH-5 also are similarly charged proteins (Fig. 1, pattern C). The mixture, therefore, contains seven separable forms of LDH. In the hybrid splake nine proteins with lactate dehydrogenase activity can be resolved on polyacrylamide gels (Fig. 1, pattern D). This is exactly the number that would be predicted on the basis of the isozyme complements of the parents. It is probable that the gene locus responsible for controlling the synthesis of LDH-1 is

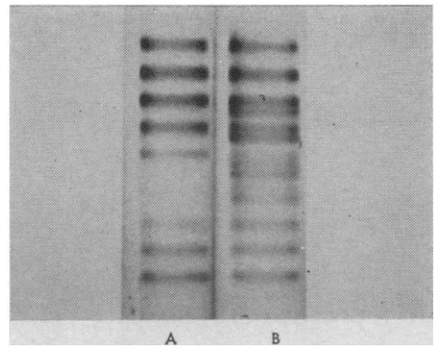


Fig. 3. LDH isozymes in eye tissue of speckled trout. Electrophoretic orientation as in Fig. 1. Pattern A, eye extract; pattern B, mixture of eye extract with recombinant.

common to *S. fontinalis* and *S. namaycush* so that this isozyme has the tetramer composition, BBBB, in both species. One of the parent *a* gene loci may be a mutant producing an *a'* allele in contrast to the *a* allele of the other parent, with LDH-5 representable as A'A'A'A' and AAAA, respectively. The genotype of the lake trout as far as LDH is concerned can be designated as *bbaa* and of the speckled trout as *bba'a'*. The splake then would have an LDH genotype of *bbaa'*, or two gene loci controlling the synthesis of three subunits, B, A, and A'. On this basis subunit compositions may be assigned for each species according to the representation in Fig. 2.

Since we know the subunit composition of the parent-type lactate dehydrogenases, we can be certain of the tetramer make-up of seven of the nine isozymes in the hybrid. The other two forms of the enzyme are assigned subunits by assuming that A and A' combinations are formed. It is possible to test this assumption by a dissociation-recombination experiment in vitro (*5*), and one could predict the formation of the hybrid splake isozyme pattern. In this experiment extracts were prepared from speckled trout and lake trout in $0.1M$ phosphate buffer at *p*H 7.0. Equivalent amounts (in terms of LDH activity) were mixed, made $0.05M$ with respect to NaCl, and slowly frozen and thawed. These results (Fig. 1, pattern E) demonstrate that the LDH complement of splake can be obtained in vitro from a combination of brook and lake trout subunits.

Since the hybrid contains, in effect, three classes of polypeptide subunits, there are 15 isozymes that can be formed in this system. The fact that only nine are demonstrable can also be readily explained. Examination of LDH-3 (A'A'BB) from *S. fontinalis* and LDH-5 (AAAA) from *S. namaycush*, which have the same electrophoretic mobility, suggests that the subunit combination A'B has the same charge in this system as AA. Therefore, by replacing an AA with A'B or vice versa, in the various tetramer combinations known from the experimental data, an additional five forms of LDH of known electrophoretic mobility can be predicted (Fig. 2).

The speckled trout from Gaspé Hatchery, which contained as many as nine LDH isozymes (*1*), were from an isolated, highly selected, inbred population (*6*). The original data were obtained from some 24 individual male and female fish, all showing the same isozyme pattern. The analyses described here were carried out with more than 100 fish from several widely separated hatchery and wild populations, and all but the hybrid had five forms of LDH. Therefore the Gaspé Hatchery trout probably maintained a mutant allele in their genotype (*7*). Preliminary experiments with other closely and distantly related species of Salmonidae indicate that all contain five forms of LDH and that LDH-1 has the same electrophoretic mobility in each. It is possible that the *b* locus is common to all members of this group of fish and, from an evolutionary standpoint, that the *b* gene represents the ancestral type from which has evolved the *a* gene and, consequently, the multiplicity of lactate dehydrogenases in the Salmonidae.

An immediate question is whether the mutant allele in *S. fontinalis* and the multiplicity of lactate dehydrogenases in the artificially produced splake are of functional significance. Some insight into this problem may be obtained from the following values for the $NADH_L$:$NADH_H$ ratios of lactate dehydrogenase activity of heart and muscle extracts of various trout: speckled trout, 1.60 (heart), 0.32 (muscle); lake trout, 1.59 (heart), 0.32 (muscle); and splake, 1.67 (heart), 0.32 (muscle). The higher the ratio, the greater the number of subunits contributed by the heart-type LDH. These results with heart and muscle LDH (*8*) suggest that there is no marked catalytic difference in the LDH (especially of the muscle preparations) as a consequence of the presence of

A and A′, or both, in the tetramers. There would appear to be no advantage in the hybrid's having all types of subunits.

The tissue specificity is particularly striking in extracts of eyes assayed for lactate dehydrogenase activity. There are at least two rapidly migrating isozymes of this enzyme in trout eyes, and they appear to be present as relatively major components (Fig. 3, pattern A). This observation is in agreement with that of Markert and Faulhauber (*9*) that a very negatively charged LDH molecule is present in eye extracts from approximately 30 species of fish. These investigators suggest that there is an independent genetic origin for these isozymes relative to the other forms of LDH in the fish. Such a conclusion is supported by the outcome of the dissociation-recombination experiment presented here, when isozymes which must contain other than an A or B subunit are formed (Fig. 3, pattern B).

Thus the molecular heterogeneity of lactate dehydrogenase in fish is relatively complex compared with the LDH isozyme complement of the more intensively investigated mammalian system (*9, 10*). Some of this complexity is clarified by the unequivocal demonstration here that in the hybrid fish heterozygosity at one genetic locus and co-dominance of alleles at this locus result in the production of three polypeptide subunits which are utilized in the synthesis of homo- and heteropolymers of a single protein. In view of these data it is necessary that I withdraw my original interpretation of the isozyme pattern in trout as representing the activity of three non-allelic genetic loci (*1*) in favor of the alternative explanation provided here. However, the original premise that three types of subunits participate in the synthesis of trout lactate dehydrogenase remains valid.

ERWIN GOLDBERG

*Department of Biological Sciences, Northwestern University, Evanston, Illinois 60201*

**References and Notes**

1. E. Goldberg, *Science* **148**, 391 (1965).
2. C. L. Markert and F. Møller, *Proc. Nat. Acad. Sci. U.S.* **6**, 753 (1959); P. G. Plagemann, K. F. Gregory, F. Wroblewski, *J. Biol. Chem.* **235**, 2288 (1960).
3. C. L. Markert, in *Cytodifferentiation and Macromolecular Synthesis*, M. Locke, Ed. (Academic Press, New York, 1963), p. 65; R. Cahn, N. O. Kaplan, L. Levine, E. Zwilling, *Science* **136**, 962 (1962); D. T. Lindsay, *J. Exp. Zool.* **152**, 75 (1963); C. R. Shaw and E. Barto, *Proc. Nat. Acad. Sci. U.S.* **50**, 211 (1963); S. H. Boyer, D. C. Fainer, E. S. Watson-Williams, *Science* **141**, 642 (1963); W. E. Nance, A. Claflin, O. Smithies, *ibid.* **142**, 1075 (1963).
4. A. C. Wilson, R. D. Cahn, N. O. Kaplan, *Nature* **197**, 331 (1963).
5. C. L. Markert, *Science* **140**, 1329 (1963).
6. J. P. Cuerrier, personal communication.
7. An analysis, completed after submission of this report, of LDH patterns in 60 speckled trout embryos from the Gaspé Hatchery reveals that a mutant *b* gene occurs in this population. Approximately 50 percent of the animals have the five isozymes described in this report, 35 percent show the nine-isozyme pattern described previously (*1*), and 15 percent have five isozymes with an electrophoretic distribution suggesting a new B monomer and therefore a mutant *b* gene. Thus the three LDH genotypes in this population are, *bbaa*, *b'b'aa*, and *b'baa*.
8. N. O. Kaplan, M. M. Ciotti, M. Hamolsky, R. E. Bieber, *Science* **131**, 392 (1960); A. C. Wilson, N. O. Kaplan, L. Levine, A. Pesce, M. Reichlin, W. S. Allison, *Fed. Proc.* **23**, 1258 (1964).
9. C. L. Markert and I. Faulhauber, *J. Exp. Zool.* **159**, 319 (1965).
10. E. Nakano and A. H. Whiteley, *ibid.*, p. 167.
11. Work was supported by NSF grant GB-1771. I thank J. P. Cuerrier, Canadian Wildlife Service, and W. R. Crowe, J. Southwick, and H. Westers of the Michigan Department of Conservation for assistance and cooperation, and Dr. R. C. King for his criticism of the manuscript.

6 December 1965

## Electron Microscopy: Two Major Synaptic Types on Spinal Motoneurons

Abstract. *Two major types of synaptic bulbs are defined on the motoneuron surface of the monkey, on the basis of content of agranular "synaptic" vesicles of two distinct kinds. Both types are present on dendritic as well as perikaryal surface. Because of the approximately equal numbers, the hypothesis that one type is excitatory and the other inhibitory naturally arises.*

In recent years the additional complexities of synaptic structure revealed by the electron microscope have fortunately coincided with the development of fundamental new evidence concerning synaptic function. The evidence for the existence of inhibitory as well as excitatory synapses, of electrical as well as chemical synaptic transmission, of a variety of possible transmitter substances, and of differential electrical properties of postsynaptic elements, whether dendrites, somata, or axons, now offers the histologist an arena of enhanced opportunity for meaningful discovery of functional anatomical relationships.

Whereas important evidence has been obtained which links structural attributes to electrotonic as contrasted to chemical synaptic transmission (*1*), the relationship of structural differentiation to excitatory or inhibitory function is much less clear. A promising lead, based upon differences of the synaptolemma (junctional interface) on dendrites as contrasted to perikaryal surface in the cerebral cortex (*2*), has not subsequently yielded a clear picture of the significance of such differences. Moreover, the distribution of granular synaptic vesicles is not such as to suggest a general correlation with excitatory or inhibitory functions (*3*). Our findings for the spinal cord are therefore of special interest; they reveal a differentiation of two major types of synaptic bulbs on the basis of the morphology of agranular vesicles.

It is probable that the fixative used (2.5 percent buffered glutaraldehyde) made possible a clearer differentiation of types of synaptic bulbs than we obtained in earlier studies with formaldehyde fixation (*4*), but other details of preparation, including hardening with osmium tetroxide, araldite embedding, and staining with lead hydroxide, were the same. Synaptic boutons were characterized according to type of vesicle location on postsynaptic surface (soma, dendrite, or dendritic spine), mode of origin (myelinated or unmyelinated telodendria, or node of Ranvier), size, and relation to subsynaptic cistern or Nissl body. Two major types, well shown in Fig. 1, and two additional types, of infrequent occurrence, were characterized in the motoneuron neuropil. The two major types were found not only on motoneuron dendritic and perikaryal surfaces but also on smaller neurons of the intermediate zone of the anterior gray column. In all locations they tend to be almost equally distributed, but there is a tendency for clustering of each type in any particular location.

One type, which tends to be slightly more numerous, contains agranular vesicles with circular profiles of about 250 to 400 Å, hereafter referred to as S vesicles. The other type contains flattened, elongated, agranular vesicles which may be 100 to 200 Å wide and 300 to 600 Å long (F vesicles). The "F" boutons may contain an admixture of S vesicles, whereas "S" boutons do