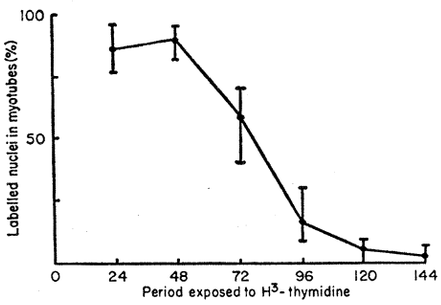


Fig. 2. Cells were treated with labeled thymidine for 24 hours on either the 1st, 2nd, 3rd, 4th, or 5th day of culture. The cells were washed and reincubated in cold medium. All were killed on the 6th day of culture and prepared for radioautography. The percentage of labeled nuclei in myotubes was determined. Each point is the average of three experiments. In each experiment 1000 myotube nuclei were counted.

duce the viability or fragment the chromosomes of some types of cells, whereas it has no demonstrable effect on other types of cells (*11*). The absence of deleterious effects of BUDR in our cultures may be due to the embryo extract in the medium; omission of embryo extract results in reduced survival and decreased mitotic rates.

That the BUDR is not selectively killing presumptive myoblasts was further demonstrated by preparing "second-generation" cultures. Control cultures and cultures treated for 5 days with BUDR were treated with trypsin and replated in normal medium for another 8 days. Second-generation cultures prepared from BUDR-treated cells formed myotubes with the same high frequency as second generation controls. These results suggest that the blockage of myogenesis depends on the incorporation of BUDR into the DNA of the involved cells. Furthermore, if BUDR-inhibited cells are permitted to synthesize new, and presumably normal, DNA their progeny again exhibit the capacity to differentiate into muscle.

The incorporation of BUDR into DNA was demonstrated by gradient centrifugation. Six million cells treated with BUDR for 5 days, and a similar number of control cells, were lysed in 1 ml of distilled water to which cesium chloride was added to produce a 7.7 molal solution. These samples were centrifuged for 24 hours at 44,770 rev/min in a Beckman model E ultracentrifuge. The DNA from untreated cultures had a density of 1.697; DNA from treated cultures, 1.733, the latter band corresponding to double strand replacement. The difference in density was equivalent to a 55 to 60 percent replacement of available thymidine positions, based on the assumption that the volume of the DNA molecule was not changed by substituting Br for the methyl group on thymine and that the DNA was composed of equal amounts of the four bases. After 3 days in culture three bands were detected by ultracentrifugation; presumably the middle band reflects replacement of thymidine by BUDR in a single strand. In other experiments cells were treated with BUDR for 5 days, washed, and then cultured for another 5 days in normal medium. The density of the DNA returned to normal. These findings confirm the biological findings, namely that the progeny of cells which had BUDR in their DNA have normal DNA when they synthesize it in medium lacking the analog.

These experiments are compatible with the possibility that, when half the thymidine positions of the DNA of presumptive myoblasts are occupied by BUDR, some functions fail to emerge (*12*) while others persist. Fusion, synthesis of myosin, and formation of myofibrils are strikingly inhibited, but the cells synthesize DNA and all those specialized molecules required for cell multiplication. It is not clear why certain synthetic functions should be inhibited by altering the structure of the DNA whereas other functions, thought to be under the same kind of genetic control, are not affected. The molecules which regulate cell division are as complex and as dependent on the proper base sequences in the DNA as are those required for cell differentiation (*13*). If the presence of BUDR interferes with proper coding of those genes required for myogenesis, the question arises as to why the BUDR does not interfere with genes regulating the events of cell division.

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## Enolase: Multiple Molecular Forms in Fish Muscle

Abstract. *Starch-gel electrophoresis shows three distinct molecular forms of enolase in each of eight different species of Salmonidae. The three enolases do not appear to be artifacts of isolation, and their electrophoretic patterns are completely reproducible. The patterns are also highly characteristic for each individual species of fish, and together with the overall myogen patterns they represent unequivocal means of taxonomical identification.*

Molecular heterogeneity of specific enzymes isolated from a given source is well known (*1*). We have recently obtained evidence for multiple forms of enolase in the muscle of several species of Salmonidae. Although the cellular origin and the physical and chemical characteristics of the different forms of enolase have not yet been determined, our data show that there are three enolases in each of the species investigated. The species investigated were rainbow trout (*Salmo gairdnerii gairdnerii*), cutthroat trout (*Salmo clarkii*), eastern brook trout (*Salvelinus fontinalis*); and sockeye

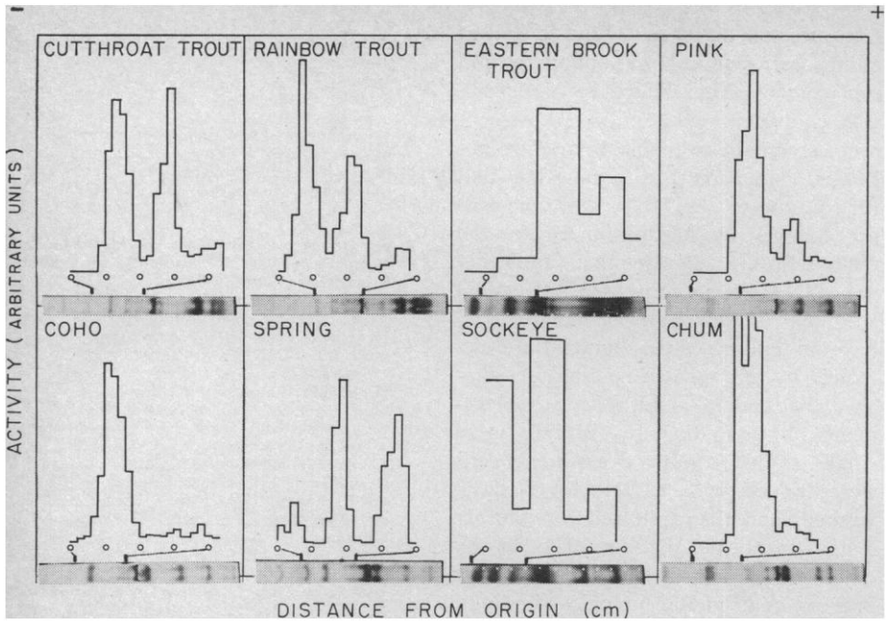


Fig. 1. The distribution of enolase activity after starch-gel electrophoresis of fish muscle extract. Only the central part of the electrophoresis patterns are shown. The circles correspond to the gel markings and indicate the areas which were sliced for elution. All the major bands were tested for enolase activity; but only the regions giving positive tests are shown. The electrophoresis was carried out in 0.023*M* sodium borate buffer, *p*H 8.5, for 12 to 20 hours at 4°C. The gel was stained with amido black. The quantities of enolase obtained from the different mature fish in units per gram of muscle was: cutthroat, 2100; rainbow, 1500; eastern brook trout, 3000; pink, 3400; coho, 1800; spring, 2400; sockeye, 2800; chum, 2700.

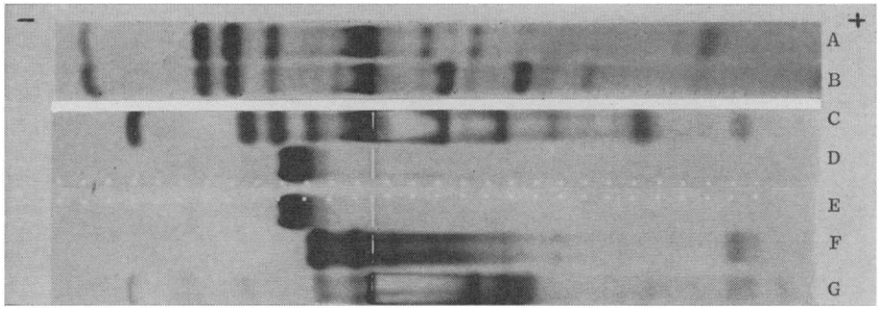


Fig. 2. Electrophoretic patterns: *A*, purified enolase from sockeye salmon (specific activity 320); *B* and *C*, muscle extract from sockeye salmon; *D* and *E*, crystalline rabbit muscle enolase; *F*, purified enolase from rainbow trout (specific activity 380); *G*, muscle extract from rainbow trout. *A* and *B* were run for 20 hours, the others for 13 hours. The conditions are given in Fig. 1.

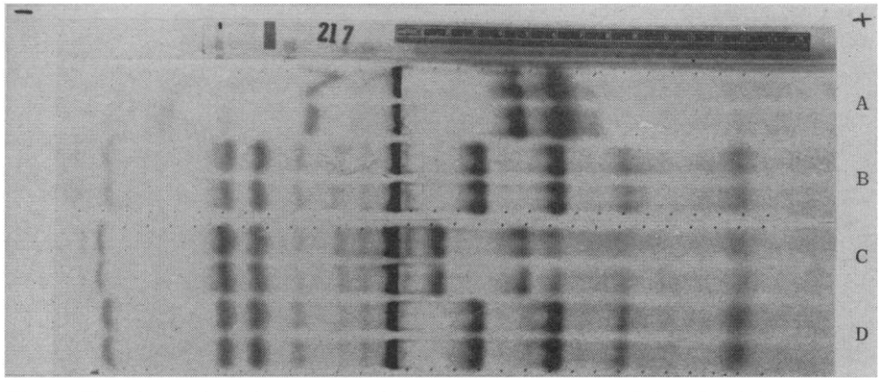


Fig. 3. The electrophoretic patterns of immature salmons. *A*, Coho; *B*, pink; *C*, chum; *D*, sockeye. The conditions are given in Fig. 1.

(*Oncorhynchus nerka*), coho (*Oncorhynchus kisutch*), pink (*Oncorhynchus gorbuscha*), chum (*Oncorhynchus keta*), and spring (*Oncorhynchus tshawytscha*) salmon.

Live fish were obtained from British Columbia coastal waters, rivers, and lakes and were used immediately or kept live in tanks. The fillets were removed from the freshly killed fish in a few minutes (no attempts were made to separate the red and the white muscle), and the flesh was immediately homogenized with 0.05 percent EDTA (ethylenediaminetetraacetic acid) solution (200 ml/100 g of muscle) in a Waring blender at 4°C for 1.5 to 2 minutes. The resulting homogenate was centrifuged at 4°C for 20 to 30 minutes at 10,000*g*; it gave an almost clear muscle extract with high enolase activity. Other extracting solutions, such as 0.05 percent EDTA containing 0.1 percent mercaptoethanol or 1.0 percent magnesium sulfate solutions, were also tested, but they gave much lower yields.

The results of starch gel electrophoresis of muscle extracts of the eight different species of Salmonidae are given in Fig. 1, together with the distribution of the eluted enolase activity in each sample. The activity was measured according to standard methods (*2*, *3*). The equipment used for both the electrophoresis and elution has been described (*4*, *5*). The existence of multiple forms of enolase is obvious. The quantitative ratio of the various enolases appeared to be independent of the method of extraction (time and temperature were the variables investigated), and it has therefore been concluded that the isozymes are not artifacts of isolation.

Simple purification steps (ammonium sulfate fractionation and heat treatment), giving a tenfold purification of the fish enolases, did not cause any significant change in the relative quantities of the different forms of enolase (Fig. 2). The sample of crystallized rabbit muscle enolase (the specific activity of this pure enzyme was 595 units per milligram under the assay conditions used for the fish enolases) was included for comparison. The isoelectric point of rabbit muscle enolase had previously been found to be 5.5 to 6 by boundary electrophoresis (*3*). The observed mobility of all the enolases toward the cathode at *p*H 8.5 (Fig. 2) thus bears no direct relationship to the molecular charge of the proteins.

Figure 3 shows the electrophoretic patterns of the muscle protein of young salmon captured at the spawning area and kept in freshwater tanks for different lengths of time. Since they were prevented from reaching the ocean, their growth was minimum. There are no significant differences in the enolase patterns or in the overall myogen patterns between the juveniles and adult samples of these species. This fact lends further support to the observation that the electrophoretic patterns of muscle proteins are unequivocal as means of classifying the different species of fish (*6*). In contrast, morphometric characteristics vary considerably during development. The same kind of species specific "fingerprints" have also been found for the hemoglobins (*5*).

A possible explanation for the multiple forms of enolase in the different fish could be that they represent molecular aggregates of a common subunit and are separated on the basis of molecular weight differences. To test this possibility, the sucrose density centrifugation shown in Fig. 4 was carried out according to the method of Martin and Ames (*7*). The fact that most of the enolase activity from rainbow trout muscle appeared in a single peak strongly indicates that the major enolases in this species have uniform molecular weight. The results in Fig. 4 do suggest, however, that a very minor fraction of higher molecular weight enolase may also be present in the preparation. The sedimentation coefficient calculated from the results in Fig. 4 was 5.75*S* (with lysozyme, $S_{20,w}$ = 2.15*S* as standard) and 6.15*S* (with catalase, $S_{20,w}$ = 11.2*S* as standard) (*7*). Yeast enolase, with a molecular weight of 67,000 (*8*), and rabbit muscle enolase with a molecular weight of 82,000 (*3*), in a parallel experiment both gave sedimentation coefficients of 6.1 to 6.3*S*, so it appears that the fish enolases have molecular weights very similar to the other two, in the range of 60,000 to 80,000.

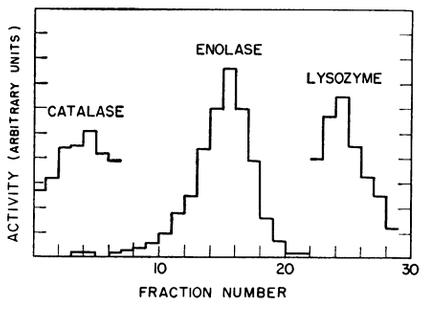


Fig. 4. Sucrose density centrifugation of rainbow trout enolase. A solution of 0.1 ml of muscle extract containing 0.3 mg each of lysozyme and catalase was layered on the sucrose gradient. The sample was centrifuged in the Spinco Model L centrifuge for 20 hours at 38,000 rev/min, and the enzymes were collected and assayed in the usual manner (*7*).

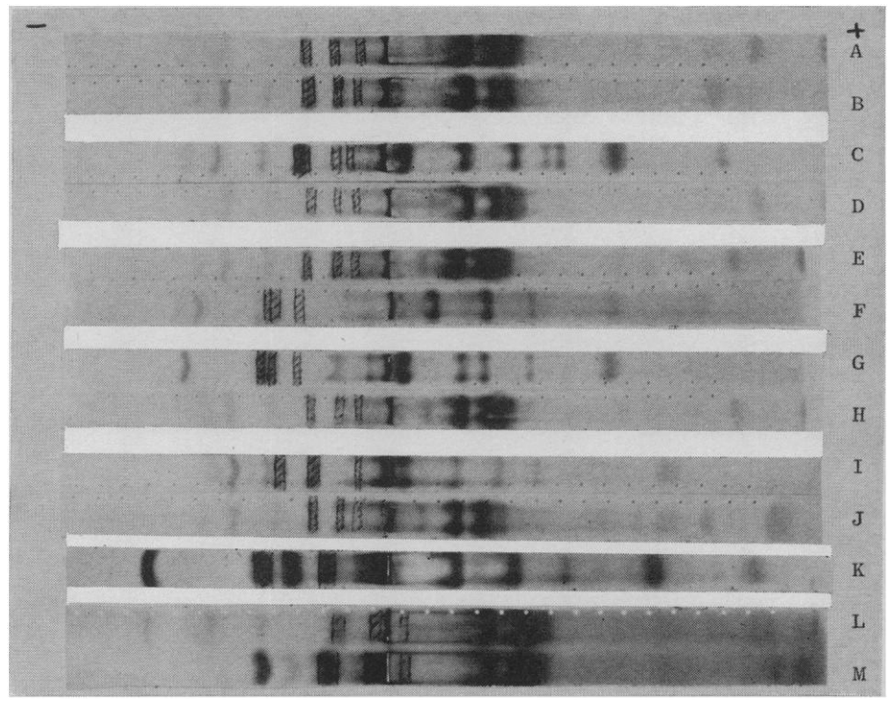


Fig. 5. The electrophoretic patterns of muscle extracts of eight species of Salmonidae. As the various gels were run for different lengths of time, rainbow trout extracts were used as reference samples. The enolase bands are indicated in each pattern. *A*, Rainbow; *B*, cutthroat; *C*, coho; *D*, rainbow; *E*, rainbow; *F*, pink; *G*, chum; *H*, rainbow; *I*, spring; *J*, rainbow; *K*, sockeye; *L*, rainbow; *M*, eastern brook trout. The conditions are given in Fig. 1.

From a comparison of the distribution of enolases in the eight species of fish (Fig. 5), it appears that there is one form of enolase with very similar electrophoretic mobility in all salmonoids, and it is thus possible that they all have this form of enolase in common. Considering all the enolase forms, it is possible to group the fishes. Thus, the rainbow and cutthroat trouts are virtually identical, and Eastern brook trout is also very similar to the other two trout species. In the salmon group, chum, pink, and sockeye have very similar enolase patterns, while the coho pattern looks much more like that of the trouts. Spring salmon occupies a position of its own, showing forms common with both the salmon group and the trout group.

It is generally accepted that the genus *Oncorhynchus* has evolved from the genus *Salmo* (*9*), and Tsuyuki *et al.* have concluded from their muscle protein patterns (*7*, *10*) that spring and coho salmons are closer to the ancestral genus than the other salmons. The enolase patterns reported here are in complete agreement with this conclusion.

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