from rats which had received infusions of both UB and Novobiocin, did not find any interaction between the 2 substances. Therefore, we used Novobiocin to confirm our hypothesis that the passage of UB occurs from the liver cell to the bile.

In our experiments, the total plasma bilirubin concentration was higher in Novobiocin treated rats than in the control group, and the concentration of TB in the bile was lower. Statistical analysis of variance showed that these differences were significant (plasma: $F = 13.8$, $p < 0.01$; bile: $F = 6.7$, $p < 0.02$). If the passage of bilirubin to the bile did not occur through the liver cell, plasma and bile bilirubin concentrations would not be affected by Novobiocin.

Intravenous infusion of CB permits biliary and urinary excretion of UB[12,13]. Our experiments suggest that under certain conditions UB may be excreted in the bile even in the absence of CB[14].

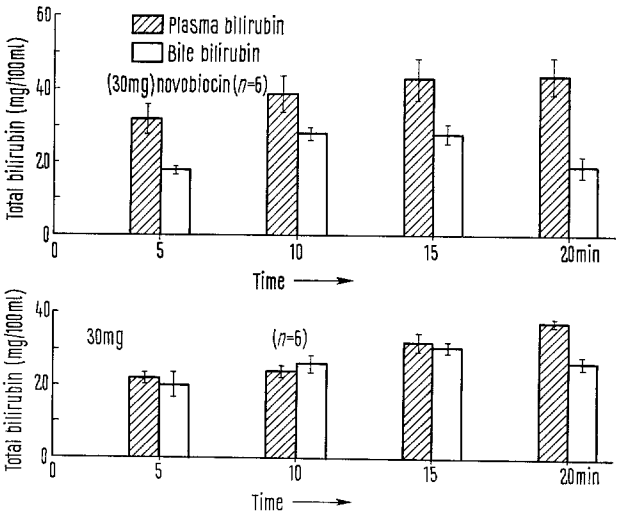

Fig. 2. Gunn rats. Infusion of 30 mg of unconjugated bilirubin. Mean changes of total plasma and total bile bilirubin after i.v. infusion of unconjugated bilirubin (below) and unconjugated bilirubin + Novobiocin (above). Vertical bars indicate standard errors.

Résumé. L'absence d'un T_m lors de perfusion en quantité croissante de bilirubine non conjuguée chez le rat Gunn, ainsi que la diminution de la concentration biliaire de la bilirubine totale lors d'infusion simultanée de bilirubine non conjuguée et de Novobiocine, suggèrent la possibilité d'un transfert passif de la bilirubine non conjuguée du sang dans la bile, à travers la cellule hépatique.

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Genetic Variation of Supernatant and Mitochondrial Malate Dehydrogenase Isozymes in the Teleost *Fundulus heteroclitus*

The study of isozymes has provided considerable insight into the subunit structure of enzymes[1] as well as providing gene markers for the analysis of problems in development[2] and evolution[3]. The isozyme system of malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37) is represented by 2 major forms in vertebrates and invertebrates: 1) supernatant malate dehydrogenase (S-MDH), and 2) mitochondrial malate dehydrogenase (M-MDH). In addition to their different subcellular locale these MDHs differ in their electrophoretic mobility[2], kinetic behavior[4], amino acid composition[5], and antigenic properties[6]. Both the S-MDH and M-MDH of vertebrates have been shown to exist as dimers by in vitro molecular hybridization[7] and allelic isozyme variation[8–10]. Malate dehydrogenases exist as conformational isozymes in some organisms[1,11].

The malate dehydrogenase isozymes of *Fundulus heteroclitus* were investigated because this species is potentially valuable for studying developmental and biochemical genetics. The present paper describes the malate dehydrogenase isozymes of *F. heteroclitus*, their subcellular distribution and discusses their possible genetic and molecular bases.

Methods. Sexually mature *F. heteroclitus* of both sexes were trapped from Mill Pond in Woods Hole, Massachusetts. The subcellular distribution of MDH isozymes from freshly killed *F. heteroclitus* was determined by differential centrifugation. Fresh unfrozen livers were homogenized in a loose-fitting Dounce homogenizer, in 2 volumes of 0.25 *M* sucrose in 0.1 *M* *Tris*-HCl, pH 7.7. Centrifugation was carried out in an SS-34 rotor in a Sorvall RC 2-B at 4 °C. Unbroken cells, cell fragments, and nuclei were removed by 480 *g* for 5 min. The mitochondria were precipitated by 12,100 *g* centrifugation for 10 min. The supernatant resulting from centrifugation at 105,000 *g* for 40 min contains the supernatant MDH (S-MDH). The isolated mitochondria were twice washed and subjected to 12,100 *g* for 10 min and then Dounce-homogenized in one volume of 0.1 *M* *Tris*-HCl, pH 7.0 followed by centrifugation at 105,000 *g* for 30 min. This supernatant contains the malate dehydrogenase released from the mitochondria (M-MDH).

The skeletal muscle from each of 245 frozen *Fundulus* was homogenized in a Dounce homogenizer in one volume of 0.1 *M* *Tris*-HCl, pH 7.0, centrifuged at 48,000 *g* for 30 min at 4 °C prior to the electrophoreses used for the population analysis.

All electrophoreses were performed in a 14% vertical starch gel at pH 6.9. The stock buffer was 0.75 *M* *Tris* + 0.25 *M* citric acid (monohydrate) adjusted to pH 6.9.

This stock buffer was diluted 1:60 for the gels and 1:20 for the electrodes. Electrophoresis was at 12 V/cm for 18 h at 4 °C. The gel was sliced and the isozymes visualized by incubating the gel at 37 °C in the dark in the following stain: 60 ml of 0.2 *M* pH 8.0 *Tris*-HCl, 12 ml of 0.5 *M* sodium malate, 6.7 ml of 1 mg/ml nitroblue tetrazolium (NBT), 6.7 ml of 1.6 mg/ml phenazine methosulfate (PMS), and 2.7 ml of nicotinamide adenine dinucleotide (NAD).

Results and discussion. The differential centrifugation reveals that the mitochondrial MDH isozymes migrate more anodally during electrophoresis than the supernatant MDH isozymes. The relative electrophoretic mobility of the *Fundulus* S-MDH and M-MDH isozymes is reversed compared to that for most vertebrates (Figure 1). This reversal has also been observed in sea urchins[12] and tuna[13], but does not occur in all fish, e.g., salmon[10]. The physiological significance of this electrophoretic reversal of mobility is not known.

The 3 different S-MDH phenotypes representing the allelic variation at the supernatant MDH locus are shown in Figure 1, left. The fast phenotype represents the normal homozygote, whereas the hybrid phenotype represents the heterozygote, and the slow phenotype represents the homozygous mutant. The number and electrophoretic mobility of the intermediate isozymes are altered concomitantly with the genetic alteration of the electrophoretic mobility of the cathodal S-MDH isozymes.

There are several hypotheses which can be brought forth to explain the S-MDH isozyme patterns of *Fundulus*.

One possibility is that there are 2 functioning S-MDH loci and that the subunits produced by each S-MDH locus (A and B) have a different net charge such that the anodal S-MDH isozyme (BB) coincides in electrophoretic mobility with the M-MDH isozymes encoded in a different locus (Figure 2, hypothesis 1). The genetic alteration of the electrophoretic behavior of the S-MDH isozymes (AA, AA′, A′A′) also simultaneously alters the number and electrophoretic mobility of the intermediate isozymes (BA and BA′). This observation suggests that 2 S-MDH subunit types (A and B) randomly assemble to form all possible dimers, AA, AB, and BB, when both loci are homozygous. However, if one of the loci is heterozygous for 2 different codominant alleles, e.g., A and A′, 6 dimeric isozymes should be seen for *Fundulus* (Figure 2, hypothesis 1). Indeed, the 3 S-MDH isozyme phenotypes in *Fundulus* are very similar to the S-MDH isozyme patterns of trout where 2 S-MDH loci have been reported[10], and allelic variation occurs at one of these loci.

Another interpretation of the isozyme phenotypes can also be illustrated schematically by hypothesis 1 of Figure 2. *Fundulus* would possess only 2 MDH loci as in most vertebrates, one for M-MDH (B), and the other for S-MDH (A). In addition to the 2 homopolymers synthesized (AA and BB), a hybrid enzyme (AB) composed of one S-MDH subunit and one M-MDH subunit is formed (Figure 2, hypothesis 1). Hybrid isozymes such as AB have been formed between supernatant MDH isozymes and mitochondrial MDH isozymes by in vitro molecular hybridization[7] which suggests that such hybrids may be formed intracellularly in some organisms.

The last hypothesis (Figure 2, hypothesis 2) is that the S-MDH isozymes can exist as dimers or monomers. Because of the molecular sieving effect of the starch gel the dimers should be more retarded in their electrophoretic mobility than the monomer. The 2 intermediate S-MDH isozymes (A and A′) in the heterozygote would represent the monomeric polypeptide contribution of 2 different codominant alleles.

The mitochondrial malate dehydrogenase isozyme phenotypes (Figure 1, center) are attributed to allelic variation at the M-MDH locus. In the hybrid (postulated heterozygote) the anodal M-MDH isozyme has diminished in intensity and a new isozyme (mit.) has appeared more cathodally. A homozygous mutant M-MDH was not detected in the 245 *Fundulus* examined presumably because of the low gene frequency. The double hybrid

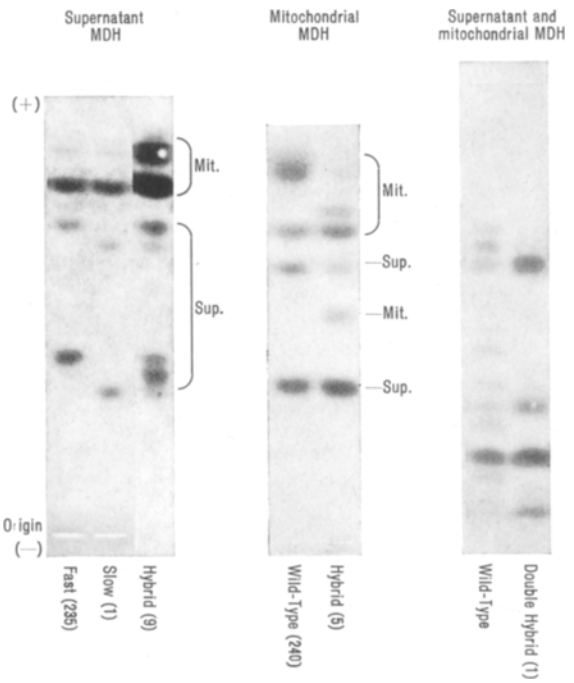

Fig. 1. Malate dehydrogenase (MDH) isozyme phenotypes from skeletal muscle extracts of *Fundulus heteroclitus*. Mit., isozymes of mitochondrial origin; sup., isozymes of supernatant origin. Left: Supernatant MDH isozyme phenotypes; fast (homozygote normal), slow (homozygote mutant), and hybrid (heterozygote). Center: Mitochondrial MDH isozyme phenotypes; wildtype (homozygote normal) and hybrid (heterozygote). Right: Double hybrid (double heterozygote), allelic variation at both the S-MDH locus and the M-MDH locus.

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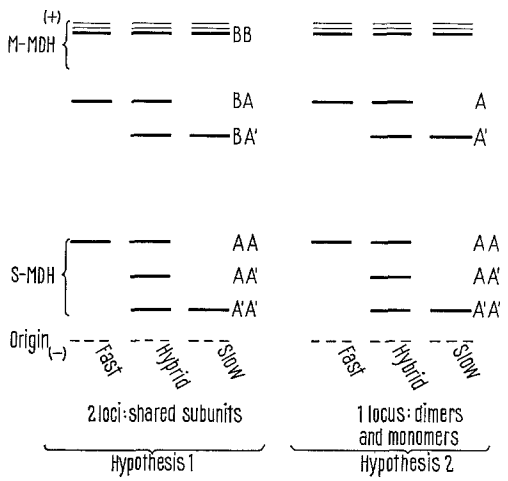

Fig. 2. Subunit composition of variant supernatant malate dehydrogenase (S-MDH) isozymes. Left: Hypothesis 1 a) both A and B subunits are encoded in duplicate S-MDH loci. The BB S-MDH isozyme coincides in electrophoretic mobility with the M-MDH isozyme or b) B subunits are encoded in the M-MDH locus and A subunits are encoded in the S-MDH locus. The appearance of BA or BA' isozymes represent sharing of subunits from different loci. Right: Hypothesis 2. The S-MDH may exist as both dimers and monomers (with the dimers possessing slower mobility because of sieving action of the gel).

phenotype (Figure 1, right) was only observed in one fish which was probably heterozygous at *both* the S-MDH locus and the M-MDH locus.

In conclusion, *F. heteroclitus* has codominant allelic variants at both the S-MDH locus and the M-MDH locus. The genetic variants encoded in both loci are evenly distributed among males and females which suggests that they are autosomally inherited. The high degree of isozyme polymorphism observed for malate dehydrogenase has also been observed for other *Fundulus* isozymes, e.g., lactate dehydrogenase[3,14] and esterases[15]. Thus, *F. heteroclitus* appears to be an excellent organism to employ for investigating the linkage relationships of genes encoding these isozymes[16].

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Active Transport in the Rabbit Blastocyst

The rabbit embryo begins to accumulate fluid to form a central cavity between 3 and 4 days after mating. Initially this process is slow but the efficiency of transport increases with time so that by the ninth day each cell transports about 20 times its own volume of fluid every hour[1]. During the earlier stages of development, fluid within the blastocyst contains high concentrations of potassium and bicarbonate and low concentrations of sodium and chloride relative to serum, but later the concentrations of all 4 ions approach those found in serum[2–4]. The present work uses cultured blastocysts to study some of the factors controlling water and ion movements across this tissue.

New Zealand white rabbits weighing 2–3 kg were killed by the i.v. injection of pentobarbiton sodium, exactly 6 days after mating. The uterus was removed and the blastocysts exposed by blunt dissection. Each blastocyst was placed on a platinum loop to be weighed on a sensitive torsion balance (0–50 $\pm$ 0.1 mg) before being transferred to 1 ml tissue culture medium '199' (Glaxo Labs. Ltd., Greenford, England) containing an additional 12 m*M* NaCl. The culture medium was maintained at 37 °C and gassed with 95% air + 5% CO_2. Blastocysts were later removed, weighed and samples taken for analysis. To analyze for bicarbonate the blastocyst was punctured and 10–20 µl fluid sucked immediately into a Natelson microgasometer. The sample was sealed with mercury, its volume read and the bicarbonate content then determined in the usual way. Other samples were analyzed for sodium and potassium by flame photometry (Evans Electro Selenium EEL) after suitable dilution with distilled water. Chloride determinations were carried out by micro-titration against silver nitrate using a potentiometric method to detect the end point[5]. Particular care was taken to obtain and dilute samples as soon as possible after removing blastocysts from the culture medium to avoid errors caused by evaporation.

The blastocyst being a closed system must produce cells by mitosis to provide space for transported fluid. The contribution new cells make to total fluid transport can be allowed for by assuming that both the cell size and water influx per unit surface area remain constant during culture. Then the total influx into a spherical blastocyst of radius r is $4\pi r^2 f$, where f is the constant influx per unit surface area. The radius increases at a constant rate and the time needed for the blastocyst to double its volume (the doubling time) can be calculated as $0.26/f \cdot r_0$, where r_0 is the initial radius. It was decided to use the doubling time as a measure of the rate of fluid transport. This time did depend however on r_0 and therefore on the initial size of the blastocyst. For blastocysts weighing from 5–25 mg, taken 6 days after mating and cultured for 7 h, the doubling time varied from 8.2 to 12.2 h. The correlation between the initial weight and doubling time was significant ($r = 0.56$, $P < 0.05$, $t = 2.8$, 19 observations). Errors from this source were minimized by ensuring that blastocysts chosen for each experimental series represented the whole range of initial weights.

Figure 1 shows the time course of blastocyst expansion in culture medium over an 8-h period. The calculated doubling time rose from 4 h, for a 1-h incubation, to 10 h

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