AN INVESTIGATION OF THE METABOLIC PATHWAYS OF GLUCOSE AND THEIR POSSIBLE REGULATION BY LACTATE DEHYDROGENASE IN THE OCULAR TISSUES OF RAINBOW TROUT (Salmo gairdneri)

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ABSTRACT

AN INVESTIGATION OF THE METABOLIC PATHWAYS OF GLUCOSE AND THEIR POSSIBLE REGULATION BY LACTATE DEHYDROGENASE IN THE OCULAR TISSUES OF RAINBOW TROUT (Salmo gairdneri)

By

Douglas B. Fonner

Pathways of glucose metabolism were investigated histochemically in rainbow trout (Salmo gairdneri) lens, cornea, and retina. Tissues were frozen in a 37.5 gm% (w/v) aqueous mixture of gum acacia, sectioned at 6-16 microns in a microtome cryostat, then incubated in a staining medium containing the respective substrate, coenzyme, and nitro blue tetrazolium chloride salt as the final electron acceptor. The presence of reduced tetrazolium salt as blue formazan granules was indicative of enzyme activity.

The kinetic characteristics of the lactate dehydrogenases (LDH) from the same ocular tissues were investigated to see if they support the hypothesis that the enzyme may be a regulator of glucose metabolism in these tissues. LDH assays were done spectrophotometrically by measuring the oxidation or reduction of the pyridine nucleotide

coenzymes with the respective substrates, i.e. pyruvate and lactate.

The histochemical identification of glucose-6-phosphate and 6-phosphogluconate dehydrogenases in the lens epithelium; corneal epithelium, endothelium, and fibroblasts; and inner retinal layers is evidence of the presence of the phosphogluconate oxidative pathway (pentose shunt). The glycolytic pathway, indicated by LDH, was found in the lens epithelium and fibers; the corneal epithelium, endothelium, and fibroblasts; and the various layers of the retina, with the photoreceptors showing the highest activity. Based on the distribution of malate, isocitrate, and succinate dehydrogenases, the only structures in which the citric acid cycle could be positively identified were the photoreceptor ellipsoids.

The high temperature characteristic for retinal LDH (13,320 ± 234 calories), combined with a low Michaelis constant (Km) (5.3×10^{-5}M pyruvate) and low pyruvate concentration necessary for substrate inhibition (8.3×10^{-4}M pyruvate) favor non-glycolytic glucose metabolism in the retina. The pyruvate and other intermediate products of the glycolytic pathway are probably shunted to the citric acid cycle instead of forming lactate.

Since the lens and cornea are avascular and presumably have a low metabolic demand, a major portion

of their energy requirement is probably derived from glycolysis. Unlike the LDH from skeletal muscle, a tissue which has a high rate of glycolysis, lens and cornea LDH have relatively high temperature characteristics (9,722 ± 417 and 9,476 ± 211 calories respectively), low Km values (9.3×10^{-5}M and 12.0×10^{-5}M pyruvate respectively), and are inhibited by low pyruvate concentrations (4.5×10^{-4}M). Since pyruvate has been reported to stimulate the pentose shunt, a hypothesis is given whereby under certain circumstances, pyruvate inhibition of LDH in these tissues would in turn stimulate the pentose shunt.

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Dedicated to my Parents, whose moral and financial support is making my education possible.

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INTRODUCTION

It is well known that in animal tissues glucose may be utilized by numerous metabolic pathways. The predominance of any one pathway is dependent upon the metabolic demands as well as the chemical environment of the tissues. Glycolysis in many tissues is inhibited by the presence of oxygen, "Pasteur effect," and in a few tissues a high glucose concentration is known to inhibit respiration, "Crabtree effect." Governing the regulation of these metabolic pathways are numerous product and metabolite feedback systems. One such mechanism that has received increased interest in the past few years is the inhibition of lactate dehydrogenase by high concentrations of pyruvate. The possible physiological significance of this substrate inhibition has only recently been realized.

Due to the avascularity of the lens and cornea and high metabolic rate of the retina, ocular tissues in general present many special problems for glucose metabolism that do not occur in other tissues. The purpose of this study was to answer the following questions concerning glucose metabolism in the teleost lens, cornea, and retina:

1. What portions of the ocular tissues are metabolically active?
2. What metabolic pathways are present for the metabolism of glucose?
3. What lactate dehydrogenase isozymes are present and do they differ from those found in non-ocular tissues?
4. What are the kinetic characteristics of the various lactate dehydrogenases and can they be correlated with the environment of the tissues?
5. Do the kinetic properties of lactate dehydrogenase support the hypothesis the enzyme may be a regulator of glucose metabolism in these tissues?

Histochemical techniques were employed to investigate the first two questions. Nitro blue tetrazolium chloride (nitro BT) was used to accept electrons (from appropriate substrates) as they passed through the various cytochromes to molecular oxygen. Enzyme activity was indicated by the precipitation of reduced tetrazolium salt as blue granules of diformazan. This is a rapid qualitative method of determining the presence and localization of particular enzymes.

The following pathways and dehydrogenases (Figure 1) were examined in the lens, cornea, and retina of rainbow trout (Salmo gairdneri).

Figure 1. Glucose metabolic pathways that were investigated histochemically in the rainbow trout (Salmo gairdneri) ocular tissues. The numerals denote the following enzymes that were stained for in this investigation.

1. Sorbitol dehydrogenase
2. Glucose-6-phosphate dehydrogenase
3. 6-Phosphogluconate dehydrogenase
4. Lactate dehydrogenase
5. Malate dehydrogenase
6. Isocitrate dehydrogenase
7. Succinate dehydrogenase
8. Xylitol dehydrogenase

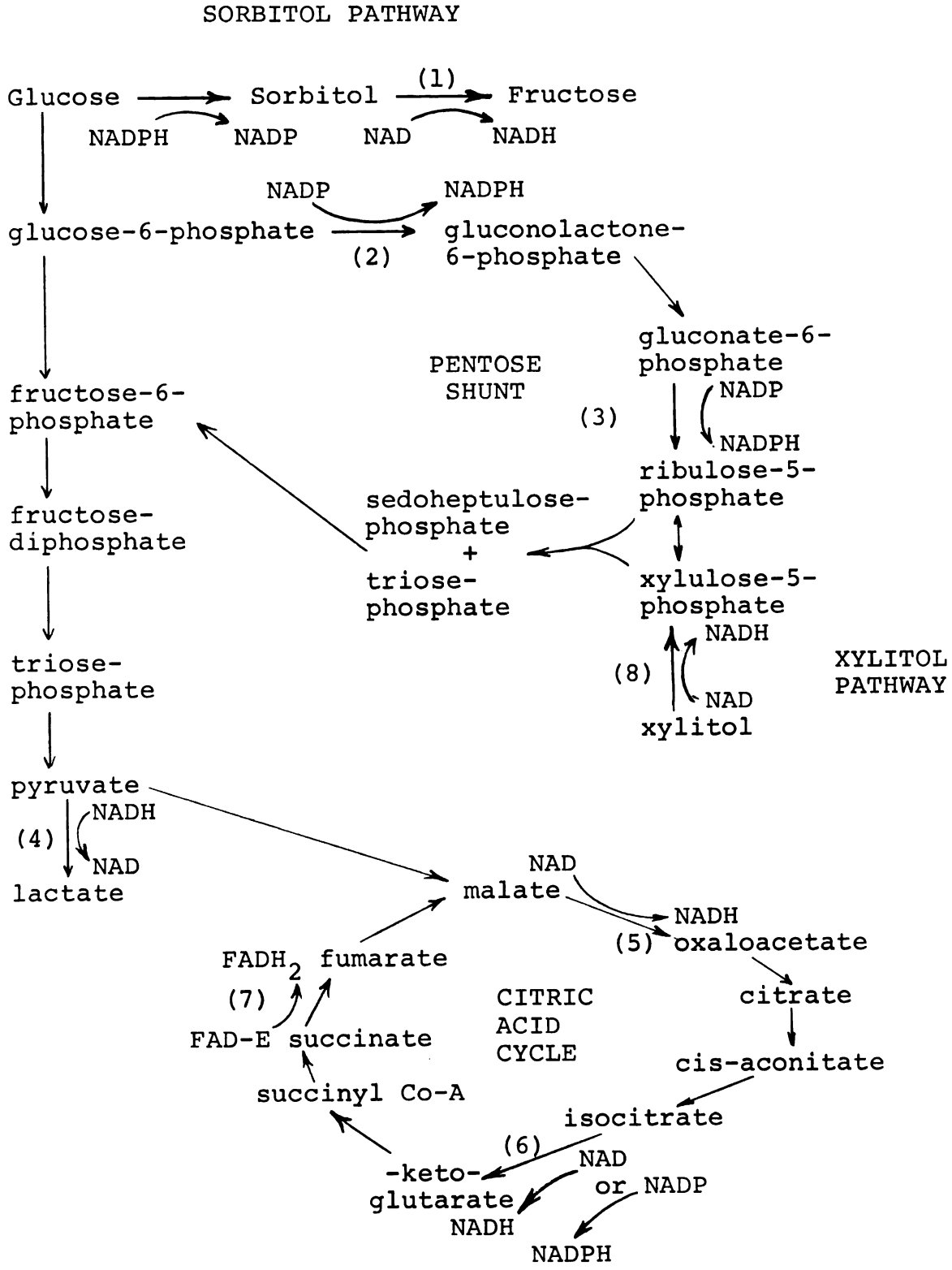

SORBITOL PATHWAY
Glucose
Sorbitol
(1)
Fructose
NADPH
NADP
NAD
NADH
NADP
NADPH
glucose-6-phosphate
(2)
gluconolactone-6-phosphate
gluconate-6-phosphate
PENTOSE SHUNT
NADP
(3)
NADPH
fructose-6-phosphate
ribulose-5-phosphate
sedoheptulose-phosphate
+
triose-phosphate
fructose-diphosphate
xylulose-5-phosphate
NADH
triose-phosphate
(8)
NAD
XYLITOL PATHWAY
xylitol
pyruvate
NADH
(4)
NAD
lactate
NAD
malate
NADH
(5)
oxaloacetate
FADH2
fumarate
(7)
CITRIC ACID CYCLE
citrate
FAD-E
succinate
cis-aconitate
succinyl Co-A
isocitrate
(6)
NAD
-keto-glutarate
or NADP
NADH
NADPH

Figure 1

1. Glycolytic Pathway (Emden-Meyerhof Pathway)
    a. Lactate dehydrogenase
2. Citric Acid Cycle (Krebs Cycle or Tricarboxylic Acid Cycle)
    a. Succinate dehydrogenase
    b. Malate dehydrogenase
    c. Isocitrate dehydrogenase
3. Phosphogluconate Oxidative Pathway (Pentose Shunt)
    a. Glucose-6-phosphate dehydrogenase
    b. 6-Phosphogluconate dehydrogenase
4. Sorbitol Pathway
    a. Sorbitol dehydrogenase (Polyhydric alcohol dehydrogenase)
5. Xylitol Pathway
    a. Xylitol dehydrogenase (Polyhydric alcohol dehydrogenase)

LDH isozymes were separated electrophoretically in polyacrlyamide gel columns. LDH isozymes from the ocular tissues were then compared with the isozymes from the other tissues of the trout.

Kinetic studies on the lactate dehydrogenase were done to determine the temperature characteristics, Michaelis constants (Km), pyruvate (substrate) concentration threshold necessary to produce inhibition, and specific activity. Assays were done spectrophotometrically

by measuring the rate of conversion of nicotinamide adenine dinucleotide (NAD) to its reduced form (NADH) with lactate as the substrate; or conversely the formation of NAD from NADH with pyruvate as the substrate. Km values were also determined using reduced nicotinamide adenine dinucleotide phosphate (NADPH).

The kinetic characteristics give an idea of the expected activity of the enzyme in the tissue. Since the "temperature characteristic" (μ) is a close approximation of the "energy of activation," this can be used to predict the relative turnover rates of the different isozymes; the less energy required by the substrate molecules to react, the higher the expected turnover rate. The Michaelis constant (Km), along with the threshold pyruvate concentration necessary to inhibit LDH, gives the optimum substrate concentrations for the LDH isozymes. A low Km value combined with a low pyruvate concentration needed to produce inhibition would show the enzyme is unable to efficiently catalyze high concentrations of pyruvate to lactate. Conversely low concentrations of pyruvate would be efficiently catalyzed to lactate. These characteristics, along with the specific activity of the enzyme, must all be taken into account when analyzing LDH to see if it may be a regulator of glucose metabolism.

LITERATURE REVIEW

Lens Metabolism

There is much evidence that mammalian lenses are able to anaerobically metabolize glucose. By studying the metabolism of various intermediate products of glycolysis, Green, Bocher, and Leopold (1955a, 1955b) clearly showed that one such route of metabolism is the Emden-Meyerhof pathway.

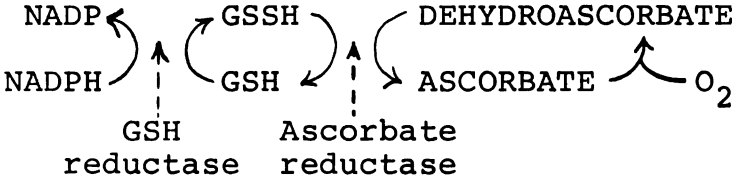
Ely (1951) found the enzymes necessary for the citric acid cycle were present in a homogenate of bovine lenses. This, along with the identification of malic and citric acids in the lens (Krause and Stack 1939) supported the idea that the citric acid cycle was responsible for the oxygen utilized by the lens.

Because of the inability of the lens to oxidize 2-C^{14} pyruvate to $C^{14}O_2$, Kinoshita (1955) reported the citric acid cycle was relatively inactive in the lens. By following the reduction of NADP in the presence of the respective substrates, he found both glucose-6-phosphate and 6-phosphogluconate dehydrogenases were present in a homogenate of lenticular capsule and epithelium; he postulated that the pentose shunt, instead of the citric

acid cycle, was the aerobic pathway of significance. But the reportedly low activity of glucose-6-phosphate dehydrogenase in comparison to malate and lactate dehydrogenases failed to support this hypothesis (Wortman and Becker 1956). It must be noted though, that the investigators of this latter study did not measure the rate limiting enzymes of the respective pathways.

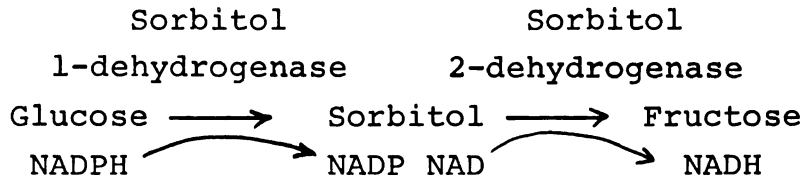
Lenses incubated with C^{14} glucose provided further evidence of the minor role of the citric acid cycle in this tissue. Kinoshita and Wachtl (1958) showed the C-1 atom is metabolized preferentially over the C-6 atom in an atmosphere of 7% oxygen. From this data and information on lactate production, they concluded that approximately 90% of the glucose was metabolized via the glycolytic (Emden-Meyerhof) pathway and 10% via the pentose shunt.

Because the pentose shunt enzymes are cytoplasmic rather than mitochondrial, and based on the reported high levels of glutathione (GSH) and ascorbic acid present throughout ocular tissues, Kinoshita (1955) postulated an electron transport system independent of the mitochondrial cytochrome system. In this system NADPH is used to reduce "oxidized glutathione" (GSH) to "reduced glutathione" (GSSH) via glutathione reductase (Eq. 1).



(1)

In a review of lenticular metabolism, Kinoshita (1965) discussed the possible role of the sorbitol pathway previously identified in several mammalian lenses. Since NADPH, from the pentose shunt, cannot be utilized by the cytochrome oxidase system, the sorbitol pathway may be a transhydrogenase system in which NADPH is utilized and NADH produced (Eq. 2).



(2)

Epithelium and cortex are primarily responsible for the oxygen utilized by the lens. Ely (1949) noted oxygen uptake by the lens increased two to four fold if the capsule was ruptured. Oxygen uptake by the lens capsule and nucleus were reportedly too low to measure. The capsule apparently is able to metabolize glucose-6-phosphate, fructose-1-6-diphosphate and glucose. Adenosine-5'-triphosphate (ATP) must be presented though in order to metabolize glucose (Dische and Ehrlich 1955). Wortman and Becker (1956) reported enzymes involved in glycolysis, the citric acid cycle, and pentose shunt were present in the lens capsule. Malate and lactate dehydrogenase activities were approximately three times higher in the lens fibers than in the capsule and attached epithelium but glucose-6-phosphate dehydrogenase had a

higher activity in the capsule and epithelium than the lens fibers.

Corneal Metabolism

Duane (1949), in a review article on corneal metabolism, reported that Fisher had estimated the oxygen uptake of corneal epithelium and endothelium was 3.5 to 4.5 times higher than the whole cornea and that both layers showed considerable aerobic and anaerobic glycolysis. Lee and Hart (1944) reported rat corneal epithelium rapidly took up oxygen and accounted for the major portion of the oxygen consumed by the cornea. Based on the effects of local anesthetics and other ophthamolic drugs, Herrman, Moses and Friendenwald, as cited by Duane (1949), concluded the metabolism of the cornea involved both glycolysis and "oxygen activation" (cytochrome-cytochrome oxidase complex and dehydrogenation with intermediate carriers). Since then, many other investigators have also shown that more oxygen is utilized by the epithelium than the stroma. Investigators at the Wilmer Institute (Duane 1949) found the stroma could utilize glucose but not oxygen. The lactate produced in the stroma could only be metabolized by the epithelium, which was observed to have both anaerobic and aerobic glycolysis.

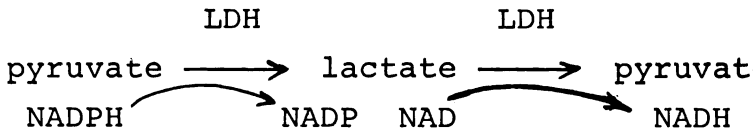
Kinoshita and Masurat (1954) demonstrated the existence of the pentose shunt in the cornea. The

activities of glucose-6-phosphate and 6-phosphogluconate dehydrogenases were determined and several intermediate steps of this pathway were also demonstrated. Comparing these two enzyme activities from the cornea with those from the liver these investigators thought the pentose shunt might play an important role in corneal metabolism. They suggested, as previously discussed for the lens, that glutathione may be involved in oxidizing the NADPH to NADP (Eq. 2).

Kinoshita, Masurat, and Helfant (1955) compared the incorporation of labeled C-1 and C-6 atoms of glucose into $C^{14}O_2$ and lactic acid. The pentose shunt produced a large amount of CO_2 and accounted for 35% of the glucose metabolized in the epithelium. The majority of the glucose, 65%, was metabolized via the glycolytic pathway.

The presence of pyruvate under anaerobic conditions greatly stimulated the extent of C-1 oxidation of glucose to CO_2 (Kinoshita 1957). Since the reoxidation of NADPH to NADP is the rate limiting step, pyruvate was postulated as a possible electron acceptor. Unlike the reoxidation of NADH, NADPH reoxidation is not associated with the formation of high energy bonds. Since there is little pyridine nucleotide transhydrogenase in corneal tissue, it was difficult to see how NADPH could be used for energy. Kinoshita postulated that corneal LDH in the presence of pyruvate and NADPH produces lactic acid and NADP. The

reverse reaction then takes place, but with NAD as the coenzyme. The NADH that is produced then enters the normal mitochondrial electron transport system to produce high energy phosphate bonds (Eq. 3).



(3)

Based on the high isocitrate dehydrogenase levels found in corneal epithelium, Kuhlman and Resnik (1958) took exception to Kinoshita's conclusions that the citric acid cycle was less important than the pentose shunt. As was the case with Wortman and Becker's study (1956) on lenticular enzymes, these investigators did not necessarily measure the rate limiting steps.

Retinal Metabolism

The importance of glucose metabolism in the adult mammalian retina was demonstrated by Cohen and Noell (1959a, 1959b). They noted there is an increase in activity of the citric acid cycle, as measured by C-6 glucose oxidation, with the maturation of the visual cells in the retina. In the adult retina, glucose metabolism accounts for 70% of the oxygen uptake, as opposed to accounting for only 30% in the immature retina (Cohen and Noell 1959b).

The glucose metabolism in the adult mammalian retina appears to be almost exclusively by way of glycolysis

and the citric acid cycle, although under suitable conditions the pentose shunt can be activated. Indeed the outstanding characteristic of mammalian retinal metabolism is its capacity for aerobic glycolysis as well as a marked Pasteur effect. In these retinas, the glycolytic pathway is believed to be associated with tissue maintenance and oxygen utilization is associated with the visual cycle (Cohen and Noell 1965).

The capacity of the pentose shunt increases with age, but on the basis of the oxidation of labeled C-1 and C-6 atoms of glucose, it remains virtually idle unless suitably stimulated with pyruvate under anaerobic conditions (Cohen and Noell 1959b; Futterman and Kinoshita 1959a).

Futterman and Kinoshita (1959b) observed that retinal LDH could serve with NADPH as the coenzyme. The coupling of NADP and LDH as previously proposed for the cornea, was also suggested for the retina (Eq. 3).

Another role of NADPH may be the reduction of Vitamin A_1 aldehyde (retinene$_1$) to Vitamin A_1 (Futterman 1963). Futterman noted aldehydes, in addition to pyruvate, could stimulate C-1 glucose oxidation. Previously Wald (1951) had demonstrated NADH was probably responsible for the reduction of retinene$_1$ to Vitamin A_1 in the outer segments of the rods, but Futterman found NADPH was possibly responsible for this reduction. This conclusion

was based on the quantitative relationship between the oxidation of C-1 atom of glucose and reduction of Vitamin A_1 aldehyde to Vitamin A_1 in dark adapted visual segment preparations exposed to light (Eqs. 4, 5, and 6).

$$\text{Rhodopsin} \xrightarrow{\text{light}} \text{Vitamin } A_1 \text{ aldehyde} + \text{opsin} \quad (4)$$

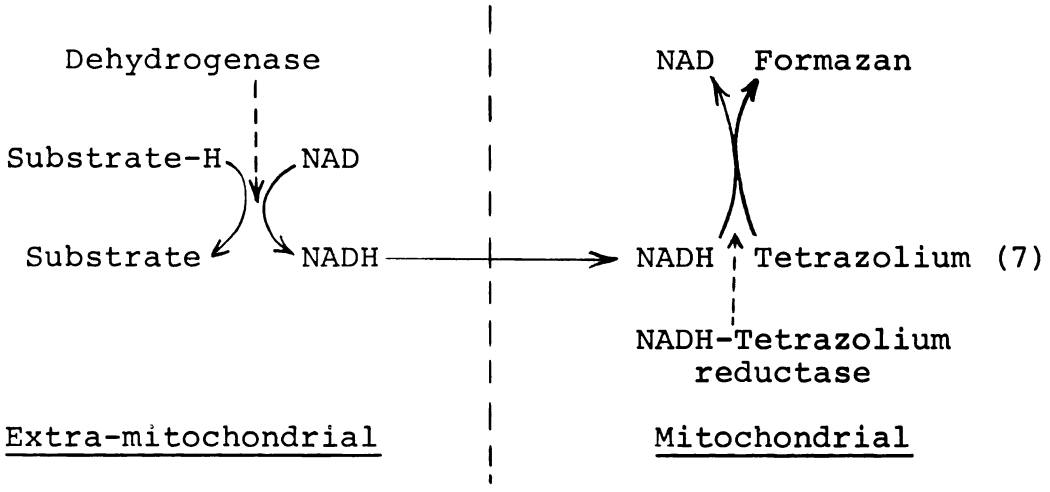
$$\text{NADPH} + \text{Vitamin } A_1 \text{ aldehyde} \xrightarrow[\text{dehydrogenase}]{\text{Alcohol}} \text{Vitamin } A_1 + \text{NADP} \quad (5)$$

$$\text{NADP then stimulates the pentose shunt.} \quad (6)$$

Histochemistry

With the recent development of the easily reducible tetrazolium salts, it has become possible to identify within tissues the dehydrogenases involved in glucose metabolism. These salts act as the final acceptor of electrons from the oxidized substrate that would normally pass through the cytochromes to molecular oxygen (Pearse 1961). Since the tetrazolium salt does not accept electrons directly from the dehydrogenase but rather from one of the electron carriers, the deposition of the colored insoluble formazan is generally a cellular localization of the diaphorase (tetrazolium reductase) involved in reconversion of the carrier to the oxidized state. Since this latter reconversion is largely a mitochondrial

phenomena, mitochondria are generally the sites of formazan precipitation (Pearse 1961; Slater 1965) (Eq. 7).



For a more detailed discussion of the theory behind the histochemical localization of dehydrogenases with tetrazolium salts, the reader is referred to Pearse (1961). While some histochemical studies involving dehydrogenase localizations have been done on mammalian ocular tissues, no extensive investigations have been conducted on ocular tissues of teleosts.

Lens. Cogan and Kuwabara (1960) reported the presence of tetrazolium reductase in the lens epithelium. In this particular study, small sections of the tissues were incubated in blue tetrazolium prior to sectioning. Yanoff and Tsou (1965) found NADH and NADPH diaphorases in the lens epithelium and fibers. Using fresh flat mounts of lens epithelium and capsule, Cotlier (1964) found that while the capsule was unreactive, the epithelium displayed high LDH and malate dehydrogenase activities. There was

minimal glucose-6-phosphate dehydrogenase and no succinate dehydrogenase activity could be detected.

Cornea. Lactate, isocitrate, and malate dehydrogenases have been stained for in corneal epithelium and endothelium (Cogan and Kuwabara 1960). In the same study, they reported LDH caused moderate formazan precipitation in the corneal stromal cells. There was possible succinate dehydrogenase activity in the epithelium, but none was found elsewhere.

In a detailed histochemical investigation of rabbit and rat corneal enzymes, Baum (1963) could identify lactate, malate, glutamate, isocitrate, succinate, glucose-6-phosphate, and 6-phosphogluconate dehydrogenases in the endothelium and epithelium. The endothelium and epithelium had approximately the same degree of activity for each enzyme, with the exception of glucose-6-phosphate dehydrogenase, which had more activity in the epithelium than endothelium. Baum also reported the presence of lactate, malate (rat only), isocitrate (rat only), and glucose-6-phosphate in the keratocytes.

Retina. Francis (1953) incubated whole retinas from frog, guinea pig, rabbit, sheep, and chick, in neotetrazolium plus succinic acid. He found in every case the blue granules were precipitated in the inner segments of the visual cells, being aggregated in the ellipsoids of

both rods and cones. Other retinal structures were relatively sparse in precipitate and the outer segments of visual cells were free of precipitate. Wislocki and Sidman (1954) found the same type of pattern in the frog. They also found a mild reaction in the plexiform layers.

The first detailed histochemical studies of the dehydrogenases involved in retinal glucose metabolism were done by Kuwabara and Cogan (1959, 1960) and Cogan and Kuwabara (1959). They incubated small portions of the retina in blue tetrazolium plus various substrates. They observed two main patterns of precipitation. LDH was principally observed in the inner layers and in particular in the glial cells and to a lesser extent in the ganglion cells. It was absent from the rods and cones. Succinate dehydrogenase was most prominent in the outer layers and precipitated formazan principally in the rod and cone ellipsoids. Isocitrate and malate dehydrogenases produced a formazan pattern which resembled the lactate diaphorase pattern, with more activity observed in the rod and cone ellipsoids. Glucose-6-phosphate dehydrogenase was distributed in the photoreceptor ellipsoids as well as inner retinal layers.

If the retina is first frozen, sectioned, and then incubated, a different pattern of precipitation is observed (Berkow and Patz 1961a, 1961b). In all the species studied,

they noted precipitation, with lactate, malate, or succinate in the media, was mainly in the ellipsoids of the inner segments of the visual cells, pigment epithelium, and to lesser extent in the plexiform and ganglion cell layers. Succinate dehydrogenase activity was limited to the rod and cone ellipsoids (Berkow and Patz 1961b). In the rat, toad, dog, pigeon and monkey retinas, succinate dehydrogenase was present in moderate amounts in the outer plexiform, ganglion cell, and nerve fiber layers. The toad, fish, and mouse retinas showed precipitation in the ellipsoids, outer plexiform, ganglion cell and nerve fiber layers.

Strominger and Lowry (1955) developed a technique of dissecting the retina into various layers and doing enzymatic studies on each layer. They reported the ganglion cells had the highest LDH activity and the next two layers, outer reticular and outer nuclear, had a much lower activity. The latter two layers though had an extremely high malate dehydrogenase activity. The inner rod segments of monkey retina had a higher malate dehydrogenase activity than the outer segments.

Using the same dissecting technique, Lowry, Roberts, and Lewis (1956) found that where malate dehydrogenase activity was high, LDH activity was low. There was more LDH and less malate dehydrogenase in the inner layers of the avascular rabbit retina than in the vascular monkey

retina. They found the outer reticular layer, which is avascular in both species, to be rich in LDH. Lowry et al. (1961) observed glucose-6-phosphate dehydrogenase was exceedingly high throughout the first neuron (receptor cell), except the outer segment (photoreceptor), which had a somewhat lower content. In general the photoreceptors had a low level of enzymatic activity.

Lactate Dehydrogenase

LDH heterogeneity was recognized by Neilands (1952) when he demonstrated LDH activity in each of two electrophoretically distinct proteins from crystalline ox-heart enzyme. He found they differed in their kinetic properties as well as electrophoretic properties. Three distinct components in human serum were found when it was subjected to starch-block electrophoresis (Vessell and Bearn 1957). Since then it has become recognized that most mammalian tissues contain five electrophoretically distinct LDH isozymes.

Vessell and Bearn (1957) suggested the different isozymes found in the serum may have originated from different sites in the body. Contrastingly, Sayre and Hill (1957) suggested LDH is a single molecular species, which can be observed as a number of components representing different states of aggregation as a result of sulfhydryl or other protein interaction.

The present view is that mammalian LDH is a tetrameric molecule that results from assembling two different monomeres, A and B, into five possible combinations, A_4, A_3B, A_2B_2, AB_3, and B_4. Evidence is that the A and B polypeptides are under the control of separate genetic loci (Cahn et al. 1962). The pure A and B forms are often termed M (muscle) and H (heart) for the organs from which they are obtained or LDH-5 and LDH-1 for their electrophoretic mobility. By convention, LDH isozymes are numbered beginning with the one closest to the anode. Thus LDH-1 is H_4, LDH-2 = H_3M_1, LDH-3 = H_2M_2, LDH-4 = HM_3, LDH-5 = M_4.

Evidence for the "subunit hypothesis" is based on the following properties of the isozymes:

1. The H_4 and M_4 polypetides are immunologically distinct from each other. Antibodies prepared against one polypeptide will not react against the other peptide, but antibodies prepared against either the H_4 and M_4 forms will react with the three hybrids (Cahn et al. 1962; Markert and Appella 1963).
2. An orderly difference among the LDH isozymes is noted in many of their kinetic properties, e.g., the greater the electrophoretic mobility the lower the pyruvate concentration required to give

inhibition and the lower the Michaelis constant (Wroblewski and Gregory 1961). The isozymes differ in a similar manner in their activity with NAD and NAD analogs, inhibition with different inhibitors, heat lability and molecular weights. For a complete review of the differences noted among LDH isozymes, the reader is referred to Wilkinson (1965).

3. Guanidine hydrochloride causes the dissociation of the LDH tetramere into four subunits with a consequent loss of activity (Appella and Markert 1961). Freezing the LDH tetramere in a 1M sodium chloride solution also causes dissociation and upon thawing the subunits are able to reassociate into tetrameres without apparent loss of activity (Markert 1963). Upon freezing the combined pure LDH-1 and LDH-5 forms and consequent thawing, Markert reported all five isozymes were formed.

Some mammalian tissues have more than the five major isozymes. Zinkham, Blanco, and Kupchyk (1963) reported a third subunit in the LDH of sperm cells which was under the control of a third gene loci. In general though, mammalian tissues contain a predominence of one or more of the five major isozymes. Although there is a great deal of species variation, homologous tissues from

different species have similar LDH patterns. But the homologous isozymes among different species are immunologically different (Markert and Appella 1963).

Teleost Lactate Dehydrogenase. LDH patterns in teleosts are quite variable and different from those of mammals. In an extensive survey of LDH isozyme patterns in fish, Markert and Falhaber (1965) grouped them into those possessing one, two, three, or five LDH isozymes. The presence of minor isozymes complicated the picture, as they do in mammals, but apparently did not obscure the primary classification. The tissue distribution of the isozymes frequently did not parallel that of homologous mammalian tissues.

Many species of teleosts have an isozyme or isozymes unique to the eye. Nakano and Whitelely (1965) found five isozymes in the medaka (Oryaias latipes) of which LDH-1 and LDH-2 were found only in the retina. Lens and scleral-corneal extracts had only LDH-5. The retina was the only source of all five isozymes. Markert and Falhaber (1965) noted fourteen of the thirty fish they examined had an isozyme unique to the eye. Goldberg (1966) observed the same phenomena in splake and lake trout (Salvelinus namaycush).

As in mammals, the different fish LDH isozymes differ in their kinetic properties. The enzyme from heart

muscle is inhibited by a lower concentration of pyruvate than is skeletal muscle enzyme (Kaplan et al. 1960). In general though pyruvate was found to be less inhibitory to LDH from teleost tissue than from mammalian tissues.

Physiological Significance. Cahn et al. (1962) first proposed that the two parent types of LDH have different functional roles. This concept, which has been discussed in more detail by Dawson, Goodfriend, and Kaplan (1964), is based on the different optimum pyruvate concentrations demonstrated for each LDH isozyme in vitro. Heart LDH in most species consists predominantly of LDH-1 (H_4) and is maximally active at lower concentrations of pyruvate than LDH-5 (M_4). The LDH-5 maintains activity at relatively high pyruvate concentrations. These facts may be related to function in the following way. The heart requires a steady supply of energy and this is maintained by the oxidation of pyruvate and lactate in mitochondria. Because relatively low concentrations of pyruvate inhibit H_4 this would favor this oxidative pathway. Skeletal muscle often requires sudden releases of energy in the relative absence of oxygen. This energy is provided by glycolysis which produces a large amount of pyruvate and requires its reduction to lactate. While this latter reaction produces no energy, it is necessary for the conversion of NADH to NAD which is in short supply and is necessary for other steps in the glycolytic pathway.

Muscle LDH allows this reaction to take place despite the high pyruvate levels that may occur. The lactate can then be removed by the blood stream to join metabolic pathways elsewhere.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout (Salmo gairdneri) were obtained from the Michigan State Conservation Department Hatchery at Grayling, Michigan. The animals were transported to the Michigan State Campus, East Lansing, Michigan, in a galvanized metal tank lined with nontoxic paint and fitted with an agitator for aeration. The holding facilities at Michigan State University were maintained at a temperature of 13-14 C with 14 hours of light and 10 hours of darkness per day. The fish were kept in fiberglass lined tanks containing aereated flowing water. The water was initially treated by passing it through an activated charcoal filter to remove the iron and chlorine. The fish were fed weekly, but were fasted several days prior to their use.

Histochemistry

Tissues. Rainbow trout were killed by a blow to the head and the tissues immediately removed and frozen in an I.E.C. Model CTD microtome cryostat (Needham Heights, Mass.). The tissues were mounted and frozen in a

37.5 gm% (w/v) aqueous mixture of gum acacia. This material was found to give more support and maintained the tissue's integrity better than ice.

The tissues were then sectioned at 6-16 microns; due to the nature of the gum mounting media and hardness of some of the tissues, some sections tended to be thicker than this. After sectioning they were transferred to previously cooled coverslips, finger thawed, then promptly refrozen until used.

Staining. With slight modifications, the staining media were derived from those compiled by Pearse (1961). The standard medium generally contained phosphate buffer, nitro blue tetrazolium chloride salt (nitro BT), magnesium chloride, respiratory inhibitor, and the respective co-enzyme and substrate (see Appendix I for complete formulas). The tissues were stained within three hours after sectioning and were never used if they were more than 48 hours old. Several drops of the staining media were placed on the sections and then incubated on the coverslips at 31-33 C for 15-40 minutes.

Control sections were incubated in a similar medium, with phosphate buffer or distilled water substituted for the substrate. After incubation the tissues were rinsed in 0.8 gm% (w/v) saline, fixed in 10% (v/v) formal-saline (0.8%) for a minimum of 10 minutes, and

then rinsed in 15% ethyl alcohol. This last procedure reduced to a low level any artifact precipitate that may have been present. The sections were then mounted on glass slides with glycerol-gel (see Appendix I for formula).

Inhibitors. Experiments were done to see what effect iodacetate (5 mM), oxalate (2.5 mM), malonate (10 mM) and cyanide (10 mM) had upon the enzymatic reactions (see Appendix I for preparation of the inhibitors). The inhibitors were incorporated into the medium prior to incubation of the tissues. In these particular experiments, two types of controls, one without the substrate and inhibitor and another control without the inhibitor but with added substrate, were incubated simultaneously for comparative purposes.

Disc Electrophoresis

Tissues. Tissues were washed in distilled water to remove any contaminating tissue and fluids, blotted and placed in a vial on ice. Tissues removed were the lenses, corneas, retinas, as well as skeletal, smooth, and cardiac muscles. Any iris or sclera that was attached to the cornea was cut away. The smooth muscle was taken from the stomach, and the skeletal muscle was removed from the mid-ventral region of the fish. In various experiments, brain, optic nerve, kidney, gill, aqueous and vitreous humor were also examined for LDH isozymes.

Enough distilled water was added to make an approximate 10% (w/v) homogenate. The tissues were then homogenized with a Branson Sonifier (Melville, N. Y.) while in an ice bath. Because of the qualitative nature of these experiments exact dilutions were not of critical importance. The homogenate was then centrifuged for 4 minutes in a Beckman 152 Microfuge (Lincolnwood, Ill.). If the supernatant fluid was not used immediately, it was frozen at -20 C. No homogenate was used if more than 48 hours old.

Apparatus. The disc electrophoretic apparatus was constructed from two 17 x 12 1/2 x 7 1/2 cm polyethelene trays. The holes for the tubes (1/2 cm diameter and approximately 8.0 cm long glass tubes) were placed in two rows of seven holes each and the electrodes aligned in the middle (Figure 2). The anode, normally in the lower bath, was a 14 1/2 cm long, 2.0 cm diameter carbon cylinder and the cathode was a 22 gauge silver wire. A carbon cylinder was substituted for the wire if the polarity was reversed. The power supply was a Heathkit Variable Voltage Regulated Power Supply, Model PS-3 (Benton Harbor, Mich.).

Polyacrylamide Gels. The isozymes were separated in 7% (w/v) polyacrylamide gel columns. The method of Ornstein and Davis (1962) was used in preparing the gels and tubes and are given in Appendix II. The individual lengths for the separating, stacking and sample gels were 4.8, 1.0, and 0.5-1.0 cm respectively. The supernatant

Figure 2. Disc electrophoretic apparatus used for separating lactate dehydrogenase isozymes

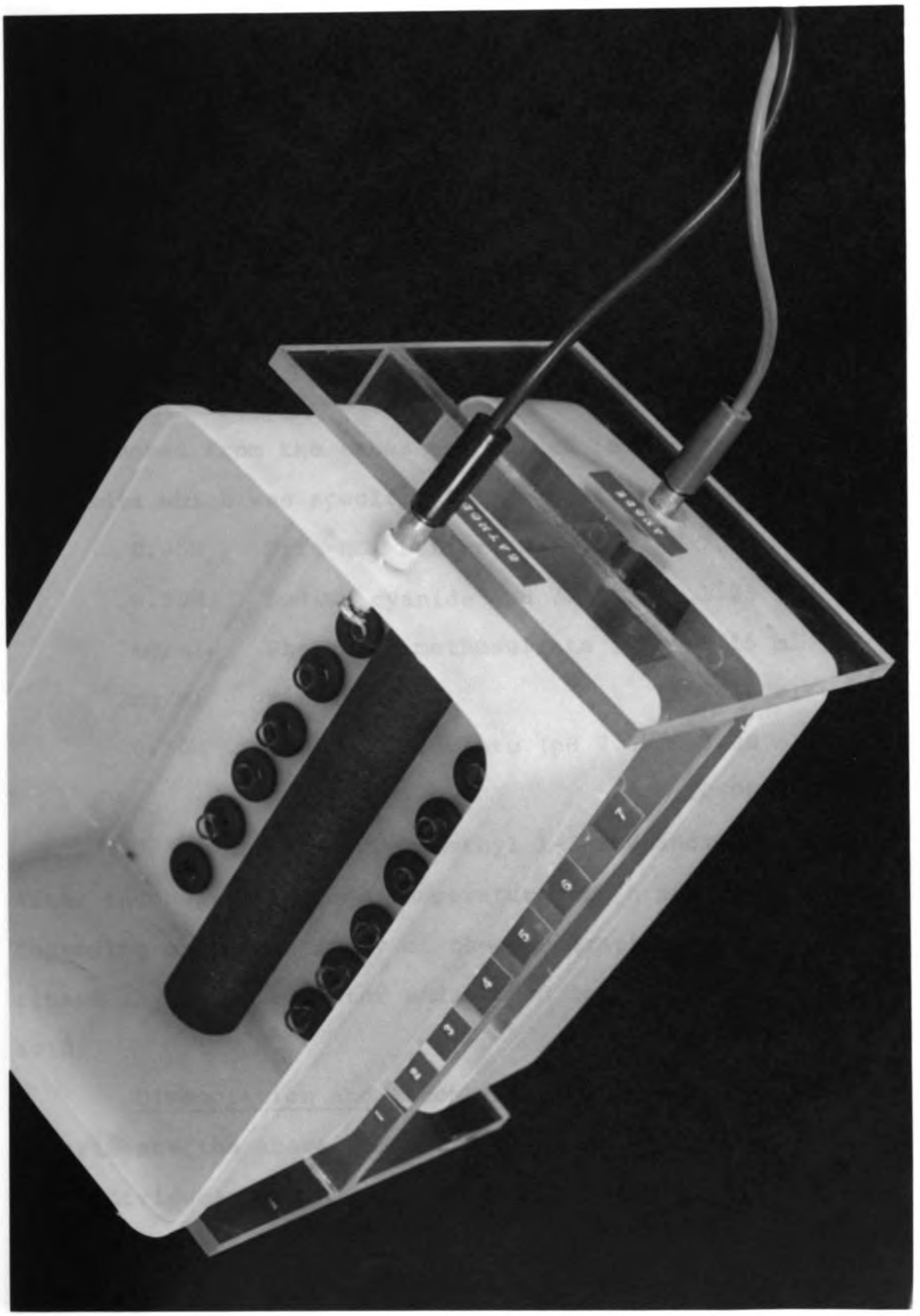


Figure 2

from the homogenate was mixed with the sample gel in an approximate 1:5 ratio (v/v). Immediately after polymerization the isozymes were separated electrophoretically in a glycine buffer (pH 8.4) at a constant current of 70 mamps (5 mamps per tube). Bromphenol blue was added to the upper bath (cathode) at the start of the run to mark the movement of the buffer front.

Incubation. After completion of the run, the gels were removed from the tubes and placed in a nitro BT staining media which was specific for LDH (Allen, date unknown).

0.05M	Tris*-HCl buffer (pH 7.5)	7.50 ml
0.50M	Sodium cyanide (pH 7.5)	1.25 ml
2mg/ml	Phenazine methosulfate	0.15 ml
2mg/ml	Nitro BT	3.50 ml
0.50M	Sodium dl-lactate (pH 7.5)	3.00 ml
	NAD	10.00 mg

*2-Amino-2-Hydroxymethyl 1-3-Propandiol

After incubation at room temperature for a period of time, depending on the activity of the preparation, the gels were rinsed in distilled water and stored in 7% (v/v) acetic acid.

Dissociation and Recombination. In several experiments the above procedure was used for preparing the individual LDH isozymes for dissociation and recombination experiments. In these preparations one gel was stained

to determine the distance traveled by the isozymes. The corresponding sections were then removed from the other columns and homogenized in an approximately equal volume of distilled water with the Branson Sonifier. The homogenized gels of two different LDH isozymes were combined and an equal volume of 2M NaCl solution added to give a final 1M NaCl solution. This was frozen at -20 C overnight and separated electrophoretically as previously described.

Enzyme Kinetics

Homogenates as described for disc electrophoresis were prepared from cornea, lens, retina, and skeletal and cardiac muscle. The homogenate was centrifuged in an I.E.C. centrifuge Model SBV at 1700 rpms for 10 minutes and the supernatant further centrifuged in the Beckman 152 Microfuge for an additional 3 minutes. Any enzyme solution or tissue not used immediately was stored at -20 C.

A Beckman DB-G (dual beam-grating) spectrophotometer (Fullerton, Calif.) measured the change in absorbance (ΔA) produced by the conversion of NAD to its reduced form (NADH) or the oxidation of NADH to NAD. The measurements were made at a wave length of 340 millimicrons (mμ) and a slit width of 1.0 mm. The change in absorbance was recorded on a Beckman (10-inch) Potentiometric Recorder. The temperature within the spectrophotometer was regulated by circulating water from a constant temperature water bath

through the sample compartment coolant unit. The reaction mixtures were kept in this same water bath. While in the spectrophotometer, media temperatures were monitored by a Tele-Thermometer (Y.S.I., Yellow Springs, Ohio) with the probe placed directly within the sample cell.

Temperature Characteristic. The Wacker (Wacker, Ulmer and Vallee 1956) method for measuring LDH activity, using lactic acid and NAD, was employed for the temperature characteristic determinations. The increase in absorbance caused by the reduction of NAD to NADH was recorded and was the measure of enzyme activity (Eq. 8).

$$\text{NAD} + \text{lactate} \xrightarrow{\text{LDH}} \text{NADH} + \text{pyruvate} \qquad (8)$$

The reaction mixture consisted of:

0.100M	Sodium phosphate buffer (pH 8.8)	1.5 ml
0.005M	NAD	0.3 ml
0.160M	Sodium dl-lactate (pH 7.3)	1.0 ml
	Tissue homogenate	0.2 ml

The enzyme (tissue homogenate) was diluted with enough distilled water to cause an approximate 0.02 absorbance increase per minute at 25 C under the specified conditions. The change in absorbance was read against a reference blank that consisted of the above mixture, but with distilled water substituted for the tissue homogenate. The sample was adjusted against the blank to give 0-0.022 absorbance (1.00-0.95 transmission) at zero time.

Fifteen test tubes containing the above reaction mixture, minus the enzyme, were placed in the water bath (33-35 C) that controlled the temperature of the spectrophotometer's sample compartment coolant unit. The tissue homogenate was kept in an ice bath and was added to the sample cell immediately prior to use. The reaction mixture was then added and the cell shaken vigorously and immediately placed in the spectrophotometer. The increase in absorbance was recorded for a minimum of 2 minutes. Ice was then added to the water bath to lower the temperature 1-2 degrees and the procedure repeated until all fourteen samples were run. Occasionally the reverse procedure was employed and the temperature was first lowered to 15-16 C and consequently raised 1-2 degrees at a time.

The logs of the change in absorbance per minute (log ΔA/min) were plotted against the reciprocals of the absolute temperatures ($\frac{1}{T}$) and a straight line fitted to the points. From the relationship

$$\text{slope} = \frac{-\mu}{4.6}$$

the temperature characteristic (μ) was determined.

Michaelis Constants. A modified Wroblewski method (Wroblewski and LaDue 1955) for determination of LDH activity was used to obtain the Km values. The oxidation of

NADH to NAD was measured at 340 mμ with pyruvate as the substrate (Eq. 9).

$$\text{NADH} + \text{pyruvate} \xrightarrow{\text{LDH}} \text{NAD} + \text{pyruvate} \qquad (9)$$

The reaction mixture consisted of:

0.0670M	Sodium phosphate buffer (pH 7.4)	2.0 ml
0.0027M	NADH	0.3 ml
	Pyruvate solution	0.3 ml
	Tissue homogenate	0.4 ml

The enzyme tissue homogenate was diluted with enough phosphate buffer to cause an approximate 0.022 absorbance decrease per minute with a 10^{-4}M pyruvate at 25 C, pH 7.4, and 2.7 x 10^{-4}M NADPH.

The enzyme and pyruvate solutions were made up in the phosphate buffer rather than distilled water. The buffer, coenzyme, and enzyme were mixed and incubated (minimum of 5 minutes) in a water bath (25 C) which was connected to the spectrophotometer as previously described. The reaction was initiated by rapidly mixing the substrate with the preincubated enzyme, coenzyme, and buffer and immediately recording the decrease in absorbance for two minutes. Final pyruvate concentrations varied from 10^{-2}M to 4 x 10^{-5}M. The reciprocals of the change in absorbance ($\frac{1}{\Delta A}$) were plotted against the reciprocals of substrate concentration ($\frac{1}{S}$) to give typical Lineweaver and Burk plots. The Km values, from the X intercepts, and the threshold for

pyruvate inhibition were determined from this graph. The Km values were also determined for LDH with NADPH (2.2×10^{-4}M) as the coenzyme. All the other conditions were as previously described.

Specific Activity. Homogenates of the tissues were prepared as previously described. LDH was assayed at pH 7.4 at 25 C. The reaction mixture consisted of:

0.0670M	Sodium phosphate buffer (pH 7.4)	2.0 ml
0.0020M	Pyruvate	0.3 ml
0.0027M	NADH	0.3 ml
	Tissue homogenate	0.4 ml

A unit of activity is that which causes an initial rate of oxidation of one micromole of NADH per minute under the conditions specified at 25 C.

Calculation:

$$\text{Units/mg protein} = \frac{\Delta A/\text{min} \times 1000}{6.2 \times 1000 \times \text{mg protein/ml reaction mixture}}$$

Proteins were assayed by a modified colorimetric method of Lowry (Oyama and Eagle 1956). Protein solutions prepared from a standard protein solution, 730 µg/ml, lot no. 2370 (Cappel Lab., West Chester, Pa.), were used to prepare a standard curve ranging from 0 µg/ml to 292 µg/ml. The tissue homogenates were then diluted with enough distilled water to give a protein concentration within this range.

RESULTS

Histochemical

Typical paraffin imbedded retinal sections, Figures 3 and 4, do not resemble the frozen sections stained for particular enzymes, Figures 5, 6, 7 and 8. In Figures 3 and 4 (standard hematoxylin and eosin stained retinal sections from rainbow trout) are listed the layers with abbreviations and nomenclature which will be used throughout this work. The cornea and lens are simpler tissues than the retina and the layers are easily observed in frozen sections. The lenticular capsule and epithelium though did not stay intact upon incubation, this resulted in poor histological sections.

Some problems were also encountered in the controls with "non-specific" staining when NADP was the coenzyme. To lower the amount of this "non-specific" staining it was necessary to use a 6.0×10^{-4}M concentration of NADP, which was 10-20% of the NAD concentration. Even at this lowered concentration, some formazan precipitation was observed in the lens epithelium of many controls. This was not a problem in the cornea or retina.

Figure 3. Photomicrograph of a hematoxylin and eosin stained section of rainbow trout retina.

A. Retinal pigment epithelium
B. Photoreceptors
C. Outer nuclear layer
D. Outer plexiform layer
E. Inner nuclear layer
F. Inner plexiform layer
G. Ganglion cell layer
H. Nerve fiber layer (X 286)

Figure 4. Photomicrograph of rainbow trout retina. (Hematoxylin and eosin, X 625)

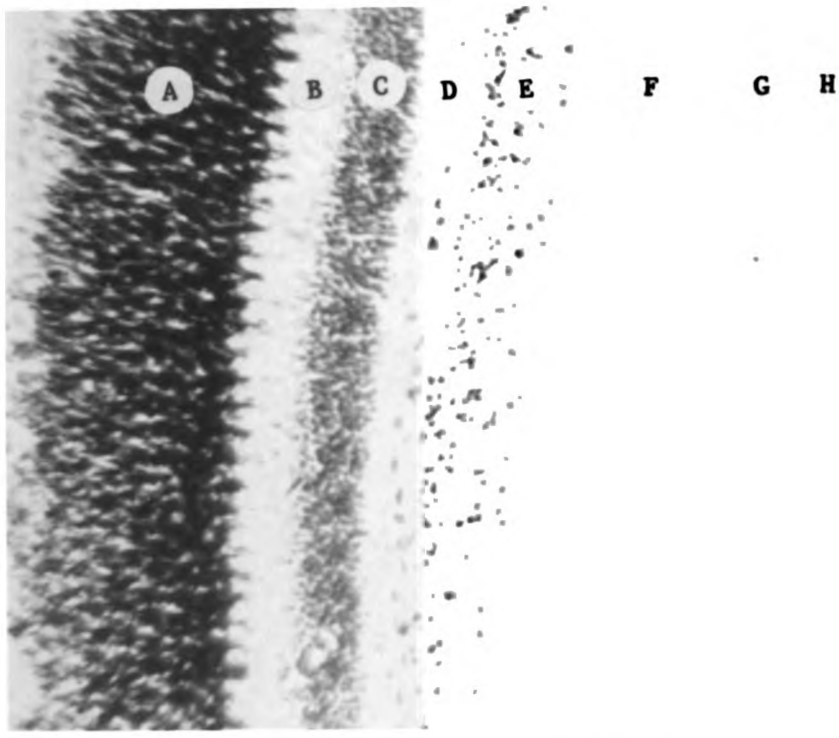


Figure 3

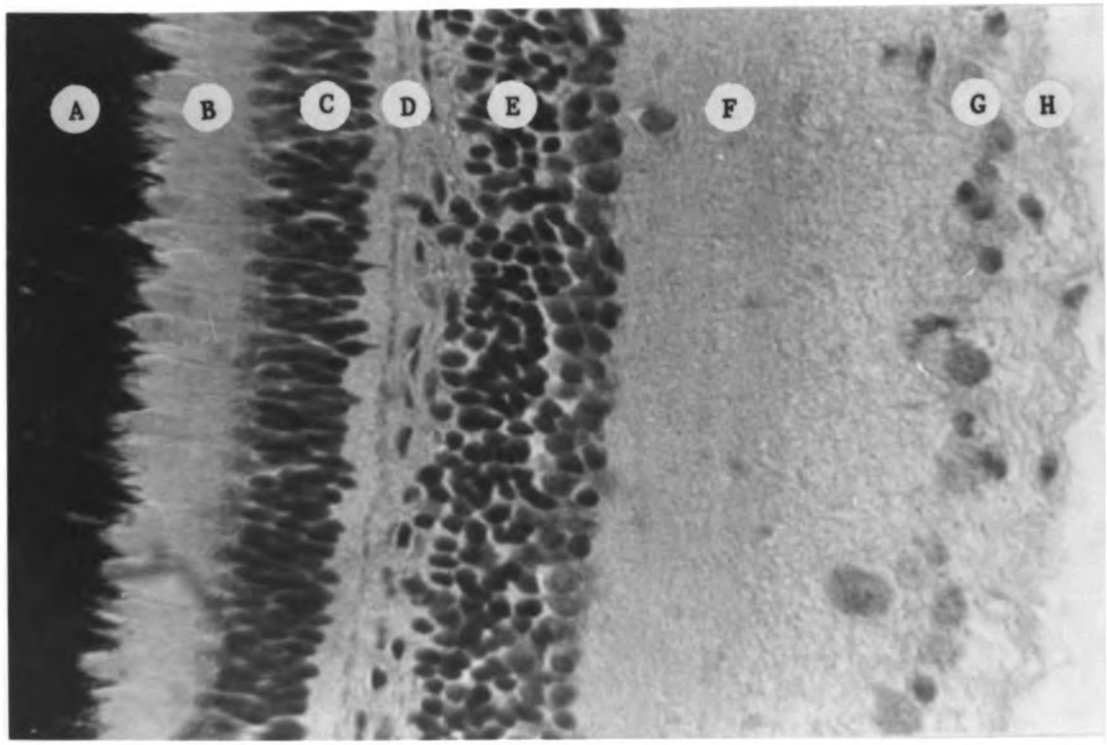
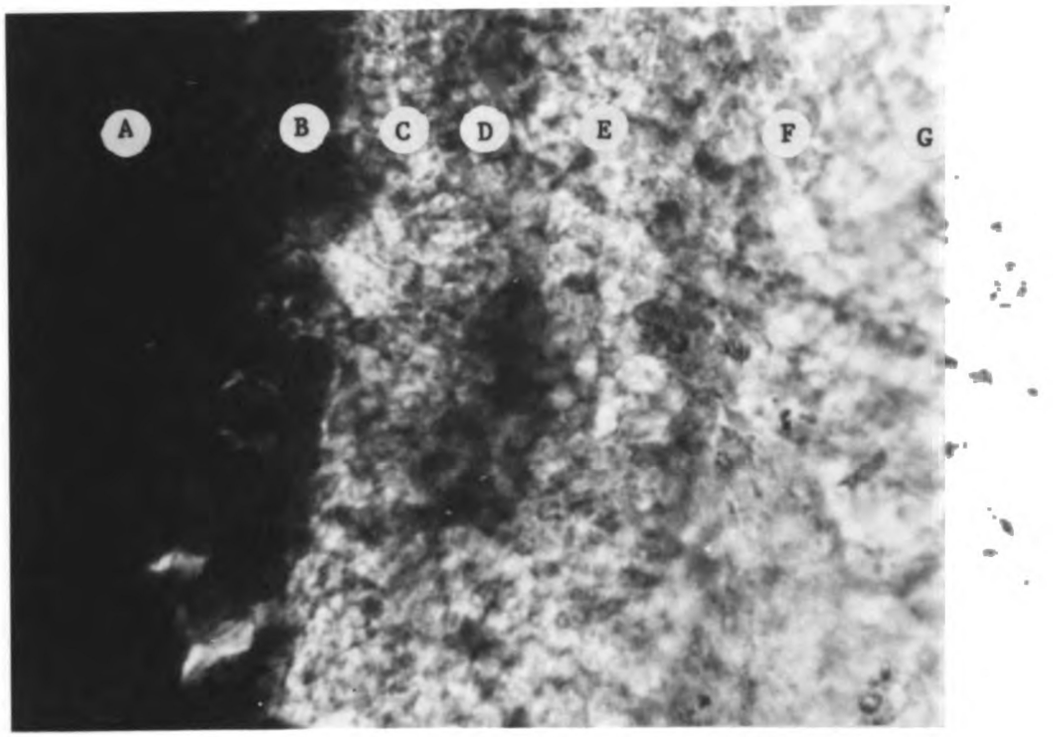


Figure 4

Figure 5. Photomicrograph of rainbow trout retina stained for NAD linked lactate dehydrogenase. The photoreceptors (B) have the greatest formazan deposition, but the inner layers (C, D, E, F, G) show some activity. Because the pigment epithelium (A) has large quantities of melanin in it, it cannot be determined if there is any formazan precipitation here. (X 625)

Figure 6. Photomicrograph of rainbow trout retina stained for NADP linked lactate dehydrogenase. The photoreceptors (B) have a moderate formazan precipitate, while the inner layers (C, D, E, F, G) show little or no activity. The reason the pigment epithelium (A) is black is due to melanin and not formazan precipitation. (X 625)



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G) show some
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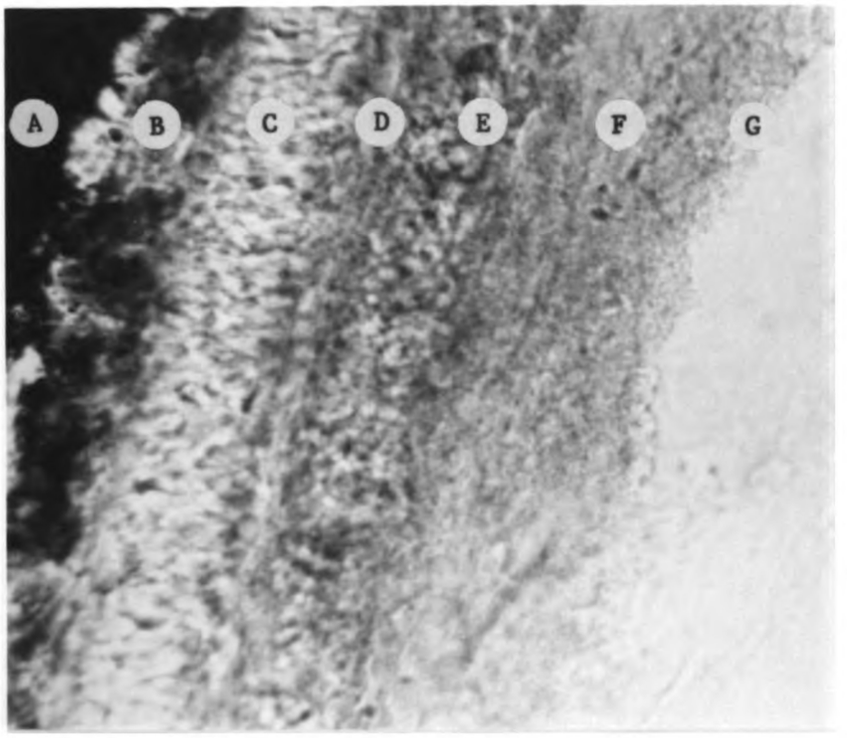
Figure 5



trout retina
tate dehydro-
(B) have a
te, while the
show little
the pigment
due to melanin
ion. (X 625)

Figure 6

Figure 7. Photomicrograph of rainbow trout retina stained for succinate dehydrogenase. The photoreceptors (B) have the greatest amount of formazan precipitation. The outer and inner plexiform layers (D, F) also have high activity. The nuclear layers (C, E) have little or no formazan precipitation. The pigment epithelium is black because of melanin and not because of formazan precipitation. (X 625)

Figure 8. Photomicrograph of rainbow trout retina stained for NADP linked glucose-6-phosphate dehydrogenase. The inner layers (C, D, E, F, G) have a light formazan deposition. The photoreceptors (B) have no formazan deposited here. The pigment epithelium is black because of melanin and not because of formazan precipitation. (X 625)

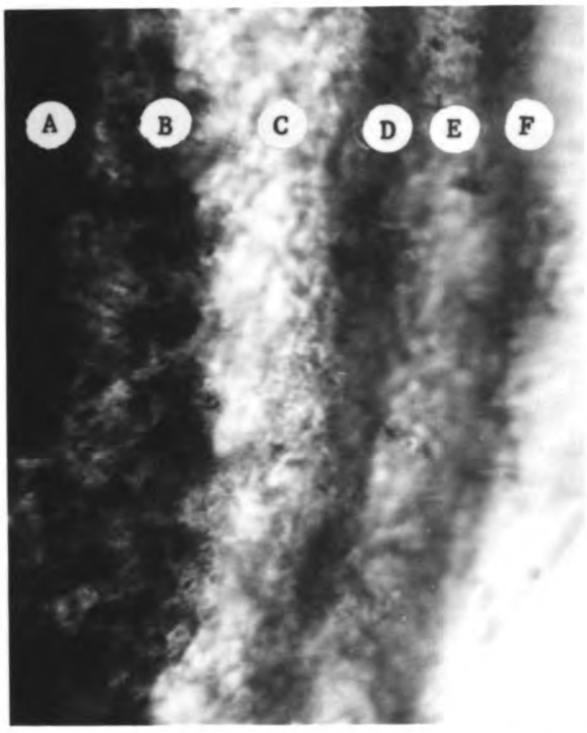


Figure 7

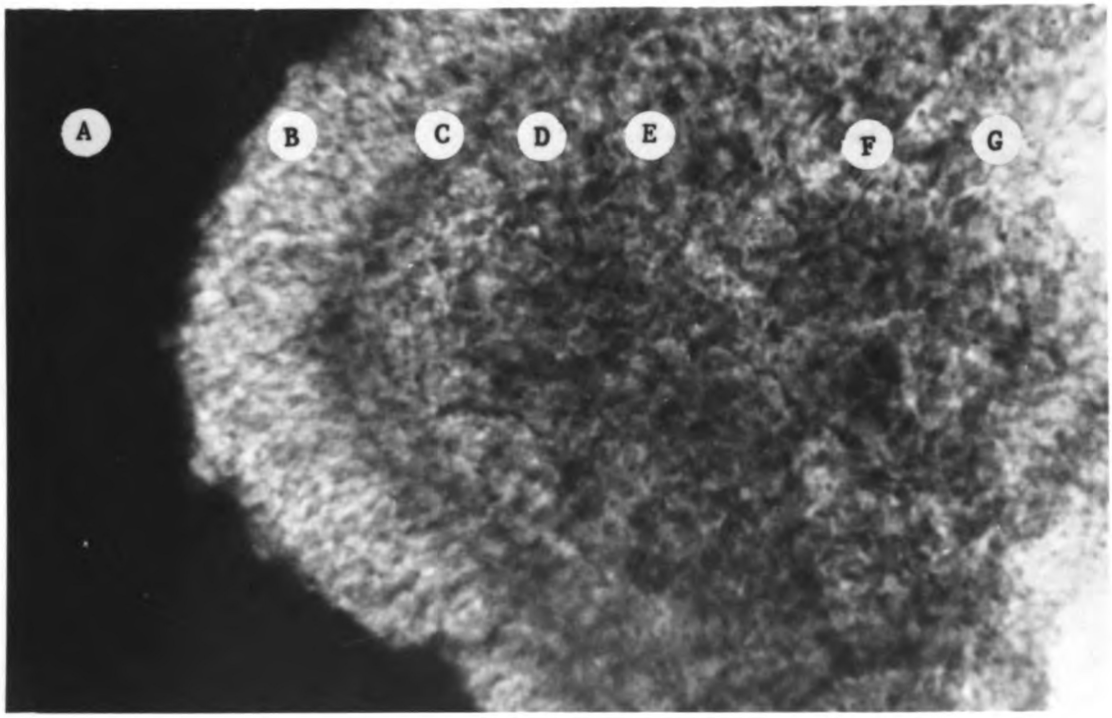


Figure 8

Figure 9. Photomicrograph of rainbow trout cornea stained for NAD linked lactate dehydrogenase. The fibroblasts in the stroma (A) show moderate activity, Bowman's membrane (B) has no formazan precipitation, and the epithelium (C) shows intense activity. (X 625)

Figure 10. Photomicrograph of rainbow trout cornea stained for NADP linked glucose-6-phosphate dehydrogenase. The epithelium (C) shows intense activity, Bowman's membrane (B) has no formazan precipitation, and the fibroblasts in the stroma (A) have a slight amount of formazan precipitation. (X 625)

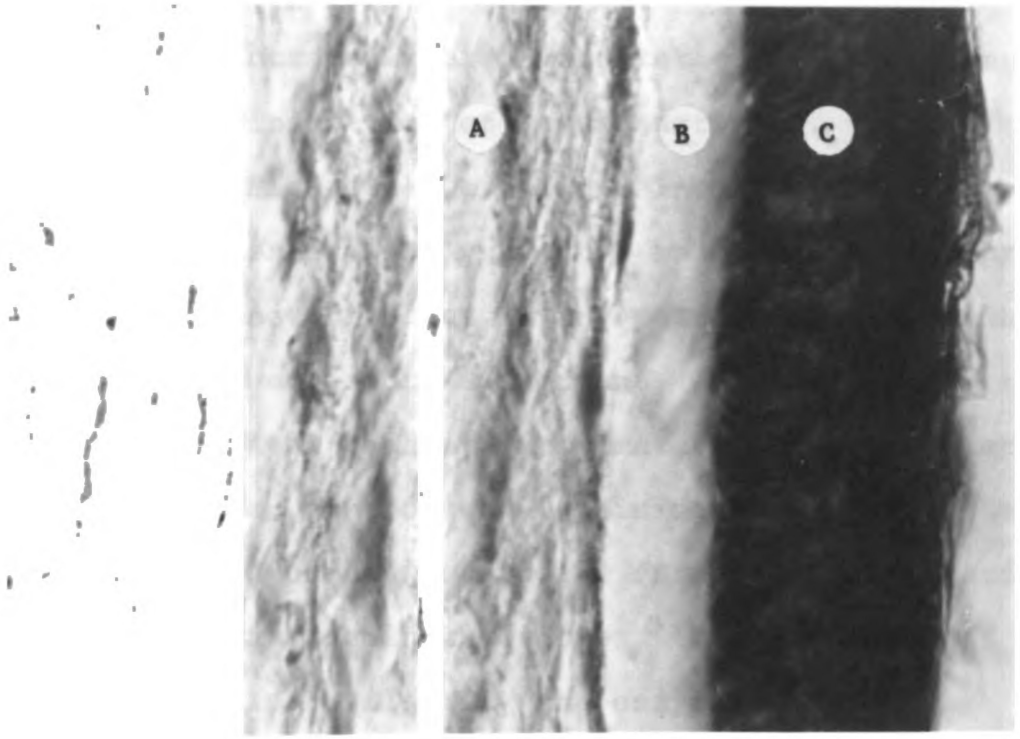


Figure 9

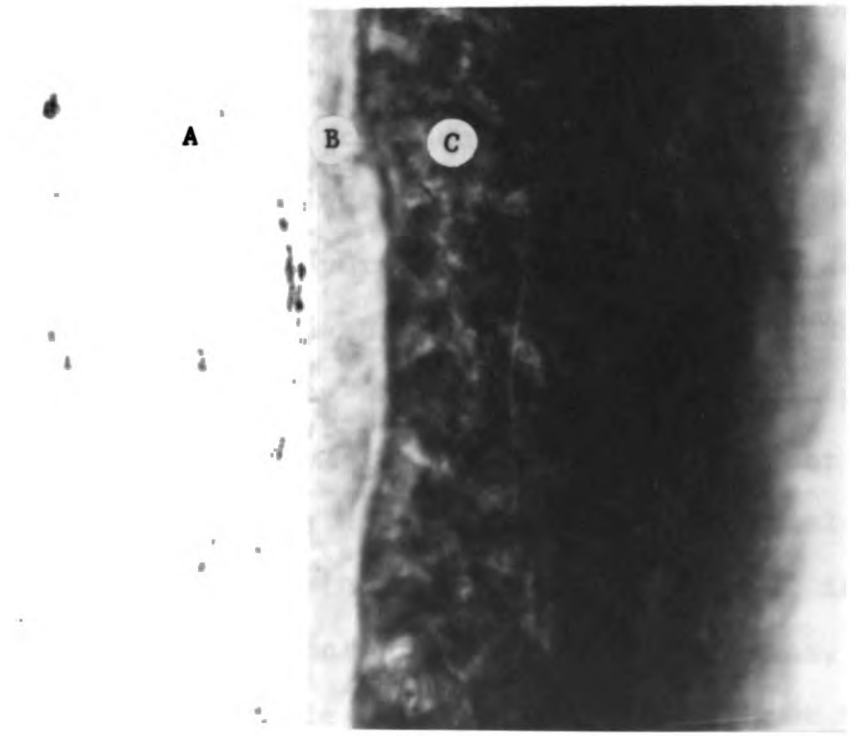


Figure 10

The intracellular precipitation of blue granular formazan was indicative of enzyme activity. Six of the enzymes (See Figure 1) were found in one or another of the ocular tissues. Only xylitol and sorbitol dehydrogenases were not detected in any portion of the ocular tissues. The accessory sex organs (which are known to have the sorbitol pathway) from mature male rats, were used as another control when staining for sorbitol dehydrogenase. Since there was a heavy formazan precipitation in the prostate and seminal vesicles, the technique was apparently able to identify the enzyme.

The most widely distributed enzyme was LDH. It was most prominent in corneal epithelium and endothelium (Figure 9), lenticular epithelium, and the photoreceptor ellipsoids (Figure 5). When NADP was substituted for NAD, essentially the same formazan pattern was observed in the cornea and retina (Figure 6).

The three dehydrogenases of the citric acid cycle were widely scattered throughout the tissues. Succinic dehydrogenase activity was found in the retinal photorecptors and plexiform layers (Figure 7) but not in the cornea nor lens. The addition of the artificial electron carrier, phenazine methosulfate (PMS), did not increase formazan precipitation in the cornea nor retina, but it did cause considerable precipitation throughout the lens.

Isocitrate dehydrogenase was found in the photoreceptors, choroid, and plexiform layers of the retina when NADP or NAD served as the coenzyme. Corneal activity was confined to the peripheral epithelium, endothelium, and scleral-corneal junction. Lenticular isocitrate dehydrogenase was found primarily in the epithelium. The choroid gland and corneal epithelium showed high malate dehydrogenase NAD linked activity, while corneal endothelium and choroid gland had considerable formazan precipitation with NADP in the media.

Glucose-6-phosphate and 6-phosphogluconate denydrogenases were observed in the corneal epithelium and endothelium (Figure 10) and in the lens epithelium. Some formazan precipitation was also seen in corneal fibroblasts and lens fibers. Enzyme activity was confined to the inner layers of the retina with the photoreceptor ellipsoids having no observable activity (Figure 8). The preceding results are summarized in Tables 1 and 2.

Addition of malonate to the succinate dehydrogenase incubation media effectively inhibited formazan precipitation. Malonate addition to the other media had little effect on precipitation. While oxalate inhibited LDH in the cornea and retina, lenticular activity was not completely inhibited. The other dehydrogenases were not significantly affected by the addition of oxalate to the media.

Table 1. Formazan deposition in the different areas of the rainbow trout retina with different substrates and coenzymes*

Substrate	Coenzyme	Choroid	Photoreceptors	Nuclear Layers	Plexiform Layers	Ganglion Cell Lay
Succinate	none	?	++++	?	+++	?
Malate	NAD	+++	++	0	?	0
Malate	NADP	+++	+	0	?	0
Isocitrate	NAD	++	++++	?	++++	?
Isocitrate	NADP	+++	++++	?	++++	?
Lactate	NAD	++	++++	?	+	?
Lactate	NADP	+++	+++	0	0	0
Glucose-6-phosphate	NADP	?	0	+	+	?
6-Phospho-gluconate	NADP	0	0	+	+	?
Sorbitol	NAD	0	0	0	0	0
Xylitol	NAD	0	0	0	0	0

*The amount of formazan deposited is rated from heavy (++++) to none (0).
(?) Designates questionable deposition when compared to the control sections.

Table 2. Formazan deposition in the different areas of the rainbow trout lens and cornea with different substrates and coenzymes*

Substrate	Coenzyme	Corneal Epith.	Corneal Endoth.	Corneal Fibro.	Lens Epith.	Lens Cap.	Lens Fibers
Succinate	none	0	+	0	?	0	0
Malate	NAD	+++	+	?	+	0	?
Malate	NADP	+	+++	0	++	0	0
Isocitrate	NAD	+++	+++	0	++	0	?
Isocitrate	NADP	+++	+++	0	+++	0	0
Lactate	NAD	++++	++++	+	+++	0	+
Lactate	NADP	++	++++	0	?	0	0
Glucose-6-phosphate	NADP	++++	++	+	+++	0	?
6-Phospho-gluconate	NADP	+++	++	+	+++	0	?
Sorbitol	NAD	0	0	0	0	0	0
Xylitol	NAD	0	0	0	0	0	0

*The amount of formazan deposited is rated from heavy (++++) to none (0).
(?) Designates questionable deposition when compared to the control sections.

Although the 0.005M iodoacetate inhibited all the enzymes to some degree, LDH and 6-phosphogluconate dehydrogenase were most notably affected. Iodoacetate also caused considerable cellular disintegration. It was difficult to judge whether reduced formazan precipitation was a result of the iodoacetate inhibiting the enzyme or the cellular destruction. The addition of cyanide to any of the incubation media, neither increased nor decreased formazan precipitation.

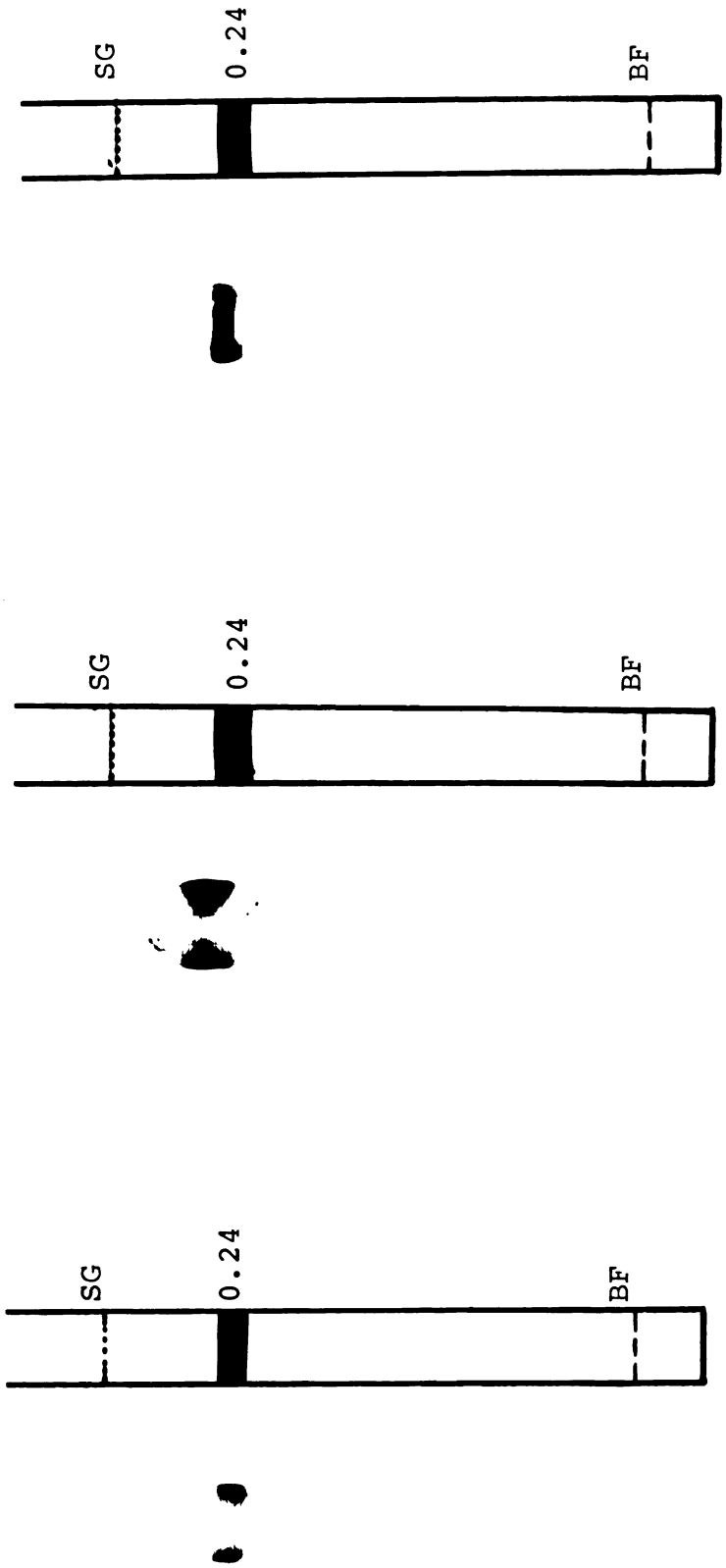
Electrophoretic Separation of Lactate Dehydrogenase Isozymes

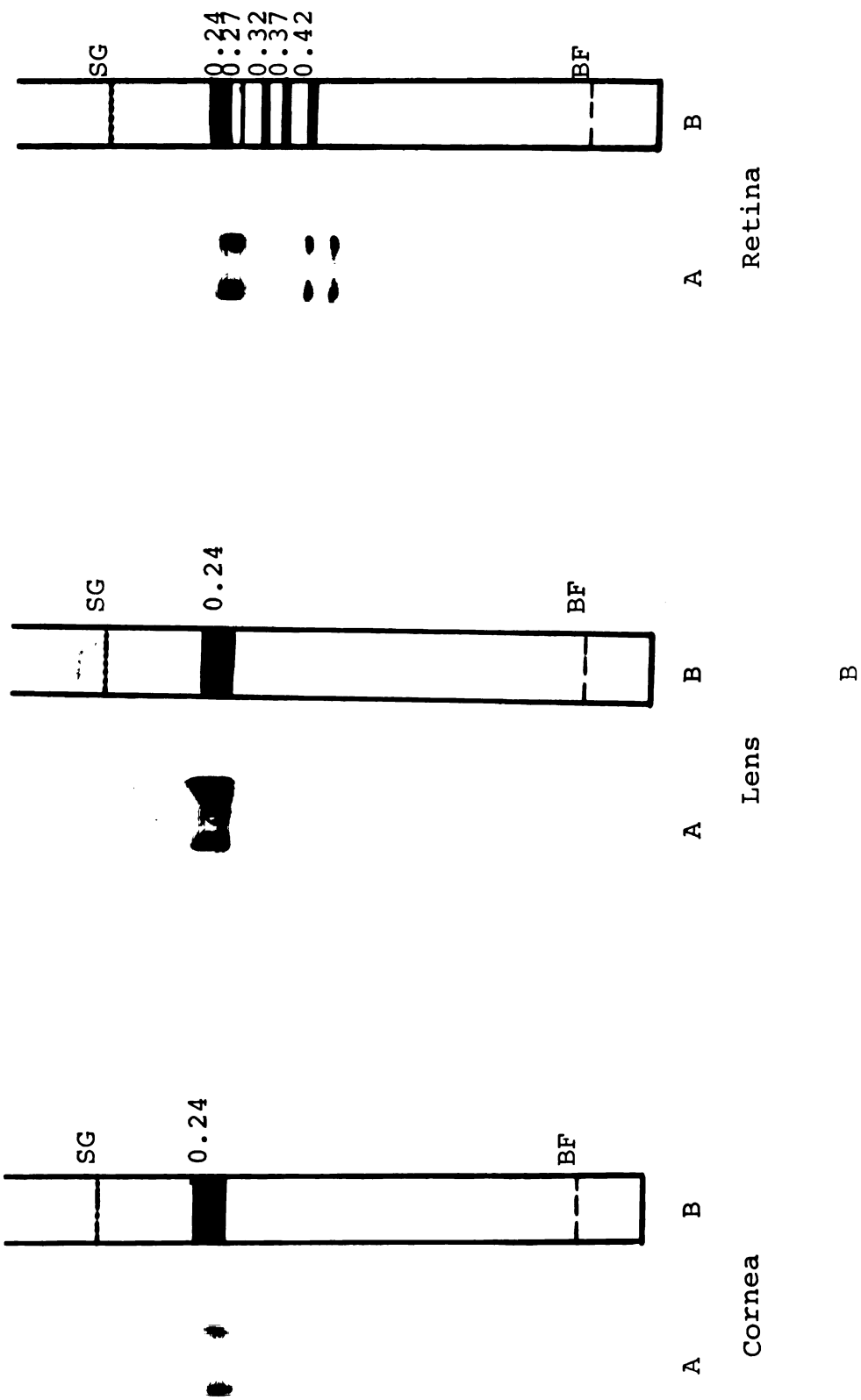
The retina showed five distinct bands of LDH activity (Figure 11B). With the exception of the gill, the other tissues had only one observable band of activity and this corresponded to the retinal LDH-5 (Figures 11A, 11B, and 11C). The gill typically showed two bands of activity that traveled in close proximity to each other (Figure 11C). Normal aqueous and vitreous humor were devoid of LDH, but in fish that had developed cataracts with degenerated ocular structures, retinal LDH isozymes were present in both the aqueous and vitreous humor.

Subjecting combinations of tissue extracts to electrophoresis confirmed that LDH from the various tissues (gill and retina excluded) had the same electrophoretic mobilities (Figure 11D). As seen by electrophoretic separation, freezing the isolated retinal LDH-1 with

Figure 11. Lactate dehydrogenase isozymes separated in polyacrylamide gel columns. The (A) figures are photographs of the gel stained for lactate dehydrogenase. (B) figures are schematic sketches of the columns which have been adjusted so the distance traveled by the buffer front (BF) is the same in all cases. The numbers on the right are Rf values. SG represents the beginning of the separating gel.

A. The different muscles of rainbow trout
B. The different ocular tissues of rainbow trout
C. Rainbow trout optic nerve, gill and human plasma
D. Combinations of tissue homogenates


SG
0.24
BF
A
B
Smooth Muscle
SG
0.24
BF
A
B
Skeletal Muscle
SG
0.24
BF
A
B
Cardiac Muscle
A


SG
0.24
0.27
0.32
0.37
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BF
B
A
Retina
SG
0.24
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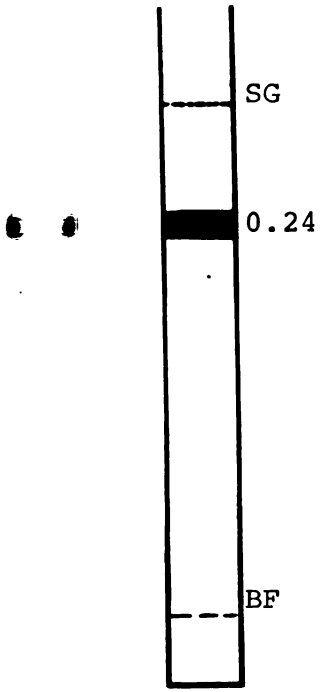
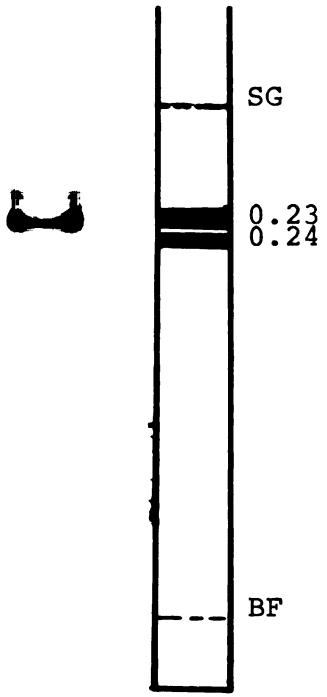
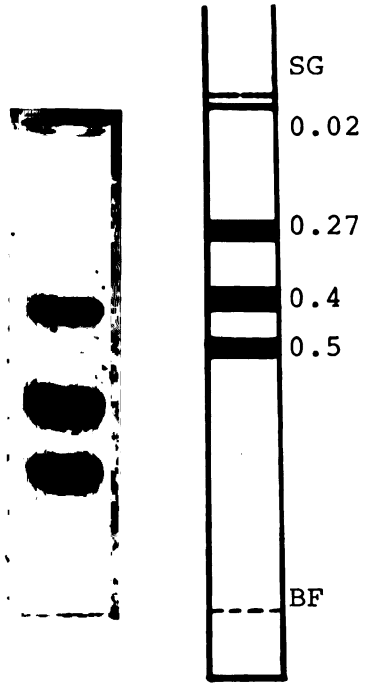


Optic Nerve

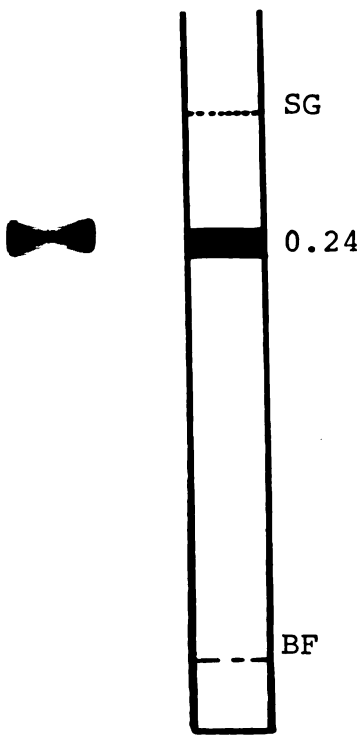


Gill

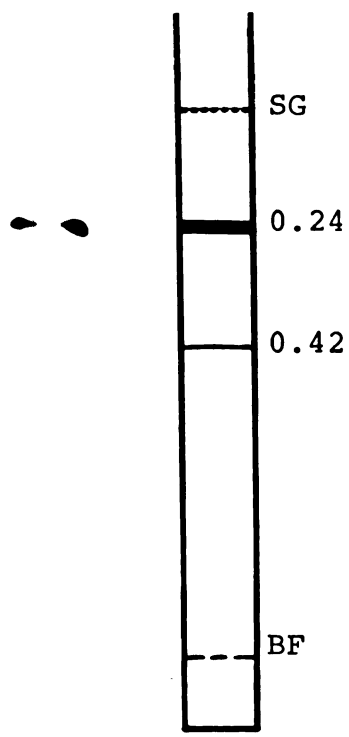


Human Plasma

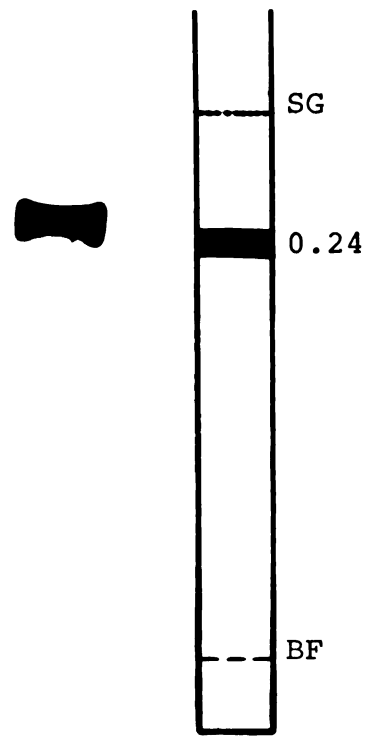
C



Cardiac + Lens



LDH-1 (retina) + Lens



Skeletal + Cardiac Muscles

D

isolated retinal LDH-5 or lenticular LDH in a 1.0M NaCl solution did not cause the dissociation and recombination of subunits (Figure 11D).

Lactate Dehydrogenases Kinetics

Figures 12A, 12B, 12C, 12D, and 12E are Lineweaver and Burk plots for the tissue LDH isozymes linked with NADH, under the specified conditions. The dotted lines on the plots are the pyruvate concentrations at which inhibition begins to occur. The Km values and pyruvate concentrations which begin to produce inhibition are found in Table 3.

Table 3. Michaelis constants (Km) of rainbow trout lactate dehydrogenase isozymes and the pyruvate threshold concentrations that produce inhibition

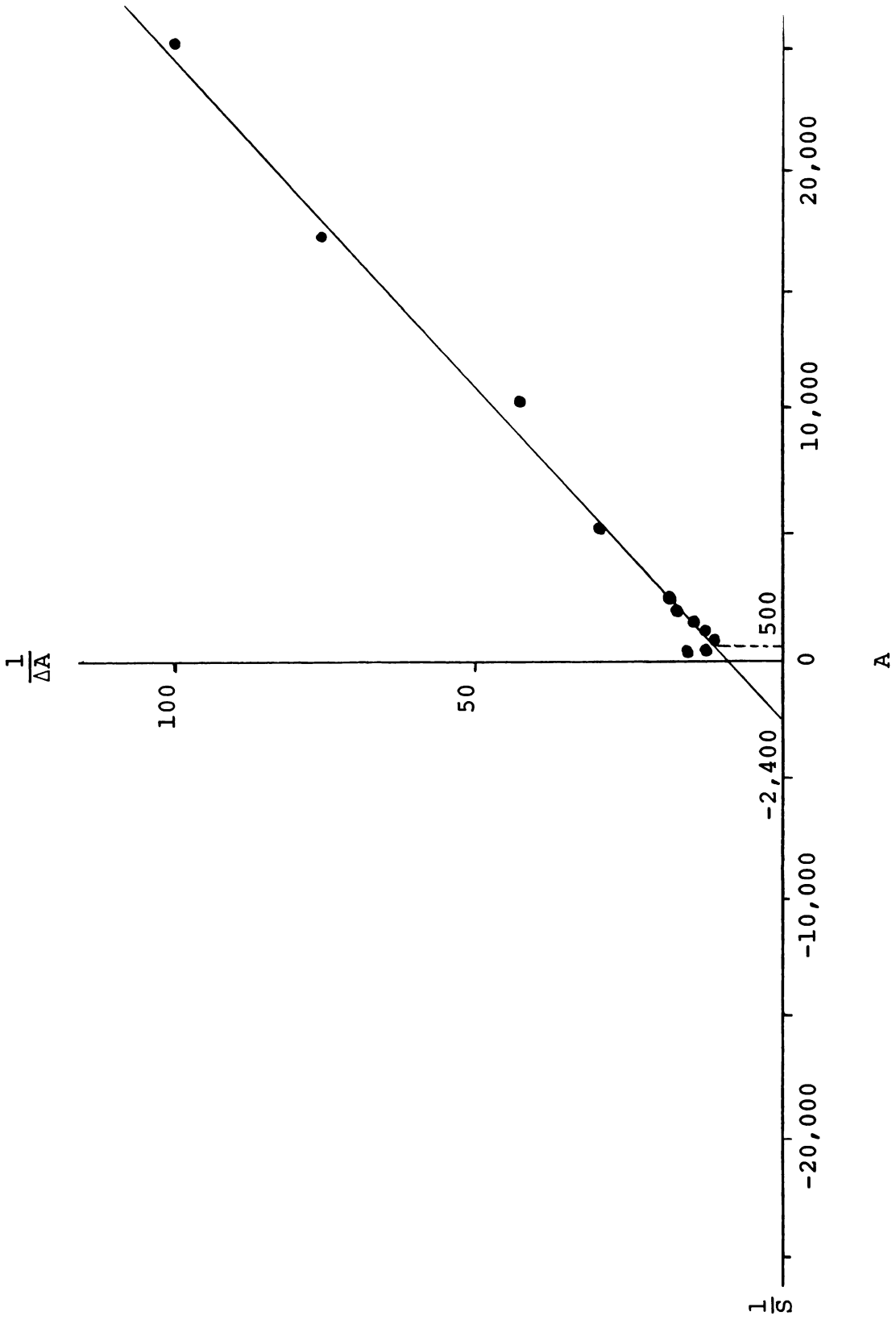
Tissue	Km* (2.7×10^{-4}M NADH)	Pyruvate Threshold*	Km* (2.2×10^{-4}M NADP)
Cardiac Muscle	4.8×10^{-5}	6.3×10^{-4}	3.3×10^{-2}
Skeletal Muscle	4.2×10^{-4}	2.0×10^{-3}	1.0×10^{-1}
Retina	5.3×10^{-5}	8.3×10^{-4}	5.7×10^{-3}
Lens	9.3×10^{-5}	4.5×10^{-4}	--------
Cornea	12.0×10^{-5}	4.5×10^{-4}	not done

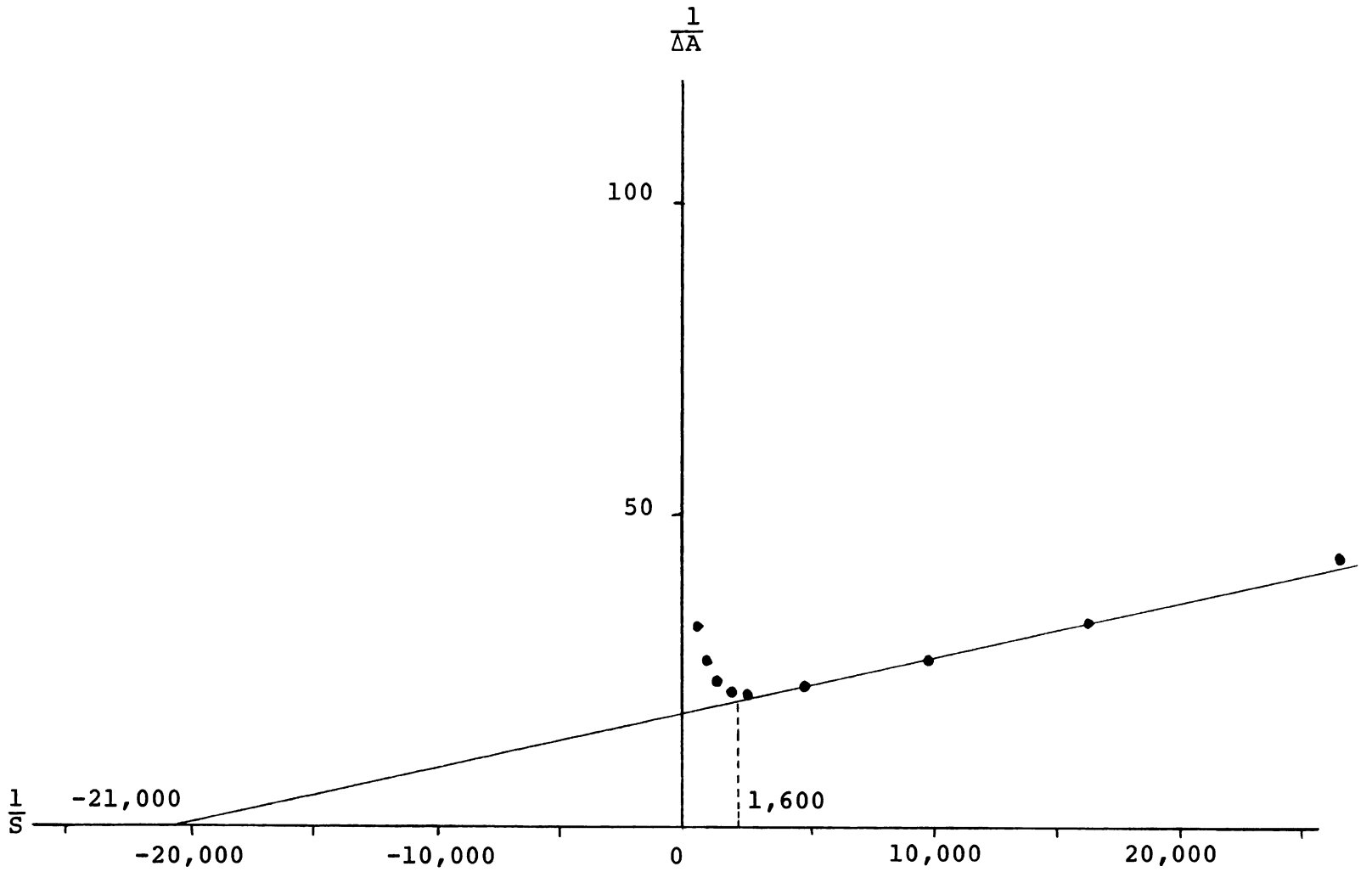
Measurements were taken at 25 C and pH 7.4

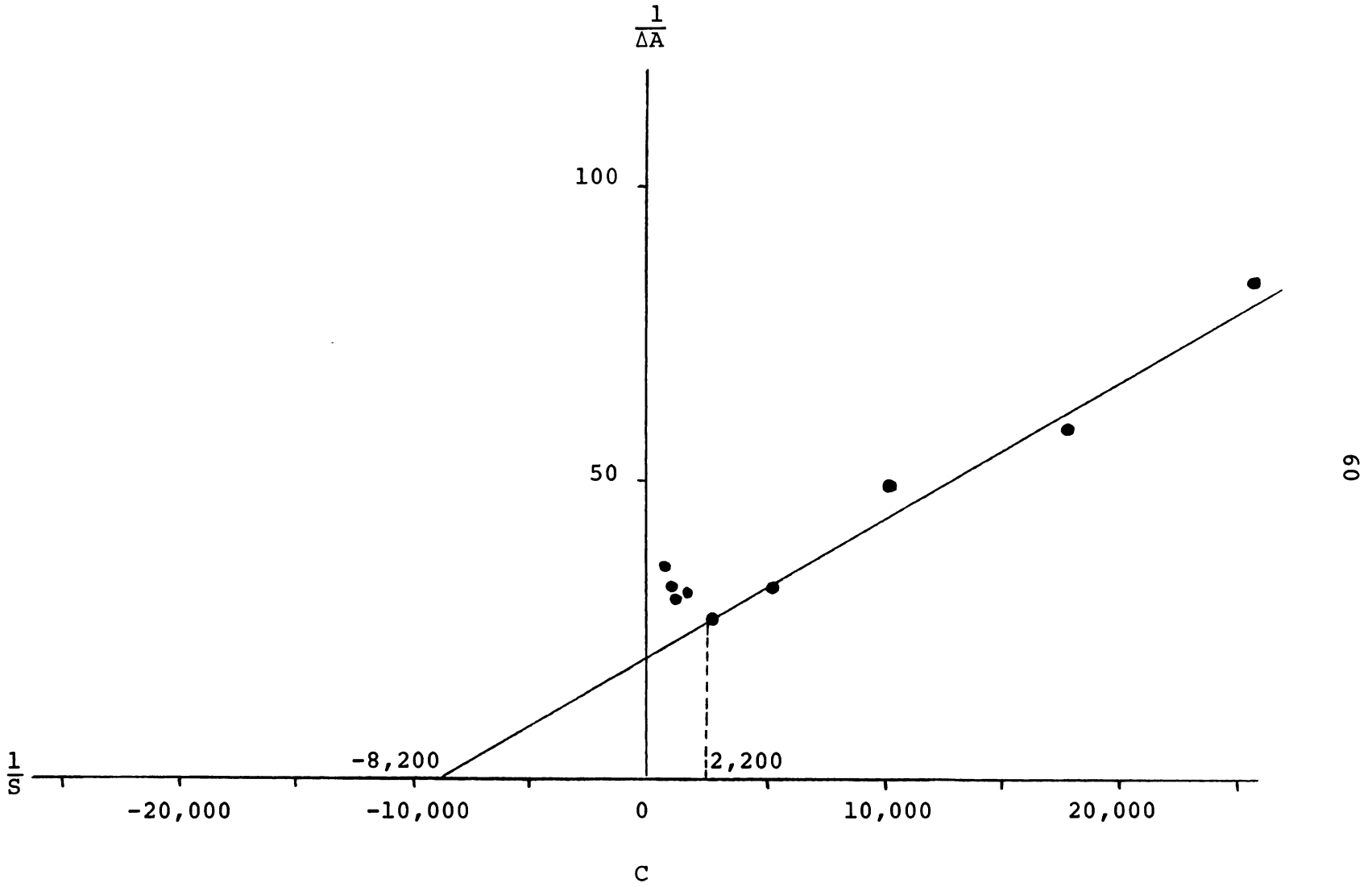
*Units are moles

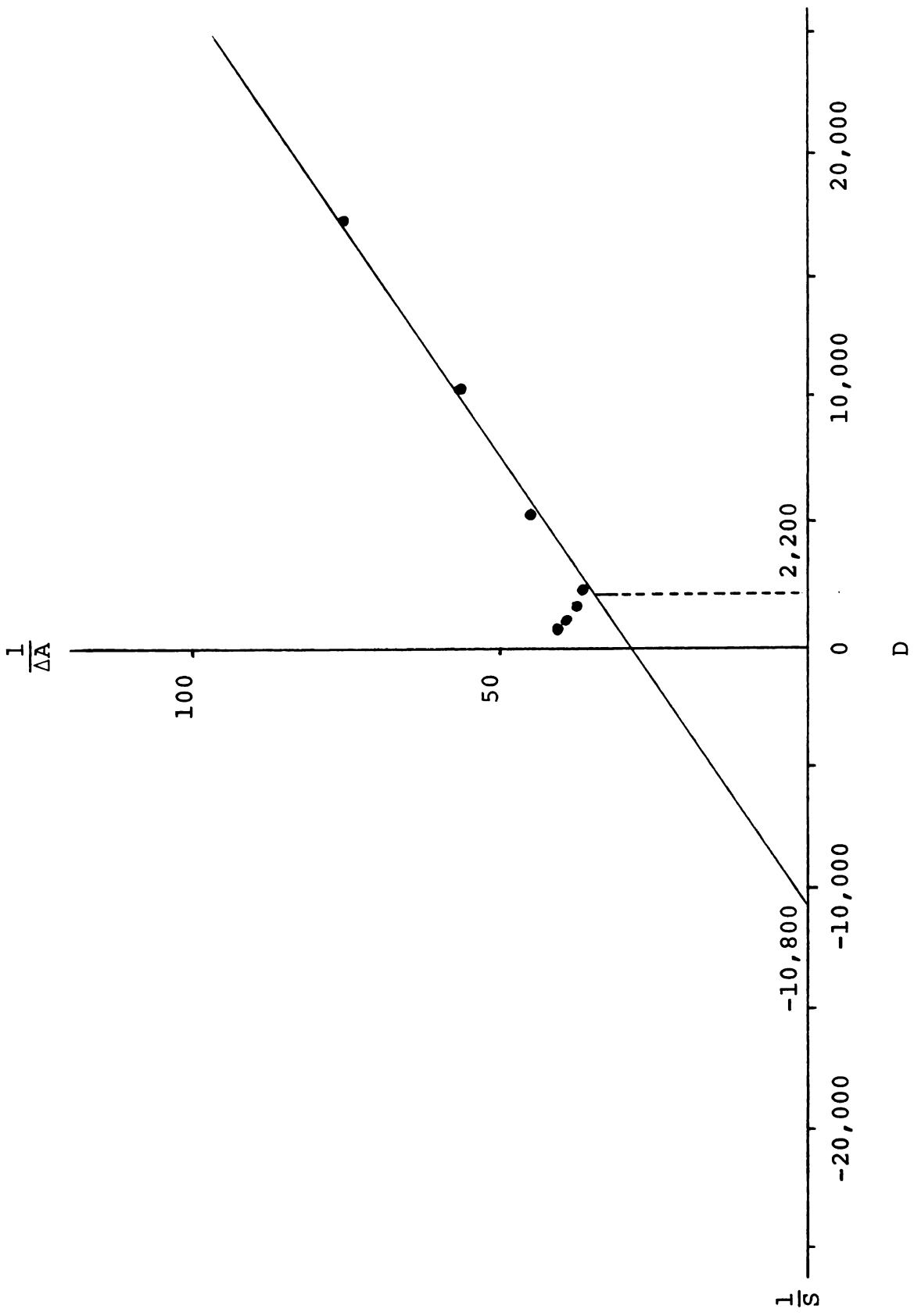
Figure 12. Lineweaver and Burk plot of lactate dehydrogenase isozymes from rainbow trout. Reaction velocities ($\Delta A/min$) were determined at 25 C, pH 7.4, 2.7×10^{-4}M NADH, and with pyruvate concentrations from 10^{-2}M to 4×10^{-5}M.

A. Skeletal muscle lactate dehydrogenase
B. Cardiac muscle lactate dehydrogenase
C. Cornea lactate dehydrogenase
D. Lens lactate dehydrogenase
E. Retina lactate dehydrogenase


1/ΔA
100
50
500
0
-2,400
-10,000
-20,000
1/S
A
20,000
10,000


1/ΔA
100
50
1/S
-21,000
1,600
-20,000
-10,000
0
10,000
20,000
B


1/ΔA
100
50
1/S
-8,200
2,200
-20,000
-10,000
0
10,000
20,000
C


$\frac{1}{\Delta A}$
100
50
0
2,200
D
$\frac{1}{S}$
-10,800
-10,000
-20,000
10,000
20,000

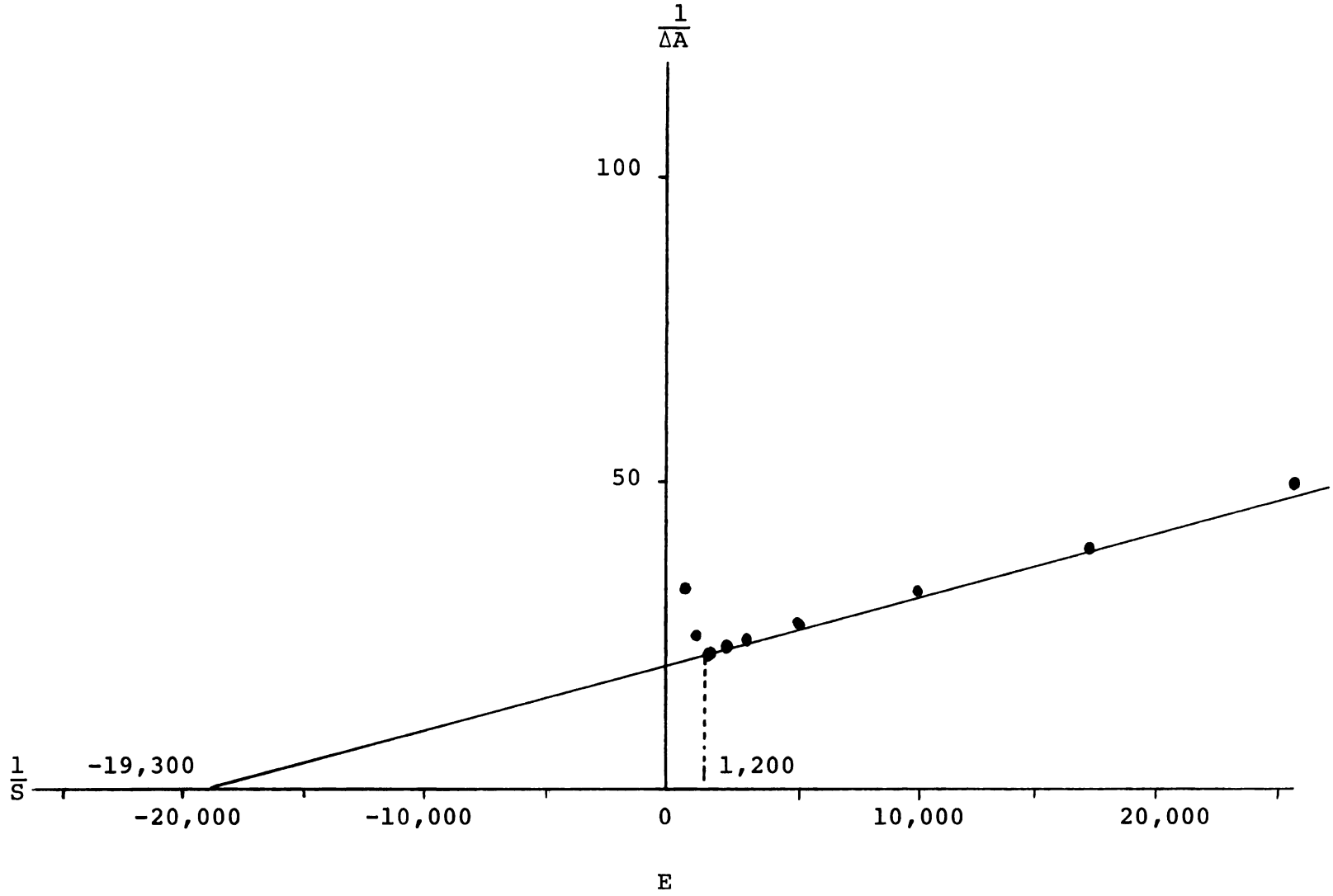

1/ΔA
100
50
1/S
-19,300
1,200
-20,000
-10,000
0
10,000
20,000
E

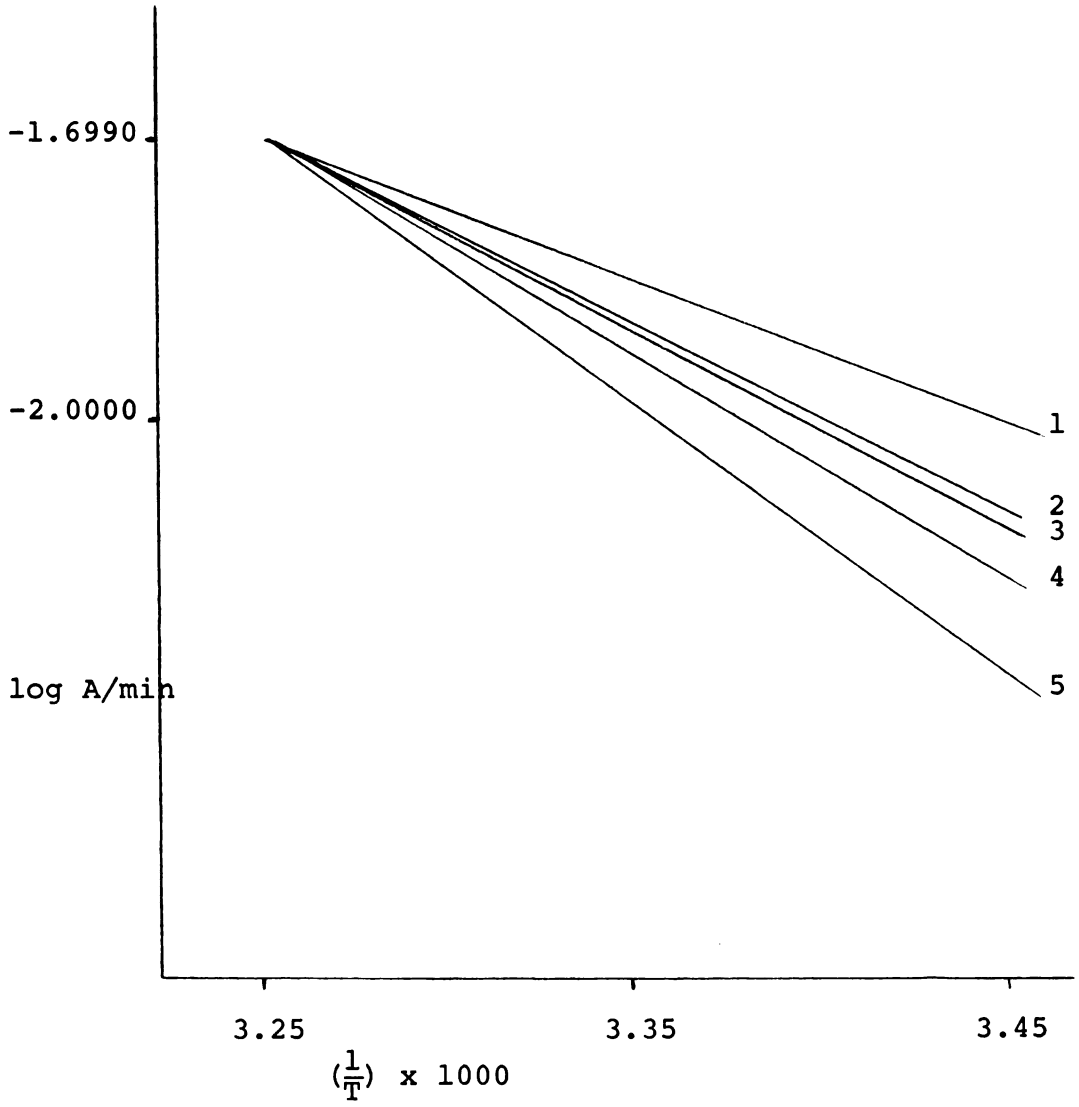
Figure 13 shows the reciprocals of the absolute temperature ($\frac{1}{T}$) plotted against the log ΔA/min. Each slope of a line represents the average of five observations. The lines have been adjusted to intersect at a common point (0.00325, -1.699). In the experiment itself, a temperature characteristic was determined for each observation (N = 5) and these were then averaged to give the values found in Table 4. The specific activity of the enzyme is also found in Table 4.

Table 4. Temperature characteristics of rainbow trout lactate dehydrogenase isozymes and their specific activity

Tissue	Temperature Characteristic Mean ± S.E. (N) Calories	Specific Activity*
Cardiac muscle	11,021 ± 241 (5)	0.9532
Skeletal muscle	7,316 ± 319 (5)	0.4674
Retina	13,320 ± 234 (5)	0.5911
Lens	9,722 ± 417 (5)	0.0047
Cornea	9,476 ± 211 (5)	0.0612

*LDH units/mg protein, where a unit of activity is that which causes an initial rate of oxidation of one micromole of NADH per minute under the conditions specified at 25 C.

Figure 13. The reciprocals of the absolute temperatures ($\frac{1}{T}$) plotted against the log of the velocity (log ΔA/min). Each slope is an average (N = 5) and the lines have been adjusted to pass through the point (0.00325, -1.6990). From similar plots the temperature characteristics were calculated (-μ = 4.6 x slope). Substrate, 0.053M dl-lactate. Coenzyme, 5.0 x 10^{-4}M NAD. pH = 8.8.



1. Skeletal muscle
2. Cornea
3. Lens
4. Cardiac muscle
5. Retina

Figure 13

DISCUSSION

Histochemical

Cornea. Numerous investigators have reported the epithelium and endothelium are the primary sites of glucose metabolism in mammalian corneas. The histochemical localization of various enzymes in these areas supports this conclusion (Cogan and Kuwabara 1960; Baum 1963). The evidence presented here shows the same is true in rainbow trout and probably teleosts in general.

The high LDH activity found in the cornea indicates the presence of the glycolytic pathway. Formazan deposition with glucose-6-phosphate and 6-phosphogluconate as substrates shows the pentose shunt is probably present in the epithelium, endothelium, as well as the fibroblasts. The inability to find succinate dehydrogenase, even with the aid of PMS, is evidence the citric acid cycle is not present in the epithelium. Inability to identify isocitrate dehydrogenase in the central portions of the epithelium supports this conclusion. Since malate, succinate and isocitrate dehydrogenases can be found in the endothelium, the citric cycle may be present and operative here.

Lens. The histochemical evidence indicates glucose metabolism by the lens is similar to corneal glucose metabolism. Apparently the glycolytic pathway and pentose shunt are present, while the citric acid cycle is incomplete. Unlike the cornea though, PMS in the succinate media caused a heavy blue formazan precipitate throughout the lens. Since malonate did not inhibit this reaction, it is doubtful succinate dehydrogenase was responsible for the precipitation.

This histochemical study on trout lenses and that of Cotlier's (1964) on rabbit lenses shows the capsule to be devoid of enzymes. Wortman and Becker (1956); and Dische and Ehrlich (1955) reported the capsule has enzymatic activity. This contradiction can be explained on the basis of the acellular nature of the capsule and absence of mitochondria and without these, there is unlikely to be any formazan formation.

Retina. Since malate, succinate, and isocitrate dehydrogenases could be found in the retina, the citric acid cycle is probably present and appears to be concentrated in the photoreceptor ellipsoids (inner segments of the visual cells). LDH was also observed principally in the ellipsoids. The pentose shunt is confined to the inner retinal layers and little evidence for its presence in the inner segments (ellipsoids) could be found. This

is a marked contrast from the observations of Kuwabara and Cogan (1960) who found the enzyme present in rabbit photoreceptor ellipsoids as well as the inner retinal layers.

The results from this and similar studies do not agree with Lowry's et al. (1961) findings. They reported photoreceptors were low in enzyme activity while histochemical evidence (Berkow and Patz 1961a) shows high activity. Cohen and Noell (1965) thought the NADPH and NADH diffused outward from the inner retinal layers where the enzymes are concentrated and were oxidized in the mitochondria of the ellipsoids; hence this is where the formazan was precipitated. The failure to find an active pentose shunt in the rainbow trout photoreceptors is evidence that NADPH is not involved in reducing retinene to Vitamin A (Eq. 4, 5, and 6) as proposed by Futterman (1963).

Inhibitors. The inhibitors were used to test the specificity of the reactions. Malonate, an inhibitor of succinate dehydrogenase, demonstrated the formazan precipitate with succinate in the media was due to succinate dehydrogenase. In a similar manner oxalate (an LDH inhibitor) showed the specificity of the LDH formazan precipitation.

Lactate Dehydrogenase

While electrophoretic evidence shows rainbow trout lens and cornea, as well as skeletal and cardiac muscles have identical LDH isozymes, kinetic studies show they are quite different. If the "subunit theory" is valid for this species, it would mean the monomers have the same net charge and molecular weight. A third gene locus could be responsible for the isozymes observed in the retina. Although the inability to disrupt and reassociate the subunits (by freezing the fastest and slowest migrating retinal forms in 1M NaCl) does not favor this explanation, other experiments (e.g., immunochemical studies) are needed to establish the relationship the different isozymes have to each other.

The temperature characteristics, μ, for rainbow trout skeletal (7,316 ± 319) and cardiac muscle (11,021 ± 241) are lower than the values for rabbit LDH, 8,285 ± 400 and 13,188 ± 74 calories respectively (Wroblewski and Gregory 1961). Although the μ can be used to predict the catalytic efficiency of the LDH isozymes, the results may be misleading. Apparently there are factors which compensate for the difference in the μ. If the entropy of activation values for both M_4 and H_4 isozymes were the same, then according to the "theory of absolute reaction rates" (at 30 C), M_4 should have a turnover number approximately 3,300 times greater than H_4.

The turnover number at 30 C, pH 7.4 x 10^{-4}M NADH and optimum pyruvate concentration for each, assuming molecular weight of 100,000 for each LDH, were found to be 43,000 and 12,000 respectively. Apparently there is some difference in entropy of the different isozymes that offset the heat of activation (temperature characteristic) (Wroblewski and Gregory 1961).

Based on μ values, retina LDH would have a turnover number considerably lower than any of the other lactate dehydrogenases. This would not favor glycolysis, which is one of the primary characteristics of mammalian retinas. This apparent difference in glucose metabolism is reflected in the LDH content of retinas. Graymore (1965) reported rat retinal LDH was predominantly the type found in skeletal muscle. The high retinal LDH μ confirms earlier evidence that teleost retinas do not carry on aerobic glycolysis (de Vincentiis 1951). de Vincentiis observed that the ratio, vitreous lactate conc./plasma lactate conc., in man, rabbit, fowl, and pigeon was 2.65, 2.68, 3.14, and 4.3 respectively. The teleost ratio though was approximately equal to one; hence the teleost retina is not a producer of lactate.

The low Km value (5.3 x 10^{-5}M pyruvate) and low threshold concentration of pyruvate necessary to inhibit rainbow trout retinal LDH (8.3 x 10^{-4}M) are two other factors which do not favor glycolysis and lactate formation.

The teleost retina is avascular, yet it has a considerable energy demand. If it were to depend on glycolysis for a major portion of this energy, considerable quantities of lactic acid would be produced and probably accumulate because the acid would have to diffuse over a great distance to the chorial capillaris to be eliminated. By depending, on the citric acid cycle for its energy requirement, the retina would be able to produce considerably more energy per glucose molecule; with the end product being CO_2. Although avascular, the rainbow trout retina has a very high oxygen tension (Fairbanks 1968) which could support the oxidative citric acid cycle. Because of the gradient in oxygen tension from the back of the retina to the vitreous humor, Fairbanks concluded that oxygen is utilized by the tissue.

Although the cornea and lens have μ's (9,476 ± 211 and 9,722 ± 417 calories respectively) and Km values (12.0×10^{-5}M and 9.3×10^{-5}M respectively which fall between the values for skeletal and cardiac muscle, both lactate dehydrogenases are inhibited by low concentrations of pyruvate. Since there is some doubt if the citric acid cycle exists in these two tissues, the LDH may regulate metabolism between the pentose shunt and glycolytic pathway. As previously reported pyruvate could stimulate oxidation by the pentose shunt (Futterman and Kinoshita, 1959b). Since the LDH from these two tissues is inhibited

by comparatively low concentrations of pyruvate, there could be a possible accumulation of substrate which would then stimulate the pentose shunt. But for the lens at least, the LDH apparently cannot utilize NADPH, hence it is doubtful pyruvate could stimulate the pentose shunt in the manner proposed by Futterman and Kinoshita (1959b). Since neither the lens nor cornea demand large amounts of metabolic energy, it is probable they rely heavily on glycolysis for their energy, and substrate inhibition may never be apparent in vivo.

The physiological significance of an operating pentose shunt in the ocular tissues is not known. The NADPH that is generated cannot be used directly by the mitochondrial cytochrome system to produce "high energy bonds." Normally the hydrogen must be transferred to NAD which is used by the mitochondria in forming the "high energy bonds." Since mammalian ocular tissues are not rich in this transhydrogenase, other alternate methods of transhydrogenation have been proposed by investigators, two of which have been discussed in this work; the sorbitol pathway and LDH linked with NADPH.

The histochemical and LDH kinetic studies conducted on rainbow trout ocular tissues support neither of these concepts. None of the ocular tissues appear to have sorbitol dehydrogenase and lens LDH cannot function with NADPH. Although retinal LDH can utilize NADPH, whether

it does *in vivo* is not certain. Histochemical evidence shows the LDH that can utilize NADPH is present in the photoreceptor ellipsoids while the pentose shunt enzymes are in the inner layers and not the ellipsoids. Possibly there could be some diffusion of the NADPH from the inner layers to the photoreceptor ellipsoids, but this would seem to be a rather inefficient method of obtaining energy.

SUMMARY AND CONCLUSIONS

1. Based on histochemical evidence, the most metabolically active portions of the examined rainbow trout ocular tissues are the lens epithelium; corneal epithelium and endothelium; and the retinal photoreceptor ellipsoids.
2. Histochemical evidence indicates the glycolytic pathway and pentose shunt are present in the rainbow trout lens and cornea. The inability to positively identify succinate dehydrogenase in the same tissues, suggests the citric acid cycle may not be present here. The glycolytic and citric cycle are present in the retina and are concentrated in the photoreceptor ellipsoids. The pentose shunt appears in the inner layers of the retina and not the ellipsoids.
3. Separation of rainbow trout lactate dehydrogenase (LDH) isozymes by disc electrophoresis shows the retina has four LDH isozymes that are not present in the other tissues examined. The gill had two LDH isozymes and the other tissues investigated had only one electrophoretically distinct LDH isozyme.

4. Although most of the rainbow trout LDH isozymes can not be distinguished electrophoretically, they do have different kinetic properties. Temperature characteristics of these electrophoretically indistinguishable LDH isozymes varied from 7,316 ± 319 (skeletal muscle LDH) to 11,021 ± 241 calories (cardiac muscle LDH); Michaelis constants (NADH as the coenzyme) were from 4.8×10^{-5}M (cardiac muscle LDH) to 4.2×10^{-4}M (skeletal muscle LDH) pyruvate; and threshold concentration for pyruvate inhibition varied from 6.3×10^{-4}M (cardiac muscle LDH) to 2.0×10^{-3}M (skeletal muscle LDH) pyruvate.
5. The temperature characteristic (13,320 ± 234 calories) and low optimum pyruvate concentration for retinal LDH do not favor glycolysis. Because relatively low concentrations of pyruvate (8.3×10^{-4}M) begin to inhibit retinal LDH, oxidation of pyruvate by the citric acid cycle would be favored.
6. This evidence, along with the reported high oxygen tension, suggests that the rainbow trout retina depends primarily on the oxidative citric acid cycle for energy.

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LITERATURE CITED

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APPENDICES

APPENDIX I

HISTOCHEMISTRY

Staining Media

Composition of standard medium used for demonstration of the respective dehydrogenases:

1.0M	Substrate	0.1 ml
3×10^{-2} to 6×10^{-2}M	NAD	
	or	
6×10^{-3}M	NADP	0.1 ml
0.1M	Cyanide (Adjusted to pH 7.2)	0.1 ml
0.06M	Phosphate buffer (pH 7.4-7.6)	0.25 ml
1 mg/ml	Nitro BT	0.25 ml
0.05M	Magnesium chloride	0.1 ml
	Distilled water	0.1 ml

Stock solutions of magnesium chloride, cyanide, and phosphate buffer were stored at 0-2 C. Aqueous nitro BT and coenzyme solutions were prepared immediately before use or kept frozen at -20 C for not longer than 48 hours. Stock substrate solutions were stored at 0-2 C with the exception that glucose-6-phosphate and isocitrate solutions were frozen.

The following variations were made for demonstrating the different dehydrogenases:

1. Glucose-6-phosphate dehydrogenase. Substrate, d-glucose-6-phosphate disodium salt; 0.01M sodium fluoride 0.05 ml; NADP.
2. 6-Phosphogluconate dehydrogenase. Substrate, 0.02M 6-phosphogluconic acid barium salt; NADP.
3. Isocitrate dehydrogenase. Substrate, dl-isocitric acid trisodium salt; NAD or NADP.
4. Malate dehydrogenase. Substrate, l-malic acid monosodium salt, pH adjusted to 7.2; NAD or NADP.
5. Lactate dehydrogenase. Substrate, sodium dl-lactic acid, pH adjusted to 7.2; NAD or NADP.
6. Sorbitol dehydrogenase. Substrate, d-sorbitol; NAD.
7. Xylitol dehydrogenase. Substrate, d-xylitol; NAD.
8. Succinate dehydrogenase. Equal volumes of 0.2M phosphate buffer, pH 7.6, and 0.2M sodium succinate salt were combined. An equal portion of this solution was added to an aqueous solution of nitro BT (1 mg/ml). Neither exogenous coenzyme, $MgCl_2$, nor respiratory inhibitor were added to this medium.

Inhibitors

In the inhibition studies, 0.1 ml of inhibitor replaced the distilled water in the staining media. The stock solutions were as follows:

0.025M Oxalate

0.050M Iodoacetate

0.100M Malonate

0.100M Cyanide

The malonate and cyanide solutions were adjusted to pH 8.2 and 7.2 respectively.

Glycerol-gel Mounting Media

Gelatin	40.0 gm
Distilled H_2O	210.0 ml
Soak 2 hours then add:	
Glycerine	250.0 ml
Phenol	5.0 ml

The mixture was then heated gently for 10 to 15 minutes, stirring all the while, until smooth. The glycerol-gel was stored at 4 C until used. It was heated to 50 C for mounting.

APPENDIX II

ELECTROPHORESIS

Stock Solutions

(A) 1N HCl 48 ml
2-Amino-2-Hydroxymethyl 1-3-Propandiol (Tris) 36.3 gm
N,N,N',N' Tetramethylethylene-diamine (Temed) 0.23 ml
H_2O to make 100 ml
(pH 8.8-9.0)

(B) 1N HCl 48 ml
Tris 5.98 gm
Temed 0.46 ml
H_2O to make 100 ml
(pH 6.6-6.8)

(C) Acrylamide 28.0 gm
N,N'-Methylene bis acyrlamide (Bis) 0.735 gm
H_2O to make 100 ml

(D) Acrylamide 10.0 gm
Bis 2.5 gm
H_2O to make 100 ml

(E) Riboflavin 4.0 mg per 100 ml H_2O

(F) Sucrose 40.0 gm
H_2O to make 100 ml

(G) Ammonium persulfate 0.14 gm
H_2O to make 100 ml

Buffer

Tris 3.0 gm
Glycine 14.4 gm
H_2O to make 1 liter

Tracking Dye

Bromphenol Blue solution 0.005 gm per 100 ml H_2O

Working Solutions

Separating Gel Solution - Lower Gel

1 part (A)
2 parts (C)
1 part H_2O
(pH 8.8-9.0)

Stacking Gel Solution

1 part (B)
2 parts (D)
1 part (E)
4 parts (F)
(pH 6.6-6.8)

Tube Preparation

The separating gel solution was prepared immediately prior to use and 1.0 ml put in each glass tube. A layer of water was then gently placed on top of the gel solution. Polymerization took 40 minutes at room temperature. After polymerization the water was removed and the stacking gel, 0.2 ml, put in each tube and then water layered. Polymerization took 20 minutes under fluorescent light.

The sample gel was prepared by mixing the supernatent from the tissue homogenate with the stacking gel in an approximately 1:5 ratio. 0.1 ml to 0.2 ml of this mixture was then added to the tubes. Fluorescent light was again

needed for polymerization. In many cases, due to the dilution and protein content of the sample, the gel failed to polymerize properly. This was not a problem in separating the LDH isozymes.

APPENDIX III

SOURCES OF COENZYMES, SUBSTRATES, AND CHEMICALS

Compound	M.W.	Source*
Acrylamide	71.08	MCB
2-Amino-2-Hydroxymethyl 1-3-Propandiol		MCB
Ammonium Persulfate	228.21	MCB
Cyanide (sodium salt)	49.02	MCB
Dihydronicotinamide Adenine Dinucleotide (NADH) Grade III (disodium salt)	709.00	SIG
Dihydronicotinamide Adenine Dinucleotide Phosphate (NADPH) (tetrasodium salt) type 1	833.00	SIG
Gum Acacia (Arabic Gum U.S.P. Powder)		MCB
Iodoacetic Acid (sodium salt)	207.94	MCB
D-Glucose-6-Phosphate (disodium salt)	304.10	SIG
DL-Isocitric Acid (trisodium salt)	276.12	NaBC
DL-Lactic Acid (sodium salt)	112.10	SIG
L-Malic Acid (sodium salt)	156	SIG
Malonic Acid (disodium salt)	148.10	SIG
N,N'-Methylene bis acrylamide (Bis)	154.17	EOC
Nicotinamide Adenine Dinucleotide (NAD) Grade III	663.50	SIG
Nicotinamide Adenine Dinucleotide Phosphate (sodium salt) Sigma Grade	765.00	SIG

Compound	M.W.	Source*
Nitro Blue Tetrasodium Chloride Salt	851.00	NuBC
Oxalate (disodium salt)	133.99	FSC
Phenazine Methosulfate	306.40	SIG
6-Phosphogluconic Acid (barium salt)	479.10	SIG
Pyruvic Acid (sodium salt)	110.00	SIG
Riboflavin (Vitamin B_2)	376.40	SIG
D-Sorbitol (glucitol) C. P. hydrate	182.17	PLI
Succinic Acid (sodium salt) A-grade	270.20	Cal
N,N,N',N' Tetramethylethylene-diamine (Temed)	116.21	MCB
D-Xylitol	152.00	SIG

*See Source List

Source List

BCC Baker Chemical Co., Phillipsburg, N. J.

Cal Calbiochem, Los Angeles, Calif.

EOC Eastman Organic Chemicals, Rochester, N.Y.

FSC Fisher Scientific Co., Pittsburg, Pa.

MCB Matheson Coleman and Bell, Norwood, Ohio

NaBC National Biochemical Corp., Cleveland, Ohio

NuBC Nutritional Biochemicals Corp., Cleveland, Ohio

PLI Pfanstiehl Laboratories, Waukegan, Ill.

SIG Sigma Chemical Co., St. Louis. Mo.