TEMPERATURE ADAPTATION IN ENZYMES FROM POIKILOTHERMS: 
ACETYLCHOLINESTERASES IN THE NERVOUS SYSTEM OF FISHES

by

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ABSTRACT

The effects of temperature upon acetylcholinesterase (AChE) from the nervous system of fish were studied to determine if such compensatory phenomena as thermal accommodation, thermal acclimation and evolutionary adaptation to temperature as displayed by this physiological system could be observed and interpreted at the level of enzyme function.

At probable physiological substrate concentrations the rate of acetylcholine (ACh) hydrolysis by AChE from rainbow trout (*Salmo gairdnerii*) and electric eel remains relatively unaffected by assay temperature over the temperature ranges normally experienced by these animals. Plots of Km versus temperature for these enzymes yield U shaped curves with minimum Km values occurring at temperatures close to the minimum habitat temperature. It is proposed that thermal accommodation of reaction rate is achieved throughout the habitat temperature range by temperature directed changes in enzyme-substrate affinity.

Thermal acclimation in rainbow trout, and probably in speckled trout (*Salvelinus fontinalis*) and lake trout (*Salvelinus namaychus*) is accompanied by alterations in the relative proportions of two electrophoretically distinct AChE variants displaying different and adaptive Km-temperature relationships. Since the minimum Km values and energies of activation of the two rainbow trout enzymes are similar, and the specific activities of the enzymes are essentially identical following acclimation
of fish to 2° and 17°C, it is suggested that rate compensation of AChE activity may not occur at different acclimation temperatures. However, the possibility remains that changes in such factors as pH, ionic environment and membrane lipids which accompany the acclimation process may act to stabilize reaction rates.

Comparisons of AChE enzymes from rainbow trout, electric eel and the Antarctic fish *Trematomus borchgrevinki* indicate that the evolutionary adaptation of AChE function in species inhabiting different thermal environments is based upon selection for a Km-temperature relationship that will allow thermal accommodation of reaction rate over the temperature range normally encountered. Shifts in the Km-temperature relationship during speciation are interpreted in terms of changes in enzyme conformation following the accumulation of amino acid substitutions. Possible mechanisms by which two AChE enzymes could be incorporated into the trout central nervous system were considered and a hypothesis involving hybridization between fish populations was tested with trout inter-species crosses. It was observed that hybrids formed between speckled and lake trout contained a greater number of electrophoretically distinct AChE variants than did either parent and further, the presence of similar thermally controlled AChE complexes in rainbow, speckled and lake trout indicated that the original incorporation of multiple AChE enzymes into the rainbow trout probably occurred prior to the evolutionary divergence of these three species.

It is concluded from this study that changes in enzyme-substrate affinity with temperature, and the temperature directed production of enzyme variants displaying adaptive
Km-temperature relationships, are both important mechanisms for controlling catalytic activity in an enzyme system which functions over a wide range of temperatures.
# TABLE OF CONTENTS

Abstract  
List of Tables  
List of Figures  
Acknowledgements  
Introduction  
  1. Statement of the Problem  
  2. Thermal Accommodation and Thermal Acclimation in the Central Nervous System of Poikilotherms  
  3. Role of Acetylcholinesterase in Nerve Transmission  
  4. Importance of Cholinergic Mechanisms in the Central Nervous System  
Methods  
  1. Experimental Animals  
  2. Enzyme Preparations  
    a. Preparation of Rainbow Trout Brain Acetylcholinesterase  
    b. Preparation of Acetylcholinesterase from Trout Brain and Spinal Cord for Electrophoresis  
    c. Electric Eel Acetylcholinesterase  
    d. Preparation of *Trematomus borchgrevinki* Brain Acetylcholinesterase  
  3. Assay of Acetylcholinesterase Activity  
  4. Gel Electrophoresis  
  5. Protein Determinations
6. Sucrose Gradient Centrifugation 16
7. Ultraviolet Difference Spectra 18
   a. Introduction 18
   b. Method 18

Results and Discussion 20

1. Partial Purification of Rainbow Trout Brain Acetylcholinesterase 20
2. Characterization of Acetylcholinesterase from the Rainbow Trout Central Nervous System 20
   a. Introduction 20
   b. Multiple Forms of Acetylcholinesterase in the Rainbow Trout Central Nervous System 20
   c. Substrate Specificity and Inhibition Studies of Rainbow Trout Brain Acetylcholinesterase 22
   d. Effect of pH on AChE Activity 27
   e. Sucrose Gradient Centrifugation of Rainbow Trout Brain Acetylcholinesterase 27

3. Characterization of Acetylcholinesterase from Trematomus borchgrevinki Brain 30
4. Effect of Assay Temperature upon the Kinetics of Acetylcholine Hydrolysis by Acetylcholinesterase 30
   a. Effect of Temperature on the Maximum Velocity of Acetylcholinesterase Hydrolysis 30
   b. Effect of Assay Temperature on Enzyme-Substrate Affinity 35
c. Relationship between Thermally Induced Changes in Km and Structural Conformation of Electric Eel Acetylcholinesterase

5. Thermal Accommodation, Thermal Acclimation and Evolutionary Adaptation to Temperature for Acetylcholinesterase from the Nervous System of Fish.
   a. Thermal Accommodation
   b. Thermal Acclimation
      (i) Adjustment of the thermal accommodation range
      (ii) Rate compensation of AChE activity
   c. Evolutionary Adaptation to Temperature
      (i) Adjustment of the thermal accommodation range
      (ii) Evolution of the rainbow trout brain AChE complex
      (iii) Regulation of the composition of the trout brain AChE complex during thermal acclimation

Summary

Abbreviations

Literature Cited
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Partial Purification of AChE from Rainbow Trout Brain</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>Summation Experiments with Choline Esters and the Effect of Inhibitors on the Hydrolysis of ACh by Rainbow Trout and Electric Eel AChEs</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>Apparent Energies of Activation (Ea) for the Rainbow Trout and Electric Eel AChEs at Several Temperatures</td>
<td>36</td>
</tr>
<tr>
<td>4</td>
<td>Rates of ACh Hydrolysis at Minimum Km Levels of ACh for AChEs from Rainbow Trout, Electric Eel and <em>Trematomus</em></td>
<td>41</td>
</tr>
<tr>
<td>5</td>
<td>Effect of Temperature upon the Km and Rate of ACh Hydrolysis for Brain AChE from <em>Trematomus borchgrevinki</em></td>
<td>44</td>
</tr>
<tr>
<td>6</td>
<td>Relationship between Km Change and $Q_{10}$ of the Rate of ACh Hydrolysis at Concentrations of ACh Approaching the Minimum Km for AChEs from Rainbow Trout, Electric Eel and <em>Trematomus borchgrevinki</em></td>
<td>46</td>
</tr>
<tr>
<td>7</td>
<td>Effect of Salts on the Km and Rate of Hydrolysis of ACh by AChE from 2°C Acclimated Rainbow Trout</td>
<td>61</td>
</tr>
<tr>
<td>8</td>
<td>Specific Activities of Brain AChE from Rainbow Trout Acclimated to 2°C and 17°C for 35 Days</td>
<td>63</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Facing Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Resolution of Rainbow Trout Brain AChEs by Acrylamide Gel Disc Electrophoresis</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>Substrate Specificity of AChE from 2°C Acclimated Rainbow Trout</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>Substrate Specificity of AChE from 17°C Acclimated Rainbow Trout</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>Influence of pH on the Activity of Rainbow Trout Brain AChEs</td>
<td>28</td>
</tr>
<tr>
<td>5</td>
<td>Sucrose Gradient Centrifugation of Rainbow Trout and Electric Eel AChEs</td>
<td>29</td>
</tr>
<tr>
<td>6</td>
<td>Arrhenius Plots of AChE Activity for the Rainbow Trout and Electric Eel Enzymes</td>
<td>33</td>
</tr>
<tr>
<td>7</td>
<td>Effect of Assay Temperature on the Km of AChE for AChEs from 17°C and 2°C Acclimated Rainbow Trout</td>
<td>38</td>
</tr>
<tr>
<td>8</td>
<td>Effect of Assay Temperature on the Km of ACh for Electric Eel AChE</td>
<td>39</td>
</tr>
<tr>
<td>9</td>
<td>Effect of Assay Temperature on the Km of ACh for <em>Trematomus borchgrevinki</em> AChE. Lineweaver-Burk Plots at 2°C and 10°C.</td>
<td>43</td>
</tr>
<tr>
<td>10</td>
<td>Sucrose Gradient Sedimentation Profiles of Electric Eel AChE at 15°C, 25°C and 33°C</td>
<td>48</td>
</tr>
<tr>
<td>11</td>
<td>Effect of Temperature on the Km of ACh for Electric Eel AChE Assayed in the Centrifugation Medium. Lineweaver-Burk Plots at 15°C, 25°C and 33°C.</td>
<td>49</td>
</tr>
<tr>
<td>Figure</td>
<td>Title</td>
<td>Facing Page</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>12</td>
<td>Effect of Temperature upon the Sedimentation Behaviour of Electric Eel AChE</td>
<td>51</td>
</tr>
<tr>
<td>13</td>
<td>Ultraviolet Difference Spectra of Electric Eel AChE as a Function of Temperature</td>
<td>52</td>
</tr>
<tr>
<td>14</td>
<td>Effect of Assay Temperature on the Km of ACh for AChEs from Rainbow Trout, Electric Eel and Trematomus</td>
<td>54</td>
</tr>
<tr>
<td>15</td>
<td>ACh Saturation Curves of Electric Eel AChE at 15°, 25° and 40°C</td>
<td>56</td>
</tr>
<tr>
<td>16</td>
<td>ACh Saturation Curves of Trematomus borchgrevinki AChE at 2° and 10°C</td>
<td>57</td>
</tr>
<tr>
<td>17</td>
<td>ACh Saturation Curves of 2°C Acclimated Rainbow Trout AChE at 0°, 2°, 12° and 18° C</td>
<td>58</td>
</tr>
<tr>
<td>18</td>
<td>Resolution of Brain AChEs from Speckled Trout, Lake Trout and Splake by Acrylamide Gel Disc Electrophoresis</td>
<td>72</td>
</tr>
</tbody>
</table>
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INTRODUCTION

1. Statement of the Problem

The ability of poikilotherms to utilize a wide range of thermal environments and in many cases to remain active throughout relatively large and often rapid changes in body temperature, raises many interesting problems at the biochemical level, particularly with respect to enzyme function.

Before discussing a number of these questions in detail, it is necessary to define several terms which will be used in this thesis to describe the reactions of biological systems to their environment. The reason for this is not so much to make claims for the correctness of certain terms, but rather to give specific meanings to terms which are often used loosely in the literature to group processes at the physiological level, so that these concepts may be extended to the molecular level.

The term "thermal accommodation" will refer to the ability of a function to proceed independently of temperature throughout a particular temperature range, the thermal accommodation range. Thermal accommodation occurs instantaneously and is an intrinsic property of the function.

"Thermal acclimation" describes the ability of a function to thermally accommodate over a different temperature range after a period of exposure of an individual organism to a new thermal regime. The time course of thermal acclimation for various physiological functions in poikilotherms is generally in the order of days or weeks.

"Evolutionary adaptation" will be used to describe changes which occur over time intervals longer than the life span of
the individual organism. Thermal evolutionary adaptation would cover such changes as those underlying the ability of a function to proceed in related stenothermic poikilotherms inhabiting different thermal environments in cases where the temperature difference is greater than can be compensated for by thermal accommodation or thermal acclimation.

The studies reported in this thesis were undertaken with the hope of providing at least partial answers to the following general questions. How are enzyme systems in many poikilotherms able to function over wide temperature ranges when the effects of temperature on the catalytic and regulatory properties of many mammalian and bacterial enzymes appear to be incompatible with the maintenance of function through such thermal extremes? In particular, can such phenomena as thermal accommodation, thermal acclimation and evolutionary adaptations to temperature as displayed by poikilothermic systems be observed and interpreted at the level of enzyme function?

The experimental approach adopted to investigate these problems can be outlined as follows:

(i) Select a critical physiological function which is known to display both thermal accommodation and thermal acclimation.

(ii) Isolate a key enzyme from this system and investigate the effects of immediate temperature changes and thermal acclimation upon the enzyme.

(iii) Compare temperature characteristics of this enzyme with similar characteristics of homologous enzymes
obtained from species inhabiting different thermal environments.

Acetylcholinesterase from the nervous system of fish was selected as a suitable enzyme for such a study. The rationale behind this choice is discussed in the following sections of the introduction.

2. **Thermal Accommodation and Thermal Acclimation in the Central Nervous System of Poikilotherms**

The importance of the central nervous system of poikilotherms in setting limits of thermal tolerance, and the probable limiting role of changes in the central nervous system in the overall acclimation process have been covered in numerous reviews (e.g. Fry, 1947; Brett, 1956; Fisher, 1958; Baslow, 1967; Prosser and Nagai, 1968). The following observations are probably the most relevant in establishing a relationship between central nervous function and the temperature tolerance (thermal accommodation range) of the whole organism. In 1908, Brecht found that heat paralysis in frogs was confined to the central nervous system, and occurred at temperatures below those at which peripheral nerve conduction and muscle contraction were inhibited. Battle (1926) determined the upper thermal limits at which various tissues in skates and flounder were able to elicit physiological responses and established that functions involving synapses and ephapses such as the heart pacemaker mechanism, conduction across the nerve muscle junction, and peristalsis in intestinal
smooth muscle, failed at, or slightly below, the lethal temperature of the organism. In a later report (Battle, 1929) it was noted that at temperatures approaching the upper thermal limit reflexes disappeared in definite sequence, and it was proposed that death resulted primarily from the failure of some central co-ordinating mechanism. Orr (1955) arrived at a similar conclusion for heat death in *Rana pipiens*. Prosser and coworkers have demonstrated by both behavioural and neuro-physiological techniques a hierarchy of temperature sensitivity in the nervous system of fish. Mid brain functions appear to be most temperature sensitive, followed by spinal cord, with peripheral nervous function being least sensitive (Roots and Prosser, 1962; Prosser and Farhi, 1965; Prosser and Nagai, 1968). Thus the thermal accommodation range of the central nervous system in fish appears to be a key factor in setting limits of thermal tolerance for the whole organism.

The ability of the central nervous system in fish to acclimatize to thermal stress has been most clearly demonstrated by Konishi and Hickman (1964). By monitoring the mid brain response to electrical stimulation of the retina in rainbow trout held at both high (16°C) and low (4°C) temperatures they were able to show compensatory changes in both nerve conduction velocity and central response time over an acclimation period of several weeks. The effect of thermal acclimation on central nervous function has also been shown in quite a different way by Roots and Prosser (1962) and Prosser and Farhi (1965). In a series of experiments utilizing the establishment of conditioned reflexes in goldfish they found that both the
lowest temperature at which a conditioned reflex could be established, and the cold blocking temperature for the conditioned response varied directly with the temperature to which the fish were acclimated.

Biochemical responses of the central nervous system in fish during thermal acclimation have been recently reviewed by Baslow (1967) and include changes in such factors as enzyme levels, structural lipids, electrolyte distribution, and various metabolites. However, little is known of the role of these changes in thermal acclimation of nervous function.

3. **Role of Acetylcholinesterase in Nerve Transmission**

The early studies which established the role of acetylcholine (ACh) as a chemical transmitter at the neuromuscular junction have been summarized in a monograph by Nachmansohn (1959), and later developments relating to the structure and function of cholinergic systems have been described in several recent reviews (Nachmansohn 1967, 1968, 1969; De Robertis, 1964). The following outline of cholinergic transmission of nerve impulses is given by Nachmansohn in his 1969 review.

ACh is released from the nerve membrane following excitation and acts as signal which is recognized by a stereospecific receptor protein located within the membrane. The reaction between ACh and the receptor induces a conformation change in the receptor molecule, releasing Ca^{++} ions bound to carboxyl groups in the protein. The free Ca^{++} ions induce further conformational changes in membrane phospholipids.
and other polyelectrolytes, leading to a change in nerve membrane permeability and the movement of from 20,000 to 40,000 ions across the membrane for each molecule of ACh initially released. Acetylcholinesterase (AChE) rapidly hydrolyses ACh, permitting the receptor protein to return to its original conformation thereby re-establishing the membrane permeability barrier. Nachmansohn proposes that the ACh-receptor protein and AChE are structurally linked and it has often been suggested that AChE may function as the ACh-receptor molecule. However, there is no general agreement on this latter point (Nachmansohn, 1959; 1969; Changeux, 1966; Ehrenpreis, 1967; Karlin, 1967; Podleski, 1969; Hasson-Voloch, 1968; Changeux et al, 1968, 1969).

While this theory of cholinergic transmission has gained wide acceptance, alternative mechanisms of ACh action have been proposed. For example, Durrell, et al (1969) suggest that ACh alters membrane permeability at the synapse by enhancing enzymatic hydrolysis of membrane phospholipids. It appears that hydrolysis of ACh by AChE would remain an essential component of this system.

4. Importance of Cholinergic Mechanisms in the Central Nervous System

In spite of a large volume of data relating to the widespread distribution of ACh, choline acetyltransferase (ChAc, the enzyme involved in synthesis of ACh) and AChE throughout the vertebrate central nervous system and to the central action of ACh (Feldberg, 1945; Feldberg and Vogt, 1948;
Burgen and Chipman, 1951; Hebb, 1963; Aprison et al, 1964; Eccles, 1964; De Robertis, 1964; Whittaker, 1965; Koelle, 1969; Krnjevic, 1969), there is still no clear evidence as to the importance of cholinergic mechanisms in the central nervous system. Although nerve ending and synaptic membrane fractions containing bound ACh, AChE and ChAC have been isolated from mammalian brain cortex preparations by sucrose gradient centrifugation (De Robertis, 1964; Rodriguez DeLores Arnaiz et al, 1967) the motor neurone-Renshaw cell synapse remains the first and only clear demonstration of a central cholinergic junction (Eccles et al, 1954).

It has been well documented that significant amounts of AChE occur within the axonal plasma membrane at regions far removed from synaptic junctions (Schlaepper and Torack, 1966; Brzin, 1966), and Nachmansohn (1959) has proposed that axonal conduction is mediated through a cholinergic mechanism essentially similar to that operating at the neuromuscular junction. If this is true, then cholinergic mechanisms would be essential for central nervous transmission. Consideration of this proposal generally has been discounted for the following reasons: 1. it is not possible in many cases to block axonal conduction by the application of potent AChE inhibitors, or if conduction is affected, the concentration of inhibitor far exceeds that required to block at the neuromuscular junction. Under these conditions blocking is assumed to result from a non-specific toxic effect of the compounds used; 2. direct application of ACh fails to elicit an action potential, although a general depolarization possibly relating to such factors as
altered pH or ionic environment may occur. Conclusions based on such results have been readily dismissed by Nachmansohn on the grounds that not enough is known about the availability of AChE and the ACh-receptor to externally applied pharmacological reagents (Nachmansohn, 1969). Despite considerable experimental evidence indicating that the outer membranes do in fact mask the plasma membrane from the action of AChE inhibitors and ACh in the external medium (Walsh and Deal, 1957; Dettbarn, 1960a;b; Armett and Richie, 1960; Rosenberg, 1965; Brzin, 1966; Martin and Rosenberg, 1968), the concept of cholinergically mediated axonal conduction has gained little support from workers in this field and the function of axonal AChE remains an open question.

The release of substantial amounts of ACh in the mammalian cerebral cortex following stimulation of afferent pathways and the mid brain reticular formation has also been cited as evidence for the importance of cholinergic mechanisms in the central nervous system (Kanai and Szerb, 1965; Phillis and Chong, 1965; Celesia and Jasper, 1966). Krnjevic (1969) has suggested that this type of slow and diffuse release is not suitable for rapid transmission but may play a more general function in cortical arousal, or in maintaining different activity levels in the central nervous system.

In recent years a number of investigators have searched for possible relationships between central cholinergic mechanisms and animal behaviour. These studies, and the problems involved in this type of approach have been reviewed by Russell (1969) and Weiss and Heller (1969). In the 1950's
Rosenzweig and coworkers investigated the effects of "enriched" and "impoverished" behavioural environments upon the levels of AChE activity in the rat central nervous system, and evidence was presented for increased AChE activities in animals subjected to such behavioural situations as maze training (Rosenzweig, 1957; Bennett et al., 1964). Russell established a dose-response relationship between inhibition of brain AChE with organophosphates, and the extinction of learned behavioural responses in rats. The relationship was not linear, but below a critical level of 40 to 50 percent inhibition of normal AChE activity, the speed of extinction was directly related to AChE inhibition (see Russell, 1969). Similar experiments by Glow and coworkers (Glow and Rose, 1966; Glow et al., 1966) demonstrated that reduction of AChE activity below 40 percent of the normal value leads to a sudden increase in brain ACh levels.

While it is difficult at present to evaluate the importance of such experiments in terms of underlying cholinergic mechanisms, this behavioural approach, together with the biochemical and neurophysiological evidence for the presence and action of ACh and AChE in the central nervous system, seems to indicate a definite role for cholinergic mechanisms in central nervous integration.

Thus AChE from the fish central nervous system appears to meet the criteria outlined at the beginning of this discussion for a suitable enzyme system with which to study thermal acclimimation and evolutionary adaptation to temperature at the level of enzyme function. It is an enzyme incorporated into a critical physiological process that is known to display both thermal
accommodation and thermal acclimation in response to changing environmental temperature.

An investigation into the effects of immediate temperature changes upon AChE activity revealed that thermal accommodation of reaction rate does occur at probable physiological substrate concentrations. The basis for this phenomenon lies in the influence of temperature upon enzyme substrate affinity.

The effects of thermal acclimation upon AChE were studied in several species of trout. Following acclimation of these fish to different temperatures, alterations in the relative proportions of two AChE variants were observed. Kinetic analysis of these enzymes showed that when the environmental temperature was maintained at a level where one form of the enzyme could no longer thermally accommodate for reaction rate, or where regulation of catalytic activity might be lost, a second form is produced for which the enzyme substrate affinity-temperature relationship is better suited for control of these functions.

Comparisons of the properties of probably homologous AChE enzymes from different species of fish inhabiting markedly different thermal environments lead to the conclusion that evolutionary adaption of AChE function to temperature is based upon selection for an enzyme-substrate affinity-temperature relationship permitting thermal accommodation of reaction rate over the temperature range normally experienced by the species.

In answer to the question initially posed in designing these experiments, it can be stated that thermal accommodation,
thermal acclimation and evolutionary adaptation to temperature as displayed by many poikilotherm systems can be observed and interpreted at the level of enzyme function.
METHODS

1. **Experimental Animals**

   Adult rainbow trout (*Salmo gairdnerii*) averaging about 250 g were obtained from the Sun Valley Trout Farm, Port Moody, B.C. The fish were held in a large outdoor tank with circulating water and fed *ad lib* on Clark's New Age 'Fish Feed' (J. R. Clark Co., Salt Lake City, Utah). Rainbow trout gill netted in Pinask Lake, B.C. during both summer and winter were used in a number of experiments. *S. gairdnerii* can tolerate temperatures in the range of 0° to 25°C.

   In acclimation experiments, groups of trout (generally 18 fish) were taken from the outdoor holding pool and placed in 60 gallon stainless steel tanks in which the temperature could be controlled accurately with heating and refrigeration units. As it was not possible to circulate water through these tanks, one quarter of the volume was changed daily. The natural photoperiod of the outdoor tank was maintained throughout the acclimation period of from 30 to 36 days, and the fish were fed daily with Clark's 'Fish Feed'.

   Speckled trout (*Salvelinus fontinalis*), lake trout (*Salvelinus namaychus*) and the speckled-lake hybrid, splake, were made available by the University of Toronto Laboratory for Experimental Limnology, Southern Research Station, Maple, Ontario. Immature fish (6-15 cm in length) were thermally acclimated in circulating water tanks at the research station.
Trematonus borchgrevinki were captured in McMurdo Sound, Antarctica during the summer of 1965 by Dr. G. N. Somero. Brains from 6 fish were freeze dried and stored at -20°C until assayed in November 1969. The temperature of the waters inhabited by this fish average -1.9°C, with annual variations in the order of 0.1°C. This species has not been recorded in waters with temperatures higher than 2°C (see Somero and DeVries, 1967).

2. Enzyme Preparations

(a) Preparation of Rainbow Trout Brain Acetylcholinesterase

Pooled brains were homogenized in a small volume of cold distilled water and freeze dried. The procedure for solubilization of the membrane bound enzyme was based on the butanol extraction technique described by Morton (1955). All steps were carried out in a 4°C cold room. Freeze dried brains were dispersed in cold n-butanol (1 g dried tissue to 50 ml solvent) by grinding with a mortar and pestle. The suspension was stirred for 2 hours, then centrifuged at 10,000 x gravity for 15 minutes. The supernatant was discarded and the pellet re-extracted with n-butanol as before. After centrifuging the pellet was dispersed in dry acetone at -20°C, stirred for 5 minutes and centrifuged at 10,000 x gravity for 20 minutes. The pellet was dried in vacuo over calcium chloride at -20°C. After complete removal of the organic solvents the acetone dried powder was taken up in cold 10^{-2} M tris-HCl buffer, pH 7.2, stirred for 2 hours and centrifuged at 30,000 x gravity for one hour to remove insoluble material. The
supernatant was brought to 20 percent saturation with solid ammonium sulphate, left to settle for one hour, then centrifuged at 10,000 x gravity for 10 minutes. The pellet was discarded and the supernatant adjusted to 50 percent saturation with solid ammonium sulphate. After precipitation overnight the sediment was collected by centrifugation at 10,000 x gravity for 20 minutes and taken up in distilled water. This preparation could be stored frozen in 5 percent saturated ammonium sulphate for at least six months without loss of activity. The enzyme solution was dialysed against 10^{-2} M tris-HCl buffer, pH 7.2 before use.

(b) Preparation of Acetylcholinesterase from Trout Brain and Spinal Cord for Electrophoresis

Brain and spinal cord were dissected out, washed and homogenized in a small volume of cold distilled water. The preparation was frozen and thawed six times then spun at 2,000 rpm for 20 minutes on a bench centrifuge. The supernatant was drawn off and used as a source of AChE for electrophoresis.

(c) Electric Eel Acetylcholinesterase

A partially purified preparation of AChE extracted from the electric organ of electric eel was purchased from the Sigma Chemical Company (Electric eel Acetylcholinesterase type V). This material had an activity of 1,000\mu Molar units/mg (one\mu molar unit will hydrolyze 1\mu Mole of acetylcholine per minute at pH 8.0 at 37°C) and gave a single band of AChE activity on acrylamide disc electrophoresis.
(d) Preparation of *Trematomus borchgrevinki* brain

Acetylcholinesterase

Freeze dried brains from 6 fish were homogenized in 4 ml of $10^{-2}$ M tris-HCl buffer, pH 7.2. This preparation was used as a source of brain AChE.

3. **Assay of Acetylcholinesterase Activity**

AChE catalyses the reaction

$$\text{acetylcholine} + \text{H}_2\text{O} \rightleftharpoons \text{Choline} + \text{acetic acid}$$

AChE activity can be conveniently determined by following the rate of hydrogen ion production. In this study, AChE activity was assayed in an automatic titrator (Radiometer, Copenhagen, type TTA 31) operated as a pH stat with sodium hydroxide as titrant. The volume of sodium hydroxide added per unit time gives a measure of the rate of acetylcholine (ACh) hydrolysis. The basic reaction mixture contained buffer, enzyme and substrate in a total volume of 2 ml. Temperature was controlled accurately with a circulating water bath (Lauda Brinkman, K-2/R) coupled to a water jacket surrounding the reaction vessel. Sodium hydroxide (generally $10^{-2}$ M) was standardized by titration against potassium biphthalate. In all experiments appropriate blanks were run to compensate for any uptake of atmospheric carbon dioxide or non-enzymic hydrolysis of substrate.

4. **Gel Electrophoresis**

Electrophoretic separation of esterases was carried out by standard acrylamide disc electrophoresis (Davis, 1964), using
a 4 percent stacking gel, a 7 percent separating gel, and $5 \times 10^{-3}$ M tris-glycine tank buffer, pH 8.7. Samples were applied to the top of the stacking gel and run for 90 minutes at 3 mA per tube and at 4°C.

Esterase activity was localized within the gel by the $\alpha$-naphthyl acetate-diazonium salt technique (Market and Hunter, 1959). After completion of the run, gels were placed in $4 \times 10^{-2}$ M tris-HCl buffer pH 7.1 for 10 minutes to inhibit precipitation of the dye during staining (Allen et al., 1965) and then transferred to a solution containing $\alpha$-naphthylacetate (Sigma Chemical Co., 40 mg/100 ml) and Fast Blue R.R. salt (Sigma Chemical Co., 70 mg/100 ml) in $4 \times 10^{-2}$ M tris-HCl buffer, pH 7.1. Gels were reacted for 20 minutes at 25°C then placed in an acid-alcohol solution (ethanol: 10 percent acetic acid, 3:2) for 30 minutes to stop the reaction and reduce nonspecific staining. After rehydration in distilled water, the gels could be stored indefinitely at 4°C.

5. **Protein Determinations**

Protein concentrations of AChE preparations were estimated by the method of Lowry et al. (1951). Samples were diluted to a concentration of approximately 20$\mu$g protein/ml. A standard curve was plotted with ovalbumin in the range 5 to 100$\mu$g/ml for each set of determinations.

6. **Sucrose Gradient Centrifugation**

Sucrose gradients were prepared in 5 ml tubes with a dual chamber gradient maker. Samples were run on the Spinco
model L preparative ultracentrifuge equipped with an SW 39 rotor.

For studies of the effect of temperature on the sedimentation of electric eel AChE, 5 to 20 percent sucrose gradients were prepared in $2 \times 10^{-2}$ M sodium barbitone buffer, pH 7.2, and contained $2 \times 10^{-1}$ M magnesium chloride. A 0.2 ml sample of AChE dissolved in the same buffer-magnesium chloride solution was layered onto the top of each gradient and run for six hours at 35,000 rpm at the specified temperature. Drop count fractions were collected by gravity and assayed for AChE activity by the standard method. Gradient densities at each temperature were calculated from refractometer readings.

In the case of trout brain AChE preparations, where the activity was too low to measure conveniently by the standard assay, the enzyme was detected in situ by the method of Jolley et al (1967). In this technique sucrose gradients are prepared in acrylamide solution and photopolymerized after completion of the run. Gradients were formed by placing 2.3 ml of a 7.5 percent acrylamide gel solution containing $2 \times 10^{-1}$ M magnesium chloride in the distal chamber of the gradient maker and 2.5 ml of the same solution containing 20 percent sucrose in the outlet chamber. The sample, dissolved in 0.2 ml of the 7.5 percent acrylamide gel solution, was applied to the top of the prepared gradient immediately before centrifuging and run for ten hours at 35,000 rpm and at 4°C. After completion of the run, water was layered onto the top of each tube to give a flat surface, and the gel polymerized under a fluorescent light. The gels were then removed from
the centrifuge tubes and stained for esterase activity as described previously for disc electrophoresis.

7. **Ultra Violet Difference Spectra**

a. Introduction

Proteins exhibit characteristic absorption patterns in the ultra violet region of the spectrum. The basis of this phenomenon has been reviewed by Wetlaufer (1962), and it is generally considered that absorption in the 260-300 nm interval is caused by electron transitions in the amino acids phenylalanine, tryptophan and tyrosine, whilst absorption in the 230 nm region results mainly from electron transitions in the carboxyl moiety of the peptide group. In a number of studies, shifts in absorption spectra have been interpreted in terms of changes in the secondary and tertiary structure of the protein molecule (Yanari and Bovey, 1960; Foss, 1960, 1961; Massey et al., 1966). Thus, by plotting the UV absorption spectra obtained at different temperatures relative to the spectrum at a reference temperature thermally induced changes in protein conformation can be detected. Ideally, the difference spectra are obtained in a twin beam spectrophotometer in which the samples can be read relative to a cell held at the reference temperature.

b. Method

UV spectra were determined with a Cary 15 recording spectrophotometer equipped with a circulating water bath. All measurements were made in 1 cm cells. Electric eel AChE
was dissolved in $2 \times 10^{-2}$ M sodium phosphate buffer, pH 7.2, to a concentration of 0.15 mg/ml, and extensively dialyzed against this buffer before use. The reference cuvette contained sample buffer only and was maintained at the sample cuvette temperature. Spectral scans were run in triplicate and a difference spectrum for each temperature was obtained by replotting the results relative to the spectrum obtained at 25°C.
RESULTS AND DISCUSSION

1. **Partial Purification of Rainbow Trout Brain Acetylcholinesterase**

   The relative activities of fractions taken throughout the extraction procedure are given in Table 1. Although the specific activity of the final preparation represents only about a four fold purification over the original crude homogenate, titration plots gave straight lines down to an AChE concentration of $2 \times 10^{-4}$ M. With crude homogenates non-linear plots were encountered and activity could not be accurately measured below about $5 \times 10^{-4}$ M ACh.

2. **Characterization of Acetylcholinesterases from the Rainbow Trout Central Nervous System**

   a. **Introduction**

      Acetylcholinesterases have been defined by Augustinsson (1957) as eserine-sensitive esterases which are inhibited by high acetylcholine concentrations (generally 3 to $5 \times 10^{-3}$M) and which split acetylcholine at a much higher rate than butyryl choline. The compound 284C51 (Burroughs Wellcome) at concentrations from $10^{-6}$ to $10^{-5}$ M gives about 100,000 fold greater inhibition of AChE than of other cholinesterases (Austin and Berry, 1953).

   b. **Multiple Forms of Acetylcholinesterase in the Rainbow Trout Central Nervous System**

      At least 7 bands of esterase activity were observed following acrylamide gel disc electrophoresis of extracts from rainbow trout brain and spinal cord. Specific AChE bands
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific Activity</th>
<th>Purification (X)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain homogenate</td>
<td>8.5</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Butanol solubilized enzyme</td>
<td>11.9</td>
<td>1.4</td>
<td>107</td>
</tr>
<tr>
<td>20% (NH₄)₂SO₄ fraction</td>
<td>23.7</td>
<td>2.8</td>
<td>53</td>
</tr>
<tr>
<td>20-50% (NH₄)₂SO₄ fraction</td>
<td>32.3</td>
<td>3.8</td>
<td>25</td>
</tr>
</tbody>
</table>

a. 2°C acclimated trout

b. Specific activity is expressed as μM ACh hydrolysed/mg protein/hour
were detected by their inhibition with $10^{-4}$ M eserine and with $10^{-5}$ M 284C51. The AChE bands obtained with brain preparations from rainbow trout acclimated to $2^\circ$, $12^\circ$ and $17^\circ$C for 32 days are shown in Figure 1. Identical results were obtained with both purified extracts and crude homogenates of brain, and with the spinal cord preparations. AChE from cold acclimated trout shows a distinctly slower migration rate than does the enzyme from warm acclimated fish. Trout acclimated to $12^\circ$C possess both enzyme types. On the basis of staining intensity it was estimated that equal amounts of the two rainbow enzymes were present in the $12^\circ$C fish. Brain extracts from wild trout populations were also examined. In fish captured during the winter, only the $2^\circ$C enzyme was present; in summer fish, both enzymes usually occurred, with the $17^\circ$C form in excess. Fish held at $9^\circ$C in the outdoor pool during autumn also had both enzymes, with the cold form predominating.

c. Substrate Specificity and Inhibition Studies of Rainbow Trout Brain Acetylcholinesterases

Substrate saturation plots for brain extracts from $2^\circ$C and $17^\circ$C acclimated rainbow trout are given in Figure 2 and Figure 3. Both enzymes show greater activity with ACh than with propionyl-choline or butyryl choline, and in each case substrate inhibition occurs at concentrations above about $3 \times 10^{-3}$ M ACh. The relative activities of rainbow trout preparations and electric eel AChE with individual and paired substrates and inhibitors are given in Table 2. No summation
Resolution of Rainbow Trout Brain AChEs by Acrylamide Gel Disc Electrophoresis.

Electrophoresis conditions: 90 minutes at 3 mA and 400 volts per gel. Tris-glycine tank buffer, pH 8.7

2°C acclimated trout
17°C acclimated trout
12°C acclimated trout
Figure 2. Substrate Specificity of AChE from 2°C Acclimated Rainbow Trout. Standard assay in $10^{-2}$ M tris-HCl buffer, pH 7.2 with $10^{-2}$ M sodium hydroxide as titrant. Assay temperature 2°C.

- Acetylcholine iodide
- Propionylcholine iodide
- Butyrylcholine iodide
Figure 3. Substrate Specificity of AChE from 17°C Acclimated Rainbow Trout. Standard assay as in Figure 2. Assay temperature 15°C.

- Acetylcholine iodide
- Propionylcholine iodide
- Butyrylcholine iodide
Table 2. Summation Experiments with Choline Esters and the Effect of Inhibitors on Hydrolysis of ACh by Rainbow Trout and Electric Eel AChEs

<table>
<thead>
<tr>
<th>Substrate</th>
<th>2°C trout AChE</th>
<th>17°C trout AChE</th>
<th>Electric eel AChE (×10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>54.0</td>
<td>43.2</td>
<td>71.0</td>
</tr>
<tr>
<td>Propionylcholine</td>
<td>9.2</td>
<td>7.3</td>
<td>61.0</td>
</tr>
<tr>
<td>Butyrylcholine</td>
<td>0</td>
<td>0</td>
<td>4.3</td>
</tr>
<tr>
<td>Acetylcholine and propionylcholine</td>
<td>22.1</td>
<td>15.9</td>
<td>64.0</td>
</tr>
<tr>
<td>Acetylcholine and butyrylcholine</td>
<td>11.9</td>
<td>5.3</td>
<td>9.9</td>
</tr>
<tr>
<td>Propionylcholine and butyrylcholine</td>
<td>0</td>
<td>3.5</td>
<td>8.0</td>
</tr>
<tr>
<td>Acetylcholine and 5 x 10⁻³ M eserine</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acetylcholine and 10⁻⁶ M 284C51</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Assays were carried out at 10°C for the rainbow trout enzymes and at 25°C for the electric eel AChE.

Substrates: Acetylcholine iodide; propionylcholine iodide; butyrylcholine iodide.
of activity occurred with any substrate pair tested, indicating that butyryl or propionyl cholinesterases are not contributing to the rate of hydrolysis. Hydrolysis of $2.5 \times 10^{-3}$ M ACh by either the $2^\circ$ and $17^\circ$C trout AChE or the eel enzyme, was completely inhibited with $5 \times 10^{-5}$ M eserine, and with $10^{-6}$ M 284C51. These results obtained with specific substrates and inhibitors indicate that essentially all of the esterase activity of the trout brain extracts detected by the assay method can be attributed to AChE.

d. Effect of pH on Acetylcholinesterase Activity

The bell shaped pH-activity curves for the rainbow trout AChEs shown in Figure 4 are similar to those obtained with AChEs from a variety of sources (Bernsohn et al, 1963; Bull and Lindquist, 1968; Silman and Karlin, 1967).

e. Sucrose Gradient Centrifugation of Rainbow Trout Brain Acetylcholinesterases

The results of the experiments in which rainbow trout brain preparations were centrifuged on sucrose gradients made up in acrylamide gel solution are shown in Figure 5. Identical results were obtained with extracts from both $2^\circ$ and $17^\circ$C acclimated fish. In each case 3 bands of esterase activity were detected, the central band sedimenting in the same position as electric eel AChE. Inhibition with $10^{-5}$ M 284C51 and $10^{-4}$ M eserine did not remove any one band, but greatly reduced the intensity of the central band.
Figure 4. Influence of pH on the Activity of Rainbow Trout Brain AChEs. Sodium phosphate buffer, $10^{-2}$ M, was used in the range pH 6-8, and tris-HCl buffer, $10^{-2}$ M, containing $10^{-2}$ M sodium chloride from pH 7.5 to 9.5.

1. $17^\circ$C acclimated rainbow trout. Assay temperature $17^\circ$C.

2. $2^\circ$C acclimated rainbow trout. Assay temperature $2^\circ$C.
Rate (μM ACh hydrolyzed /mg protein/hr)
Figure 5. Sucrose Gradient Centrifugation of Rainbow Trout and Electric Eel AChEs. A 5 ml 0 to 20 percent sucrose gradient was formed in 7.5 percent acrylamide gel solution containing $2 \times 10^{-1}$ M MgCl$_2$. Samples of 0.2 ml were layered on top. Centrifugation conditions: 10 hours at 100,000 x gravity at $4^\circ$C. After centrifugation the gels were photopolymerized and stained for esterase activity.
electric eel AChE

2°C acclimated trout

17°C acclimated trout

2° or 17°C acclimated trout. Stained in the presence of $10^{-6} M \text{ 284C51}$
The following conclusions can be made on the basis of these results. 1. Rainbow trout brain esterases occur in 3 molecular size classes, AChE probably sharing a class with at least one other esterase. 2. The AChEs from both warm and cold acclimated trout and from electric eel are of similar molecular weight, about 260,000 (Leuzinger et al, 1969).

3. **Characterization of Acetylcholinesterase from Trematomus borchgrevinki Brain**

Because of the small amount of material available it was not possible to characterize this enzyme system as fully as was done for the rainbow trout AChE's. It was concluded from the following results that the total activity detected by the assay could be attributed to a single AChE species.

1. The presence of $2 \times 10^{-6}$ M 284C51 in the assay completely inhibited ACh hydrolysis.

2. Acrylamide gel electrophoresis of the crude brain homogenate gave four bands of esterase activity; only one of these bands was inhibited by $5 \times 10^{-6}$ M 284C51 and $10^{-4}$ M eserine.

4. **Effect of Assay Temperature upon the Kinetics of Acetylcholine Hydrolysis by Acetylcholinesterase**

a. **Effect of Temperature on the Maximum Velocity of Acetylcholine Hydrolysis**

Numerous attempts have been made to establish relationships between the thermal tolerances of poikilotherms and temperature dependent characteristics of their enzymes. The
characteristics most commonly investigated are the temperatures for optimal enzyme activity at V max levels of substrate, and enzyme thermostability. Studies of this type have been reviewed recently by Licht (1967), Read (1967), and Ushakov (1967) among others. In most cases both thermal optima and thermal denaturation occur at temperatures above those encountered in the environment. Of particular interest with reference to the present study are results obtained for fish cholinesterases. Kusakina (1963) has demonstrated a positive relationship between the temperature at which muscle excitability is lost, the temperature of muscle cholinesterase inactivation, and the thermal environment of cottoid fish. The temperature at which 50 percent of cholinesterase activity was lost after incubation for 30 minutes increased with increasing habitat temperature, and occurred at temperatures 12° to 15°C above those at which muscle excitability ceased. Similarly, Baslow and Nigrelli (1964) found no difference in the levels of cholinesterase activity of control and heat killed killifish. Alexandrov (1969) has proposed that in cases where a correlation exists between habitat temperature and the resistance of proteins to thermal denaturation, but where thermostability is clearly not setting temperature limits for the organism, the property subject to selection may not be thermostability per se, but rather, a certain conformational flexibility of the protein molecule required for such functions as catalytic activity and allosteric regulation. Changes in thermally dependent conformational flexibility may in turn alter the thermal denaturation character-
istics of the molecule. Certain aspects of thermally dependent conformational changes in protein structure are discussed in detail in following sections of this thesis.

Criticism against the use of thermal optima for maximum enzyme activity and thermostability as measures of thermal adaptation is generally leveled at the non-physiological nature of the experimental methods. For example, the actual temperature at which enzyme activity is maximal depends on such factors as assay time, and the V max levels of substrate employed are generally far in excess of probable physiological substrate concentrations. Similarly, protein thermostability is highly dependent upon such factors as presence or absence of substrate, pH, ionic environment, and binding of the enzyme to membranes and other cellular structures (Vessell and Yielding, 1966, Bowen and Kerwin, 1956; Cheeseman et al, 1967). Thus any correlation between in vitro enzyme thermal optima or thermostability with environmental temperature, may be simply fortuitous.

Arrhenius plots of log V opt. versus 1/T for AChEs from rainbow trout acclimated to 2° and 17°C and for electric eel are shown in Figure 6. In each case the maximum velocity of the reaction increases with temperature beyond the thermal range of the fish. With brain AChE from Trematomus borchgrevinki, the activity of the enzyme at high levels of substrate (10⁻³ M ACh) is twice as great at 10°C as it is at 2°C (see Table 5). This fish has not been recorded in waters above 2°C. Thus, the optimal temperature for enzyme activity, and thermal stability of enzyme structure, are probably not important factors in
Figure 6. Arrhenius Plots of AChE Activity for the Rainbow Trout and Electric Eel Enzymes. Standard assay in $10^{-2}$ M tris-HCl buffer, pH 7.2 with $V_{opt.}$ levels of ACh.

- 17°C acclimated trout AChE
- 2°C acclimated trout AChE
- Electric eel AChE
setting thermal limits for AChE activity in these species.

It has been argued that rates of enzyme activity in cold adapted organisms where available thermal energy is low may be maintained through a lowering of the energy of activation (Ea). Although such a correlation between Ea and the environmental temperature has been demonstrated in a number of cases (Vroman and Brown, 1963; Kwon and Olcott, 1965; Somero and Hochachka, 1968) it is by no means universal (Read, 1964; Hochachka and Somero, 1968).

In the case of the rainbow trout and electric eel AChEs the curved Arrhenius plots yield energies of activation which decrease as the temperature is raised. Similarly curved plots have been reported previously for an electric eel AChE (Wilson and Cabib, 1956), and for cholinesterases from a variety of sources (Chadwick, 1957). Wilson and Cabib (1956) interpreted the decrease in Ea with temperature for electric eel AChE in terms of a change in the rate limiting step for the overall reaction. They proposed that Arrhenius plots would give a straight line through the temperature range where one of the steps is rate limiting, and a different straight line in the range where the other step is slower. These two lines would be joined by a curved portion over the thermal range where both rates are comparable. The curve would flatten at higher temperatures as the rate approached the step which had the lower energy of activation. It is also suggested that log V max versus 1/T plots need not give straight lines when both V max and Km vary with temperature (see below). Non-linear Arrhenius
plots obtained with amino oxidase have been related to a
temperature dependent transition of the enzyme between two
conformations (Koster and Veeger, 1968; Massey et al, 1966).
Recently, evidence has been presented for similar temperature-
dependent transitions between multiple forms of serum cholin-
esterases and AChE from erythrocytes (Main, 1969).

Energies of activation for the trout and electric
eel AChEs were calculated from the relationship \( \log (K_2 - K_1) = \frac{E_a}{4.6} \left( \frac{1}{T_1} - \frac{1}{T_2} \right) \) where \( K_1 \) and \( K_2 \) are reaction velocities at
absolute temperatures \( T_1 \) and \( T_2 \) and \( E_a \) is the energy of
activation. \( E_a \) values at a specific temperature was obtained by
drawing a tangent to the Arrhenius curve at that temperature.
The relationships between \( E_a \) and temperature are given in Table 3.

For the rainbow trout enzymes the \( E_a \) values at any
given temperature are essentially the same. The results are
difficult to interpret in terms of adaptive advantage, as the
value for the 17°C enzyme is lower at 17°C than the value of the 2°C
enzyme at 2°C. The value of 1.9 K cal/mole obtained for the
electric eel enzyme at the probable habitat temperature
(25°C) is lower than either of the trout enzymes at 2°C.
It must be concluded that no clear relationship exists between
the apparent energies of activation of AChE and habitat
temperature in the species studied.

b. Effect of Temperature on Enzyme-Substrate Affinity

Current models of enzyme regulation stress the importance
of effectors in modifying enzyme-substrate affinities (Atkinson,
Table 3. Apparent Energies of Activation (Ea) for the Rainbow Trout and Electric Eel AChEs at several temperatures

<table>
<thead>
<tr>
<th>AChE source</th>
<th>Temperature (°C)</th>
<th>Ea (kcal/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2°C acclimated trout</td>
<td>2</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>1.8</td>
</tr>
<tr>
<td>17°C acclimated trout</td>
<td>2</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>2.1</td>
</tr>
<tr>
<td>Electric eel</td>
<td>25</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>3.0</td>
</tr>
</tbody>
</table>
1966; Stadtman, 1966). It has been suggested recently that in the case of enzymes from poikilotherms, temperature may play a role analogous to that of positive and negative effectors by altering enzyme-substrate affinities in such a way as to bring about compensatory changes in the rates of enzyme reactions throughout the biological thermal range of the organism (Hochachka and Somero, 1968).

The relationships between the affinity of rainbow trout and electric eel AChE for ACh (as measured by the reciprocal of the apparent Michaelis constant, $K_m$) and assay temperature, are shown in Figures 7 and 8.

In the case of the trout AChEs it is apparent that over the upper part of the biological thermal range of each enzyme, the affinities of the enzymes for the ACh vary with temperature and approach maximal values (minimum $K_m$) at temperatures corresponding to those at which the fish were acclimated. This relationship between habitat temperature and $K_m$ also holds for the electric eel enzyme; the minimum $K_m$ in this case occurs at about $25^\circ C$, a temperature corresponding closely to the probable minimum habitat temperature. A similar correspondence between minimum $K_m$ and environmental temperature has been observed for pyruvate kinases from rainbow trout and the antarctic fish *Trematomus bernacchii* (Somero and Hochachka, 1968) for lactate dehydrogenases from trout, tuna, lungfish, *Trematomus* (Hochachka and Somero, 1968) and king-crab (Somero and Hochachka, 1969), for glucose-6-phosphate dehydrogenase and 6-phospho-gluconate dehydrogenases from king-crab (Somero, 1969), for salmon and lungfish fructose diphosphatases (Behrisch,
Figure 7. Effect of Assay Temperature on the Km of ACh for AChEs from 17° and 2°C Acclimated Rainbow Trout. Standard assay in 10^{-2} M tris-HCl buffer, pH 7.2. The enzymes were assayed at ACh concentrations in the range 10^{-4} to 5 \times 10^{-3} M and Km values were determined from double-reciprocal plots ($1/V$ versus $1/\text{ACh}$).

- 17°C acclimated trout AChE
- 2°C acclimated trout AChE
Figure 8. Effect of Assay Temperature on the Km of ACh for Electric Eel AChE. Standard assay in $10^{-2}$ M tris-HCl buffer, pH 7.2. The enzyme was assayed at ACh concentrations in the range $10^{-4}$ to $5 \times 10^{-3}$ M and Km values were determined from double-reciprocal plots ($1/V$ versus $1/ACh$).
1969; Behrisch and Hochachka, 1969), for rainbow trout NADP isocitrate dehydrogenases (Moon, personal communication) and citrate synthases (Hochachka and Lewis, 1970).

At temperatures above that at which $K_m$ is minimum, the physiological significance of the $K_m$-temperature relationship is clear. Although a rise in temperature would be expected to increase the velocity of the enzyme reaction, this effect is counteracted by a decrease in enzyme-substrate affinity. Hence, the overall reaction rate remains relatively independent of temperature. This type of temperature-independence is characteristic of both forms of trout AChE. The 'warm' form shows this relationship at temperatures above about $17^\circ C$; the 'cold' form shows the relationship above about $2^\circ C$. This effect is reflected in the rates of acetylcholine hydrolysis at substrate concentrations approaching the minimum $K_m$ (Table 4). In this connection, a recent estimate of $2.16 \times 10^{-4}$ M for the concentration of ACh released into the synaptic space of the vertebrate motor endplate corresponds closely to the minimum $K_m$ value of both trout AChEs (Namba and Grob, 1969).

A considerable overcompensation for the accelerating effects of temperature upon reaction rate is observed with the $2^\circ C$ trout enzyme at $18^\circ C$, and with the electric eel AChE at $40^\circ C$ (Table 4). However, this effect may be of little physiological importance. In the case of the $2^\circ C$ trout enzyme the decrease in reaction rate is presumably compensated for in vivo by the appearance of the $17^\circ C$ enzyme, while the electric eel may not encounter water temperatures as high as $40^\circ C$. 
Table 4. Rates of ACh Hydrolysis at Minimum Km Levels of ACh for AChEs from Rainbow Trout, Electric Eel and Trematomus

<table>
<thead>
<tr>
<th>AChE source</th>
<th>ACh (M)</th>
<th>Temperature at minimum Km (°C)</th>
<th>Assay temperature (°C)</th>
<th>V/V(^a) (minimum Km)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2°C acclimated trout</td>
<td>2.5 x 10(^{-4})</td>
<td>2</td>
<td>0</td>
<td>0.79</td>
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<tr>
<td></td>
<td></td>
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<td>17°C acclimated trout</td>
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<td></td>
<td>40</td>
<td>0.65</td>
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<tr>
<td>Trematomus borchgrevinki</td>
<td>1.5 x 10(^{-4})</td>
<td>10</td>
<td>10</td>
<td>0.51(^b)</td>
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</tbody>
</table>

a. Rate of hydrolysis at assay temperature/rate at temperature of minimum Km

b. The rate of ACh hydrolysis at 10°C is expressed relative to the rate at 2°C
At lower thermal extremes the $K_m$-temperature relationship is reversed. For the 'warm' form of the trout enzyme the $K_m$ rises sharply as the temperature falls below 15°C, reaching a value at 10°C that is about 4 times the minimum value. Thus, at 2°C the $K_m$ of the enzyme is probably so high as to make the enzyme essentially inactive at low and presumably physiological substrate concentrations. Similarly, a fall in temperature from 20°C to 15°C leads to a 3 fold increase in the $K_m$ of the electric eel enzyme. This decrease in enzyme-substrate affinity is reflected in a reduction of the rates of ACh hydrolysis at low substrate concentration at temperatures below the minimum $K_m$ point (Table 4).

For *Trematomus borchgrevinki*, the amount of enzyme available was sufficient only for $K_m$ determinations at two temperatures. Lineweaver-Burk plots obtained at 2°C and 10°C with the crude brain homogenate are presented in Figure 9. The effect of assay temperature on $K_m$, and on reaction rates at several substrate concentrations, are given in Table 5. As with the trout and electric eel AChEs, the affinity of the *Trematomus* enzyme for ACh changes markedly with temperature. The reaction rates given in Table 5 show that at low substrate concentrations (below $5 \times 10^{-4}$ M ACh) there is considerable overcompensation for reaction rate. For example, at $1.5 \times 10^{-4}$ M ACh the rate of ACh hydrolysis at 10°C is only 52 percent of the rate at 2°C. Again, this effect is of little importance in vivo as *Trematomus borchgrevinki* does not inhabit waters above 2°C, and dies within 81 minutes at 10°C (Somero and DeVries, 1967).
Figure 9. Effect of Assay Temperature on the Km of ACh for *Trematomus borchgrevinki* AChE. Lineweaver-Burk Plots at 2°C (□) and 10°C (●). Standard assay in 10⁻² M tris-HCl buffer, pH 7.2.
Table 5. Effect of Temperature upon the Km and Rate of ACh Hydrolysis for Brain AChE from *Trematomus borchgrevinki*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reaction rate (µM ACh hydrolysed/ml/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2°C</td>
</tr>
<tr>
<td>ACh (M)</td>
<td></td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>46</td>
</tr>
<tr>
<td>$5 \times 10^{-4}$</td>
<td>49</td>
</tr>
<tr>
<td>$2.5 \times 10^{-4}$</td>
<td>39</td>
</tr>
<tr>
<td>$1.5 \times 10^{-4}$</td>
<td>$31^{a}$</td>
</tr>
<tr>
<td>Km</td>
<td>$1.5 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

\(^a\) Values obtained by extrapolation from Lineweaver-Burk plots.
Clearly, more data must be obtained before the significance of Km-temperature relationships can be assessed for AChE from such a remarkably stenothermal species.

The relationships between change in Km and reaction rate, expressed as $Q_{10}^c$, over temperature ranges above the temperature of minimum Km for the rainbow trout, electric eel and Trematomus AChEs are given in Table 6. For the trout and electric eel AChEs a 2 to 3 fold change in Km over 10°C will maintain an approximately constant rate of ACh hydrolysis at substrate concentrations approaching the minimum Km value.

c. Relationship between Thermally Induced Changes in Km and Structural Conformation of Electric Eel Acetylcholinesterase

Main (1969) has presented evidence indicating that the kinetic properties of bovine erythrocyte AChE are dependent upon thermally induced changes in molecular aggregation of the solubilized enzyme. If the Km-temperature relationships observed with the solubilized fish AChEs are dependent upon such aggregation it is difficult to see how the Km-temperature effect could occur in vivo where the enzyme forms a structural component of the neural membrane. Thus, the possibility exists that the Km-temperature relationship is simply an artifact of the in vitro assay system. To examine this question, the effect of temperature upon the molecular conformation of electric eel AChE over the range where Km is markedly affected by temperature was investigated by sucrose gradient centrifugation and ultraviolet difference spectroscopy.
Table 6. Relationship between Km Change and $Q_{10}$ of the Rate of ACh Hydrolysis at Concentrations of ACh Approaching the Minimum Km for AChEs from Rainbow Trout, Electric Eel and *Trematomus borchgrevinki*

<table>
<thead>
<tr>
<th>AChE source</th>
<th>ACh (M)</th>
<th>Temperature range ($^\circ$C)</th>
<th>$Q_{10}$</th>
<th>Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>2$^\circ$C acclimated trout</td>
<td>$2.5 \times 10^{-4}$</td>
<td>2 - 12</td>
<td>0.9</td>
<td>2.2</td>
</tr>
<tr>
<td>17$^\circ$C acclimated trout</td>
<td>$2.5 \times 10^{-4}$</td>
<td>17 - 27</td>
<td>1.2</td>
<td>2.4</td>
</tr>
<tr>
<td>Electric eel</td>
<td>$10^{-4}$</td>
<td>25 - 35</td>
<td>1.1</td>
<td>3.0</td>
</tr>
<tr>
<td><em>Trematomus borchgrevinki</em></td>
<td>$1.5 \times 10^{-4}$</td>
<td>2 - 10</td>
<td>0.4</td>
<td>26.8</td>
</tr>
</tbody>
</table>

$Q_{10}$ values were calculated from the relationship

$$Q_{10} = \left( \frac{V_1}{V_2} \right)^{10/(t_1 - t_2)}$$

where $V_1$ and $V_2$ are reaction rates at temperatures $t_1$ and $t_2$ respectively.
AChE from the electric organ of the eel Electrophorus electricus has a molecular weight of 260,000 and possess a subunit structure of the $2\alpha_2\beta$ type (Leuzinger et al, 1969). Studies by Changeux (1966) and Grafius et al (1968) have shown that the sedimentation behaviour of electric organ AChEs is dependent upon the ionic environment, polydispersity of the system increasing at ionic strengths below that of $2 \times 10^{-1}$ M MgCl$_2$. Sucrose gradient centrifugation patterns obtained at $15^\circ$, $25^\circ$ and $33^\circ$C for electric eel AChE in the presence of $2 \times 10^{-1}$ M magnesium chloride show a single major component accounting for the majority of the total activity, with some polydispersity about this fraction (Figure 10).

As the Km of AChE is known to be dependent upon the ionic environment (Changeux, 1966) Km determinations were made in the centrifugation medium at $15^\circ$, $25^\circ$ and $33^\circ$C. The Lineweaver-Burk plots in Figure 11 indicate a significant change in the Km of the enzyme with temperature under these conditions.

In sucrose gradient studies of this type sedimentation coefficients of proteins are generally determined by running known standards with the test sample. The method could not be used in this study as sedimentation behaviour of both the test sample and the standard might be expected to change with temperature. However, since the sedimentation coefficient of a protein is directly proportional to the viscosity of the centrifugation medium (see Martin and Ames, 1961), and the viscosity of the sucrose solution is essentially a linear function of temperature over the temperature range investigated,
Figure 10. Sucrose Gradient Sedimentation Profiles of Electric Eel AChE at 15°C (■), 25°C (●) and 33°C (▲). Experimental conditions are given in the methods section. Activities are expressed relative to the fraction with the highest activity in each experiment.
Figure 11. Effect of Temperature on the Km of ACh for Electric Eel AChE Assayed in the Centrifugation Medium. Lineweaver-Burk Plots at 15°C (■), 25°C (●) and 33°C (▲).
it was argued that if the sedimentation coefficient of the protein does not change significantly with temperature, plots of sedimentation distance versus temperature, or viscosity, should yield a straight line for a standard set of experimental conditions. Marked changes in protein conformation or molecular aggregation with temperature would be expected to alter the sedimentation behaviour of the enzyme and, unless the sedimentation coefficient changed in a linear fashion with temperature, plots of sedimentation distance against temperature would be non-linear. The plot of sedimentation distance (as a function of fraction number) against temperature for the electric eel AChE is shown in Figure 12, and is clearly linear. One is left to conclude that either, (a) no structural change large enough to be detected by the method has occurred or, (b) changes may have occurred in such a way that the sedimentation coefficient of the enzyme altered in a linear fashion with temperature over a temperature range where the Km temperature relationship shows a sharp change. Thus, although the Km changes may be accompanied by small conformational changes in the enzyme, the large Km difference between 15° and 25°C cannot be attributed to such major changes as alterations in molecular aggregation or dissociation of subunits. Similar conclusions can be drawn from the ultraviolet difference spectra plotted in Figure 13. Although changes presumably resulting from conformational alterations in the enzyme occur in the ultraviolet spectra with changing temperature, the actual absorbance changes are small in comparison to those accompanying such events as protein denaturation.
Figure 12. Effect of Temperature upon the Sedimentation Behaviour of Electric Eel AChE. Each point represents the mean of three determinations. The error bars indicate the range about this mean, and are in the order of ± 0.5 fractions (approximately ± 0.08 ml). The fraction number is expressed as a function of total fraction number to compensate for variation in the number of fractions collected in each group of experiments (27 to 32 fractions). Experimental conditions are given in the methods section.
Figure 13. Ultraviolet Difference Spectra of Electric Eel AChE as a Function of Temperature. Spectra are plotted relative to the spectrum obtained at 25°C.
5. Thermal Accommodation, Thermal Acclimation, and Evolutionary Adaptation to Temperature for Acetylcholinesterase from the Nervous System of Fish

a. Thermal Accommodation

The basis for the observation that at low and probably physiological substrate concentrations the enzyme reaction may remain relatively unaffected by temperature over a particular temperature range appears to lie in the Km-temperature relationships shown in Figure 14. For the rainbow trout and electric eel AChE's, a 2 to 3 fold change in Km over 10°C at temperatures above the minimum Km point will maintain an approximately constant rate of ACh hydrolysis (Table 6). With the Trematomus enzyme insufficient data were available to determine if rate accommodation and thermally induced Km changes occur over temperature ranges normally encountered by the animal.

The minimum temperature for thermal accommodation of reaction rate is set by the temperature at the minimum Km. At temperatures below this point the reaction rate falls rapidly (Table 4) as both the enzyme-substrate affinity and the thermal energy of the system decrease.

In the trout AChE system the reaction rate at low substrate concentrations is maintained at a relatively constant level to temperatures beyond the upper thermal tolerance (about 25°C) of the animal. Thus, if the central nervous system is the limiting system in setting the temperature range of the organism, the rate of ACh hydrolysis by AChE is probably not an important factor in setting the upper thermal limit.
Figure 14. Effect of Assay Temperature on the Km of ACh for AChEs from Rainbow Trout, Electric Eel and *Trematomus borchgrevinki*.

- □ 2°C acclimated trout
- ◇ 17°C acclimated trout
- ▲ electric eel
- ○ *Trematomus borchgrevinki*
It is possible, however, that enzyme characteristics other than reaction rate are affected by the thermally induced Km changes, and that these factors are involved in setting thermal limits for the enzyme system in vivo. One mechanism which may be important in this respect is the ability of small fluctuations in substrate concentration to regulate enzyme activity. The effect of thermally induced Km changes upon this form of enzyme regulation is demonstrated in the substrate saturation plots for electric eel AChE shown in Figure 15. At 25°C, the temperature at which the Km reaches a minimum value, small fluctuations in AChE concentration about this minimum Km value \((0.98 \times 10^{-4})\) can greatly affect the rate of enzyme activity, acting essentially as an all-or-nothing switch. As the Km value increases at temperatures above and below 25°C, much greater changes in ACh concentration are required to achieve the same effect. A similar situation is observed with the Trematomus and rainbow trout AChEs (Figures 16 and 17). Thus, as the Km of the enzyme increases at temperatures above the minimum Km point, the advantages to be obtained from rate stabilization may be offset by loss of control over the reaction. At temperatures below the minimum Km point, increasing Km is accompanied by a decrease in both reaction rate and control over the reaction. If this form of control is important in the AChE system, then both enzyme regulation and rate accommodation may be important in setting thermal limits for the enzyme system in vivo. As discussed previously, many investigators have attempted to correlate the upper thermal tolerance or organisms with the thermostabilities of enzymes.
Figure 15. ACh Saturation Curves of Electric Eel ACh at 15° (●), 25° (■), and 40°C (▲).
Figure 16. ACh Saturation Curves of *Trematomus borchgrevinki* AChE at 2°C ( ■ ) and 10°C ( ⊙ ).
Figure 17. ACh Saturation Curves of 2°C acclimated Rainbow Trout AChE at 0°C (■), 2°C (●), 12°C (▲) and 18°C (○).
from possibly limiting physiological systems. The Km-temperature relationships observed with the fish AChE's provide an alternate mechanism by which enzymes may be thermally inactivated in vivo in the absence of protein denaturation.

b. Thermal Acclimation

(i) Adjustment of the thermal accommodation range

In the trout brain AChE system adjustment of the thermal accommodation range following thermal acclimation is achieved by regulating the relative proportions of the two enzyme types in response to changing environmental temperature. It is proposed that when the environmental temperature is altered to a range where one form of the enzyme can no longer thermally accommodate for reaction rate, or where regulation of catalytic activity is lost, a second form is produced for which the Km-temperature relationship is better suited for control of these functions. The two enzymes occur singly at thermal extremes (2°C and 17°C) and together at intermediate temperatures where the relative amounts of each enzyme varies with the acclimation temperature. The production of enzyme variants with altered and apparently adaptive changes in the Km-temperature curve following changes in environmental temperature has also been observed with trout pyruvate kinases (Somero and Hochachka, 1968), lactate dehydrogenases (Hochachka and Somero, 1968), isocitrate dehydrogenases (Moon, personal communication) and citrate synthases (Hochachka and Lewis, 1970).
(ii) Rate compensation of AChE activity

An interesting feature of the two rainbow trout AChE enzymes is that the minimum Km values are similar although they occur at widely different temperatures. Thus, at low and presumably physiological substrate concentrations the reaction catalysed by the 'cold' enzyme at 2°C will proceed at a slower rate than the reaction catalysed by an equal amount of the 'warm' enzyme at 17°C if the two enzymes have similar turnover numbers.

There are a number of factors which may act to raise the rate of ACh hydrolysis in the cold acclimated state. Hickman et al (1964) observed changes in brain Na⁺, K⁺ and Cl⁻ levels in rainbow trout transferred from 16°C to 6°C. A study of the effects of salts on brain AChE from trout acclimated to 2°C (Table 7) shows that at high ACh concentrations increasing ionic strength leads to a marked increase in both Km and maximum velocity of ACh hydrolysis. A similar relationship has been reported for AChE from the electric organ of Torpedo marmorata (Changeux, 1966). However, at lower ACh concentrations the rate of the reaction generally decreases as ionic strength is increased. Thus, the rate of ACh hydrolysis in vivo may be modulated by changes in the ionic environment that occur during the thermal acclimation process.

An increase in intracellular and blood pH on lowering environmental temperature (about 0.014 pH units per °C) has been observed in several poikolootherms (Rahn, 1965; Reeves and Wilson, 1969). With the 2°C trout AChE, a fall in temperature
Table 7. Effect of Salts on the Km and Rate of Hydrolysis of ACh by AChE from 2°C Acclimated Rainbow Trout

<table>
<thead>
<tr>
<th>Salt</th>
<th>Km x 10^-4</th>
<th>relative activity^a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACh (M)</td>
<td>2.5 x 10^-4</td>
</tr>
<tr>
<td>10^-3 M NaCl</td>
<td>3.3</td>
<td>0.36</td>
</tr>
<tr>
<td>5 x 10^-3 M NaCl</td>
<td>4.1</td>
<td>0.35</td>
</tr>
<tr>
<td>10^-2 M NaCl</td>
<td>7.7</td>
<td>0.31</td>
</tr>
<tr>
<td>10^-2 M KCl</td>
<td>4.9</td>
<td>0.35</td>
</tr>
<tr>
<td>2 x 10^-3 M MgCl2</td>
<td>4.4</td>
<td>0.45</td>
</tr>
<tr>
<td>5 x 10^-3 M MgCl2</td>
<td>7.0</td>
<td>0.32</td>
</tr>
<tr>
<td>Control</td>
<td>2.7</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Standard assay in 10^-2 M tris-HCl buffer, pH 7.2 (control) at 2°C.

a. Activities are expressed relative to the activity of the control at saturating ACh levels.
from $17^\circ$ to $2^\circ$C could result in an increase in the rate of ACh hydrolysis if the relationship between pH and activity (Figure 4) holds at physiological levels of substrate.

It was considered possible that rate compensation might be achieved through an increase in the total amount of enzyme present in the cold acclimation state, as proposed by Baslow and Nigrelli (1964) to account for rate compensation of brain cholinesterase activity in thermally acclimated killifish. However, estimations of the specific activity of AChE from trout acclimated to $2^\circ$ and $17^\circ$C for 35 days (Table 8) failed to show any significant difference.

On the other hand, it can be argued that it may not be necessary to maintain the same rate of ACh hydrolysis in the warm and cold acclimated states because of altered ACh concentrations, or changes in properties of neural membranes which may affect cholinergic mechanisms. Although no information is available on altered brain ACh levels in fish, a number of investigators have reported significant changes in membrane structure following thermal acclimation.

Johnston and Roots (1964) found a trend towards increased unsaturation of the total brain fatty acids with decreasing acclimation temperature in the gold fish, and in a later study (Roots, 1968) this same trend was established for the brain phospholipids, particularly choline and ethanolamine glycerophosphotides. These two phospholipid types account for over 60 percent of the total lipid in rat brain synaptic plasma membranes (Cotman et al, 1969). It is generally agreed that
Table 8. Specific Activities of Brain AChE from Rainbow Trout Acclimated to 2°C and 17°C for 35 days

<table>
<thead>
<tr>
<th>Acclimation temperature (°C)</th>
<th>Number of fish assayed</th>
<th>Assay temperature (°C)</th>
<th>Specific activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>12</td>
<td>18</td>
<td>10.1 ± 0.1 8.6 ± 0.3 6.2 ± 0.3</td>
</tr>
<tr>
<td>17</td>
<td>11</td>
<td>10</td>
<td>10.1 ± 0.3 8.4 ± 0.1 6.2 ± 0.1</td>
</tr>
</tbody>
</table>

Whole brain homogenates (100 mg brain/ml) were assayed in 10<sup>-2</sup> M tris-HCl buffer, pH 7.2, with 2 x 10<sup>-3</sup> M ACh as substrate.

<sup>a</sup> Specific activities are expressed as µM ACh hydrolyzed per mg protein per hour. ± values indicate the range about the mean.
the lipid constituents of biological membranes play a critical role in regulation of ionic permeability, although most evidence for this has been somewhat indirect. For example, Watlington and Harland (1969) have found a correlation between the phospholipid content of frog skins and properties of the Na\(^+\) and Cl\(^-\) transport systems while other workers have reached similar conclusions from studies with artificial membranes (Fast, 1967; Tobias et al., 1962), and from the binding of ions to various phospholipid species (Vanatta, 1969; Papahadjopoulos, 1968). Thus, there is considerable support for the suggestion by Roots (1968) that changes in the lipid component of neural membranes during thermal acclimation may be necessary for the maintenance of membrane permeability characteristics underlying nerve conduction. Alteration in the ionic composition in tissues from fish exposed to rapid changes in temperature may reflect thermally induced changes in membrane permeability that can not be compensated for by the lipid species present. Continued maintenance at the altered temperature generally leads to a gradual stabilization of the ionic balance over a time course similar to that followed by changes in tissue lipids (Hickman et al., 1964).

It should be stressed that phospholipids do not occur free in neural membranes, but rather as lipoprotein complexes possibly incorporating AChE. This may be of some importance when considering the effects of thermal acclimation upon AChE, as it has been found in other membrane systems that enzyme activity can be altered by removal of the phospholipid
component. For example, phospholipids are essential for respiratory activity in mitochondria, (Fleischer et al., 1962) and form an absolute requirement for such mitochondrial enzymes as cytochrome oxidase (Tzagoloff and MacLennan, 1965), \( \beta \)-hydroxy-butyric dehydrogenase (Sekuzu et al., 1963) and cytidine diphosphocholine transferase (Fiscus and Schneider, 1965), and for membrane bound Na-K ATPase (Tanaka and Strickland, 1965). Thus the possibility exists that changes in membrane lipids following thermal acclimation might have some effect upon AChE activity. With the rainbow trout AChEs, comparisons made between crude homogenates and lipid free butanol-acetone extracts of brain from warm and cold acclimated fish failed to show any changes in specific activity that could be attributed to the lipid component.

One point that should not be overlooked in considering the need for rate compensation during thermal acclimation is the relationship between in vivo enzyme and substrate concentrations. If AChE was present in great excess in the warm acclimated state then a reduction in enzyme activity following a drop in environmental temperature might have little physiological effect if it was still possible to maintain the rate of ACh hydrolysis above critical levels with a large concentration of catalytically less efficient enzyme.

It is difficult to determine enzyme and substrate concentrations within such discrete systems as mitochondria with any degree of accuracy. With brain AChE these difficulties are compounded by lack of precise knowledge as to the action of the enzyme in the central nervous system. If it is assumed
that cholinergic mechanisms in the central nervous system and
neuromuscular junction are essentially similar, some estimate
can be made of the level at which ACh hydrolysis must be main-
tained for the transmission of nerve impulses. Namba and Grob
(1969) have calculated that the postsynaptic membrane from rat
intercostal muscle contains $10^8$ molecules of AChE, and that one
synapse hydrolyses $5 \times 10^{-8}$ molecules of ACh per millisecond.
They estimate that $10^7$ molecules of ACh are released into the
synaptic space per action potential, a quantity of substrate
which could be hydrolysed within 0.02 milliseconds by the
estimated available AChE. If the time taken for ACh to cross the
synaptic space, and the duration of transmitter action at the post
synaptic membrane were known, it would be possible to make some
estimate of the amount of AChE required to hydrolyse $10^7$ molecules
of ACh within the time available. Eccles (1957) states that the
latency period of synaptic transmission as measured from the
time of arrival of a presynaptic impulse to the time of post-
synaptic response is generally in the order of 0.3 to 0.5 milli-
seconds. However, the duration of transmitter action is
considerably longer, in the order of 50 milliseconds for the
ACh mediated transmission at the Renshaw cell synapse.
Calculations based upon the rate of free diffusion of ACh
indicated that ACh levels should become negligible at the post-
synaptic membrane within one millisecond of release, and it
was concluded from this that some barrier to free diffusion exists
in the synaptic space (Eccles, 1957). Using 0.5 to 50 milli-
seconds as extreme values for the time available for ACh
hydrolysis, one arrives at an estimated 25 to 2500 fold functional
excess of AChE. Clearly this is a rough calculation at best,
but it does indicate that postsynaptic AChE may never
be saturated with substrate but rather, is exposed to ACh concentrations at or below the Km of the enzyme. Similar conclusions have been arrived at for mitochondrial enzyme systems (Srere, 1968; Vegotsky and Frieden, 1958). These estimates serve to underline the importance of enzyme-substrate affinities in the regulation of enzyme activities in vivo.

There is some experimental data which could be interpreted as evidence of a functional excess of AChE in the central nervous system. For example, Glow and Rose (1966) have shown in bioassays of rat brain that ACh levels do not increase significantly until AChE activity has been reduced below 40 to 50 percent of normal values. A sharp drop in neve conduction also occurs at this level of AChE inhibition (Wilson and Cohen, 1963). If there is a functional excess of AChE in the trout central nervous system there would seem little point in stabilizing reaction rates during temperature fluctuations. However, the adaptive advantages inherent in the relationships between habitat temperature, minimum Km values, probably physiological ACh concentrations, thermally induced Km changes and stabilization of reaction rates for the trout AChEs, seem too compelling to be dismissed as simply coincidental.

From the data at hand, it is proposed that while thermal accommodation of reaction rate is necessary in both the cold and warm acclimation states it may not be necessary to maintain the same rates of ACh hydrolysis at different acclimation temperatures. In this situation, selection for a particular minimum Km value may be determined by in vivo substrate
concentrations, for it is only when Km values are at or below physiological substrate levels that small fluctuations in substrate concentration can effectively regulate enzyme activity.

c. Evolutionary adaptation to temperature

(i) Adjustment of the thermal accommodation range

The adaptation of AChE function to temperature in species inhabiting different thermal environments appears to be based upon selection for a Km-temperature relationship that will allow thermal accommodation for reaction rate over the temperature range normally experienced by the species. As suggested for the trout enzymes, selection of a particular range of Km values may be determined primarily by physiological ACh concentration.

If the Km-temperature relationship is dependent upon thermally induced conformational changes in the enzyme molecule, shifts in the Km-temperature curve during speciation can be readily interpreted in terms of changes in conformation resulting from the gradual accumulation of amino acid replacements. Clearly it would be an advantage at this point to present amino acid sequence data to support the view that the trout and electric eel AChEs are in fact homologous enzymes which have diverged from a common ancestral gene. In the absence of such information, however, the best that can be done is to list similarities between the AChEs which are at least compatible with this hypothesis of a common origin. 1. Both the trout and electric eel AChEs have molecular weights of about 260,000, and are probably similar in net charge and configuration as judged
from electrophoresis on acrylamide gels. While by no means conclusive, this data is consistent with the proposition that both the trout and eel enzymes have similar subunit structures.

2. The AChEs display similar substrate and inhibitor specificities and pH-activity relationships. The curved Arrhenius plots for the enzymes have essentially the same form, approaching very low energies of activation at higher temperatures. It can be argued from these properties that both the active sites and the mechanisms of enzyme hydrolysis are probably similar in the trout and eel AChEs.

(ii) Evolution of the rainbow trout brain AChE complex

While the accumulation of amino acid substitutions will account for changes in the properties of a single enzyme, further mechanisms must be proposed to explain the presence of the two AChE variants in the trout CNS. One explanation is that the two trout enzymes arose by gene duplication, and that they subsequently diverged through amino acid replacements. Selection of different Km-temperature relationships for the two enzymes would be based upon advantages accruing to individuals who could extend their thermal ranges. Gene duplication, followed by the independent evolutionary fates of the duplicates, has been implicated in the original divergence of many structurally homologous proteins (Smithies et al, 1962; Ingram, 1963; Rutter, 1964; Cohen and Milstein, 1967; Augustinsson, 1968; Watts, 1968; Watts and Watts, 1969). In salmonids at least four duplicated genetic loci are known; A and B lactate dehydrogenases (Holmes and Markert, 1969; Massaro and Markert, 1968), enolase (Cory and Wold, 1966), and
supernatant malate dehydrogenase (Bailey et al, 1969), and recently multiple forms of several enzymes in addition to AChE have been found in the rainbow trout (Hochachka and Somero, 1968; Somero and Hochachka, 1968; Somero, 1969; Hochachka and Lewis, 1970). In fact, it appears likely that duplication of the entire genome has occurred in these fish as salmonids possess about twice as much DNA per nucleus and approximately twice as many chromosomes as other fish in the same order (Ohno and Atkin, 1966; Ohno et al, 1968; Hinegardner, 1969). Thus there is considerable support for the view that the two trout AChEs arose following duplication of an ancestral gene.

An alternative hypothesis is that the two trout AChEs may have diverged from a single molecular type in the absence of gene duplication, following the reproductive isolation of two populations that were exposed to different thermal regimes. Interbreeding between the populations at a later time might have led to the presence of the two divergent enzymes within the one individual. If conditions were such as to favour this genotype it might have been established in the population, eventually becoming the dominant form. Massaro and Markert (1968) have in fact suggested that the tetraploid salmonids may have arisen from hybrids formed between ancestral diploid species that possessed different alleles for a number of enzymes.

There is experimental evidence to support the view that hybridization among trout can lead to the incorporation of enzyme variants from both parents within the hybrid. For example, Bouck and Ball (1968) found that speckled trout-brown trout F₁ hybrids possessed at least 27 electrophoretically
distinct lactate dehydrogenases, whilst only 15 could be detected in each of the parental stocks. Goldberg has described a similar situation with splake, the speckled trout-lake trout hybrid, and was able to show that the lactate dehydrogenase profile in splake was maintained through subsequent generations (Goldberg, 1966; Goldberg et al, 1967).

An investigation of temperature tolerance in trout hybrids is being conducted by Ihssen at the University of Toronto (Ihssen, personal communication). Speckled trout and lake trout have different upper thermal tolerances. The hybrid, splake, has a temperature tolerance lying between the two parental species, and a range of thermal tolerances have been found in crosses between different generations of speckled, lake and splake trout hybrids. It was considered of particular interest with respect to the evolution of the rainbow trout AChE system to study the inheritance of AChEs in these fish.

The results obtained for acrylamide gel disc electrophoresis of brain homogenates from groups of speckled trout, lake trout and splake F1 hybrids acclimated to 4°C, 9°C and 20°C are shown in Figure 18, and can be summarised as follows: After acclimation to 4°C speckled and lake trout each show one AChE band, whereas splake has one major AChE band and two minor bands. Following acclimation to 9°C, speckled and lake trout each give two AChE bands, while splake has two major bands and two minor bands. After 14 days at 20°C, speckled trout show a single AChE band, while splake has one major band and one minor band of AChE activity. Unfortunately, 20°C lake
Figure 18. Resolution of Brain AChEs from Speckled Trout, Lake Trout and Splake by Acrylamide Gel Disc Electrophoresis. Specific AChE bands were identified by inhibition with 284C51 and eserine. The diagram shows only the number of bands resolved and does not indicate the relative migration rates of the fractions, a property which can only be accurately determined by running mixtures within the one gel. The electrophoretic conditions and staining technique are given in the methods section.

Acclimation periods were as follows: 4°C fish for 6 weeks, 9°C fish for 6 weeks, and 20°C fish for 2 weeks.
4°C acclimated trout

9°C acclimated trout

20°C acclimated trout
trout brains were not obtained. As only a small number of fish were available it was not possible to compare the electrophoretic mobilities of the different AChEs by running mixtures of brain homogenates, or to kinetically characterize the enzymes. Thus, it is not known if this increase in the number of AChE types present in splake involves a simple summation of the parental types, breakdown of the thermal switching mechanism, or possibly the formation of hybrid molecules containing polypeptide chains from each parental AChE. However, it is apparent from the results obtained that interbreeding between trout species which are normally reproductively isolated in nature can lead to an increase in the number of AChE enzymes present in the hybrid. Further, the presence of similar thermally inducible AChE systems in rainbow, speckled and lake trout leads one to suggest that the original incorporation of multiple AChE types into one species occurred prior to the evolutionary divergence of these three trout.

(iii) Regulation of the composition of the trout brain AChE complex during thermal acclimation

While such events as gene duplication and hybridization may underly the presence of multiple forms of AChE within one species, the development of some form of thermally controlled switching mechanism must be postulated to explain changes in enzyme profile during thermal acclimation of trout. At present it is not known if these changes result from altered rates of enzyme synthesis, enzyme degradation, or both, or possibly even modification of the gene products following
synthesis, thus any discussion of regulation of the trout AChE complex must be speculative. Even so, it would seem of value at this point to outline possible control mechanisms if only to indicate important areas for future investigation.

Although the effects of temperature upon total protein synthesis have been studied in a variety of poikilotherms (Mews, 1957; Jankawsky, 1960; Das and Prosser, 1967; Dean and Berlin, 1969; Haschemeyer, 1969) and probable thermally rate limiting steps have been identified (Haschemeyer, 1969), information relating to the rates of synthesis and degradation of specific proteins during thermal acclimation is completely lacking.

If the amounts of each AChE enzyme present at particular acclimation temperatures in trout are determined by regulation of protein synthesis, one can postulate that changes in the properties of regulator and operator genes may have accompanied the divergence of the structural genes, thereby regulating enzyme synthesis at the level of transcription. Regulation could also be achieved through thermally induced changes at numerous steps subsequent to messenger RNA production. With reference to the trout system, Somero (person communication) has found clear differences in the melting profiles of ribosomal preparations from the livers of warm and cold acclimated rainbow trout. These differences may reflect changes in ribosomal structure and function during thermal acclimation. Other possibilities include the modulation of such enzymes as the amino acyl transferases which appear to be a rate limiting step
in protein synthesis following the transfer of warm acclimated fish to lower temperatures (Haschemeyer, 1969).

Alternately, the activation and inactivation of preformed protein through modification of amino acid side groups, addition and removal of amino acids, or changes in subunit composition could be important methods of regulation.

Thermal switching of the 'warm' and 'cold' AChEs in enzyme mediated mechanisms might be achieved directly through $K_m$-temperature relationships similar to those observed with the AChEs, or indirectly by such factors as thermally induced changes in hormone balance. The influence of hormones in controlling both synthesis and activity of enzymes is well established (Metzenberg et al., 1961; McKearns, 1963; Varner and Ramchandra, 1964; Kim et al., 1966; Oki et al., 1966; Tomkins et al., 1969).

It is hoped that these thermally controlled enzyme complexes in poikilotherms will provide a valuable experimental system for studies of the regulation of gene expression in vertebrates.
SUMMARY

The effects of temperature upon AChE from the nervous system of fish were investigated with the object of interpreting thermal accommodation, thermal acclimation and evolutionary adaptation to temperature at the level of enzyme function.

At probable physiological ACh concentrations, the rate of ACh hydrolysis by AChE can remain relatively unaffected by assay temperature throughout a temperature range corresponding to that experienced by the animal in nature.

Plots of Km versus temperature for AChE enzymes from rainbow trout and electric eel yield U shaped curves with minimum Km values occurring at temperatures close to the habitat temperatures. Studies utilizing sucrose gradient centrifugation and ultraviolet difference spectra suggest that changes in Km with temperature probably result from small alterations in enzyme conformation, and that sharp breaks in the Km-temperature curve are not associated with changes in molecular aggregation or the dissociation of subunits.

Energies of activation for the trout and electric eel enzymes decrease with increasing temperature and show no clear relationship with habitat temperature.

It is concluded that the observed thermal accommodation of AChE reaction rate is achieved through changes in enzyme substrate affinity with temperature.

Arrhenius plots of log Vopt versus 1/T for the rainbow trout and eel AChEs continue to increase throughout the temperature range experienced by the animals, indicating that
thermal denaturation is probably not a factor in setting upper limits for thermal accommodation. A similar conclusion was reached for AChE from the Antarctic fish *Trematomus borchgrevinki*. It is proposed that the Km-temperature relationship may be important in setting both upper and lower limits for thermal accommodation of AChE activity through an interaction between rate stabilization and the maintenance of enzyme regulation.

Thermal acclimation of AChE activity in the rainbow trout, and probably in speckled and lake trout, is achieved by regulating the relative proportions of two AChE variants displaying different Km-temperature relationships. When the environmental temperature is altered to a range where one form of the enzyme can no longer thermally accommodate for reaction rate, or where regulation of catalytic activity is lost, a second form is produced for which the Km-temperature relationship is better suited for control of these functions.

In the case of the rainbow trout, both AChE variants have similar minimum Km values although these minima occur at markedly different temperatures. Thus at probable physiological substrate concentrations the reaction catalysed by the 'cold' enzyme at 2°C will proceed at a slower rate than the reaction catalysed by the 'warm' enzyme at 17°C, unless other factors associated with the cold acclimation process act to increase enzyme activity. With this in mind, the effects of ionic environment, pH and membrane lipids upon AChE activity were considered. It was found that while changes in both ionic environment and pH may be of importance, the presence of membrane lipids in the enzyme extract had no significant effect upon
AChE activity. Determinations of specific activity for brain AChE from cold (2°C) and warm (17°C) acclimated trout indicated that rate compensation is not achieved through alterations in the total amount of AChE present. It is concluded that although thermal accommodation of reaction rate is probably necessary in both the cold and warm acclimation states it may not be necessary to maintain the same rates of AChE hydrolysis at different acclimation temperatures.

The evolutionary adaptation of AChE function to temperature in species inhabiting different thermal environments appears to be based upon selection for a Km-temperature relationship that will allow thermal accommodation of reaction rate over the temperature range normally encountered by the species. Shifts in the Km-temperature curve during speciation are interpreted in terms of changes in enzyme conformation following the gradual accumulation of amino acid substitutions. Physical and kinetic evidence is presented which, while not conclusive, is consistent with the view that rainbow trout and electric eel AChEs were derived from a common ancestral gene.

Possible mechanisms by which two AChE enzymes could be incorporated into the trout central nervous system were also considered. While there is considerable evidence implicating gene duplication in this process, an alternative hypothesis involving hybridization between fish populations was also suggested. This theory was examined with trout interspecies hybrids, and it was observed that crosses between trout species which are normally reproductively isolated could result in an
increased number of AChE enzymes present in the hybrid. Further, the presence of similar AChE complexes in rainbow trout, speckled and lake trout indicated that the original incorporation of multiple AChEs into one species probably occurred prior to the evolutionary divergence of these trout.

It is concluded from this study that thermal accommodation, thermal acclimation and evolutionary adaptation to temperature as displayed by many physiological systems in poikilotherms can be observed and interpreted at the level of enzyme function.
ABBREVIATIONS

ACh - acetylcholine
AChE - acetylcholinesterase
ChAc - choline acetyltransferase
Ea - energy of activation
°K - degrees absolute
Km - Michaelis constant
△O.D. - change in optical density
T - absolute temperature
tris - tris(hydroxymethyl)aminomethane
Vmax - maximum velocity
Vopt - optimum velocity
284C51 - dimethobromide of 1:5 - di(p-N-allyl-N-methyl-aminophenyl)-pentan-3-one


Rosenzweig, M. R. 1957. La chimie du cerveau et la comporte individual. (Brain chemistry and individual behaviour.) Psychol. Franc. 1: 10-11.


