THE UNIVERSITY OF MIAMI

ANOXIC PHYSIOLOGY IN THE GREEN TURTLE *CHELONIA MYDAS MYDAS*

BY

Edward D. Scura

A DISSERTATION

Submitted to the Faculty
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the requirements for the degree of
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Subject

Anoxic Physiology in the Green Turtle Chelonia mydas mydas

By

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PREFACE

I wish to express my sincere gratitude to Dr. Charles E. Lane, chairman of my dissertation committee for his enthusiastic interest, support and guidance. I am also indebted to Dr. Ronald Samson for his continued advice and assistance. The other members of my committee: Dr. Eugene F. Corcoran, Dr. Robert A. Stevenson, Dr. Lowell P. Thomas and Dr. Martin A. Roessler are kindly thanked for their constructive discussion and criticisms.

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INTRODUCTION

Modern reptiles are noteworthy for their ability to survive prolonged apnea: sea snakes spend most of their lives submerged and alligators have been reported to remain active during dives of more than 90 minutes duration (Andersen, 1961). Altland and Parker (1956) showed that box turtles (Terrapene carolina) could survive exposure to hypoxia for 10 days with no apparent injury. Robin et al. (1964) described forced dives of the freshwater turtle Pseudemys lasting for 120 hours and Ingle and Smith (1949) reported that marine turtles stay submerged in captivity for as long as three hours without breathing. Diving, air-breathing vertebrates have developed several mechanisms to increase their diving capabilities (for review see Irving 1939; Scholander, 1940). Scholander (1962) gives a comparative discussion of the physiological responses of vertebrates to anoxia.

Johlin and Moreland (1933) exposed turtles to 100% N_2 for as long as 27 hours and reported time-dependent increases in blood lactate levels. Others have attributed some of the diving capability of turtles to their ability to extract dissolved oxygen from the water through the bucco-pharyngeal mucosa or to a respiratory function they proposed for the lateral bladders (Nicol, 1960; Musacchia and Chladek, 1961). However, Robin et al. (1964) provided turtles with gas tight

masks and sealed the cloaca surgically with no significant effect on their ability to remain submerged. These investigators restricted turtles to an atmosphere of pure nitrogen and recorded survival for as long as 48 hours. They concluded that the basic mechanism responsible for prolonged diving in turtles is their ability to exploit energy sources anaerobically.

Belkin (1962) inhibited anaerobic glycolosis in the musk turtle, Sternothaerus minor, with monoiodoacetate. Animals left in air survived but submerged animals died quickly. Berkson (1966) showed that the Pacific green turtle, Chelonia mydas agassizii, develops an extreme bradycardia during prolonged dives. Lactates, blood gases and tracheal gases were monitored throughout experimental dives. Berkson interpreted his data to mean that decreased metabolic rate and selective ischemia resulting from vasoconstriction were the principal physiologic changes during the first 30 to 60 minutes of the dive. By the time the oxygen storage depots (lung, blood, muscle) were exhausted, the metabolic rate was so reduced that the animal's energy requirements were satisfied by anaerobic pathways.

Wilbur (1960) and Andersen (1961) observed severe diving bradycardia in the alligator. Andersen (1961) found that the alligator lung was an impressive oxygen storage depot. The diving air volume (lungs and anatomical dead space) ranged from 76 to 102 ml/kg of body weight and the alligator appeared to be relatively insensitive to high levels of

alveolar carbon dioxide and low levels of oxygen.

Berkson (1966) found that the lung of the Pacific green turtle was also an important oxygen storage depot. However, it is generally agreed that the resting aerobic metabolic rate can not be sustained during a dive using only depot oxygen (Scholander, 1940; Andersen, 1961; Berkson, 1966). These investigators all stressed the importance of reducing metabolic rate during a dive.

Robin et al. (1964) concluded that in Pseudemys sp., the accumulation of lactic acid and the decrease in plasma HCO_3 concentration during a dive illustrates the importance of anaerobic glycolysis as an energy source. The conversion of pyruvate to lactate (Pyruvic acid + NADH → lactic acid + NAD^+) is an energy yielding reaction that is catalyzed by the enzyme lactate dehydrogenase (LDH). Vertebrate LDH exists as a complicated isozymic system. It is a tetramer formed by at least two different subunits, A and B, which combine in all possible combinations to form five isoenzymes (for review see Markert, 1963) that can be written B_4 (LDH-1), A_1B_3 (LDH-2), A_2B_2 (LDH-3), A_3B_1 (LDH-4), A_4 (LDH-5). These isoenzymes differ in charge and can be separated by electrophoresis or ion-exchange chromatography.

During ontogeny vertebrate tissues show characteristic changes in distribution of the five isoenzymes. These variations may be directly related to the functional significance of the tissue or organ and to the availability of oxygen to

the developing animal. For example, some suggest that the molecular structure of the LDH molecule determines the pathways used for energy production (Dawson et al., 1964; Fondy and Kaplan, 1965). Tissues that contain predominantly B-isoenzymes (such as cardiac muscle) have been presumed to metabolize exclusively by aerobic pathways. The B_4 tetramer (LDH-1) is more sensitive to substrate (i.e. pyruvate) inhibition that is the A_4 tetramer (LDH-5) (Cahn 1962). Vertebrate skeletal muscle from various animals has been found to contain a higher concentration of the A-isoenzymes. It is thought that this isoenzyme type may contribute to the ability of skeletal muscle to accumulate an oxygen debt during periods of stress (for reviews see Markert 1968; Kaplin et al., 1968).

Altman and Robin (1969) suggested that an unusual adaptation of LDH isoenzymes enable the pond turtle, Pseudemys scripta elegans, to survive long periods of diving anaerobiosis. They found that the LDH isoenzymes in the heart and skeletal muscle of the pond turtle were similar in kinetic and electrophoretic properties and seemed to resemble mammalian A_4 isoenzymes.

Markert and Faulhaber (1965) found that in several species of fish there was a reversal of A and B subunit electrophoretic mobility. Markert and Holmes (1969) stress the importance of identifying A and B subunits by comparing their immunochemical and kinetic properties with authentic vertebrate

isoenzymes. Thus, electrophoretic mobility is not the only criterion used to establish the identity of unknown isoenzymes.

Markert and Holmes (1969) report that representatives of eighteen species of flat-fish belonging to the order Pleuronectiformes contain a predominance of A_4 tetramers and A' hybrid isoenzymes. The A' hybrid is similar to the A subunit in kinetic and immunochemical properties but is capable of combining with A subunits to form a hybrid tetramer of A and A' subunits. A, B and A' can be made to combine and form 15 hybrid tetramers. Markert and Holmes (1969) attempt to relate the predominance of A_4 and A' hybrids to the environmental conditions and life style of the flat-fish. However, most common flat fishes are bathed in oxygen rich water and live a relatively inactive life, punctuated by occasional bursts of vigorous activity. These conditions and mode of life would confer only modest adaptive advantage to mechanisms that would improve anaerobic efficiency.

Until now no investigator has attempted to correlate LDH isoenzyme distribution with the considerable diving ability of the Atlantic green turtle, Chelonia mydas mydas. The objectives of this study were to determine the LDH isoenzyme distribution pattern in the different organ systems of the green turtle and to clarify the role played by specific LDH isoenzymes in the response to anoxia. Various immunochemical, kinetic and hybridization techniques were employed to characterize these isoenzymes.

A detailed examination was made of the turtle during the anaerobic challenge presented by a prolonged dive and during the post-dive recovery period. Blood chemistry studies included: blood lactate, blood pyruvate, blood pH and blood gas analysis to determine pO_2 and pCO_2. Heart rate was also monitored continuously throughout the dive.

An antibody was developed against isolated sea-trout LDH-A_4 in the rabbit and used to inactivate turtle A subunits for positive identification of LDH isoenzymes. Two turtles in this study were innoculated with LDH-A_4 antiserum in an effort to inactivate A subunits *in vivo*. After treatment with antiserum, these animals were forced to make prolonged dives to clarify the importance of this isoenzyme in their diving ability. Goldberg and Lerum (1972) showed LDH antiserum to be effective *in vivo*. They were able to develop an antiserum specific to LDH-X, which is a unique isoenzyme composed of a third subunit different from A and B and found only in spermatozoa. Female white mice treated with LDH-X antiserum after mating had a significant reduction in pregnancies.

There is a limited information on the blood chemistry of *Chelonia mydas mydas* (Khalid, 1947; Lewis, 1964; Holmes and McBean, 1964; Thorson, 1968). Because of the availability of a Technicon SMA 12/60 Autoanalyzer, several normal values for the turtles employed in this study were established.

METHODS AND MATERIALS

EXPERIMENTAL ANIMALS

The green turtles, Chelonia mydas mydas, used in this study were wild specimens captured in the Florida Keys (Fla. Dept. of Natural Resources Permit #S-372) or animals reared in captivity from eggs collected in Costa Rica, Surinam or Ascension Island. The latter animals were provided by Mariculture Ltd., Grand Cayman Island, British West Indies. All animals were fed a diet of Green Turtle Feed formulated by Central Soya Co. for Mariculture Ltd. Test animals were maintained in 200 gallon concrete aquaria with running sea water. Experimental animals were used within a few weeks after being placed in the aquaria. The turtles used in the study ranged in size from 5 to 12 kg.

TISSUE HOMOGENATES

Experimental animals were placed under light anaesthesia with sodium pentobarbital. The plastron was quickly removed and several blood volumes of heparinized saline were then flushed through the circulatory system as the animal bled by the still beating heart. After exsanguination, the desired organs were removed. Tissue samples were homogenized in 3 volumes of 0.01M Tris-HCL buffer, pH 7.6 at 0-2°C. Homogenates were centrifuged at 0-2°C for 1 hour at 30,000 g in a Sorvall refrigerated centrifuge. The supernatant

solutions were recentrifuged for 1 hour.

ELECTROPHORESIS

Tissue extracts were separated by vertical gel electrophoresis (EC Apparatus Co.). The solid phase was 5% Cyanogum-41 gel (95% acrylamide and 5% bis-acrylamide, EC Apparatus Co.) and the liquid phase was the tris buffer system of Peacock et al. (1965). Reagents for electrophoresis were made up as follows:

Buffer:	43.1 gms
Tris (Hydroxymethyl) Aminomethane (Fisher Scientific Co.)	
Na_2EDTA (Ethylenedinitrilo-tetraacetic Acid, Disodium Salt) (Matheson Coleman & Bell)	3.7 gms
Boric Acid (Fisher Scientific Co.)	22.0 gms
Water to	4 liters
Solid Phase:	
Cyanogum-41	10.0 gms
Buffer to	200.0 ml
TMED (N,N.N',N'-tetramethylethylenediamine) (EC Apparatus Co.)	0.2 ml
Ammonium Persulfate (EC Apparatus Co.)	0.2 gms

The buffer was continuously circulated and cooled to minimize local changes in pH and overheating the gel slab.

Depending on LDH activity 10-25 microliter samples were placed in the sample slots and allowed to migrate for 5-7 hours at 300V. LDH isoenzyme bands in the resulting gel were visualized by the tetrazolium method described by Markert and Faulhaber (1965).

Tris-HCL buffer	0.2 M, pH 8.0	45 ml
Sodium lactate (DL-Lactic Acid) (Sigma Chemical Co.)	0.5 M, pH 7.0	9 ml
Nitro Blue Tetrazolium (Sigma Chemical Co.)	1 mg/ml	5 ml

Phenazine Methosulfate (Nutritional Biochemicals Corp.)	1.6 mg/ml	5 ml
NAD+ (Nutritional Biochemicals Corp.)	10 mg/ml	2 ml

The gels were incubated in the above mixture overnight in the dark at room temperature.

LDH ACTIVITY

LDH activity was measured spectrophotometrically by the method of Markert and Faulhaber (1965) in a Coleman Hitachi Model 124 spectrophotometer. LDH catalyzes the reduction of pyruvate to lactate in the presence of NADH. Activity is determined at 30 ^{o}C by measuring the change in absorbance per minute at 340 millimicrons during the formation of NAD+ from NADH in a cuvette with a 10 mm light path. The reaction mixture contains:

Sodium phosphate buffer	0.1 M, pH 7.0	2.9 ml
Sodium Pyruvate (Type II) (Sigma Chemical Co.)	0.02 M	0.05 ml
NADH (Grade III) (Sigma Chemical Co.)	10 mg/ml	0.02 ml
Enzyme solution		0.01 ml

The reaction was initiated by adding the enzyme solution. Absorbance readings were taken after 1 minute. Activity of LDH in International Units was calculated according to the following equation:

$$\frac{\text{Change in O.D./minute X 3 X dilution factor}}{6.22} = \text{I.U./ml}$$

where:

3.01 = total volume of reaction mixture
dilution factor = 300 (reciprocal of enzyme to reaction

mixture dilution)
6.22 = extinction coefficient of NADH at 340 nm

IN VITRO HYBRIDIZATION

The breakdown and recombination of LDH subunits with known mammalian A and B subunits is often helpful in characterizing unknown isoenzyme assemblages. Turtle isoenzymes were hybridized *in vitro* with purified LDH-A_4 (Sigma #1-2875 rabbit muscle type V) and LDH-B_4 ($HLDH_4$, beef heart, Worthington Biochemical) by the methods of Markert (1963) and Markert and Massaro (1966). Enzymes were diluted with 0.1 M phosphate buffer, pH 7.0 in 1 M NaCl and frozen for 18 hours. After thawing the hybridized enzymes were differentiated electrophoretically as described above.

SUBSTRATE INHIBITION

Enzymes in turtle heart, brain and skeletal muscle homogenized in 0.1 M phosphate buffer at pH 7.0 were assayed in reaction mixtures containing pyruvate concentrations ranging from 10^{-2}M to $5X10^{-5}$M. Using the same range of pyruvate concentrations, pure LDH-A_4 and LDH-B_4 were assayed for comparison.

FORCED DIVES

Turtles were immobilized by crossing the front flippers and securing them with wide cheesecloth shrouds. The animal was then securely tied to a concrete block and lowered into a test tank immediately after a breath. The depth of the

water was just sufficient to keep the turtle's head submerged.

Heart rate was recorded continuously throughout the dive on a Grass Polygraph model #7. Heart rate was visualized in one of two ways: 1) Arterial pressure changes were recorded from the brachial catheter connected through a 3-way valve with a Statham P23AC physiological pressure transducer, or 2) lead 3 electrocardiographs were taken directly using platinum intradermal needle electrodes suitably waterproofed.

BLOOD SAMPLING

Blood samples were taken through a chronic indwelling polyethylene catheter introduced surgically into a brachial artery and passed centrally towards the heart. After closure of the incision the plugged catheter filled with heparinized saline was fixed to the dorsal surface of the carapace with a wide rubber band that encircled the entire animal.

After discarding 2 ml of mixed blood and heparinized saline, the next 3 ml of arterial blood were collected in a heparinized 3 ml syringe. Two ml of the sample were discharged into 6% perchloric acid for lactate and pyruvate analysis and the remainder was immediately analyzed for pO_2, pCO_2 and pH using the I.L. Blood Gas Analyzer, model #213. All measurements were made at turtle ambient dive temperature which ranged between 23-25°C.

After each sample 2 ml of heparinized saline were flushed through the catheter. Between blood sample withdrawals, the

open end of the catheter was resealed with a polyethylene plug.

LACTIC AND PYRUVIC ACID

Lactic and pyruvic acids were determined with only slight modifications in the enzymatic methods described by Neville and Gelder (1971). Results were expressed in millimoles of lactate or pyruvate per liter of blood water. Per cent blood water was determined by drying weighted blood samples for 24 hours at 100°C. Arterial blood was immediately mixed with chilled 6% perchloric acid in the proportion of one volume of blood water to 5 volumes of perchloric acid. Mixed samples were centrifuged and from the supernatant solution a 5 ml aliquot was chilled and partially neutralized with 5 M KOH to a pH of 1.6 to 2.5. The chilled sample was recentrifuged and 0.3 ml of the supernatant solution was added to the following mixture for lactate analysis:

3.0 ml of glycine buffer, pH 9.5 X number of analyses

4.5 mg of NAD^+ X number of analyses

20.0 units of LDH X number of analyses (LDH, beef heart Type III, Sigma)

The solutions were mixed and incubated at 37°C for 90 minutes rather than the 60 minutes prescribed by Neville and Gelder (1971). The concentration of NAD^+ was increased from 4.0 mg/sample to 4.5 mg/sample to facilitate recovery at the high lactate levels found in this study. Also, 20 units of LDH activity were added for each analyses instead

of the 10 units described by Neville and Gelder (1971). Standard solutions of zinc lactate (1.0, 5.0, 10.0, 20.0, 25.0 mM/liter) were run with each set of unknowns. Recoveries of lactate were somewhat reduced at the higher concentrations so lactate levels in the samples were read from a standard curve.

Absorbance (E_2) of the sample is compared with that of the reagent blank (E_1) after incubation. Lactate is calculated from the formula:

mM Lactate = ΔE X K when:

E_1 = absorbance of the reagent blank
E_2 = absorbance of unknown
ΔE = E_2-E_1
K = dilution factor/e x d
e = extinction coefficient of NADH at 340 nm (6.22/micromole/sq.cm)
d = light path of the cuvette, 1 cm.
dilution factor = reciprocal of total dilutions.

Pyruvic acid is measured by adding 2.0 ml of the partially neutralized supernatant solution from above to a reaction mixture containing 1.0 ml TEA buffer, pH 7.4 and 0.1 ml of NADH (1.4 mg/ml). Absorbance (E_1) is read before the addition of 25 I.U. of LDH. Abosrbance (E_2) is read when a stable value is achieved after addition of LDH. Pyruvate concentration in the sample is calculated from the following formula:

mM pyruvate/liter blood water = ΔE X K where:

E_1 = absorbance before adding LDH
E_2 = abosrbance after addition of LDH
ΔE = (E_1-E_2) - correction of blank
K = dilution factor/e x d
e = extinction coefficient of NADH at 340 nm (6.22/micromole/sq.cm.)
d = light path of the cuvette, 1 cm
dilution factor = reciprocal of total dilution

Standard solutions of pyruvate served as controls for all determinations.

ISOLATION OF SEA-TROUT LDH-A_4

Sea-trout (Cynoscion regalis) LDH-A_4 was isolated and partially purified according to a modification of the methods of Markert and Holmes (1969). The sea-trout were purchased from a local fish market in Miami, Florida. Several kilograms of beheaded sea-trout were ground in an approximately equal volume of 0.01 M Tris-HCl buffer, pH 7.6. The ground fish mixture was squeezed through cheesecloth and the fluid collected was centrifuged at 5°C at 10,000 g for 30 minutes. The combined supernatant solutions were precipitated with 80% ammonium sulfate and recentrifuged. The precipitate was collected and dissolved in a minimum volume of buffer. This solution was dialyzed for three days at 4°C against several changes of 0.01 M Tris-HCl buffer, pH 7.6. After centrifugation of the contents of the dialysis sac, 1,200 ml of the supernatant solution was applied to a DEAE cellulose column (35 x 750 mm) equilibrated with 0.01 M Tris-HCl buffer, pH 7.6. The column was run at a constant flow rate of 100 ml/hour maintained by a peristaltic pump. The effluent was monitored at 280 nm by a LKB uvicord. The column was washed with buffer until the optical density trace stabilized at the baseline. The eluate collected during this wash showed a high concentration of A_4 isoenzymes when examined by vertical gel electrophoresis. This fraction was

dialyzed against 0.01 M phosphate buffer, pH 6.0 and 450 ml was applied to a carboxy methyl cellulose column (15 x 400 mm) equilibrated with the same buffer. After washing, LDH-A_4 was eluted in a 1,000 ml linear gradient of 0.01 M phosphate buffer, pH 6.0 to 0.1 M Tris-HCl buffer, pH 7.6. Fractions of 10 ml were collected during the gradient and one fraction contained a high concentration of essentially pure LDH-A_4. This fraction was dialyzed against 0.9% NaCl in 0.02 M phosphate buffer pH 7.6 and frozen.

ANTIBODY PRODUCTION

Antibodies to the purified sea-trout LDH-A_4 were produced in healthy New Zealand white female rabbits by the methods of Markert and Holmes (1969). Approximately 2,000 I.U. of the LDH-A_4 preparation were mixed with complete Freund's adjuvant by forcing the mixture back and forth between two 3 ml syringes fitted with a double ended hub. This was repeated a minimum of 50 times. This mixture was injected on day 1 into two hind foot pads and into the dorsal thoracic muscles. On day 8, an additional 2,000 I.U. were mixed with incomplete Freund's adjuvant as described above. This mixture was injected into the same areas. On day 15 and 17 an additional 1,000 units were injected into the dorsal thoracic muscles and blood was withdrawn from the heart after day 19. Blood samples were centrifuged and serum collected using sterile procedure. Antiserum samples were frozen for future use.

ANTIBODY INHIBITION

The enzyme inhibiting capacity of the antiserum to sea-trout LDH-A_4 was determined by the methods of Markert and Holmes (1969). Increasing quantities of antiserum were added to 1 ml of a specific enzyme and allowed to incubate for 15 minutes before residual enzyme activity was determined spectrophotometrically. Serum from a normal, untreated rabbit was also tested to determine decreases in activity due to dilution and possible rabbit enzymes. The enzyme inhibiting capacity of the sea-trout LDH-A_4 antiserum was tested on isolated sea-trout LDH-A_4 (1.4 I.U./ml), turtle skeletal muscle homogenate (1.8 I.U./ml) and purified LDH-B_4 (2.0 I.U./ml). Equivalance points were determined as in Markert and Holmes (1969).

IN VIVO ANTIBODY TESTING

Two turtles were injected with sea-trout LDH-A_4 antiserum one hour before a forced dive to see if A-subunit inhibition would affect the animals diving capabilities. Both animals were approximately 10 kg and were given an injection of 20 ml of antiserum through the brachial catheter.

BLOOD CHEMISTRY

Turtle serum was analyzed on a Technicon SMA 12/60 Autoanalyzer in order to obtain normal values for several chemical parameters. Serum was analyzed for Na^+, K^+, Cl^-, CO_2, glucose, blood urea nitrogen, calcium, total bilirubin, total protein, albumin, alkaline phosphatase and serum glutamic oxaloacetic

transaminase. Standard operating procedures as outlined in Technicon Technical bulletins were used.

RESULTS

DIVING BRADYCARDIA

Chelonia mydas mydas develops a severe diving bradycardia during prolonged involuntary dives (Table 1, Fig. 1). The heart rate decreases rapidly after submergence and usually reaches a minimum rate within 60 minutes. The lowest rate recorded was 0.4 beats/minute after the turtle was submerged for 54 minutes (Table 1, dive 3). In two of the experimental dives the heart rate increased after reaching a minimum rate (Fig. 1; Table 1, dives 3 & 5). This increase was also reported by Berkson (1966) in *C. mydas agassizii*.

Electrocardiography was quite unsatisfactory during the dive. Although the intradermal needle electrodes were carefully waterproofed, electrical leakage was not entirely eliminated. As the dive progressed, the amplitude of the heart signal decreased steadily as Berkson (1966) reported earlier. To improve the accuracy of heart rate measurements arterial blood pressures were recorded continuously during some dives. The heart rate of one experimental animal was much higher than all the other turtles tested (Table 1, dive 5). The lowest rate recorded during this dive (4.5/min.), was 20% of the pre-dive heart rate. In all other experiments the heart rate during the dive was less than 5% of the pre-dive rate.

BLOOD pO_2

The partial pressure of oxygen in the arterial blood of resting C. mydas mydas ranged from 110 to 130 mm Hg immediately after a breath (Dives 1,2,3,4,7; Figures 2,3,4,5,8). During a prolonged dive the pO_2 decreased precipitously, usually reaching 0 mm Hg within 90 minutes (Dives 1,2,3,7; Figures 2,3,4,8). The pO_2 levels decreased more rapidly in dives number 4 and 5 (Figures 5,6). However, these animals were anemic, having hematocrits of 12% and 6% in contrast to "normal" values of about 30%. Decreased oxygen tension in the blood of anemic turtles on the surface combined with rapid depletion of blood oxygen during involuntary dives suggests that erythrocytes may be an important oxygen depot in this animal. Test animals survived blood pO_2 of 0 mm Hg for several hours. However, turtles showing blood pO_2 of 0 mm Hg for longer than approximately 1 hour failed to breathe voluntarily immediately upon surfacing (Dives 4 & 5, Figures 5 & 6; Table 1). In dive number 4 the turtle failed to breathe after a post-dive recovery period of 1 hour so it was intubated and resuscitated. After 15 minutes of resuscitation normal independent breathing was reestablished. The turtle in dive number 5 was resuscitated when it failed to breathe for 15 minutes after emergence.

Berkson (1966) reported that in one experimental dive with C. mydas agassizi, the animal survived after maintaining apnea for 5 hours after a 60 minute dive. He suggests

that this was a voluntary apnea. It is my opinion that metabolism is so reduced by the end of a prolonged dive that it is likely to be irreversible. In this study, if animals did not breathe voluntarily after a reasonable post-dive period they were resuscitated. Turtles that were submerged for 2 hours or less began to breathe immediately when they were brought to the surface. Respiration rates were increased (Table 1) and pO_2 recovery was rapid, usually reaching pre-dive levels within 10 minutes.

BLOOD pCO_2

The partial pressure of carbon dioxide in arterial blood of a resting green turtle immediately after a breath ranged between 50 and 70 mm Hg (Dives 1,2,3,4,5,7; Figures 2,3,4,5,6,8). The pCO_2 gradually increased during the dive to a maximum between 100-135 mm Hg. After a prolonged dive the pCO_2 returned to pre-dive levels within 10 minutes after breathing was resumed (Dives 2,3,5; Figures 3,4,6).

BLOOD pH

The pH of arterial blood in resting C. mydas mydas ranged from 7.33 to 7.45 (Dives 1,2,3,4,5,7; Figures 2,3,4,5,6,8). The pH slowly decreased during a prolonged dive. The lowest pH recorded was 6.680 after 215 minutes of apnea (Dive 4, Figure 5). Recovery to pre-dive pH values occured within minutes when breathing was resumed.

LACTIC AND PYRUVIC ACIDS

Lactic acid accumulated rapidly in arterial blood during the dive, reaching levels near 20 mM/liter blood water within 2 hours (Dives 7,9; Figures 11,13). This response agrees with that observed by Robin et al. (1964) in the freshwater turtle, Pseudemys sp. Lactic acid continued to rise for some time after the dive was terminated and then slowly returned to normal levels. After one dive of 60 minutes duration (Dive 7, Figure 11) blood lactate had not returned completely to pre-dive levels 6 hours after termination of the dive. Blood pyruvate levels were variable during diving but seemed generally to decrease during a prolonged dive. A rapid increase in pyruvate level was noted during the first 90 minutes after emergence (Dive 7, Figure 11) after which the concentration of pyruvate in the blood fell slowly, requiring 4.5 hours to return to the elevated level prevailing at the end of the dive.

ISOLATION OF SEA-TROUT LDH-A_4

LDH-A_4 was isolated from a crude extract of sea-trout, Cynoscion regalis, using DEAE and CM cellulose chromatography (Table 2). LDH-A_4 was not retained on a DEAE column equilibrated with 0.01 M Tris-HCl buffer, pH 7.6 but was found in the buffer wash after adding the sample (Figure 14). Isoenzymes containing B subunits seemed to be retained by the anion exchanger effecting a partial purification of the A_4 isoenzyme. The partially purified LDH-A_4 from DEAE was

retained on a CM cellulose column equilibrated with 0.01 M phosphate buffer, pH 6.0. Fractionation in a linear gradient from 0.01 M phosphate buffer, pH 6.0 to 0.01 M Tris-HCl buffer, pH 7.6 eluted a sharp peak of protein containing LDH-A_4 with essentially no B subunits (Figures 15 & 16). This fraction was assumed to contain other non-LDH proteins.

ANTIBODY PRODUCTION

An antibody to sea-trout LDH-A_4 that inhibited LDH-A_4 specifically with little effect on LDH-B_4 activity was developed in New Zealand white female rabbits (Figures 17 & 18). The rabbit antiserum inactivated 91% of the purified sea-trout LDH-A_4 activity but inactivated only 35% of the LDH activity in turtle muscle homogenate. The equivalence point calculated for purified sea-trout LDH-A_4 was 6.7 times greater than that calculated for turtle muscle homogenate. Since turtle muscle LDH contains primarily A_4, a greater degree of inactivation was expected. However, Goldberg (1971), found equivalence points 30 times higher with antiserum produced from pure-crystalline LDH-X than from LDH-X that was presumably of less purity. Perhaps the protein impurities in the isolated sea-trout LDH-A_4 were sufficienctly antigenic to reduce the activity of the antiserum towards LDH-A_4 of other animals.

LDH PATTERNS IN CHELONIA MYDAS MYDAS

Extracts from tissue homogenates of the green turtle contain representatives of all five LDH isoenzymes commonly found

in mammalian tissues (Figure 19). Treatment of turtle brain, heart and skeletal muscle homogenates with LDH-A_4 antiserum prior to electrophoretic separation confirms the identification of the turtle A subunit. Figures 20 through 25 show LDH isoenzyme distributions before and after treatment with antiserum. These zymograms clearly show a decrease in LDH activity in the isoenzymes containing A subunits. There was no effect of LDH-B_4 activity.

HYBRIDIZATION

Using the freeze-thaw technique of Markert (1963) and Markert and Massaro (1966), turtle muscle LDH was dissociated into subunits and recombined with purified LDH-B_4 (Figure 26). This recombination yielded four distinct isoenzyme bands.

SUBSTRATE INHIBITION

The results of substrate inhibition studies are shown in figures 27 and 28. Extracts from turtle heart, skeletal muscle and brain were tested for LDH activity at various pyruvate concentrations. Turtle heart shows inhibition characteristics very similar to pure LDH-B_4. Brain and muscle show characteristics midway between LDH-A_4 and LDH-B_4.

IN VIVO RESPONSE TO SEA-TROUT LDH-A_4 ANTISERUM

Two turtles weighing approximately 10 kg each were injected with 20 ml of antiserum against sea-trout LDH-A_4 60 minutes before an involuntary prolonged dive. The first animal (Dive 6, Figure 7) remained submerged for 50 minutes. The

dive was terminated at this point when the brachial catheter was inadvertently withdrawn. The animal was brought to the surface and a post-dive blood sample was drawn after 20 minutes by cardiac puncture immediately after the first post-dive breath. It was impossible to determine if this sample was arterial or venous. The turtle used in this experiment had a hematocrit of 35%. The pre-dive respiration rate was 1.2 breaths/minute (Table 1).

The changes in arterial blood gases and pH during the dive were distinctly different from those recorded during experimental dives of untreated turtles. The pO_2 decreased from 95 to 11 mm Hg within the first 10 minutes of apnea. Arterial pO_2 was essentially 0 mm Hg after 30 minutes. Arterial pCO_2 increased from 76 to 113 mm Hg within the first 10 minutes of the dive and reached a maximum of 121 mm Hg. Untreated turtles in all the other experimental dives never increased pCO_2 by more than 15 mm Hg within the first 10 minutes. (Dives 1,2,3,4,5,7; Figures 2,3,4,5,6,8). In dive number 6 arterial pH decreased more rapidly reaching much lower levels (7.279 to 6.630 in 70 minutes) than were seen in dives of untreated turtles.

The lactic acid level rose rapidly during dive number 6, reaching 21.6mM/liter blood water within 50 minutes. Pyruvic acid levels followed no pattern during the dive but the expected post-dive increase did occur. The turtle was sacrificed after the dive and samples of heart, brain and

skeletal muscle were collected for electrophoresis. The LDH isoenzyme electrophoresis of these tissues showed no changes in subunit activity that could be ascribed to the antiserum.

The remaining animal treated with LDH-A_4 antiserum served as its own control. This turtle was injected with 20 ml of normal rabbit serum prior to a 60 minute dive (Dive 7, Figure 8,11; Table 1) while arterial blood gases, pH, lactate and pyruvate levels were continuously monitored. Lactate and pyruvate were measured for 6 hours following the dive. Sampling was spaced at greater intervals than usual to minimize blood loss. This animal exhibited a typical diving pattern.

On the following day the same animal was injected with 20 ml of sea-trout LDH-A_4 antiserum and dived for 2 hours. At this time the hematocrit was 30% (Table 1). The results of this dive can be seen in Figures 9, 12 and Table 1. Pre-dive pO_2 and pH were lower after treatment with antiserum and respiration rate was increased. In dive number 7 arterial pO_2 fell to 36 mm Hg within 30 minutes. During dive number 8 arterial pO_2 dropped to zero during the same period. During dive number 7 pH decreased to 6.945 after 60 minutes, while pH of the same animal decreased to 6.720 within the same time period during dive number 8. The pH continued to decrease during dive number 8, reaching a value of 6.611 after 120 minutes, the lowest pH value

recorded for any experimental dive. Lactic acid increased to 13.8 mM/liter blood water after 60 minutes during dive number 7. After treatment with antiserum, the same animal reached a lactic acid level of 22.1 mM/liter blood water after 60 minutes. Dive number 8 was terminated after 2 hours. The turtle began breathing spontaneously immediately upon surfacing at a rate of 5 breaths/minute. This animal is alive and well to this day.

BLOOD CHEMISTRY

Normal values and range for several blood components are listed in Table 3. Each experimental animal was shown to have a serum chemistry profile that was within this normal range before being selected for an experimental dive.

DISCUSSION

The data from this study demonstrates that the Atlantic green turtle can survive extreme anoxia and suggests some mechanisms contributing to this ability. The physiological adjustments made by this animal permit survival with no measurable oxygen in the blood. Berkson (1966) stresses the importance of selective ischemia and reduced metabolic rate during anoxia in the Pacific green turtle. I have observed the severe diving bradycardia described by Berkson and agree that marked reduction in metabolic rate must occur. Selective ischemia is also suggested by the delayed release of lactic acid from tissue after the dive that is shown by the continued high levels of lactate in the blood for 6 hours after emergence. However, my observations do not support the pronounced selective ischemia Berkson (1966) described during the first 45 minutes of the dive in C. mydas agassizii.

Berkson detected only a slight increase in blood lactate during the early minutes of the dive indicating selective ischemia. My results illustrate steady increases in lactic acid level from the beginning of the dive. There is good evidence in my study and in the literature (Robin et al. 1964; Altman and Robin 1969) that substantial oxygen debts accumulate during periods of apnea in the turtle. Lactic acid levels reached during these periods take several hours to return to normal after a prolonged dive.

Anaerobic energy sources are important from the onset of the dive. Animals treated with antiserum known to inactivate 35% of the LDH activity in turtle skeletal muscle showed more rapid depletion of oxygen reserves. The exact site of this antibody-antigen reaction is not clear at this time. LDH is a cytoplasmic enzyme and it is difficult to believe that the rabbit antibody is capable of penetrating cellular membranes to inactivate LDH-A_4 within the cell. Moreover, tissue zymograms of a turtle treated with antiserum showed insignificant reduction in LDH-A_4 activity on the cellular level. Nonetheless, this experiment demonstrates that the ability of the turtle to sustain a significant arterial pO_2 during more than 60 minutes of apnea is severely impaired by inhibiting LDH-A_4 activity in the blood.

The occurrence of 5 isoenzymes in Chelonia mydas mydas indicates that the LDH isoenzyme spectrum results from only 2 subunits that are associated in all possible combinations to yield 5 different tetrameric molecules. The identity of the A_4 tetramer was verified by specifically reducing LDH activity in isoenzymes containing A subunits with sea-trout LDH-A_4 antiserum. The identity of the B subunit was verified by hybridization with purified LDH-B_4. Disassociation and recombination of subunits from turtle tissue with purified B subunit resulted in the formation of four bands. If the turtle contained an additional subunit besides the A and B, then disassociation and recombination of these units

with purified LDH-B_4 would yield 15 distinct bands. Three monomers polymerized in all possible combinations yield 15 different tetramers. Since the turtle LDH tetramer has been shown to contain A as one of its subunits and hybridization of turtle LDH with authentic B yields only four bands then the other turtle subunit must be B. The occurrence of only four bands in the hybridization experiments instead of the expected five is still unexplained. This experiment was repeated three times and each time the A_4 band failed to appear in the zymogram. However, I feel that it is still safe to assume that the green turtle LDH spectrum results from the random association of subunits, A and B. The absence of LDH-A_4 in the hybridization experiments indicates some modification of the expected binomial distribution but since the other four bands are present, recombination of one other subunit besides B must have occurred.

In general, LDH distribution in green turtle tissue is skewed towards LDH-A_4. Skeletal muscle contains predominantly LDH-A_4 and brain is particularly interesting because of the high levels of A subunits. In most other vertebrates tested, brain is usually rich in B subunits (for reviews see Markert, 1968; Kaplan et al., 1968).

Substrate inhibition studies also confirm the high levels of A subunits in the brain of this animal. LDH activity in brain tissue homogenate demonstrates a tolerance to high levels of pyruvate which is comparable to that of skeletal

muscle. This characteristic distribution of isoenzymes would seem to facilitate anaerobic pathways in nervous tissue. Dawson et al. (1964) showed that tissues that depend on anaerobic energy sources have higher LDH-A_4 concentrations. Miller and Hale (1968) compared homogenates of turtle (Pseudemy elegans) brain and skeletal muscle for substate inhibition and found both tissues to be similar.

Vesell (1965; 1966) has argued against attributing physiological significance to results obtained under highly artificial in vitro conditions. He also contends that substrate levels high enough to cause inhibition are not likely to occur in a living organism. The data of Stambaugh and Post (1966) are consistent with those of Vessel but they also show that product inhibition could play an important role in vivo. According to their data, a concentration of 21.9 mM L-lactate caused 55% product inhibition of the LDH-B_4 isoenzyme but only 13% inhibition of the LDH-A_4 isoenzyme. This concentration of lactate was routinely reached during prolonged dives in this study.

Most vertebrates depend upon the oxygen supply to the central nervous system for the production of the high energy bonds in ATP (Robin et al., 1964). ATP provides the energy to maintain ion concentration gradients across cell membranes that are essential to the functional integrity of the central nervous system. Schilb et al. (1962) found the Na^+ pump in isolated turtle bladder to be relatively insensitive to

lack of oxygen. Perhaps the turtle brain and nervous system can also derive sufficient energy anaerobically to maintain ion gradients as suggested by Robin et al. (1964).

Cserr (1967) found that the blood-brain barrier in the turtle is more permeable to sucrose than in the mammals. This anatomical feature may serve as an additional advantage facilitating the exchange of metabolites in the brain. Belkin (1968) found that turtles (Pseudemys concinna) tolerated anoxic anoxia about 14 times longer than stagnant anoxia. He suggests that rapid exchange of glucose and lactic acid by the brain is essential to survival during anoxia.

SUMMARY

Observations of the responses of the green turtle to prolonged involuntary dives and during the post-dive recovery period suggests that in addition to metabolic rate reduction and selective ischemia, anaerobic metabolic pathways play an important role in this animals ability to survive prolonged anoxia. The turtle accumulates an oxygen debt which takes several hours to repay. The characteristic distribution of LDH isoenzymes in the green turtle facilitate anaerobic metabolic mechanisms when oxygen levels are reduced.

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TABLES AND FIGURES

TABLE 1

Changes in breathing and heart rates during prolonged involuntary dives.

	Pre-dive Breathing Rate	Post-dive Breathing Rate	Pre-dive Heart Rate	Dive Heart Rate	Time (min)	Post-dive Heart Rate	Time (min)
Dive 1 hematocrit=25%	0.3/min		25/min	1.4/min	10		
				1.8/min	39		
Dive 2 hematocrit=30%	0.7/min		19/min	0.9/min	10		
				0.7/min	78		
		5/min					
Dive 3 hematocrit=35%			16/min	1.5/min	9		
				0.6/min	26		
				0.4/min	54		
				8.5/min	88		
				7.0/min	97		
						5.0/min	3
		4/min				12.0/min	10
Dive 4 hematocrit=12%	0.7/min		21/min	2.8/min	2		
				2.8/min	7		
				1.1/min	19		
				0.9/min	43		
				1.0/min	69		
				0.8/min	117		
				0.7/min	140		
				0.5/min	172		
				0.7/min	180		
						0.5/min	15
		0 for 60 min intubated & resuscitated				0.7/min	60
Dive 5 hematocrit=6%			22/min	8.0/min	5		
				4.5/min	28		
				8.5/min	47		
				10.0/min	68		
				13.0/min	87		
				15.0/min	118		
						16.0/min	4
		0 for 15 min intubated & resuscitated				22.0/min	10
Dive 6 hematocrit=35%	1.2/min						
Dive 7 hematocrit=33%	.6/min	4.5/min					
Dive 8 hematocrit=30%	1.1/min	5/min					

TABLE 2

LDH activity during purification of *Cynoscion regalis* tissue extract.

STAGE	ACTIVITY (I.U./ml)
Crude extract	420
DEAE cellulose wash	2,700
CM cellulose	12,200

TABLE 3

Serum chemistry of normal Chelonia mydas mydas determined by Technician SMA 12/60 Autoanalyzer.

Chelonia mydas mydas (results from 28 animals)

	Mean	Standard Deviation	Normal Range (±2 S.D.)
Na^+ meq/l	151	±5.8	139-163
K^+ meq/l	4.3	±1.3	2.1-6.5
CO_2 meq/l	31.0	±3.65	23.8=38.2
Cl^- meq/l	117	±5.2	107-127
Glu. mg%	90	±28.8	52-138
BUN mg%	35	±19.3	6-64
Ca^{++} mg%	6.4	±0.75	5.2-7.6
T.Protein mg%	3.9	±0.92	2.1-5.7
Alb. mg%	0.4	±0.2	up to 0.8
T. Bili mg%	0.1	±0.2	up to 0.5
Alk.Phos.mu/ml	47.5	±22.8	1.9-93.1
SGOT mu/ml	242	±61.8	120-364

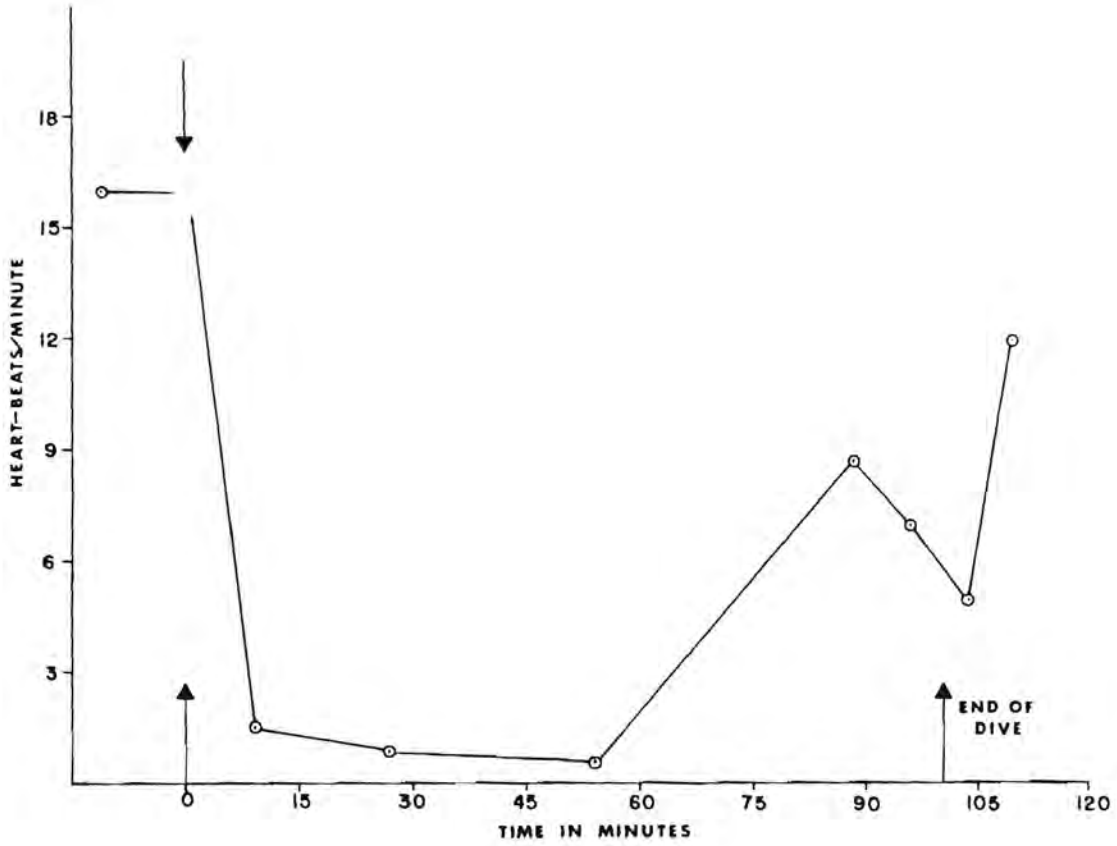

FIGURE 1

Heart rate during dive number 3.

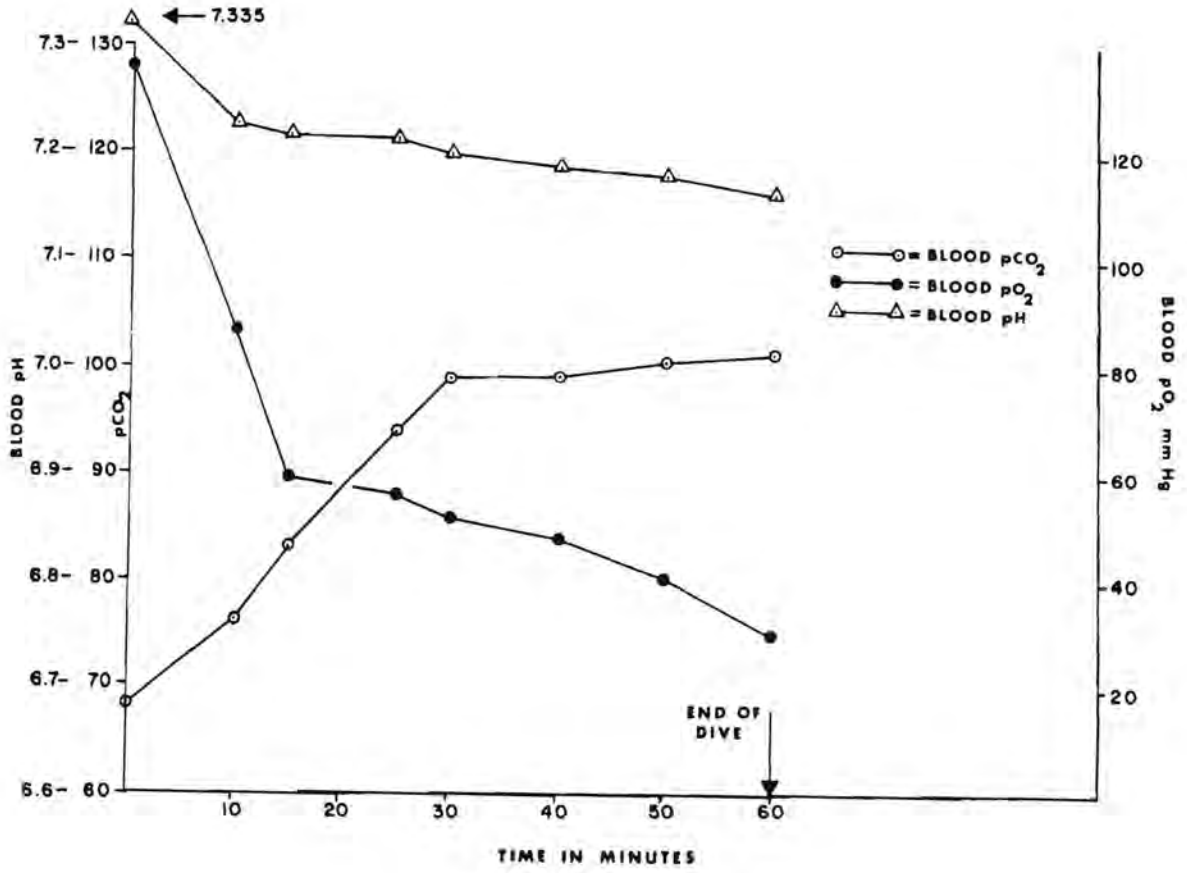

FIGURE 2

Changes in blood gases and in blood pH during dive number 1.

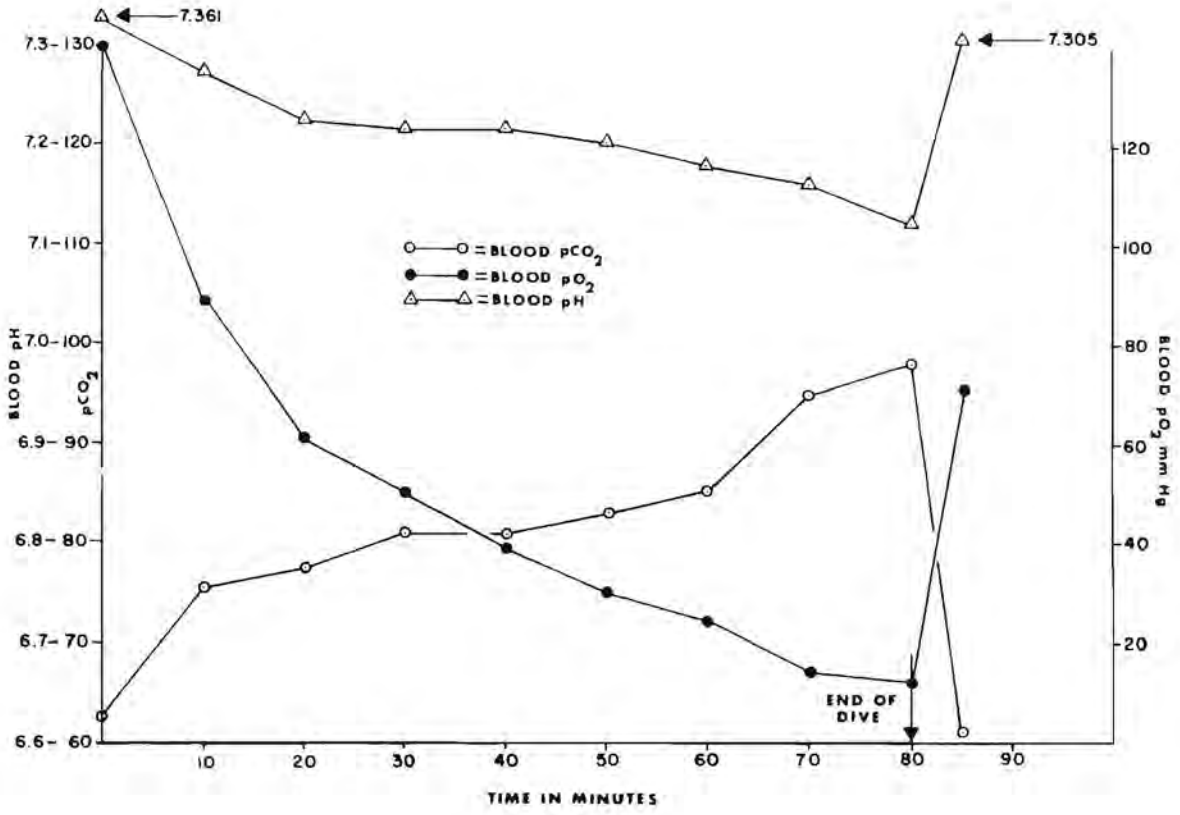

FIGURE 3

Changes in blood gases and in blood pH during dive number 2.

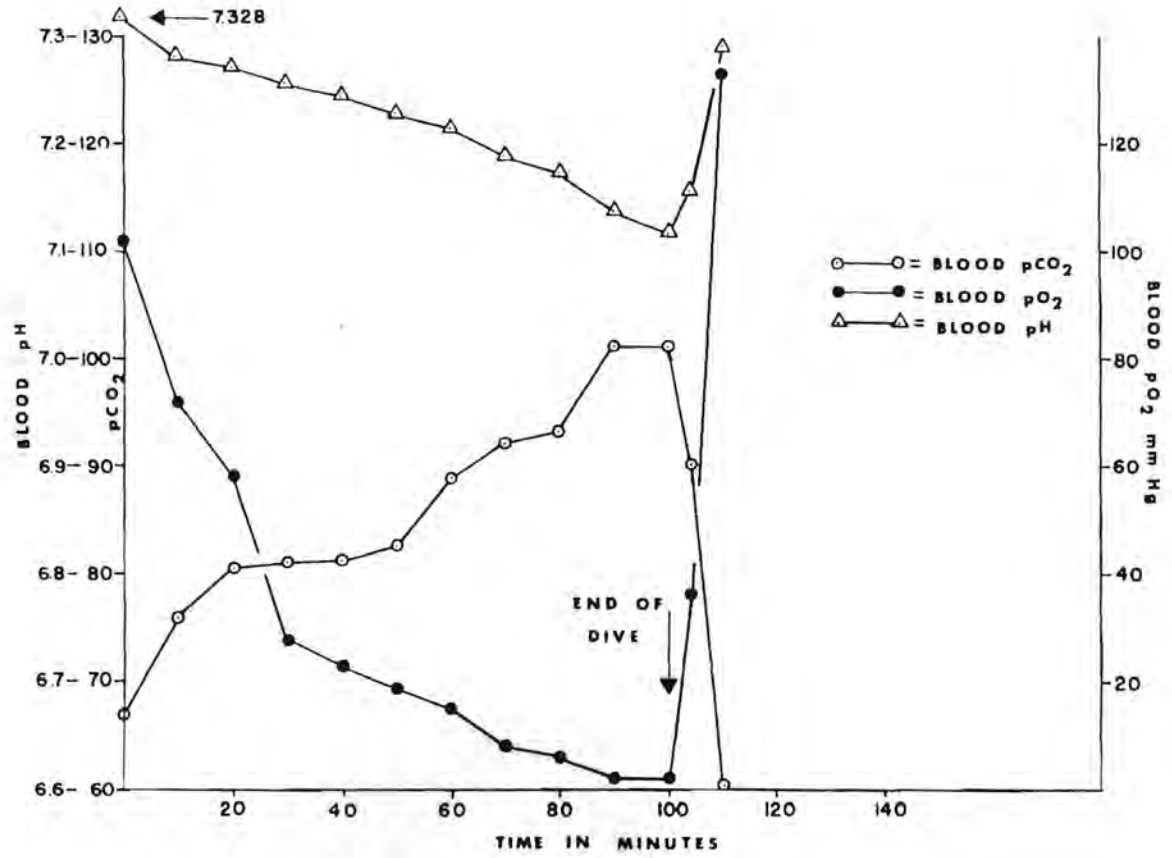

FIGURE 4

Changes in blood gases and in blood pH during dive number 3.

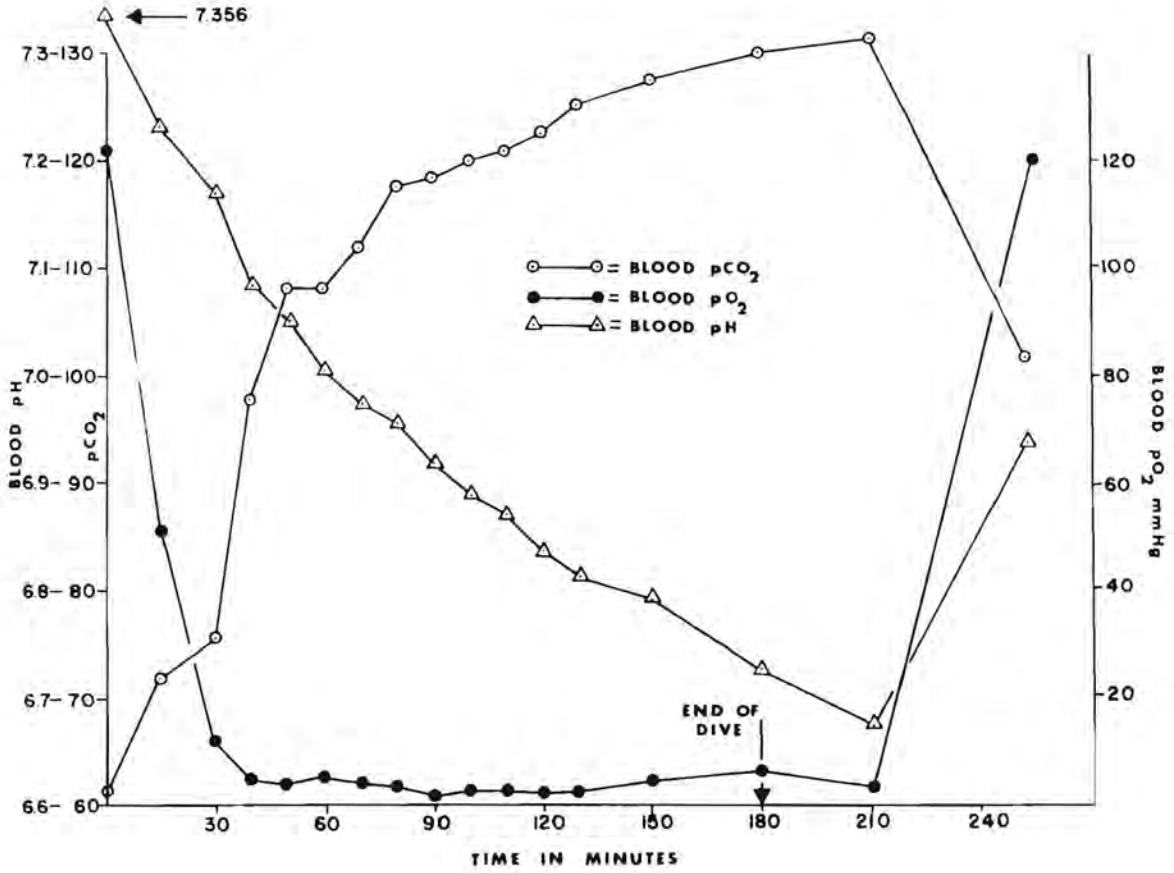

FIGURE 5

Changes in blood gases and in blood pH during dive number 4.

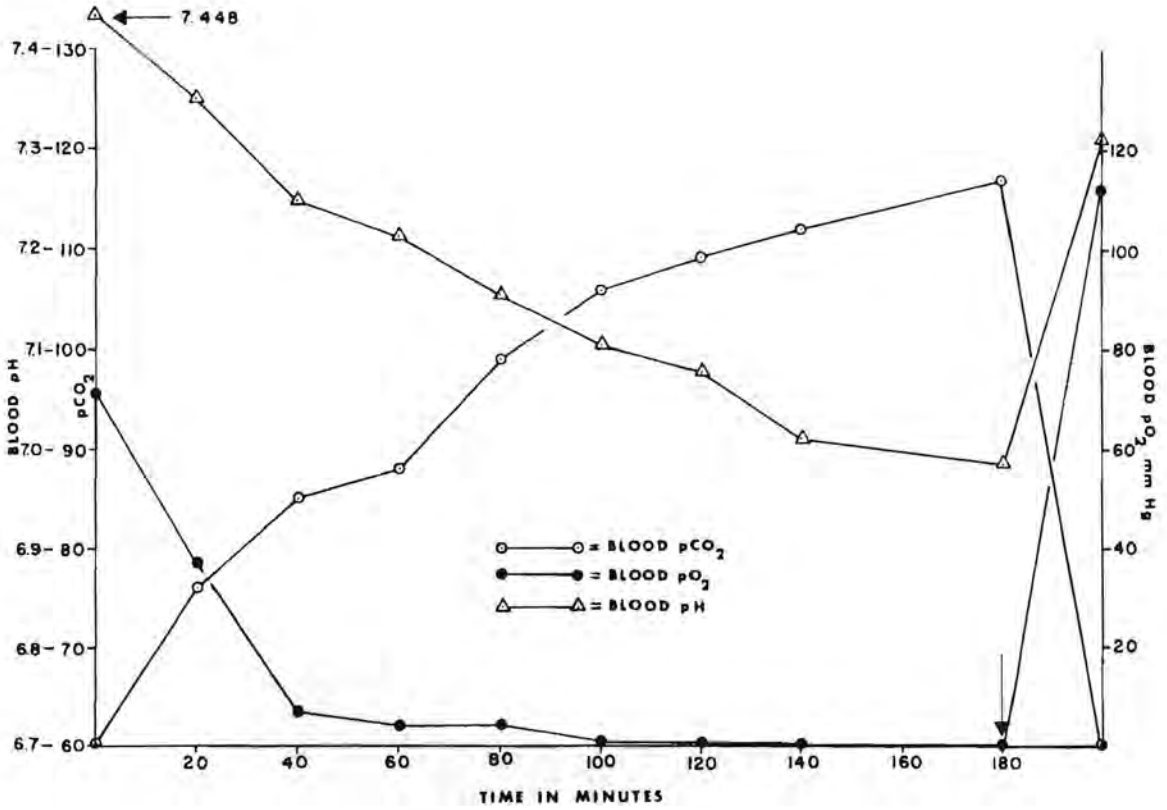

FIGURE 6

Changes in blood gases and in blood pH during dive number 5.

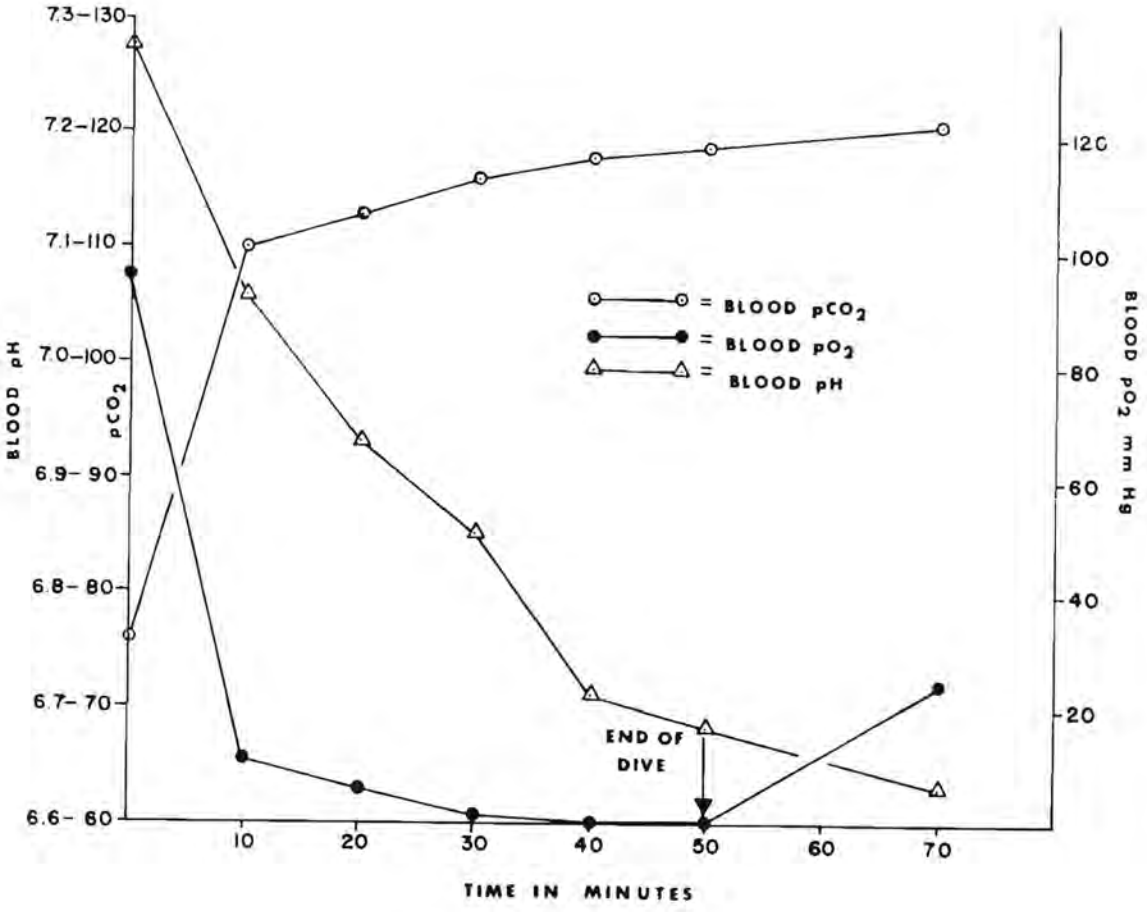

FIGURE 7

Changes in blood gases and in blood pH during dive number 6.

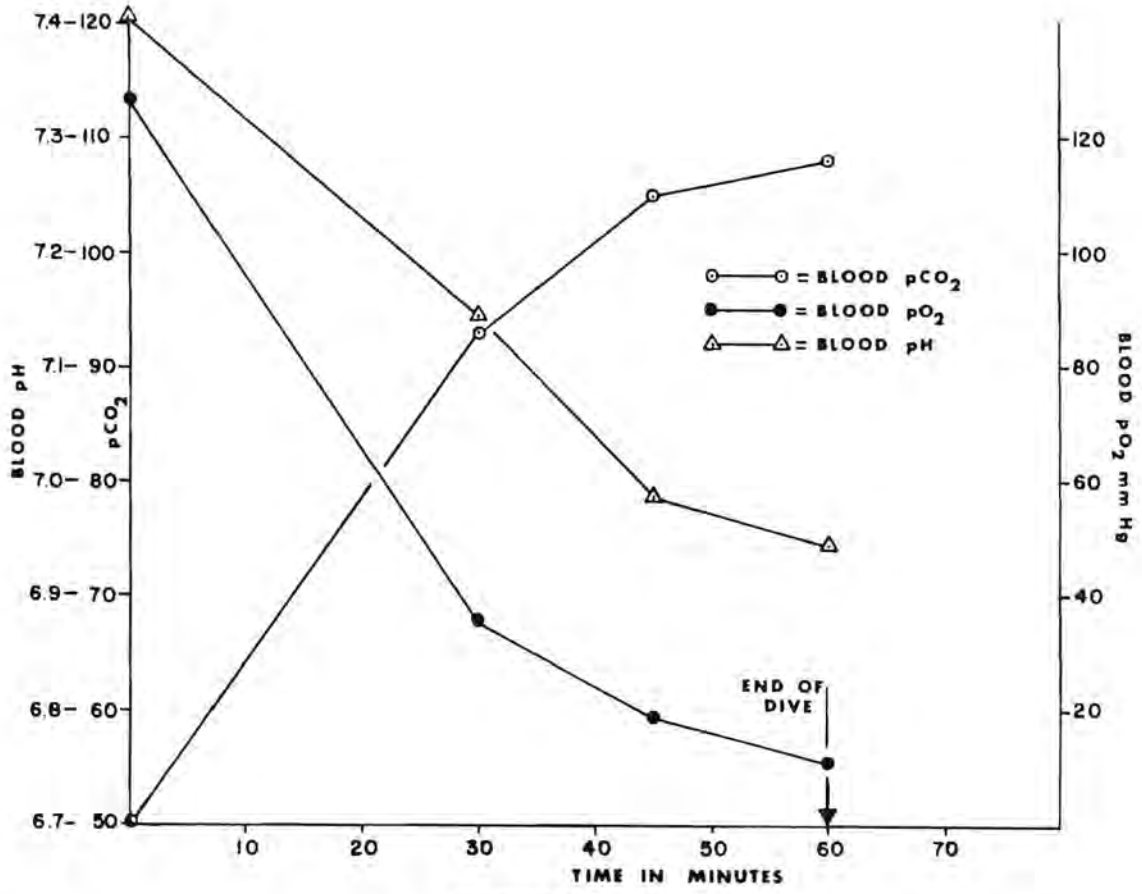

FIGURE 8

Changes in blood gases and in blood pH during dive number 7.

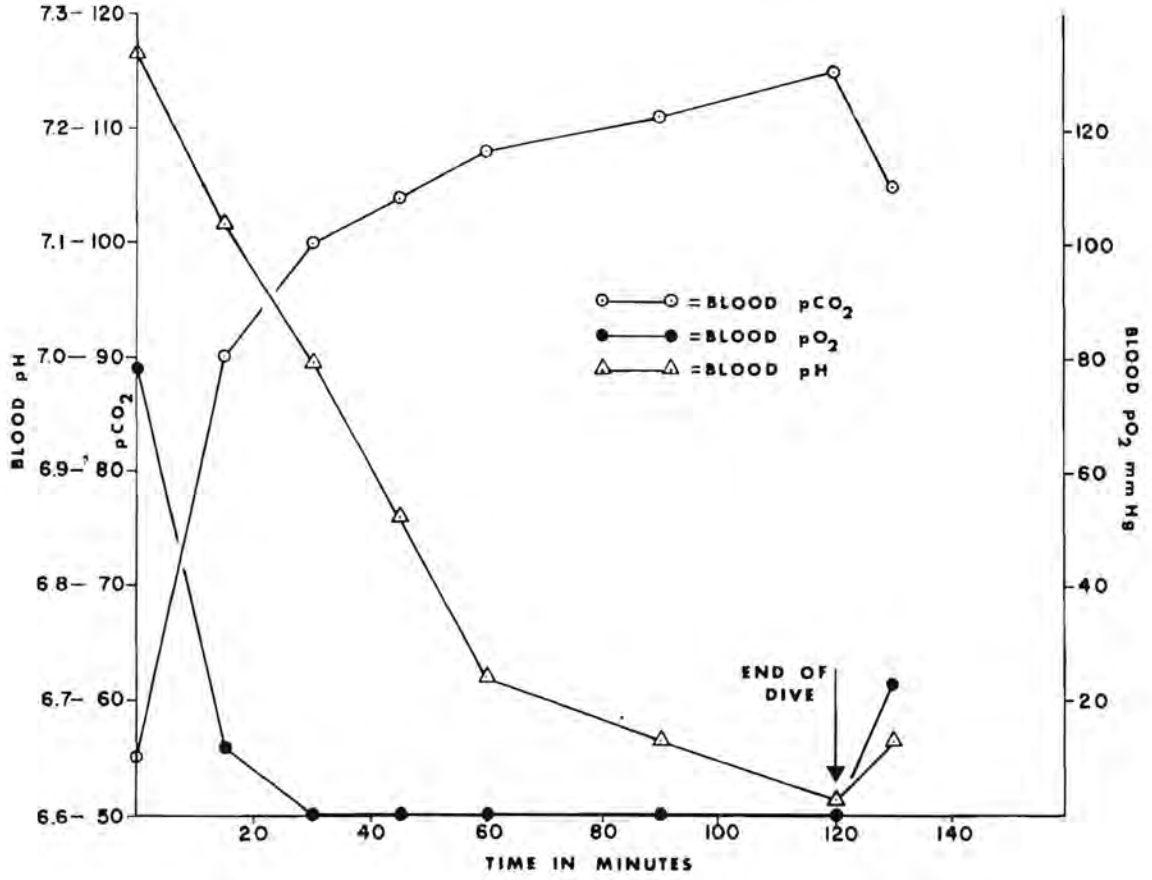

FIGURE 9

Changes in blood gases and in blood pH during dive number 8.

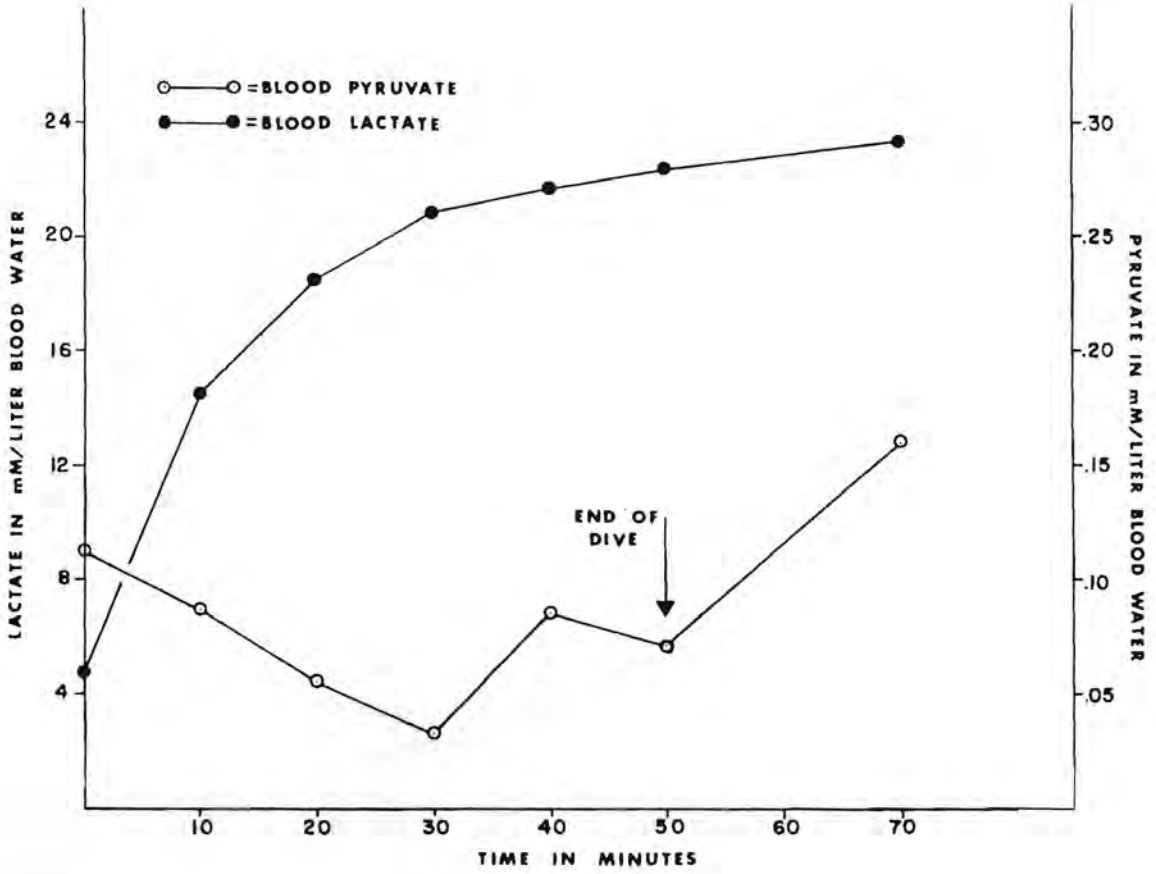

FIGURE 10

Changes in blood lactate and pyruvate during dive number 6.

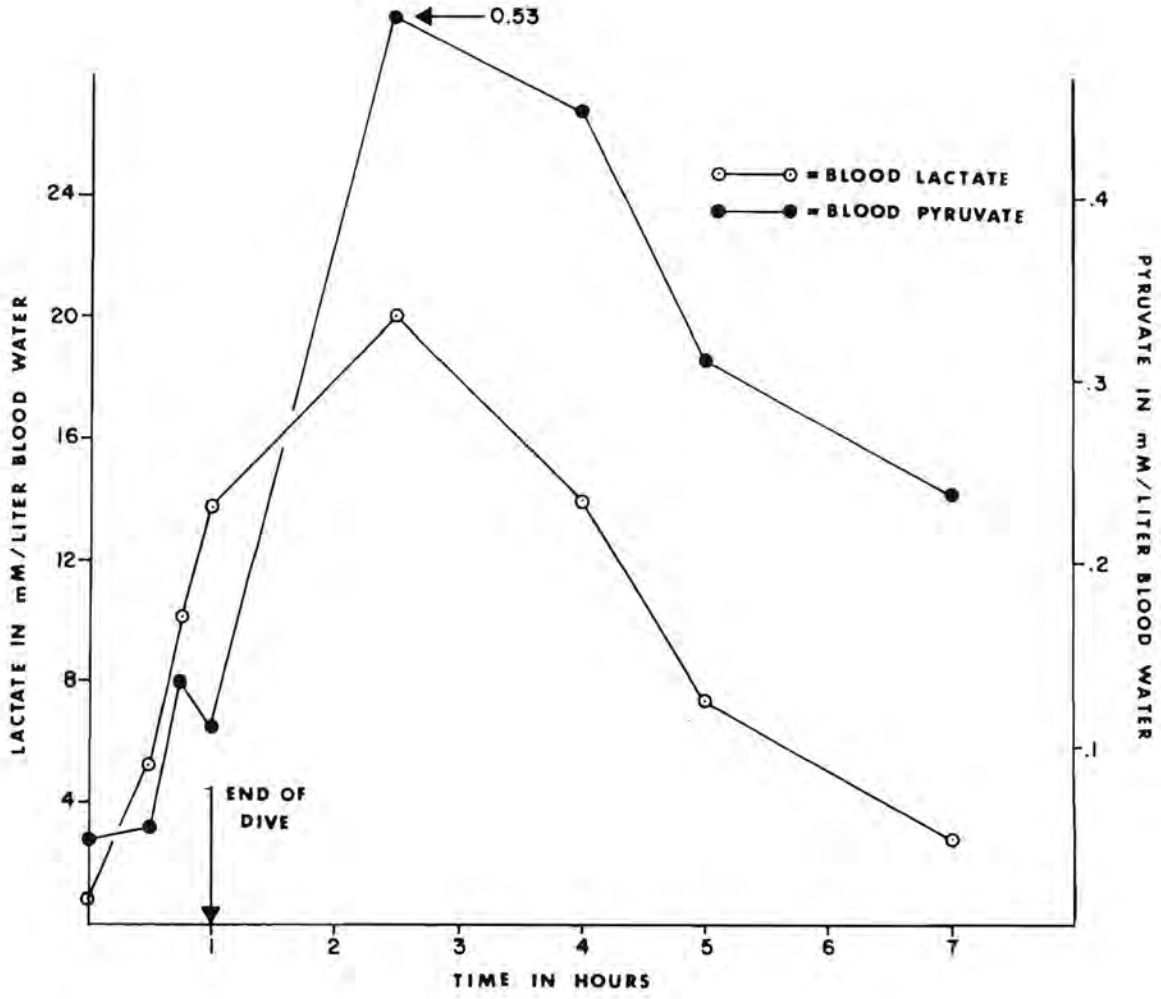

FIGURE 11

Changes in blood lactate and pyruvate during dive number 7.

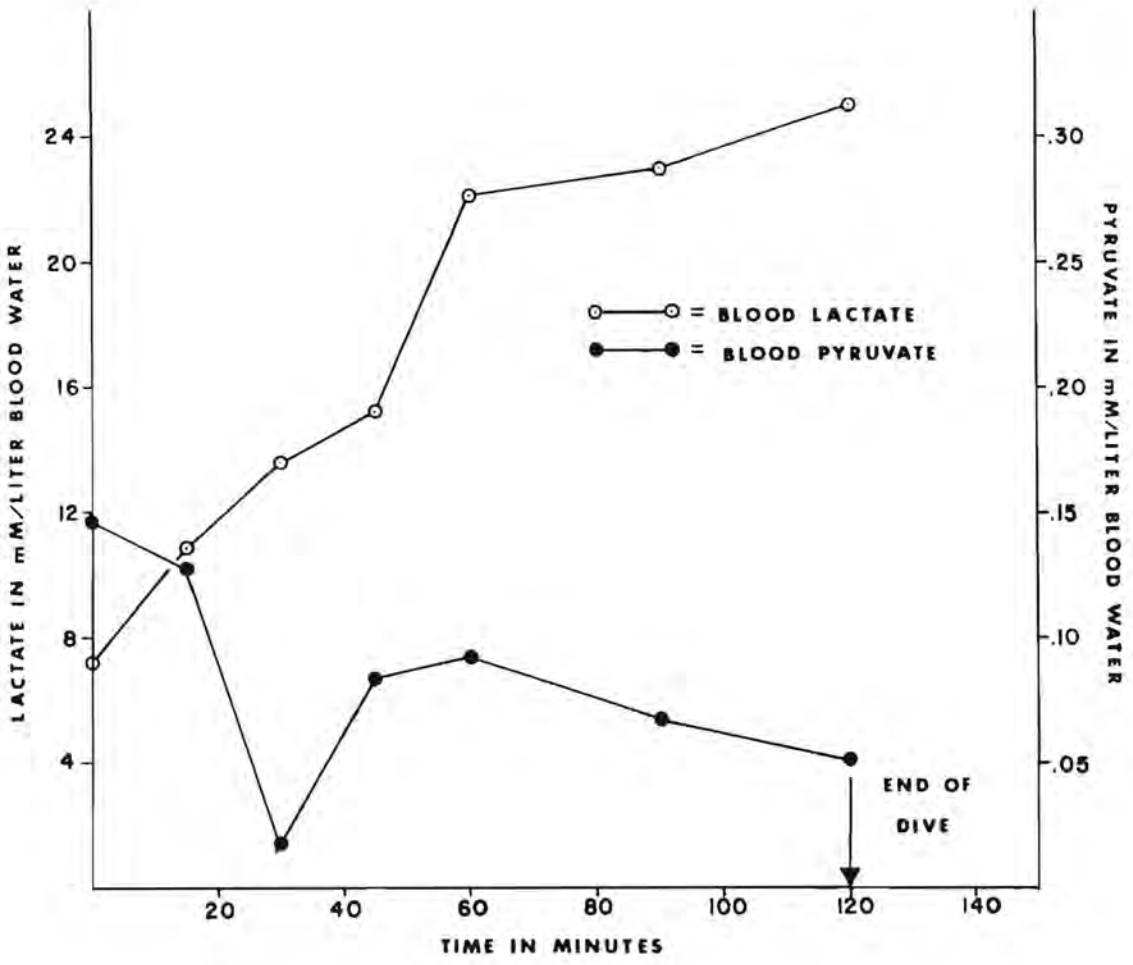

FIGURE 12

Changes in blood lactate and pyruvate during dive number 8.

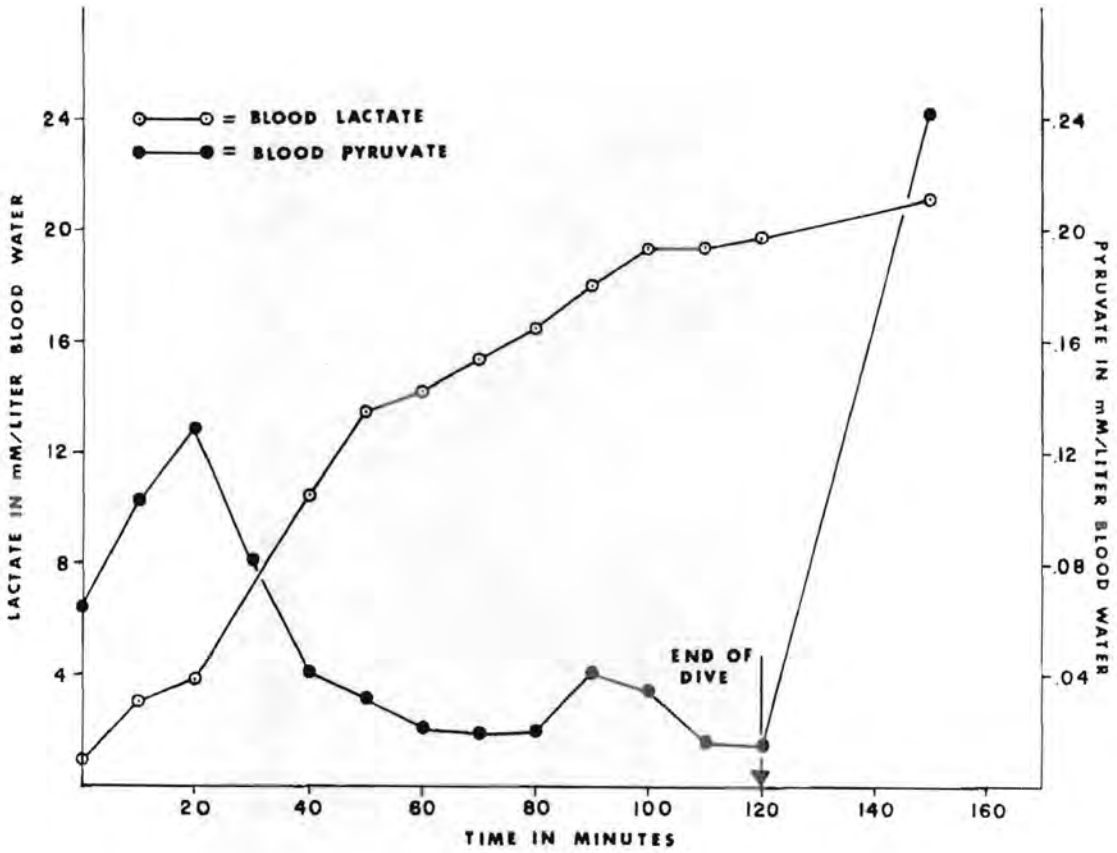

FIGURE 13

Changes in blood lactate and pyruvate during dive number 9.

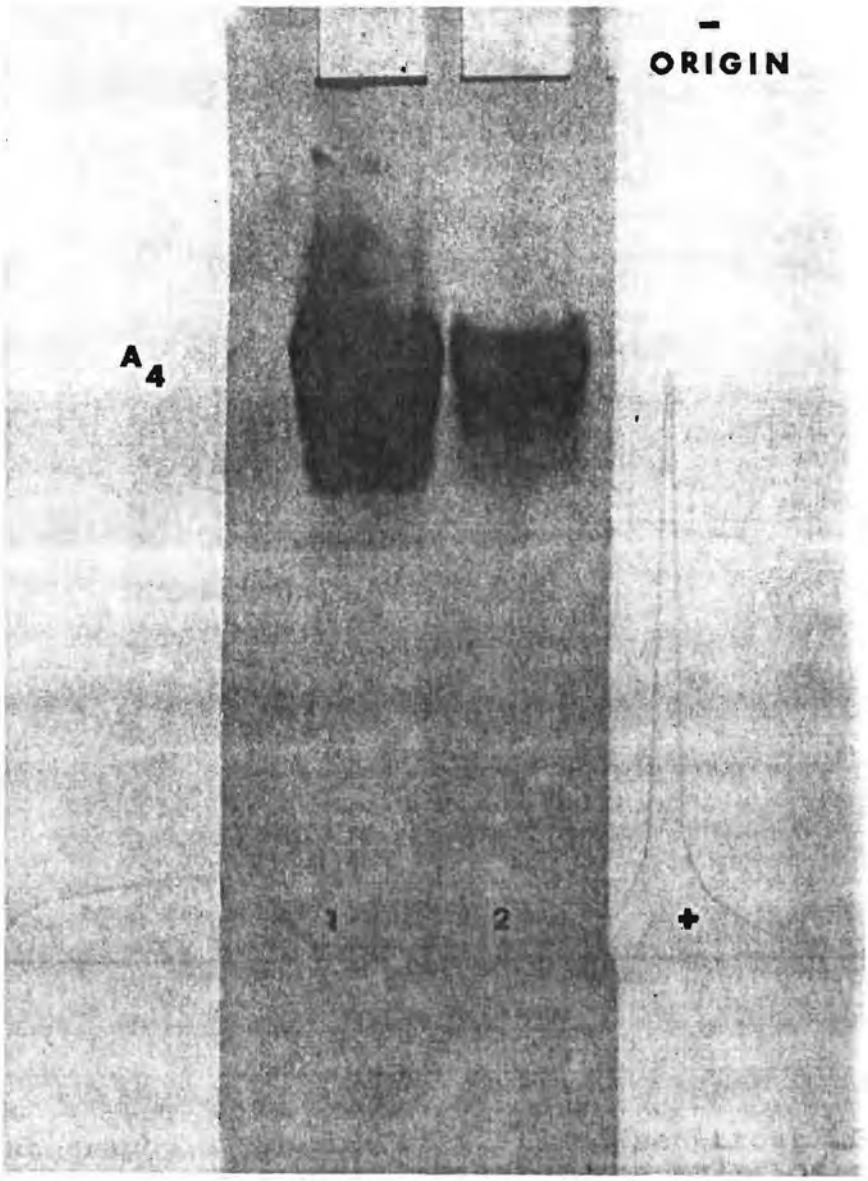

FIGURE 14

LDH isoenzyme electrophoresis of sea-trout LDH. 1) Crude tissue homogenate. 2) After partial purification on DEAE cellulose.

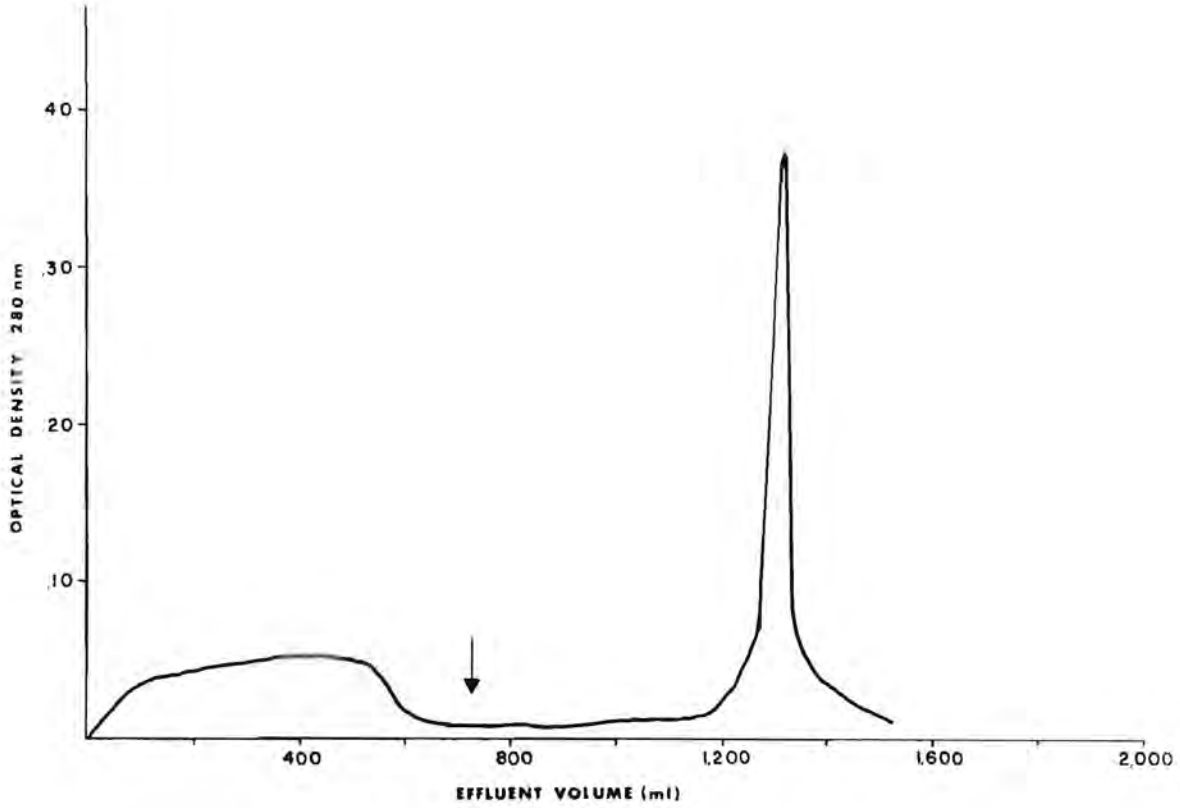

FIGURE 15

Chromatography of partially purified sea-trout LDH-A_4 on CM cellulose. The arrow indicates the start of the gradient. The sharp peak contained 12,200 I.U./ml of LDH activity.

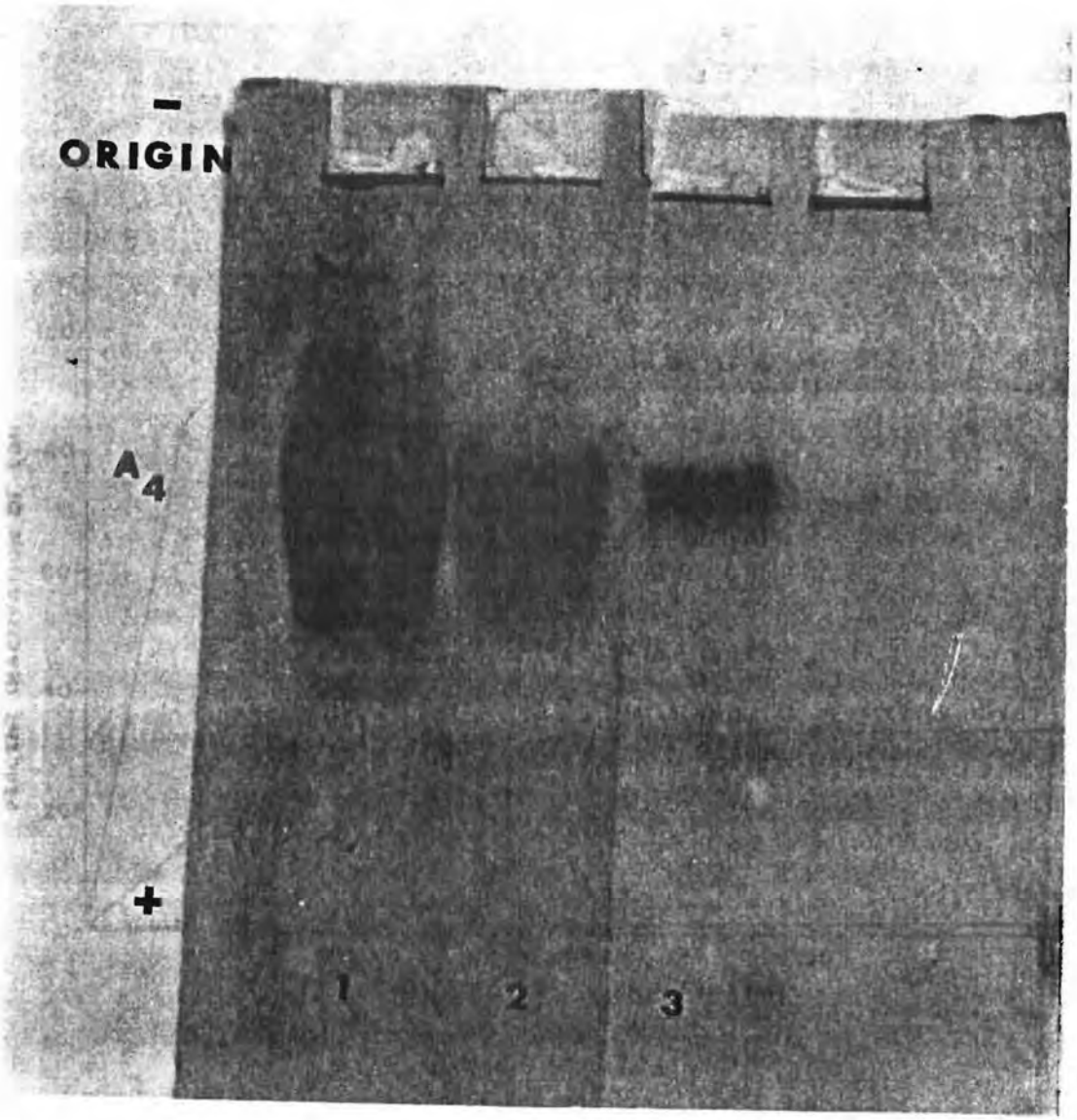

FIGURE 16

LDH isoenzyme electophoresis of sea-trout LDH. 1) crude tissue homogenate. 2) After partial purification on DEAE cellulose. 3) Pure LDH-A_4 after chromatography on CM cellulose.

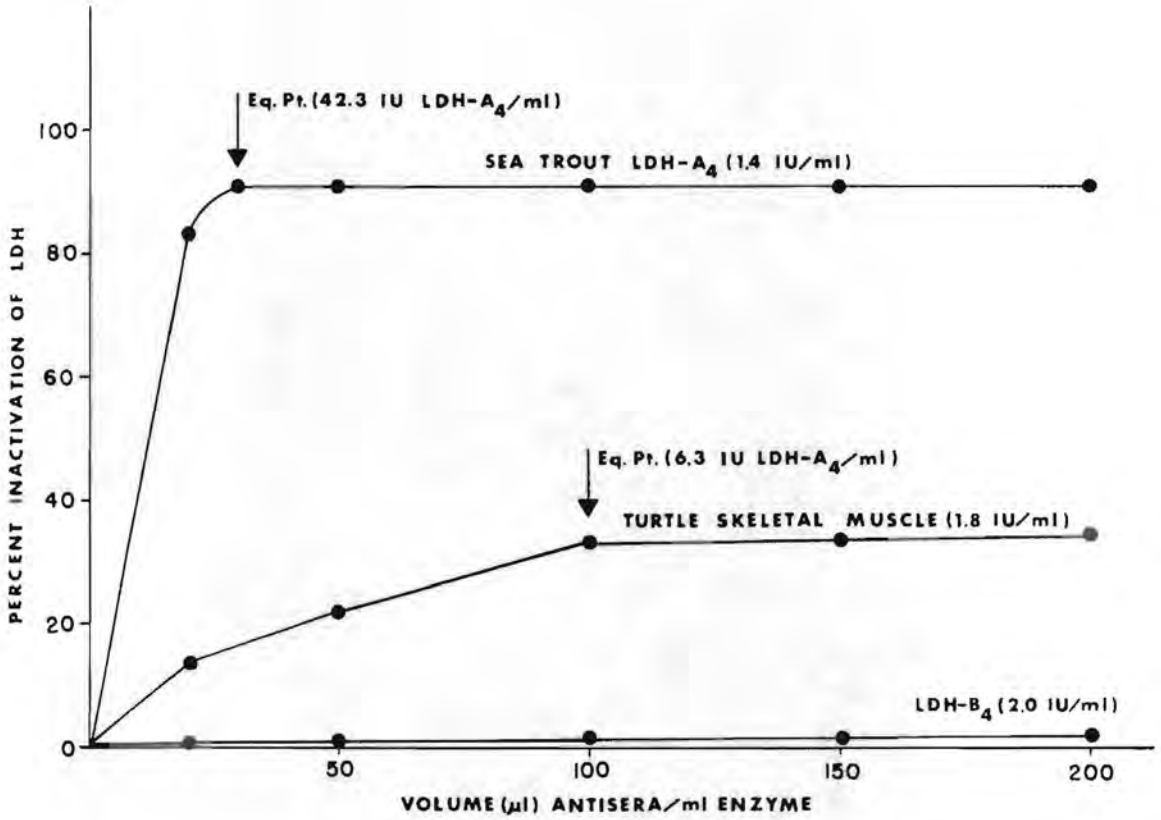

FIGURE 17

Inactivation of LDH activity by sea-trout LDH-A_4 antiserum.

FIGURE 18

LDH isoenzyme electrophoresis. 1) Sea-trout LDH-A_4 2) Sea-trout LDH-A_4 incubated with antiserum for 15 minutes prior to electrophoresis. Note lack of any LDH activity.

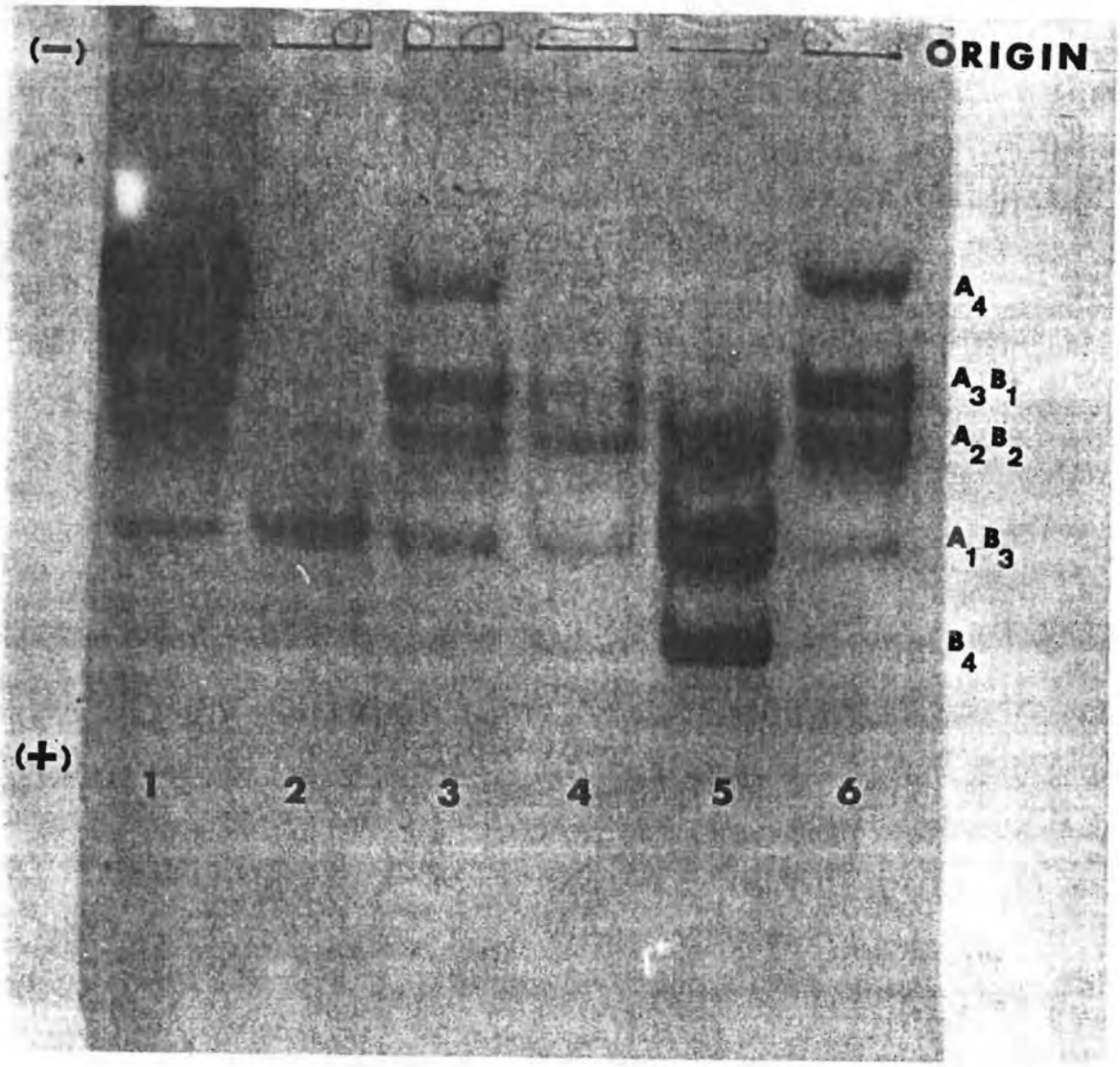

FIGURE 19

LDH isoenzyme patterns of the green turtle. 1) Skeletal muscle. 2) Liver. 3) Kidney. 4) Spleen. 5) Heart. 6) Brain.

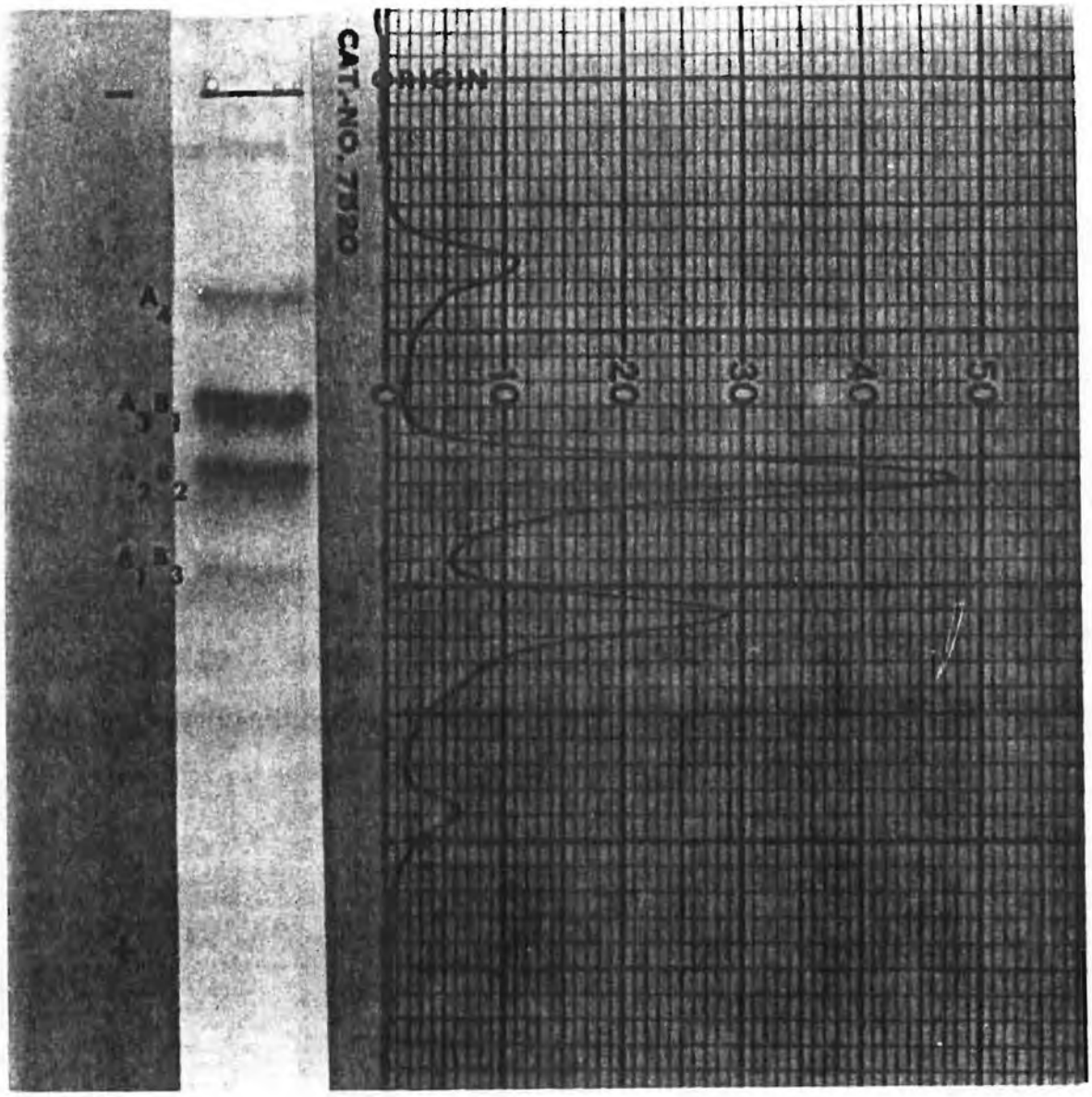

FIGURE 20

LDH isoenzyme electrophoresis of turtle brain showing optical density of each band.

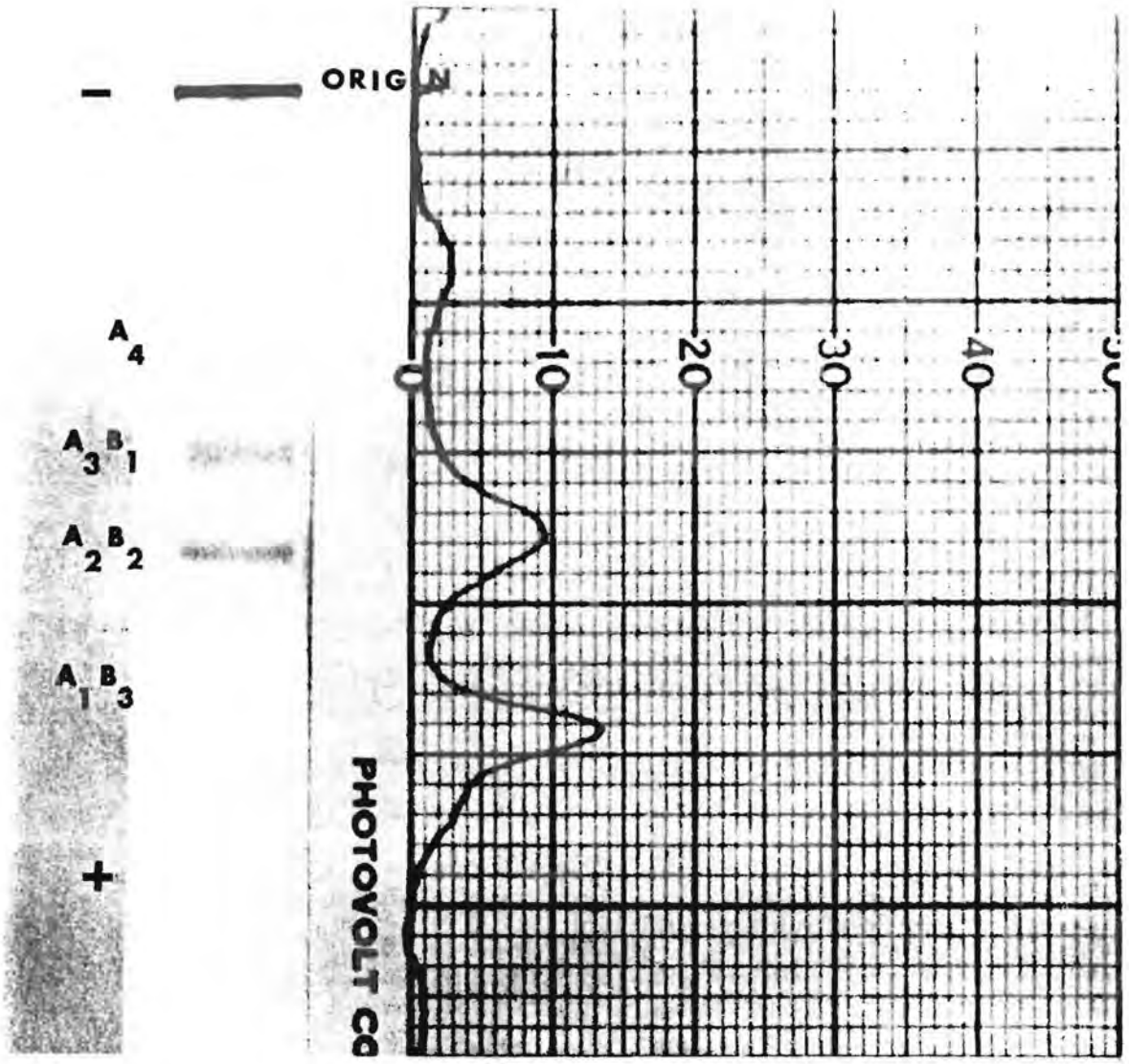

FIGURE 21

LDH isoenzyme electrophoresis of turtle brain homogenate after 15 minute incubation with A_4 antiserum 1:1. Note reduction of LDH activity in the isoenzymes containing A subunits.

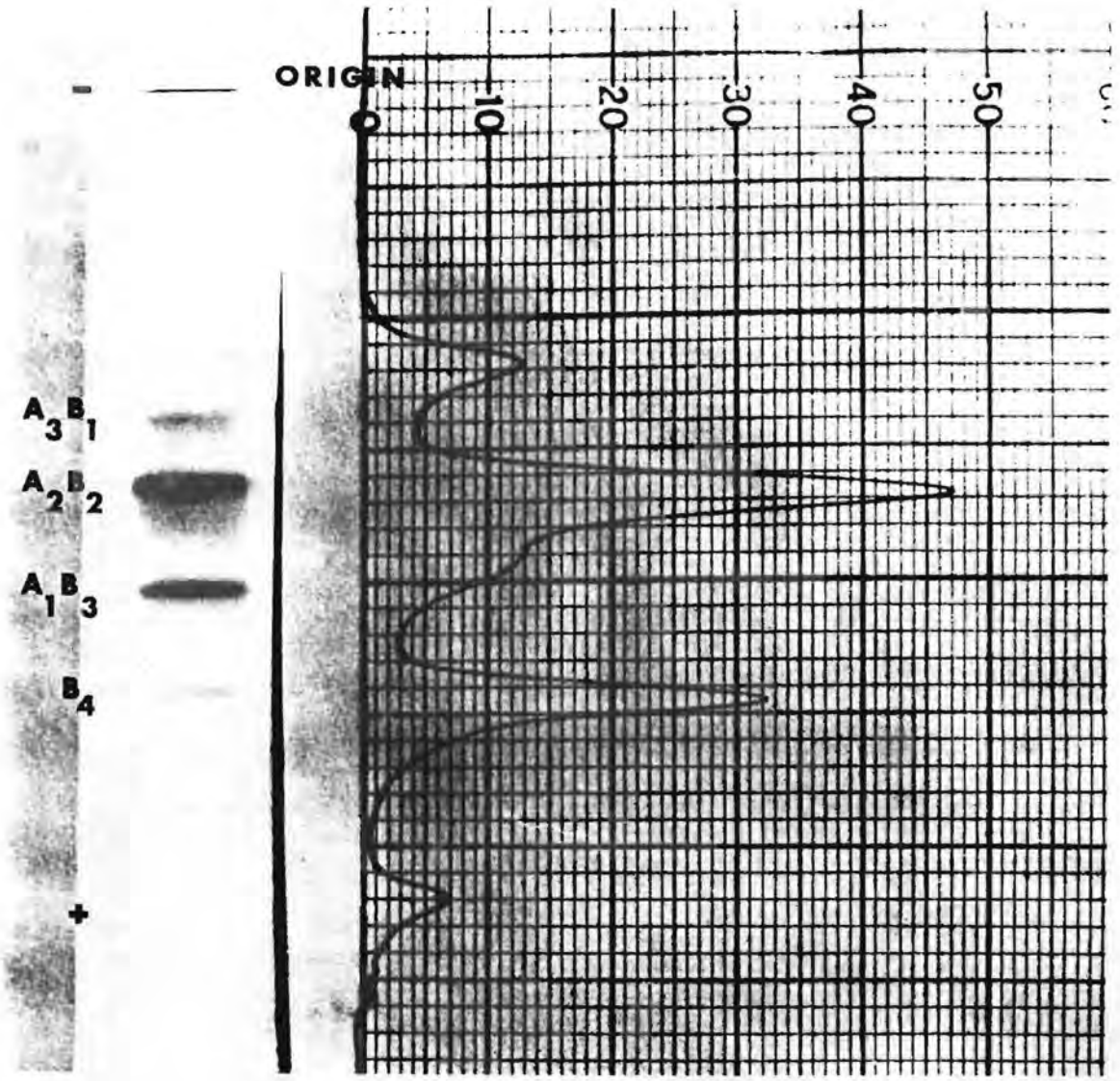

FIGURE 22

LDH isoenzyme electrophoresis of turtle heart homogenate showing optical density of each band.

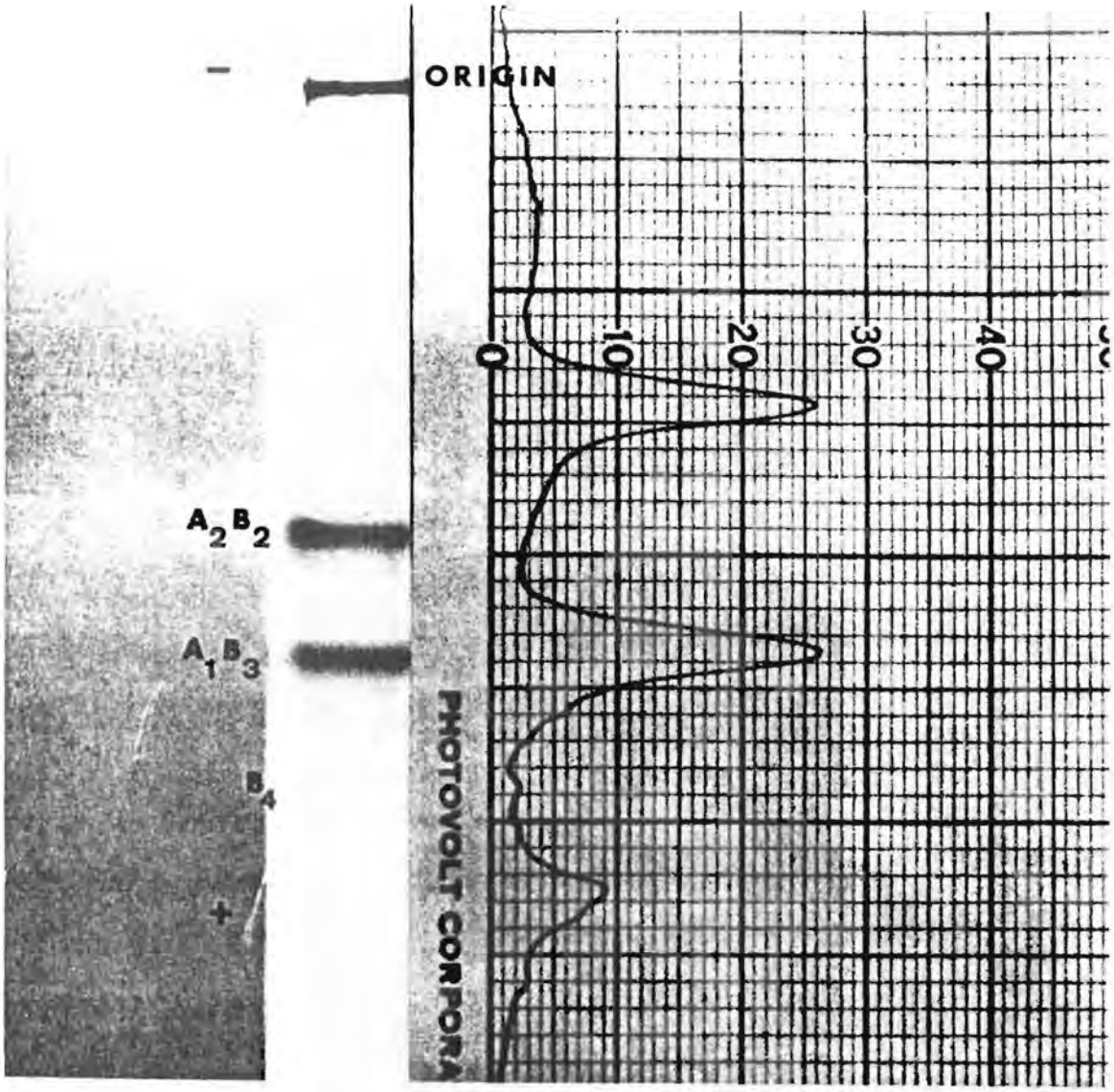

FIGURE 23

LDH isoenzyme electrophoresis of turtle heart homogenate after 15 minute incubation with A_4 antiserum 1:1. Note reduction of LDH activity in the isoenzymes containing A subunits.

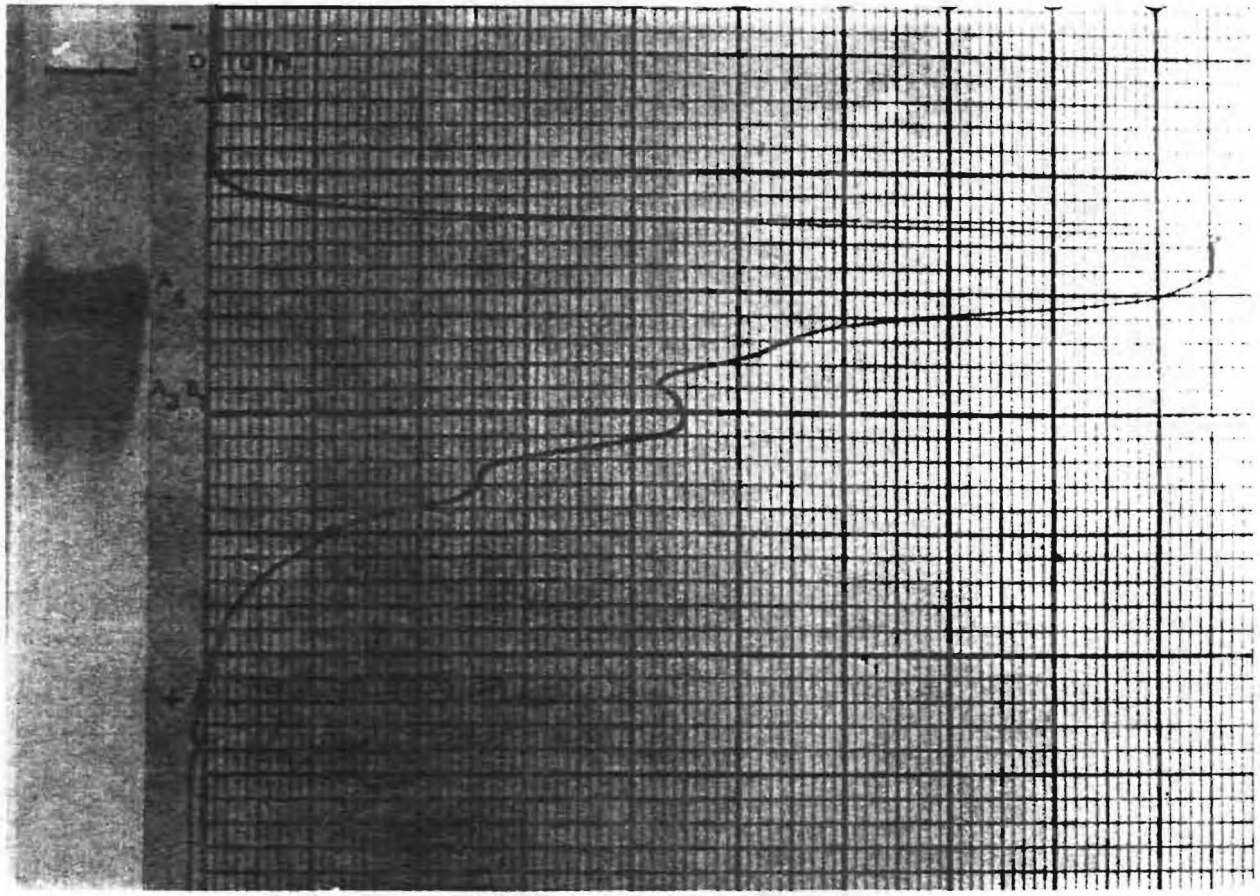

FIGURE 24

LDH isoenzyme electrophoresis of turtle skeletal muscle homogenate showing optical density of each band.

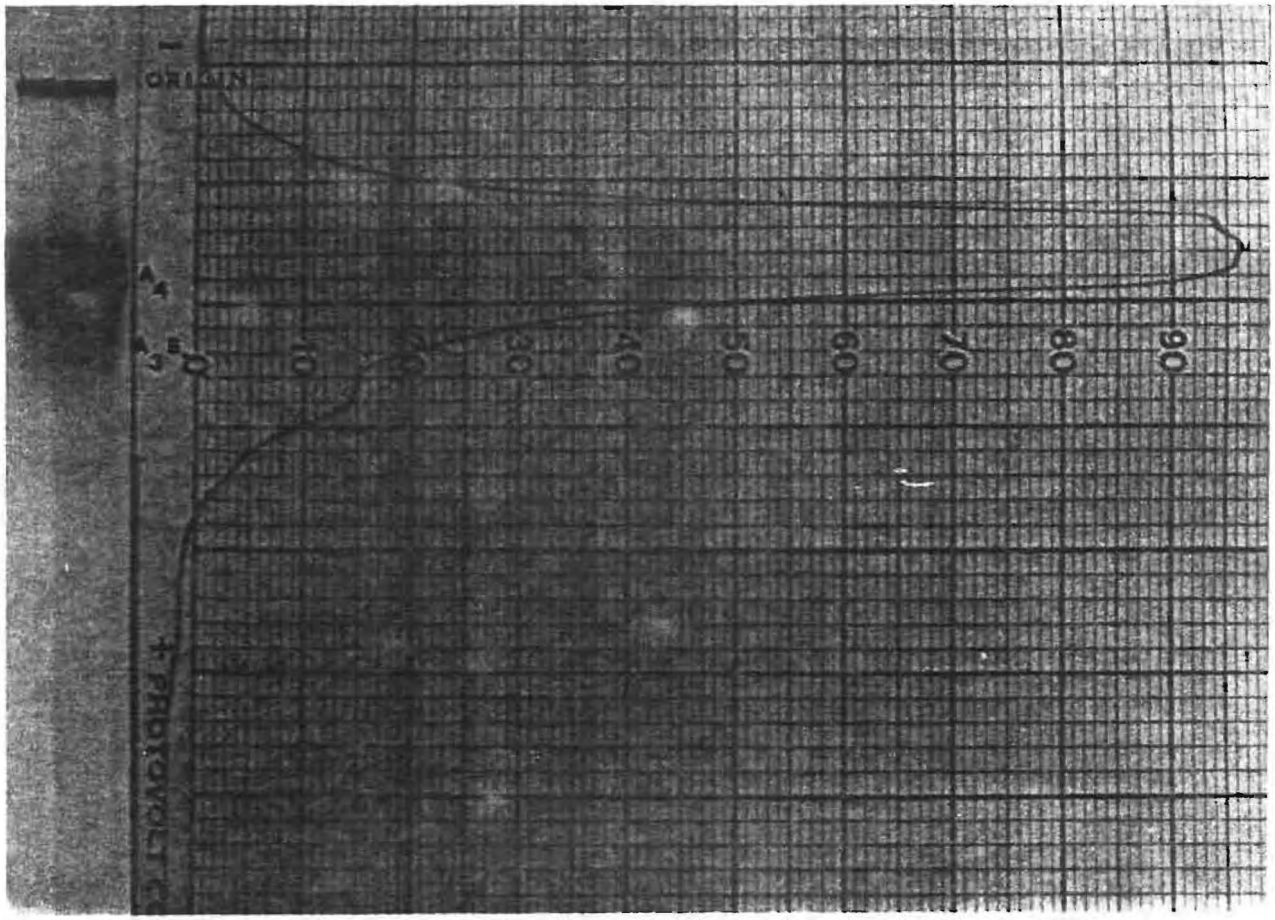

FIGURE 25

LDH isoenzyme electrophoresis of turtle skeletal muscle homogenate after 15 minute incubation with A_4 antiserum. Note reduction in A_3B_1 activity. The A_4 band still shows full scale deflection after treatment with antiserum because it is present in such large amounts.

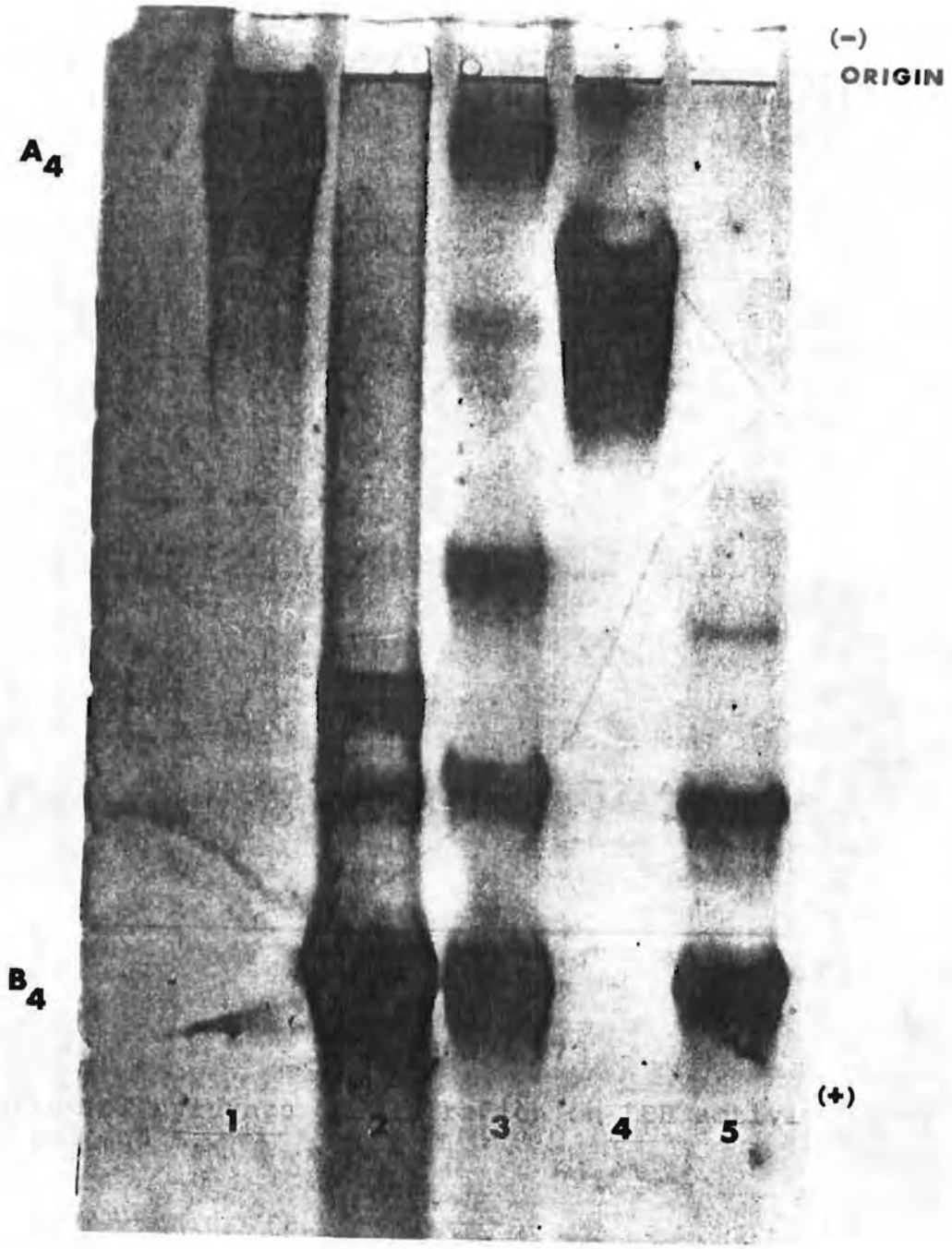

FIGURE 26

Hybridization of turtle skeletal muscle homogenate with purified LDH-B_4. 1) Pure A_4. 2) Pure B_4. 3) Hybridization of A_4 and B_4. 4) Turtle skeletal muscle homogenate. 5) Hybridization of turtle skeletal muscle homogenate with B_4.

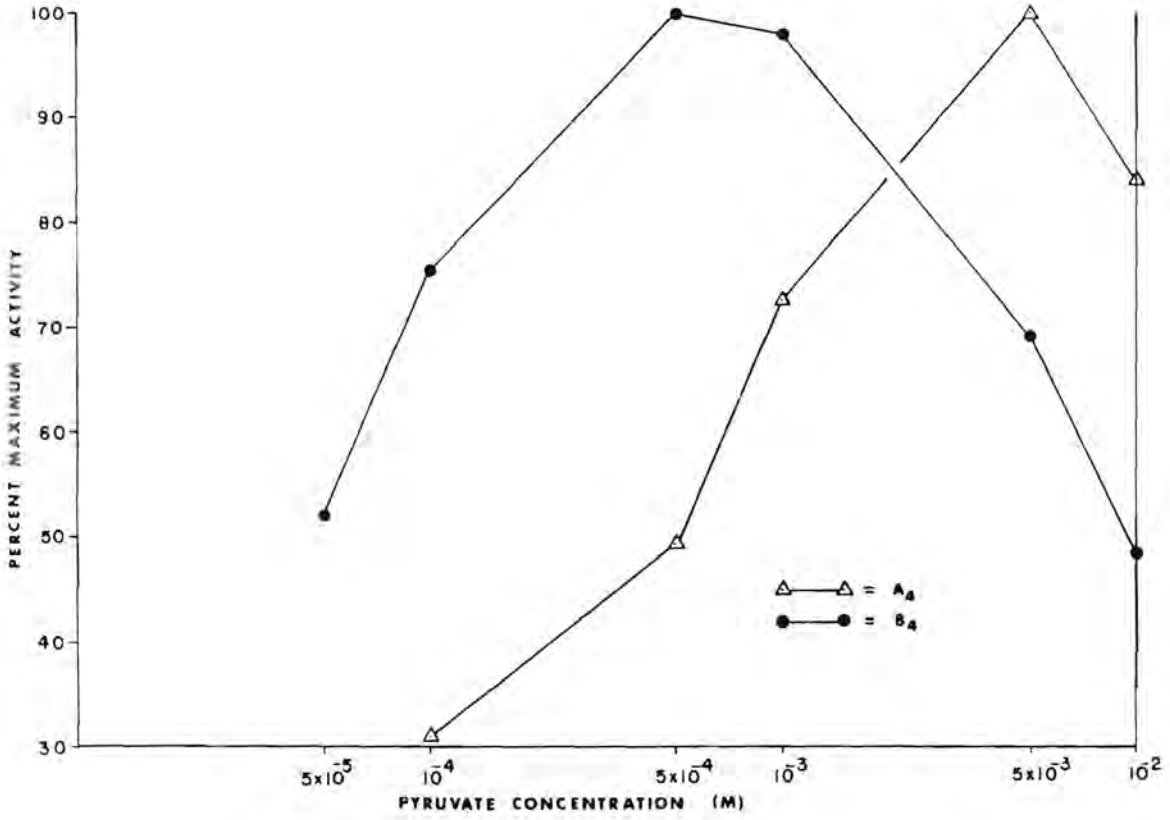

FIGURE 27

Effect of pyruvate concentration on LDH activity of pure LDH-A_4 and B_4.

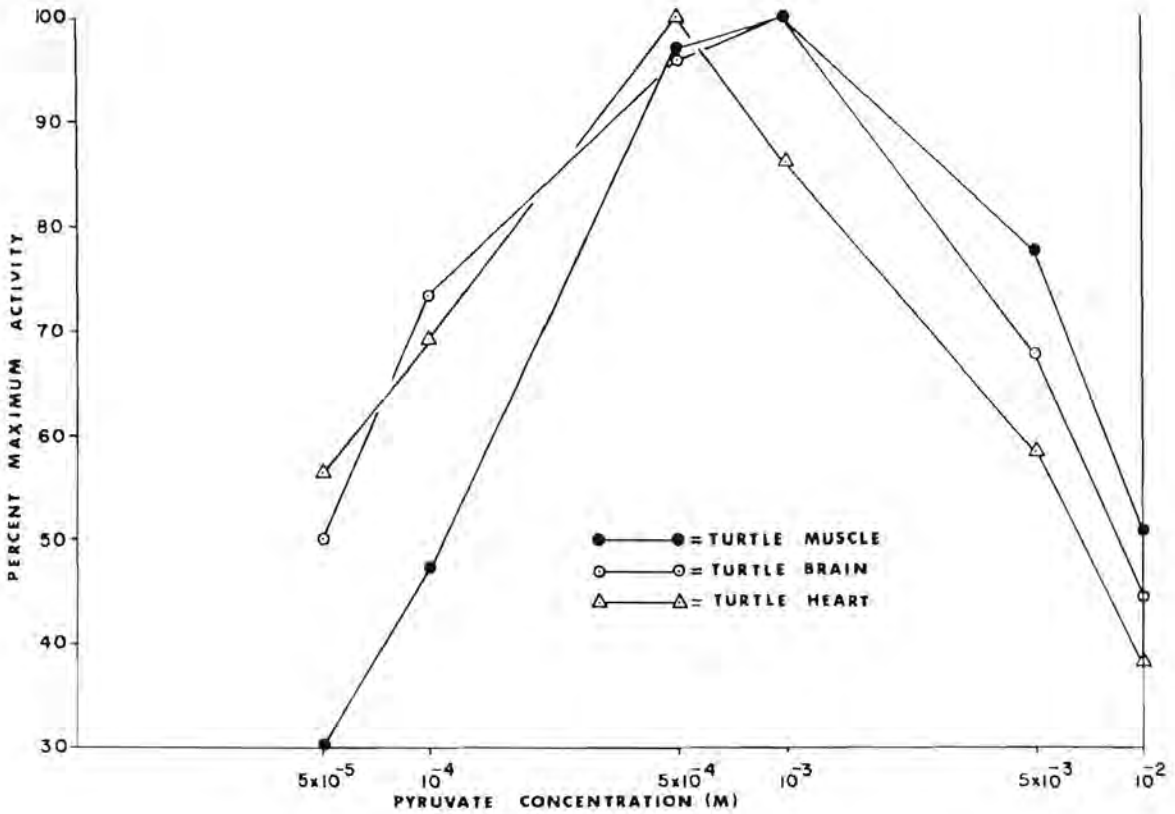

FIGURE 28

Effect of pyruvate concentration on LDH activity in tissue homogenates of turtle heart, brain and skeletal muscle.

VITA

Edward D. Scura was born in Passaic, New Jersey on June 11, 1944. He graduated from De Paul Diocesan High School, Wayne, New Jersey in 1962. He majored in biology and chemistry at Villanova University and graduated with a Bachelor of Science degree in 1966. From 1966 to 1968, he was in the United States Army Medical Corps. In 1968, he entered graduate school at the University of Miami Rosenstiel School of Marine and Atmospheric Sciences, majoring in marine biology and was granted the degree of Doctor of Philosophy in January, 1973.

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THE UNIVERSITY OF MIAMI

ANOXIC PHYSIOLOGY IN THE GREEN TURTLE *CHELONIA MYDAS MYDAS*

BY

Edward D. Scura

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida

Januray, 1973

UNIVERSITY OF MIAMI
Coral Gables, Florida

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THE UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

Subject

Anoxic Physiology in the Green Turtle Chelonia mydas mydas

By

Edward D. Scura

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