CHANGES IN THE OUABAIN-SENSITIVE, SODIUM AND POTASSIUM-ACTIVATED ADENOSINE TRIPHOSPHATASE OF THE GILLS OF COHO SALMON Oncorhynchus kisutch, DURING THE FRY TO SMOLT STAGES OF ITS LIFE HISTORY AND UPON EXPOSURE TO SEA WATER

by

MICHAEL ARTHUR GILES

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Department of Zoology

The University of British Columbia
Vancouver 8, Canada

Date July 29/69

ABSTRACT

Some of the kinetic characteristics of the sodium and potassium-activated adenosine triphosphatase of the fragmented cell membranes of cells from the gills of sea water adapted coho salmon Oncorhynchus kisutch, and changes in this enzyme upon exposure to sea water and during the fry to smolt stages of fresh water reared juvenile coho were investigated. Inhibition with 4×10^{-4} moles/liter ouabain was used to assay the activity of this enzyme since this ATPase is specifically inhibited by ouabain (Skou, 1957).

The following assay conditions were found to result in maximal hydrolysis of ATP in enzyme preparations from sea water adapted coho: pH, 7.4; incubation temperature, 40°C; NaCl and KCl concentrations of 100.0 and 20.0 mmoles/liter, respectively, and Mg^{2+}-ATP, 5.0 mmoles/liter. The Km for ATP was 0.2 mmoles/liter. The enzyme activity recorded with magnesium ions as the only cation present (Mg^{2+}-ATPase) was not affected by any concentration of ouabain, although the addition of sodium ions (100 mmoles/liter) appeared to inhibit this activity slightly. The additional hydrolysis of ATP observed when sodium, potassium and magnesium ions were present was inhibited by ouabain. The Ki for ouabain was 7×10^{-6} moles/liter when sodium and potassium ion concentrations were 100.0 and 20.0 mmoles liter, respectively.

The ($Na^+ + K^+$)- activated ATPase of sea water adapted coho was characterized by its high ouabain-sensitive activity and the large activating effect of potassium ions in the presence

of magnesium and sodium ions compared to the activity observed with the latter two ions alone. This enzyme in preparations from the gills of fresh water reared fish was characterized by a high activating effect of sodium ions when present with magnesium ions. This sodium activation often comprised over 60% of the total ouabain-sensitive activity.

Considerable increases in the total activity, and activating effects of potassium ions and decreases in the activating effects of sodium ions alone were observed when fresh water reared coho were transferred directly to sea water. The changes in the activating effect of the ions were noticable after 5 days exposure to sea water although no changes in the total activity of the enzyme occurred until after 10 days exposure.

On a seasonal basis changes in enzyme activity occurred which were apparently linked to the stage of development of the parr-smolt transformation in fresh water reared juvenile coho. Activities during the period of October 1, 1968 to late November, 1968 were generally quite low. A sharp peak in activity occurred in December, 1968 to late January, 1969 which decreased to a low level by mid-February. Up to and including this last period the activity of enzymes from the gills of both fresh water and sea water reared coho were qualitatively similar although the seawater fish always had a higher enzyme activity. During the period of mid-February to late April, 1969 the enzyme from fresh water reared coho changed in total activity and characteristics of sodium activation and potassium activation and became very similar to that of sea water reared fish of the same age.

W. A. Hoar

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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INTRODUCTION

The parr-smolt transformation of young salmon prior to and during their seaward migration is accompanied by changes in form and by many biochemical, physiological and behavioral changes (Baggerman, 1960; Hoar, 1965; Vanstone and Markert, 1968). Recently, changes in the sodium and potassium-activated, ouabain-sensitive adenosine triphosphatase enzyme of the gills of fish transferred to sea water have been reported (Epstein, Katz and Pickford, 1967; Kamiya and Utida, 1968). The present investigation was undertaken to determine whether this enzyme changed in the gills of juvenile coho salmon exposed to sea water and if there were any preadaptive changes in the enzyme in freshwater fish during the parr-smolt transformation.

Since the microsomal preparation from coho gill tissue contained phosphatases which do not require sodium and potassium ions for activity and are not inhibited by ouabain (Skou, 1957), the sodium and potassium-activated ATPase was assayed in appropriate media with and without ouabain.

The stage of development of the juvenile coho was determined by visual observation of external changes (Houston and Threadgold, 1963), and by changes in the weight - length relationship (Vanstone and Markert, 1968).

MATERIALS AND METHODS

General Methods

Source and maintenance of fish

Approximately 3500 coho fry in fresh water were obtained from the Washington State Hatchery at Skaget, Washington, U.S.A. in May of 1968 and transported in large tanks to the laboratory in West Vancouver. Additionally, approximately 400 coho post smolts in sea water were obtained from the Biological Station of the Fisheries Research Board of Canada at Nanaimo, B.C. and transported to West Vancouver. No mortalities related to transportation were observed. The fish were placed in two fibreglass aquaria 1.8 m in diameter containing 5000 litres of water in which a circular flow was maintained by means of recirculating pumps. Chlorinated municipal water or sea water was supplied to each aquarium at a rate which replaced the water in each tank at least four times in every 24 hr. These fish were fed once daily to satiation with a diet composed of beef liver, 4500 g; beef heart, 4500 g; canned salmon, 4500 g; salt, 110 g; and Pablum, (Meade-Johnson Canada Ltd.), 40 g.

In August of 1968 approximately 1000 fry were placed in each of three 1.8 m aquaria and were designated seasonal freshwater fish, freshwater adaptation fish and seawater adaptation fish respectively. Of the two freshwater groups of fish, which were maintained in chlorinated fresh water until April, 1969 one, the seasonal freshwater fish, was used to determine the seasonal changes in gill ATPase while the other, the fresh-

water adaptation fish, was used for the sea water adaptation experiments. The seasonal seawater fish were maintained in chlorinated tap water for two days and then adapted to sea water over a 10 day period during which the salinity was increased approximately 3 $^{o}/_{oo}$ per day resulting in a final salinity of 30 $^{o}/_{oo}$ equal to 100% sea water. Mortality during this procedure was less than 1%. These seasonal seawater fish served as "sea water controls" in the determination of seasonal changes in gill ATPase and were the source of fish used in the investigation of the effects of ouabain on this enzyme system.

Length and weight records

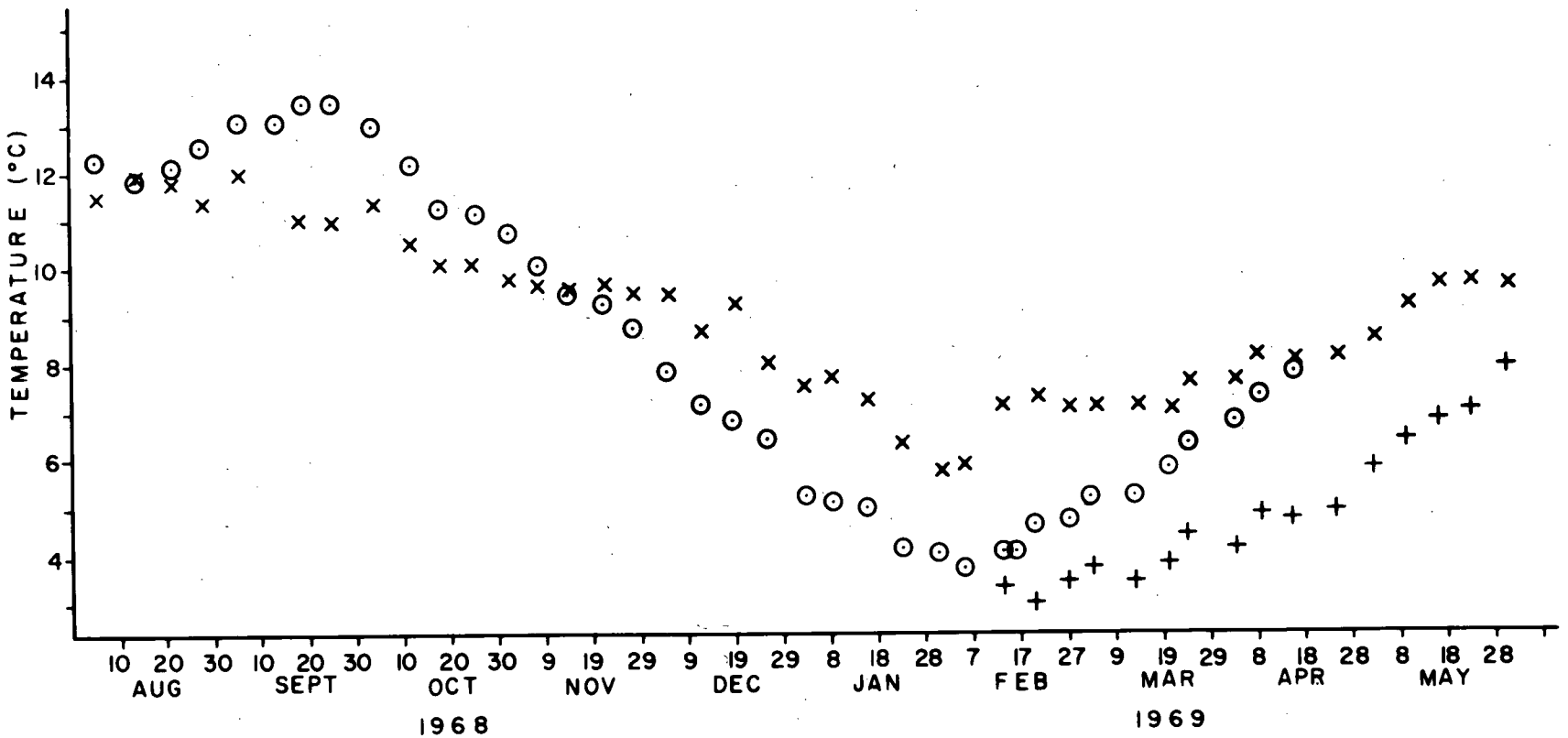
Length and weight data were obtained periodically on samples of seasonal freshwater and seasonal seawater fish. The fish were starved for 24 hours, anaesthetized lightly with 2-phenoxyethanol, measured individually for fork length and wet weight (Mettler P-120 torsion balance) and returned to their respective aquaria. In some instances fish for the enzyme assays were measured in this manner and then maintained in their respective aquaria, without food, for 24 hours before use. In other cases fish were starved for 24 hours and then measured immediately prior to use. These latter fish were immobilized by severing the spinal chord just posterior to the head region prior to measurement.

Water temperature and salinity records

Continuous temperature records of fresh and sea water were obtained with a recording thermograph (Taylor Instruments of

Legend for Figure 1.

Mean weekly water temperatures in degrees Centigrade of sea water, (X), municipal fresh water, (⊙), and Cypress Creek water,(+).


TEMPERATURE (°C)
14
12
10
8
6
4
10 20 30 10 20 30 10 20 30 9 19 29 9 19 29 8 18 28 7 17 27 9 19 29 8 18 28 8 18 28
AUG SEPT OCT NOV DEC JAN FEB MAR APR MAY
1968
1969

Canada Ltd.). The salinity of the sea water was measured daily with a hydrometer. The mean weekly temperatures of fresh and sea water are presented in Figure 1. The salinities averaged $30^{o}/_{oo}$, range $28\text{-}32^{o}/_{oo}$, throughout the experimental period.

Enzyme preparation

Fish were decapitated at the level of the pectoral fin insertion and the head pinned, ventral side up, to a disecting board. The heart was exposed and 2.0 ml of ice cold homogenizing solution (composition in mmoles/liter; sucrose, 250; imidazol, 2.0; disodium ethylenediaminetetraacetate, 2.0; pH 7.4), was perfused through the gills via the conus arteriosus. Each gill arch was then disected out and placed in cold homogenizing solution. The gills of two or more fish were pooled for each enzyme preparation. The gills were then dried lightly on cellulose wipes to remove any external blood clots and extraneous moisture, weighed to the nearest 0.01 g and placed in fresh homogenizing solution in the proportion of 1 g gill to 10 ml solution. The tissue was then homogenized in a grinding tube with a loose fitting, motor-driven, teflon pestle for 45 sec, in an ice bath. A few glass beads (0.01 to 0.02 mm in diameter) or glass wool were added to facilitate grinding of the cartilage. This crude homoginate was then centrifuged in a Servall refrigerated centrifuge, SS 1 rotor, at 1-4°C for 10 min at 1000 X g, and then 2000 X g for an additional 10 min. The supernatant from this procedure was centrifuged at 10,000 X g for 30 min and the resulting supernatant was decanted and centrifuged at 37,000 X g for 60 min. The supernatant of this

final centrifugation was discarded and the pellet resuspended in 4-5 ml of ice cold homogenizing solution. The particles of membrane fragments thus obtained were stored in capped tubes for 2-3 days at 0-2°C to facilitate dissociation of the membrane fragments. After storage a vortex mixer was used to uniformly suspend the fragments. With this technique the cell membrane fragments did not noticeably settle out after standing 7 days.

Enzyme assay

Magnesium-ATP and disodium-ATP, (yeast crystal) were obtained from Mann Research Laboratories, New York, N.Y., and ouabain from Nutritional Biochemicals Corp., Cleveland, Ohio. All other reagents were purchased from Fisher Scientific Inc. In preliminary experiments to determine optimum assay conditions the incubation media contained various concentrations of ATP, TRIS-HCl, NaCl and KCl. Magnesium concentration was always equimolar to ATP concentration and ouabain concentration was 4×10^{-4} moles/l (Table I). The composition of the incubation media used in the long term investigation of the changes in the ouabain-sensitive ATPase of coho gill tissue during their first year of fresh water residence and upon exposure to sea water are presented in Table II.

Both the enzyme suspension and substrates were preincubated at 40°C for 5 minutes before 0.2 ml of the enzyme was pipetted into 1.0 ml of the appropriate substrate. The reaction was allowed to proceed for 10 minutes in a constant temperature shaking water bath (Blue M Electric Co.) and then stopped by the addition of 0.5 ml of cold 17.5% trichloroacetic acid. Normally duplicate or triplicate assays

were performed.

The contents of each incubation tube were then assayed for inorganic phosphorus by the Fiske-SubbaRow method (Hawk, Oser, and Summerson, 1947), and the membrane nitrogen concentration of the enzyme suspension determined, in duplicate, by the microkjeldahl method (Association of Official Agricultural Chemists, 1960).

Methods for Section I

Optimal assay conditions

The optimal assay conditions of incubation time, pH, temperature, ATP concentration and sodium and potassium ion concentrations were determined on enzyme preparations from the gills of 20 month old post smolts which had been living in sea water for 3 months. Since these parameters were studied consecutively no single substrate composition could be provided. The actual substrate composition for each experiment is given in Table I. All data are presented as the specific activity in μmoles P_i liberated/hour/mgN, of the ouabain-sensitive ATPase and have been corrected to the standard assay conditions presented in Table II.

Effect of ouabain on the $(Na^+ + K^+)$-activated adenosinetriphosphatase

In April 1969, 16 seasonal seawater coho were used as a pooled source of gill tissue. Only one enzyme preparation was made and each value presented represents the mean of triplicate assays.

Legend for Table I.

Composition of the incubation media used in the determination of the optimal assay conditions for the ouabain sensitive hydrolysis of ATP.

Variable	Concentration (mM/l) Tris	Mg-ATP	Na^+	K^+	Ouabain	pH	Incubation Temperature (°C)	Incubation Time (minutes)	Number of Replicates
Linearity with Time	30.0	5.0	I00.0	20.0	± 0.4	7.4	40	var.	I
pH	30.0	5.0	I00.0	20.0	± 0.4	var.	40	I0	I
Incubation Temperature	30.0	5.0	I00.0	20.0	± 0.4	7.4	var.	I0	2
ATP Concentration	30.0	var.	I00.0	20.0	± 0.4	7.75	45	5	2
Sodium and Potassium Ion Concentration	30.0	5.0	var.	var.	± 0.4	7.75	45	I0	I

Methods for Section II

Changes in gill ($Na^+ + K^+$)-activated ATPase upon transfer to sea water

In February 1969, 240 freshwater adaptated fish (10.6-11.5 cm in length), were randomly divided into 4 groups and placed in oval aquaria, 48 X 110 cm containing 225 litres of water. These aquaria were provided with flowing Cypress Creek water but with no recirculating pumps. Two days later two of the groups were transferred directly to 100% sea water, salinity $30^o/_{oo}$, while the remaining groups served as freshwater controls. The gills of fish from both environments were assayed periodically for 50 days after transfer for ouabain-sensitive, ($Na^+ + K^+$)-activated, ATPase in the media outlined in Table II.

Seasonal changes in ($Na^+ + K^+$)-activated ATPase

The seasonal freshwater and seasonal seawater fish were sampled at intervals from October 15, 1968 to April 21, 1969 for analysis of the gill enzyme. Approximately equal sizes were taken from each stock to insure that the population densities remained equal. On April 21, 1969, 80 seasonal freshwater fish were divided equally between two oval aquaria which were provided with flowing fresh water. On April 22, the fish of one tank were adapted to sea water over an 18 hour period. The second aquaria was kept as a source of freshwater fish. Enzyme preparations from the gills of all these fish were assayed in the media presented in Table II.

Definitions of the enzyme components

The following definitions faciliated the presentation of

Legend for Table II.

Composition of the various incubation media used in the study of seasonal changes and changes upon exposure to sea water in the ouabain-sensitive ATPase of the gills of juvenile coho salmon. The values presented are final concentrations after the addition of 0.2 ml of enzyme suspension. The pH of the media was 7.4.

	Final Concentration (mmoles/l.)					
	Tris	ATP	Mg^{2+}	Na^{+}	K^{+}	Ouabain
Medium I	30.0	5.0	5.0	100.0	20.0	0.0
Medium 2	"	"	"	"	0.0	"
Medium 3	"	"	"	"	20.0	0.4

the results of Section II and the discussion of the data:

TS-ATPase activity is the total ouabain-sensitive, sodium and potassium ion activated, magnesium-dependent, adenosine triphosphatase activity and was calculated as the specific activity, expressed in μmoles inorganic phosphorus released from ATP per hour per milligram nitrogen, measured in medium No.1 minus the specific activity measured in medium No.3 of Table II.

Ouabain-sensitive sodium activation is the portion of the TS-ATPase activated by sodium ions in the absence of potassium ions and was calculated as the specific activity measured in medium No.2 minus the specific activity measured in medium No. 3 of Table II.

Ouabain-sensitive potassium activation is the additional phosphatase activity recorded when both sodium and potassium ions are present in the incubation medium over that recorded when only sodium ions are present and is calculated as the difference between TS-activity and sodium activation.

RESULTS

General

Growth in length and weight

The patterns of growth in length and weight with time for sea water and fresh water reared fish are presented in Figure 2. In both groups the rates of growth declined during the period of December 1, 1968 to mid-January 1969 which corresponded to the latter part of the period of declining water temperatures as shown in Figure 1. Growth was accelerated from mid-January to April 1969 as water temperature began to increase.

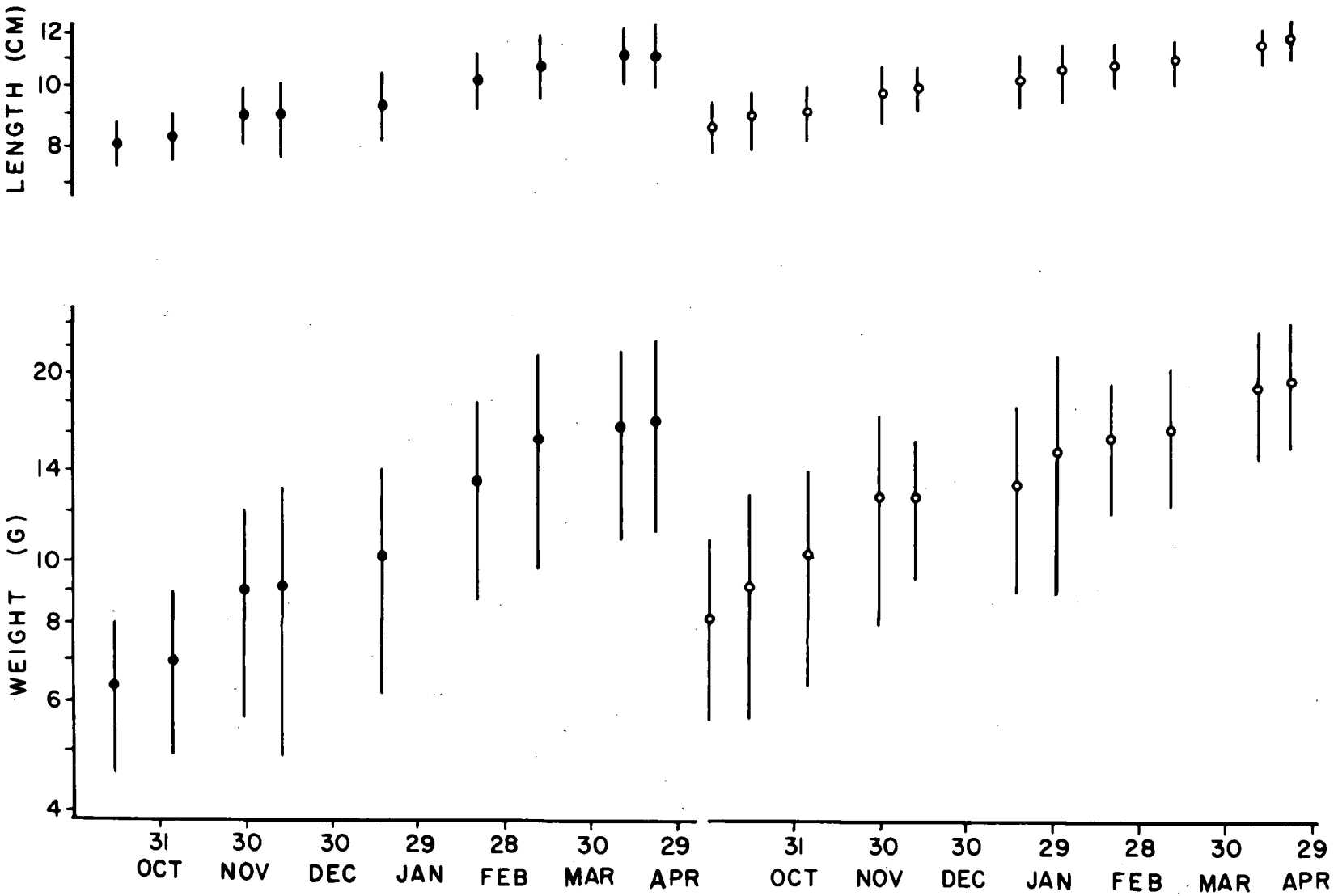
Weight-length relationship

The parameter "a" and "b" of the weight-length formula $W = aL^b$ for sea water and fresh water reared fish are presented in Table III. Although the "a" parameters varied widely in both groups the "b" parameter tended to cluster around a value of 3.20.

The plot of mean weights against mean lengths for sea water and fresh water reared coho at each sampling time together with a short section of least square fit relationship of each sample is presented in Figure 3. The broken lines are included for reference purposes and have a slope of 3.2. In both groups a gradual decrease in the "a" intercept with increasing size was observed while the slope "b" tended to remain at 3.20. The transition from the upper broken line to the lower broken line for fresh water reared coho resulting in a more streamlined fish occurred during the period November 29, 1968 to January 16, 1969 when the fish were experiencing

Legend for Figure 2.

Plot of mean weight and mean fork length of individual samples of juvenile coho from October 1, 1968 to April 21, 1969. Solid circles refer to sea water reared fish and open circles to fresh water reared fish.


LENGTH (CM)
12
10
8
WEIGHT (G)
20
14
10
8
6
4
31 OCT
30 NOV
30 DEC
29 JAN
28 FEB
30 MAR
29 APR
31 OCT
30 NOV
30 DEC
29 JAN
28 FEB
30 MAR
29 APR

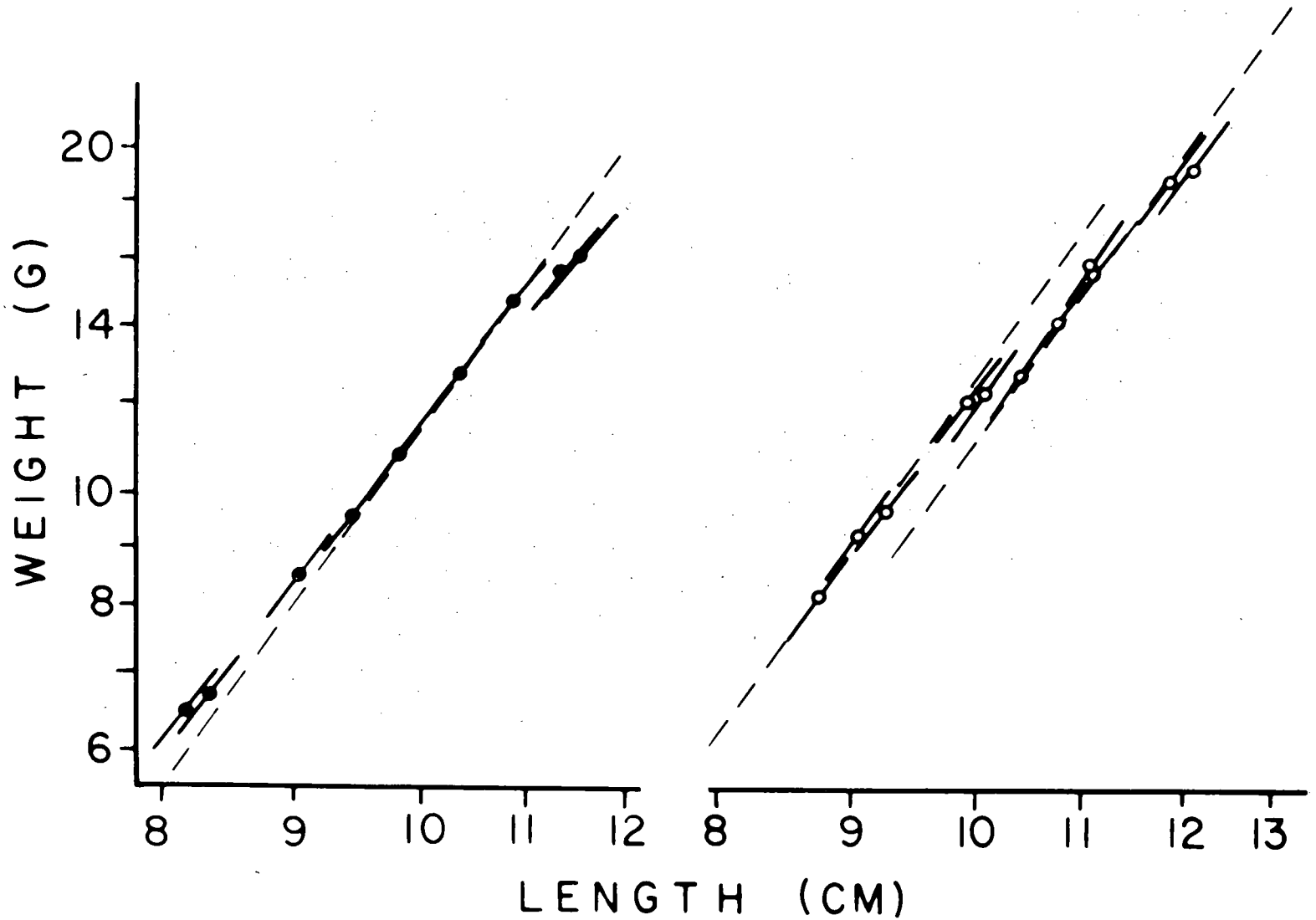
Legend for Table III.

Parameters and type 2 errors of the least-square fit relationships of fish weight on fork length of sea water and fresh water reared juvenile coho salmon (log weight, g = log a + b log length, cm).

Date	Sample size (n)	Least-square fit of weight-length ($W = aL^b$)			
		a (mg)	Error	b	Error
Sea water reared					
Oct. 15, 1968	24	12.0	2.4	2.99	0.09
Nov. 4, "	52	7.3	1.2	3.22	0.08
Nov. 29, "	65	7.2	0.8	3.23	0.05
Dec. 12, "	77	6.6	0.7	3.27	0.04
Jan. 17, 1969	66	9.0	1.1	3.12	0.05
Feb. 18, "	67	6.9	0.9	3.22	0.06
Mar. 11, "	53	5.7	1.0	3.31	0.07
Apr. 9, "	84	7.1	1.0	3.17	0.06
Apr. 21, "	106	13.3	2.5	2.92	0.07
Fresh water reared					
Oct. 1, 1968	23	9.8	3.3	3.09	0.15
Oct. 15, "	24	5.8	1.0	3.34	0.08
Nov. 4, "	52	6.3	1.0	3.29	0.07
Nov. 29, "	70	8.4	1.1	3.16	0.05
Dec. 12, "	45	9.7	2.3	3.10	0.10
Jan. 16, 1969	58	5.2	0.9	3.34	0.07
Jan. 31, "	66	5.4	0.6	3.32	0.04
Feb. 18, "	67	8.5	1.9	3.13	0.09
Mar. 11, "	52	7.3	2.0	3.20	0.11
Apr. 10, "	71	8.3	1.6	3.13	0.08
Apr. 21, "	92	4.6	0.8	3.35	0.06

Legend for Figure 3.

Relationship between mean weight and mean fork length of samples taken from October 1, 1968 to April 21, 1969. Solid circles refer to sea water reared coho and open circles to fresh water reared fish. The short solid lines are the slopes of the individual weight/length relationship of each sample. The broken lines are included for reference purposes and have a slope of 3.20.


WEIGHT (G)
20
14
10
8
6
8
9
10
11
12
8
9
10
11
12
13
LENGTH (CM)

a period of decreased rate of growth as demonstrated in Figure 2.. The length-weight relationship of sea water reared coho showed changes similar in form and time but smaller in magnitude in comparison to fresh water reared fish.

Section I

Optimal assay conditions

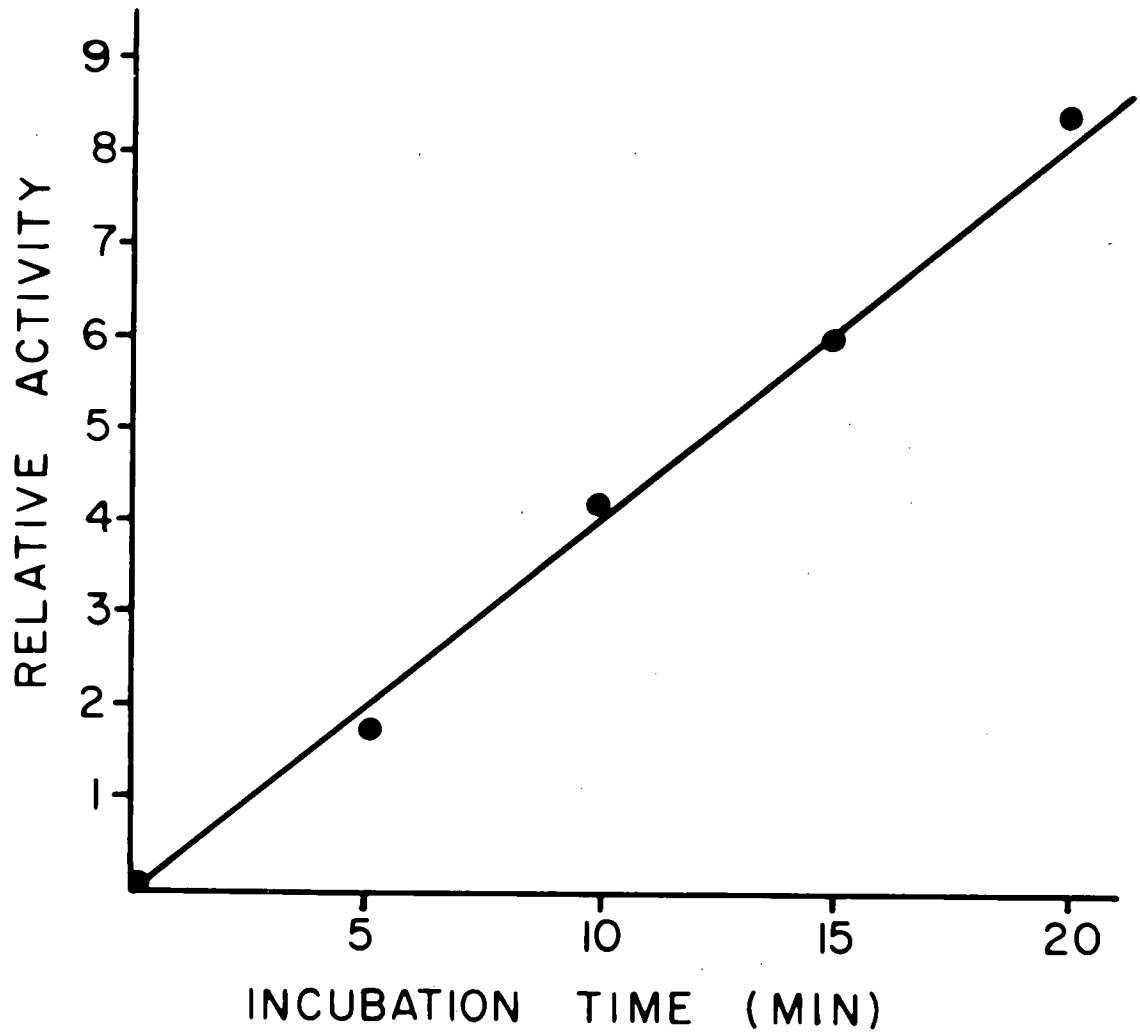
The ouabain-sensitive and potassium-stimulated hydrolysis of ATP as a function of incubation time was found to be linear from 0 to 20 minutes as demonstrated in Figure 4. The effects of pH and incubation temperatures on this enzyme are shown in Figure 5. The optimum pH was found to be 7.4 and enzyme activity declined sharply on either side of this value especially on the more alkaline side where activity at pH 8.0 was only 50% of that recorded at pH 7.4. The optimum incubation temperature was 40°C and a rapid decline in activity at higher temperatures was observed.

The effect of increasing Mg-ATP concentration from 0.02 to 8.0 mmoles/l was an increase in enzyme activity to a maximum at 0.5 - 1.0 mmoles/l and a slight decline in activity at higher concentrations. The apparent K_m for ATP of the ouabain-sensitive ATPase was 0.2 mmoles/l. The specific activity a V_{max} was 260. This data is presented in Figure 6.

Figure 7 demonstrates the effect of increasing potassium ion concentration at three concentrations of sodium ions. The TS-enzyme activity increased with increasing potassium ion concentration to a maximum activity which was dependent upon

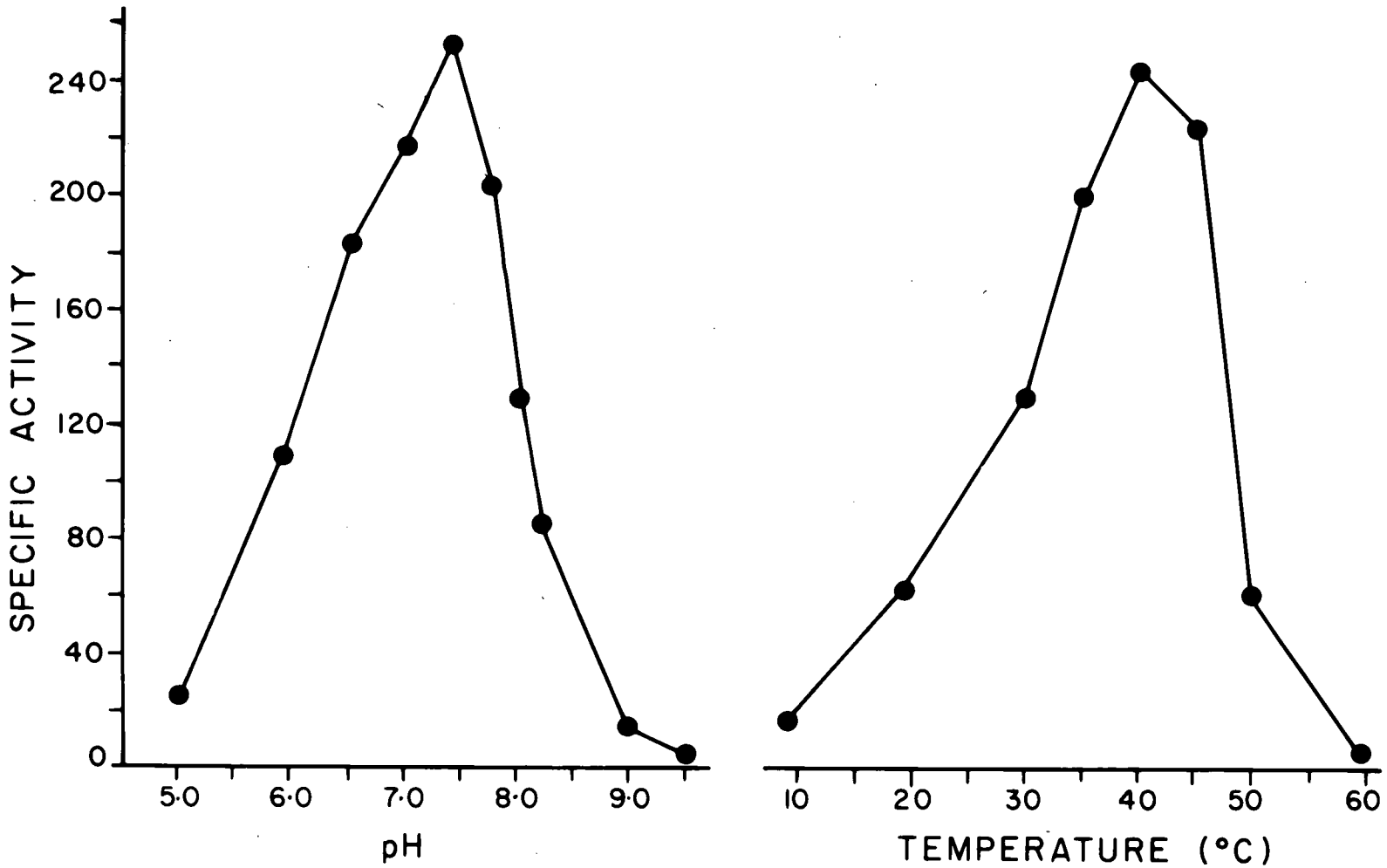
Legend for Figure 4.

Plot of ouabain-sensitive hydrolysis of ATP against incubation time for the assay conditions presented in Table I. The data are presented as relative activities with no units. Membrane nitrogen concentration was 0.0588 mg/ml.


RELATIVE ACTIVITY
9
8
7
6
5
4
3
2
1
5
10
15
20
INCUBATION TIME (MIN)

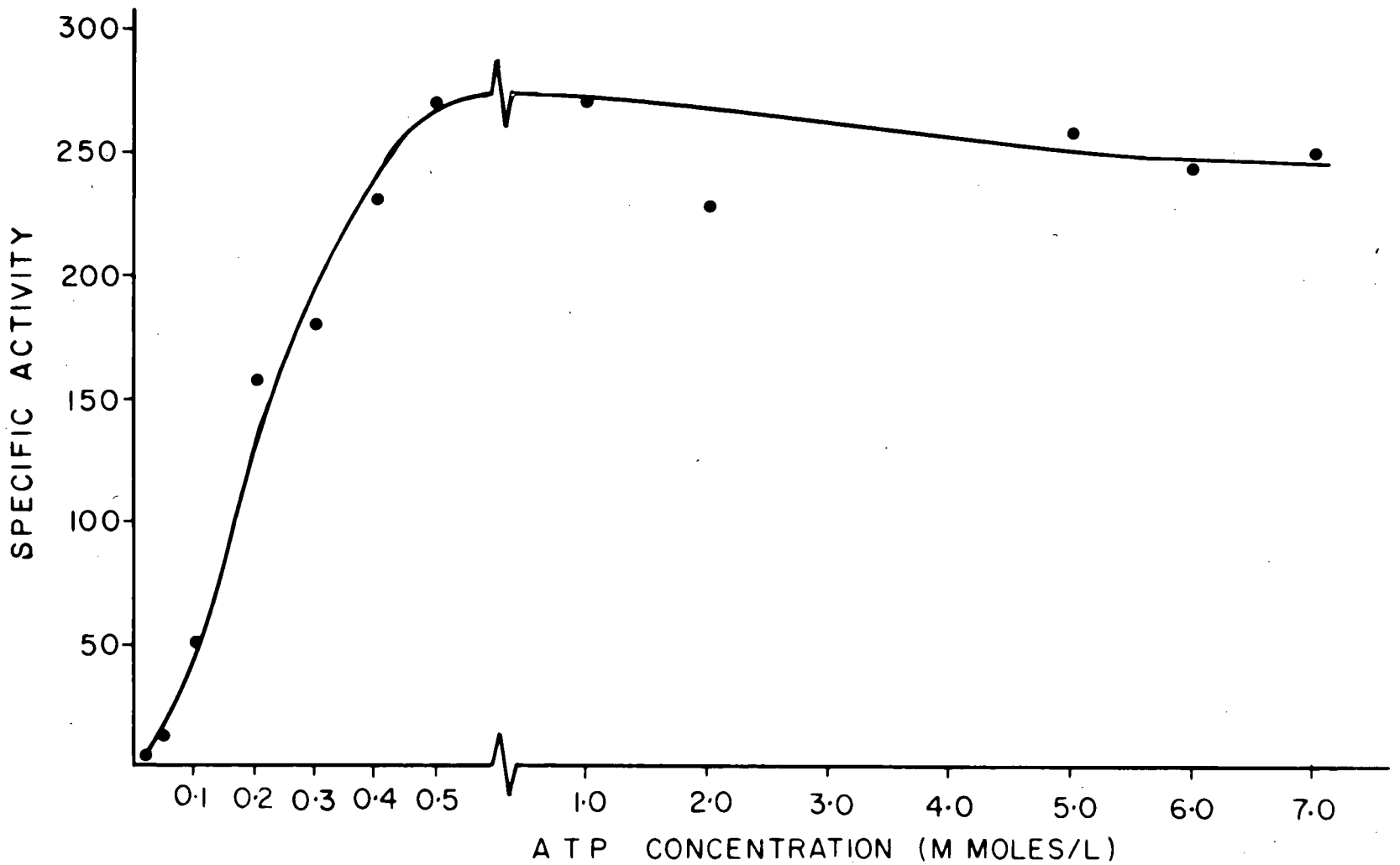
Legend for Figure 5.

The effect of pH (left scale), and incubation temperature (right scale), upon the ouabain-sensitive hydrolysis of ATP. The assay conditions are presented in Table I, and all data have been corrected to the standard assay conditions presented in Table II. The membrane nitrogen concentration for both parts of Figure 5 was 0.0719 mg/ml.


SPECIFIC ACTIVITY
240
200
160
120
80
40
0
5·0
6·0
7·0
8·0
9·0
pH
10
20
30
40
50
60
TEMPERATURE (°C)

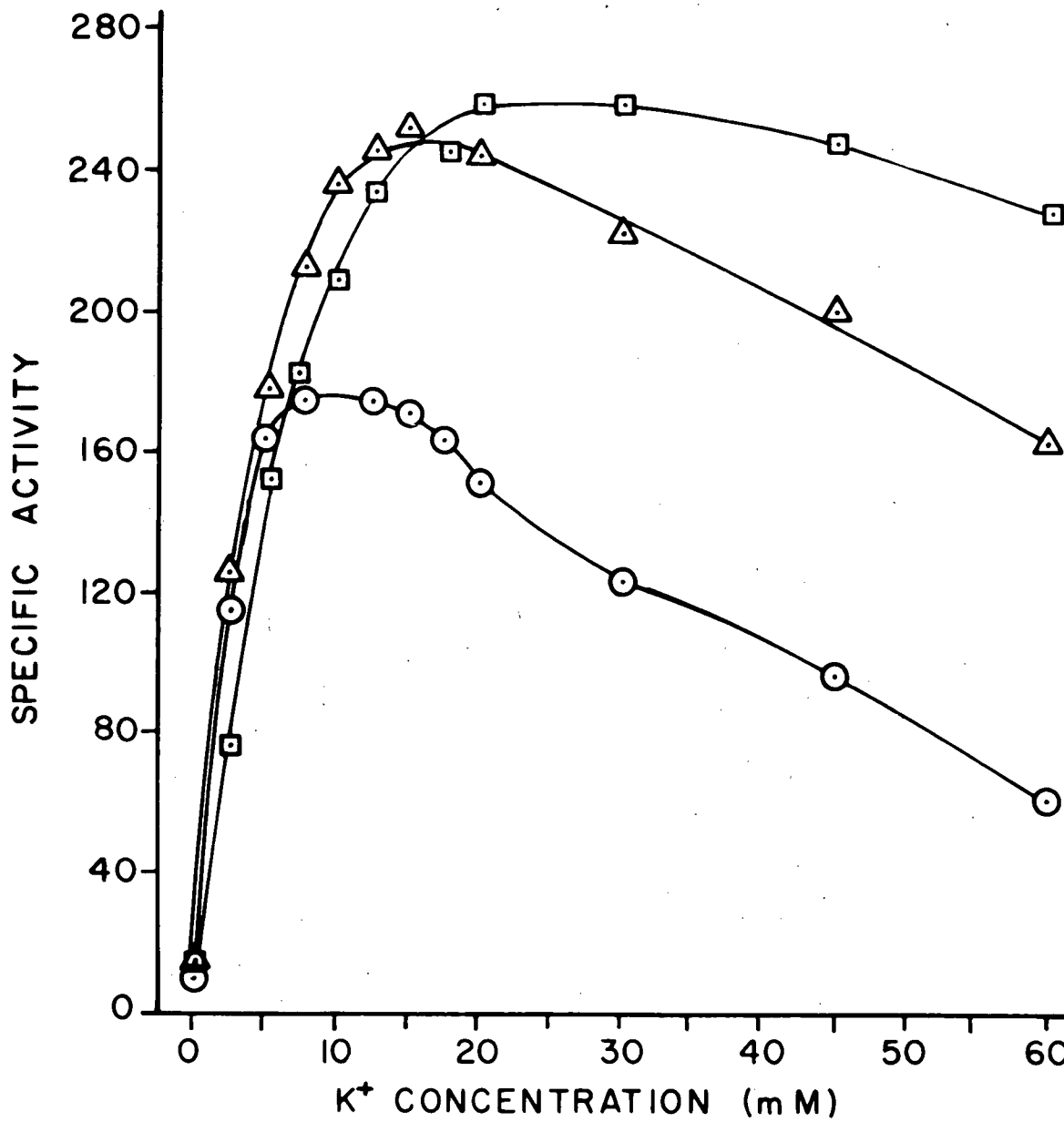
Legend for Figure 6.

The effect of ATP concentration on the ouabain-sensitive hydrolysis of ATP. The assay conditions used are presented in Table I and the data corrected to the standard assay conditions presented in Table II. The membrane nitrogen concentration was 0.1607 mg/ml.


SPECIFIC ACTIVITY
300
250
200
150
100
50
0·1
0·2
0·3
0·4
0·5
1·0
2·0
3·0
4·0
5·0
6·0
7·0
A T P CONCENTRATION (M MOLES/L)

Legend for Figure 7.

The effect of potassium ion concentration on the ouabain-sensitive hydrolysis of ATP at three concentrations of sodium ions. Assay conditions used are presented in Table I and the data have been corrected to the standard assay conditions presented in Table II. The open circles, triangles, and squares, refer to sodium ion concentrations of 10.0, 50.0, and 100.0 mmoles/l, respectively. The membrane nitrogen concentration was 0.1008 mg/ml.


280
240
200
160
120
80
40
0
SPECIFIC ACTIVITY
0
10
20
30
40
50
60
K+ CONCENTRATION (m M)

sodium ion concentration, and then decreased at higher potassium ion concentrations. Higher sodium ion concentrations mitigated, somewhat, the inhibitory effect of high potassium ion concentrations as enzyme inhibition by potassium ions at sodium ion concentrations of 10.0, 50.0, and 100.0 mmoles/l first occurred at potassium ion concentrations greater than 12.5, 15.0 and 20.0 mmoles/l respectively. The latter values corresponded to the ion concentrations at which maximal hydrolysis of ATP was observed. Half maximal activity for sodium ion concentrations of 10.0, 50.0 and 100.0 mm/l occurred at potassium ion concentrations of 2.0, 2.5 and 5.0 mm/l, respectively. Sodium ions in the absence of potassium ions also had a slight but measurable stimulating effect on the ouabain-sensitive ATPase.

Effect of ouabain on Mg^{2+}-ATPase and (Mg^{2+} + Na^{+} + K^{+})-stimulated ATPase

The results of the experiment to determine the effect of various concentrations of ouabain on the phosphatases activated by magnesium ions alone and those activated by sodium and potassium ions in the presence of magnesium ions are presented in Table IV. The incubation media consisted of MgATP, 5.0 mm/l; tris 30.0 mmoles/l; and various combinations of NaCl, KCl and ouabain as indicated in the table. Each value presented represents the mean of three identical assays of the same enzyme preparation which had a nitrogen concentration of 0.0593 mg/ml.

The data in the first row of Table II indicate that ouabain in concentrations of 10^{-9} to 10^{-3} moles/l had no effect

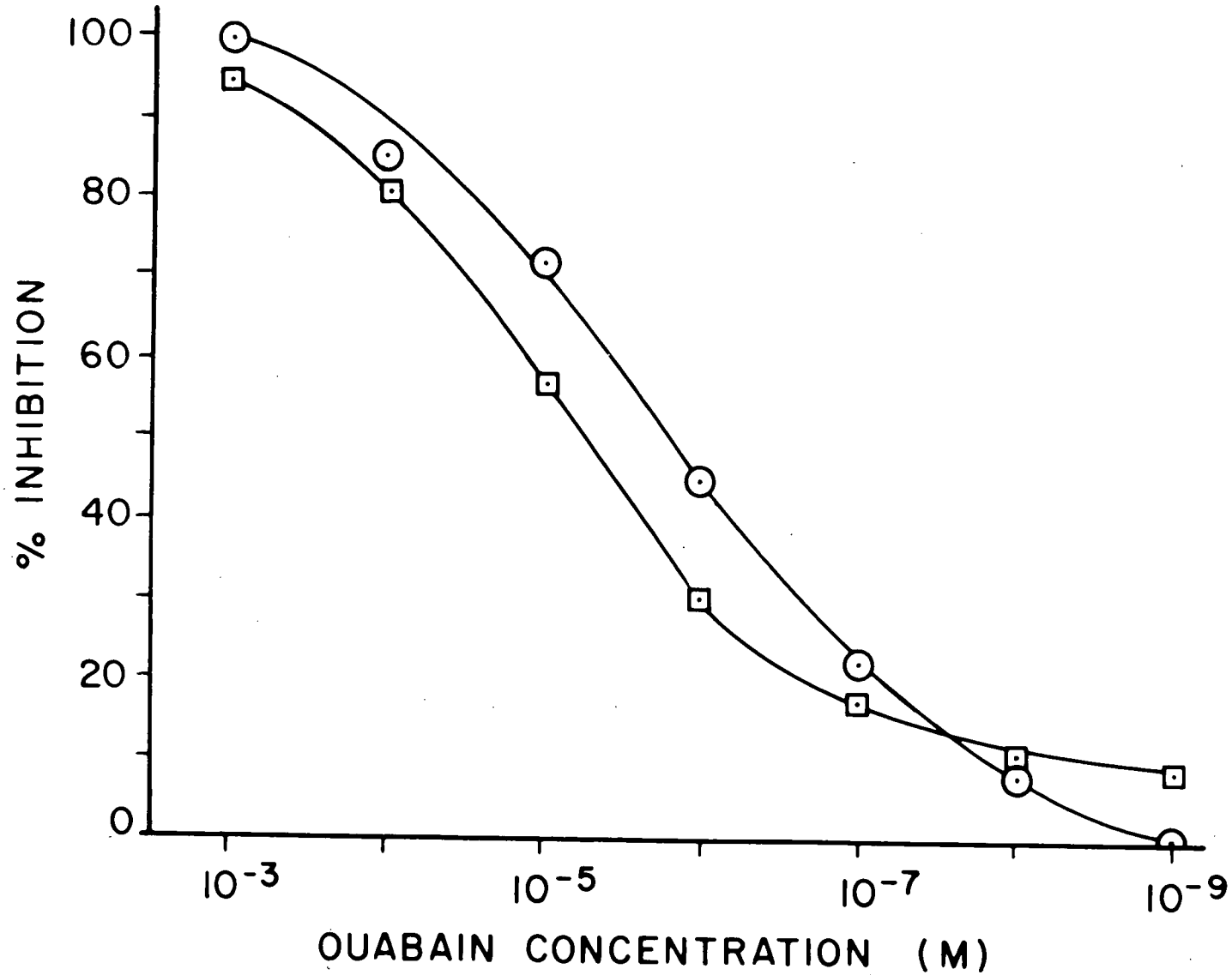
Legend for Table IV

The effect of various concentrations of ouabain on ATPases activated by magnesium ions and various concentrations of sodium and potassium ions. All data are expressed as specific activities in micromoles P_i/Hr./mg nitrogen. The percent inhibition by ouabain was calculated as follows: the $(Na^+ + K^+)$-activated ATPase was considered to be 100% inhibited when incubated in a medium containing 5.0 mm Mg , 100 mm NaCl, and 10^{-3}M ouabain. The activity recorded with each concentration of KCl with no ouabain was considered to be 100% activity for the enzyme under those conditions and all intermediate values are expressed as a percentage of 100% activity.

	Cation Concentration (mmoles/l.)			Ouabain Concentration (moles/l.)							
	Mg^{2+}	Na^{+}	K^{+}	0	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Specific Activity	5.0	0.0	0.0	126.5	128.3	--	128.3	--	135.4	--	130.0
Specific Activity	5.0	100.0	0.0	112.2	106.9	110.4	110.4	108.7	103.3	105.1	99.8
Specific Activity	5.0	100.0	10.0	169.2	172.8	165.7	155.0	138.9	119.3	110.4	99.8
Percent Inhibition by Ouabain	5.0	100.0	10.0	0	0	7.5	22.5	45.0	72.5	85.0	100.0
Specific Activity	5.0	100.0	20.0	199.5	190.6	188.8	181.7	169.2	142.5	119.3	105.1
Percent Inhibition by Ouabain	5.0	100.0	20.0	0	8.9	10.7	17.9	30.4	57.1	80.4	94.6

Legend for Figure 8.

Relationship between ouabain concentration and percentage inhibition of the ouabain-sensitive hydrolysis of ATP in media containing Tris, 30.0; Mg-ATP, 5.0; NaCl, 100.0; and KCl, 10.0, (open circles), or 20.0 mmoles/l, (squares), at a pH of 7.4 and incubation temperature of 40° Centigrade. Membrane nitrogen concentration was 0.0593 mg/ml.


100
80
60
40
20
0
% INHIBITION
10^{-3}
10^{-5}
10^{-7}
10^{-9}
OUABAIN CONCENTRATION (M)

upon the phosphatases requiring magnesium ions alone, (Mg^{2+}-ATPase). Sodium chloride (100 mmoles/l) appeared to inhibit the Mg^{2+}-ATPase by 13.6%. With sodium chloride, (100 mmoles/l), and ouabain (10^{-3}moles/l) an additional 9.6% decrease in activity from that recorded with magnesium ions alone was observed. Potassium ions, when present together with magnesium and sodium ions exerted a stimulating effect on phosphatase activity much greater than that observed with the latter two cations alone. This activation by potassium ions was inhibited by increasing concentrations of ouabain and the inhibition by any concentration of ouabain, at constant concentrations of magnesium and sodium ions, decreased as potassium ion concentration increased. Since the media containing magnesium ions, 5.0 mmoles/l, sodium ions, 100 mmoles/l; potassium ions, 10mmoles/l; and ouabain, 10^{-3}moles/l resulted in the same hydrolysis of ATP as the same media with no potassium ions, 99.8 μmoles P_i per hour per mg N, it was considered that this value represented complete inhibition of the sodium and potassium-activated ATPase. Figure 8 presents graphically the ouabain inhibition data of Table IV for potassium ion concentration of 10.0 and 20.0 mmoles/l calculated with this assumption. The K_i for ouabain was 2×10^{-6} and 7×10^{-6} moles/l for potassium ion concentrations of 10.0 and 20.0 mmoles/l respectively.

Section II

Changes in ouabain-sensitive ATPase upon transfer to sea water

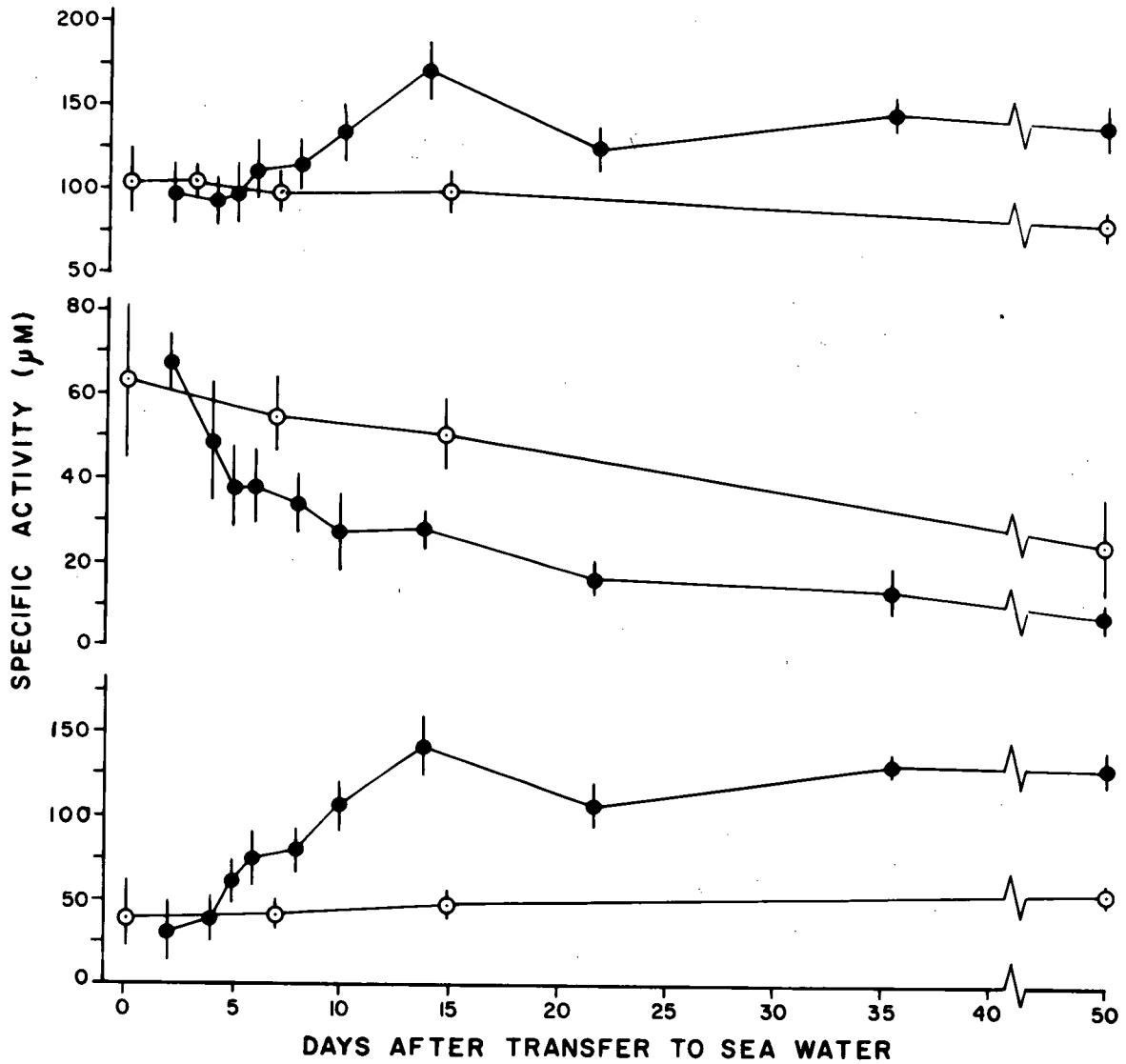
The changes in TS-ATPase, sodium activation and potassium activation with time of exposure of the fish transferred directly from fresh water to 100% sea water, salinity $30^{o}/_{oo}$, in February, 1969 are presented in Figure 9. Each point between 0 and 22 days exposure represents the mean, $\pm$ 1 standard deviation, of 3 to 4 replicate preparations. Figure 9 demonstrates that although no significant changes in TS-activity occurred until 10 days after transfer, significant changes in sodium activation and potassium activation occurred after 5 days exposure to sea water. Sodium activation declined steadily at a rapid rate from day 2 to day 6 and continued declining at a slower rate thereafter to a value of 7 µmoles P_i/hr/mg N at day 50.

Potassium activation increased steadily after 4 days exposure to a maximum of 142 µmoles P_i/hr/mg N at day 14, then declined slightly and levelled off at approximately 130 µmoles P_i/hr/mg N. The changes in TS-activity parallelled changes in potassium activation after 8 - 10 days exposure as the latter comprised 80 - 90% of the total ouabain-sensitive activity. The times in sea water required for the initial 50% of the changes in potassium and sodium activations to occur were 9 and 5 days respectively.

Although changes in the sodium activation and potassium activation of the fresh water controls were observed over the the experimental period these changes were much less than those observed in sea water exposed animals and may have represented normal changes observed during the smolting period (see Figure

Legend for Figure 9.

Changes in TS-activity, (upper scale), sodium activation, (middle scale), and potassium activation, (lower scale), with time of exposure to sea water. The mean $\pm$ 1 standard deviation is presented for sea water exposed fish, (solid circles), and controls maintained in fresh water, (open circles).


SPECIFIC ACTIVITY (μM)
200
150
100
50
80
60
40
20
0
150
100
50
0
0
5
10
15
20
25
30
35
40
50
DAYS AFTER TRANSFER TO SEA WATER

10). No difference in the degree of silvering of the skin or loss of parr marks was observed between fish exposed to sea water and the fresh water controls.

Seasonal changes in ouabain-sensitive ATPase of juvenile coho gill

The changes in the TS-activity, sodium activation, and potassium activation of the ouabain-sensitive ATPase during the fry, presmolt and smolt stages of the life cycle are presented in Figure 10. Although these life stages are not well defined and tend to overlap in time within a population of fish (Houston 1963), the following general descriptions may be valid for the juvenile coho salmon used in these experiments:

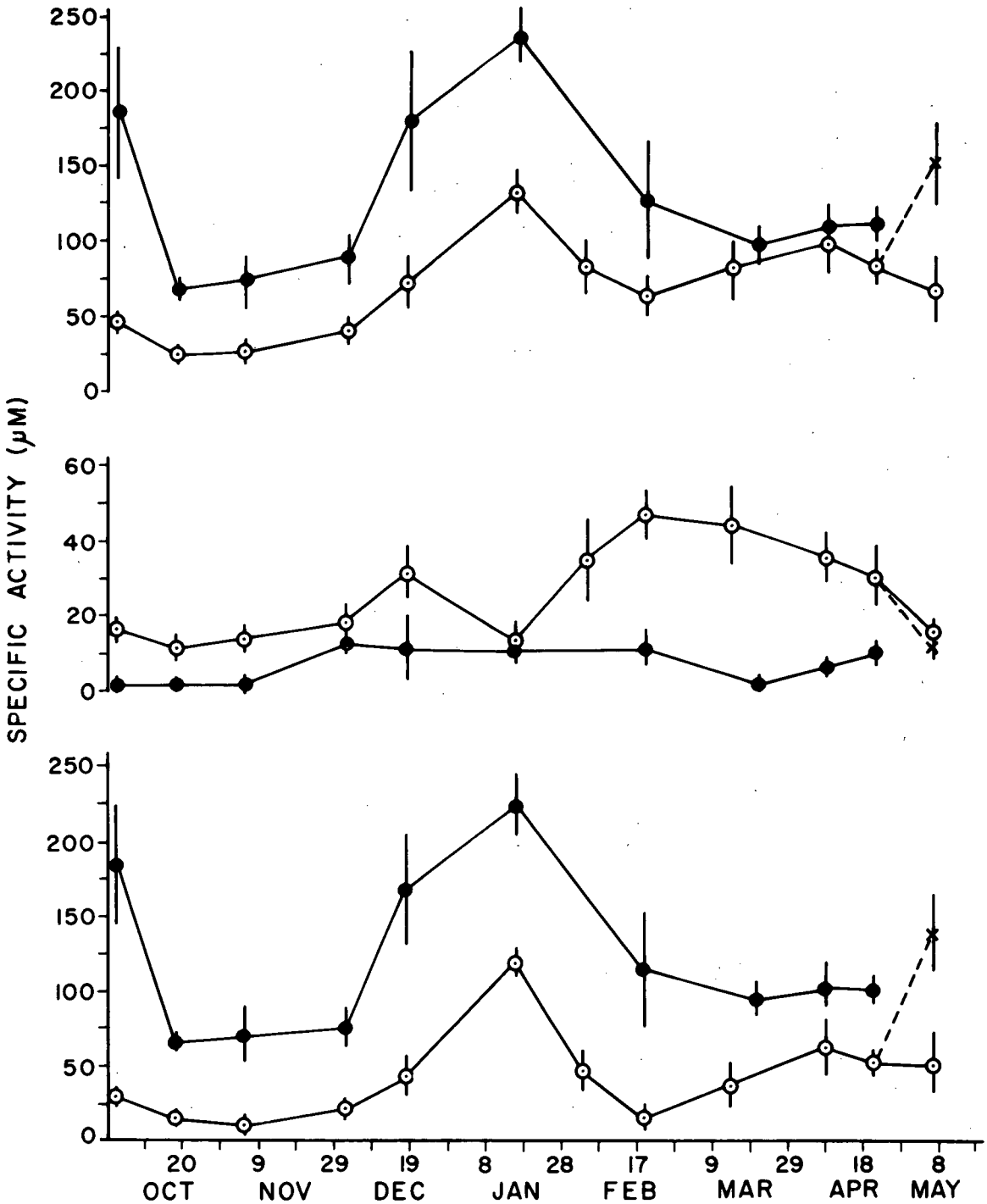
Fry stage: parr marks dark and sharply delineated; no noticeable darkening of pelvic, anal, dorsal or caudal fins; white coloration on the first few rays of the anal fin; weight-length relationship approximated the upper broken line of Figure 3; was included in the period ending in late November, 1968.

Pre-smolt stage: parr marks dark but borders less distinct, slight darkening of the tips of the pelvic, anal, dorsal and caudal fins; noticeable silvering of the ventral and ventro-lateral skin; weight-length relationship in the transition phase between the two broken lines of Figure 3; and occurred in the period of mid-December, 1968 to late January, 1969.

Smolt stage: parr marks visible only in oblique light, intense darkening of the extremities of the pelvic, anal, dorsal and caudal fins; intense silvering of the ventral and

Legend for Figure 10.

Changes in TS-activity, (upper scale), sodium activation, (middle scale), and potassium activation, (lower scale), from October 1, 1968 to May 8, 1969. The mean $\pm$ 1 standard deviation is presented for sea water reared fish, (solid circles), and fresh water reared fish, (open circles). The broken line from April 21 to May 6 represents fish transferred from fresh to sea water on April 21.


SPECIFIC ACTIVITY (μM)
250
200
150
100
50
0
60
40
20
0
250
200
150
100
50
0
20 OCT
9 NOV
29
19 DEC
8 JAN
28
17 FEB
9 MAR
29
18 APR
8 MAY

ventro-lateral skin; weight-length relationship approximates the lower broken line of Figure 3; and occurred in the period of mid-February to mid-April, 1969.

Sea water reared fish also exhibited the characteristics of these stages but differentiation was less distinct than in fresh water reared coho. The data for sea water reared coho are included in Figure 10 as a reference to indicate whether or not the changes observed are the result of physiological changes occurring independently of the salinity of the aquatic environment.

The fry stage, ending in late November 1968, was characterized by relatively low, uniform levels of TS-activity, and potassium activation in both groups of fish although the values were 3 to 7 times higher in the sea water reared fish. The high values of these activities observed in sea water reared coho on October 3, 1968 may have been the result of adaptation of these fish to sea water as preliminary sea water adaptation experiments performed in this period showed a large initial increase and subsequent decline in TS-activity, (data not presented). Sodium activation was also low in this period but was always higher in fresh water reared coho than in sea water reared fish.

The first portion of the pre-smolt period (early December 1968, to mid-January 1969) was characterized by 3-fold increase in both TS-activity and potassium activation for sea water reared fish and 3.7 and 4.6 fold increases respectively for fresh water reared fish. These activities then declined

from mid-January to mid-February in both groups of fish. Initially sodium activation changed erratically in fresh water reared coho but reached a low in mid-January then increased sharply until mid-February. In sea water reared coho the sodium activation was relatively constant during the pre-smolt stage.

In the smolt stage, mid-February to April 1969, the levels of TS-activity, sodium activation and potassium activation in sea water reared fish did not change significantly from the values observed at the end of the pre-smolt stage. The fresh water reared coho, however, demonstrated marked decreases in sodium activation and increases in TS-activity and potassium activation until the first week in April. Thus during this period the characteristics of the ouabain-sensitive ATPase of these fish tended to approach those of coho residing in sea water. Freshwater fish transferred to sea water on April 18 (broken line of Figure 10) showed the same increases in TS-activity and potassium activation as seen in Figure 9 while the former activity continued to decline in fish held in fresh water. This decline in TS-activity in the freshwater fish during the period of April 8 to May 6 was the result of decreasing sodium activation which declined to a value near that observed in fish during the fry stage.

DISCUSSION

Section I

As Skou (1965), points out, the assay conditions of pH, temperature, ATP concentration and sodium and potassium concentrations which result in maximal hydrolysis of ATP by the ouabain-sensitive, ($Na^+ + K^+$)-activated ATPase vary widely in enzyme preparations from different tissue; moreover the pattern of inhibition of this enzyme by ouabain is also different in different preparations. Since the determination of optimal assay conditions in these experiments employed sea water adapted fish as an enzyme source, a question could be raised as to whether these assay conditions were optimal for enzyme preparations from fresh water fish. It would appear, however, that since the TS-activities of enzyme from fish adapted to both environments were very similar during the period of March and April, 1969 (Figure 10) the optimal assay conditions may be similar for enzymes from both sources. Only a detailed analysis of the enzyme kinetics at closely spaced intervals during fresh water development would provide satisfactory answers to these problems.

The optimal pH of 7.4 for the coho gill ouabain-sensitive ATPase was similar to that of the Japanese eel, reported as 7.5 by Kamiya and Utida (1968), although it is slightly different from that used by other workers, (Skou, 1957, Epstein et al. 1967). Although the incubation temperature of 40°C has no physiological significance in fish it was the temperature at which maximum ATP hydrolysis occurred.

The Km of the enzyme for the ATP substrate, 0.2 mmoles/l is very similar to that recorded by Skou (1960), for the crab nerve ($Na^+ + K^+$)-stimulated ATPase although the present experiments employed equimolar magnesium and ATP concentrations while Skou varied the magnesium/ATP ratio. Skou (1957) demonstrated, however, that maximal ATP hydrolysis occurred when the magnesium and ATP concentrations were equal. The V_{max} occurred at an ATP concentration of 0.5 mmoles/l which is in agreement with the value of 0.75 mmoles/l reported for nasal gland homogenates of fresh water and saline adapted ducks (Fletcher et al, 1967).

In experiments utilizing the gills of sea water adapted Japanese eels as a source of enzyme Kamiya and Utida (1967), determined the concentrations of potassium and sodium ions which when present with magnesium ions and ATP gave maximal hydrolysis of the ATP substrate. These authors found that maximal hydrolysis occurred at potassium ion concentrations of 15, 20 and 40 mmoles/l when the sodium concentrations were 10, 50 and 100 mmoles/l, respectively. In sea water adapted coho, however, maximal ouabain-sensitive ATP hydrolysis occurred at potassium ion concentrations of 12.5, 15.0 and 20.0 mmoles/l respectively for these sodium concentrations. These data indicate that the ouabain-sensitive ATPase of coho gills may have a higher affinity for potassium ions than does this enzyme in Japanese eels. This suggestion is supported by the fact that for cation concentrations of 100 mmoles Na and 20 mmoles K per litre and ouabain concentrations of 10^{-6}, 5×10^{-6}, and 10^{-5} moles/l respective inhibitions of 15, 30, and 55% of

maximal ouabain-sensitive activity were recorded in Japanese eels (ibid) compared to 30, 44 and 56% respectively in coho salmon (Figure 8). Thus, the sea water adapted coho gill enzyme not only has a higher affinity for potassium ions but this activation by potassium ions is more sensitive to low concentrations of ouabain (Table IV) than this enzyme in the gills of sea water adapted Japanese eels.

The Mg^{2+}-ATPase was not affected by any concentration of ouabain. This finding is in agreement with the results on other tissues (Bonting and Caravaggio 1963, Kamiya and Utida, 1968, Post et al 1960 and Skou 1960). The cause of the apparent inhibition by sodium ions of a fraction of the Mg^{2+}-ATPase (Table IV) is unknown. Since the enzyme preparation used in the present investigations was relatively impure it can be speculated that sodium ions may act as a control mechanism which deactivates certain Mg^{2+}-ATPases in order to insure adequate ATP substrate is available for the $(Na^+ + K^+)$-activated enzyme. Another possible explanation suggested by Skou (1965) is that the Mg^{2+}-ATPase and the $(Na^+ + K^+)$-activated ATPase may be part of the same enzyme system; the activity of this changes in different ionic environments of with different methods of purification.

In general then the characteristics of the partially purified ouabain-sensitive $(Na^+ + K^+)$-activated adenosine triphosphatase prepared from the gills of sea water adapted coho are similar if not identical to those of other tissues and animals.

Section II

Relatively little research into the effect of transfer from a hypotonic to a hypertonic environment on the $(Na^+ + K^+)$-activated ATPase of fish gills has been performed. Epstein et al (1967) found that a 7-fold increase in the activity of gill microsomal preparations of this enzyme occurred when young killifish Fundulus heteroclitus were adapted to sea water as compared to fresh water controls. Kamiya and Utida (1968) reported 5-fold increases in activity of NaI treated microsomal preparations when Japanese eels Anguilla japonica were transferred from fresh to sea water. The latter authors also found that a rapid increase in the activity of this enzyme occurred in the first seven days of sea water exposure followed by a more gradual increase to the thirtieth day of exposure. In an analogous investigation, Fletcher et al, (1967), using inhibition by 0.1 millimolar ouabain to measure the $(Na^+ + K^+)$-activated ATPase in crude homogenates of duck nasal glands, found that the total ouabain-sensitive activity reached a maximum 8 to 10 days after a saline solution (284 mmoles/l NaCl) had been substituted for fresh water as the sole source of drinking water and that changes in the excretion of sodium and chloride by this gland were correlated to increases in the enzyme activity. The changes in TS-activity for coho transferred directly from fresh to sea water (Figure 9) are generally in agreement with these results from other fish gills and duck nasal glands. The changes in sodium activation and in potassium activation presented in Figure 9, however, have not

been presented elsewhere. If the hypothesis that intracellular sodium is exchanged for extracellular potassium (Skou 1957, 1960, 1965, Bonting and Caravaggio 1963, Post et al. 1960), and that the effect of potassium ions is to activate this transfer of sodium (Willis 1968 a, b,), is accepted, then a measure of the activating effect of potassium ions would describe the potential for activity of the enzyme system. This proposition is supported by the observation that sea water adapted coho which have an absolute requirement to excrete sodium ions and retain water have a high potassium activation which exceeds 90% of the TS/activity. Fresh water adapted coho without this requirement have a low potassium activation which may be as low as 22% of the TS activity, (Figure 10, February 19, 1969). When coho are transferred from fresh to sea water the initial decrease in sodium activation and concurrent rise in potassium activation would permit the enzyme system to more effectively remove internal sodium ions to the external medium long before the actual concentration of enzyme showed any increase. In this respect it has been found (Conte and Lin, 1967) that the turnover time of 50% of labelled DNA, which is a measure of the rate of de novo synthesis of DNA, was 15.8 and 5.8 days for the gill cells of fresh and sea water adapted juvenile coho respectively. In addition some salt-inducible changes in antigenicity have been found between gill microsomes of fresh and sea water adapted chinook salmon which may reflect changes in the $(Na^+ + K^+)$-activated ATPase (Conte and Morita, 1968). It is important to emphasize that in fish many passive changes in the distri-

bution of chloride, extracellular phase volume and permeability of the body surface to ions, which tend to decrease the rate of change in the ion concentrations of the body fluids during exposure to a hypertonic environment but that these changes can only function to allow regulatory processes sufficient time to become operative (Houston, 1964).

The seasonal changes observed in the levels and characteristics of activation by sodium and potassium ions of the ouabain-sensitive ATPase of coho gills (Figure 10), appear to be related to the stage of development of these fish. These stages are related to changes in the weight-length relationship, deposition of guanine in the belly skin (Vanstone and Markert, 1968), loss of parr marks, changes in internal ion concentrations and distribution (Houston and Threadgold, 1963; Conte and Wagner, 1965), and to several endocrine and behavioral changes (Hoar, 1965). The present data indicate that during the period of October, 1968 to mid-February 1969 the changes observed in enzyme levels were a response to changes in internal factors independent of the aquatic environment, since the pattern of changes in TS-activity were similar in fresh water and sea water reared fish. The characteristics of activation by sodium ions and potassium ions, however, were different in the two groups of fish (see previous discussion).

The peak in TS-activity and potassium activation during the period December 1968 and January 1969 may reflect the observation that juvenile coho can osmoregulate in a shorter

time when transferred directly to sea water in this period than during the months immediately preceeding and following this interval (Conte, Wagner, Fessler and Gnose, 1966). Houston and Threadgold (1963) found significant decreases in plasma and tissue chloride during the parr to silvery-parr transition stage of Atlantic salmon which may reflect changes in sodium ion concentrates in this period.

The smolt stage in fresh water fish was characterized by increasing levels of TS-ATPase, decreasing levels of sodium activation and increasing levels of potassium activation. These results agree with the findings of Conte et al. (1966), that the time required for coho to initiate osmoregulation in sea water decreases to a minimum of 30 hours as the smolting period progresses. Thus the levels and characteristics of the activation components of the ouabain-sensitive ATPase of smolting fresh water coho as well as the ability to osmoregulate in sea water tend to approach those observed in sea water adapted fish of the same age.

It has been demonstrated that the gills are the major site of ion excretion in seawater fish (Motais, Garcia Romeu, and Maetz, 1966). If the sodium and potassium activated adenosine triphosphatase of the gills is in fact an integral part of the osmoregulatory apparatus in seawater fish then the significance of preadaptive changes in the enzyme activity in fish migrating from fresh water to sea water is obvious. The ability to osmoregulate quickly upon exposure to sea water would avoid the stresses associated with exposure of freshwater fish to a hypertonic environment. The preadapted fish could

leave the estuarine environment and move directly to full strength sea water, thus avoiding concentrations of potential predators and making available the abundant food resources of the sea.

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