

AN ABSTRACT OF THE THESIS OF

LAVON LEE PARTEL for the degree of MASTER OF SCIENCE

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Title: SULFONAMIDE SENSITIVITY OF BRINE SHRIMP, ARTEMIA  
SALINA, NAUPLII: AN APPROACH TO ANION TRANSPORT  
MECHANISM

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Abstract approved:                      Frank P. Conte                     

This study established the sulfonamide-sensitivity of brine shrimp (Artemia salina) nauplii; measurements of which indicated both dose and salt dependence. It was shown that sulfonamide sensitivity does not appear to be due to a crustacean carbonic anhydrase, as evidenced by the lack of acetazolamide inhibition of esterase activity. Bicarbonate transport into the cytoplasm is affected by sulfonamides as shown by <sup>14</sup>C-bicarbonate labeling methods, which suggests the possibility of a sulfonamide-sensitive mechanism of ionic movement across the cell membrane. In addition CO<sub>2</sub> fixation from the bicarbonate substrate is unaffected by acetazolamide. However, at high salinities there appears to be a build-up of α-Keto acids when nauplii are exposed to sulfonamides. Oxidative metabolism, as measured by oxygen

consumption, was unaffected by the sulfonamides. It has, therefore, been postulated that sulfonamide sensitive sites lie within a facultative anerobic pathway for the production of ATP.

Sulfonamide Sensitivity of Brine Shrimp, Artemia salina,  
Nauplii: An Approach to Anion Transport Mechanism

by

Lavon Lee Bartel

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Dean of Graduate School

Date thesis is presented August 15, 1975

Typed by Julia Bruce for Lavon Lee Bartel

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SULFONAMIDE SENSITIVITY OF BRINE SHRIMP, ARTEMIA SALINA,  
NAUPLII: AN APPROACH TO ANION TRANSPORT MECHANISM

I. INTRODUCTION

A wide tolerance of salinity concentrations has been reported for the branchiopod crustacean, Artemia salina. For this reason the hardy crustacean is commonly referred to as the brine shrimp. It has been found throughout the world to live in permanent or temporary salt lakes which may contain salinitation ranging up to 220 parts per thousand salt. It has been known for some time that these animals are hypoosmoregulators and can regulate their hemolymph concentrations between 150 to 275 meg Na/L (Mangos, 1974). In addition they are permeable to water (Krogh, 1939) which brings to mind certain fundamental questions regarding the mechanism of osmotic regulation. Both adult (Croghan, 1958) and larval (Conte, et al., 1972) developmental stages of this crustacean have been shown to possess the ability to hypoosmoregulate to the same degree. However, anatomical structures responsible for osmoregulatory capability are quite different. In the adult, the metepipodite segments of the branchiae together with the gut epithelium seem to be active sites for water and electrolyte metabolism. The gut provides for sodium uptake and the concomitant water of hydration. The metepipodites are associated with secretion of excess salts. In the early nauplii the alimentary canal (Hootman, et al., 1972) is incomplete and is differentiating.

The epithelium may be the site of water and sodium uptake, but this has not been proven. In contrast to the adult, branchial anlage are not developed and it is obvious that some organ other than the metepipodite is responsible for salt secretion. It has been postulated that the neck organ has this responsibility.

Since nauplii possess embryonic structures capable of maintaining water and electrolyte metabolism in the absence of other organs (i.e., endocrine organs, digestive glands, neural ganglion, etc.), they serve as an ideal model for the investigation of biogenesis of subcellular structure responsible for transepithelial movement of ions. The nature of molecular events employed by the embryo to attain electrolyte regulation has been revealed through the use of inhibitors.

Ewing et al., (1972) using the cardiac glycoside, ouabain, revealed not only the presence of the cationic transport enzyme  $\text{Na}^+ + \text{K}^+$ -activated ATPase, but also found that this enzyme is an absolute requirement in the development of sodium ion regulation. Subsequent studies by Peterson (1975) on the isolation, characterization, and purification has shown the nauplius to be one of the richest sources of this enzyme.

Accompanying these findings was the result that acetazolamide, a sulfonamide that blocks carbonic anhydrase (C.A.), interfered with survival of nauplii incubated in

environmental salinities of 2.5M NaCl and greater. Thus was developed the rationale for pursuing the salt dependent effects of sulfonamide toxicity. Experimentation in this study was initially based upon the hypothesis that the sulfonamides would reveal the role of C.A., if any, in the transport of anions across the epithelial membrane.

Acetazolamide has been used medically as a diuretic because of its inhibition of mammalian kidney carbonic anhydrase (Stecher, et al., 1968). In fact much of the physiological knowledge about the role of carbonic anhydrase in electrolyte transport stems from research with acetazolamide (Maren, 1967). In terms of cellular transport, carbonic anhydrase is the enzyme responsible for an increase in rate of the reversible conversion of carbon dioxide plus water to form carbonic acid (Roughton, 1935) which will yield high levels of bicarbonate ions. In the case of gastric secretion, bicarbonate is exchanged for chloride ions by active transport (Hogben, 1955; Imamura, 1970). Cerebrospinal fluid also shows differential chloride concentration from that of plasma. Carbonic anhydrase accelerates chloride entry into cerebrospinal fluid (Leusen, 1972) which can be inhibited by acetazolamide. The result of inhibition is a loss of ionic concentration differences between plasma and cerebrospinal fluid (Maren, 1967).

As well as these investigations, others also point toward active  $\text{Cl}^-/\text{HCO}_3^-$  exchange. The aqueous humor of the

vertebrate eye has a higher content of  $\text{Cl}^-$  than serum and a lower than serum content of  $\text{HCO}_3^-$ . These serum-humor differences can be abolished by acetazolamide (Davson and Luck, 1957; Maren, 1967). The skin of the frog actively transports  $\text{Cl}^-$  independent of  $\text{Na}^+$  transport (Martin and Currans, 1966). This transport is apparently in exchange for bicarbonate (Garcia Romeu, *et al.*, 1969) and can be inhibited by acetazolamide (Maren, 1967). The  $\text{Cl}^-/\text{HCO}_3^-$  exchange system thus seems to be dependent upon carbonic anhydrase for generation of  $\text{HCO}_3^-$  although the ion distribution mechanisms are not understood.

Evidence for  $\text{Cl}^-/\text{HCO}_3^-$  exchange is numerous. Chloride transport mechanisms biochemically are little known. The brine shrimp's salt regulating abilities on an organismic level as well as a cellular level generate an easily manipulable and readily available biological system. A chloride-bicarbonate exchange mechanism could be responsible for maintaining the electrochemical equilibrium necessary for the brine shrimp electrolyte balance. If this exchange is dependent upon carbonic anhydrase-generated bicarbonate, then the apparent toxicity of the sulfonamide, acetazolamide, would be understood.

## II. MATERIALS AND METHODS

Dessicated cysts of Artemia salina, provided by Longlife Products (Harrison, N.J.), were utilized and are of the Great Salt Lake, Utah, variety. Dry cysts were kept in tightly covered cans at  $-20^{\circ}\text{C}$  until needed. Nauplii were prepared by hydrating cysts for three hours in distilled water at  $4^{\circ}\text{C}$  to minimize metabolic activity. Extraneous materials (sand, debris, etc.) were washed from the cold hydrated cysts using cold distilled water. Cold hydrated cysts were filtered from the wash water and ten gram portions were routinely placed in Fernbach flasks containing 500 ml of artificial sea water and were gently shaken at  $26^{\circ}\text{C}$  on a platform rotator for the desired length of time. The developing embryos were harvested at 18, 24, or 33 hours post incubation as indicated by the method of Finamore and Clegg (1968). Embryos were not staged according to molting cycles, but were timed from beginning of incubation since they can be reared with a high degree of synchrony in the population.

Samples of nauplii were removed from culture flasks and used according to four experimental protocols; II A) Sulfonamide Mortality, II B) Carbonic Anhydrase Inhibition, II C) Respiratory Energy Production and Inhibition, and II D) Bicarbonate Uptake and Carboxylase Inhibition.

### General Acclimation Procedure

In all of the protocols the acclimation of nauplii to different salinities followed the general method of hatching cysts. Newly hatched nauplii, after harvesting, were filtered on Miracloth to remove excess incubation media and then were washed with the appropriate acclimation media in which they would be reared for the desired length of time. Various salines were prepared from Instant Ocean Salts in which they were either fortified with NaCl to achieve concentrations greater than 0.5M NaCl or were diluted with distilled water to achieve concentrations below 0.5M NaCl.

#### A. Sulfonamide Mortality

The effectiveness of various sulfonamides as brine shrimp inhibitors was determined by transferring a small number (200-400) of newly hatched nauplii to chemical spot plates containing 0.5 mls of acclimation media in which inhibitor was dissolved to an exact concentration. Complete solubility of several sulfonamides could not be achieved using distilled water. Therefore, they were prepared in either a) aqueous-salt or b) aqueous-ethanolic mixture to obtain desired solubility. This solubility was due to a salting-in effect (Stecker, et al., 1968) from the sodium salt concentration of the medias. Sometimes best solubility could be obtained, in higher concentrations ( $>10^{-3}M$ ) as

in the case of Cyclothiazide<sup>1</sup>, Dichlorphenamide<sup>2</sup> or Ethoxzalamide<sup>3</sup>, with an aqueous-ethanolic solution and for this reason brine shrimp viability was measured in various ethanolic solutions. Ethanolic solutions less than four percent provided survival within  $\pm$  1% of saline controls in salinities from 0.05M NaCl to 2.5M NaCl. In summary, all sulfonamides, which were sparingly soluble in distilled water, were made as a 4% ethanolic solution and added to the acclimation media.

Spot plates containing nauplii were incubated at 26°C and examined at hourly intervals to determine the number of dead animals. The criterion for death was identical to that reported earlier by Conte, et al. (1972). Control nauplii were those animals exposed to the various salinities and 4% ethanol that had no inhibitor. The survival was greater than 95% for all tests; however, the percentage of nauplii dying in control media was subtracted from the experimental group. Each assay contained duplicates of several (> two) plates for each saline media. Three salinities used in this study were 0.05M, 0.5M, and 2.5M NaCl because they represented the major range of osmotic regulation that gave

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<sup>1</sup>Generously supplied by Lilly Research Laboratories, Indianapolis, Indiana.

<sup>2</sup>Generously supplied by Merck, Sharp and Dohme, Rahway, New Jersey.

<sup>3</sup>Generously supplied by The Upjohn Company, Kalamazoo, Michigan.



greater than 95% survival after 24 hours (Conte, et al., 1972).

### B. Carbonic Anhydrase Inhibition

Carbonic anhydrase (C.A.) catalyzes the hydration of carbonyl groups as well as catalyzing the reversible dehydration of  $\text{HCO}_3^-$  and the hydration of  $\text{CO}_2$  (Coleman, 1973). The assay described here utilized the enzymatic hydrolysis of an ester, p-nitrophenol acetate (Pocker and Storm, 1968). The esterase activity of C.A. is inhibited by the sulfonamides which do not inhibit the catalysis of other esterases.

To measure sulfonamide-sensitive C.A. a modification of the esterase activity method reported by Armstrong, et al., (1966) was used. p-Nitrophenol, released as the reaction product, was measured at a wavelength of 348 m $\mu$  in a diethylmalonic acid buffer, pH = 7.0. The substrate, p-nitrophenol acetate, was dissolved in 1.5 ml acetone and made to a final concentration of 3mM p-nitrophenol acetate by adding diethylmalonic acid buffer prior to each assay. The reaction mixture was placed in a spectrophotometric cell and contained: 1 ml substrate, an aliquot of enzyme (0.1-0.5 ml) and 1.5 ml diethylmalonic acid buffer. A final volume of 3 ml for the reaction mixture was always used and the contents were mixed with rapid stirring. An inhibitor of C.A., the sulfonamide acetazolamide was substituted for an equal volume of buffer. Acetazolamide was dissolved in

0.05M NaCl and made to a concentration of 1mM. A Beckman Quartz spectrophotometer with a Gilford attachment 2000 Multiple Sample Absorbance Recorder was used to record enzymatic activity and p-nitrophenol absorbancy. Two sources of C.A. were utilized in these experiments. Bovine erythrocyte C.A.<sup>4</sup> was used as the standard enzyme assay. The crustacean C.A. was prepared from a crude homogenate made from brine shrimp nauplii.

Cell fractionation protocol of the brine shrimp nauplii is as follows. Brine shrimp, 24 hours post incubation, were taken from either 0.05M or 0.5M NaCl media since 2.5M media does not allow for hatching of cysts (Clegg, 1964). In addition nauplii were sometimes acclimated such that after 24 hours post incubation in 0.5M NaCl, they were transferred for 4 hours into 0.05M NaCl or 2.5M NaCl. The homogenizing media contained either a.) 0.25M sucrose + 0.1M diethylmalonic buffer pH = 7.0 or b.) 0.25M sucrose + 0.1M phosphate buffer at pH = 7.2 with 10mM EDTA. Nauplii were placed in a Potter-Elvehjem homogenizer with several ml of homogenizing media and stroked ten times to fractionate the embryos. An aliquot of homogenate was removed for assay. The crude homogenate was spun at 6,500 x g for ten minutes to remove unbroken cysts, cells, and debris. The pellet was removed and discarded while the supernatant was utilized for

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<sup>4</sup>Purchased from Sigma Chemical Company.

enzymatic assay. In addition other homogenates were spun at 15,000 x g for 15 minutes to remove mitochondria and 44,000 x g for 120 minutes to remove large fragments of membrane. Postmicrosomal and postribosomal supernatants were prepared as reported in an earlier study (Peterson, 1975).

### C. Respiratory Energy Production and Inhibition

Earlier physico-chemical properties of the sulfonamides have shown that metalloenzymes containing  $Zn^{++}$  and  $Co^{++}$  metals are tightly bound into chemical complexes with the  $-SO_2NH_2$  group (Coleman, 1973). It was considered important in this study to ascertain whether the oxidative phosphorylation of metabolic intermediates from the tricarboxylic acid cycle, via the electron transport chain of mitochondria could be sulfonamide-sensitive sites. Manometric measurement of gaseous oxygen utilization in a Gilson respirometer was the method employed to determine if oxidative phosphorylation was inhibited.

Nauplii (0.25 gm) were filtered from incubation media at 24 hours post incubation and placed in 15 ml respirometer flasks having a  $CO_2$  trap and containing 5 ml of the experimental media. Experimental media consisted of several salt concentrations with and without inhibitors such as  $10^{-3}M$  acetazolamide,  $10^{-3}M$  ferrous cyanide or  $10^{-3}M$  cyanide. Control flasks contained the media and inhibitors, but were without nauplii. The flasks were attached to the respirometer

and the system was gassed with air or oxygen (2 psi for 5 seconds). Each flask was allowed to equilibrate for 15 minutes before measurements with manometers began. Readings were taken every five minutes during the course of one-half hour or every ten minutes for an hour to determine the rate of  $O_2$  consumed, then the flasks were reopened and flushed with new gases ( $O_2$  or air). These operations were repeated until a total of six hours had elapsed.

#### D. Bicarbonate Uptake and Carboxylase Inhibition

Earlier studies by Clegg, et al., (1967) have shown the capability of nauplii to incorporate  $^{14}C$ -labeled bicarbonate ( $^{14}C-HCO_3^-$ ) into precursors of pyrimidines, purines, and protein. Therefore it is important in this study to determine if the anionic transport of  $HCO_3^-$  across the membrane into the cytosol and its subsequent incorporation into these precursor molecules is sulfonamide-sensitive. Acidification of the cellular extracts with 1N HCl and trapping of released  $^{14}CO_2$  by NaOH was the method employed in assaying for  $^{14}C$ -bicarbonate transport. Liquid scintillation counting was used to measure radioactivity. However, wet oxidation with hydrogen peroxide of the acid soluble extracts was utilized to assay for  $^{14}C$ - $\alpha$ -Ketodicarboxylic acids formed from the bicarbonate incorporation by PEP carboxylase and/or pyruvate carboxylase.

Thirty-three hour old nauplii were placed in an

acclimation media consisting of varied concentrations of NaCl in a 5mM phosphate buffer pH = 7.75 with 0.05% NaHCO<sub>3</sub> and 0.13% CaCl<sub>2</sub> (Sato, 1966) in the presence and absence of inhibitor (10<sup>-3</sup>M acetazolamide). A series of disposable petri dishes, each containing 5 ml of acclimation medium and 0.5 gm of nauplii were assayed. Prior to the <sup>14</sup>C-bicarbonate placement in the medium, the nauplii remained for 5 hours to allow the inhibitor to penetrate the organisms. The pH of each incubation medium was monitored before and after pre-incorporation acclimation to provide for identical specific activity of <sup>14</sup>C-HCO<sub>3</sub><sup>-</sup> in the external medium. If pH decreases there would be a loss of available HCO<sub>3</sub><sup>-</sup> to be unincorporated.

After pre-incorporation acclimation a spike (1μC-5μC) of <sup>14</sup>C-HCO<sub>3</sub><sup>-</sup> was added to each petri dish. At various times following the spike (0, 5, 10, 15, and 30 minutes), nauplii from individual petri dishes were transferred to a filtering apparatus. The <sup>14</sup>C-HCO<sub>3</sub><sup>-</sup> external medium was removed by filtration and saved for radioactive assay. The labelled nauplii, which were on a Miracloth filter, were washed quickly with distilled water. The filter was removed and nauplii transferred into a Potter-Elvehjem homogenizer with a volume of 5 ml of homogenizing media. In one group of experiments nauplii were homogenized with ten strokes in the presence of HClO<sub>4</sub>. This was to determine the amount of radioactivity incorporated into acid insoluble proteins and

nucleic acids. The suspension was transferred to chilled 15 ml Corex centrifuge tubes. 0.2ml of this suspension was replaced on each of two 3MM Quantitative Whatman filter discs and air dried. Subsequently, free precursors were washed from the discs by placing them in a 600ml beaker of cold 10% trichloroacetic acid (T.C.A.), followed by cold ethanol to remove the excess TCA, and cold acetone to remove excess ethanol and water which effectively quenches the scintillation fluid. The discs were dried to evaporate acetone and placed in a scintillation vial with 15 ml or a 3/5 (v/v) methylcellulosolve/BBOT-toluene scintillation mixture. All radioactivity measurements were made in a Packard liquid scintillation counter.

The second group of experiments were designed to assay unincorporated  $^{14}\text{C-HCO}_3^-$  and  $^{14}\text{C}$  labeled  $\alpha$ -Keto acids. Filtered nauplii were homogenized in a 0.1M sucrose, 0.1M phosphate buffer (pH - 7.78), and 1mM EDTA solution, using 10 strokes of the pestle. The suspension (3ml) was transferred to aliquots of homogenizing media (1 ml each) into a 25ml Erlenmeyer flask. Final volume of suspension was 5.0 ml and the reaction flask was sealed with a serum cap containing a specialized polyethylene  $\text{CO}_2$ -trap attachment. The  $\text{CO}_2$ -trap contained 0.2ml of 1.0M NaOH. The reaction flask was acidified to release  $^{14}\text{CO}_2$  from free  $^{14}\text{C-HCO}_3^-$  by injection of 1ml of 1N HCl through the serum cap. The injection of acid immediately followed the time course of 0, 5, 10,

15, and 30 minutes after the bicarbonate spike. The gaseous  $^{14}\text{C}$ - $\text{CO}_2$  was allowed to equilibrate for 30 minutes and then the cap was removed together with the trap.

Distilled  $\text{H}_2\text{O}$  (0.2ml) was added with thorough mixing to each trap to dilute 1.0M NaOH in order that it be applied directly to scintillation counting fluid. Two 0.15ml samples were taken from the  $\text{CO}_2$ -trap and placed directly into 5ml Aquasol.<sup>5</sup> Afterwards, Erlenmeyer flasks containing the acidified homogenate were recapped with fresh serum caps with new  $\text{CO}_2$ -trapping media. The reaction flasks were injected with 1.0ml of 30%  $\text{H}_2\text{O}_2$  and placed in a water bath and heated for 1 hour at  $70^\circ\text{C}$  to decarboxylate the  $\alpha$ -Keto acids. After the heating, the reaction flasks were cooled at room temperature for 30 minutes and then the  $\text{CO}_2$ -trap was removed. Again, distilled water was added to the  $\text{CO}_2$ -traps to dilute the sodium hydroxide and all samples were handled in the same manner as above.

Following the wet oxidation, the  $^{14}\text{C}$ -soluble and insoluble residues were assayed for radioactivity. 1.0 ml samples of the acidified-oxidized suspension were placed in 10.0 ml of Aquasol and counted in duplicate. All radioactive assays were counted to a 1% counting error.

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<sup>5</sup>Scintillation counting fluid from New England Nuclear.

### III. RESULTS

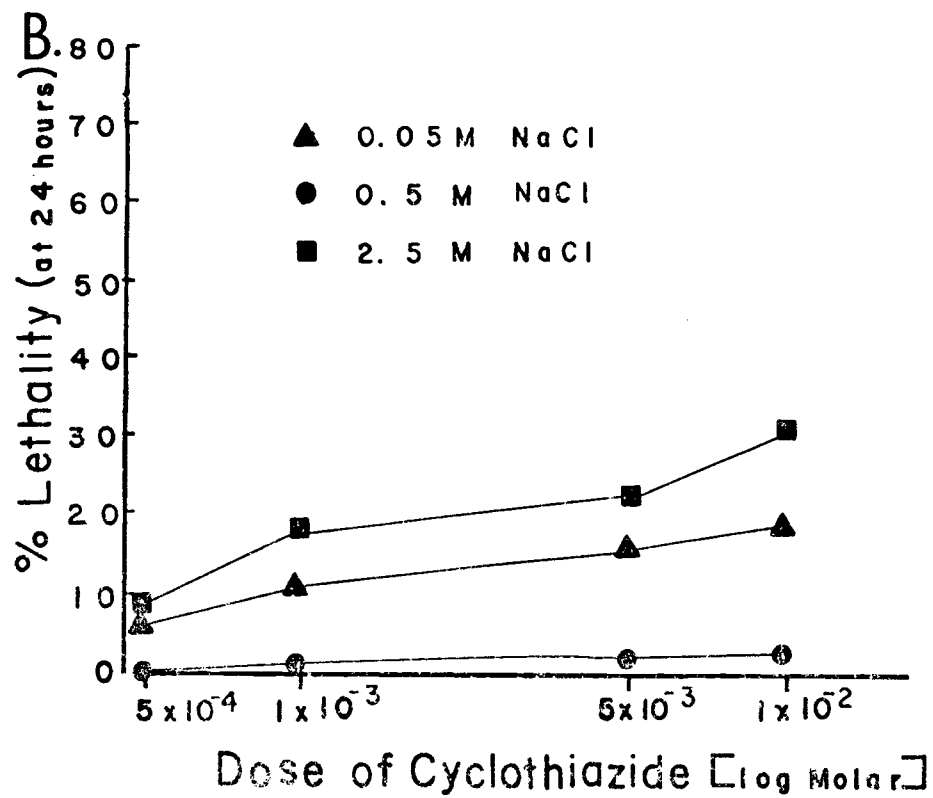
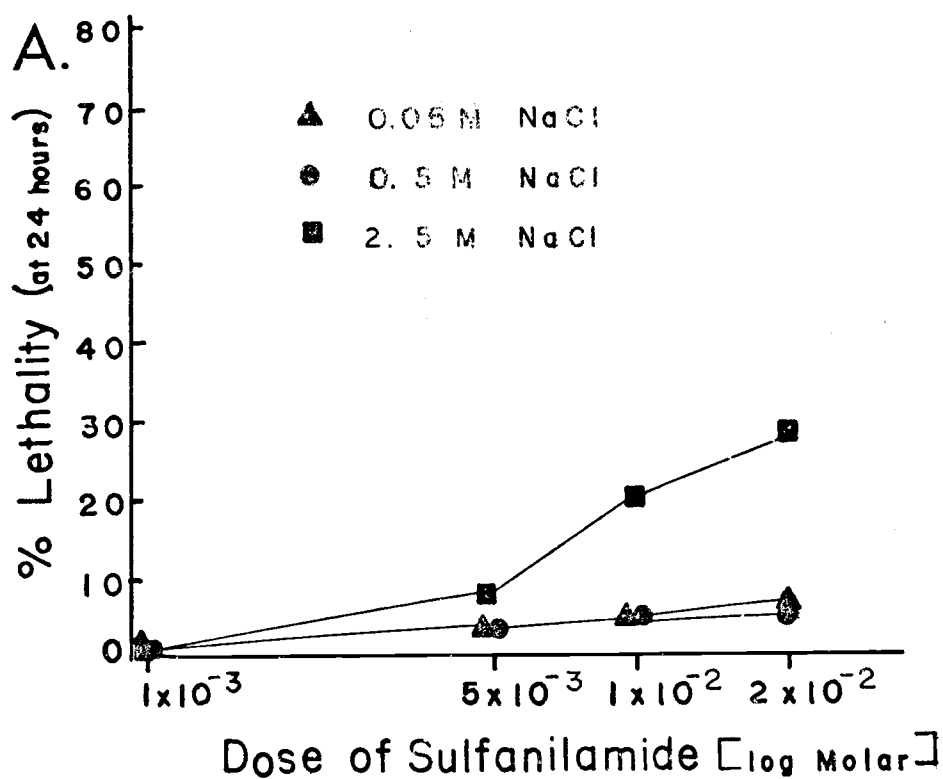
#### A. Sulfonamide Mortality

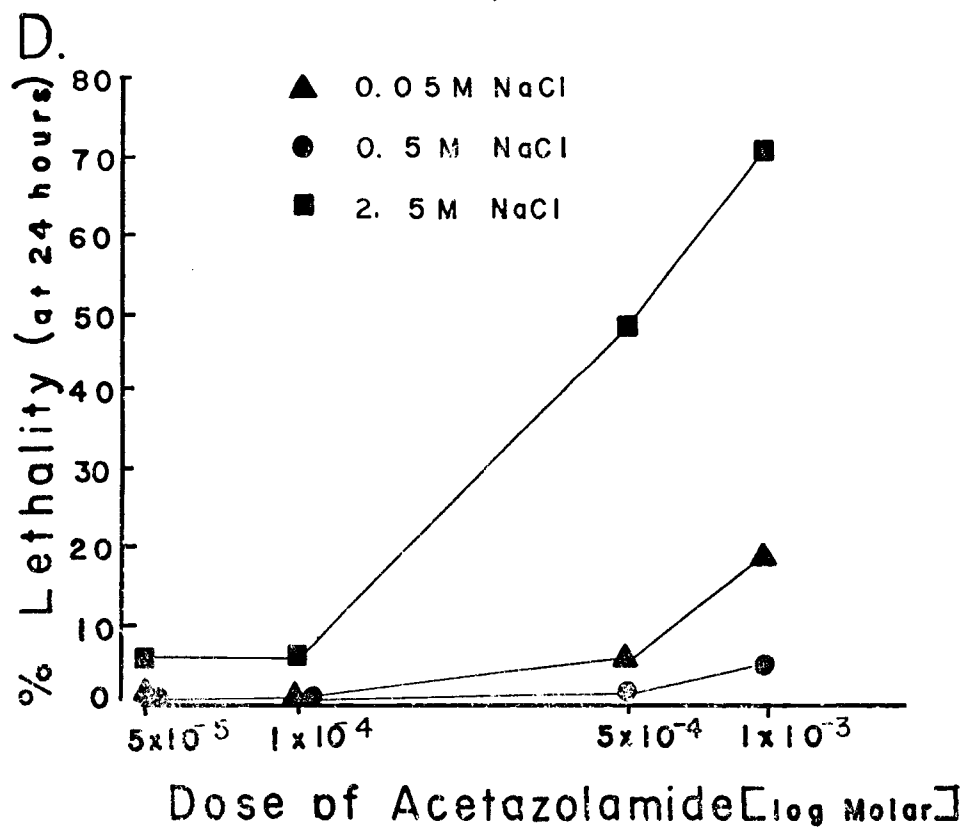
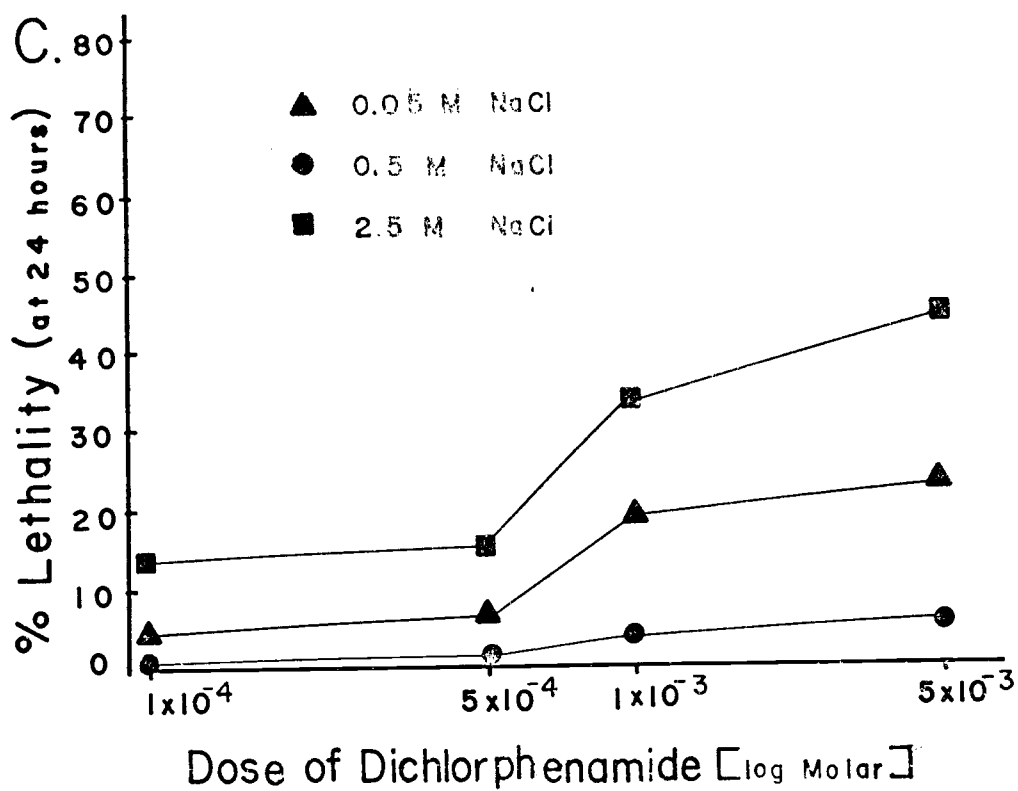
The pursuit of this study was made feasible by the previously reported observation of salt-dependent lethality of acetazolamide (Ewing, et al., 1972). This observation was verified in the present study, as shown in Figure 1-D. The hypertonic media, 2.5M NaCl, bathed nauplii had a high mortality to this compound as did the hypotonic media, 0.05M NaCl, bathed nauplii. Both osmotically stressful environments show increased toxicity to sulfonamides (Figure 1 A-E). However, the 0.5M NaCl is relatively non-toxic even at concentrations greater than  $10^{-3}M$ . Interestingly, ethoxzolamide had the greatest salt-dependent effect as shown in Figure 1-E where a dose of  $5 \times 10^{-5}M$  produced 50 percent mortality in 2.5M NaCl and no death occurred for 0.5M or 0.05M NaCl. Accompanying the salt dependency was the observation that lethality appeared to correlate with the binding constant of specific sulfonamides in their affinity for the  $Zn^{++}$  containing enzyme, carbonic anhydrase (Table 1 and Figure 2).

Sulfanilamide ( $K_C = 2.8 \times 10^{-6}$ ) produced the least mortality in all salinities, whereas it is the most water-soluble. In marked contrast acetazolamide and ethoxzolamide, both having similar binding constants ( $K_C \sim 10^{-9}$ ), effected the greatest toxicity to the nauplii and were the



Figure 1. Salinity and dose dependency of  
sulfonamides: a. sulfanilamide  
b. cyclothiazide c. dichlorphenamide  
d. acetazolamide e. ethoxzolamide





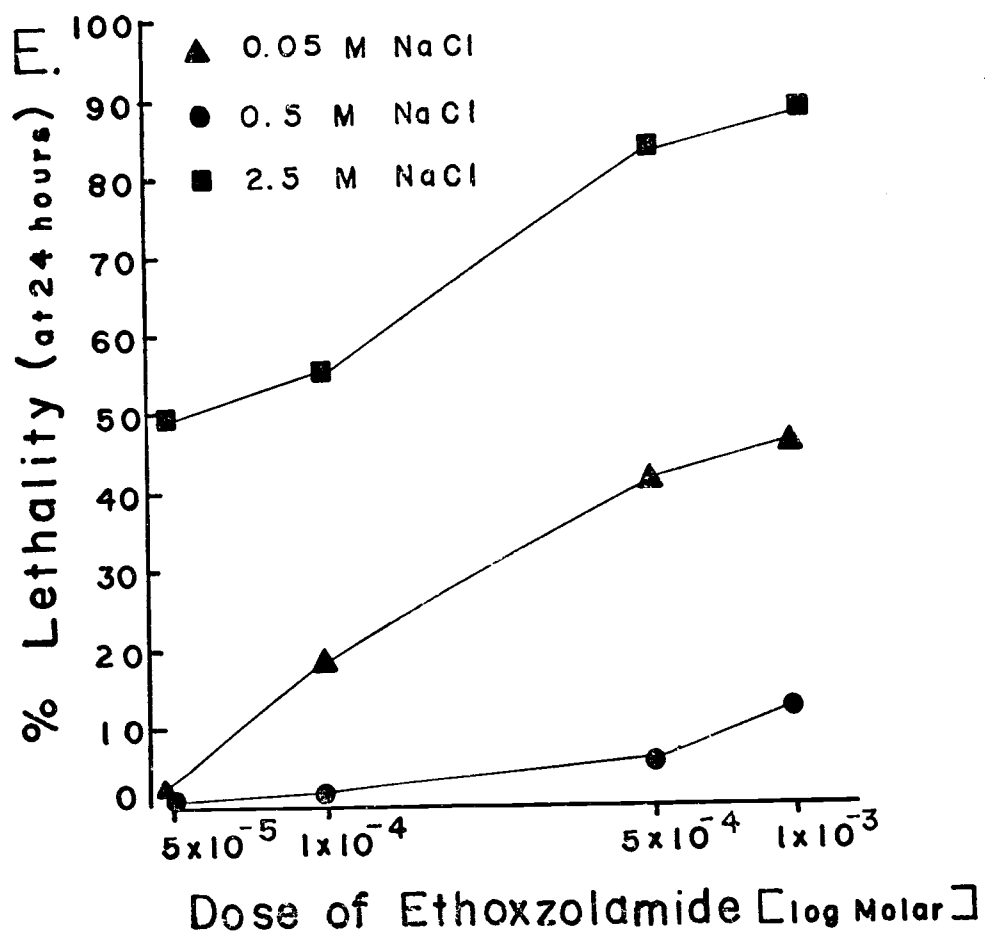


Table 1: Lethality ( $LT_{50}$ ) in Hours After Exposure to  $10^{-3}M$  Sulfonamide in Different Salines

COMPOUND ( $K_C$ = Dissociation Const.)	n*	SALINITY (M NaCl)		
		<u>0.05M NaCl</u>	<u>0.5M NaCl</u>	<u>2.5M NaCl</u>
Sulfanilamide ( $K_C = 2.8 \times 10^{-6}$ )	4	36 <sup>≠</sup>	36	36
Cyclothiazide ( $K_C = 6.0 \times 10^{-7}$ )	4	36	36	33.8 $\pm$ 2.3
Dichlorphenamide ( $K_C = 2.5 \times 10^{-8}$ )	3	30.0 $\pm$ 3.2	36	29.5 $\pm$ 3.0
Acetazolamide ( $K_C = 8.0 \times 10^{-9}$ )	4	24.1 $\pm$ 0.4	36	23.8 $\pm$ 3.0
Ethoxzolamide ( $K_C = 1.0 \times 10^{-9}$ )	3	23.3 $\pm$ 0.9	36	23.0 $\pm$ 2.6

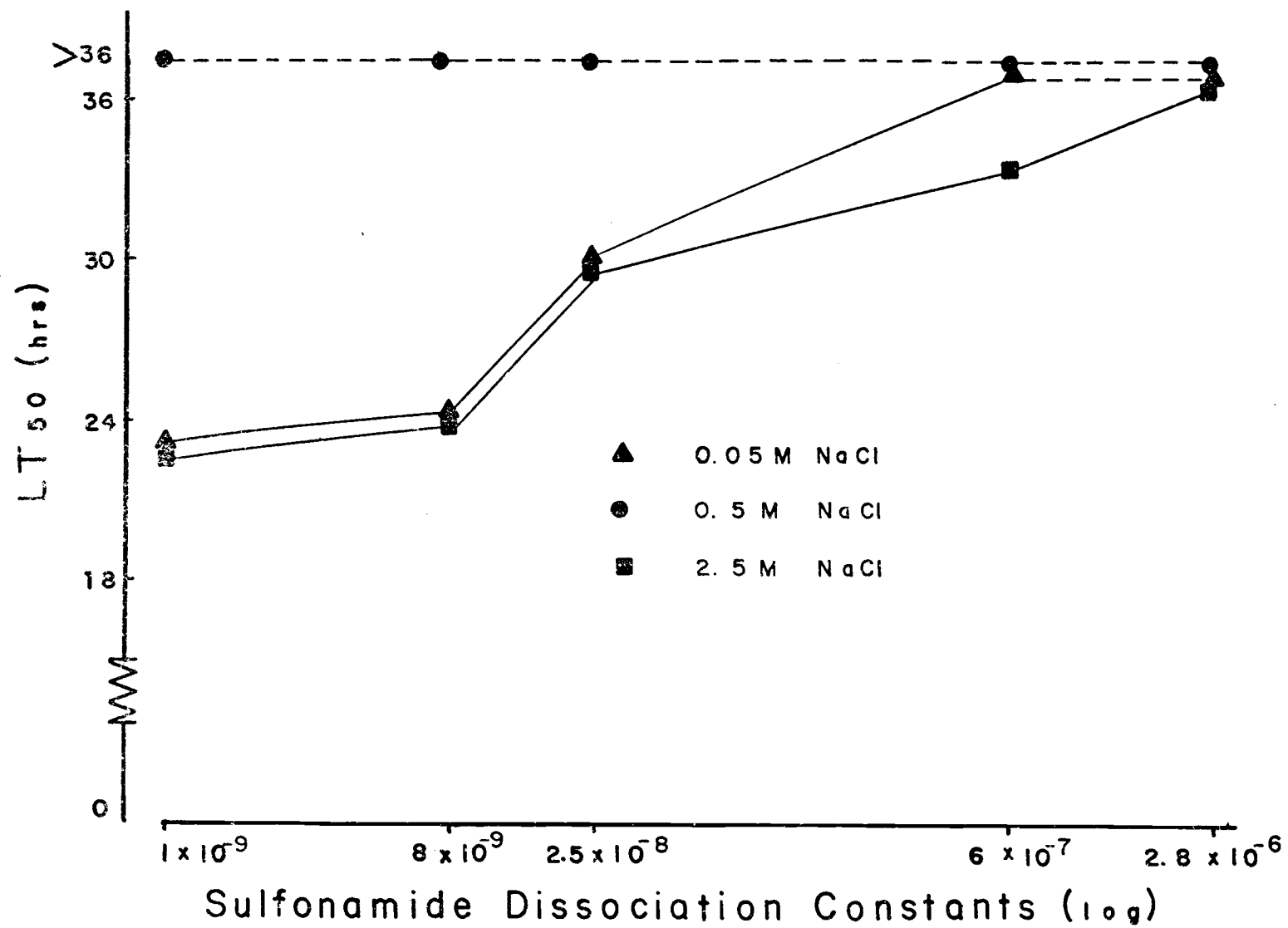
\*n = number of experiments and 400-800 animals exposed per dose

<sup>≠</sup>Time in hours is mean value  $\pm$  standard error

LT = Length of time for population to reach fifty-percent mortality

$K_C$  = Dissociation constant of sulfonamide complex with  $Zn^{++}$  under equilibrium dialysis conditions (Coleman, 1973)

Figure 2. Dissociation constants of sulfonamides from  $\text{Zn}^{++}$  enzyme as related to  $\text{LT}_{50}$  at  $10^{-3}\text{M}$  concentration.



most water-insoluble. The salt-dependent toxicity, together with the correlation to the  $K_C$  in regard to C.A. inhibition, suggest that the site of action of the sulfonamide might lie with the inhibition of an unknown crustacean carbonic anhydrase utilized in the transport of electrolytes.

### B. Crustacean Carbonic Anhydrase

The analyses for carbonic anhydrase (C.A.) are numerous and they include manometric assays of  $\text{CO}_2$ , titrimetric assays of  $\text{H}^+$  ion, and enzymatic fixation of radioactive carbon dioxide. However, one of the easiest and most specific assays is the inhibition of esterase activity of C.A. as measured by spectrophotometric analysis of the end product p-nitrophenol (Pocker and Storm, 1968). Bovine erythrocyte C.A. is readily available as a purified enzyme and was utilized as the standard enzyme assay. As shown in Table 2, the enzyme readily catalyzes the reaction and is completely inhibited at  $10^{-5}\text{M}$  concentration of acetazolamide, as shown in Figure 3. Since the substrate p-nitrophenyl acetate, yields a yellow product (p-nitrophenol), it was thought prudent to ascertain if the carotenoid pigments contained in the embryo would interfere with the analysis. The results presented in Figure 4 show the lipid pigments to have very little, if any, effect upon the stoichiometry of the color reaction. Table 3 contains the results of the esterase activity assay from a wide variety of cell fractions which



Table 2: Bovine Carbonic Anhydrase Esterase Activity and Its Inhibition by Acetazolamide

Substrate concentration p-nitrophenol acetate	Enzyme concentration* U = Wilbur- Anderson units	Inhibitor concentration Molar	Esterase Activity** umole/ml/min p-nitrophenol formed	% Residual Esterase Activity
1mM	166.7 U	--	.071	100%
1mM	166.7 U	$1.67 \times 10^{-5}$	.000	0%
1mM	83.3 U	--	.075	100%
1mM	83.3 U	$1.67 \times 10^{-5}$	.000	0%
1mM	41.7 U	--	.058	100%
1mM	41.7 U	$1.67 \times 10^{-5}$	.000	0%
1mM	20.8 U	--	.023	100%
1mM	20.8 U	$1.67 \times 10^{-5}$	.000	0%

\*Unit = Wilbur-Anderson unit which will cause pH of a 0.012M Veronal buffer to drop from 8.3 to 6.3 per minute at 0°C. Approximately equal to one Roughton-Booth unit.

\*\*All values corrected for continuous hydrolysis of p-nitrophenol acetate to p-nitrophenol of .003µmoles/ml/min taking place.

Figure 3. Acetazolamide inhibition of bovine erythrocyte carbonic anhydrase expressed in terms of activity remaining with variable inhibitor and enzyme concentrations.

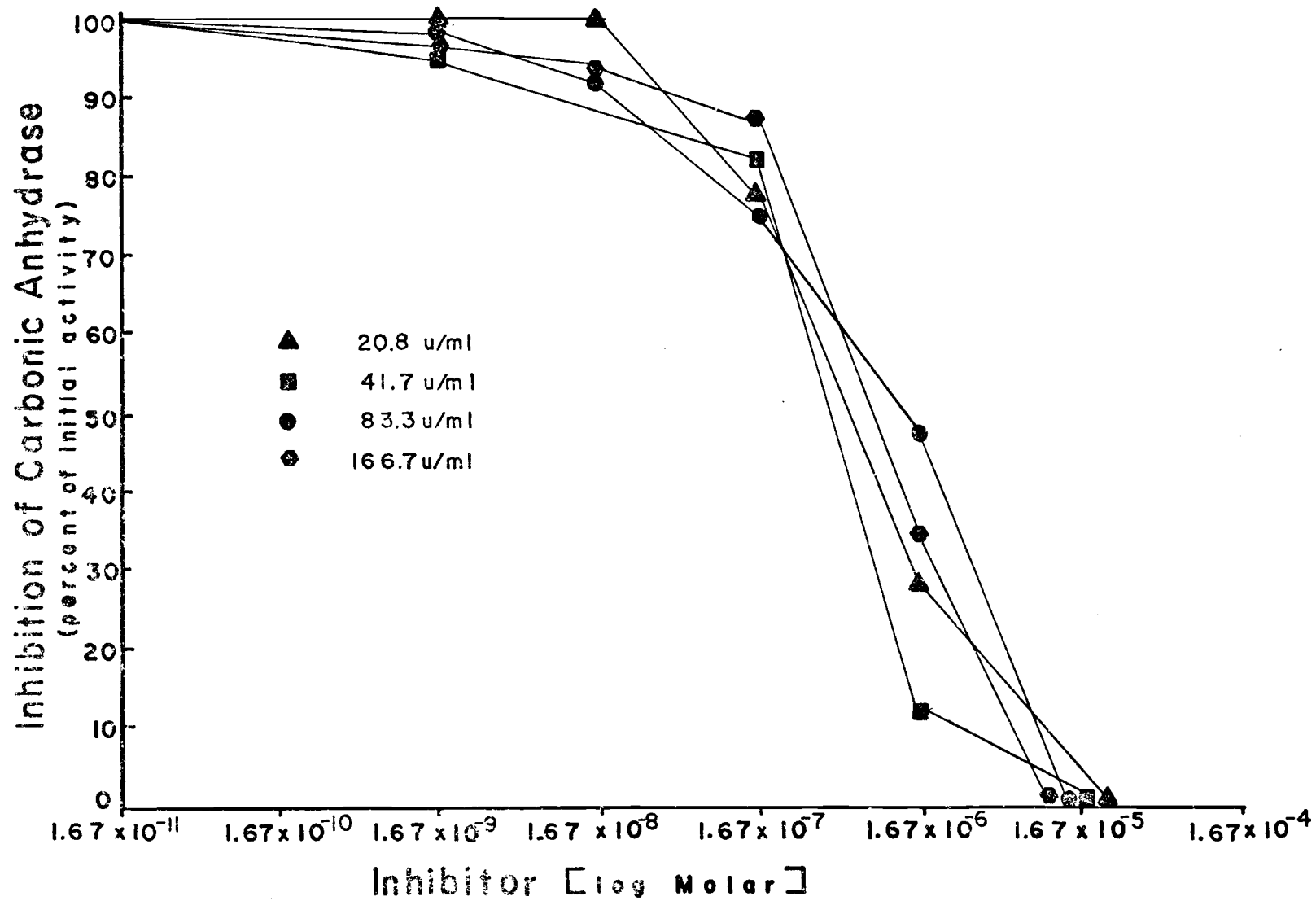


Figure 4. The effect of lipid pigments on the spectrophotometric visualization of p-nitrophenol:

- p-nitrophenol
- p-nitrophenol + 15,000 x g homogenate
- ▲ p-nitrophenol + 44,000 x g homogenate

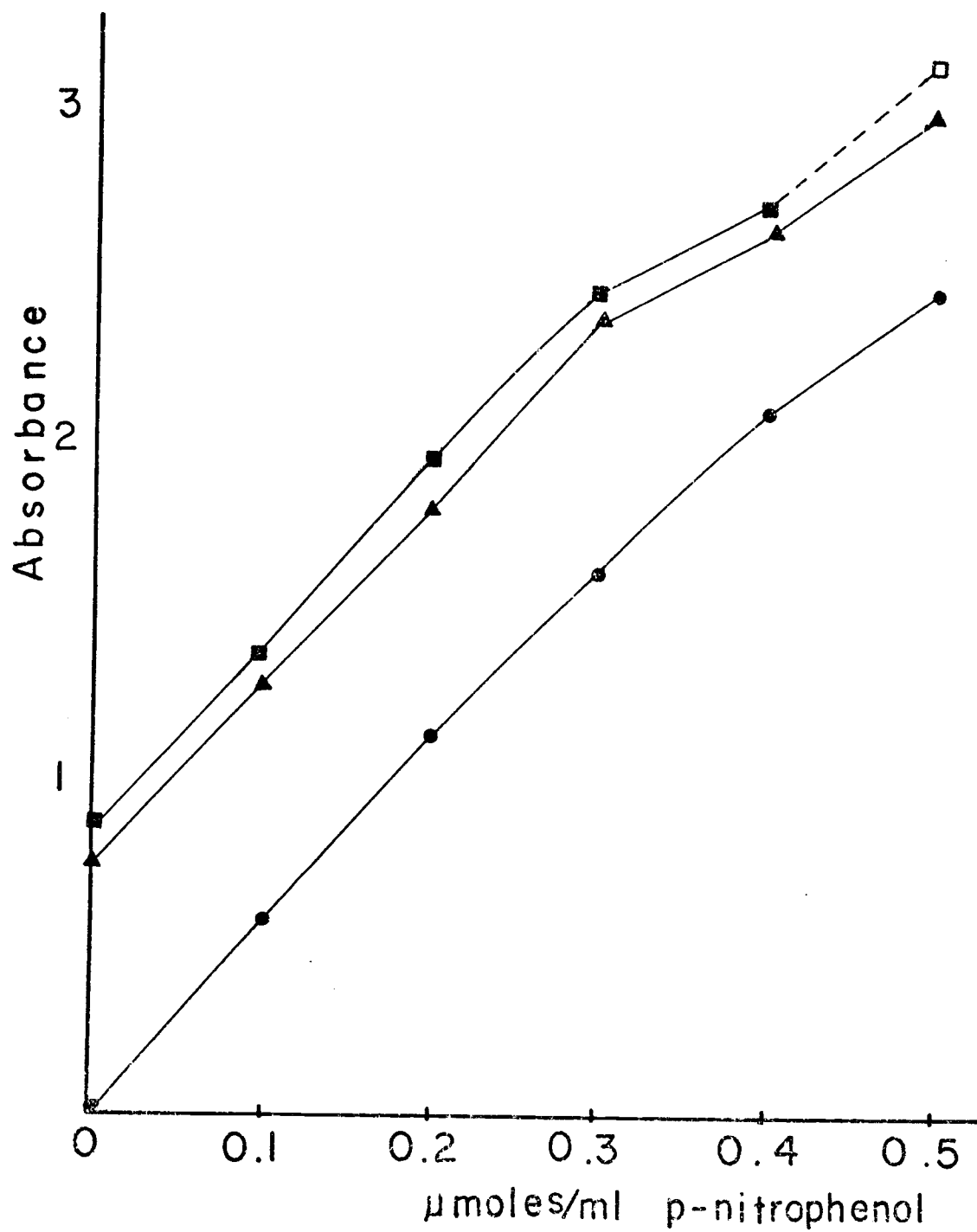


Table 3: Crustacean Esterase Activity in the Presence and Absence of Acetazolamide

Homogenizing media	Treatment (salinity incubated)	Processing	Homogenate	Inhibitor (10 <sup>-4</sup> M Acetazolamide)	Esterase Activity* $\mu$ mole/ml/min p-nitrophenol formed	% Activity remaining
0.1M PO <sub>4</sub> <sup>-</sup> buffer 0.25M sucrose 1.0mM EDTA  1gm/5ml	0.5M NaCl 24 hr post incubation	full homogenate	0.5ml	--	.008	
			0.5ml	0.5ml	.009	>100%
		15,000xg	0.5ml	--	.005	
			0.5ml	0.5ml	.005	100%
		44,000xg	0.5ml	--	.002	
			0.5ml	0.5ml	.002	100%
		postmicrosomal supernatant	0.5ml	--	.001	
			0.5ml	0.5ml	.001	100%
		postribosomal supernatant (Na DOC added)	0.5ml	--	--	
			0.5ml	0.5ml	--	--

Table 3 (cont.)

Homogenizing media	Treatment (salinity incubated)	Processing	Homogenate	Inhibitor (10 <sup>-4</sup> M Acetazolamide)	Esterase Activity* $\mu$ mole/ml/min p-nitrophenol formed	% Activity remaining
0.1M PO <sub>4</sub> <sup>-</sup> buf 0.25M sucrose  0.5gm nauplii 1 ml homogenizing media	0.05M NaCl acclimation for 4 hours after 24 hr post incubation in 0.5M NaCl	6,500xg	0.5ml	--	.025	
			0.5ml	0.5ml	.027	>100%
			0.1ml	--	.019	
			0.1ml	0.5ml	.020	>100%
	0.5M NaCl- acclimation for 4 hours		0.05ml	--	.013	
			0.05ml	0.5ml	.013	100%
			0.5ml	--	.010	
			0.5ml	0.5ml	.010	100%
			0.1ml	--	.001	
			0.1ml	0.5ml	.001	100%
			0.5ml	--	.015	
			0.5ml	0.5ml	.016	>100%
	2.5M NaCl- acclimation for 4 hours		0.1ml	--	.005	
			0.1ml	0.5ml	.005	100%

Table 3 (cont.)

Homogenizing media	Treatment (salinity incubated)	Processing	Homogenate	Inhibitor (10 <sup>-4</sup> M Acetazolamide)	Esterase Activity* $\mu$ mole/ml/min p-nitrophenol formed	% Activity remaining
0.1M diethyl-malonic acid 0.25M sucrose 0.5gm nauplii 3ml homogenizing media	0.05M NaCl 28 hr post incubation	6,500xg	0.5ml	--	.024	
			0.5ml	0.5ml	.024	100%
			0.1ml	--	.006	
			0.1ml	0.5ml	.008	>100%
	0.5M NaCl 28 hr post incubation		0.5ml	--	.031	
			0.5ml	0.5ml	.032	>100%
			0.2ml	--	.017	
			0.2ml	0.5ml	.018	>100%
			0.1ml	--	.010	
			0.1ml	0.5ml	.010	

\*All values corrected for continuous hydrolysis of p-nitrophenol acetate to p-nitrophenol of .003  $\mu$ moles/ml/min taking place.



could possibly contain crustacean C.A., if it were present. A substantial amount of crustacean esterase activity was observed, but it is clear that none of it is inhibited by acetazolamide. To determine if an endogenous inhibitor to crustacean C.A. was released during cell fractionation, bovine C.A. was added to the homogenizing media. Embryos were added to the media and the cell fractionation protocol carried out in an identical manner. C.A. activity which was acetazolamide inhibited was found to be present and was nearly 100% recoverable. Therefore, it appears that a crustacean C.A. is not present in the nauplii. Of course, it may be undetectable by the esterase analysis or it may not show esterase activity that is sulfonamide sensitive.

The lack of a crustacean C.A. posed a paradox concerning the observed toxicity of sulfonamides to the nauplii. A possible site of inhibition is the interruption of metabolism of high energy intermediates since it is known that osmotic gradients present an energy demand upon the organism. Therefore, an investigation of the sulfonamide-sensitivity of metabolic pathways was begun.

### C. Respiratory Energy Production and Its Inhibition

The ultimate proton and electron acceptor is oxygen and the metabolic requirements of an organism can generally be evaluated by the consumption of oxygen. Any one of several key metabolic enzymes and metalloenzymes, such as 1,3-

phosphoglyceric dehydrogenase, or malic dehydrogenase (Jacoby and Laties, 1971), together with the cytochromes and cytochrome oxidase of the electron transport system (E.T.S.), might account for the sulfonamide sensitivity. Therefore, a general evaluation of alterations of oxygen consumption in the presence of acetazolamide was undertaken. Other inhibitors ( $\text{CN}^-$ ) known to block cytochrome oxidase without salt dependency were used as standard E.T.S. inhibitors.

Oxygen consumption measurements of 24 hour post-incubation nauplii reveal that there is very little difference in oxygen consumption during the six hour period, but the rate varied for different salinities. Oxygen consumption in the presence of  $10^{-3}\text{M}$  acetazolamide is not appreciably altered for the three salinities (Figure 6). In fact, the 0.05M salinity shows an increased response in oxygen consumption, indicating a possible uncoupling of mitochondria. Cyanide, the nonsalt-dependent inhibitor, immediately depressed the oxygen consumption of the nauplii. Therefore, it appears that the mitochondria containing the E.T.S. is sparingly sulfonamide sensitive and can not be the major target of these inhibitors.

Since the oxidative metabolic cycle of high-energy intermediates is unaffected, the alternative metabolic pathways of glycolysis and the tricarboxylic cycle are suspect. In addition, the facts that this organism can fix  $\text{CO}_2$  (in

Figure 5. Oxygen consumption of nauplii in various incubation medias and salinities.

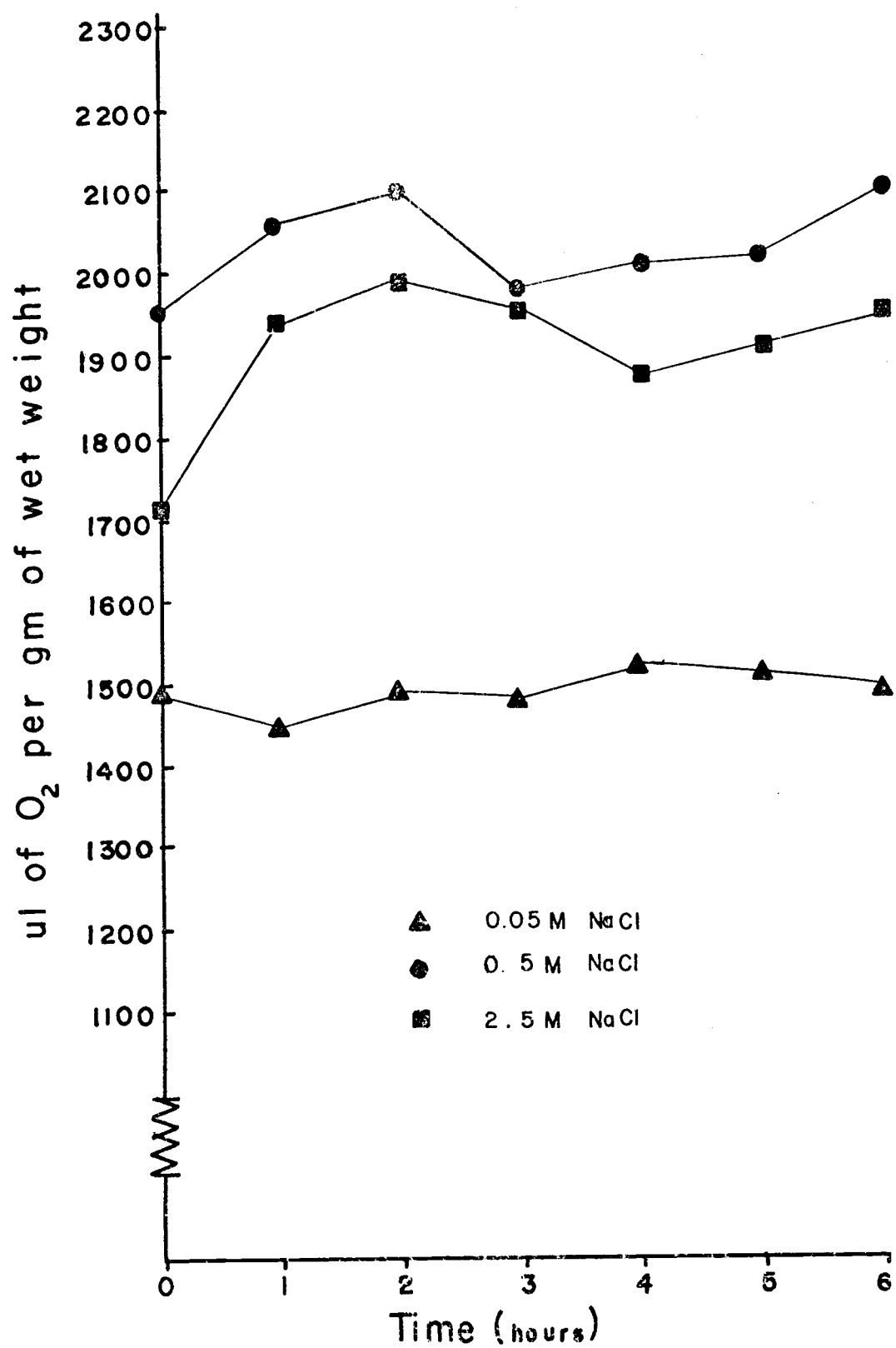
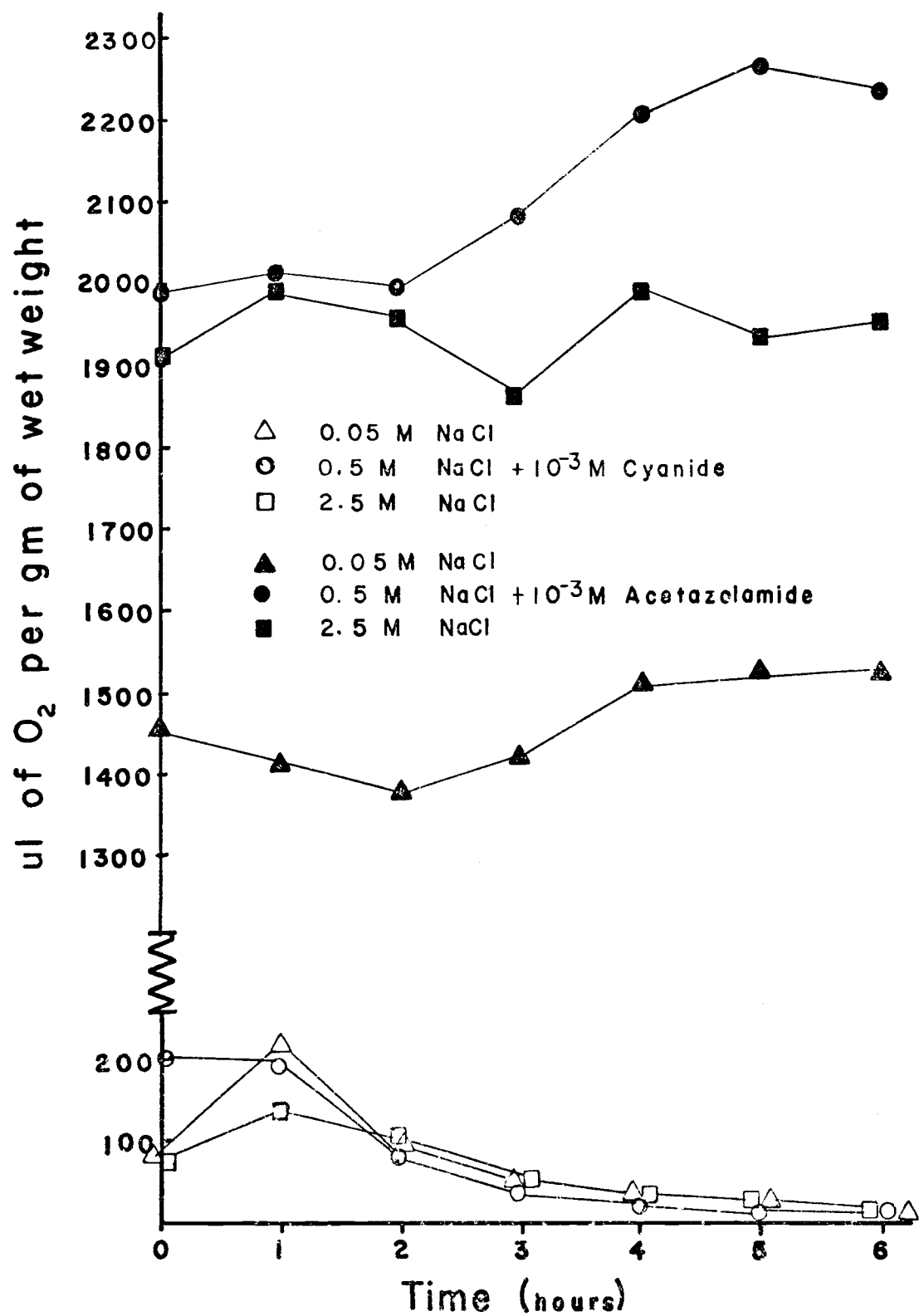


Figure 6. Oxygen consumption of nauplii in various incubation medias and salinities with acetazolamide or cyanide inhibition.



the form of  $\text{HCO}_3^-$ ) into organic compounds and that during salinity adaptation the organism secretes acids suggest the possibility that the target of inhibition may be the transport of bicarbonate ion and its incorporation into essential precursors for energy production and/or macromolecule biosynthesis.

#### D. Bicarbonate Uptake and Carboxylase Inhibition

The general hypothesis that is offered for the study of bicarbonate uptake is taken from experimental evidence obtained in eucaryotic plant cells, in that salt-induced organic acid synthesis by phosphoenolpyruvate carboxylase is controlled by the levels of cytoplasmic bicarbonate (Jacoby and Laties, 1971). These cytoplasmic bicarbonate levels are regulated in response to the exchange of  $\text{H}^+$  for external cations, such as  $\text{K}^+$  absorption or the metabolism of cytoplasmic  $\text{NO}_3^{--}$  with the consequent formation of  $\text{NH}_3$  (Burstom, 1945). In addition, bicarbonate exchange for active transport of chloride has been found in the epithelial cells of the vertebrate cornea in the absence of C.A. It was suggested that the energy required for the transport was derived from anerobic glycolysis, which is the main metabolic process in the cornea. Interestingly, despite the lack of C.A., the electrical potential across the membrane is susceptible to sulfanilamide (Kitahara, et al., 1967). The kinetics of  $^{14}\text{C}$ -bicarbonate uptake from the media in the presence and

absence of inhibitor is shown in Figure 7. The rate of entry and levels of  $^{14}\text{C}$ -bicarbonate are markedly affected by acetazolamide in all salinities but are inhibited most drastically in 2.5M NaCl as shown in Figure 8. Also, as indicated in Figure 9, the cytoplasmic levels of bicarbonate are sensitive to the presence of sulfonamide even though the external level of bicarbonate is constant and the specific activity of  $^{14}\text{C}$ -bicarbonate in the incubation medium is maintained at a constant level between the three salinities (see Section II-D). The evidence that cytoplasmic  $^{14}\text{C}$ -bicarbonate becomes incorporated into organic acids is shown in Figure 10. The lack of inhibition by acetazolamide suggests that the carboxylase activity is sulfonamide-insensitive, but that the subsequent utilization of this precursor(s) in the metabolic pathway is blocked since there occurs a substantial build-up of  $^{14}\text{C}$ -labeled Keto acids. The results indicate that this metabolic step is salt-dependent and might be regulated by cytoplasmic cations and/or anions. Furthermore, the results in Figure 11 show that  $^{14}\text{C}$ -labeled insoluble products, such as protein and nucleic acids, are markedly affected at the higher salinity (2.5M NaCl). This effect would be produced if either formation of  $^{14}\text{C}$ -labeled amino acids or nucleotides from  $^{14}\text{C}$ - $\alpha$ -Keto acids is under feedback inhibition by high levels of cytoplasmic  $\alpha$ -Keto acids. The assay of radioactivity in biopolymers yielded consistent results, as shown in Figure 12 and 13, where



Figure 7. The entry of  $^{14}\text{C}$ -bicarbonate into the internal fluids of nauplii at various salinities.

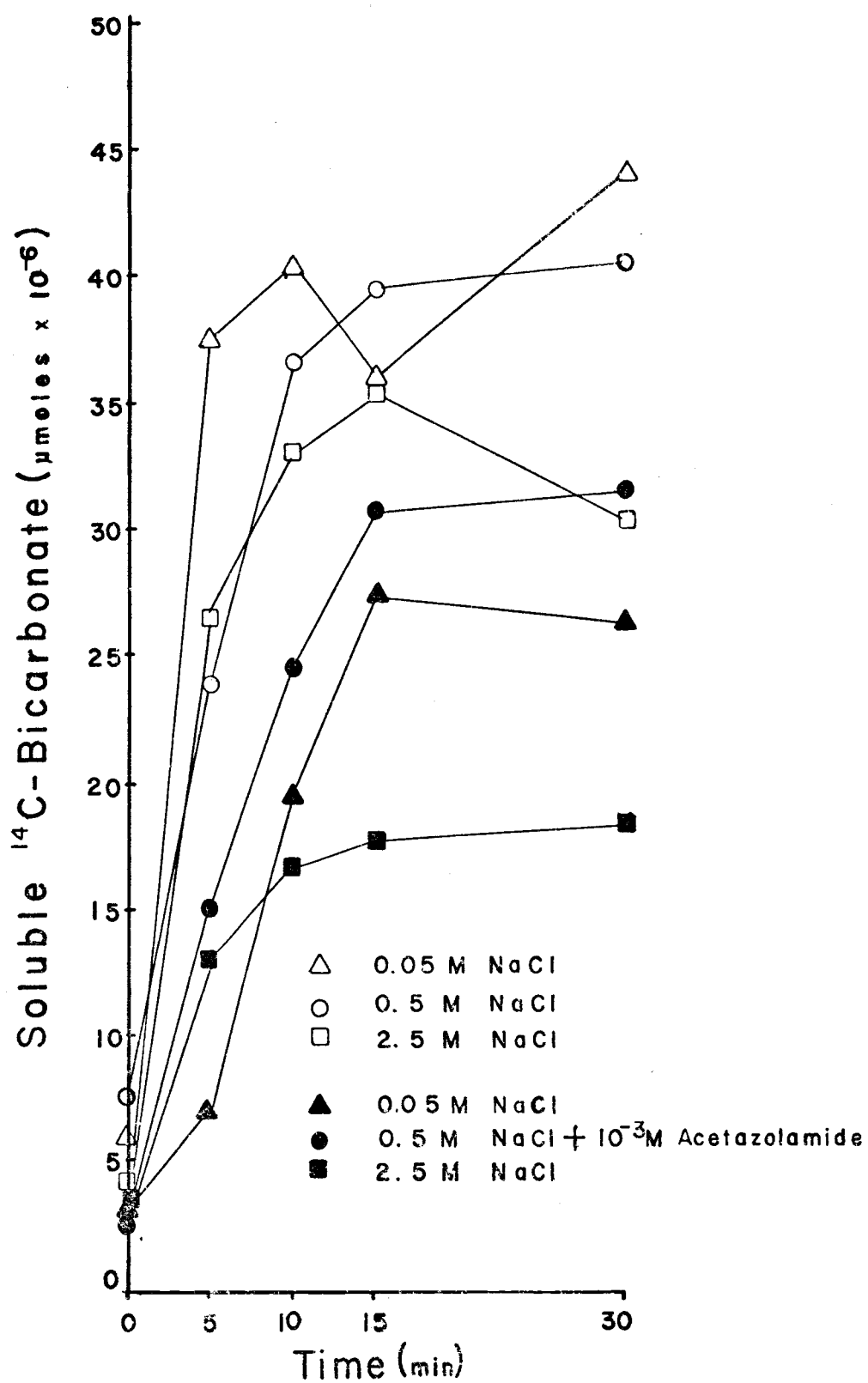


Figure 8. Salinity variable inhibition of initial uptake of free  $^{14}\text{C}$ -bicarbonate into the internal fluids of nauplii by acetazolamide.

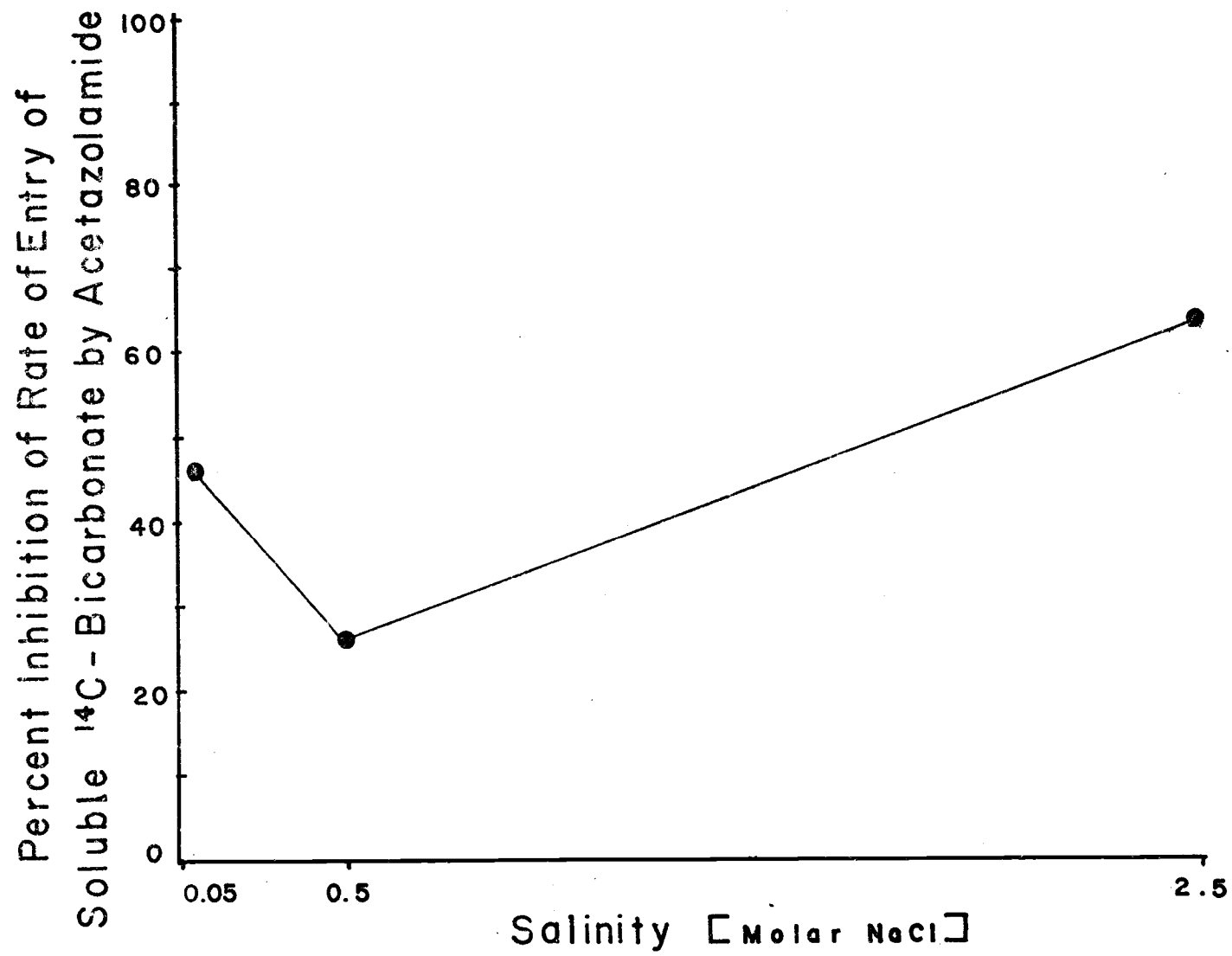


Figure 9. Salinity variable inhibition of saturation pool size of free  $^{14}\text{C}$ -bicarbonate into internal fluids of nauplii by acetazolamide.

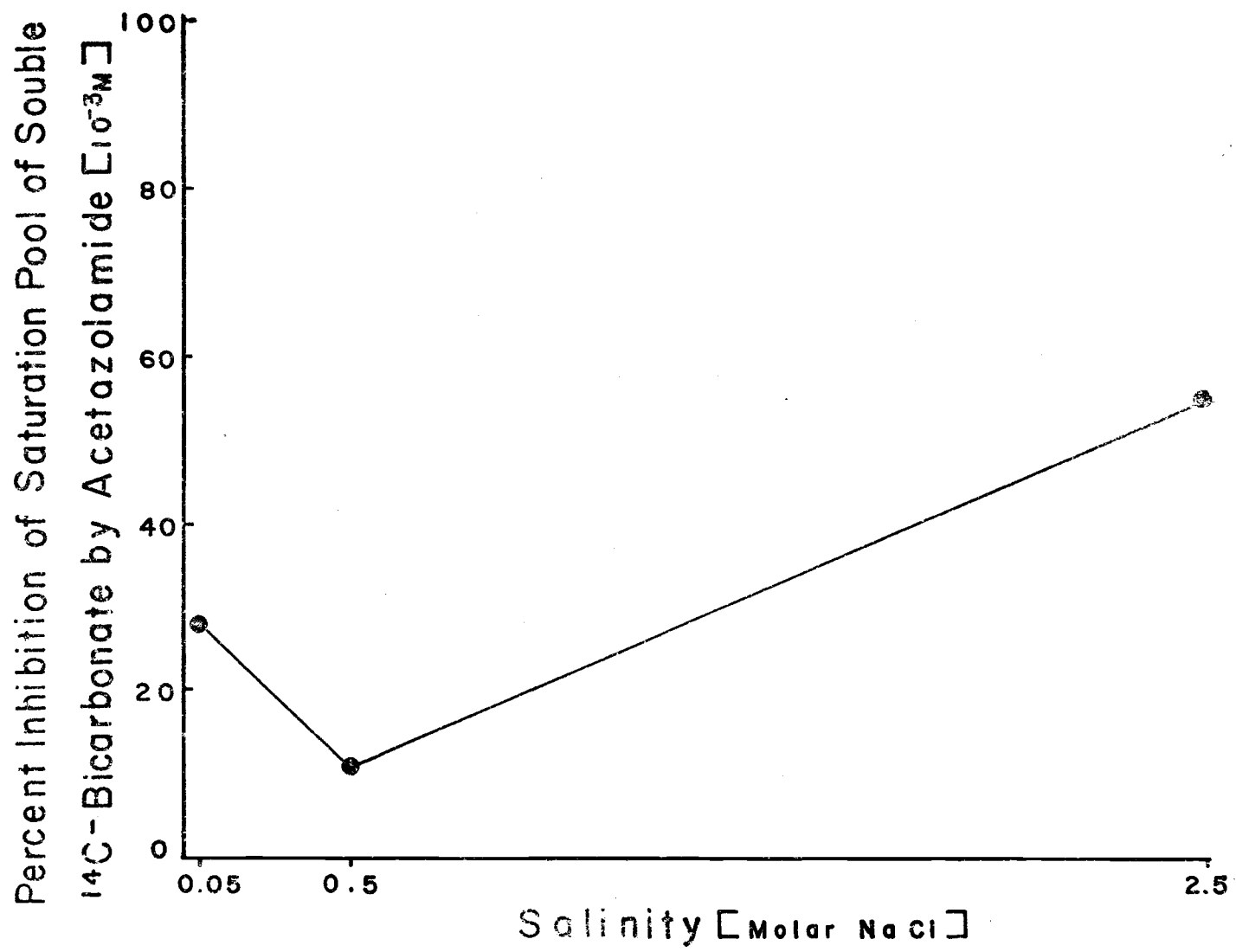


Figure 10. The incorporation of  $^{14}\text{CO}_2$  into  $\alpha$ -ketoacids of nauplii at various salinities. Open symbols are control media without inhibitor and shaded symbols are media with  $10^{-3}\text{M}$  acetazolamide.

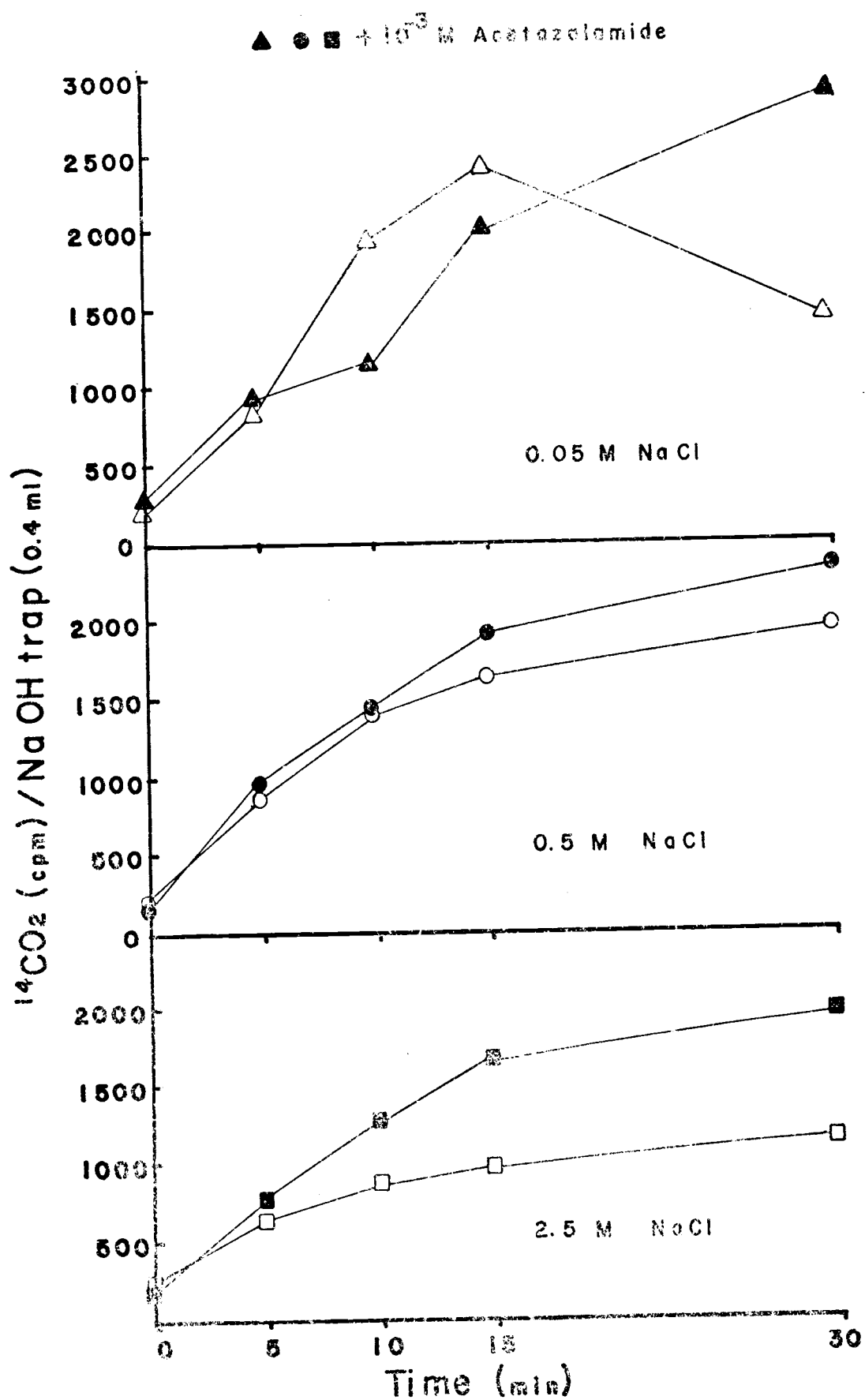




Figure 11. The entry of  $^{14}\text{CO}_2$  into naupliar bio-polymers.

# Incorporation of $^{14}\text{CO}_2$ in P.C.A. Insoluble Portion of Nauplii

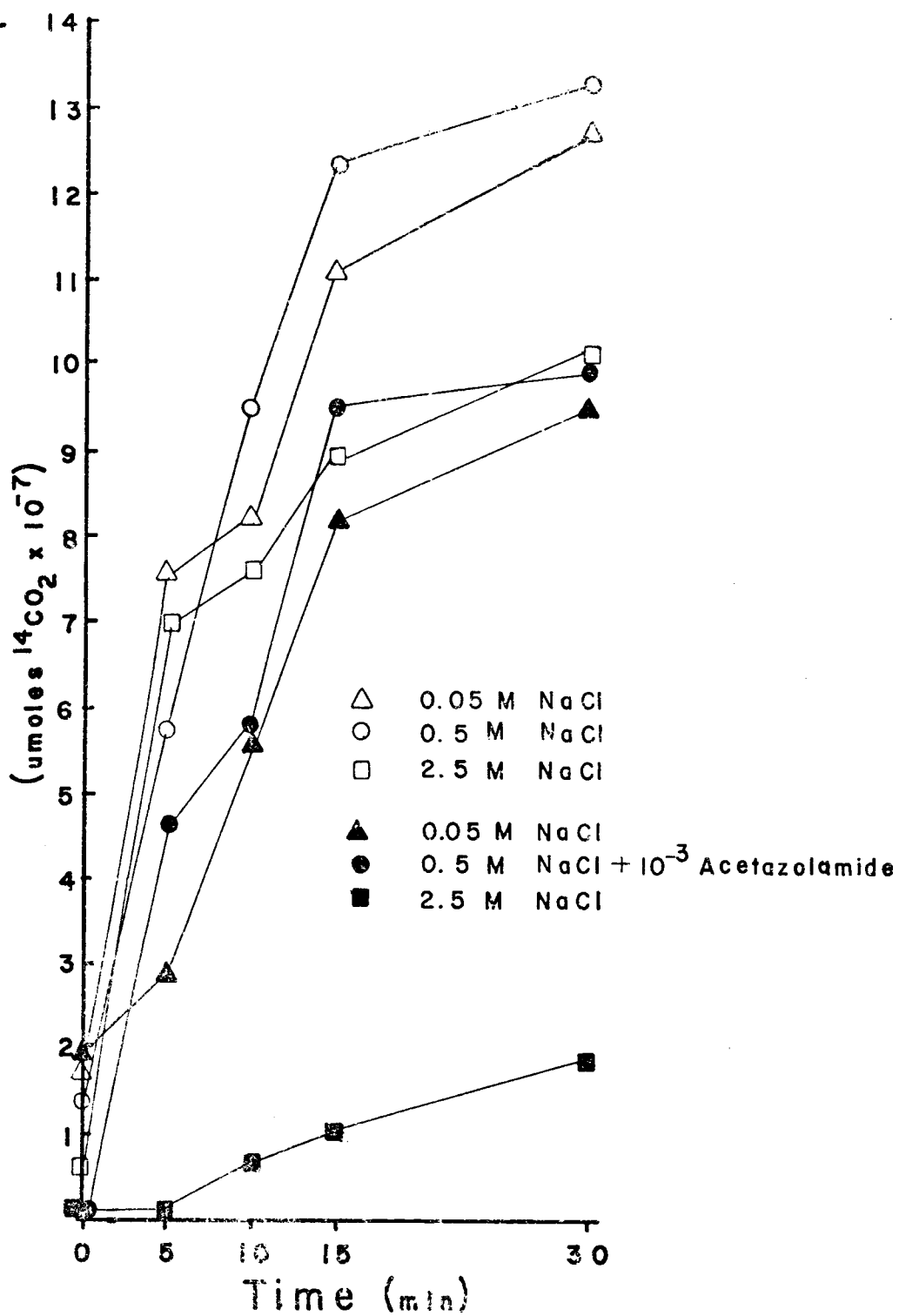


Figure 12. Salinity variable inhibition of rate of incorporation of  $^{14}\text{CO}_2$  into naupliar biopolymers by acetazolamide.

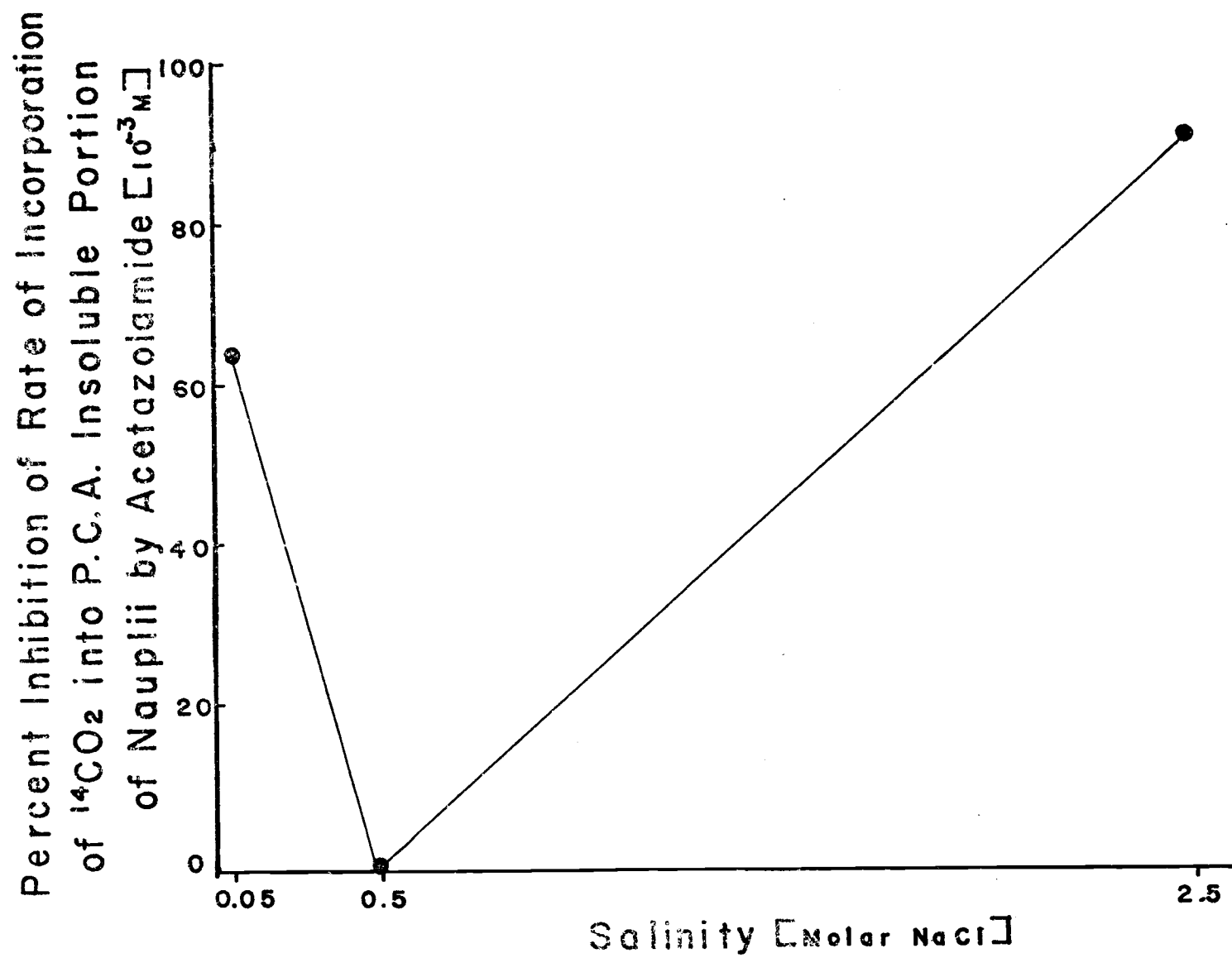
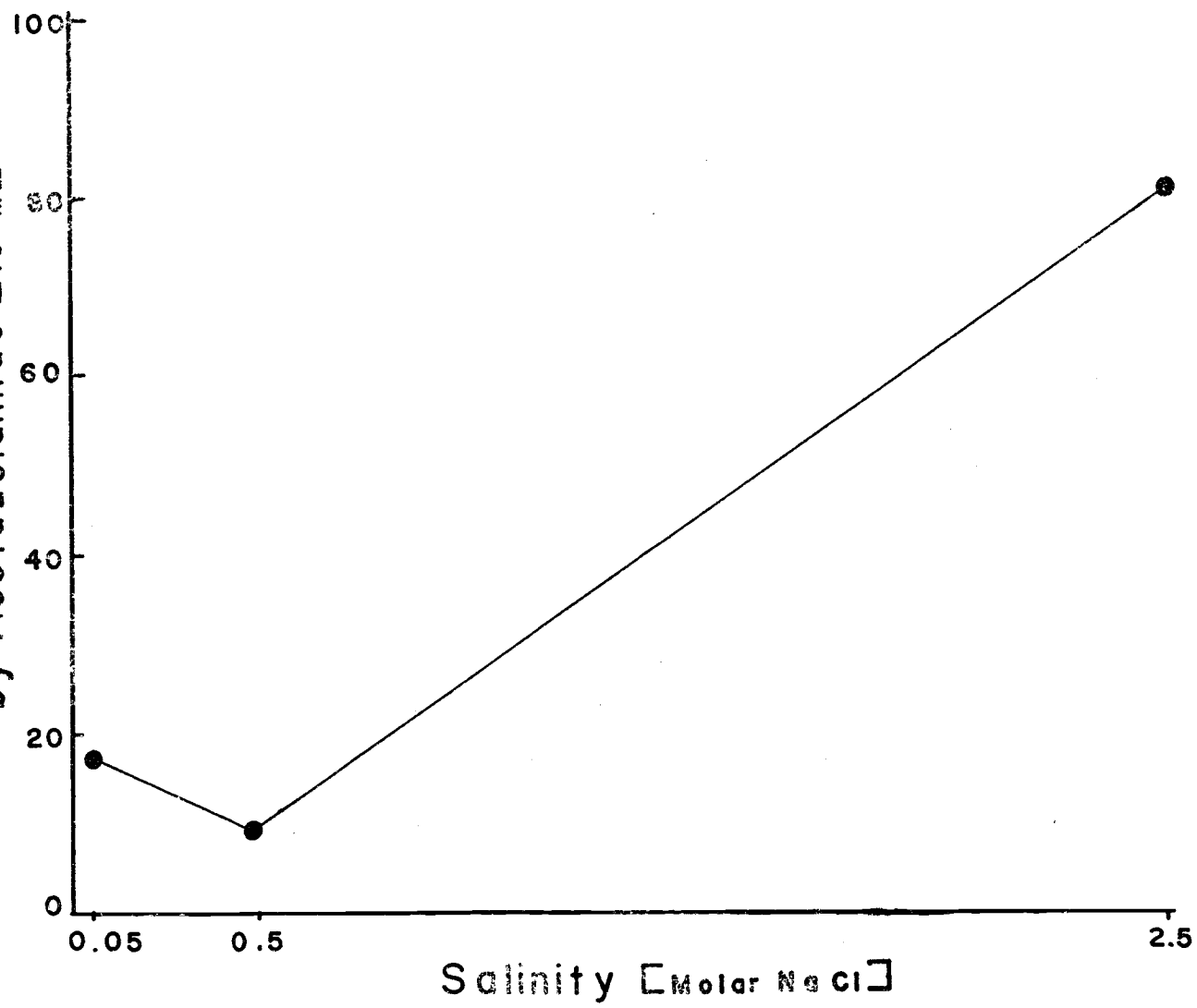


Figure 13. Salinity variable inhibition of saturation pool of  $^{14}\text{CO}_2$  naupliar incorporation in biopolymers by acetazolamide.

Percent Inhibition of Saturation Pool of  $^{14}\text{CO}_2$   
in P.C.A. Insoluble Portion of Nauplii  
by Acetazolamide [ $10^{-3}\text{M}$ ]



$10^{-3}$ M acetazolamide produced an inhibition of 80-90% of the control values. Therefore, the sulfonamide sensitive site appears to be at either the oxidation-reduction step of the  $\alpha$ -Keto acids or their transamination reactions into  $\alpha$ -amino acids.

## IV. DISCUSSION

The discovery of sulfonamide sensitivity in Artemia salina nauplii presented the interesting problem as to the mechanism of action. The molecular pathology suggests that interference occurs with the homeostatic processes involved in the water and electrolyte balance of the organism. The apparent specificity of sulfonamides in their inhibition of carbonic anhydrase (C.A.) is unique and is found throughout the biological domain (Mann and Keilen, 1940; Maren, etal., 1961, Maren, 1967; Coleman, 1973). The biological role of C.A. has been to increase the rate of CO<sub>2</sub> hydration which facilitates systems requiring a carrier for CO<sub>2</sub>, or to couple the active transport of bicarbonate with chloride movement across epithelial membranes (Imamura, 1970).

Since C.A. is a metalloenzyme that requires zinc as an integral part of the catalytic site, the inhibition of the enzyme by sulfonamides has been related to its interaction with the zinc atom. The sulfonamides show a coordination with the zinc atom such that the -SO<sub>2</sub>NH<sub>2</sub> group is a ligand to the Zn<sup>++</sup> moiety, Zn-SO<sub>2</sub>NH<sub>2</sub> (Lindskog, 1963). The CO<sub>2</sub> binds in an unstrained manner to the site next to the metal atom. Removal of the metallo-complex (i.e., treatment of the enzyme with 1,10 phenanthroline) results in failure of the apoenzyme to bind to sulfonamide (Coleman, 1973). Restoration of Zn<sup>++</sup> to the apoenzyme results in an active enzyme. The sulfonamides, including acetazolamide, during

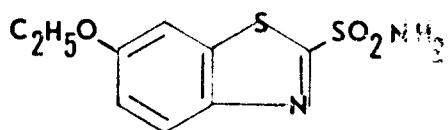


inhibition of C.A. behave as classic non-competitive inhibitors of the hydration reaction which allows for treatments in vivo to be done without rigorous knowledge of the  $\text{CO}_2$  concentration in the organism. To act as effective inhibitors the sulfonamides must have unsubstituted sulfonamide groups ( $-\text{SO}_2\text{NH}_2$ ) and must have bulky aromatic or heterocyclic R-groups attached to the free sulfonamide (Coleman, 1973). Maren (1967) systematized all of the sulfonamides which fulfill these initial requirements as to effectiveness against C.A. activity. He suggests that 1) heterocyclics are more active than benzyl groups, 2) in closely related sulfonamides the acetylated forms are more active (i.e., acetazolamide is more active due to acetylation of  $\text{NH}_3$ ), and 3) saturation of the 3-4 bond reduces activity against C.A.

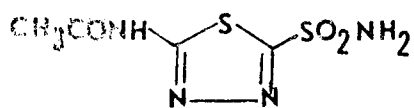
The antibacterial sulfonamides, with the exception of sulfanilamide, do not have C.A. inhibitory activity since there is a substitution of the primary amine group to a secondary amine group ( $\text{RSO}_2\text{NH}_2$  to  $\text{RSO}_2\text{NHR}'$ ). Also, there is a pH dependency of binding, in that the highest sulfonamide binding of C.A. is near neutrality, but with either high acidification or high alkalination binding of sulfonamide to C.A. decreases rapidly (Coleman, 1967a).

The present study evaluated five types of sulfonamides of known inhibition to C.A. The chemical structure of each of the compounds is shown in Figure 14. One finds that the

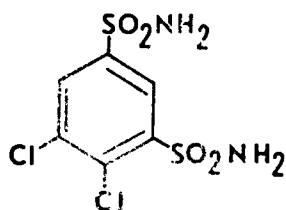
Figure 14. The structures of various  $\text{Zn}^{++}$  enzyme inhibitors used - sulfonamides.



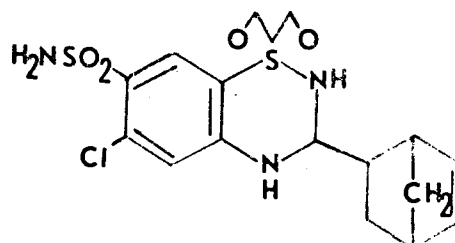
Ethoxzolamide



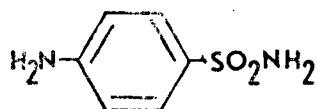
Acetazolamide



Dichlorophenamide



Cyclothiazide



Sulfanilamide

inhibitor with the least affinity for C.A. is sulfanilamide. It is not possible to assay for C.A. in an in vitro system with this weak inhibitor (Maren, et al., 1961). Dichlorophenamide and cyclothiazide are more potent inhibitors of C.A. which have greater affinity for the enzyme. These drugs are seldom used commercially but occasionally are found in drug compounds.

Acetazolamide, one of the commonly used C.A. inhibitors, is readily available and is a potent inhibitor with a high affinity for vertebrate C.A. Chronic administration in mammals causes general anorexia and metabolic acidosis. However, in most mammals, high doses lack toxicity because C.A. inhibition is essentially complete in all organs at very low doses (5-20mg/Kg animal). Apparently C.A. is not essential for life or physiological function in these organisms (Maren, 1967).

Ethoxzolamide is a more potent inhibitor of C.A. than acetazolamide in mammalian systems and the most potent sulfonamide assayed in Artemia salina nauplii. In the dog and rat ethoxzolamide can be lethal, although its only known property is C.A. inhibition (Maren, 1967). Ethoxzolamide distributes in enzyme-laden areas and in fat. The lipid sojourn of the drug may be responsible for the greater effect observed for low doses ( $5 \times 10^{-5} \text{M}$ ) than for acetazolamide in Artemia.

The use of the sulfonamides, particularly acetazolamide,

in C.A. studies is widespread. Localization of C.A. is often based on the  $Zn^{++}$  or  $Co^{++}$  metal ion-dependence of acetazolamide binding through the use of  $^3H$ -acetazolamide (Maren, 1967). Carbonic anhydrase plays an important role in gaseous exchange and electrolyte transport in aquatic gills (Van Goor, 1948; Maren, 1967). In fresh water fish gills Garcia-Romeu and Maetz (1964) found that  $Na^+$  exchanged with  $NH_3^+$  and  $Cl^-$  exchanged with  $HCO_3^-$  and that both utilized  $H^+$  produced by the hydration of  $CO_2$  catalyzed by C.A.

Some invertebrates utilize C.A. in the synthesis of  $CaCO_3$  from a  $CO_2$  substrate for shell formation (Polya and Wirtz, 1965). The mantles of molluscs, arthropods, and coelenterates contain C.A. Acetazolamide has been found to behave as a non-competitive inhibitor with oyster C.A., as it does with mammalian C.A. (Van Goor, 1948).

Plants also contain C.A. which is involved in water and electrolyte absorption, although initial reports indicated that plant C.A. did not contain zinc and was not inhibited by sulfonamides (Fellner, 1963). Tobin (1970) has presented new evidence which shows that parsley C.A. has one gram-atom zinc per 29,000 grams C.A. In addition, it is inhibited by acetazolamide. The amino acid composition of parsley C.A. is similar to that in mammalian forms although it differs by being composed of sub-units.

Experiments dealing with esterase activity of C.A. have shown the esterase to be effectively inhibited by

acetazolamide with a few exceptions. Sulfonamide sensitive esterase activity has been found in bovine erythrocyte C.A. (Pocker and Storm, 1968), guinea pig C.A. (Maren, 1967), and primate erythrocyte C.A. (Tashian, et al., 1964). The turnover rate for p-nitrophenol acetate is much slower ( $\times 10^{-4}$ ) than the hydration rate of  $\text{CO}_2$  (Armstrong, et al., 1966). Acetate has only a mild inhibitory effect on C.A. activity (Coleman, 1967b). It has been shown, however, that some C.A. preparations lack esterase activity, such as the primate kidney C.A. (Tashian, et al., 1964), canine erythrocyte C.A. (Maren, 1967), and plant C.A. (Tobin, 1970). These findings would indicate that crustacean C.A. might exist in a similar esterase activity-lacking form and that it is quite possible for brine shrimp nauplii to contain carbonic anhydrase. Further work utilizing manometric methods for measuring the hydration of  $\text{CO}_2$  should be done to clarify this point.

The presence or absence of C.A. in the nauplius is an important factor since it has been found that some tissues lacking C.A. are also sulfonamide-sensitive. There are receptors which bind sulfonamides in the frog cornea with the apparent physiological mechanism of maintaining chloride transport. When sulfonamide concentrations were brought to a level of  $10^{-3}\text{M}$  there was a decrease in electrical potential and a reduction of chloride transport (Kitahara, et al., 1967). In the frog gastric mucosa C.A. plays a role in acid secretion ( $\text{HCl}$ ). However, the rate of secretion of  $\text{H}^+$  is not

stoichiometric with the sulfonamide binding capability. It suggests that sulfonamide can be bound to other substances along with C.A. Again it appears that chloride transport is reduced with inhibition (Hogben, 1967). Another unresolved site of sulfonamide binding has been reported by Maren (1967), in that, red blood cells of the canine bind three times as much sulfonamide as expected when compared to C.A. content. In conclusion, it is quite possible that the sulfonamide mechanism of action can be associated with another type of metalloenzyme and not with C.A.

The lack of an effect by sulfonamides upon the oxidative metabolic cycle suggests the importance of the anerobic pathway in furnishing the necessary energy for maintaining the active transport of ions out of the nauplii. In addition, the uptake of bicarbonate into the cytoplasm and its conversion into  $\alpha$ -Keto acids is an ATP utilizing reaction. In both of these instances, the importance of sulfonamide-metal binding must be kept in mind. Therefore, an analysis of enzymes which contain  $Zn^{++}$  or  $Co^{++}$  that exist within these metabolic pathways is very important. Several distinct possibilities appear: a) malic dehydrogenase is a  $Zn^{++}$  containing enzyme found in the cytoplasm and mitochondrial matrix which catalyzes the conversion of malic acid to oxaloacetate, b) transcarboxylase is a  $Zn^{++}$  enzyme catalyzing the carboxylation of pyruvate by methylmalonyl CoA to form oxaloacetate and propionyl CoA (Northrop and Wood,

1969), c) phosphoenol pyruvate (PEP) carboxylase catalyzes the fixation of  $\text{HCO}_3^-$  with PEP to form oxaloacetate, and d) glutamic dehydrogenase catalyzes the conversion of glutamic acid to  $\alpha$ -ketoglutaric acid.

Phosphoenol pyruvate carboxylase is a  $\text{Co}^{++}$  containing enzyme but the lack of inhibition by acetazolamide upon the conversion of  $^{14}\text{C}$ -labeled bicarbonate into  $\alpha$ -ketoacids eliminates this enzyme as the site of action. The rate of entry of  $^{14}\text{C}$ -bicarbonate into the internal fluids of nauplii at various salinities was seen to be inhibited, particularly at low and high salinities. The inhibition of the entry rate could be due to a number of factors. It is not likely that acetazolamide is acting on an enzyme which establishes the electrical gradient. If movement inward were due to exchange diffusion, formation of bicarbonate internally could be accomplished by carbonic anhydrase. This would not result in the net change indicated. If the entry is carrier-mediated then there should be a compound coupled to bicarbonate entry. This seems to be the case, for the only requirement on the compound coupled to bicarbonate entry is that of being an anion that sequesters protons. There is evidence that brine shrimp secrete acid, but it is not proven whether the secretion by the nauplii is of a strong acid nature ( $\text{HCl}$ ) or the secretion is of a weak acid nature such as the monoketo acids (pyruvate or lactate) or the dicarboxylic acids. An analysis of  $\alpha$ -Keto acids in the media would be



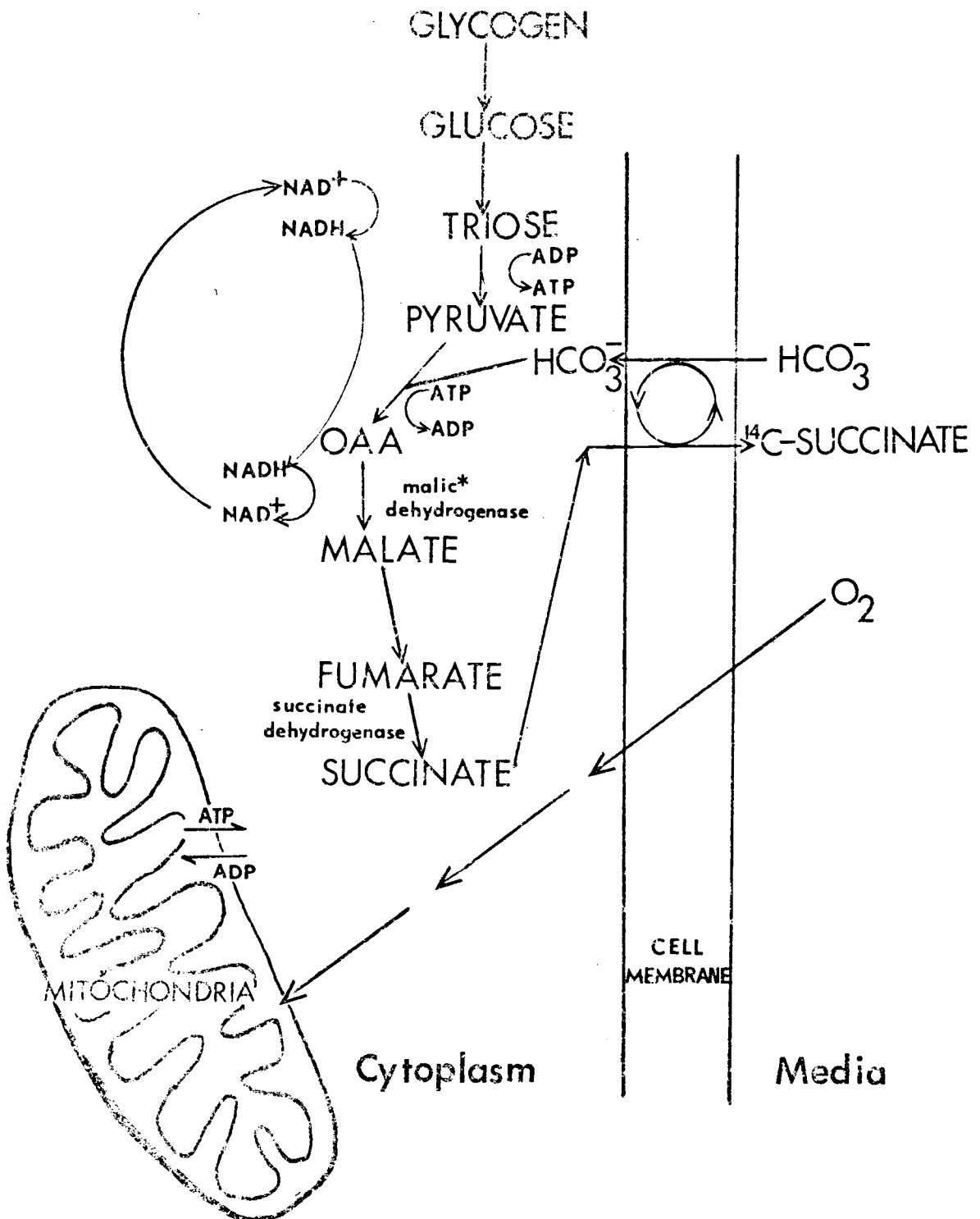
very useful in revealing the role of bicarbonate uptake.

After entry, the bicarbonate is metabolically acted upon and is fixed into organic precursors. The  $\alpha$ -Keto acids formed are seen to have accumulated quickly (Figure 10) and in the presence of inhibitor have begun to concentrate above control levels. The conversion of the keto acids (oxaloacetate and  $\alpha$ -ketoglutarate) and succinate to amino acids requires glutamine which results from decarboxylation. The carboxylase is not affected adversely by the acetazolamide since the  $^{14}\text{CO}_2$  is quickly and efficiently converted into the precursors for the formation of biopolymers. However, the rate is reduced (Figure 11) greatly at high salinities, which could be accounted for by the lack of ATP, producing the buildup of the  $\alpha$ -Keto acids. The formation of the purine and pyrimidine precursors requires a ligase reaction and ATP utilization. Unless ATP can be synthesized, the synthesis of the precursors is not possible.

It has been shown by Ewing and Conte (1975, to be published) that when the nauplius is under osmotic stress, ATP becomes limiting in nucleic acid synthesis and ADP builds up. The salt-dependent utilization of glycogen in Artemia nauplii, as shown by Edwards (1973), may be due to the facultative use of bicarbonate in anerobic mechanisms to synthesize ATP. For instance, in the synthesis of triose from glycogen,  $\text{NAD}^+$  is a cofactor required for ATP synthesis and is usually provided by the conversion of pyruvate to lactate

(Figure 15). In the presence of oxygen lactate synthesis is inhibited (Pasteur effect); therefore, there is a demand for a pathway to substitute for the pyruvate-lactate shunt to provide the  $\text{NAD}^+$ . The conversion of pyruvate to oxaloacetic acid provides the alternate route for the regeneration of the  $\text{NAD}^+$  cofactor. This permits recycling in the system and the production of ATP. It should be noted that of the enzymes in the facultative pathway of the cytosol, the only  $\text{Zn}^{++}$  containing enzymes are malic dehydrogenase and glutamic dehydrogenase. Glutamic dehydrogenase plays a role in the deamination of glutamate to form  $\alpha$ -ketoglutarate which, through decarboxylation, goes to succinate. This process, which is useful for ridding the animal of ammonia, would produce a basic media. The media in actuality became acidic. In summary, the site of action of the sulfonamides in Artemia salina nauplii appears to be at the malic dehydrogenase step which results in the buildup of oxaloacetate in the cytoplasm.

Figure 15. Facultative metabolic adaptation in response to changes in salinity (modified from Hochachka and Somero, 1973).



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