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Effects of Phenol on ATPase Activities in Crude Gill Homogenates of Rainbow Trout (*Salmo Gairdneri* Richardson)

T. M. Poston

January 1979

Prepared for the U.S. Department of Energy
under Contract EY-76-C-06-1830

Pacific Northwest Laboratory
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ABSTRACT

The purpose of this study was to examine the ATPase enzyme systems in crude gill homogenates of rainbow trout. These activities were used as indicators of potential toxicity with phenol, a significant by-product of the solvent refined coal process. Additionally, an anesthetic (MS-222) was tested with this enzyme system. The three activities assayed were Mg-baseline, total NaK-ATPase (non-ouabain sensitive) and "sodium pump" (ouabain sensitive NaK-ATPase).

Mg-baseline activity was assayed at 3.25 mM Mg^{+2}, pH 7.0 and 20°C. Total NaK-ATPase was assayed at 3.25 mM Mg^{+2}, 100 mM Na^{+}, 20 mM K^{+}, pH 7.0, 20°C and 37°C. Sodium pump activity was detectable at 37°C under the assay regime for total NaK-ATPase plus 0.5 mM ouabain.

Under these assay conditions, total NaK-ATPase was inhibited 50% by 18 mM phenol. Mg-baseline was inhibited 55% by 25 mM phenol. Sodium pump activity did not exhibit any sensitivity to phenol at concentrations as high as 25 mM. Inhibition of Mg-baseline and total NaK-ATPase was uncompetitive.

Concentrations of phenol which produced significant inhibition of ATPase activities exceed reported EC_{50} values by 2-3 orders of magnitude. This suggests that the toxic mode of action of phenol is not related to ionic or osmotic regulation. An experiment with a 96 hour *in vivo* exposure of rainbow trout to sublethal concentrations of phenol (2.3 $mg \cdot liter^{-1}$ and 4.7 $mg \cdot liter^{-1}$) was also conducted. Test fish became infested with an external parasite (*Gyrodactilus*) which precluded any concrete conclusion from the experiment. The toxic action of phenol is most likely unrelated to effects on these ATPase activities.

All ATPase activities were unaffected by MS-222 at concentrations ranging from 0.38 to 3.83 mM.

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INTRODUCTION

The advent of increasing energy demands has placed increasing burdens upon man and the environment. New energy technologies such as solar and geothermal are rapidly being developed. Major emphasis towards development of alternative energy (i.e., other than oil) sources is being directed at our vast coal and oil shale deposits. Development of these resources has been deemed necessary if our country is to become self sufficient in its energy requirements. Concomitant with the development and implementation of these technologies is the threat of accidental release of dangerous and toxic wastes to the environment. Chronic releases of low-levels of nondegradable pollutants may also adversely affect the environment.

Phenol is of particular interest in that the increased utilization of coal as an energy source may lead to higher environmental burdens of this toxic compound. Ongoing research at Pacific Northwest Laboratory (Petersen el al., 1976) has identified many of the lower molecular weight organic constituents of solvent refined coal (SRC) effluents. The SRC process involves the dissolving and subsequent demineralization of high mineral coal in a solvent (Howard-Smith and Werner, 1976). Organic sulphur is removed by hydrogenation to form hydrogen sulfide, which is then precipitated as a salt. The end product is a higher BTU coal which is essentially free of sulphur. SRC untreated effluents are characterized by several isomers of cresol and xylenol as well as methylated and ethylated phenolic compounds. Pure phenol is the predominant species, and the best candidate for initial investigations.

To evaluate the effects of industrial and energy production related waste and pollutants, initial efforts centered on determining acute doses capable of producing 50% mortality for a specified length of time. The acute bioassay approach circumvents any consideration of sublethal effects usually attributed to chronic long-term exposures of toxicants. Sprague (1976) recently criticized the present status of aquatic pollution assessment as inadequate with insufficient emphasis on the sublethal parameters, such as swimming performance, avoidance reactions, reproduction and internal physiological parameters.

The basic objectives of this study are: 1) to develop an assay procedure for adenosine triphosphatase (E.C.3.6.1.3; hereafter abbreviated as ATPase) in crude gill homogenate of rainbow trout, 2) to characterize the ATPase enzyme systems in crude gill homogenates; investigate parameters including cation stimulation, pH, temperature, linearity, and optimal substrate concentration, 3) to determine the *in vitro* effects of MS-222 on ATPase activities, 4) to determine the *in vitro* effects of phenol on the ATPase activities, and 5) to determine the *in vivo* effects of phenol. Enzyme activities reflected by ATPases have a physiological significance in osmoregulation in both the kidneys and gills of teleosts (Hochachka and Somero, 1973). They are also responsible for maintaining the Na^+ and K^+ gradient in nerve tissue which is required for neurotransmission (Prosser, 1973). ATPase has been shown to be coupled with oxidative phosphorylation in the mitochondria (Lehninger, 1975); hence, it affords a major source of ATP generation in metabolically active cells. The ubiquitous nature and diverse physiological functions of ATPase make this enzyme ideal for physiological and toxicologic investigations.

This assay procedure was applied towards the determination of effects of phenol and MS-222 (tricaine methanesulfonate, a fish anesthesia) on these activities. Classically, gill ATPase activities in teleosts emphasized the osmoregulatory function associated with transitions from freshwater to saltwater habitats. In this study, these activities are treated as general indicators of metabolic stress. Moreover, gill activities were selected because the gill is the initial site of absorbtion of toxicants in teleosts.

LITERATURE REVIEW

TELEOST ENZYMOLOGY AND TOXICOLOGY

One approach to the assessment of sublethal stress in fish is the inhibition (or activation) by pollutants on enzyme systems of aquatic organism. Bell (1968) and Racicot et al.(1975) examined the stimulation of asparate aminotransferase (EC 2.6.1.1) by intraperitoneal injection of CCl_4 and bromobenzene to *Oncorhynchus* spp. and *Salmo gairdneri*, respectively. Aberrations of enzyme activity were related to hepatic damage elicited by the toxicants.

However, intraperitoneal injection of any toxicant fails to realistically represent natural exposure conditions relative to effluent discharges.

Several investigators have looked at the effect of trace metals on different enzyme systems. Jackim (1973) reports a reduction in δ-amino levulinic acid dehydratase activity upon exposure to sublethal concentrations of lead in *Fundulus heteroclitus* and *Psuedopleuronectes americanus*. Hodson (1976) observed the same response in *Salmo gairdneri* in erythrocytes whereas Jackim (1973) studied liver and kidney extracts. Other trace metals studied include copper (Christensen et al., 1972; Jackim et al., 1970; Jackim, 1973), cadmium (Jackim, 1973; Jackim et al., 1970; Gould and Karolus, 1974; Wekell and Brown, 1973) and zinc (Jackim et al., 1970; Jackim, 1973; Brown, 1976).

Enzyme assays have also been indicative of pesticide contamination in the aquatic environment. Abou-Donia and Menzel (1967) have demonstrated 50% inhibition of fish brain (*Cymatogaster aggregata*) acetylcholinesterase (EC 3.1.1.7) by six carbamate insecticides. The *in vitro* assay concentrations of insecticides covered a range of $0.49 - 39.0 \times 10^{-7}$ molarity. Similar work with adenosine triphosphatase (EC 3.6.1.3) has been carried out with DDT, kepone and related compounds (Koch,1969/70; Cutkomp et al., 1970; Desaiah et al., 1975).

ATPASE ACTIVITIES

NaK-ATPase refers to the enzyme which transports sodium (Na^+) ions across a membrane in exchange for potassium (K^+) ions. This reaction also requires magnesium (Mg^{+2}) and is inhibited by ouabain, a cardiac glycoside. Bonting (1970) has summarized the relationship of NaK-ATPase and the energy coupled transport of Na^+ and K^+ across membranes. Both systems are characterized by the following similarities:

(1) localized in the membrane,
(2) Na^+ and K^+ are essential in combination (NH_4^+ can substitute for K^+),
(3) ATP is utilized (inosine triphosphate will not substitute for ATP),

(4) cardiac glycosides with an unsaturated lactone group inhibit both systems equally,

(5) cardiac glycoside inhibition is antagonized by K^+,

(6) half maximal activation concentrations of Na^+ and K^+ are the same.

The physiological significance of gill NaK-ATPase activities is attributed to ionic regulation. Changes in activity levels are classically associated with euryhaline (*Fundulus*), catadromous (*Anquilla*) on anadromous (*Salmo, Oncorhynchus*) species. Increased NaK-ATPase activities in salmonid sea bound juveniles (during parr-smolt transformation) have been related to seasonal variations (Zaugg and McLain, 1970), temperature (Adams et al., 1973; Zaugg and Wagner, 1973) and photoperiod (Zaugg and Wagner, 1973). This relationship of elevated NaK-ATPase activities and parr-smolt transformation has been utilized as a research tool for demonstrating copper toxicity at sublethal concentrations.

Lorz and McPherson (1976) have adequately demonstrated a significant inhibition of NaK-ATPase in *Oncorhynchus kitsutch* juveniles. Copper ($CuCl_2$) at concentrations of 20 $\mu g \cdot liter^{-1}$ induced a 50% *in vivo* inhibition in activity in freshwater adapted fish. Efforts to correlate this enzymatic inhibition with survival in acclimation from freshwater to seawater were significant; however, there was no demonstratable suppression of the ability to elevate NaK-ATPase activities once the exposed fish were transferred to seawater. NaK-ATPase was unaffected by sublethal concentrations of zinc in similar studies ran concurrently with the copper experiments.

Pfeiler and Kirschner (1972) have investigated the different alkali metal requirements of saltwater and freshwater adapted rainbow trout ATPases. Optimal conditions for saltwater adapted fish were 100 mM Na^+ and 20 mM K^+ (Mg^{+2} at 5.0 mM). This activity was ouabain-sensitive at 13°C. Freshwater adapted trout exhibited a requirement for 200 mM Na^+ and essentially no requirement of K^+ at 13°C. Ouabain sensitivity was demonstrated at 37°C, but not at 13°C. Enzyme preparations utilized deoxycholate and involved a microsomal preparation rather than a crude homogenate. Generally, Na^+ concentrations range from 100-125 mM and K^+ ranges from 18-25 mM in most other studies dealing with branchial NaK-ATPases.

NaK-ATPase activities are associated with chloride cells found in the branchial epithelium. In studies with the Japanese eel (*Anquilla japonica*), Kamiya (1972) and Shirai (1972) demonstrated that the higher levels of NaK-ATPase are associated with the smooth endoplasmic reticulum and plasma membrane of cholride cells.

There is some evidence that stimulation of NaK-ATPase activity is under hormonal control. Hypophysectomy in the euryhaline *Fundulus heteroclitus* reduced the levels of gill NaK-ATPase (Epstein et al., 1967). Pickford et al. (1970) were able to reverse this effect with injections of cortisol. In contradiction, Kamiya and Utida (1968) could not demonstrate a reduction in branchial NaK-ATPase related to hypophysectomy in *A. japonica*.

EFFECTS OF ORGANOCHLORIDE COMPOUNDS ON ATPASE

Desaiah and Koch (1975) assayed *in vitro* brain ATPase activities of *Ictalurus punctatus* to assess the effects of kepone and decachloropentacyclodecan-5-ol (DCPN), an organochlorine pesticide and its reduction product. Mitochondrial Mg-ATPase activities were found to be more sensitive than both NaK-ATPase and nonmitochondrial Mg-ATPase. A greater than 50% inhibition was observed at 2.5 μM kepone for mitochondrial Mg-ATPase and 10 μM kepone for NaK-ATPase. Cutkomp et al. (1971) showed a distinct *in vitro* inhibition of Mg-ATPase and NaK-ATPase in *Lepomis macrochirus* in several tissue homogenates by DDT and TDE. A synergistic effect was noted when DDT and dicofol were combined in muscle ATPases. Kelthane (a miticide) and chlordane have shown to be inhibitory to the ATPase systems of brain, liver and muscle tissue in lake trout (Koch, 1969/70).

In vivo experiments with DDT have been conducted with rainbow trout (Campbell et al., 1974; Leadem et al., 1974) and fathead minnow (Desaiah et al., 1975) demonstrating both inhibition and stimulation of ATPase activities. Gill NaK-ATPase activities were inhibited 33% and 67% by 0.3 mg DDT (added by gelatin capsule in alternating days for 2 weeks) in fresh and saltwater adapted fish, respectively. Kidney NaK-ATPase activities exhibited significant inhibition; however, there was no apparent correlation with salinity. Leadem et al. (1974) demonstrated a negative correlation between gill NaK-ATPase and serum sodium in saltwater adapted rainbow trout.

Microsomal Mg-baseline and NaK-ATPase activities in brain tissue of the fathead minnow were stimulated by long-term chronic exposure to DDT (Desaiah et al., 1975). However, gill NaK-ATPase (mitochrondial and microsomal) appeared to be inhibited *in vivo* by DDT at 225 and 266 days exposure.

ASSAY CONDITIONS

There are three basic approaches to assaying NaK-ATPase. Total activity is determined initially with adequate amounts of Na^+, K^+, and Mg^{+2} ions (from their respective chloride salts). A second assay conducted concurrently determines the activity of Mg-ATPase. Subtracting the Mg^{+2} stimulated ATPase activity from the total activity (Na^+, K^+, Mg^{+2}) would yield NaK-ATPase activity. Mg-ATPase activity may be determined in three ways. Ouabain can be added to one assay to determine the Mg-ATPase activity (Post and Sen, 1967). Another method utilized by many researchers in gill ATPase involves removing Na^+ and K^+ (or just K^+ ions) from one assay solution to isolate Mg-ATPase activity (Epstein et al., 1967; Jampol and Epstein, 1970; Kamiya and Utida, 1969; and Zaugg and McLain, 1970). Pfeiler and Kirschner (1972) report an independent Na^+-stimulated (Na-ATPase) activity which does not require potassium. This Na-ATPase activity was found to be insensitive to ouabain inhibition at a 5×10^{-4} Molar concentration. They in turn found that a 30 minute thermal preincubation of the assay mixture (prior to the addition of ATP to initiate the reaction) effectively removed the Mg-ATPase baseline activity allowing for the assay of NaK-ATPase (and Na-ATPase) activities.

Other assay parameters vary quite noticeably. Teleost ATPase activities have been assayed at temperatures ranging from 13°C (Pfeiler and Kirschner, 1972) to 37° (Epstein et al., 1967). Sodium ion concentrations range from 50 mM (Zaugg and McLain, 1970) to 200 mM (Pfeiler and Kirschner, 1972) and potassium ion concentrations ranged from 10 mM (Zaugg and McLain, 1970) to

20 mM (Jampol and Epstein, 1970). Assay pH ranged from 7.0 (Zaugg and McLain, 1970) to 7.8 (Jampol and Epstein, 1970). Obviously, this great variation in assay conditions reported in the literature restricts the comparison and interpretation of the results.

MS-222

MS-222 (tricaine methanesulfonate) has been implicated in the inhibition of nueronal discharge by preventing the selective increase in sodium permeability of the neural membrane observed during excitation (Strobel and Wollman, 1969). In many of the above studies, MS-222 was used as an anesthesia with no reference as to its effect (or lack of effect) on ATPase activities. The fact that ATPase activities are associated with cellular membrane (lipid) material coupled with the demonstrated sensitivity to highly lipid soluble organochloride pesticides indicates a possible vulnerability of this enzyme to other lipid soluble organic coumpounds. Ethyl m-amino benzoic acid, the anesthetic component of MS-222, is insoluble in aqueous solutions at neutral pH, hence, lipid bound enzyme systems may display an increased sensitivity to MS-222 due to its high degree of lipid solubility.

LITERATURE SUMMARY

Pertinent aspects of the reviewed literature are presented below:

- Gill ATPase activities are generally associated with freshwater-saltwater transitions of anadramous, catadramous and euryhaline species. NaK-ATPase activities increase during this transition, and this phenomenon may be under hormonal control.
- *In vitro* and *in vivo* sensitivity of ATPase activities has been demonstrated in a number of teleosts for organochloride compounds and copper. ATPase activities were inhibited by both these compounds in the micromolar range.
- MS-222 may affect the permeability of nueronal membranes to Na^+ and/or K^+. This observation may indicate a potential effect of MS-222 on NaK-ATPase activities.

- Methodologies vary between investigators on assay procedures for ATPase activities. Determination of NaK-ATPase has involved either addition of ouabain or elimination of K^+ in the reaction medium. These major differences coupled with the possible existence of a non-ouabain sensitive NaK-ATPase activity confuses the interpretation of the literature.

NOMENCLATURE

It is obvious from reviewing the literature that there are inconsistencies in the use of terms which describe ATPase activities. For this reason, I have defined the terms for target ATPase activities as follows:

- Mg-Baseline--the amount of activity stimulated solely by magnesium ions.
- Total NaK-ATPase-- the total amount of detectable activity observed in the presence of magnesium, sodium and potassium ions.
- Sodium pump activity--the fraction of activity stimulated by sodium, potassium and magnesium which is inhibited by 0.5 mM ouabain, a cardiac glycoside.

METHODS AND MATERIALS

EXPERIMENTAL ORGANISMS

Two separate stocks of rainbow trout (*Salmo gairdneri* Richardson) were utilized during this study. Enzyme characterization, as well as *in vitro* studies with MS-222 and phenol, used a stock raised at Hanford. This stock hatched in December of 1976 and was maintained on ambient Columbia River water until August of 1977, at which time the temperature was maintained at 10-13°C for the remainder of the study. A subsample (n=10) of this stock of fish averaged 18.7 cm (standard deviation of 1.2 cm) fork length and weighed 79 grams (standard deviation of 12 g) when the study terminated.

Smaller fish were required for the *in vivo* exposure due to space limitations of the exposure system. These fish were obtained from the Trout Lodge Hatchery in Soap Lake, Washington, one month prior to the *in vivo* exposure. This stock was acclimated from 9°C to 15°C over a 10-day period and maintained at that temperature on Columbia River water. The average weight and length (± one standard deviation) of 10 fish subsampled from this stock was 32 ± 7 grams and 14.7 ± 1.3 cm, respectively. Both stocks were fed Silver Cup Trout Feed during the course of the study.

PREPARATION OF CRUDE HOMOGENATES

Each fish was stunned with a light blow to the head. The spinal column was immediately severed and the head was tipped forward exposing the branchial basket. Blood was removed by perfusing, via the ventral aorta, with 2.5 ml of chilled homogenizing buffer (0.3M sucrose, 0.03 M Mg_2EDTA, 0.1M imidazole, 0.001 M 2-mercaptoethanol, pH 7.5). Each gill arch was removed separately. The filaments were trimmed from the arch, blotted to remove excess homogenizing buffer and weighed.

The tissue was then homogenized in the homogenizing buffer at a ratio of 1.0 g tissue to 10.0 mls homogenizing buffer. A 10 ml Thomas tissue grinder operated at approximately 1,700 rpms was used to grind the tissue samples. Coarse filaments were removed by centrifuging the sample at 1600 x g for one minute. The supernatant was then assayed as the crude homogenate. All of the above operations were conducted at 4°C. All enzyme assays were conducted the same day of sampling.

PREPARATION OF TRIS-ATP

Sodium-free preparations of ATP were obtained by anion exchange at 4°C. One and six tenths grams of Na_2ATP (Sigma, equine muscle) was loaded on to 9.0 mls of wet anion exchange resin (Bio-Rad AG1-X4, 100-200 mesh, chloride form). Disposable 10 ml serological pipets plugged with glass wool were used for columns. ADP and inorganic phosphate (P_i) residues were eluted with 80 mls of 0.02 N NH_4Cl in 0.02 N HCl. The column was rinsed with 80 ml of glass distilled water prior to elution of ATP with 50 mls of 0.3 N HCl. The ATP solution was adjusted to a pH of 7.0 with tris (hydroxymethyl) aminomethane. ATP concentrations were determined at 259 nm with a molar absorption coefficient of 14,500.

ASSAY CONDITIONS

All reaction mixtures were buffered with 10 mM imidazole. Tests prior to the establishment of a pH optimum were conducted at pH of 7.5; whereas, those following this test were conducted at pH of 7.0. All assays were conducted in duplicate.

The three target ATPase activities were sampled with cations of the following concentration:

- Mg-baseline activity: Preliminary tests and the *in vivo* exposure were conducted at 6 mM Mg^{+2}. All other tests were conducted at 3.25 mM Mg^{+2}. Magnesium ion (2.73 mM-theoretical) was derived from the homogenizing buffer. Any additional contribution of Mg^{+2} was derived from the chloride salt. Mg-baseline tests were conducted at 20°C.
- Total NaK-ATPase activity: Concentrations of Na^+ and K^+ were 100 mM and 20 mM, respectively. Each was added as the chloride salt. Mg^{+2} was derived in the same manner as described for Mg-baseline activity. Total NaK-ATPase activities were measured at 20°C and 37°C.
- Sodium pump ATPase activity: This reaction mixture was identical with the reaction mixture for total NaK-ATPase activity except that 0.5 mM ouabain was present. Sodium pump activity was expressed as the difference between total NaK-ATPase activity and the activity observed in the presence of 0.5 mM ouabain in a duplicate set of assay tubes. Sodium pump activity was assayed at 37°C.

Aliquots of 1.7 ml of the appropriate reaction mixture were dispensed into 15 ml disposable polystyrene centrifuge tubes. Initially, 0.1 ml of the crude homogenate was added and the reaction was initiated by the addition of 0.2 ml of 30 mM Tris-ATP. Tests involving the effect of increasing temperature showed that Mg-baseline activity was thermally labile. Tests subsequent to thermal tests were initiated by the addition of 0.1 ml crude homogenate to the reaction mixture with Tris-ATP already added (final concentration of ATP was 3.0 mM).

The duration of the assays ranged from 15 to 25 min depending upon the quantity of tubes required to perform the test. The reactions were terminated by the addition of 1.0 ml of 18% trichloroacetic acid and by transferring each reaction vessel to an ice water bath.

Inorganic phosphate (P_i) was determined by the method of Gomori (1942). The chilled tubes were removed from the ice bath and 3.5 mls of sodium molybdate-sulfuric acid reagent was dispensed to each tube followed by 1.0 ml of Elon's reagent (1.0 g of p-methylaminophenol sulphate per 100 mls of 3% sodium bisulfite). The final volume for P_i determinations was 7.5 mls whereas Gomori's (1942) procedure resulted in a final volume of 15.0 mls. Figure 1 represents standard curves for both the 15 ml and 7.5 ml final volume.

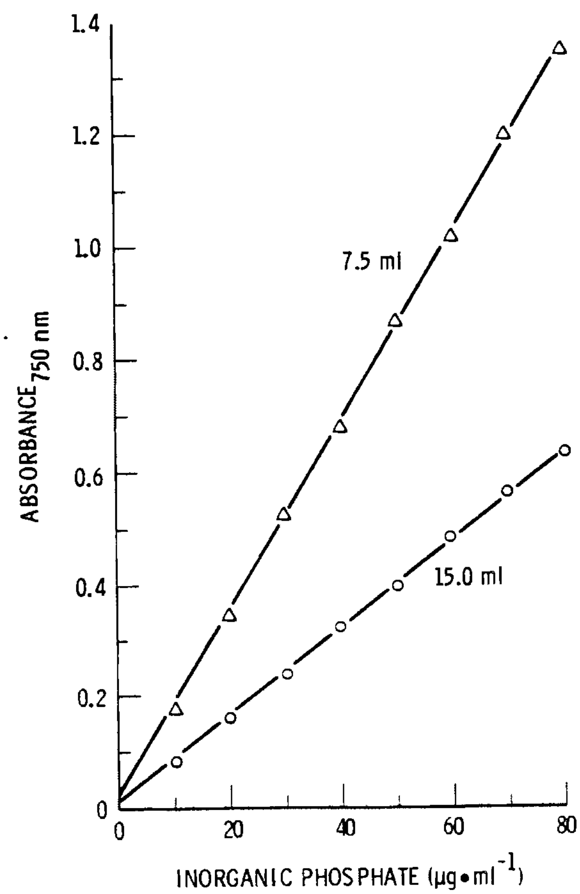


FIGURE 1. Standard curves for inorganic phosphate at final volumes of 7.5 mls and 15.0 mls.

Each tube was thoroughly mixed and centrifuged at 1600 x *g* to sedimentate the precipitated protein. Spectrophotometric measurements for P_i assays and protein determinations (Lowry et al., 1951) were conducted with a Beckman DB25 spectrophotometer (750 nm) equipped with a clinical sipper. The crude homogenates were diluted 1:125 and the amount of protein per assay ranged from 0.3 to 0.5 mg protein per assay tube. All assay tubes were blanked against boiled crude homogenates.

EXPRESSION OF ACTIVITY

ATPase activities are expressed in terms of μ mole P_i liberated per mg protein per hr ($\mu m\ P_i \cdot mg^{-1}$ protein $\cdot\ hr^{-1}$). In practice, P_i in μg liberated during the course of the reaction was multiplied by a specific activity factor (SAF).

$$SAF = \frac{1 \quad \mu \text{ mole P}}{30.9738 \quad \mu\text{g P}} \times \frac{60 \text{ min}}{\text{duration of assay (15-25 min)}} \times \frac{1}{\text{mg protein per assay}}$$

ENZYME CHARACTERIZATION

Modifications of the assay procedure were required to perform some of the enzyme characterization experiments.

- pH Optimum: Magnesium ion concentrations for pH optimums were 6 mM. The assay system included 60 mM tris in addition to 10 mM imidazole. NaK-ATPase activities and sodium pump activities were buffered with 10 mM imidazole. Alkaline pH values were achieved by using sodium hydroxide as a source of sodium ions rather than sodium chloride.

- Cation Stimulation: Magnesium stimulation was initially tested on homogenates in which Mg_2EDTA had been excluded from the homogenizing buffer. A second test was performed to demonstrate the effect of Mg^{+2} ion in the presence of EDTA to determine the chelating effect of EDTA. Both tests were performed at 20°C. Sodium ion was varied between 0 and 300 mM in the presence of 20 mM KCl and potassium was varied from 0 to

40 mM in the presence of 100 mM NaCl for alkali metal stimulation experiments. Magnesium ion was 6 mM for both alkali metal tests. These tests were performed at 37°C.

- Temperature Effects: Assays for all activities were determined at 13°C, 20°C, 25°C, 30°C and 37°C. Results were graphically expressed with Arrhenius plots.

In Vitro EFFECTS OF MS-222

Reaction mixtures were prepared with MS-222 at concentrations of 0 to 1000 mg•liter^{-1} (0 to 3.83 mM). One-tenth ml aliquots of MS-222 stock solutions were dispensed to assay tubes containing 1.8 ml of reaction mixture and tris-ATP. Disassociation of MS-222 inparts one mole of H^+ per mole of MS-222 added to the reaction mixture. This imparted a final pH to the complete reaction mixture of 6.8; however, a pH of 7.0 was attained by adjusting the homogenizing buffer to 7.6 rather than 7.5.

In Vitro effects of MS-222 were assayed at 20°C for Mg-baseline and total NaK-ATPase activity and at 37°C for total NaK-ATPase and sodium pump activity.

In Vitro EFFECTS OF PHENOL

Demonstration of the inhibitory effects of phenol was conducted over the range of 0 to 25 mM phenol. Mg-baseline activities were determined at 20°C. Total NaK-ATPase was assayed at 20°C and 37°C whereas sodium pump activities were determined at 37°C. A preliminary screening test (Test I) and a confirmatory replication test (Test II) were performed. The mode of inhibition was determined for Mg-baseline and NaK-ATPase activities at 20°C in subsequent experiments (Eadie-Hofstee plots).

In Vivo EFFECTS OF PHENOL

Data from the *in vitro* experiments indicated little chance of detecting changes in ATPase activity at sublethal concentrations of phenol. However, a preliminary exposure with one control and two levels of phenol was conducted.

Figure 2 is a schematic of the toxicant delivery system. Each 51.5 liter aquaria received 2000 $ml \cdot min^{-1}$ of Columbia River water. A Mariotte bottle was set at an average drip rate of 2.5 $ml \cdot min^{-1}$ into a mixing chamber which received 1500 $ml \cdot min^{-1}$ from the head tank. Flows from the mixing chamber were set at 1000 $ml \cdot min^{-1}$ and 500 $ml \cdot min^{-1}$ and were combined with 1000 $ml \cdot min^{-1}$ and 1500 $ml \cdot min^{-1}$ river water, respectively, from the head tank prior to delivery to the aquaria. Stock solutions of phenol were prepared at 7.2 $g \cdot liter^{-1}$ resulting in theoretical concentrations of 12 $mg \cdot liter^{-1}$ in the mixing chambers and 6 $mg \cdot liter^{-1}$ (high concentration) and 3 $mg \cdot liter^{-1}$ (low concentration) in the respective aquaria. The foil-wrapped Mariotte bottle was replenished daily with fresh stock solution. The aquaria were shielded from laboratory distractions with a black plastic curtain for the duration of the exposure.

Temperature, pH and dissolved oxygen (determined by YSI Model 51-B, D.O. meter) were monitored daily in each aquarium. Diluent (Columbia River water heated to 15°C) was also checked on a daily basis for alkalinity (APHA, 1975) and EDTA total hardness (APHA, 1975). Phenol concentrations were monitored daily by spectrophotometry (Method 510 C, APHA, 1975). In unpublished data, Schneider (personal communication) has demonstrated that the chloroform extraction step may be bypassed for relatively clean water systems such as the upper and mid Columbia River.

Ten trout were loaded into each aquaria. Five fish from each aquaria were to be sampled at 24 hr and again at 96 hr. This stock of fish incurred an infestation of *Gyrodactylus* which became manifested in those fish exposed to phenol. This resulted in two mortalities at the high concentration and severe loss of equilibrium of one fish at the low concentration (by 96 hr). Control fish also displayed some stress from *Gyrodactylus* and some mortalities were observed in the original stock of fish. Surviving fish were sampled as originally scheduled.

All activities were assayed as described for the *in vitro* exposures with the exception that 6 mM magnesium was inadvertently used in the reaction mixture.

Water quality monitoring data are reported in Table 1.

STATISTICAL ANALYSIS

Two separate hypotheses were examined with a Student's t test. Mean specific activities for test groups were tested against control groups for Mg-baseline and total NaK-ATPase activities. Sodium pump activity, due to its low level of detectable activity, was tested to see if sample means were significantly greater than zero (Mendenhall, 1971).

Population variances were tested with an F test statistic to determine homoscedastity of the test groups. Data for the *in vivo* exposure were L_e transformed due to heteroscedastity. Significance was reported at $\alpha \leq 0.05$ for all tests.

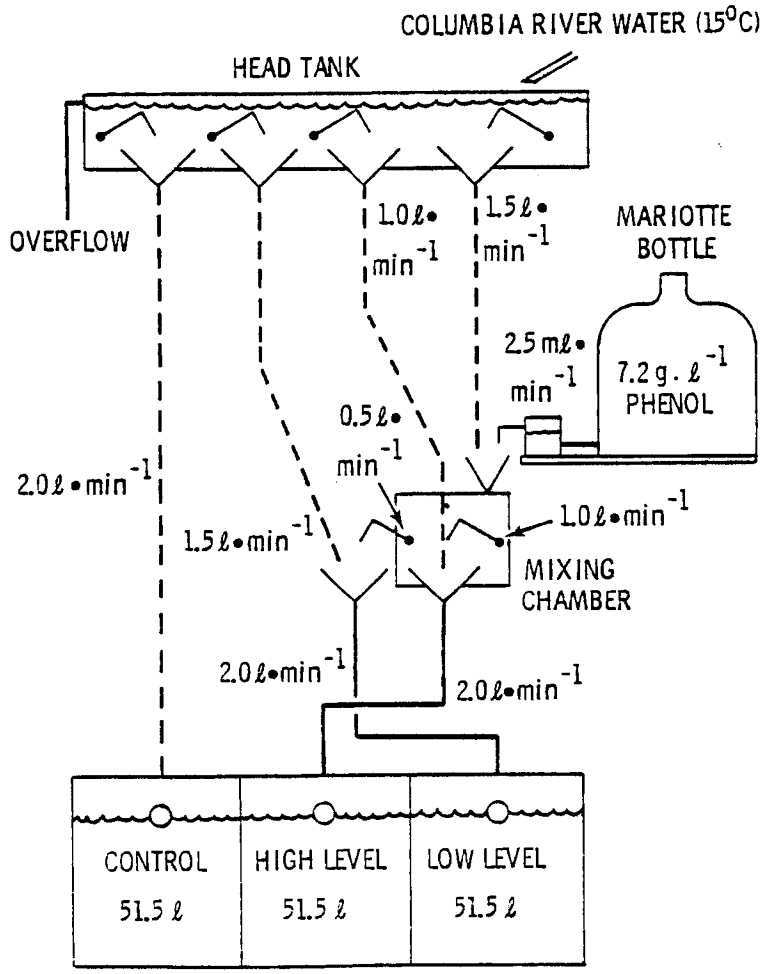


FIGURE 2. Schematic of the toxicant delivery system employed for exposure of trout to phenol.

TABLE 1. Water Quality Parameters of *in vivo* Exposure

Date	3-15	3-16	3-17	3-18	3-19	Average or Range
Diluent (Columbia River Water) Monitoring						
Alkalinity ($mg \cdot l^{-1} CaCO_3$)	63.2	61.9	64.5	64.7	65.2	63.9
EDTA Hardness ($mg \cdot l^{-1} CaCO_3$)	75.5	77.0	79.0	75.1	76.1	76.5
Aquarium Monitoring						
Control						
pH	7.7	8.2	8.1	8.1	8.2	7.7 - 8.2
DO ($mg \cdot l^{-1}$)	10.0	10.2	9.7	9.9	10.1	10.0
Temp (°C)	14.6	15.4	14.9	15.0	15.2	15.0
Low						
pH	7.7	8.2	8.1	8.0	8.1	7.7 - 8.2
DO ($mg \cdot l^{-1}$)	10.1	10.2	9.6	10.0	10.1	10.0
Temp (°C)	14.6	15.4	14.9	15.0	15.2	15.0
High						
pH	7.7	8.2	8.1	8.0	8.1	7.7 - 8.2
DO ($mg \cdot l^{-1}$)	10.1	10.2	9.6	9.9	10.1	9.9
Temp (°C)	14.6	15.4	14.9	15.0	15.2	15.0
Phenol						
Control	0	0	0	0	0	0
Low	2.3	2.6	2.4	2.2	1.9	2.3
High	4.5	5.5	4.9	4.6	4.0	4.7

RESULTS

RANGE OF ACTIVITIES

An example of the range of specific activity values of ATPases rainbow trout (Hanford raised) is given in Table 2. These data are drawn from several experiments and indicates the variabilities associated with the activities under investigation. Note that sodium pump activity was not detectable at 20°C. At 37°C, the activity was demonstratable but excessively small with high relative variability. Total NaK-ATPase activity was demonstratable at 20°C with 3.25 mM Mg^{+2} and not at 37°C. At 6 mM Mg^{+2}, total NaK-ATPase could not be demonstrated.

PRECISION OF TECHNIQUES

One sample of gill homogenate was assayed for Mg-baseline and total NaK-ATPase (n=10 for each activity) to determine the precision of the organic phosphate assay (Gomori, 1942). Ten samples were also analyzed for protein to determine the precision of the Lowry method (Lowry et al., 1951). Results are listed in Table 3. Figure 3 shows the effect of increasing dilution on protein determination. The optimal dilution ranged between 1:100 to 1:250. Within this range, six replicate samples had 5.8 ± 0.2 $\mu g \cdot ml^{-1}$ protein (mean ± 1.0 S.D.).

LINEARITY OF REACTIONS

Figure 4 and 5 exhibit linearity of the absorbance (750nm) over a 25 min span for Mg-baseline (20°C), NaK-ATPase (37°C) and sodium pump (37°C) activities. Lines were drawn by inspection.

TEMPERATURE EFFECTS

At 13°C, 20°C and 25°C, total NaK-ATPase activity significantly exceeds Mg-baseline activity (Table 4). However, as temperatures increased, these activities converge upon the same value (Figure 6). Sodium pump activity was solely demonstratable at 37°C. A Student's t test performed on sodium pump activity (H_0:μ=0) was significant (α=0.05) at 37°C. All other temperatures produced nonsignificant t-statistics (Table 5).

TABLE 2. Specific Activities (μ mole $P_i \bullet mg^{-1}$ Protein$\bullet hr^{-1}$) for Hanford Raised Rainbow Trout (*Salmo gairdneri*)

Temp.	pH	Specific Activity[a]	RSD[d]
		Mg Baseline	
20°C	7.5	8.3 ± 1.5 (5)[b]	18%
37°C	7.5	6.4 ± 1.0 (5)[b]	16%
20°C	7.0	1.9 ± 0.4 (6)[c]	23%
20°C	7.0	2.1 ± 0.2 (8)[c]	10%
20°C	7.0	4.0 ± 0.5 (4)[c]	12%
37°C	7.0	5.8 ± 1.2 (6)[c]	21%
		Total NaK-ATPase	
20°C	7.5	7.1 ± 1.1 (5)[b]	15%
37°C	7.5	7.3 ± 1.2 (5)[b]	16%
20°C	7.0	2.6 ± 0.4 (8)[c]	16%
20°C	7.0	3.9 ± 0.6 (8)[c]	16%
20°C	7.0	2.9 ± 0.5 (8)[c]	18%
37°C	7.0	5.9 ± 1.2 (6)[c]	20%
37°C	7.0	4.6 ± 0.5 (8)[c]	12%
37°C	7.0	6.4 ± 0.5 (8)[c]	8%
		Sodium Pump	
20°C	7.5	-0.3 ± 0.8 (5)[b]	-267%
37°C	7.5	0.8 ± 0.4 (5)[b]	50%
37°C	7.0	0.3 ± 0.2 (6)[c]	67%
37°C	7.0	0.6 ± 0.3 (8)[c]	48%
37°C	7.0	0.8 ± 0.4 (8)[c]	50%

(a) mean value ± 1.0 standard deviation (n)
(b) Mg^{+2} concentration was 6 mM
(c) Mg^{+2} concentration was 3.25 mM
(d) RSD = relative standard deviation

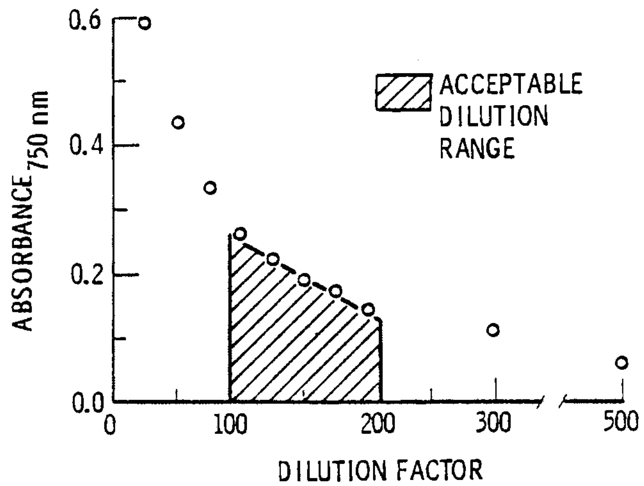


FIGURE 3. Effect of Increasing Dilution Factor on Protein (Lowry et al., 1951).

TABLE 3. Precision of P_i Assays (Gomori, 1942) and Protein Determinations (Lowry et al., 1951). P_i assays conducted at pH 7.5 and 6 mM Mg^{+2}. Dilution factor for Lowry's was 125.

P_i Assays	$\mu g\ P_i$ (a)	RSD
Mg- Baseline	35.1 ± 0.7	2%
NaK-ATPase	32.1 ± 0.3	1%
Lowry	**$\mu g \bullet m\ell^{-1}$ Protein (a)**	**RSD**
	44.1 ± 6.0	14%

(a) mean ± 1.0 standard deviation, n = 10

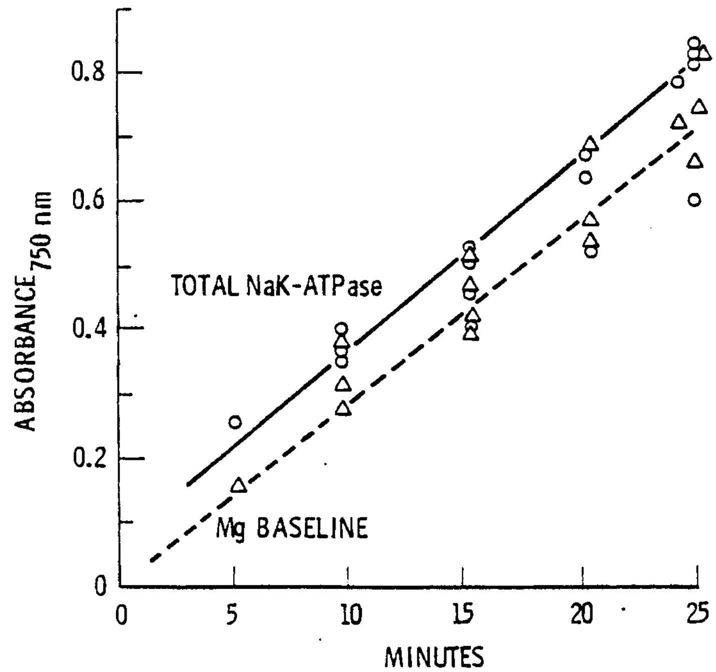


FIGURE 4. Linearity of Mg-baseline (20°C,---) and total NaK-ATPase (37°C,——) activities for 4 fish.

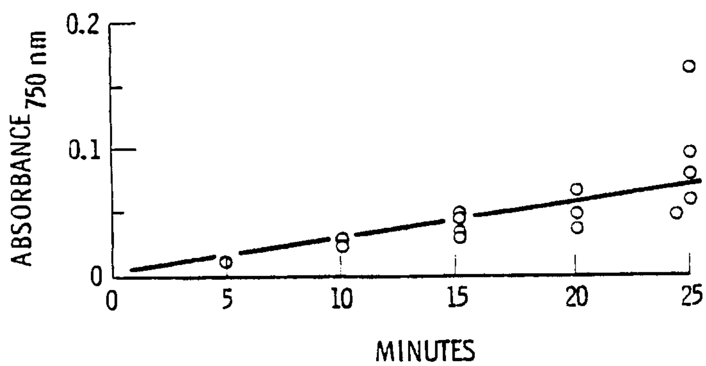


FIGURE 5. Linearity of sodium pump activity (37°C,-o-) for 4 fish.

Thermal lability of Mg-baseline and total NaK-ATPase activities was demonstrated with Arrhenius plots (Figure 7) at temperatures greater than 30°C and 20°C, respectively. No change in specific activity was observed over a 25-min span for Mg-baseline (20°C, $[Mg^{+2}] = 6$ mM) or sodium pump (37°C) activities. Total NaK-ATPase activities (37°C) did exhibit a decrease in specific activities from 10 to 25 min (Figure 8). Although the Arrhenius plot for sodium pump activity is erratic; it supports the hypothesis that increasing temperature favors the detection of activity.

The energy of activation (E_a) at 25°C was 10,500 cal•mole^{-1} for total NaK-ATPase activity and 15,600 cal•mole^{-1} for Mg-baseline activity.

pH OPTIMUM

Mg-baseline activity exhibited a broad optimal pH range between 6.0 and 7.0 (Figure 9). Mg^{+2} concentration was 6 mM and the assay temperature was 20°C.

Total NaK-ATPase (37°C) displayed an optimal pH range of 6.0 to 7.3 (Figure 10). Sodium pump activity was demonstratable over a pH range of 5.8 to 7.4 with an apparent optimum of 7.0 (Figure 11).

CATION STIMULATION

Figure 12 exhibits the effect of Mg^{+2} on Mg-baseline activity on gill tissue homogenized in the absence of Mg_2EDTA. Optimal activity was observed at 4 mM Mg^{+2}. Figure 13 shows the effect of increasing Mg^{+2} concentration on both Mg-baseline and total NaK-ATPase (20°C) on gill tissue homogenized with 0.03 M Mg_2EDTA. Contribution of Mg^{+2} to the assay medium from the gill homogenate was calculated at 2.73 mM. Note that at a Mg^{+2} concentration of 6.0 mM, Mg-baseline activity exceeds NaK-ATPase activity; while at concentrations below 5.0 mM total NaK-ATPase activity exceeds Mg-baseline. This observation is born out in data presented in Table 2.

Alkali metal stimulation was not detectable at 37°C (Figures 14 and 15). Assay conditions were 6.0 mM Mg^{+2} and pH 7.0.

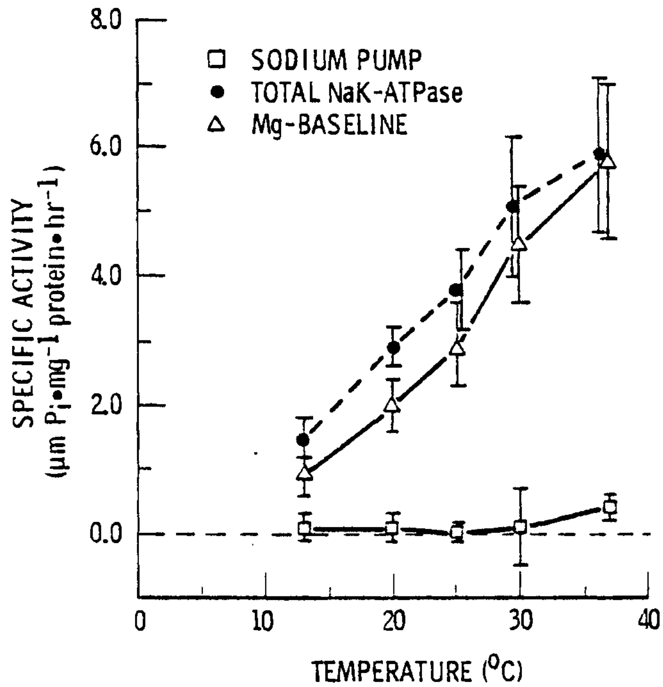


FIGURE 6. ATPase activities as a function of temperature. Values represent the average (± 1.0 standard deviation) for 6 fish.

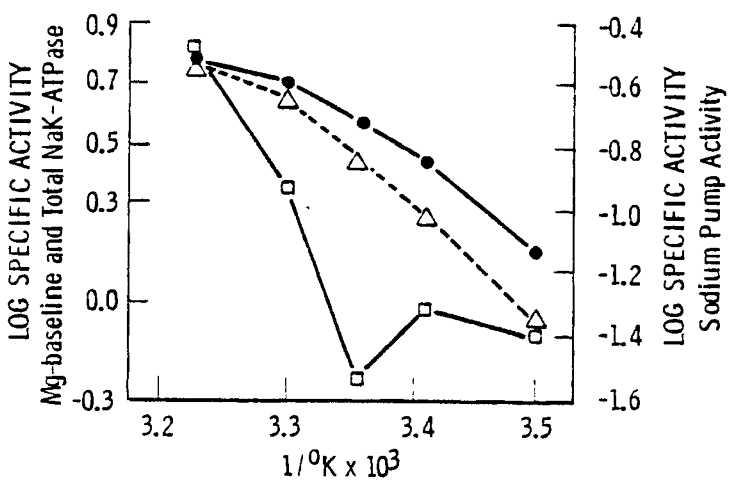


FIGURE 7. Arrhenius plot of Mg-baseline (-Δ-), total NaK-ATPase (-•-) and sodium pump (-□-) activities.

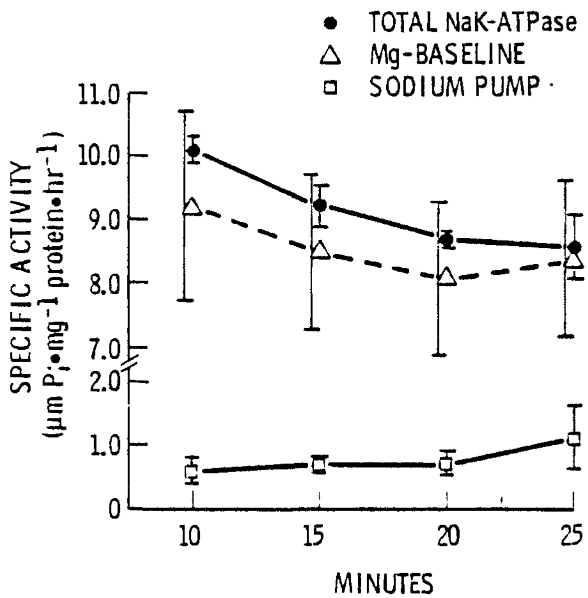


FIGURE 8. Specific activity as a function of time; Mg-baseline (20°C) total NaK-ATPase (37°C) and sodium pump (37°C) activities. Values are means (± 1.0 standard deviation) for 3 (at 10 and 20 minutes) or 4 (at 15 and 25 minutes) fish.

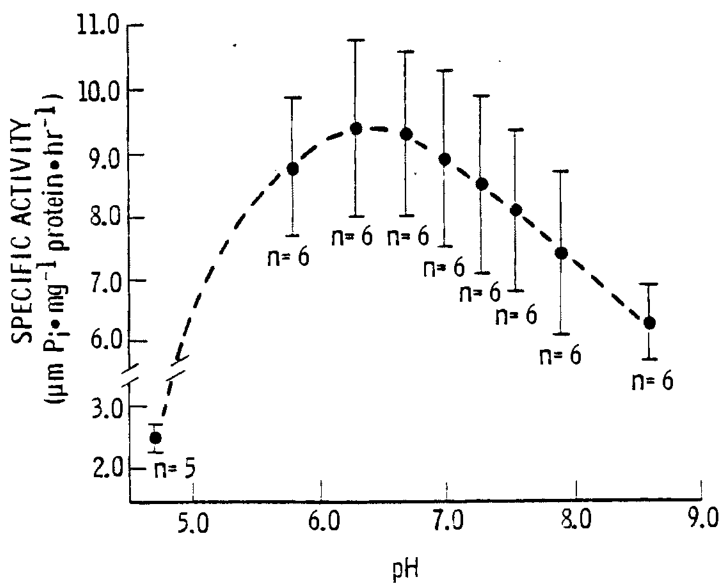


FIGURE 9. Optimum pH of Mg-baseline activities (20°C: 6 mM Mg^{+2}).

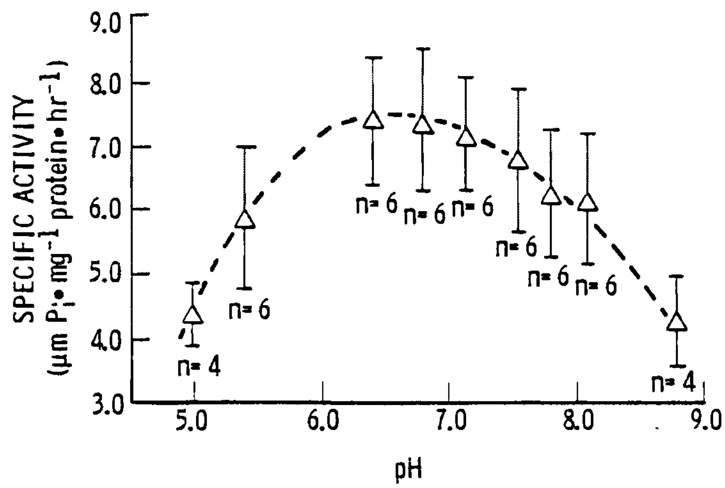


FIGURE 10. Optimum pH of total NaK-ATPase activity (37°C: 6 mM Mg^{+2}, 100 mM Na^+ and 20 mM K^+).

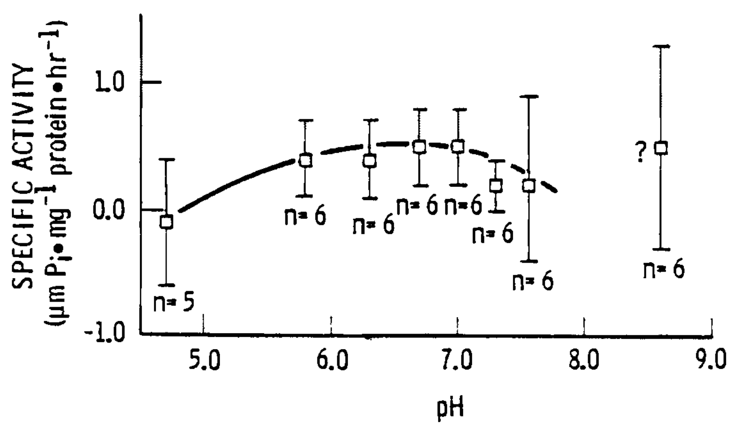


FIGURE 11. Optimum pH of sodium pump activities at 37°C. Magnesium concentration was 6.0 mM in these assays.

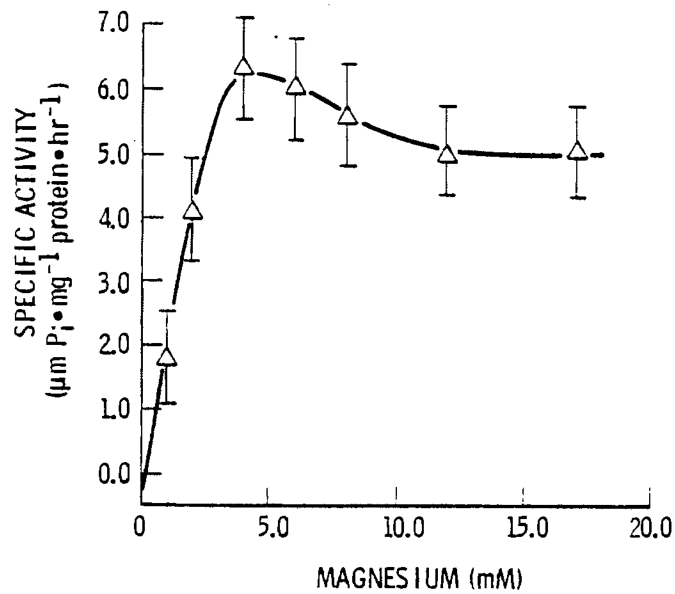


FIGURE 12. Optimum Mg^{+2} concentration for Mg-baseline (20°C) activity. Reaction medium lacks EDTA. Each point represents the mean (± 1.0 standard deviation) for 6 fish.

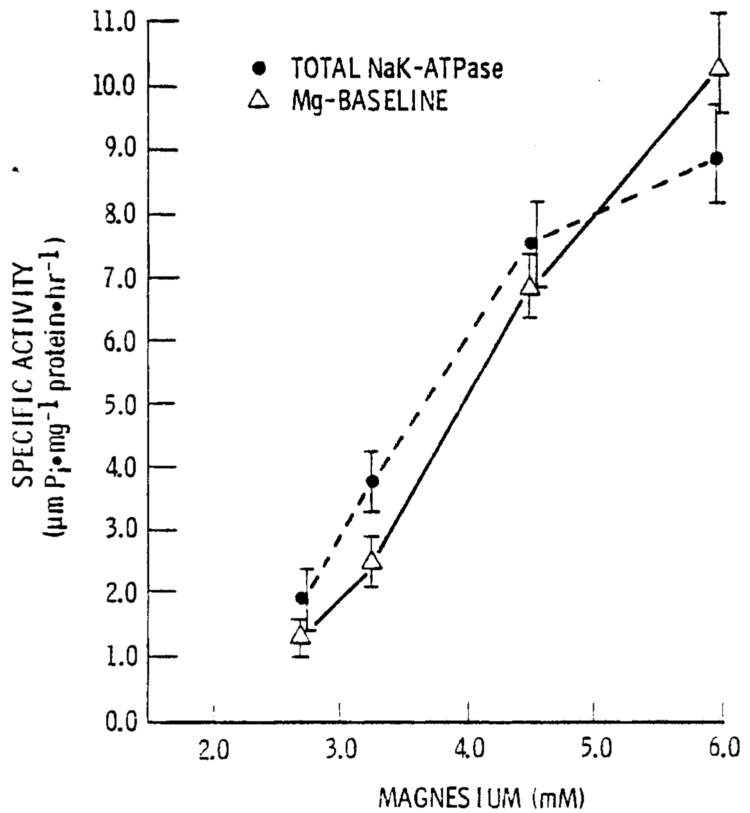


FIGURE 13. Effect of increasing Mg^{+2} ion on Mg-baseline (20°C) and total NaK-ATPase (20°C) in the presence of 1.36 mM EDTA. Each point represents the mean (± 1.0 standard deviation) of 4 fish.

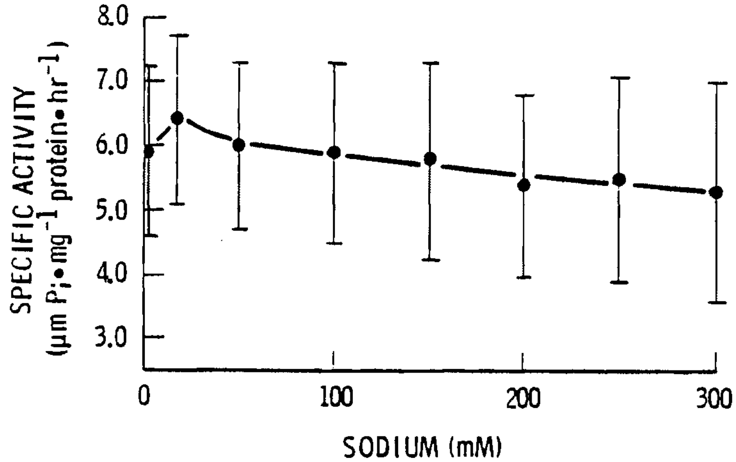


FIGURE 14. Sodium stimulation of specific activities at 37°C. Reaction medium contained 6.0 mM Mg^{+2} and 20 mM K^{+}, pH 7.0. Each point represents the mean (± 1.0 standard deviation) for 6 fish.

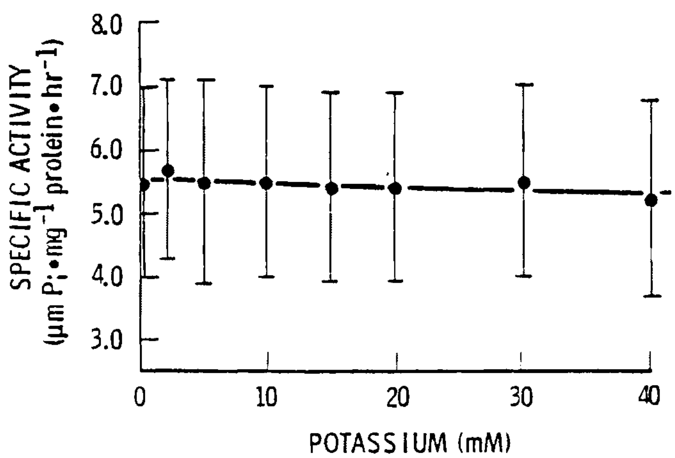


FIGURE 15. Potassium stimulation of specific activities at 37°C. Reaction mixtures contained 6.0 mM Mg^{+2} and 100 mM Na^{+}, pH 7.0. Each point represents the mean (± 1.0 standard deviation) for 6 fish.

TABLE 4. Difference Between Mg-Baseline and Total NaK-ATPase. Student's t test was employed to detect differences between the two activities.

	Temperature, °C				
Activity	13	20	25	30	37
Mg-Baseline	0.9±0.3	2.0±0.4	2.9±0.7	4.5±0.9	5.8±1.2
Total NaK-ATPase	1.5±0.3	2.9±0.3	3.8±0.6	5.1±1.1	5.9±1.2
t Statistic	2.996[b]	4.315[c]	2.444[b]	1.073	0.122

(a) average specific activity (± 1.0 standard deviation), n = 6
(b) significant at $\alpha = 0.05$
(c) significant at $\alpha = 0.01$

TABLE 5. Demonstrability of Sodium Pump Activity as a Function of Temperature.

Temperature	13°C	20°C	25°C	30°C	37°C
Specific Activity (n = 6)	0.1±0.2	0.1±0.2	0.0±0.1	0.1±0.6	0.4±0.2
t Statistic	0.700	0.645	0.525	0.498	3.514[(a)]

(a) significant $\alpha = 0.05$, mean greater than 0.

ATP CONCENTRATION

Figure 16 portrays the effects of increasing substrate concentration on specific activities. Results for sodium pump activity were inconclusive due to high variability (Table 6). Mg-baseline activity was irregular; whereas, total NaK-ATPase activity exhibited typical kinetic parameters.

In Vitro EFFECTS OF MS-222

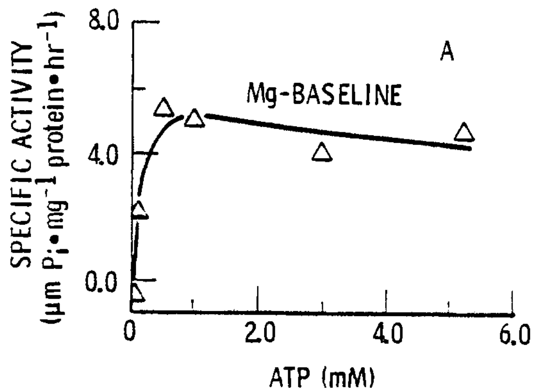
MS-222 failed to produce any detectable change in any of the activities under investigation (Figure 17 and Table 7). The difference between total NaK-ATPase (20°C) and Mg-baseline appears to be half of the control value. Differences between these activities for controls and 3.83 mM MS-222 were determined. These data were transformed [L_e (1.0 + specific activity)] and tested (Student's t test). The two levels were not significantly different ($t = 1.98$, significant at $t = 2.36$, $\alpha = 0.05$).

In Vitro EFFECTS OF PHENOL

Two tests were conducted on the *in vitro* effects of phenol (Tables 8 and 9). These data are graphically expressed as percent of control activities in Figures 18 and 19. Mg-baseline activity (20°C) appears to be less sensitive to phenol than does total NaK-ATPase activity. Twenty millimolar phenol produced 57 ± 11% and 65 ± 8% of control activities, whereas total NaK-ATPase activities were 42 ± 9% and 43 ± 7% of controls, respectively for Tests I and II. All alkali metal stimulation at 20°C was inhibited at 10 mM phenol (Table 9). The mode of inhibition was determined to be uncompetitive for both Mg-baseline and total NaK-ATPase at 20°C (Figure 20).

In Vivo EFFECTS OF PHENOL

Data from the *in vivo* exposure are tabulated in Table 10. The low-level (2.3 $mg \cdot liter^{-1}$) was significantly different from controls at 24 hr for Mg-baseline. These data are suspect due to abnormalities in sample variation which necessitated a L_e transformation. In spite of this adjustment, variances for Mg-baseline and total NaK-ATPase (20°C and 37°C) were still significantly different (Table 11) for certain tests. This aberration may be



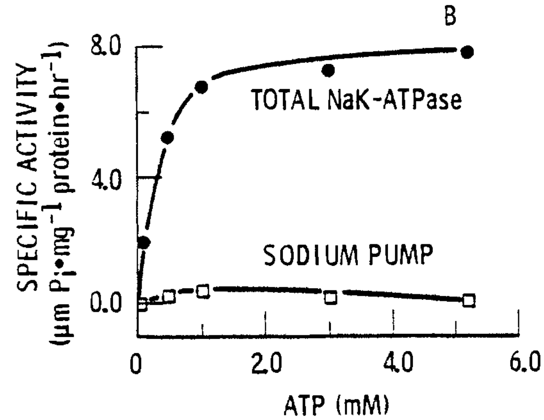


FIGURE 16. Effect of ATP concentration on ATPase activities. Mg-baseline (A) was assayed at 20°C, pH 7.0. Total NaK-ATPase and sodium pump were assayed at 37°C, pH 7.0 (B).

TABLE 6. Effect of Substrate (ATP) Concentration on ATPase Activities[(a)]. Sodium Pump t test is for Ho: $\mu = 0$.

ATP Concentration (mM)	Activity (Temp) Mg-Baseline (20°C)	Total NaK-ATPase (37°C)	Sodium Pump (37°C)	t Test Statistic Sodium Pump
0.3	-0.3 ± 0.7	0.2 ± 0.1	0.5 ± 0.6	0.667
0.1	2.3 ± 0.2	2.0 ± 0.2	0.1 ± 0.1	1.273
0.5	5.5 ± 0.2	5.3 ± 0.4	0.3 ± 0.1	4.00[(b)]
1.0	5.0 ± 0.3	6.9 ± 0.7	0.6 ± 0.5	2.120
3.0	4.0 ± 0.5	7.4 ± 0.8	0.2 ± 0.4	0.744
5.2	4.6 ± 0.7	7.9 ± 0.6	0.1 ± 0.1	1.750

(a) average ± 1.0 standard deviation for 4 fish
(b) significant at $\alpha = 0.05$

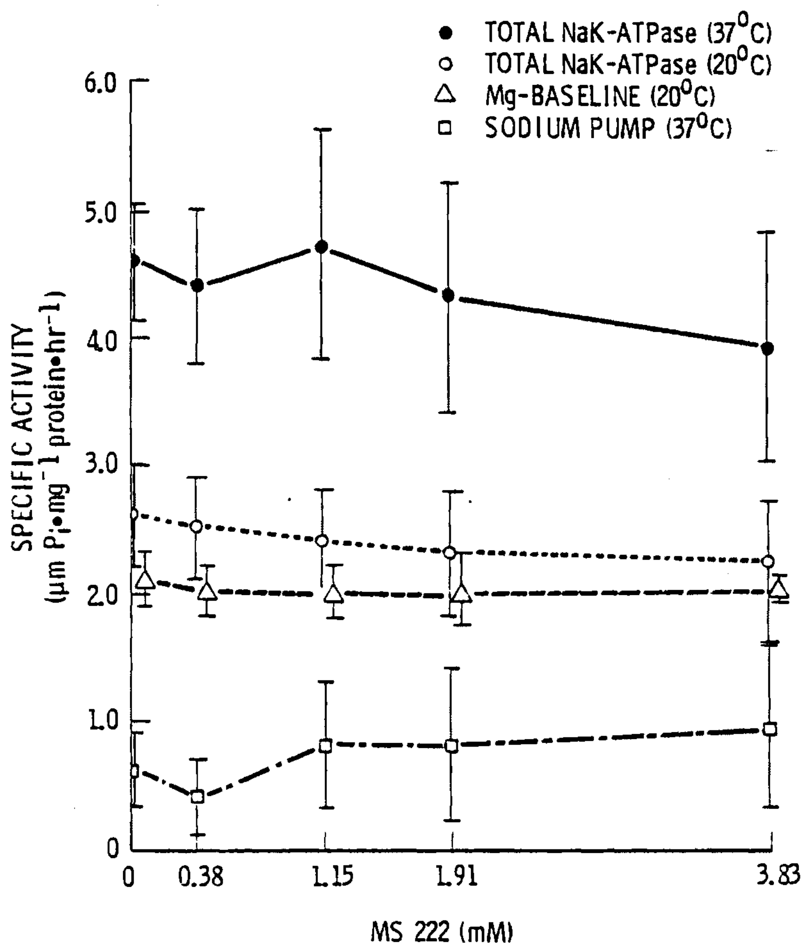


FIGURE 17. *In vitro* effects of MS-222 on ATPase activities; total NaK-ATPase (20°C and 37°C), Mg-baseline (20°C) and sodium pump (37°C). Concentrations of MS-222 in mg·liter^{-1} are 0, 100, 300, 500 and 1000 respective to increasing values on the abscissa.

TABLE 7. *In Vitro* Effects of MS-222. Specific activities are reported as the mean (± 1.0 standard deviation) for 8 fish.

Activity (Temp)	Concentration of MS-222 (mM/mg liter)				
	0/0	0.38/100	1.15/300	1.91/500	3.83/1000
Mg-Baseline (20°C)	2.1 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	2.0 ± 0.3	2.0 ± 0.1
Total NaK-ATPase (20°C)	2.6 ± 0.4	2.5 ± 0.4	2.4 ± 0.4	2.3 ± 0.5	2.2 ± 0.5
Total NaK-ATPase (37°C)	4.6 ± 0.5	4.4 ± 0.6	4.7 ± 0.9	4.3 ± 0.9	3.9 ± 0.9
Sodium Pump (37°C)	0.6 ± 0.3	0.4 ± 0.3	0.8 ± 0.5	0.8 ± 0.6	0.9 ± 0.7
t Test Statistic Sodium Pump	5.575[b]	3.663[b]	4.103[b]	3.616[b]	3.367[a]

(a) significant at $\alpha = 0.05$
(b) significant at $\alpha = 0.01$

TABLE 8. *In Vitro* Effects of Phenol, Test I

Concentration of Phenol (mM)	Activity[a] (Temp) Mg-Baseline (20°C)	Total NaK-ATPase (20°C)	Total NaK-ATPase (37°C)	Sodium Pump (37°C)
0	3.9 ± 1.2(8)	3.9 ± 0.6(8)	6.4 ± 0.5(8)	0.8 ± 0.4(8)
0.1			6.4 ± 1.5(4)	0.8 ± 0.6(4)
0.5	3.0 ± 0.6(4)	3.2 ± 0.3(4)		
1.0	3.4 ± 1.0(6)	3.6 ± 0.6(6)	6.0 ± 1.3(4)	0.6 ± 0.2(4)
5.0	3.3 ± 0.7(6)	3.4 ± 0.5(6)	5.6 ± 0.9(8)	0.7 ± 0.4(8)
10.0	3.8 ± 0.4(4)	3.3 ± 0.4(4)	4.8 ± 0.4(4)	0.6 ± 0.4(4)
15.0	3.2 ± 0.5(4)	2.4 ± 0.3(4)	4.0 ± 0.4(4)	0.7 ± 0.5(4)
20.0	2.2 ± 0.4(8)	1.6 ± 0.3(8)	2.7 ± 0.5(6)	0.2 ± 0.3(6)

(a) mean (specific activity) ± 1.0 standard deviation, (n)

TABLE 9. *In Vitro* Effects of Phenol, Test II. Data were analyzed with Student's t test against controls, t values appear directly beneath specific activity values. Specific activities are the mean ± 1.0 standard deviation for 8 fish.

Activity (Temp)	Phenol Concentration (mM) 0	10	15	20	25
Mg-Baseline (20°C)	2.3±0.5	2.3±0.2 t=0.205	2.0±0.5 5=1.499	1.6±0.4 5-3.306[a]	1.3±0.5 t=4.365[b]
Total NaK-ATPase (20°C)	2.9±0.5	2.4±0.5 t=2.118	1.8±0.5 t=4.612[b]	1.3±0.4 t=6.868[b]	1.0±0.4 t=8.068[b]
Total NaK-ATPase (37°C)	5.2±0.3	4.0±0.4 t=6.948[b]	3.2±0.4 t=11.75[b]	2.6±0.4 t=15.10[b]	2.1±0.4 t=19.49[b]
Sodium Pump (37°C)	0.5±0.2	0.6±0.2 t=-0.972	0.6±0.2 5=-0.358	0.5±0.2 t=-0.248	0.3±0.3 t=1.499

(a) significant at $\alpha = 0.01$
(b) significant at $\alpha = 0.001$

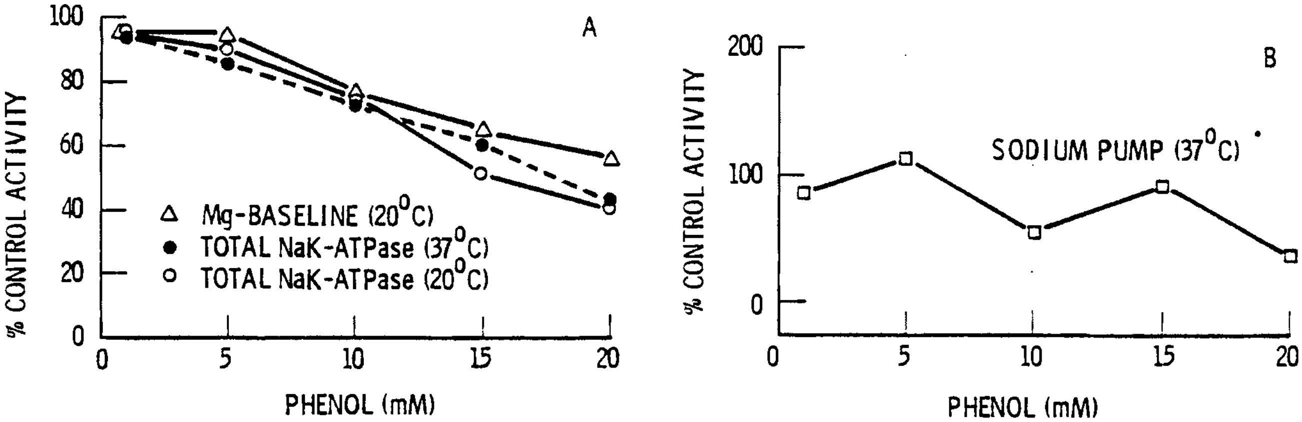


FIGURE 18. *In vitro* effects of phenol, Test I. Mg-baseline (20°C) and total NaK-ATPase (20°C and 37°C) are above (A), sodium pump (37°C) is below (B). Specific activity values and sample size are located in Table 8. Each point represents the average % specific activity of the control fish.

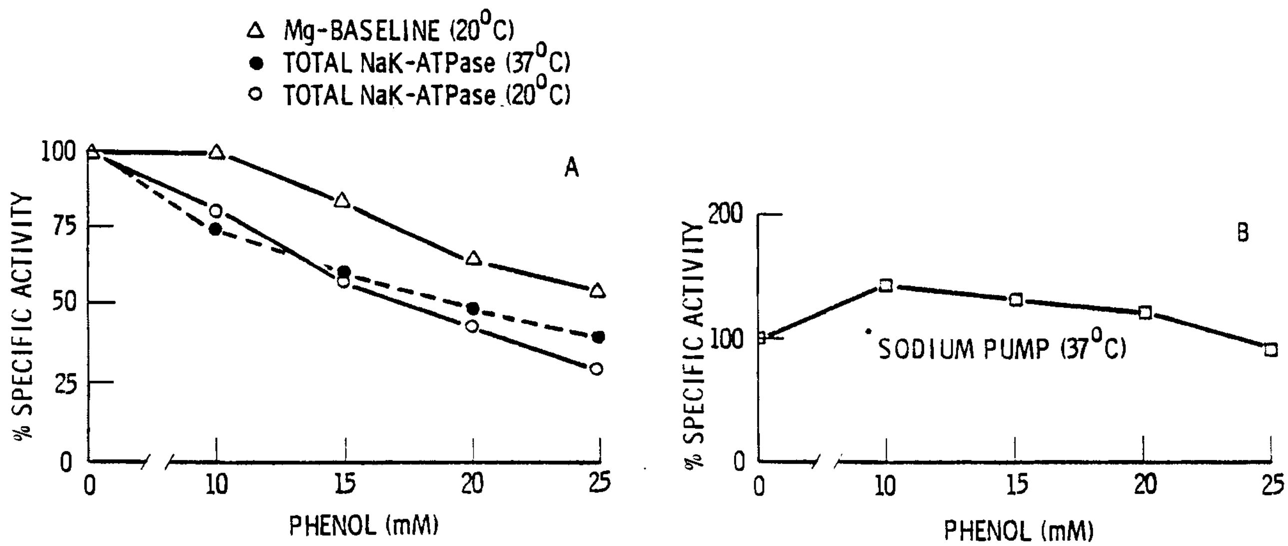


FIGURE 19. *In vitro* effects of phenol, Test II. Mg-baseline (20°C), and total NaK-ATPase (20°C and 37°C) are above (A). Sodium pump (37°C) is below (B). Specific activity values are located in Table 9. Each point represents the average % specific activity of the controls, n=8.

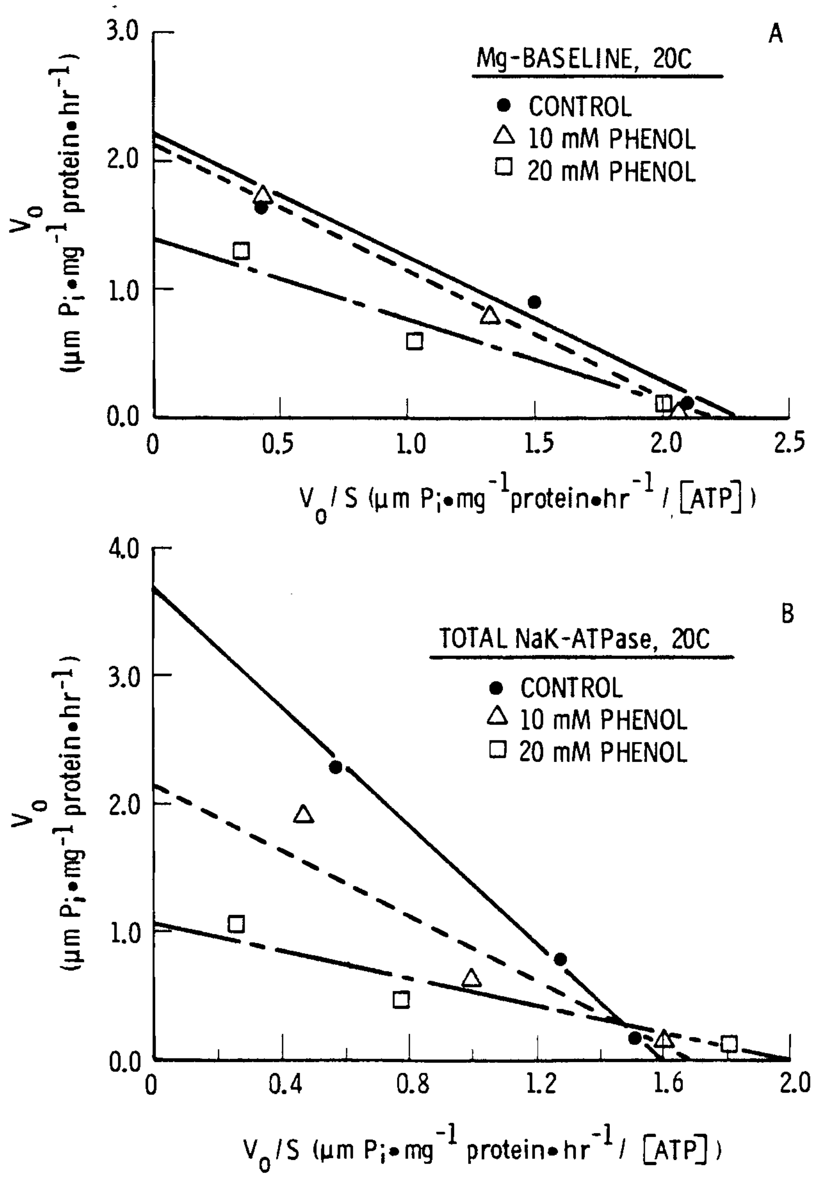


FIGURE 20. Inhibition of Mg-baseline (A) and total NaK-ATPase (B) activities at 20°C. Solid dots and solid lines represent controls (i.e., no phenol). Ten mM and 20 mM phenol are represented by triangles (dashed line) and squares (alternating dashed line) respectively. ATP concentrations were 0.1, 0.6 and 4.0 mM. Each point represents the mean of 3 fish.

TABLE 10. Specific Activities[a] of *in vivo* Exposure of Trout Lodge Strain of Rainbow Trout to Phenol. Fish exposed to phenol were tested with a Student's t test against control fish with L_e transformed data. Test statistics appear immediately below specific activity values.

Phenol Concentration	Mg-Baseline[d] (20°C)	NaK-ATPase[d] (20°C)	NaK-ATPase[d] (37°C)	Sodium Pump[d] (37°C)
24 Hrs				
$0.0\ mg \cdot \ell^{-1}$	7.5 ± 1.8 (5)	6.8 ± 1.7 (5)	9.1 ± 2.1 (5)	0.7 ± 0.3 (5)
$2.3\ mg \cdot \ell^{-1}$	5.5 ± 0.6 (5) t=2.327[b]	5.1 ± 0.6 (5) t=0.689	7.0 ± 0.8 (5) t=1.650	0.5 ± 0.3 (5) t=1.355
$4.7\ mg \cdot \ell^{-1}$	5.8 ± 0.8 (5) t=1.895	5.2 ± 0.7 (5) t=1.795	7.3 ± 0.7 (5) t=1.998	0.9 ± 0.6 (5) t=-0.478
96 Hrs				
$0.0\ mg \cdot \ell^{-1}$	7.9 ± 0.3 (4)	6.7 ± 0.3 (4)	7.8 ± 0.4 (4)	0.6 ± 0.4 (4)
$2.3\ mg \cdot \ell^{-1}$	8.8 ± 1.5 (4) t=-1.175[c]	8.1 ± 1.4 (4) t=-2.047[c]	11.2 ± 2.7 (4) t=-2.655[c]	1.4 ± 1.6 (4) t=-1.155
$4.7\ mg \cdot \ell^{-1}$	7.2 ± 0.7 (3) t=1.841	6.5 ± 0.7 (3) t=0.511	8.7 ± 0.6 (3) t=-2.059	0.6 ± 0.1 (3) t=-1.029

(a) μmoles $P_i \cdot mg^{-1}$ protein $\cdot hr^{-1}$, mean ± 1.0 standard deviation, sample size in parenthesis
(b) significant at $\alpha = 0.05$
(c) F ratios's on L_e transformations were significant, hence, no inference can be drawn from t tests
(d) Mg^{+2} present at 6 mM in all reaction mixtures

TABLE 11. F Ratios of Variance Estimates (L_e Transformed) from *In Vivo* Exposure to Phenol

Phenol Concentration	Mg-Baseline[d] (20°C)	Total NaK-ATPase[d] (20°C)	Total NaK-ATPase[d] (37°C)	Sodium Pump[d] (37°C)
24 Hrs				
2.3 $mg \cdot \ell^{-1}$	5.43[c]	5.16[c]	4.63[c]	1.01
4.7 $mg \cdot \ell^{-1}$	4.06	1.16	5.77[c]	1.83
96 Hrs				
2.3 $mg \cdot \ell^{-1}$	16.01[a]	14.25[b]	17.95[a]	2.08
4.7 $mg \cdot \ell^{-1}$	4.77	5.21	1.34	17.39[c]

(a) significant at $\alpha = 0.025$
(b) significant at $\alpha = 0.05$
(c) significant at $\alpha = 0.10$
(d) Mg^{+2} concentration was 6 mM in all reaction mixtures

attributable to the infestation by *Gyrodactilus* on the test fish. For these reasons, nothing conclusive concerning *in vivo* effects is drawn from this experiment.

DISCUSSION

ACTIVITY LEVEL

The activity levels of crude gill homogenates are lower than values reported in the literature (Kamiya and Utida, 1969, Davis and Wedemeyer, 1971 and Pfeiler and Kirschner, 1972). This discrepency is in part due to the fact that values reported by others are based upon experiments with microsomal preparations which have been treated with a dispersing agent, either NaI or deoxycholate. I refrained from the use of such dispersing agents on the grounds that crude homogenates represent a less modified system. Specifically, lipid bound enzymes may exhibit differing sensitivities to different compounds on the basis of that compound's lipid solubility. Possibly greater sensitivity could be obtained with a "cleaner" system, but it may not represent the "*in vivo*" mode of inhibition.

As a consequence of the overall lower activity in my experiments, the variation for my activities was higher. Additionally, the use of crude homogenates as opposed to microsomal preparations introduces an additional amount of variability. Sodium pump activity at best may be defined as demonstrable with high variability. This low level of activity is in agreement with the results of Pfeiler and Kirschner (1972) for freshwater adapted rainbow trout. The variability is derived in part from the expression of this activity as the difference of two activities which had their own inherent variability.

Similarly, others (Davis and Wedemeyer, 1971; Kamiya and Utida, 1969, and Epstein et al., 1967) have reported NaK-ATPase (representing sodium pump) as the difference between two reaction mixtures, one of which lacks an essential component of NaK-ATPase (sodium pump) activity. In these studies, the lack of K^+ rather than ouabain inhibition was used as a criteria for distinguishing sodium pump activity.

ASSAY CONDITIONS

Variations in assay temperatures had marked effects on activities. The thermal lability of the Mg-baseline activity has previously been reported by

Pfeiler and Kirschner (1972). This reduction of Mg-baseline activity fails to explain the apparent reduction of NaK-ATPase activity at 37°C; while sodium pump (ouabain sensitive) ATPase activity was detectable only at 37°C. Charnock et al. (1971) reported thermal dependence of ouabain sensitive ATPase at 24°C and residual (NaK) ATPase at 24°C in rabbit renal cortex. Thermal sensitivity in their studies was hypothetically attributed to conformation changes in the enzymes. Interactions with other lipoprotein membrane components were not discounted. It is quite probable that temperature may play an important role in the effects of a pollutant on an enzyme, particularly if it induces a conformation change *in vivo*. In these studies, the degree of inhibition by phenol on total NaK-ATPase was quite similar at both 37°C and 20°C.

Cation stimulation experiments were performed prior to optimal temperature experiments; hence, Na^+ or K^+ stimulated studies were assayed only at 37°C. Pfeiler and Kirschner (1972) have reported optimal concentrations of 200 mM Na^+ (in the presence of 20 mM K^+ and 5 mM Mg^{+2}) and essentially no requirement of K^+ (in the presence of 200 mM Na^+ and 5 mM Mg^{+2}) at 13°C. The lack of any apparent stimulation by either Na^+ or K^+ indicates that total NaK-ATPase activities are solely Mg^{+2} stimulated at 37°C. This observation agrees with the findings of my temperature experiments. Confusion is added when one considers that sodium pump activity is detectable only at 37°C. These systems were also assayed with 6 mM Mg^{+2} which heightens the amount of confusion.

The effects of increasing Mg^{+2} concentration are perplexing. A slight inhibition of Mg-baseline activity was observed at 6 mM Mg^{+2} when compared to an optimum at 4 mM Mg^{+2}. This system lacked Mg_2EDTA. In a subsequent test in which Mg_2EDTA was included in the homogenizing buffer, specific activities at 6 mM Mg^{+2} were higher than at 4 mM Mg^{+2}, indicating a possible sequestering of Mg^{+2} by the EDTA. Final theoretical EDTA concentration in the reaction medium was 1.36 mM; hence the degree of chelation would be notable ($\sim$ 23% at 6 mM Mg^{+2} assuming no competition with Ca^{+2}). Calcium has been reported to inhibit ATPase activities (Epstein and Whittam, 1966). In addition, the apparent inhibition observed in NaK-ATPase at 6 mM Mg^{+2} was in excess of the degree of inhibition observed in the original experiment. This may indicate some sort of synergistic mode of inhibition involving Na^+, K^+ and Mg^{+2} ions. It is unlikely that the slight increase in ionic strength would cause such a marked degree of inhibition. This point is very apparent if one views Na^+ stimulated specific activity values (37°C) as a function of increasing ionic strength. Specifically, the total ionic strength attributed to $MgCl_2$, NaCl and KCl at 300 mM Na^+ was 0.338. At 50 mM Na^+, the calculated ionic strength was 0.088. A near fourfold increase in ionic strength failed to produce a significant decrease in specific activity.

Final selection of assay conditions was greatly influenced by these observations as well as other investigator's results. Selection of assay concentrations of 100 mM Na^+ and 20 mM K^+ was a compromise of the demonstrability of sodium pump activity at 37°C and the apparent existence of a Na-stimulated activity proposed by Pfeiler and Kirschner (1972). This Na-stimulated activity may be represented by the total NaK-ATPase activity in excess of Mg-baseline activity observed at 20°C. It was also insensitive to 0.5 mM ouabain. Davis and Wedemeyer (1971) also noted the same ouabain insensitive activity in rainbow trout at 35°C. The physiological significance of a "Na-ATPase" is not understood.

Magnesium ion concentration was selected to maximize the demonstrability of total NaK-ATPase activity at 20°C while sacrificing to some degree the maximal attainable activity of Mg-baseline activity.

Additionally, pH (7.0) and ATP concentrations were selected to optimize sodium pump activity. The possibility exists that total NaK-ATPase activities might also have different pH optima at 20°C than at 37°C as determined in this study. Irregularities in Mg-baseline activities and sodium pump activities as a function of ATP concentration are attributed to "noise" in the assay system. Sodium pump activity was consistently demonstrable at 3.0 mM ATP in all other assays.

In Vitro EFFECTS OF MS-222

The range of MS-222 tested covered the range of reported dosages (recommended dosages for salmonids are 80-135 mg•liter^{-1}). Although these results indicated no effect on the gill ATPase activities, the mechanical method of anesthesia was maintained to preserve continuity of the sampling scheme.

In Vitro EFFECTS OF PHENOL

Phenol inhibited both Mg-baseline and total NaK-ATPase activities significantly; however, the levels of phenol responsible for 50% *in vitro* inhibition are several orders of magnitude greater than the EC_{50} (50% Effective Concentration) for rainbow trout. Reported values of EC_{50} for teleosts range from 0.079 to 1,900 mg•liter^{-1} depending on species, water quality, length of exposure and age of the fish (McKee and Wolf, 1971). Additionally, McKee and Wolf (1971) postulated a threshold effect at approximately 1.0 mg • liter^{-1}. EPA (1976) recommends a a limit of 1 μg•liter^{-1} for domestic water supplies. McKee and Wolf (1971) proposed allowable concentrations of phenol at 1 μg•liter^{-1} for domestic water, 50 mg•liter^{-1} for irrigation water, 1000 mg•liter^{-1} for stock watering and 0.2 mg•liter^{-1} for fish and aquatic life. To put things into perspective, approximately 18 mM phenol (1,694 mg•liter^{-1}) produced 50% inhibition of total NaK-ATPase (20°C). Less sensitive Mg-baseline (20°C) exhibited 55% inhibition at 25 mM phenol (2,353 mg•liter^{-1}). Sodium pump activity was unaffected by 25 mM phenol (2,353 mg•liter^{-1}). Ratios of inhibitor concentration (in mg•liter^{-1}) to the threshold limit (1.0 mg•liter^{-1}, McKee and Wolf, 1971) are equal to the above listed inhibitory values for the respective ATPase activities. Assuming an LD_{50} (lethal dose for 50% of the test population) for phenol of 10 mg•liter^{-1}, ratios of inhibitor concentration (50%) to the LD_{50} would be ∿170 for total NaK-ATPase and greater than 235 for Mg-baseline.

The discrepancy between EC_{50} (of LD_{50} values) and inhibitory levels of pollutants is not uncommon to other investigations of ATPase activities. Lindane (Koch, 1969/70) and DDT (Cutkomp et al., 1971) exhibit significant *in vitro* inhibition on ATPases associated with muscle, brain and kidney of selected teleosts. Mg-ATPase activities in the muscle of the bluegill sunfish were inhibited 51% by 10.4 μM DDT (3.68 mg•liter^{-1}). Assuming a 96 hr TL_m (median tolerance limit) of 0.016 mg•liter^{-1} (McKee and Wolf, 1971), a theoretical ratio of inhibitor to TL_m is 235.

Similarly, 70.6 μM Lindane (20.5 mg•liter^{-1}) produced ∿25% *in vitro* inhibition on muscle Mg-ATPase in the lake trout (Koch, 1969/70). The 96 hr TL_m for trout is listed by McKee and Wolf (1971) in the range of 0.056-0.1 mg•liter^{-1}. Calculated ratios of inhibitor concentration to TL_m were 205-367. It should be emphasized that LD_{50} or TL_m values greatly exceed acceptable levels of pollutants and that threshold limits are more applicable. The lower threshold limit also produces much higher ratios as initially observed for phenol.

The mode of *in vitro* inhibition was determined to be uncompetitive. An uncompetitive inhibitor combines with the enzyme substrate complex forming an inactive enzyme substrate inhibitor complex. The increase in substrate concentration is followed by an increase in inhibition reflected in a lower maximum velocity. Uncompetitive inhibition is common to multisubstrate reactions; hence, ATPase activities are likely candidates for this type of inhibition. Organochloride pesticides have been shown to demonstrate significant levels of *in vitro* inhibition at μM concentrations (Koch, 1969/70; Desaiah and Koch, 1975, Cutkomp et al., 1971) for ATPase activities associated with several different tissues. These references did not report the type of inhibition; however, I would speculate that the mechanism would most likely be uncompetitive and correlated with the greater degree of lipid solubility of organochloride pesticides.

In Vivo EFFECTS OF PHENOL

The levels of phenol required to produce significant *in vitro* inhibition of ATPase activities in rainbow trout so greatly exceed EC_{50} values, that the likelihood of detecting *in vivo* inhibition are quite remote. The exposure I performed with two levels of phenol failed to demonstrate any *in vivo* effects on ATPase activities by phenol. In all other experiments, sample variances of ATPase assays were shown to be similar by nonsignificant F tests. The F ratios of untransformed and L_e transformed data for this *in vivo* exposure were significant for some activities. This increased variation may be attributed to the *Gyrodactilus* infection which also resulted in two mortalities at the high level at 48 hr and loss of equilibrium of one fish at the low level (at 72 hr). No control fish were lost; however, all appeared stressed by the infection. A second experiment with healthy fish was not conducted.

ATPASES AS TOXICITY INDICATORS

Demonstration of suppressed ATPase activities (*in vivo*) in long term exposures to sublethal concentrations of toxicants may be misleading. Lower enzyme activiites may be reflecting an overall reduction in protein (i.e., enzyme) synthesis rather than a direct biochemical inhibition of the enzyme. For toxicants to effectively inhibit an enzyme *in vivo*, it must accumulate at concentrations great enough to produce at least a "significant" degree of inhibition on a particular enzyme. Toxicants subject to metabolic degradation may not be able to accumulate at levels which produce *in vitro* inhibition. What exactly constitutes a significant level of inhibition remains undecided and is a very serious limitation of *in vitro* studies. Small degrees of inhibition distributed over any number of enzymes may have a devastating effect on the homeostasis of the organism resulting in reduced fitness or death.

Temperature is also of concern in this work in that sodium pump activity was demonstrable only at a physiologically unrealistic temperature (37°C). I was unable to demonstrate either Na^+ or K^+ stimulation at this temperature. These observations suggest that the sodium pump activity assayed under the prescribed conditions may be an artifact of the assay regime rather than an enzymatic activity with physiological significance.

Phenol doesn't appear to have the potential to adversely affect gill ATPase activities *in vivo*. This conclusion is based upon the great discrepancy observed between the EC_{50} values reported earlier and my *in vitro* results. I am cautious about the application of results from the *in vivo* experiment. I feel that the infestation most likely wouldn't affect the sensitivity of these gill enzymes to phenol, but I lack any data to support this hypothesis.

FUTURE RESEARCH NEEDS

One of the objectives of this study was to develop an assay procedure for ATPase activities associated with crude gill homogenates. The possibility exists that inhibition may be influenced by the degree of enzyme purification, i.e., crude homogenates versus microsomal preparations. As noted earlier, microsomal preparations usually exhibited higher specific activities. The use of dispersing agents may also increase activity levels. Enzyme sensitivity may also increase with purification. It may be worthwhile to investigate the effect of enzyme purification as it is related to sensitivity of inhibitory pollutants.

Phenol was selected as a test toxicant due largely to its potential by a pollution problem associated with the increased utilization of fossil fuel technology. Other effluent constituents may exert a more significant effect on ATPase activities. Criteria for the selection of potential test toxicants should include the relative lipid solubility of the compound. Constituents with high lipid solubility may concentrate in the cellular organelles which house ATPase activities to a level where they may affect these enzymes.

A further partitioning of Mg-baseline activity is possible. The addition of oligomycin which inhibits mitochondrial Mg-ATPase or differential centrifugation (removal of mitochondria) may be used to separate microsomal and mitochondrial magnesium stimulated ATPase activities. These two activities may exhibit different sensitivities to selected toxicants. Implementation of a partitioning step during enzyme preparation would broaden the scope of the assay procedure.

Additionally, the ATPase activities could be investigated in other tissues. Higher levels of ouabain sensitive NaK-ATPase (sodium pump) have been shown in fish muscle, brain and liver (Cutkomp et al., 1971). Other organs of interest would be kidney, spleen and erythrocytes.

SUMMARY

1. ATPase specific activities from crude gill homogenates of rainbow trout were lower than those from microsomal preparations reported in the literature.

2. Sodium pump activity (ouabain sensitive NaK-ATPase) was demonstrable at 37°C. A ouabain insensitive NaK-ATPase was demonstrable at temperatures below 30°C and may represent a Na-ATPase activity reported by others.

3. Energy of activation at 25°C for total NaK-ATPase was 10,500 cal•mole^{-1}. Mg-baseline activity had an energy of activation at 25°C of 15,600 cal•mole^{-1}. Mg-baseline activity was thermally labile at temperatures in excess of 30°C.

4. Concentrations of Mg^{+2} in excess of 5 mM appeared to inhibit total NaK-ATPase activity. At 37°C, Na^{+} and K^{+} exerted little, if any, stimulatory effect on ATPase activities, in spite of the fact that 37°C was the only temperature at which sodium pump activity was demonstrable.

5. MS-222 failed to produce any discernible changes in any of the demonstrable ATPase activities in crude gill homogenates.

6. Total NaK-ATPase activities were more sensitive than Mg-baseline activities to *in vitro* inhibition by phenol. Concentrations of phenol which produce 50% inhibition in total NaK-ATPase produced only 35% inhibition in Mg-baseline activity. Essentially, all alkali metal ion stimulated (20°C) present in total NaK-ATPase activity was inhibited at 10 mM phenol. The nature of *in vitro* inhibition was uncompetitive. Sodium pump activity was unaffected by phenol at concentrations as high as 25 mM. Large error values and low levels of activity may have masked any effects.

7. An effort was made to demonstrate an *in vivo* effects of phenol on rainbow trout gill ATPase activities. An infestation of a parasite (*Gyrodactilus*) on the experimental fish precludes any definative assessment of *in vivo* effects.

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