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Heat Shock Proteins in Branchial Tissue in Atlantic Salmon (*Salmo salar*)

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HEAT SHOCK PROTEINS IN BRANCHIAL TISSUE
IN ATLANTIC SALMON (SALMO SALAR)

BY

JENNIFER L. ALLEN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
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MASTER OF SCIENCE THESIS
OF
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ABSTRACT

Lamellae isolated from gill arches of Atlantic salmon were incubated in 15, 25 and 27°C. Five heat shock or stress proteins (hsps) with molecular weights of 54, 71, 72, 82 and 87 kDal appeared after incubation at 25°C. At 27°C all five proteins were induced in greater quantity and an additional protein of 67 kDal was observed. The time required for the induction of hsps at 25°C was determined by labeling the gills in vitro for one hour after intervals of up to four hours of heat shock. All five proteins were apparent after one hour of heat shock and maximal by two hours. The lamellae continued to synthesize hsps throughout the four hours.

Stress proteins were not induced in lamellae exposed to 25 to 300 µM of sodium arsenite or 50 to 500 mM sodium chloride. Although viability was high under these conditions, overall protein synthesis was suppressed.

Lamellae proteins induced by heat shock at 25°C were incubated with monoclonal and polyclonal antibodies to hsps 60, 70 and 90. Only the antibodies to constitutive/inducible hsp 70 and the polyclonal antibody to hsp 70 exhibited different degrees of binding in control and shocked samples.

October and January fish were subjected to osmotic shock in vivo by transfer from freshwater to 27 ppt and 35

ppt seawater. Five stress proteins with molecular weights of 39, 40, 41, 54, and 82 kDal were induced in non-smolting fish in October upon transfer to 27 ppt for 48 hours. Fish reared under a constant daylight photoperiod did not exhibit induction of hsps when transferred to 27 ppt seawater in January.

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PREFACE

Atlantic salmon are an important species for commercial aquaculture and are reared throughout North America, South America, Europe, Asia, New Zealand and Australia. Presently, aquaculture is being used to restore Atlantic salmon to the rivers of New England. Adult returns numbered in the tens of thousands in New England rivers before the runs were decimated by overfishing, pollution and destruction of the habitat in the early 1800's (U.S. Fish and Wildlife Service, 1989). Restoration of salmon runs in these rivers would return a once native species to the region and would aid the economy through development of a sport and commercial fishery. Fish farming has also increased to meet the growing human consumption of salmon. In 1983, the U.S. imported approximately 3,968 metric tons of Atlantic salmon. Six years later, imports increased nearly 4.5 times to more than 17,000 metric tons (Bettencourt and Anderson, 1990). The increased demand and higher prices could allow domestic aquaculturists to profitably produce and market salmon.

In commercial salmon aquaculture fish are typically reared in seawater netpens because they cannot be produced cost effectively in terrestrial-based facilities. The fish are directly transferred from freshwater tanks to seawater

net pens. The fish are reared in these units at relatively low cost to market size in about 18 months. Salmon can only be transferred to seawater during a short period of time called the parr-smolt transformation or smoltification (Hoar 1988, 1976). Many morphological and physiological changes occur during smoltification and enable the fish to successfully make the transition from a hydrating (freshwater) to a dehydrating (seawater) environment. Smoltification occurs in the spring during the increase in day light and water temperature. When fish are transferred outside the two to four week period of smoltification, there are high mortalities and poor growth of survivors (Hoar, 1976). In restoration programs non-smolting salmon released into the rivers remain in the stream where they are exposed to predators and food availability is low. These fish are not likely to survive and return to the river to spawn.

Smoltification must be identified accurately for commercial aquaculture and restoration programs. For successful transfer and rearing of salmon in seawater net pens, a better understanding of smoltification is necessary to clearly identify the proper time for transfer. At the present time, several indicators are used to assess the time of smoltification. These, however, are labor intensive, require specialized equipment or are not precise. The techniques currently used are not readily adapted for routine hatchery use and require the sacrifice of many fish.

In this thesis a class of polypeptides, called heat shock proteins (hsps), which might serve as potential identifiers of smoltification, are investigated. Stress proteins have been found to confer protection to organisms exposed to various environmental stressors. If hsps are induced in either fish capable of surviving seawater transfer or those individuals unable to tolerate seawater, it may be possible to use their induction as a non-lethal and more accurate identifier of smolts.

The following manuscript has been prepared in accord with guidelines for submission to the Journal of Experimental Zoology.

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INTRODUCTION

When an organism is subjected to elevated temperatures, a group of polypeptides called heat shock proteins (hsps) is induced. These proteins are thought to protect the organism from further heat shock. Other environmental stresses, such as exposure to sodium arsenite, heavy metals, ethanol and hypoxia also induce the synthesis of hsps and additional stress proteins (Lindquist 1986, Lindquist and Craig 1988).

Many species of plants (Czarnecka et al, 1988), animals (Welch and Suhan, 1986) and bacteria (Perisic 1989; Taura et al. 1989; Skowrya et al. 1990) have been examined and all have been shown to produce heat shock proteins (for review see Nover, 1991; Sanders, 1993). Hsps share certain characteristics among species. Most organisms produce similar stress proteins which have some of the most highly conserved amino acids sequences found to date. The human hsp 70 molecule has an amino acid sequence which is 73 percent homologous to Drosophila hsp 70 and 50 percent homologous to the equivalent of hsp 70 from *E. coli* (Lindquist, 1986). The high degree of homology suggests a low degree of tolerance for mutation and a vital role in sustaining life.

A large variety of stresses induce heat shock proteins. Members of the hsp 70 family are induced in most organisms by anoxia and nutrient starvation (Nover, 1991). A

multitude of chemicals, such as hydrogen peroxide, arsenite and ethanol also induce hsps. However, species-specific responses and threshold levels of induction have been observed. Several organisms synthesize stress proteins in response to various stresses that are not induced by heat shock. These proteins and those induced upon heat shock are broadly termed stress proteins.

The hsps synthesized by these organisms fall into five categories based on molecular size. The family with the greatest molecular weight is the hsps 90. This group of polypeptides is abundant during non-stressed conditions, but during exposure to sublethal or lethal temperatures there is an increase in synthesis of these proteins (Collier and Schlesinger, 1986). These constitutive proteins are located primarily in the cytoplasm with a slightly larger member (hsp 94; also called grp 94) found in the endoplasmic reticulum (Sorger and Pelham, 1987). Hsp 94 is induced during glucose starvation, exposure to heat, steroids and other agents. The cytosolic members of hsp 90 have been found to associate with steroid hormone receptors (Dalman et al. 1989; Ohara-Nemoto et al. 1990). Hsp 90 prevents the receptors from binding to DNA when the hormone is not attached and enhances the affinity of the receptor for the hormone.

The most abundant and genetically conserved group of heat shock proteins is the hsp 70 family. This group has been extensively studied. Hsp 70 has both constitutive and

inducible members with various functions. Hsps 72 and 73 also interact with maturing polypeptides, folding the polypeptide into the active form (Beckman et al, 1990). Hsp 70 also acts to chaperone the newly synthesized proteins to the appropriate cell destination by preventing improper binding to other proteins (Beckmann et al, 1990; Ellis, 1987). Researchers have hypothesized that hsp 70 also acts to signal the cell of temperature changes (Craig and Gross, 1991).

Hsp 60 has only recently been observed and its functions have not been fully determined. Hsp 60 is constitutive and is synthesized in the cytosol then translocated into the mitochondria. Researchers have shown that hsp 60 prevents the inactivation of dihydrofolate reductase during heat shock (Martin et al, 1992).

The small hsps have molecular weights ranging from 16 to 40 kDal. This family is very diverse and not highly conserved among species (Mansfield and Key, 1987). Although the small hsps have not been well studied, some members have been found to be induced during different stages of development without exposing the organism to stress (Cheney and Shearn, 1983). Several investigators suggest they may be important to ontogeny.

The smallest group of heat shock proteins are the ubiquitins (Bond and Schlesinger 1985, Collier and Schlesinger 1986). These are small polypeptides (about 76 amino acids) found in all eukaryotic cells examined

(Matthews and VanHolde, 1990). During stressful conditions, such as elevated temperatures, damaged proteins form denatured aggregates that may impair cellular functions. Under these conditions ubiquitin is induced at a high rate and binds to the damaged protein signalling the cell to degrade the protein with proteolytic enzymes.

Stress proteins are thought to protect cells from damage caused by environmental shock. Although the evidence is not definitive, there is a strong correlation between hsp induction and thermotolerance. If an organism is first exposed to a sub-lethal temperature and capable of inducing of hsps, it can survive subsequent exposure to an otherwise lethal temperature (Lindquist, 1986). Exposure to a non-thermal hsp induction stressor, such as sodium arsenite, causing synthesis of stress proteins, confers protection to the organism against both the stressor and lethal temperatures. Mutations in the hsp 70 genes in yeast (*S. cerevesiae*) have been demonstrated to suppress growth at sublethal temperatures (Craig and Jacobsen, 1984). During embryonic development of many organisms, hsps are not yet synthesized and the organisms are very sensitive to thermal killing. When the organism is capable of synthesizing hsps, it will tolerate temperatures that would be lethal at the embryonic stage. These findings support the hypothesis that stress proteins protect cells (Brown et al, 1992).

Generally the upper lethal limit is the temperature at which vital organs fail (Prosser and Nelson, 1981). One

potential mechanism explaining this is the destruction of cellular membranes as they become fluid and loose structure. The loss of membrane integrity impairs membrane permeability and interferes with the stability of membrane enzymes (Bowler, 1981). Heat labile proteins are denatured at elevated temperatures and may bind non-specifically, causing formation of large aggregates. The cell also responds to heat shock by decreasing synthesis of non-hsp polypeptides.

As ectotherms, physiological processes of fish are greatly affected by temperature fluctuations. Heat shock proteins may be of particular significance in ectotherms because of the wide fluctuations in body temperature that parallel environmental changes. At high temperatures fish react with hyperexcitability, uncoordinated swimming, loss of equilibrium and muscle spasms (Prosser and Nelson, 1981).

Hsps have been found in all eight species of fish examined to date. Tail fin of medaka (Oryzias latipes) synthesizes three major heat shock proteins at elevated temperatures in vitro (Oda et al, 1991). Gill, muscle and brain tissues have been shown to synthesize hsps in fathead minnow (Pimephales promelas) (Dyer et al, 1991, 1993) and goldfish (Carassius auratus) tail fin cells synthesize four groups of hsps in vitro (Sato et al, 1990). Rainbow trout hepatoma (Oncorhynchus mykiss) and chinook salmon embryonic cell (Oncorhynchus tshawytscha) lines also produce hsps in response to temperature shock and heavy metals (Kothary et al. 1984; Misra 1989). Winter flounder (Pleuronectes

americanus) renal proximal-tubule cells produce hsps from three families at mild and severe heat shock (Brown et al., 1992)

Previously, researchers had not determined if Atlantic salmon respond in a similar manner. The natural range of Atlantic salmon is limited to the north Atlantic Ocean. However, they are cultured in various areas of the world and often encounter stressful thermal conditions in both native and hatchery environments. Although, Atlantic salmon are tolerant of temperatures ranging from freezing to 26°C, they grow better at temperatures below 16°C (Piper et al, 1982) and are frequently exposed to elevated water temperatures, especially during migration. It seems probable that synthesis of hsps might serve as a defense mechanism in many species of ectotherms, including Atlantic salmon, during exposure of close to sublethal temperatures.

Salmon are also exposed to another significant environmental stress, osmotic shock. Juveniles migrate from freshwater to seawater during smoltification and adults return from seawater to freshwater to spawn. In culture situations, juvenile salmon are moved abruptly from freshwater to seawater without the benefit of gradual acclimation. Salmon transferred to seawater experience a transient increase in blood sodium concentration from 175 mM/l in freshwater to ≥ 200 mM/l in seawater after 18 hours. To survive this rise in environmental osmolarity, salmon must regulate the influx of sodium and maintain circulating

levels at < 200 mM in the face of environmental sodium levels > 550 mM (Stagg et al, 1989). Functional smolts with hypoosmoregulatory ability are able to reduce sodium and chloride concentrations, while sodium levels in parr remain elevated (Birt et al., 1990). It seems likely that a mechanism is present to prevent cellular damage and death during the transitory osmotic shock the fish face upon transfer to seawater. Elevated plasma ion concentrations may induce stress protein synthesis and protect the organism from the damage of osmotic shock while long term adaptive mechanisms develop. Fish incapable of synthesizing stress proteins may be unable to survive osmotic shock during the early stages of seawater exposure. Alternately, salmon unable to tolerate a seawater transfer may synthesize stress proteins in response to osmotic shock when smolts may not need to. Other investigators have shown that bacteria (Hecker et al, 1988; Apte and Bhagwat, 1989) synthesize stress proteins in response to changes in salt concentrations. Accordingly, I have examined the effect of osmotic shock on branchial tissue of Atlantic salmon, the results of which are herein.

MATERIALS AND METHODS

FISH AND REARING CONDITIONS

Heat shock proteins were examined throughout the year in two to three year old Atlantic salmon (Penobscot strain, North Attleboro National Fish Hatchery, North Attleboro, MA) reared in the Aquaculture Center at the University of Rhode Island (Kingston, RI). The salmon were reared in two meter diameter fiberglass tanks supplied with aerated, single pass freshwater. The temperature naturally ranged from 6°C to 18°C during the course of each year. Tanks were illuminated with overhead fluorescent lights controlled by timers to mimic the natural photoperiod. The fish were fed to satiation three times daily with commercial feed (Silver Cup, Murray, UT).

IN VITRO SYSTEM -PREPARATION OF BRANCHIAL FILAMENTS

Induction of heat shock proteins was investigated in isolated branchial tissue. Fish were killed with a sharp blow to the head and partially exsanguinated by removing the caudal fin. Gill arches were excised with scissors and cut just above the septa with methanol-cleaned razor blades to separate the lamellae (McCormick and Bern, 1989). Using a plastic transfer pipet, approximately 0.3 gram of tissue was transferred to a 25 ml Erlenmyer flask containing ten

volumes of minimum essential medium (MEM with Earle's salts, Gibco BRL, Gaithersburg, MD) with 100 U/ml penicillin and 100 ug/ml streptomycin to eliminate bacterial growth and adjusted to pH 7.3 with sodium bicarbonate. The flasks were capped and gassed with 99%O₂:1%CO₂.

CELL VIABILITY

Viability of the tissue was tested prior to radiolabeling by measuring the leakage of lactate dehydrogenase (LDH) into the medium. Fifty microliters of cell-free media was taken at intervals during the six hour incubation period. LDH activity was measured on a spectrophotometer by following the oxidation of NADH with added pyruvate. At the end of the six hour period, the lamellae were homogenized for five 10-second pulses with a Tissuemizer (Tekmar, Cincinnati, OH) and LDH measured to obtain the total amount of LDH in the cells. The ratio of LDH in the total incubation medium over the sum of medium and tissue LDH was used to estimate the fraction of cell death at any given time. Only conditions providing greater than 90 % viability were used.

IN VITRO RADIOLABELING

Heat Shock Induction, Half-life and Time of Induction.

Proteins synthesized by isolated gill lamellae in the first four hours of heat shock were examined. Trans-label [³⁵S]-methionine (ICN Biomedicals, Irvine, CA) was used to

label newly synthesized proteins. Initially, protein synthesis was quantified by calculating CPM per mg of gill tissue. CPM per mg of tissue was calculated by counting the TCA insoluble fraction and dividing by the amount of sample counted. However, CPM per ug of total protein was found to give a more reproducible measure of protein synthesis and this measure was used in later experiments.

Tissue samples were transferred to a 15°C incubator with shaking to bring all the samples to a uniform temperature prior to heat shock. Heat shock was achieved by immersing the flask containing tissue in a shaking water bath at the temperatures of 25°C and 27°C. Tissue samples were then transferred with a transfer pipet to a centrifuge tube, rinsed in 10 volumes of ice cold 0.1M ammonium carbonate to remove radiolabel and then homogenized with five 10-second pulses (Tissuemizer, Tekmar Company, Cincinnati, OH). The cartilage and unbroken cells were sedimented by centrifuging (Sorvall RC-5C, Wilmington, Delaware) at 800 x g for 10 minutes at 4°C and the pellet was discarded. Protein was precipitated from 0.5 ml of the supernatant fluid by the transfer to 2 ml of 10% trichloroacetic acid (TCA). After 10 minutes, the suspension was vortexed and filtered on a 0.45 micron cellulose filter (Micron Separations, Westboro, MA). The filtered precipitate was rinsed once with 2 ml of ice cold 10% TCA. The TCA insoluble fraction was rinsed until [³⁵S] was not detectable in the wash. The washed precipitate and

filter was immersed in 10 ml of scintillation cocktail (Ecolume, ICN Biomedicals, Irvine, CA) and the amount of [^{35}S] was quantified in a scintillation counter to less than 2 sigma. The remaining unacidified supernatant was frozen 0.1 M ammonium carbonate, lyophilized and stored at -20°C for 2 to 7 days before electrophoretic resolution of the proteins.

The time required for induction of hsp's was determined by heat shocking tissue at 25°C for one, two, three or four hours. Induction was followed by adding [^{35}S]-methionine during the last hour of incubation. The half-life of induced proteins was determined by prelabeling tissue proteins with [^{35}S]-methionine for two hours, rinsing the tissue twice with fresh MEM lacking [^{35}S], and transferring to fresh MEM supplemented with carrier methionine for intervals up to six hours at 25°C . The rate of disappearance of [^{35}S] from labeled proteins was used to calculate the half-life of each.

RESOLUTION OF LABELED PROTEINS

Radiolabeled proteins were visualized by fluorography. The proteins were separated by molecular weight on sodium dodecyl sulfate (SDS) 8 to 15% linear gradient polyacrylamide gels (Van Berklom, 1978). Molecular weight markers (Sigma Chemicals, St. Louis, MO) containing Lysozyme, Carbonic Anhydrase, Albumin from Chicken Egg, Albumin from Bovine Serum, B-Galactosidase and Myosin were

run on each gel. To allow comparisons, all gels contained lanes with equal CPM unless otherwise specified. After electrophoresis, gels were fixed in a solution of 5% acetic acid and 7.5% ethanol. The gels were impregnated with fluors (Resolution, EM Corp, Chestnut Hill, MA) and then soaked in chilled 5% glycerol for 30 minutes. Gels were dried for one hour and overlaid with X-OMAT film (Kodak, Rochester NY) within an exposure cassette and stored at -70°C for one to four weeks before developing. Gels from different treatments and individuals were compared visually.

INDUCTION OF HSPS BY SODIUM ARSENITE AND SODIUM CHLORIDE

Sodium Arsenite

A 10 mM stock solution of sodium arsenite was added to the MEM to produce concentrations of 25, 50, 100 and 300 μM . Induction of stress proteins was examined over a four hour exposure using the radiolabeling method described for heat shock.

Sodium Chloride

A 4 M solution of sodium chloride was added to the culture medium to produce concentrations of 116 mM (control), the concentration in MEM, 166mM, 216 mM, 316 mM, 366 mM, 516 mM and 666 mM, the concentration of sodium chloride in seawater. Induction of stress proteins was examined over a four hour exposure using the radiolabeling method described for heat shock.

STATISTICAL ANALYSIS

Four of five fish were euthanized and the gill lamellae were separated into several samples and then subjected to treatments. Values were statistically compared using the within-groups analysis of variance (ANOVA) with an alpha level of 0.05 and a Tukey post hoc test.

ANTIGEN SPECIFICITY

Atlantic salmon hsps were tested for antigenic homology to known hsps from other species using Western blotting (Towbin et al, 1979; Dalman et al, 1989). Branchial proteins from control and shocked fish were separated on a gradient gel as described previously and transferred to Immobilon-P membranes (Millipore, Bedford, MA) using a semi-dry electrotransfer system (American Bionetics PolyBlot, Hayward, CA). Transferred proteins were exposed to several antibodies: mouse monoclonal antibody specific for HeLa hsp 72, 73 and Achlya ambisexualis hsp 90 (StressGen Biotechnologies Corp., Victoria, B.C. Canada); rabbit polyclonal antibodies to E. coli dnaK (MW= 70Kd) and GroEL (MW= 60Kd) (D. Nelson, University of Rhode Island); rabbit polyclonal antibodies to mammalian hsps 60 and 70 (R. Gupta, McMaster University, Ontario, Canada).

Membranes with bound proteins were cut into strips, wetted with methanol and rinsed for five minutes in deionized water. Membranes were treated with 0.15%

dehydrated skim milk and 5% goat serum in TBS (0.09% NaCl, 20 mM Tris/HCl, pH 7.4) for two hours at 37°C to block non-specific antibody-antigen interaction. The strips were washed three times in TBS between all steps. Strips were incubated with primary antibody for two hours at room temperature. The primary antibody was detected by then exposing the strips to a secondary antibody attached to an agent that can be seen specific for the organism of the primary antibody. The strips were incubated overnight with secondary antibody with colloidal gold (Zymed Laboratories, San Francisco, CA) at 1:1000 with constant shaking. Visibility was increased with silver enhancement (Zymed Laboratories, San Francisco, CA).

Only primary antibodies that produced bands were tested for specificity. Specificity of antibody binding and silver enhancement was determined by cutting and removing the region of the strip containing the antigen and exposing the remaining proteins to primary and secondary antibodies and silver enhancement. Non-specific binding of the secondary antibody was determined by exposing the strip to secondary antibody and silver enhancement. Specificity of the silver enhancement treatment to the secondary antibody was tested by exposing the strip to the primary antibody and enhancement. Non-specific binding of silver to the membrane or proteins was determined by excluding primary and secondary antibodies from the procedure.

IN VIVO OSMOTIC SHOCK

To determine if Atlantic salmon produce stress proteins in response to salt shock in vivo, two year old fish were radiolabeled and exposed to low and high salt concentrations in aquaria supplied with aerated, freshwater or seawater (27 ppt or 35 ppt) at 15°C. There were five fish per treatment and were sampled at four, 24 and 48 hours. Following exposure under these conditions, fish were anesthetized in a 75 mg/L solution of tricane methane sulfenate (MS-222) and injected intraperitoneally with [³⁵S]-methionine diluted 10 times in phosphate buffered saline (PBS) at a dose of one uCi per gram of fish bodyweight. The fish were returned to the aquaria of the same salinity for an additional two hours. The gill lamellae were then excised and examined for hsp's as described previously. Blood was collected from each individual to determine plasma chloride concentrations using colorimetric determination (Sigma Diagnostics, St. Louis, MO). Incorporation of radiolabel into TCA perceptible material was measured as described under In vitro Radiolabeling and proteins were separated and visualized by one-dimensional electrophoresis and fluorography as described above.

Two sets of experiments were conducted with fish weighing approximately 100 g each. The first experiment was conducted in October with post-smolt fish exposed to salinities of 0 ppt, 27 ppt and 35 ppt under simulated natural photoperiod.

In January, the experiments were repeated because technical problems prevented the development of high quality fluorographs. Fish from a constant light photoperiod (L24) fish were chosen based on previous studies indicating this regimen prevented salmon from smolting (Saunders et al., 1985). The second set of experiments, five fish per treatment group were subjected to 0 ppt and 27 ppt salinity based on results from the first set of experiments.

RESULTS

CELL VIABILITY

Heat Shock

Survival of branchial tissue preparations markedly decreased with increased time and temperature (Figure 1). At the control temperature (15°C), less than 5% of the branchial cells died. At 25°C, cell death remained low increasing to approximately 5% in six hours. When the temperature was raised to 27°C, few cells died by four hours of exposure. Cell death increased rapidly to about 15% by the next sampling time at six hours. At 28°C, cell death increased rapidly to 17% within four hours. When the temperature was raised to 30°C or higher, the cells began to die rapidly with 10% mortality within the first hour and 22% by two hours.

In preliminary investigations, branchial tissue displayed a very low rate of protein synthesis (Figure 2). Less than 0.5% of the radiolabeled methionine that crossed into the cells was incorporated into branchial proteins, whereas greater than 30% of the radiolabel was incorporated into hepatic proteins (Wang, J., unpublished data). The efficiency of incorporation was determined by adding 0.025 uCi to a flask containing approximately 300 mg of branchial lamallae and 10 volumes of MEM for four hours. Approximately 40,000 CPM/ul of total tissue homogenate was

present in the TCA soluble fraction representing [^{35}S] that crossed into branchial cells. The TCA insoluble fraction, comprised of proteins, contained approximately 60 CPM/ul of homogenate. Of the 40,000 CPM/ul of homogenate in branchial tissue, only 60 CPM/ul were actually incorporated into proteins. In order to obtain a sufficient quantity of radiolabeled proteins for electrophoresis, a four hour labeling time was chosen. The maximum temperature at which 90% of the cells remained viable, 27°C, was used for in vitro radiolabeling experiments.

Sodium Arsenite

Survival of branchial tissue remained above 90% during a six hour exposure to concentrations ranging from 25 uM to 300 uM of arsenite (Figure 3). A four hour exposure time was chosen for radiolabeling experiments for consistency with heat shock experiments. All concentrations tested for viability were used in radiolabeling experiments.

Sodium Chloride

Tissue viability was measured during exposure of isolated lamellae to sodium chloride concentrations ranging from 250 mM to 500 mM. Greater than 90% of branchial cells tolerated sodium chloride concentrations less than 500 mM (Figure 4). With exposure to 500 mM of sodium chloride, cell death reached 10% in four hours and almost 20% by six hours. A four hour exposure time with a maximum

concentration of 500 mM was chosen for in vitro radiolabeling.

INDUCTION OF PROTEINS IN VITRO

Induction of Proteins by Heat Shock.

Total protein synthesis did not differ significantly between temperatures (Figure 2), but five specific proteins became noticeably more prominent as incubation temperature was elevated to 25°C (Figure 5). The approximate molecular weights of the induced proteins were 85, 82, 72, 71 and 54 (kDal). At 27°C, these proteins became much more prominent and an additional band became apparent with an approximate molecular weight of 67 kDal. The majority of the constitutive proteins became lighter or vanished completely. An increase in protein synthesis of hsps and a decrease of other constitutive proteins is characteristic of the heat shock response. Proteins induced in shocked branchial tissue will be called heat shock proteins or stress proteins for the remainder of this thesis.

The time required for induction of hsps was examined over a four hour period. At 25°C, all four hsps became apparent during the first hour with hsp 72 being the most prominent (Figure 6). By the second hour, all the stress proteins appeared to increase to maximal levels and production was sustained through the fourth hour.

The half-life of hsps was measured by following the disappearance of the induced proteins after the removal or

continuance of the shock. The quantity of radiolabeled proteins did not change significantly over the six hour period for lamellae kept at 25°C (Figure 7). The time point of one hour was eliminated from statistical analysis of protein quantity because not all fish had enough tissue for five samples. Eliminating the time point allowed an increase in sample size. Fluorography showed additional bands of proteins appeared at one hour and remained unchanged over six hours after removal of the medium containing radiolabel (Figure 8). Determining the half-life of hsps was also attempted when the lamellae were returned to control temperatures. When medium containing radiolabel was removed and the lamellae were returned to 15°C, the quantity of radiolabeled protein did not change significantly (Figure 9). Fluorographs of these samples indicated that hsps continued to be radiolabeled, even after the addition of medium lacking [³⁵S], similar to the results observed with continuous shock (Figure 10). Prominent bands became darker over the six hour period.

Induction of Proteins by Sodium Arsenite

Incorporation of radiolabel into total branchial protein decreased as the concentration of sodium arsenite increased (Figure 11). Protein synthesis during exposure to 300 uM of sodium arsenite was significantly lower than at the lesser concentrations. Proteins from all four concentrations were separated by electrophoresis and

visualized by fluorography (Figure 12). As the concentration of sodium arsenite increased, all protein bands became lighter. No new polypeptides were induced.

Induction of Proteins by Sodium Chloride

Sodium chloride concentrations, used for radiolabeling ranged from 166 mM to 666 mM. Incorporation of radiolabel into branchial protein decreased as concentrations of sodium chloride increased (Figure 13). Incorporation of radiolabel into TCA perceptible material decreased significantly when the concentration of sodium chloride reached 316 mM. All higher concentrations tested produced similar results. Fluorography showed all bands faded with increased concentrations of sodium chloride (Figures 14 and 15). No new proteins were induced.

ANTIGEN SPECIFICITY

Non-specific binding of primary and secondary antibodies was tested. Specificity of the secondary antibody to the primary was determined by excluding the primary antibody from the normal procedure (Figure 16, lane 1). Non-specific binding of the secondary antibody was detected, but the bands stained lightly and were distinguishable from specific binding to the primary antibody. When both primary and secondary antibodies were eliminated from the procedure, no staining was observed (Figure 16, lane 2). When the secondary antibody was

excluded from the procedure, no bands were detected, regardless of the primary antibody used (Figure 16, lanes 3-5). Non-specific binding of the primary antibody was determined by cutting the antigen from the strips and exposing the remainder of the polypeptides to the described procedure. The results of the individual antibodies will be described below in the appropriate section.

Monoclonal and Polyclonal Antibodies to HSP 70, HSP 60 and HSP 90

When proteins from non-shocked branchial tissues were probed with monoclonal antibody to hsp 72\73, one prominent and two faint polypeptide bands were detected (Figure 17, lane 1). The prominent band had a molecular weight of 72 Kdal and the two faint bands had molecular weights of 42 and 40 kdal. When proteins from heat-shocked tissue were exposed to the same antibody, the prominent band of the control lane stained a band with a greater width (Figure 17, lane 2). The band width extended downward and had two different intensities indicating that the band may consist of two separate proteins. This smaller protein has a molecular weight of 71 kdal. When the region of the immunoblot strips containing proteins in the 70-80 kDal range was excised two bands of lower molecular weight (Figure 18, lane 1).

When the monoclonal antibody for inducible hsp 70 was tested against proteins from shocked and non-shocked

tissues, no prominent binding occurred (Figure 17, lanes 3-4). Very faint binding was detectable at the same relative mobility as the hsp 72\71 bands. When the shocked proteins were probed with the same antibody and no blocking buffer was applied, no proteins greater than 14 Kdal were detected (Figure 19, lane 6).

One dark polypeptide band was detected when heat-shocked proteins were exposed to rabbit polyclonal hsp 70 antibodies. When control proteins were probed with the same antibody, a narrow band was detected with the same molecular weight as the shocked band. The lanes probed with polyclonal Hsp 70 antibody are almost identical to lanes probed with monoclonal Hsp 70 antibody. In addition to the prominent bands, very faint bands with low molecular weights stained. When the shocked and control proteins were probed with polyclonal hsp 70 antibody and no blocking buffers were used, the hsp 71/72 bands were detected with the addition of several low molecular weight polypeptides (Figure 19, lanes 2-3).

Monoclonal hsp 90 antibodies did not bind strongly to polypeptides from heat-shocked or control tissues (Figure 20, lanes 1-2). A very faint band stained with the same relative mobility as hsp 70. When hsp 90 antibody was applied with no blocking buffer, only small molecular weight proteins stained (Figure 20, lane 3). These low molecular weight bands were identical to the bands detected with

exposure to the inducible hsp 70 antibody and no blocking buffer (Figure 19, lane 6).

Heat-shocked and control polypeptides were probed using polyclonal hsp 60 with various intensities of blocking. When the strips of protein were treated with the milk/goat serum block or milk blocking buffer, only very faint bands were noted (Figure 21, lanes 2-5). Many bands were detected when the strips were not treated with blocking buffer (Figure 21, lanes 6-7). The latter strips were compared to control strips probed with monoclonal and polyclonal hsp 70 (Figure 19, lanes 1-2). Numerous bands stained, but two bands with molecular weight approximately 54 kdal were distinguishable with hsp 60 antibodies and not visible when staining with hsp 70 antibody.

Antibodies to E. coli hsps

Heat shocked branchial tissue polypeptides were also probed with dnaK antibody (Figure 22, lanes 1-2). Only very faint bands could be detected at various areas of the strip. One very faint band detected in the shocked tissue was not present in the proteins from control tissue. This band had the same molecular weight as hsp 70. However, this band was not more prominent than the other bands detected.

When heat-shocked and control proteins were incubated with GroEL antibodies, no bands were detected (Figure 22, lanes 3-4). The strips were virtually blank.

IN VIVO OSMOTIC SHOCK

The first of the in vivo osmotic shock experiments (IV1) were conducted with salinities of 0 ppt, 27 ppt, and 35 ppt. Fish subjected to 0 ppt and 27 ppt survived through the 48 hour experimental period. When fish were exposed to 35 ppt, 100% of the fish died after 24 hours. Plasma chloride levels rose significantly from 122 mEq/L to 180 mEq/L upon exposure to 27 ppt for 24 hours and remained at this elevated level at 48 hours (Figure 23). When the fish were subjected to 35 ppt, plasma chloride rose to 152 mEq/L by hour four and reached 218 mEq/L by hour 24. Levels of control fish (122 mEq/L) did not change significantly throughout the testing period. Protein synthesis based on [³⁵S] incorporated/ug of protein, significantly changed during exposure to seawater (Figure 24). Protein synthesis increased from four hours to 24 hours and again from 24 hours to 48 hours in the control group. It did not change significantly during 24 hour exposure to 35 ppt. At 27 ppt, protein synthesis did not increase from 4 to 24 hours, but increased significantly between 24 and 48 hours. Protein synthesis in control fish increased between 4 and 24 hours and again at 48 hours (Figure 24).

Technical problems with fluorography prevented the development of high quality gels. A high percentage of glycerol (10%) was used during the drying process to prevent cracking. Unfortunately, this concentration of glycerol

also created blackened areas on the film, which prevented detection of the protein bands. Figure 25 shows the only gel with enough resolution to see both salinities and most of the control lanes. It was not possible to identify molecular weights, due to the inability to clearly determine the position of the molecular weight standards. These fluorographs were compared to previous fluorographs where molecular weights had been calculated. Apparently, at least three proteins with molecular weights of 40, 41 and 54 kdal, were induced in fish subjected to 27 ppt salinity for 24 hours. When the fish were exposed to 27 ppt for 48 hours, two additional proteins were detected with approximate molecular weights of 39 and 82 kDal.

The second of the in vivo osmotic shock experiments (IV2) involved subjecting the fish to 0 ppt and 27 ppt for four, 24 and 48 hours. Exposure of fish to 35 ppt was not repeated in this set of experiments because the fish from the earlier experiments did not survive for more than 24 hours and no significant protein changes occurred in 24 hours.

Plasma chloride levels increased significantly from 112 mEq/L to 150 mEq/L when fish were transferred to seawater (Figure 26). The rise in plasma chloride occurred within the first four hours and did not change significantly through the 48 hour testing period, consistent with the previous experiment.

Protein synthesis changed upon exposure to osmotic shock (Figure 27). Protein synthesis, after four hours in seawater was 17 CPM/ ug of protein and plateaued at 27 CPM/ ug of protein by 48 hours. Protein synthesis in control fish did not change significantly from the initial level of 11 CPM/ ug of protein. Fluorographs containing one sample from each treatment group, showed no change in the protein profiles between treatments (Figure 28). The control group at four hours revealed numerous bands with a high concentration between 50 and 90 Kdal. This protein profile is identical to the protein profiles of the other groups.

DISCUSSION

Exposure of branchial tissue of Atlantic salmon to elevated temperatures caused the induction of six heat shock proteins. These proteins were readily discernible using radiolabeling and SDS PAGE and had apparent molecular weights of 54, 67, 71, 72, 82 and 85 kDal. The hsps became strikingly prominent at 25°C in comparison to proteins synthesized at control temperatures, and continued to be synthesized to an even higher levels at 27°C. Synthesis of most constitutive proteins became suppressed upon the induction of hsps. This finding is in agreement with the accepted characteristic patterns associated with induction of heat shock proteins.

Only two other papers have been published concerning hsps in gill tissue of teleosts. Dyer et al. (1991) found six hsps induced in gill tissue of fathead minnow (Pimephales promelas) with molecular weights of 63, 68, 70, 78, 90 and 100 kDal. They also found the Hsp 70 doublet to consist of hsp 68 and 70, which is slightly lower than the doublet found in Atlantic salmon. Fathead minnows synthesize only one stress protein smaller than Hsp 70, whereas Atlantic salmon synthesize two. Koban et al. (1991) only examined the Hsp 70 class in Fundulus heteroclitus and found two proteins in gill tissue with molecular weights of

74 and 76 kDal, higher than the Hsp 70 doublet in Atlantic salmon. There are several possible explanations for differences in the molecular weights of the hsps reported in these investigations and those of the present study. Dyer et al. (1991) radiolabeled proteins synthesized after tissues were returned to non-inducing control conditions as opposed to the radiolabeling during stress in this study. It is possible that different polypeptides are synthesized during recovery than during the actual period of shock. Variations in molecular weight determinations could also account for small differences in molecular weight. Lambin (1978) determined that the reliability of determining molecular weights above 70 Kdal from polyacrylamide gels with 10% acrylamide or greater was low. Dyer et al. (1991) used 12.5% and Koban et al. (1991) used 10% acrylamide gels which could account for different molecular weight estimations of Hsp 70. Another possibility is that hsp 70 is species specific. Although these polypeptides have similar amino acid components, small differences could alter the molecular weight. To date, 21 members of the Hsp 70 family characterized (Nover, 1991). The Hsps 70 expressed in Atlantic salmon could simply be different proteins than those of the fathead minnow and killifish.

The stress proteins induced at 25^oC were synthesized within the first hour of shock, reached maximal levels by the second hour and remained at this level for the four hours tested. Levels were estimated by visual comparisons

of fluorographs. Gedamu et al. (1983) found hsp 70 was detectable within 30 minutes after shock and maximal at two hours in chinook salmon embryo cells. Synthesis of hsp 70 protein was sustained for 10 hours during shock before returning to control levels. Results reported for the first four hours by Gedamu et al. are consistent with those obtained in this study.

The half-life of Atlantic salmon gill hsps could not be determined in the present study. The intensities of the induced protein bands continued to increase for a period of six hours after the radiolabel was removed from the media. In the present study, a large amount of radioactive methionine crossed the gill membrane into the cells but less than 0.5%, was incorporated into proteins. Even when the radioactive medium was removed from the tissue, the intracellular pool of radiolabel appeared sufficient for continued incorporation into polypeptides. New proteins synthesized from the remaining ^{35}S pool would be indistinguishable from those produced prior to removal of radiolabel. Therefore, attempts to determine the half-life of hsps synthesized were not successful under my experimental conditions.

Sodium arsenite did not induce hsps in Atlantic salmon gill tissue. Kothary and Candido (1982) found sodium arsenite effectively induced hsps in a rainbow trout cell line when the cells were allowed a recovery time. Five hsps were induced upon exposure to increased temperatures with

molecular weights of 30, 32, 42, 70 and 87 kDal (Kothary and Candido, 1982). Upon exposure to 50 uM of sodium arsenite, the same hsps were induced with the addition of a 62 kDal polypeptide. With no recovery time, very few and faint bands were detected and normal protein synthesis was reduced. When fathead minnows were exposed to sodium arsenite various tissue specific proteins were synthesized (Dyer et al., 1993). Gill tissue synthesized five stress proteins with molecular weights of 20, 40, 70, 72 and 74 kDal in response to arsenite. Hsps were evident by two hours, but did not reach maximal synthesis until six to 10 hours of exposure. Four hours may not be long enough for stress proteins to be detected in Atlantic salmon.

Protein synthesis during recovery time differs from protein synthesis during exposure to the stress (Welch and Suhan, 1985). Kothary and Candido (1982) and Dyer et al. (1991) labeled proteins synthesized during recovery from sodium arsenite. Atlantic salmon did not synthesize any new proteins during the four hour exposure to 25 uM to 300 uM arsenite, but no recovery time was examined. Normal protein synthesis, measured by CPM/mg of gill tissue, was reduced significantly (ANOVA, $p \leq .05$). This was also evident when visually comparing the fluorographs. Atlantic salmon gill tissue was affected by exposure to sodium arsenite without the induction of stress proteins. Atlantic salmon may not respond to stress identically to other species of fish.

Immunoblotting was used to demonstrate antigenic similarities of stress proteins among Atlantic salmon and other species. The antibody to constitutive and inducible HeLa hsp 70 (hsp 72/73) and the polyclonal antibody to mammalian hsp 70 bound strongly to Atlantic salmon branchial proteins with prominent differences between control and treatment groups. Two separate bands were observed with molecular weights of 71 and 72 kDal. However, antibody to HeLa hsp 72, the inducible member of the hsp 70 family, did not bind differentially between control and shocked groups. This could be due to technical problems with the antibody. It could also show that the lower molecular weight protein of Atlantic salmon has limited homology to hsp 72 found in humans.

Polyclonal antibodies to mammalian hsp 60, did exhibit slight binding to Atlantic salmon polypeptides in the 60 kDal molecular weight range when blocking buffer was not used. These bands were not detected by any other antibody. However, they did not appear to change between control and heat shocked groups. In radiolabeling experiments, a polypeptide band was detected with a molecular weight of 54 kDal. This band was relatively faint and may not have been present in sufficient quantity to enable visualization of differences between control and shocked tissues. Alternatively, hsp 54 from Atlantic salmon may not share antigenic similarities with human hsp 60.

Monoclonal hsp 90 did not bind to proteins in either the control or heat shocked lamellae even when blocking agents were omitted from the buffers. No inducible proteins with molecular weights greater than 87 kDal were detected during in vitro radiolabeling. The hsp 87 in Atlantic salmon may not be homologous to HeLa hsp 90. However, this antibody has not tested positively in many species (Lee Mizzen, Stress Gen, personal communication). It is possible that a polyclonal hsp 90 might cross-react with hsp 87 in Atlantic salmon. However, no polyclonal hsp 90 was commercially available for the present investigation.

The effect of salinity on the induction of hsps in fish has not been investigated previously. When Atlantic salmon branchial tissue was exposed to osmotic shock, protein synthesis was suppressed. Concentrations of greater than 100 mM in the media caused a drop in protein synthesis from 400 CPM to less than 200 CPM/ mg of tissue. However, no stress proteins were detected with fluorography. Although, 1500 CPM of radiolabeled denatured proteins were loaded in each lane of the gel, bands became fainter when the tissue was exposed to higher concentrations of sodium chloride. This may be due to protein synthesis suppression and high protein levels (100 ug) per lane. If protein synthesis was low, small amounts of radiolabel might have been incorporated into a large variety of proteins, and this would spread the radiolabel throughout the electrophoretic lane instead of forming a few discrete bands.

In vitro osmotic shock may not be a true reflection of the conditions lamellae would encounter during exposure to saltwater. In vitro experiments subject the lamellae to high salt concentrations from both the apical and basilar sides. Even if fish were transferred directly to seawater, cellular levels of sodium chloride would rise relatively slower. It takes 18 hours for plasma sodium levels to rise from 175 mM/L to 200 mM/L (Stagg et al. 1989).

When fish were subjected to an in vivo osmotic shock of 35 ppt salinity, there was a slow rise in plasma chloride levels which reached almost twice the control levels over 24 hours. When gill tissue was exposed to comparable sodium chloride levels in vitro, protein synthesis dropped drastically. In fish from the first in vivo experiment (IV1) protein synthesis of the control group rose significantly. Protein synthesis in the treatment group did not exhibit a similar change, and remained significantly lower than the control by 24 hours. When the fish were subjected to 27 ppt, plasma chloride levels rose to above 170 mEq/L and remained elevated.

SNP fish in October (IV1) displayed stress protein synthesis upon exposure to 27 ppt for 24 hours and stronger hsp induction by 48 hours. This was in contrast with the results from the second set of in vivo experiments, IV2.

When fish from IV2 were subjected to osmotic shock, no inducible proteins were observed. Plasma chloride levels from fish exposed to 27 ppt for 4 to 48 hours rose above

control levels, but did not reach the levels of fish shocked with 27 ppt from IV1. Time of year, photoperiod and stage of development of fish may account for differences in the two in vivo experiments. Typically, smoltification occurs in late April for fish reared at our facility. However, numerous investigations suggest that many of the changes associated with the parr-smolt transformation may begin to appear many months prior to smoltification (Wagner, 1974, Saunders and Henderson, 1978). Constant light photoperiod (L24) is believed to suppress smoltification, however, it may not inhibit smoltification completely (Saunders et al, 1985). Recent studies indicate constant light may stimulate an early, prominent peak of Na^+/K^+ ATPase activity, one of the indicators of smoltification. The L24 fish used in the January experiment (IV2) may have developed some ability to maintain plasma chloride levels just below the threshold level of plasma osmolarity for stress protein synthesis, and therefore, not synthesize stress proteins like the SNP fish in October.

This research was conducted to identify the effects of sodium chloride on stress proteins in branchial tissue of non-smolting salmon. These experiments were done as preliminary data for testing the effects of sodium chloride on stress protein synthesis of smolting Atlantic salmon.

The aim of my thesis was to identify and characterize hsp's synthesized upon exposure to heat, sodium arsenite and sodium chloride. Stress proteins are present in Atlantic

salmon branchial tissue upon heat shock in non-smolting salmon, and might protect the fish during the freshwater to seawater transition until plasma ion levels can be reduced.

The identification of hsps in Atlantic salmon in this study will allow other researchers to test for the presence of stress proteins in branchial tissue during smoltification. Ultimately, this may lead to the development of a simple procedure to allow the aquaculturists to properly identify smolts and possibly manipulate smoltification by enhancing the survivability of salmon transferred to seawater.

Figure 1. Viability of branchial tissue exposed to heat shock determined by lactate dehydrogenase leakage.

Gill Viability Heat Shock (°C)

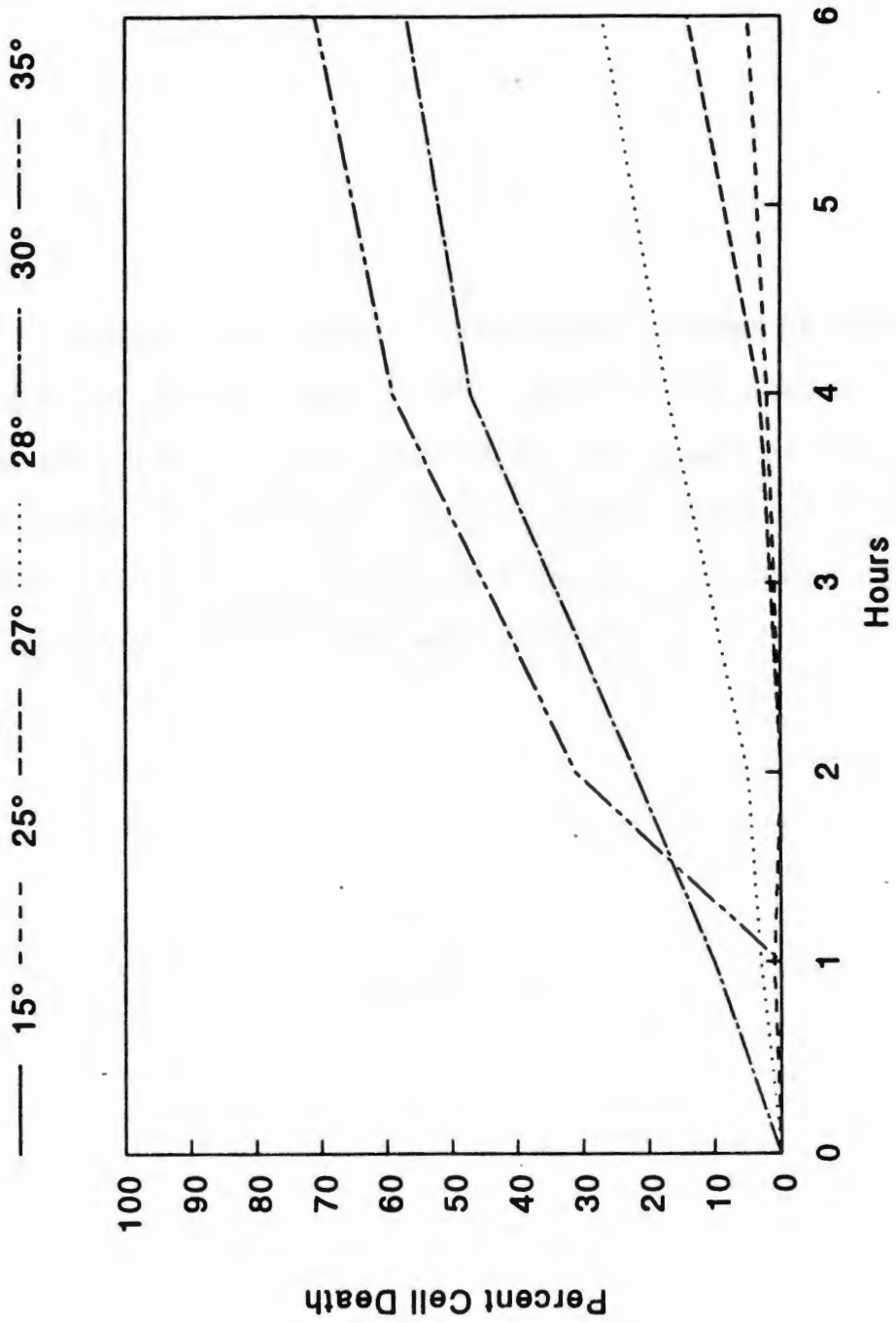


Figure 2. Protein synthesis of branchial tissue at three temperatures over four hours. Data represents mean and standard deviation of four fish. Analysis of variance and Tukey post-hoc test with an alpha level of 0.05 was used to detect differences between the means. No statistical differences were detected.

Protein Synthesis Response to Heat Shock

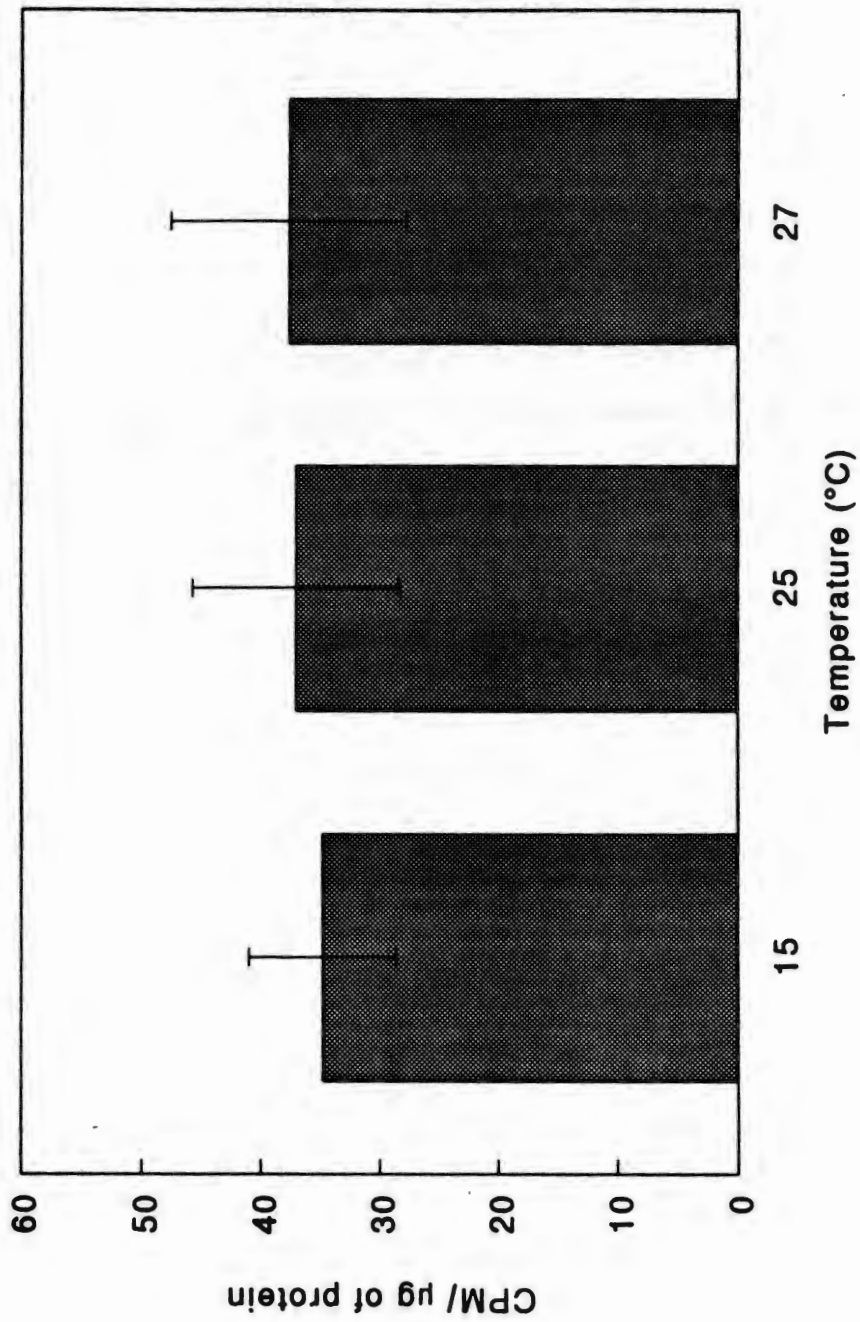


Figure 3. Viability of branchial tissue exposed to various levels of sodium arsenite, as determined by lactate dehydrogenase leakage.

Gill Viability

Sodium Arsenite (uM)

— 25 - - - 50 - - - 100 ····· 200 - · - · 300

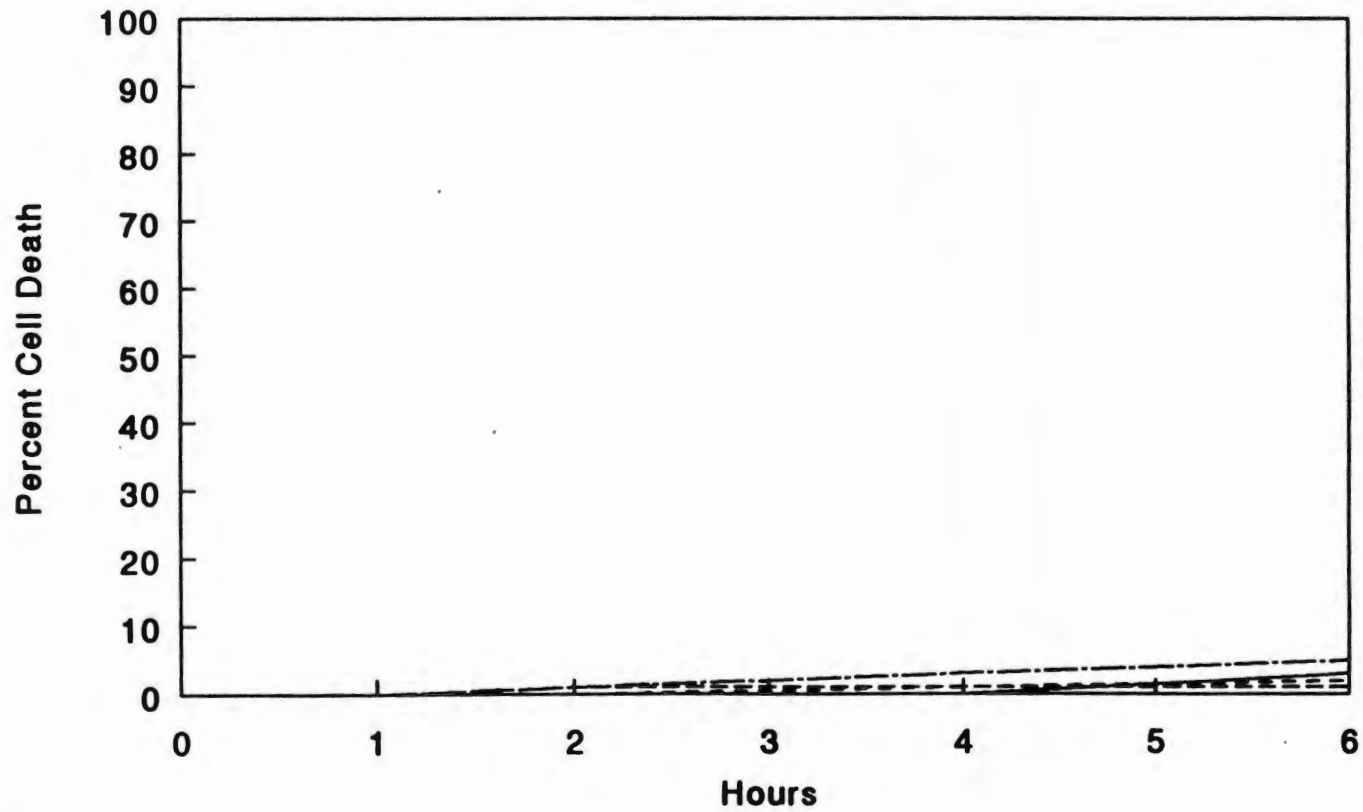
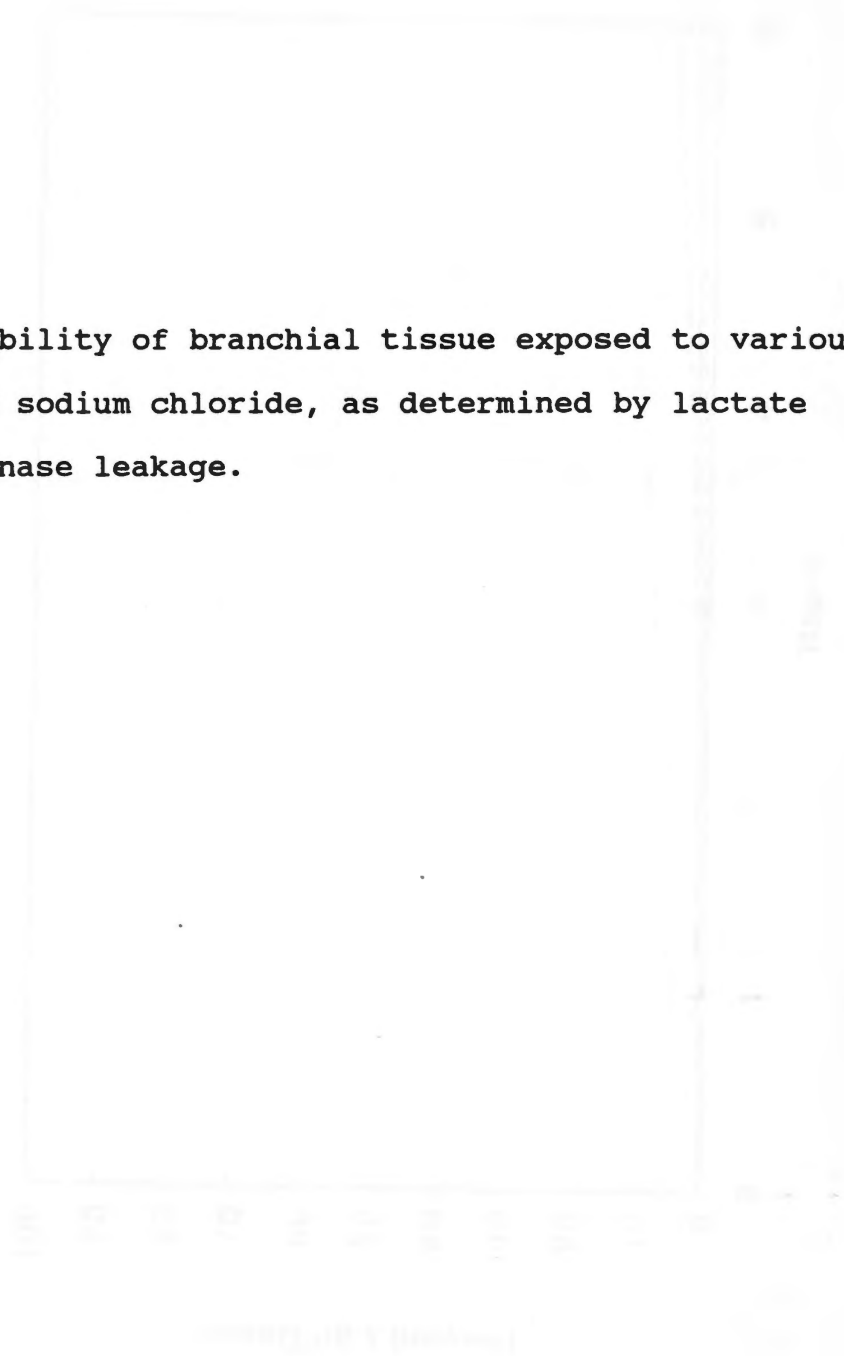


Figure 4. Viability of branchial tissue exposed to various levels of sodium chloride, as determined by lactate dehydrogenase leakage.



Gill Viability Sodium Chloride

— 366mM - - - 466mM - - - 566mM 616mM

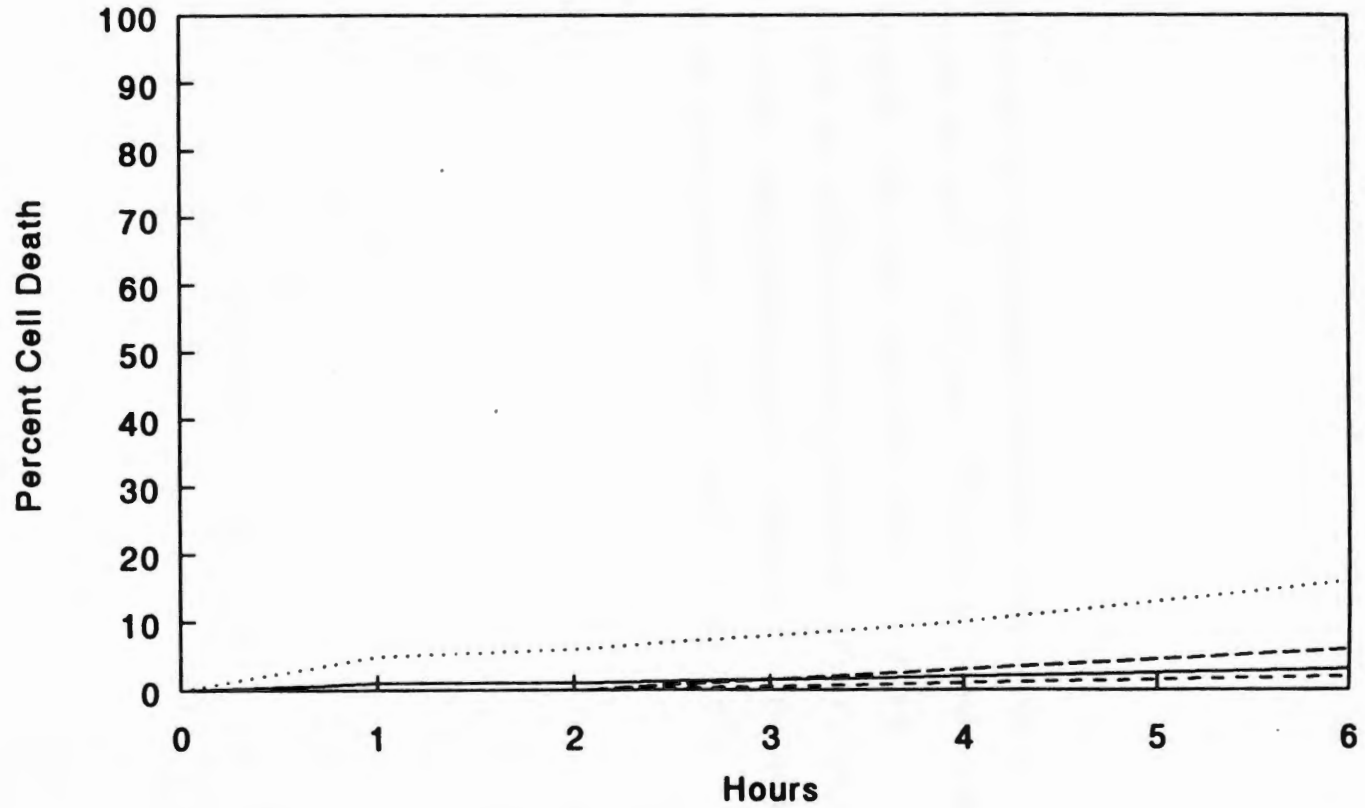


Figure 5. Fluorograph of branchial proteins produced at three temperatures (15° , 25° and 27°C) with duplicates. Gels were loaded with 1500 CPM per lane. Arrows designate proteins with molecular weights of 87, 82, 72, 71, 67 and 54 kDal from top to bottom. Lane and temperature of heat shock: (1,2) 15°C , (3,4) 25°C , (5,6) 27°C .

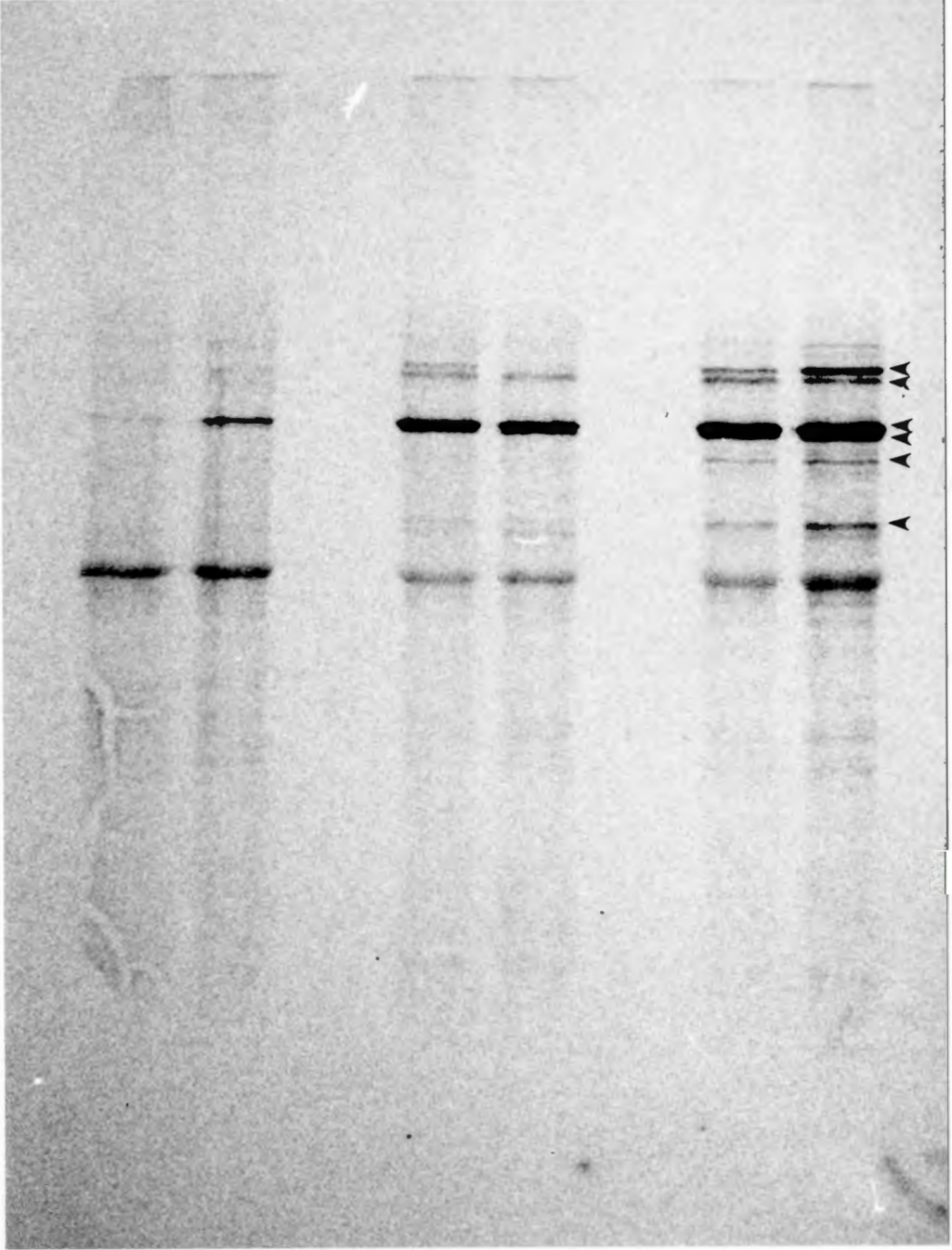


Figure 6. Fluorograph depicting time of induction of hsps in branchial tissue exposed to 25°C and labeled during the last hour. 3000 CPM were loaded per lane. Arrows designate proteins with approximate molecular weights of 87, 82, 72/71, 54 kDal in descending order. Lane, temperature and hours of heat shock: (1) 15°C, one hour; (2,3) 25°C, one hour; (4,5) 25°C, two hour; (6) molecular weight markers; (7,8) 25°C, three hour; (9,10) 25°C, four hour.



Figure 7. Protein concentrations of lamallae exposed to 25°C and labeled for two hours then incubated without radiolabel for various times at 25°C. Data represents mean and standard deviation of three fish. No statistically significant differences were detected.

Protein Decay at 25°C

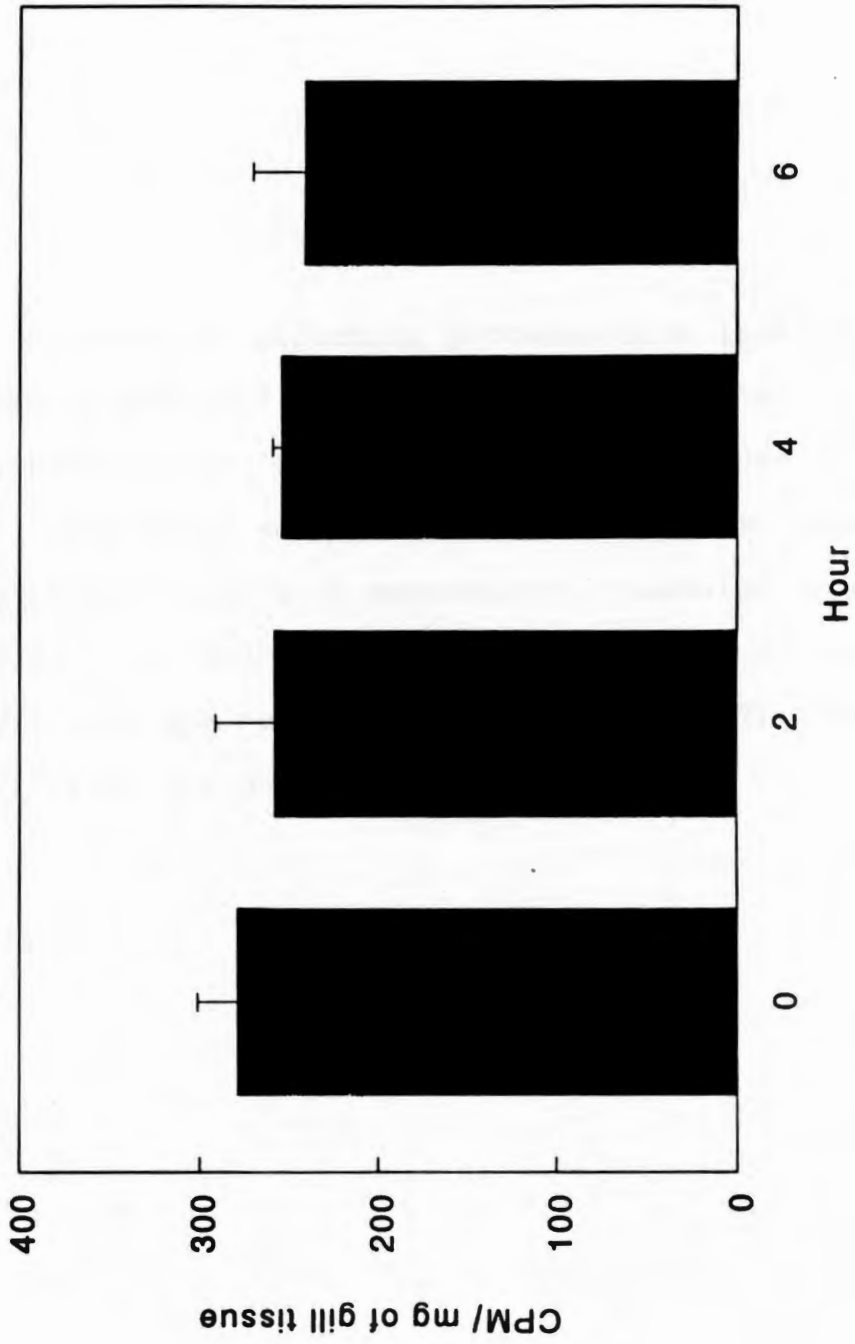


Figure 8. Fluorograph depicting proteins from lamallae exposed to 25°C and labeled for two hours then incubated without radiolabel for various times at 25°C. 150 ug of protein were loaded into each lane. Arrows designate proteins with approximate molecular weights of 87,82 72/71 and 54 kDal. Lane and hour: (1) zero hours; (2,3) one hour; (4,5) two hours; (6,7) four hours; (8,9) six hours.

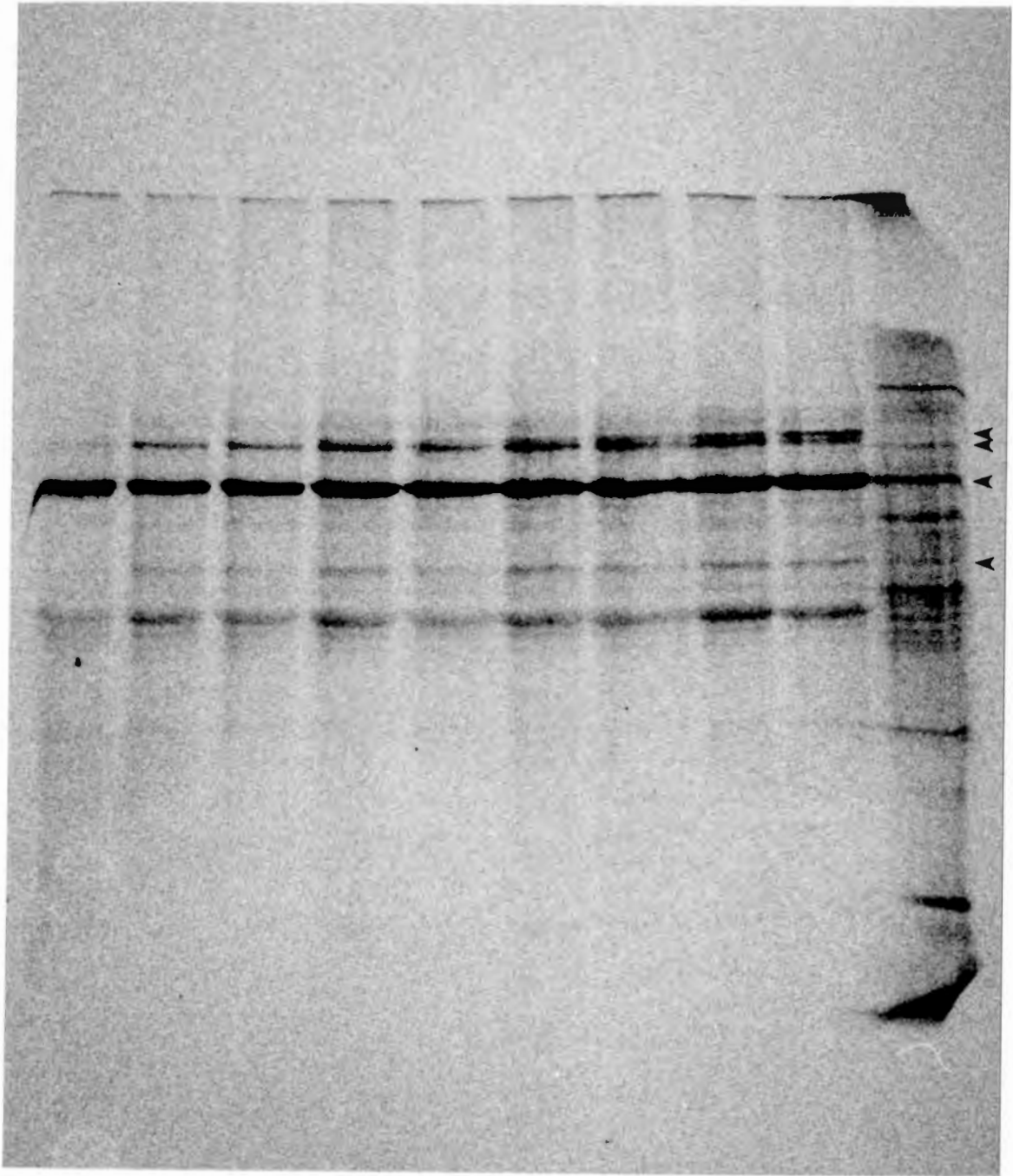


Figure 9. Protein concentrations of lamallae exposed to 25⁰C and labeled for two hours then chased for various times at 15⁰C. Data represents mean and standard deviation of three fish. Within groups analysis of variance and Tukey post-hoc test with an alpha level of 0.05 was used to detect differences between the means. No statistical differences were detected.

Protein Decay at 15°C

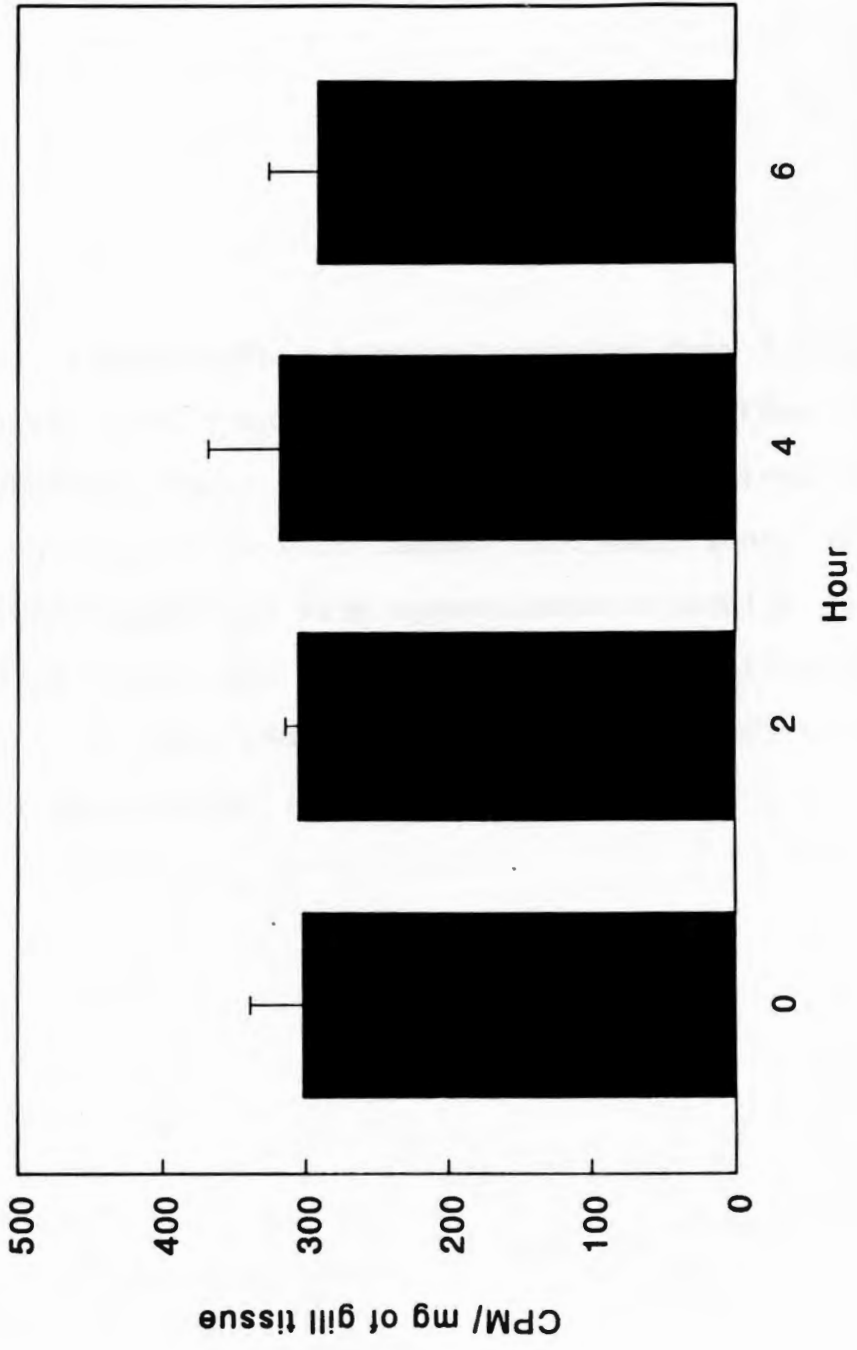


Figure 10. Fluorograph depicting proteins from lamallae exposed to 25⁰C and labeled for two hours then incubated without radiolabel for various times at 15⁰C. 150 ug of protein were loaded into each lane. Arrows designate proteins with approximate molecular weights of 87,82 72/71 and 54 kDal. Lane and duration of heat shock: (1) zero hours, (2,3) one hour, (4,5) two hours, (6,7) four hours, (8,9) six hours.

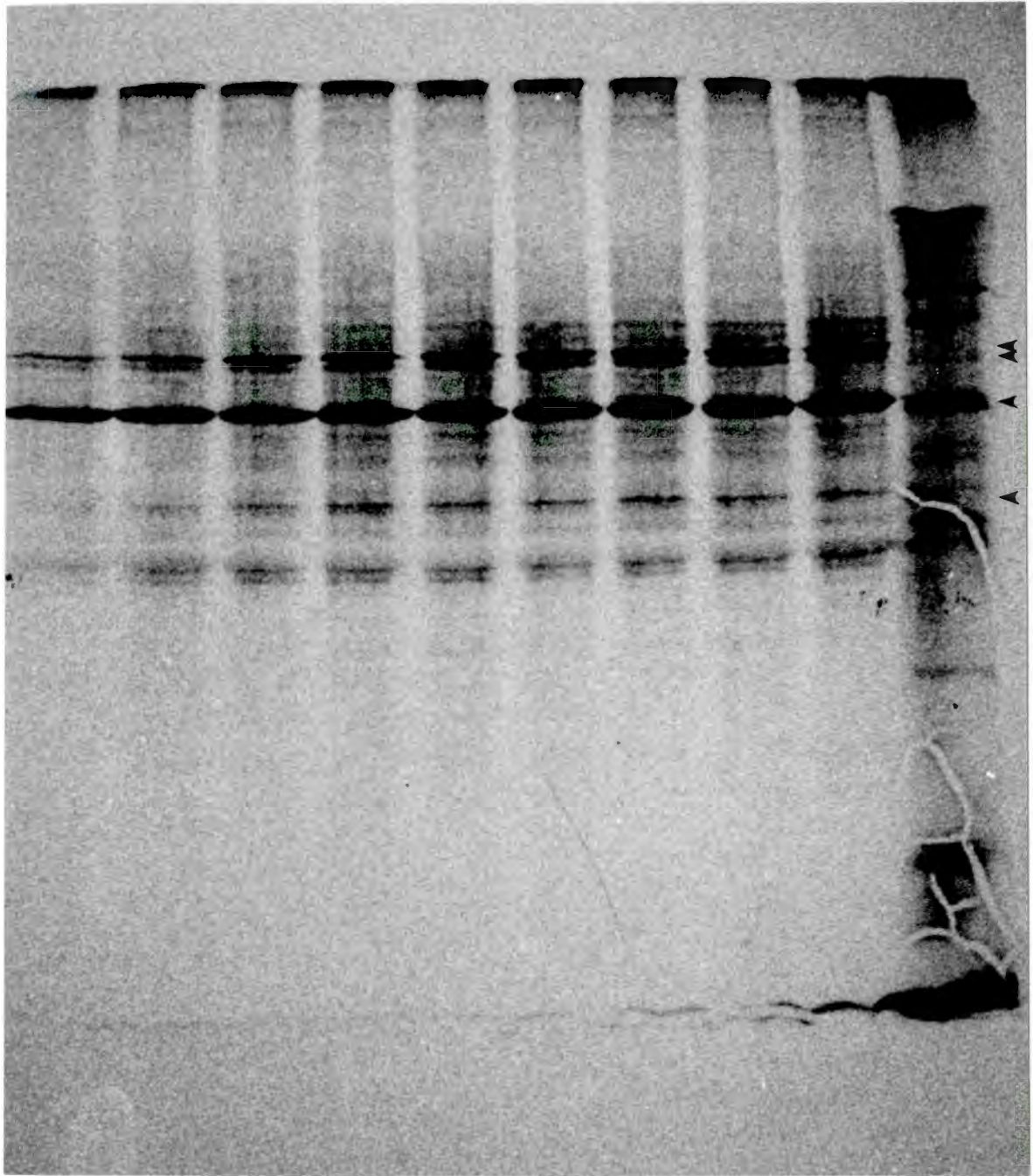


Figure 11. Protein synthesis of branchial tissue in response to exposure to sodium arsenite. Data represents mean and standard deviation of four fish. Analysis of variance and Tukey post-hoc test with an alpha level of 0.05 was used to detect differences between the means. Values with the same letters are statistically different.

Protein Synthesis Response to Sodium Arsenite

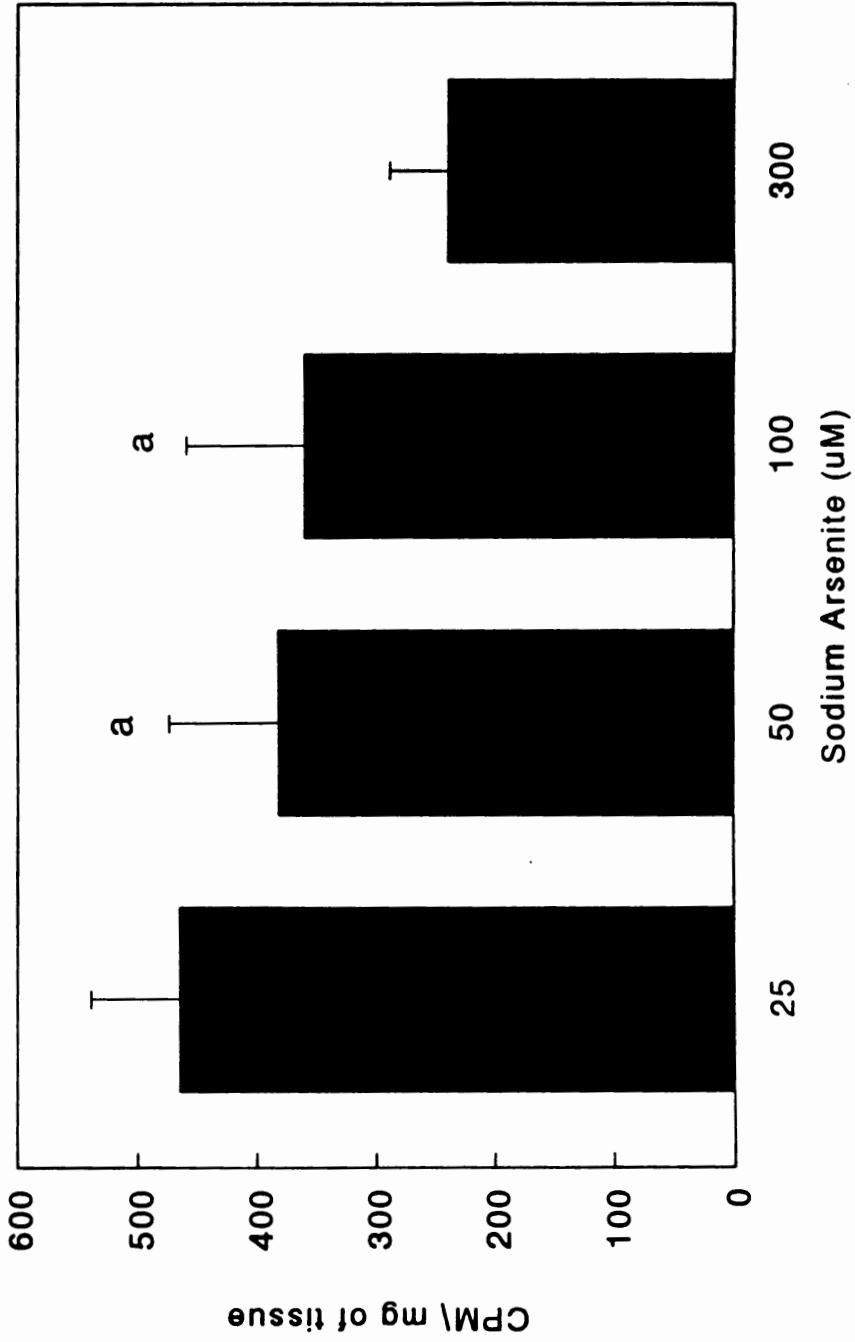
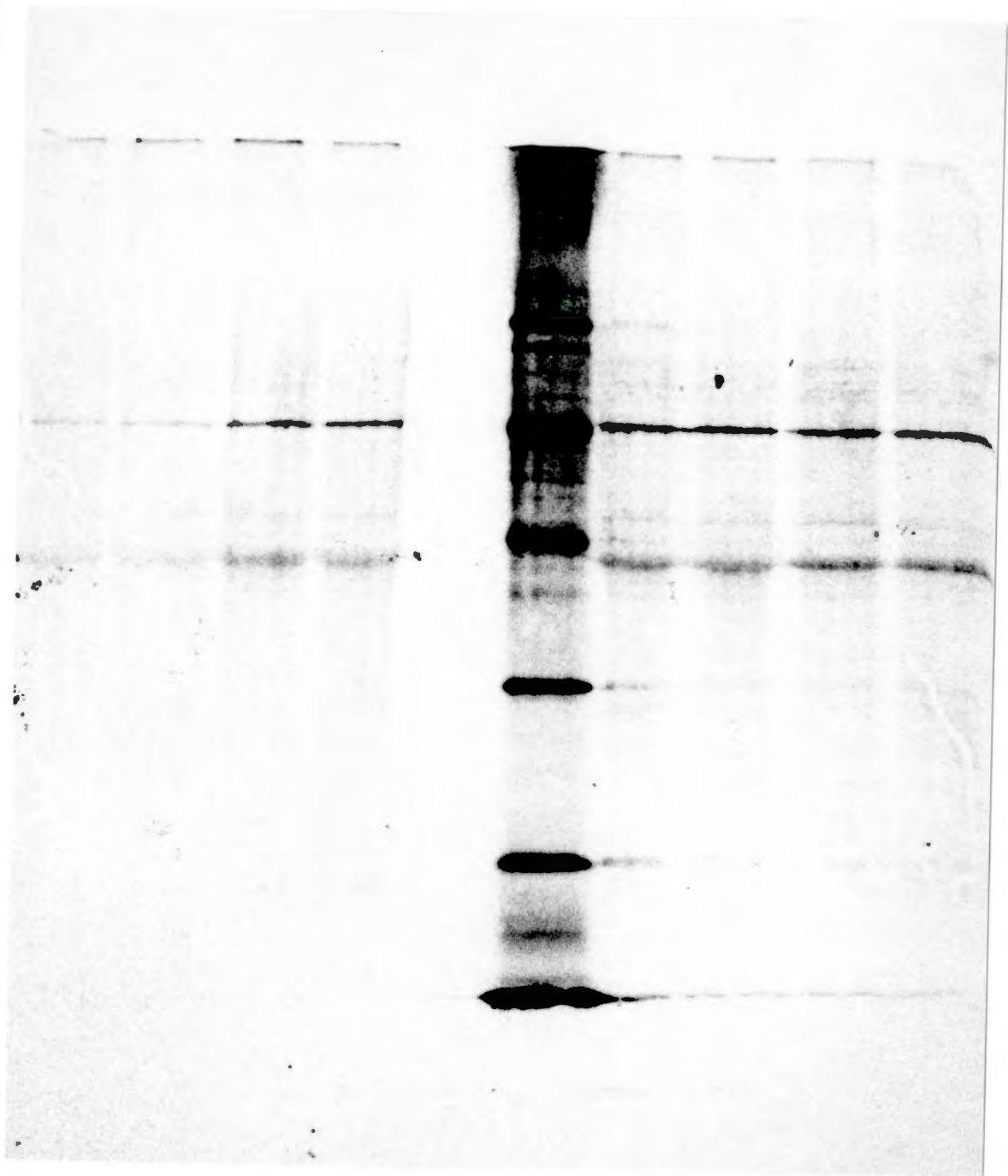


Figure 12. Fluorographs depicting proteins from lamallae exposed to sodium arsenite. Lane and concentration of sodium arsenite: (1,2) 300 μ M, (3,4) 100 μ M, (6) molecular weight standards, (7,8) 50 μ M, (9,10) 25 μ M.



Protein synthesis
Exposure to sodium chloride

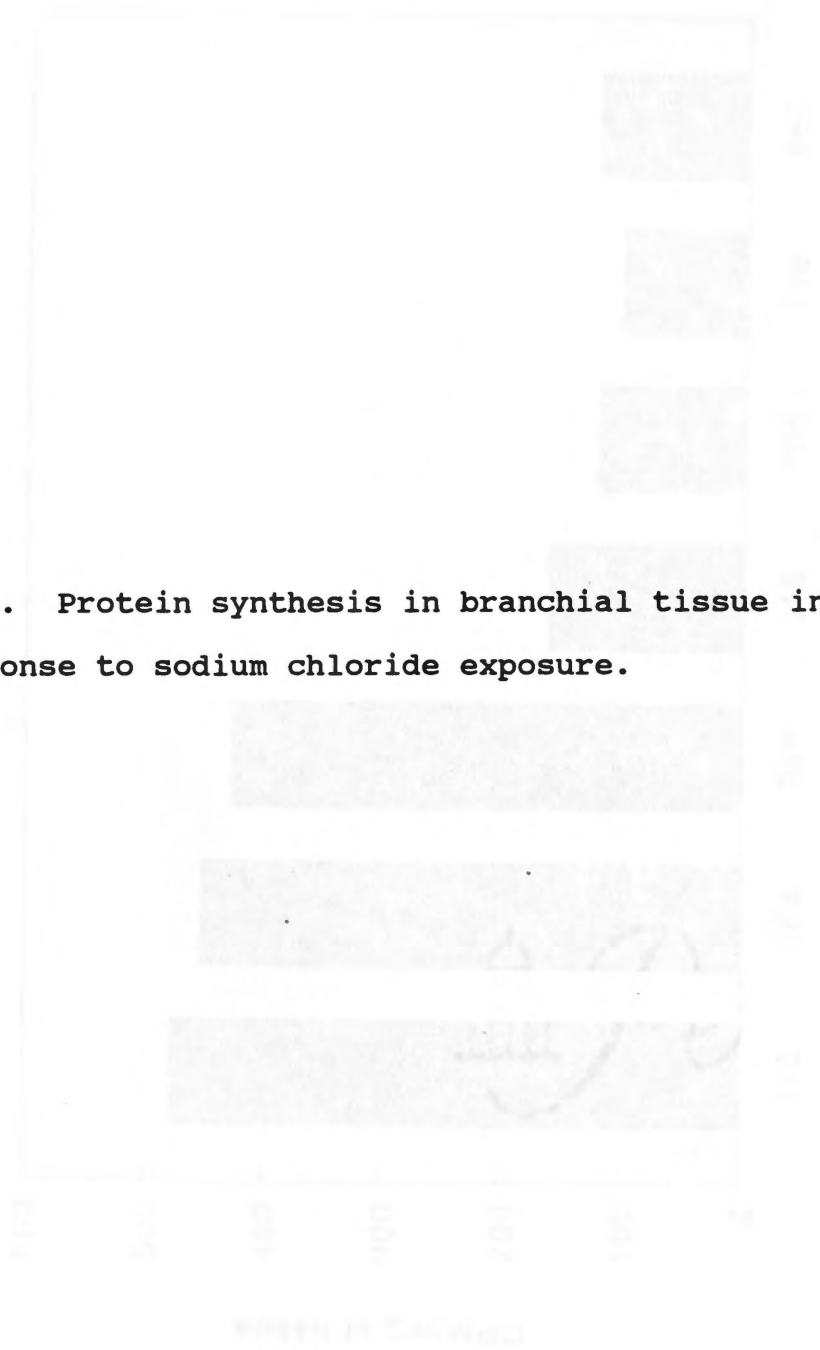


Figure 13. Protein synthesis in branchial tissue in response to sodium chloride exposure.

Protein Synthesis

Exposure to sodium chloride

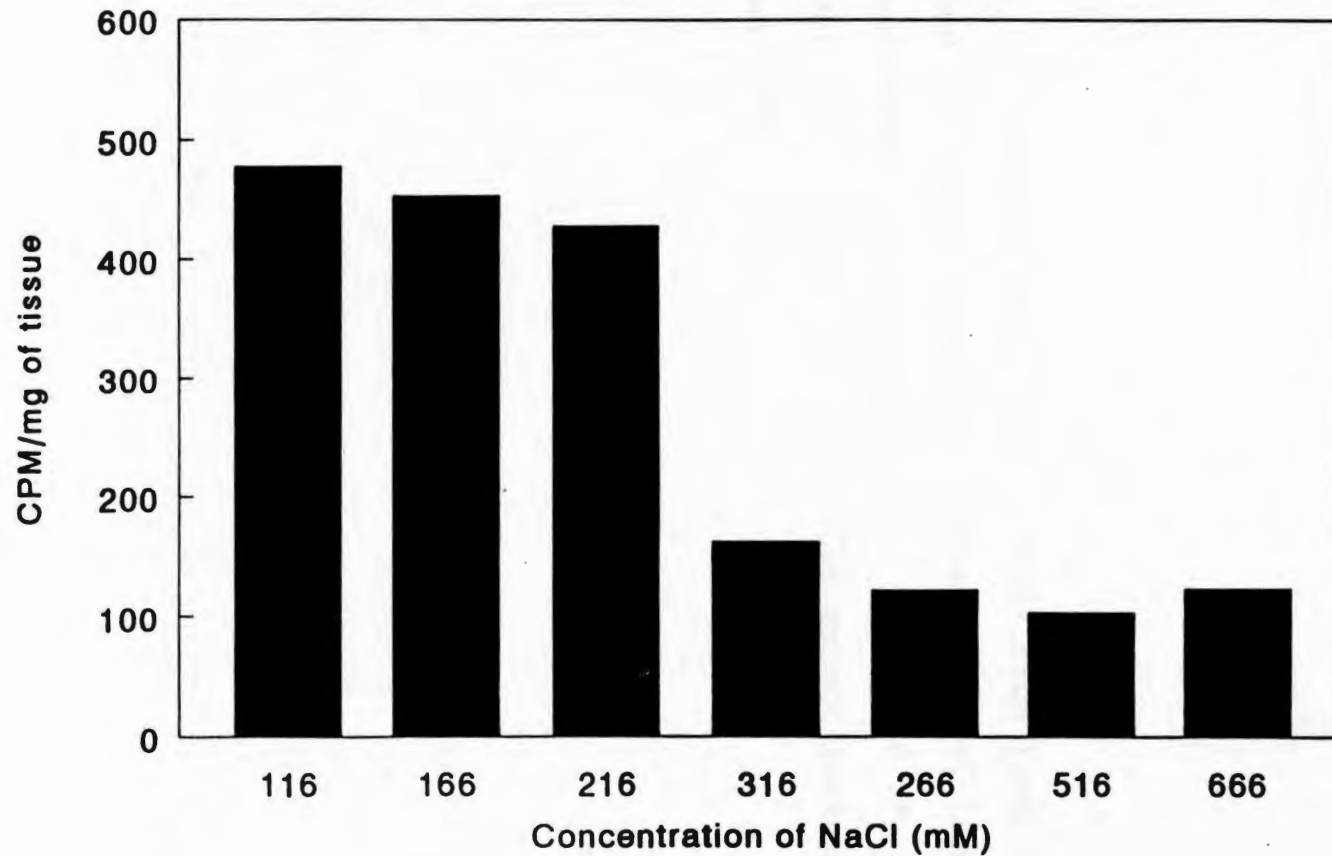


Figure 14. Fluorograph depicting proteins from lamallae exposed to various levels of sodium chloride. Lane, concentrations of sodium chloride: (1,2) 116 mM, (3,4) 366 mM, (5,8) 516 mM, (6) molecular weight markers, (9) 666 mM, (10) 766 mM.

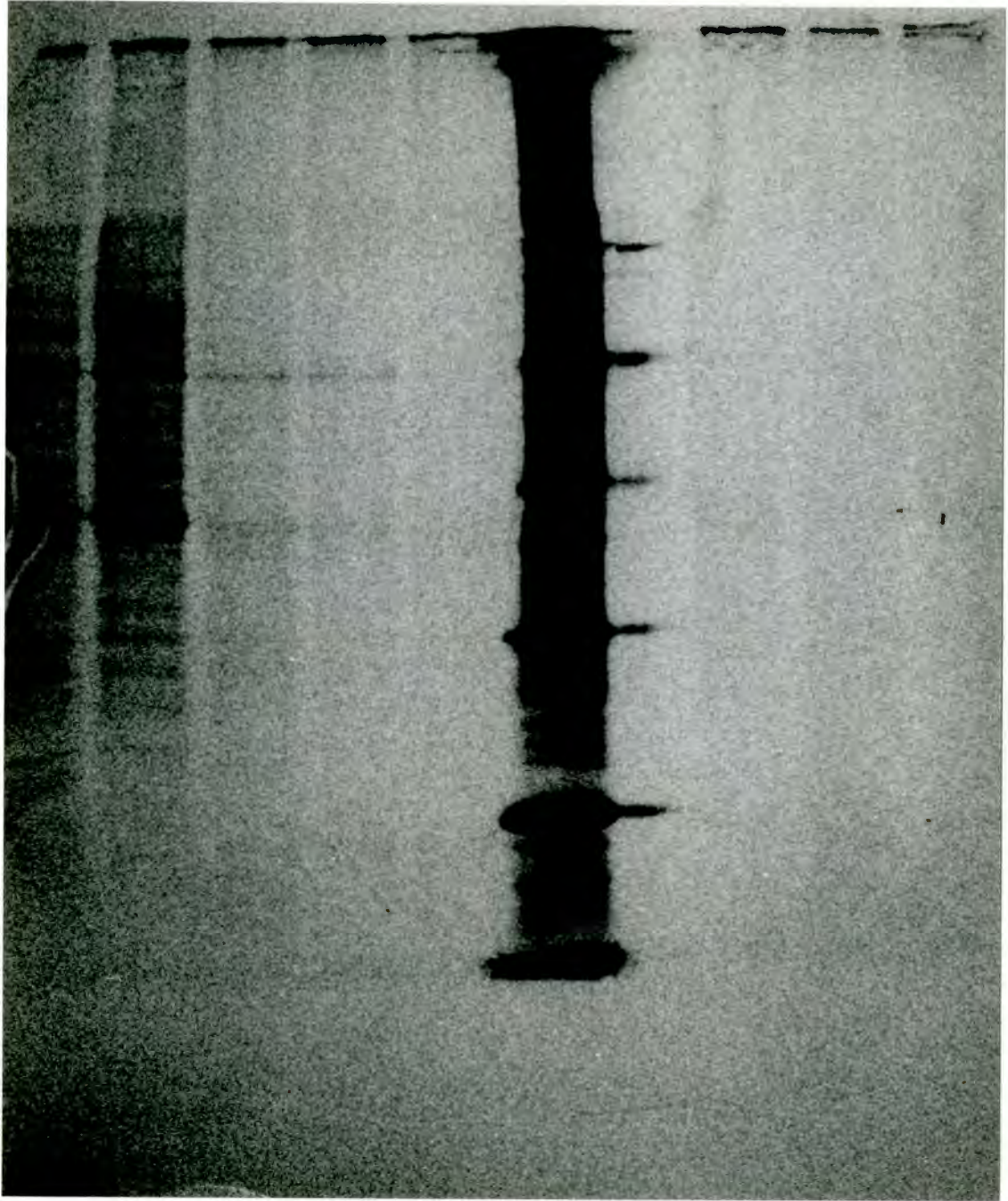


Figure 15. Fluorograph depicting proteins from lamallae exposed to various levels of sodium chloride. Lane, concentration of sodium chloride: (1,2) 116 mM, (3,4) 166 mM, (5,6) 216 mM, (7,8) 316 mM, (9) 366 mM.

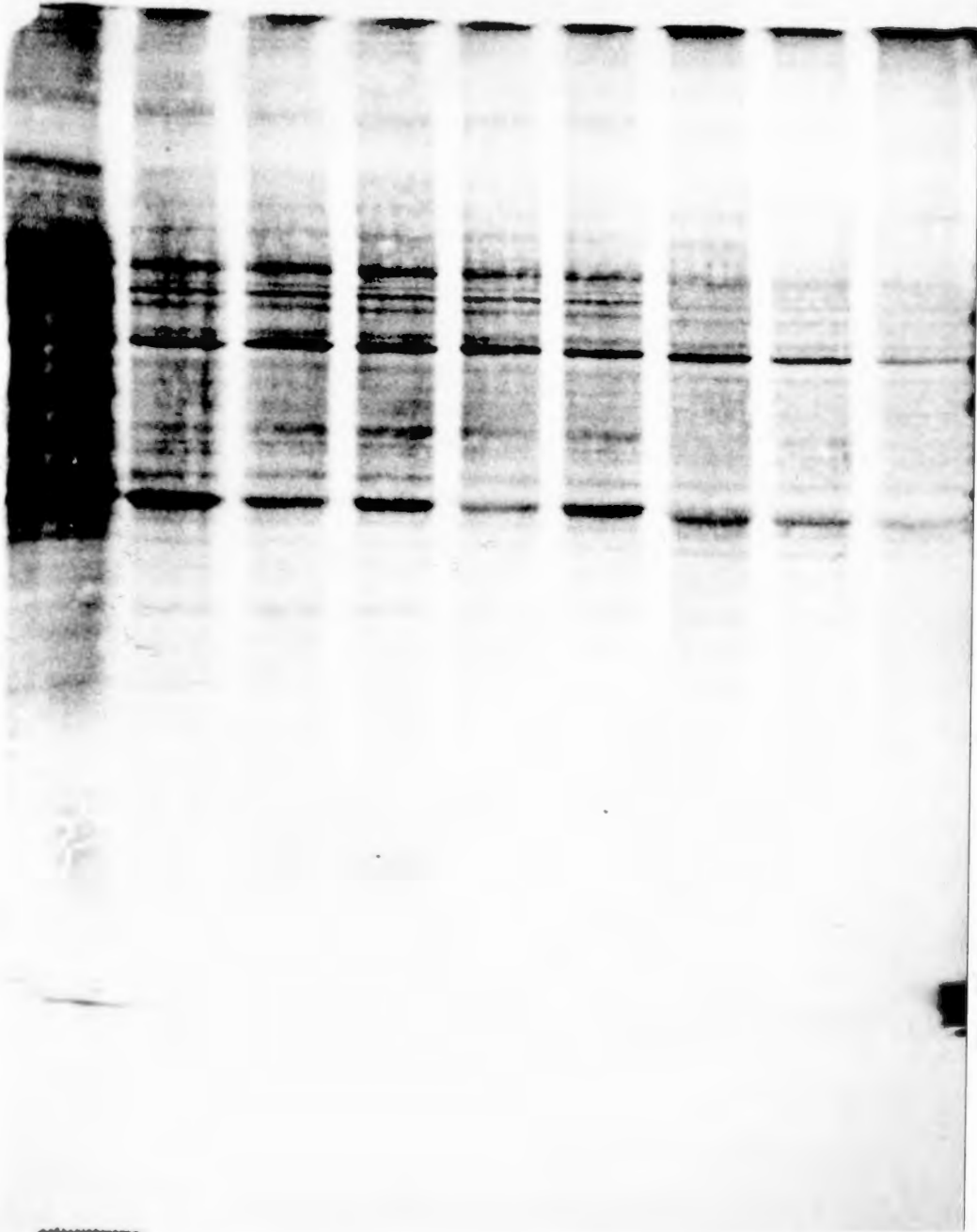
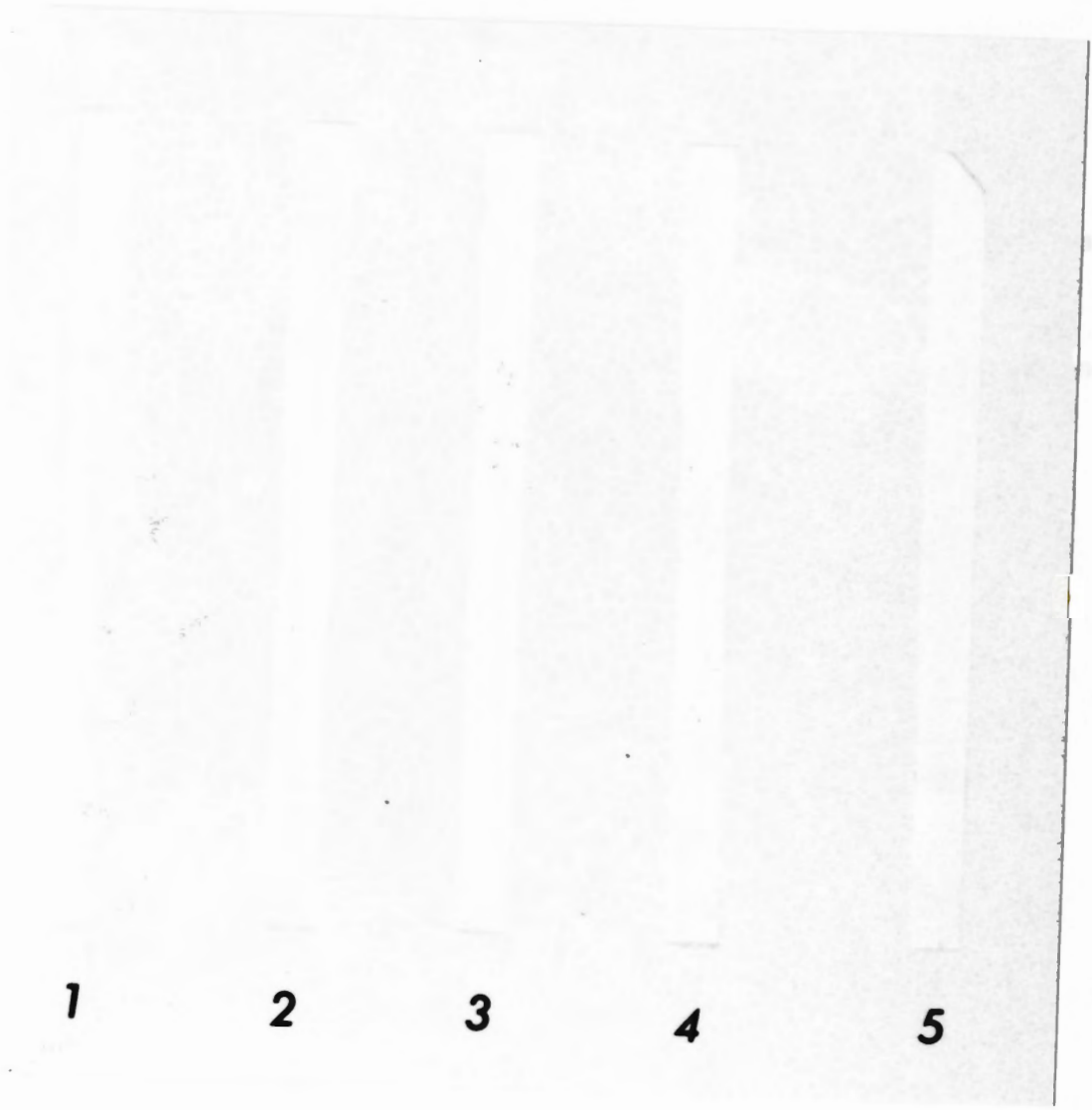


Figure 16. Six negative controls are shown in which non-specific binding and specificity of the different antibodies were tested with heat shocked polypeptides for the protein immunoblotting system.

Lane:

- 1) Primary antibody was omitted to test non-specific binding of secondary antibody and silver enhancement
- 2) Primary and secondary antibodies were omitted to detect non-specific binding of silver enhancement
- 3) Proteins were probed with monoclonal constitutive and inducible hsp 70 and secondary antibody was omitted to test non-specific binding of silver enhance to primary antibody
- 4) Proteins were probed with polyclonal hsp 70 and secondary antibody was omitted
- 5) Proteins were probed with polyclonal hsp 60 and secondary antibody was omitted



1

2

3

4

5

Figure 17. Heat shocked, 25°C (S) and control (C) branchial polypeptides were probed with monoclonal hsp 70 antibodies, constitutive and inducible hsp 70 (con) and the inducible hsp 70 (ind). Total protein was determined in lane five with the stain amido black.

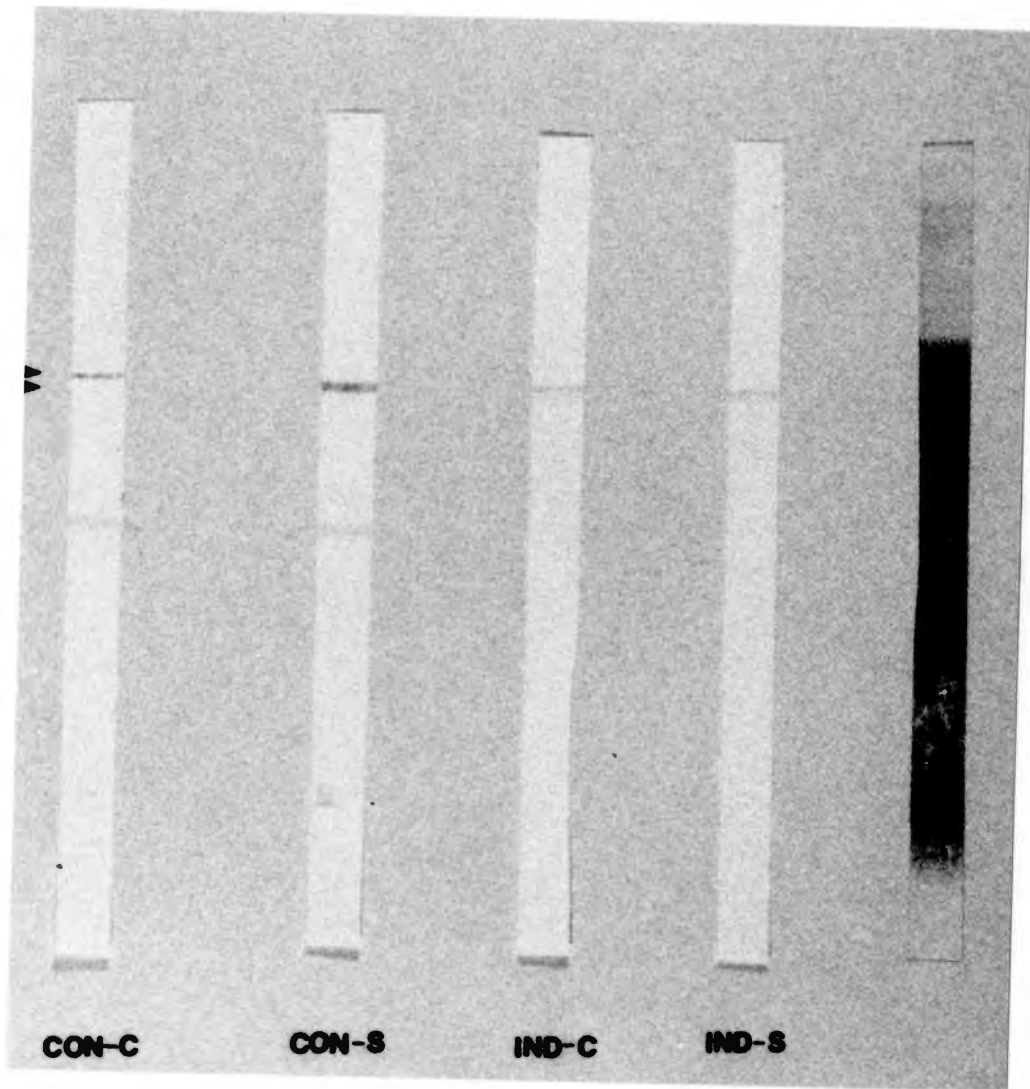


Figure 18. Immunoblot showing specificity of secondary antibody to the primary antibody. The area with a molecular weight corresponding to the respective protein was cut from the lane and constitutive and inducible hsp 70 (con 70), polyclonal hsp 70 (pol 70) and polyclonal hsp 60 (pol 60) antibodies were used to probe the remaining proteins.



Figure 19. Proteins probed with polyclonal hsp 60 (pol 60), polyclonal hsp 70 (pol 70), constitutive and inducible hsp 70 (con 70) and inducible hsp 70 (ind 70) antibodies with no blocking buffer is shown. Arrows designate 71/72 kDal and 54 kDal protein bands.

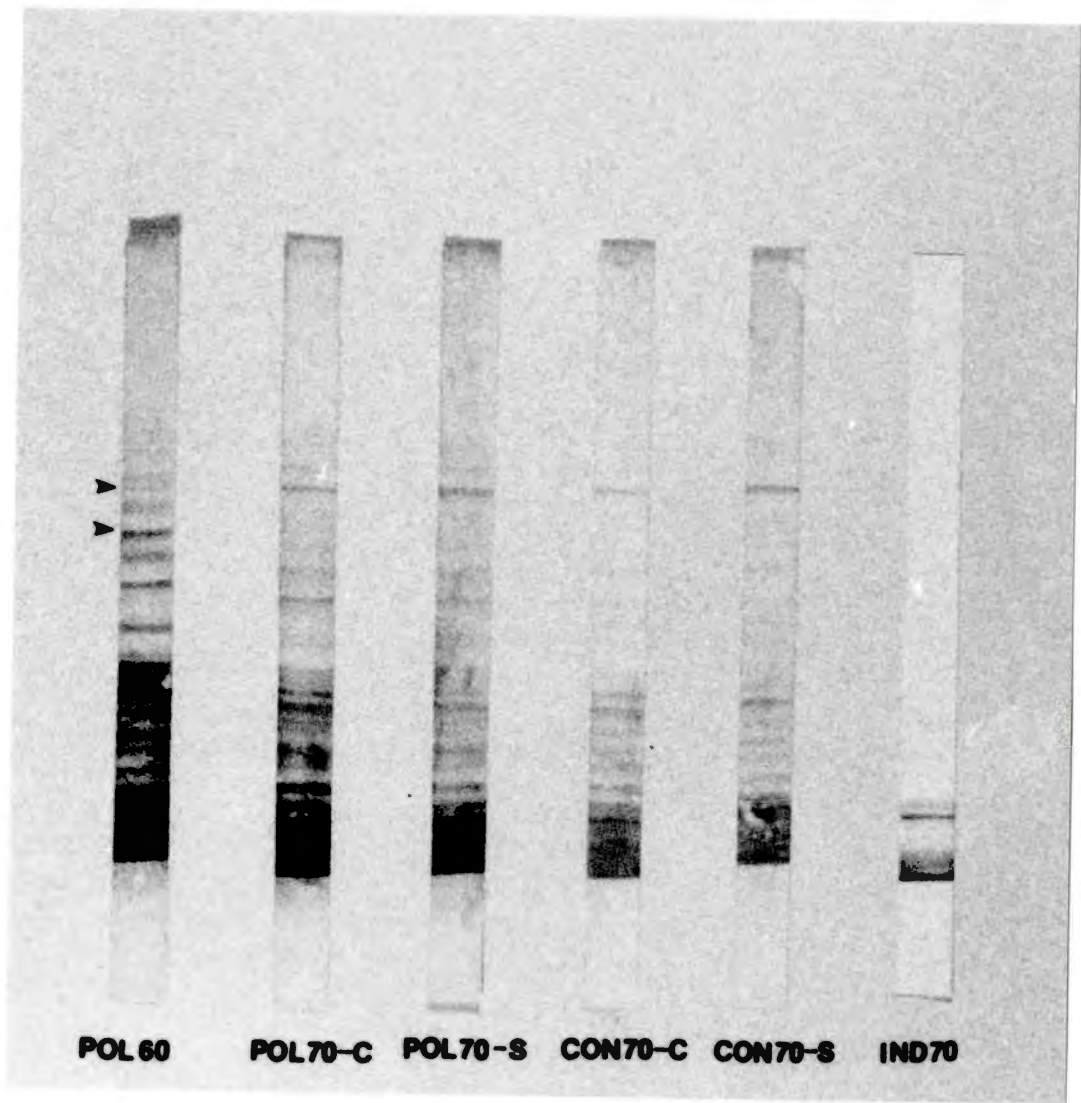


Figure 20. Control (c) and heat shocked, 25°C (s) polypeptides from branchial tissue probed with monoclonal hsp 90. Blocking buffer was omitted from lane three (nb). Total protein (lane four) was detected with amido black.

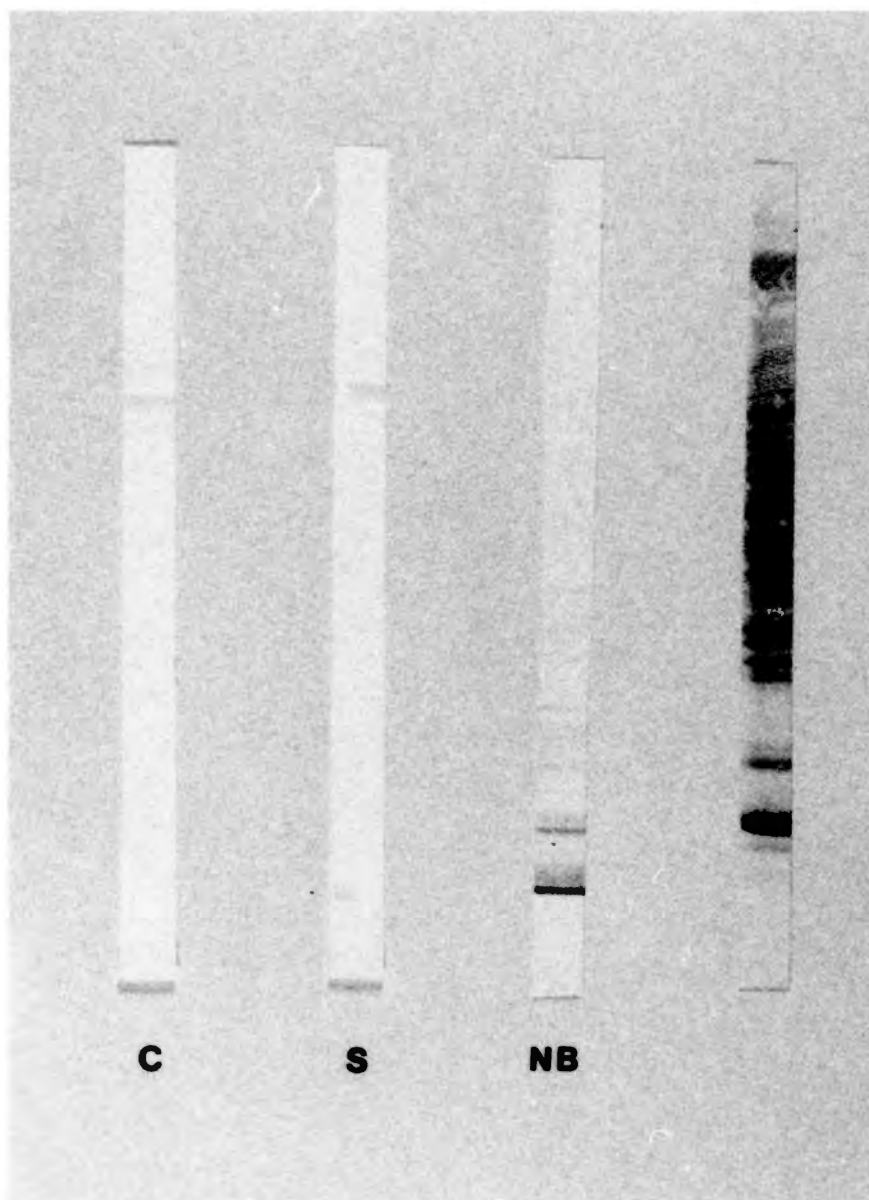


Figure 21. Control and heat shocked (25°C) branchial proteins probed with polyclonal Hsp 60. Arrow designates 54 kDal protein.

Lane

- 1) Total protein detected with amido black
- 2) Control proteins blocked with 0.15% dried skim milk and 5% goat serum
- 3) Heat shocked polypeptides blocked with 0.15% dried skim milk and 5% goat serum
- 4) Control proteins blocked with 0.15% dried skim milk
- 5) Heat shocked polypeptides blocked with 0.15% dried skim milk
- 6) Control proteins with no blocking buffer
- 7) Heat shocked polypeptides with no blocking buffer

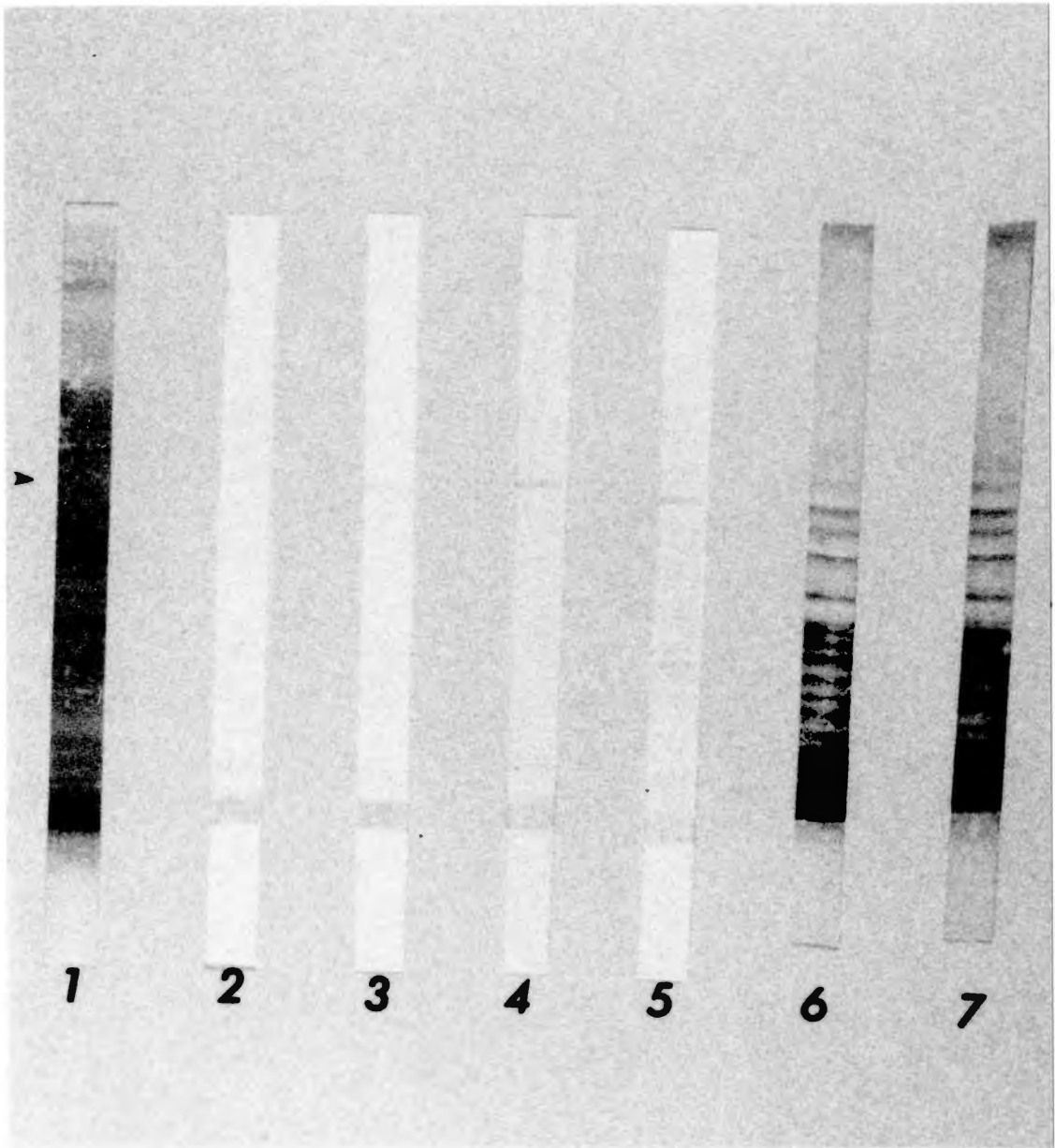


Figure 22. Control (c) and heat shocked, 25°C (s) branchial polypeptides probed with antibodies to E. coli dnaK (MW = 70 kDal) and GroEL (MW = 60 kDal).

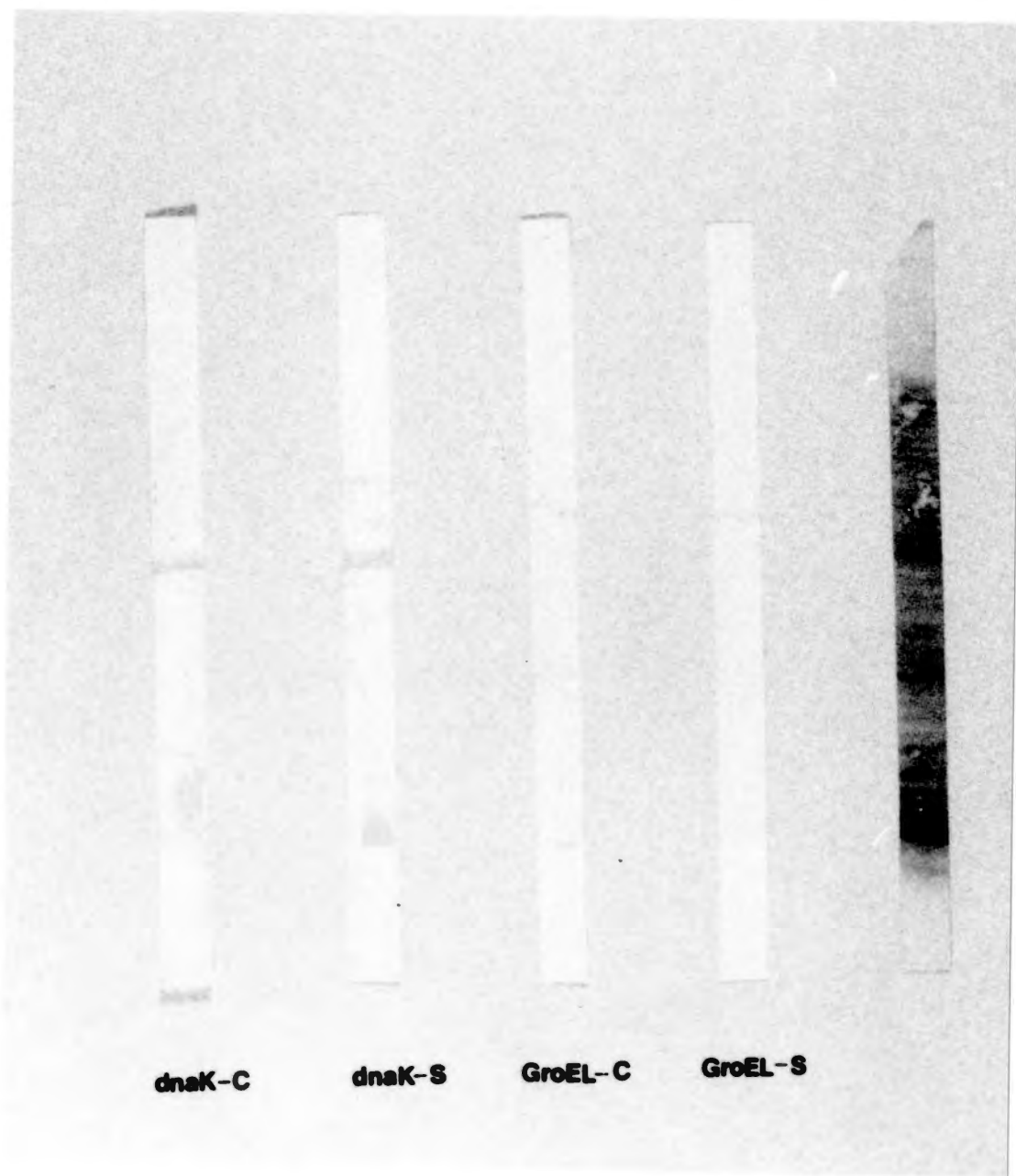


Figure 23. Mean plasma chloride level and standard deviation in fish exposed to in vivo osmotic shock (IV1). Varying sample sizes between three and five were analyzed for differences in means using a within groups analysis of variance and a Tukey post-hoc test ($\alpha = 0.05$). Same letter denotes no statistical differences between group means.

In vivo Osmotic Shock IV1

Plasma Chloride Levels

Control 27 ppt 35 ppt

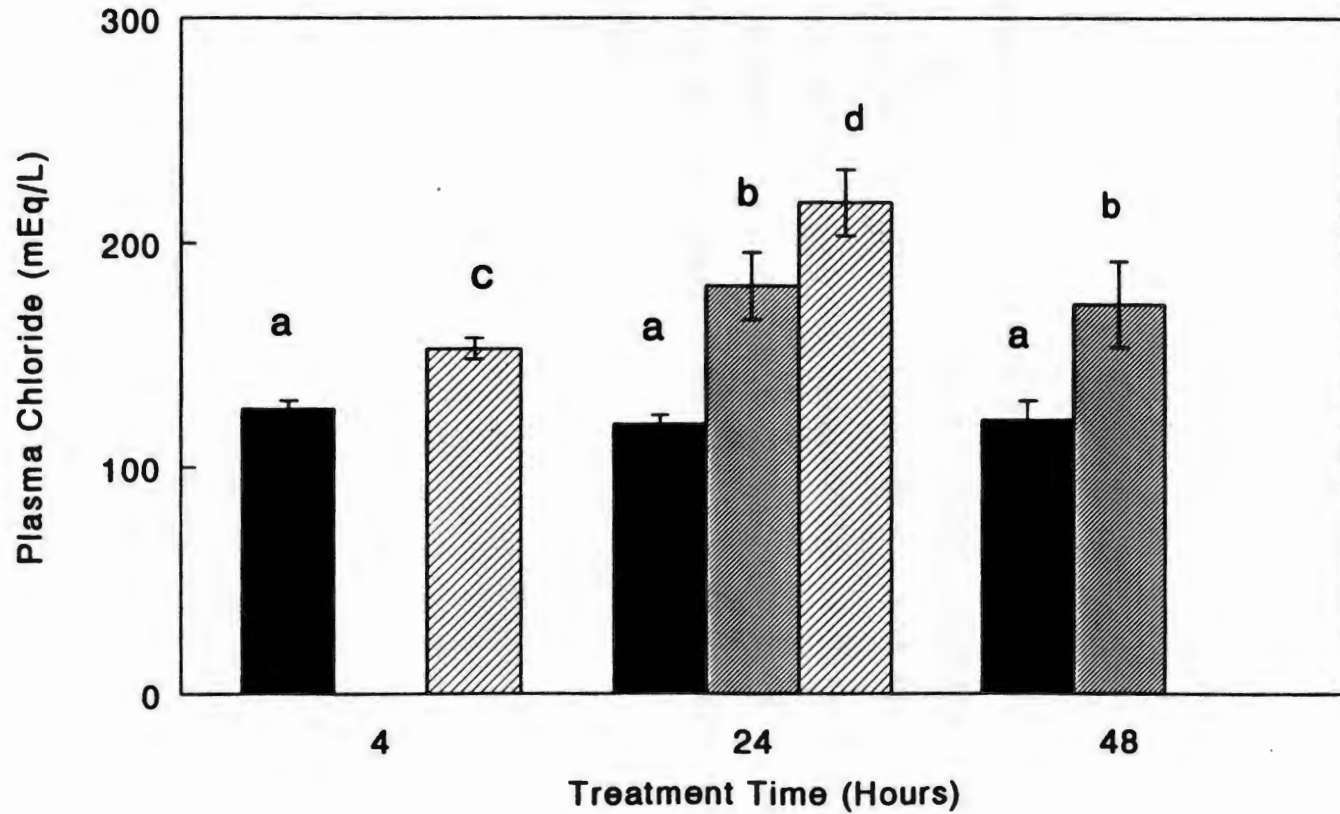


Figure 24. Incorporation of radiolabel into protein in fish exposed to in vivo osmotic shock (IV1). Values are the means \pm SD of three to five individuals and were analyzed for differences in means using a within groups analysis of variance and a Tukey post-hoc test ($\alpha = 0.05$). Same letter denotes no statistical differences between group means.

In vivo Osmotic Shock IV1 Protein Synthesis

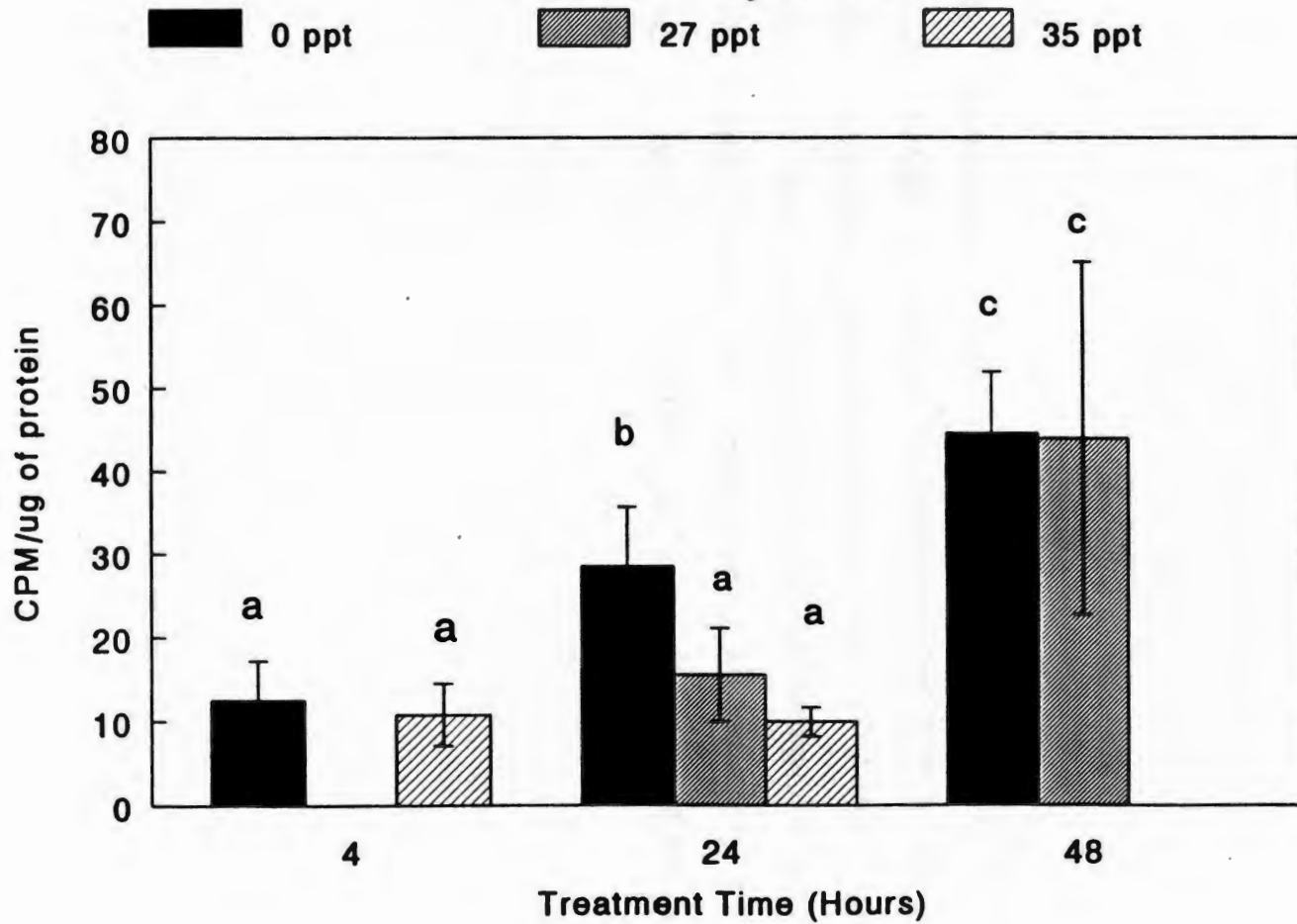


Figure 25. Fluorograph depicting branchial proteins from fish subjected to various salinities for two hours. Arrows designate proteins of 54, 41 and 40 kDal. Lane, duration of exposure, salinity: (1) 24 hours, 0 ppt; (2) 48 hours, 0 ppt; (3) four hours, 35 ppt; (4) 24 hours, 35 ppt; (5) 24 hours, 27 ppt; (6) 48 hours, 27 ppt.



Figure 26. Mean plasma chloride level and standard deviation in fish exposed to in vivo osmotic shock (IV2). Varying sample sizes between four and five were analyzed for differences in means using an within groups analysis of variance and a Tukey post-hoc test ($\alpha = 0.05$). Same letter denotes no statistical differences between group means.

In vivo Osmotic Shock IV2

Plasma Chloride Levels

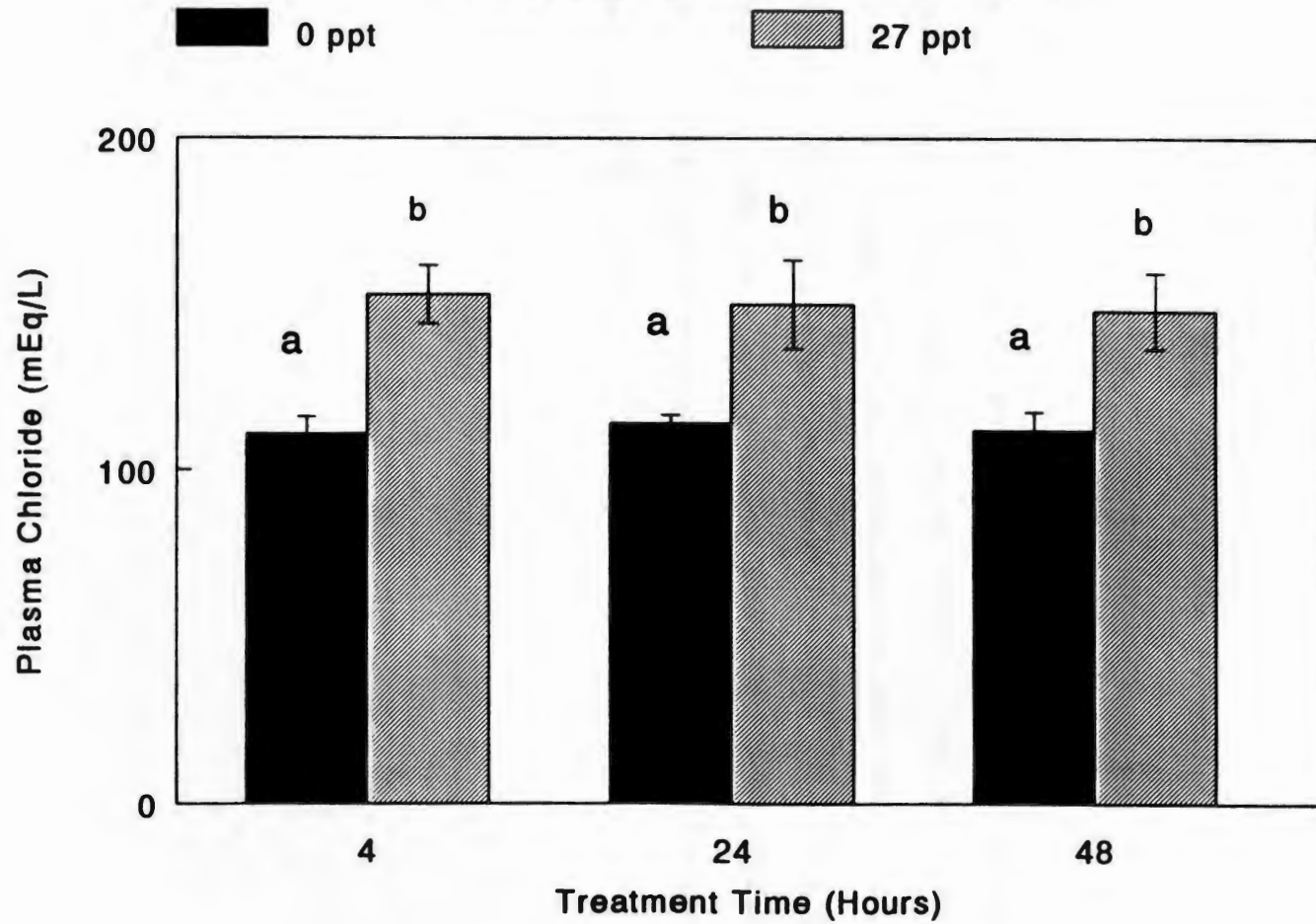


Figure 27. Incorporation of radiolabel into protein fish exposed to in vivo osmotic shock (IV2). Values are the mean \pm SD of three to five individuals and were analyzed for differences in means using a within groups analysis of variance and a Tukey post-hoc test (alpha = 0.05). Same letter denotes no statistical differences between group means.

In vivo Osmotic Shock IV2 Protein Synthesis

0 ppt 27 ppt

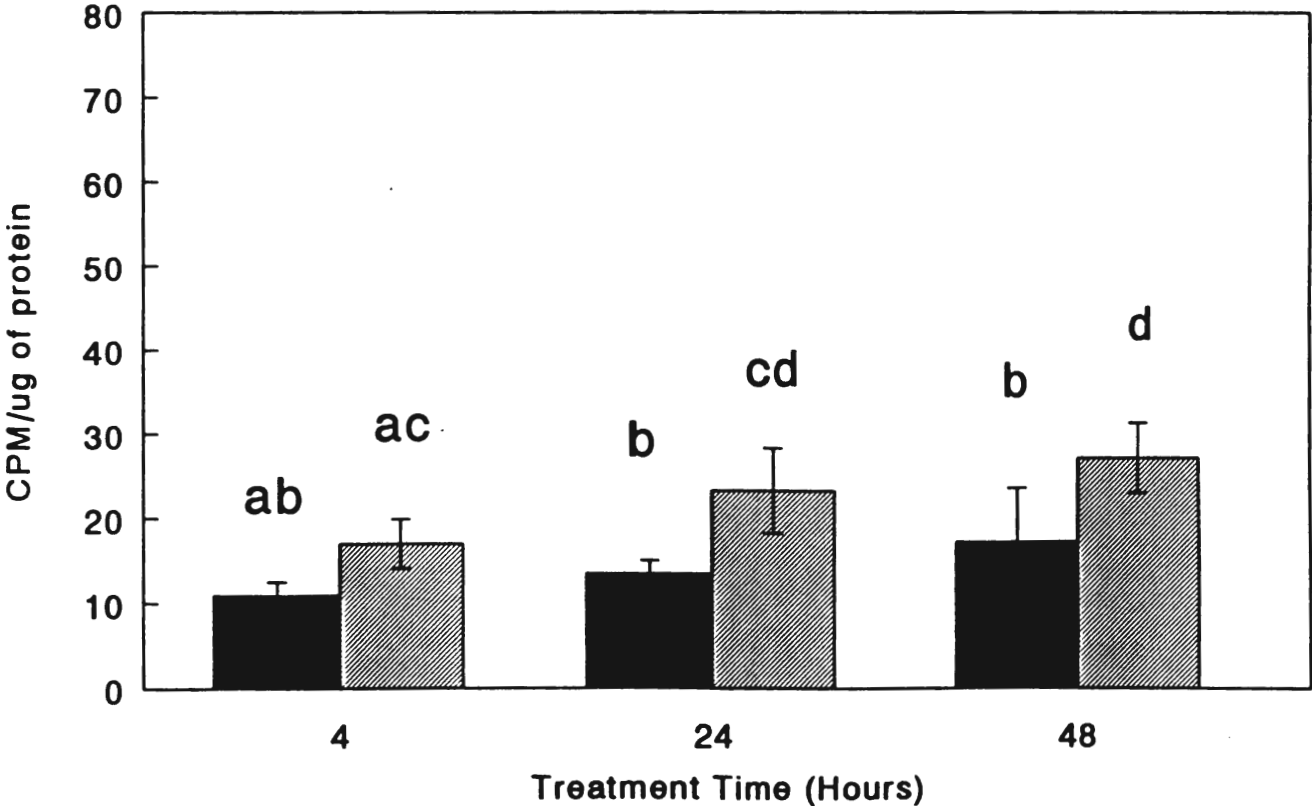


Figure 28. Fluorograph depicting branchial proteins from fish subjected to various salinities for two hours. Lane, duration of exposure, salinity: (1) 4 hours, 0 ppt; (2) 4 hours, 27 ppt; (3) 24 hours, 0 ppt; (4) 24 hours, 27 ppt; (5) 48 hours, 0 ppt; (6) 48 hours, 27 ppt, (7) molecular weight standards.

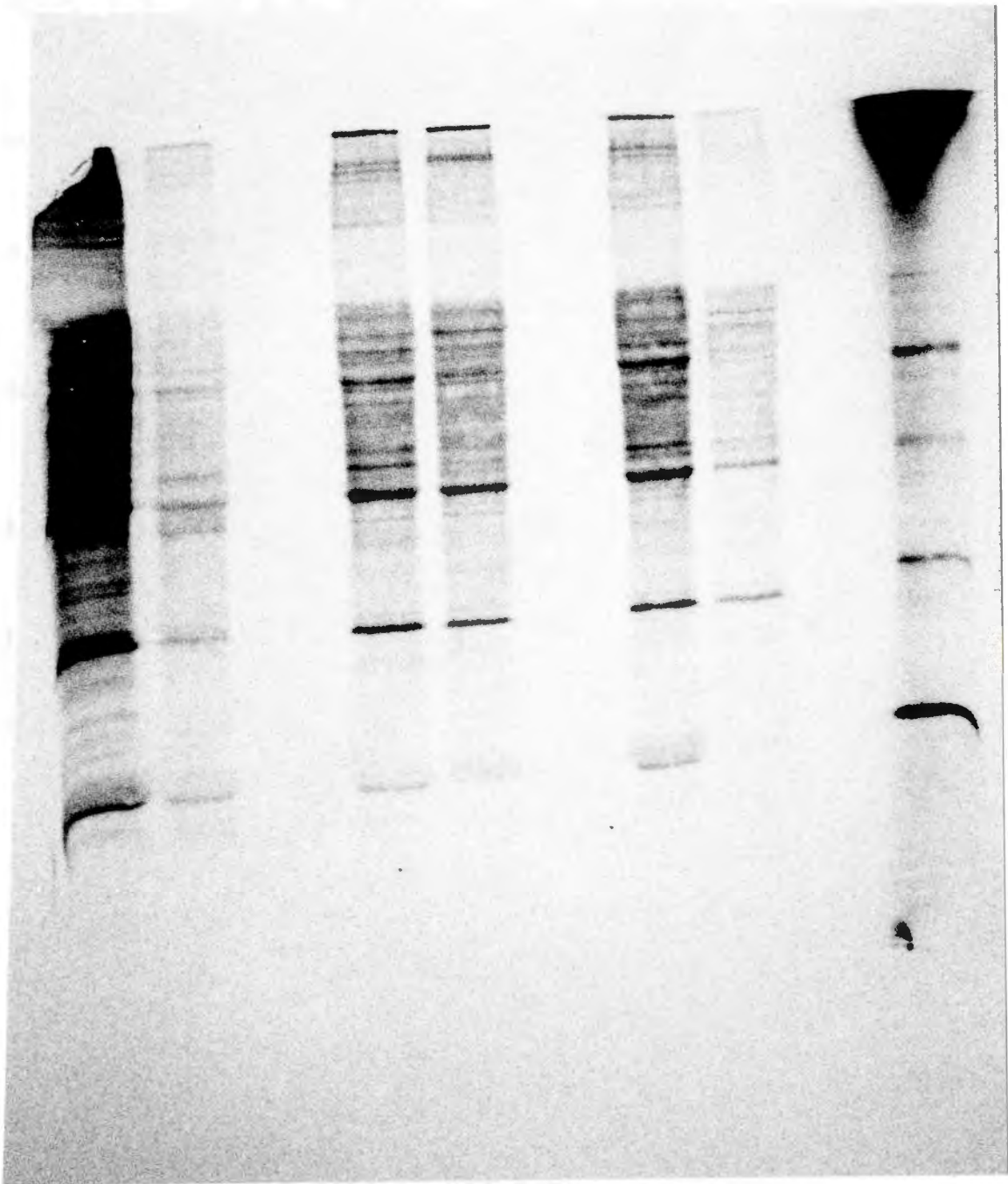


Figure 1. Gel electrophoresis image showing multiple lanes with various bands.

Figure 1. Gel electrophoresis image showing multiple lanes with various bands. The bands are arranged in vertical columns, with some lanes showing more prominent bands than others. The image is somewhat blurry and has a high-contrast, grainy appearance.

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