CHARACTERIZATION OF THE HEAT SHOCK PROTEINS

IN CULTURED TROUT CELLS

MachII

by

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Abstract

Despite considerable investigation, the function of the heat
shock proteins (hsps) remains obscure. In this thesis, the heat
shock proteins of _Salmo gairdnerii_ RTG-2 cells were examined by
two-dimensional gel electrophoresis, and the induction of the 70,000
dalton hsp (hsp70) was studied. As in _Drosophila melanogaster_ each
trout hsp detected by one dimensional analysis separated into several
isoelectric variants following two-dimensional gel electrophoresis.
Post-translational modification of a single polypeptide could have
given rise to charge variants similar to those observed here, but no
evidence for either the phosphorylation of the hsps or for changes in
the phosphorylation of other proteins was obtained. Messenger RNA was
isolated from heat shocked cells and translated in the _in vitro_
rabbit reticulocyte system. Most of the isoelectric variants were
detected in the _in vitro_ translation suggesting that either
different transcripts encode the isoelectric variants or that post-
translational modification is occurring in the _in vitro_ system.
Heat shock mRNA was isolated by hybridization to a cDNA clone of the
70K hsp and translated _in vitro_. Two-dimensional gel
electrophoresis of the translation products revealed that all 70K
isoelectric variants were present.

The induction of hsp70 shortly after the addition of sodium
arsenite was examined using an antibody to chick hsp70. Some
cross-reaction was observed with a polypeptide of 70,000 in

unshocked cells, which could indicate the presence of hsp70 in non-shocked cells. This is not conclusive since the antibody was polyclonal. It was still possible to detect the induction of hsp70 above this background. A sharp increase in the level of antibody binding was detected after 15 minutes.

The relationship of histone acetylation to hsp induction was also examined using sodium butyrate under conditions which cause a high degree of histone hyperacetylation. Synthesis of the heat shock proteins was neither induced nor blocked. Therefore, the state of histone acetylation does not affect the heat shock response.

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Abbreviations.

ACS: Aqueous counting scintillant.

bis-acrylamide: N,N'-methylene-bis-acrylamide.

BSA: Bovine serum albumin.

DNA: Deoxyribonucleic acid.

DNAase I: Deoxyribonuclease I.

EDTA: (Ethylenedinitrilo)tetracetic acid.

EGTA: Ethyleneglycol-bis-(2-aminoethylether)N,N'-tetracetic acid.

HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid.

Hsp: Heat-shock protein.

Hsp30: 30,000 dalton heat shock protein.

Hsp70: 70,000 dalton heat shock protein.

Hsp87: 87,000 dalton heat shock protein.

Hsp100: 100,000 dalton heat shock protein.

NP-40: Nonidet P40.

PBS: Phosphate buffered saline, 8 g NaCl, 0.2 g KH_2PO_4, 13 g
$Na_2HPO_4-7H_2O$ per liter.

RNA: Ribonucleic acid.

RNAase: Ribonuclease.

RNP: Ribonuclear protein particle.

Sarcosyl: N-lauryl sarcosine (sodium salt)

Standard saline citrate, 0.15 M NaCl, 0.015 M sodium citrate,

pH 7.0

TEMED: N,N,N',N'-Tetramethylethylenediamine.

Tris: Tris(hydroxymethyl)aminomethane.

INTRODUCTION

Part I.   The Heat Shock Response.

A.   Introduction.

The phenomenon of heat shock has been extensively studied in recent years. When the growth temperature of an organism is elevated, normal protein synthesis decreases and synthesis of a discrete set of polypeptides begins (1). Ritossa (2) first observed the heat shock response in the polytene chromosomes of Drosophila melanogaster. The puffing pattern of salivary gland chromosomes from heat shocked flies was different from the pattern of chromosomes from flies which had not been shocked: nine new puffs are induced upon heat shock, while all other puffs regress. Later, heat shock was shown to induce the synthesis of nine polypeptides (3). The heat shock response is ubiquitous; similar sets of polypeptides are synthesized in organisms ranging from E. coli to man (4,5). These polypeptides, called heat shock proteins (hsps), can be induced by a variety of stressors, such as metabolic inhibitors, uncouplers of oxidative phosphorylation and recovery from anoxia. Although the response to the different agents is not always identical, the term "heat shock" has been applied to the effects of these various inducers. Because so many compounds induce the hsps, a protective role during times

of stress has been proposed for the hsps (63). However, despite much investigation, the actual functions of these polypeptides remain unclear.

Besides the interest in the hsps themselves, the regulation of the heat shock response has provoked much investigation. Temperature elevation causes a rapid shift in protein synthesis such that, in D. melanogaster, over 70% of all protein synthesis is devoted to the synthesis of heat shock proteins after 1 hour of heat treatment (6). This shift to heat shock protein synthesis is caused by both transcriptional and translational regulation (2,7). In D. melanogaster, heat shock induces a unique set of puffs on the salivary gland chromosomes, which suggests that transcription is occurring at these sites (1). Translational control is also important. Under certain conditions, the translation of normal cellular mRNA is repressed, although the messages remain stable in the cytoplasm and can be translated in in vitro systems (7,8). These effects are fully reversible; upon return to normal temperature, heat shock protein synthesis decreases and normal protein synthesis resumes.

Thus, the heat shock phenomenon is of interest from several points of view. The functions and modes of action of the polypeptides themselves remain to be elucidated and the regulation of the response itself is unclear. Finally, the heat shock phenomenon provides an excellent model system for studies of gene activation.

B. Regulation of the heat shock response.

1. Regulation of transcription.

After heat treatment of cells or tissues, transcription of the heat shock genes is markedly increased, while control gene transcription is repressed. Later, during recovery, this process is reversed and control genes are activated while heat shock genes are repressed. In this thesis, genes other than the heat shock genes and their transcripts and products will be referred to as "control" genes. The transcriptional changes accompanying heat shock have been demonstrated in several ways. In D. melanogaster, in which the heat shock response has been extensively studied, activation of the heat shock genes is reflected in the puffing pattern of the polytene chromosomes (2). A unique set of puffs is induced by elevation of the temperature to 35°C while puffs present at the normal temperature, 25°C, regress. The heat shock puffs incorporate ^{3}H-uridine indicating that RNA synthesis is occurring at these sites (3,9). Newly synthesized RNA, isolated from heat shocked cells, hybridizes to the heat shock puffs (10). Incubation with actinomycin D prevents the accumulation of heat shock RNA and also prevents the induction of the heat shock puffs. Since, inter alia, actinomycin D inhibits RNA synthesis, this suggests that heat shock induces transcription of the genes located at the sites of the heat shock puffs and that RNA synthesis causes

puffing. In agreement with this, immunological studies have shown that, during heat shock, RNA polymerase II migrates out of control puffs and accumulates in heat shock puffs (11,12). Finally, isolated genes have been used to detect the changes in transcription after heat shock (13). RNA was pulse-labelled with ^{3}H-uridine, isolated and hybridized to either a cloned actin gene or a cloned heat shock gene. While the levels of actin mRNA were reduced during heat shock, the levels of the heat shock mRNA were increased. Upon recovery, the levels of heat shock mRNA decreased and actin mRNA transcription resumed. Thus, during heat shock, transcription of heat shock genes is activated while that of other genes is repressed.

2. Regulation of translation.

A severe heat shock almost completely stops control protein synthesis (3,10). However, actinomycin D treatment after heat shock does not prevent general protein synthesis during recovery (14) and if mRNA is isolated and translated in vitro, control proteins are synthesized although they were not made in vivo (15). This means that mRNA translation is also controlled during heat shock. Heat shock mRNA is translated while other transcripts are not. The control mRNA is not degraded, but remains in a form which is functional in vitro. Lysates made from heat shocked D. melanogaster cells translate heat shock messages (6,7) but not other mRNA. Lysates from unshocked cells can translate all

mRNA, as does the cell itself during recovery. Heat shock lysates (16) have been fractionated in an attempt to isolate the factors responsible for the discrimination between heat shock and control transcripts, but the factors are not soluble and seem to be bound to the ribosomes. Thus, in D. melanogaster at least, the heat shock response is maximized by translational control: transcription of heat shock genes is rapidly induced while translation of other genes is repressed such that only hsps are made.

Since heat shock results in the disappearance of pre-existing polysomes and the formation of new polysomes (10,15), translational control may be mediated by changes in the polysomes. On sucrose gradients, polysomes from unshocked cells have a unimodal distribution of 12 to 13 ribosomes per mRNA. The distribution of polysomes from heat shocked cells on the other hand, is bimodal, with peaks at 9 ribosomes per mRNA and 20 ribosomes per mRNA. However when actin and β-tubulin genes are hybridized to RNA from heat shock polysomes, control transcripts are detected (17). Each control transcript carries the expected number of ribosomes, but only 1/3 to 1/2 as many ribosomes are associated with the control polysomes as in the unshocked cells. Therefore, polysomes containing control transcripts are present in heat shocked cells but at a lower level than in unshocked cells. Since these polysomes, which are indistinguishable from polysomes in control cells, are present in lower amounts in heat shocked cells, heat shock must block translation of control mRNA at an

initiation step. Basal protein synthesis in heat shocked cells is 5% to 10% that of control cells. Since there are 1/3 to 1/2 as many ribosomes with control mRNA in heat shocked cells as in control cells, elongation steps must also be blocked. Whether these inhibitions are caused by the same factors which block translation of control transcripts in heat shocked lysates is unknown.

3. Role of hsp70 in recovery from heat shock.

Both transcriptional control (6) and translational control (18) are dependent on the length and severity of the shock. Transcription increases more rapidly with a severe shock than with a mild shock while the translation of control mRNA is not repressed during a mild shock. In addition, each heat shock protein is induced and repressed with different kinetics (6). Control proteins appear to behave as a cohort with identical induction and repression kinetics. Studies on the induction of the 70,000 dalton heat shock protein (hsp70) in both D. melanogaster (19) and E. coli (20) have shown that the recovery of basal protein synthesis correlates with the synthesis of hsp70. In addition, an active hsp70 is required for repression of the heat shock response during recovery. Thus, if cells are incubated with amino acid analogues, such as canavanine, which are incorporated into hsp70, and thereby inactivate it, heat shock transcription is not repressed after a return to normal

temperature, and normal protein synthesis does not resume (19).
Hence, hsp70 regulates its own synthesis and promotes the recovery
of normal protein synthesis.

4. Evolutionary conservation of the transcriptional regulation.

The isolation of genes coding for the hsps has made possible
the identification of sequences controlling the expression of
these genes. The cloned gene coding for the D. melanogaster
hsp70 has been transfected into monkey cells (21), rat cells
(22), mouse cells (23) and Xenopus oocytes (24,25). In all cases,
the transcription of the gene is induced by heat shock and, in
oocytes, arsenite treatment will also induce the gene. This means
that the system regulating the transcription of heat shock genes
has been conserved in all of these organisms. Hsp70 genes with
various deletions were injected into a monkey cell line (21) to
determine the sequences required for heat-regulated expression of
the gene. If nucleotide sequences from -47 to -66 are deleted,
(nucleotide 1 is the transcription start site) then the gene is no
longer expressed upon incubation at increased temperature. This
region is conserved in all heat shock genes and lies within an
inverted repeat. The consensus sequence is CTGGAATNTTCTAGA and it
may be located a variable distance upstream from the TATA box,
usually within 28-35 nucleotides of it. However, although
transcriptional control of the heat shock genes depends upon a
certain sequence which is highly conserved among different

organisms, translational control appears to be species specific.
In Xenopus oocytes, the transcription of the D. melanogaster
hsp70 gene increases upon heat shock, but the D. melanogaster
transcript is not translated at high temperature (25). Rather, it
behaves as a control mRNA and its translation is repressed. This
indicates that the translational control is not dependent upon
some highly conserved sequence in the heat shock transcript.

5. Effect of heat shock on chromatin structure.

The cloned heat shock genes have also been used as probes for
chromatin structure. As in many other genes, DNAase I
hypersensitive sites are located at the 5' end of the genes for
hsp70 (26,27), hsp83 (26,27,28), and the small hsps of D.
melanogaster (29). The hypersensitive sites are present whether
or not the gene is active, although the coding region of the gene
becomes much more sensitive to DNAase I when the gene is
activated. Similar studies have been performed using micrococcal
nuclease (30). Again, when the gene is activated, the coding
region becomes much more sensitive to the nuclease although it is
more resistant than the surrounding, non-transcribed regions, when
repressed. It has recently been suggested that this is because of
removal of the histones from the coding region during
transcription. Karpov et al. (31) cross-linked histones to DNA
to show that the coding region of hsp70 is depleted of histones
when activated. Micrococcal nuclease appears to cleave the same

sites as DNAase I, but this is difficult to determine because of the preference of micrococcal nuclease for certain sequences (30). These nuclease sensitive sites appear to be required but not sufficient for transcription (30). Their presence at the 5' end of inactive heat shock genes may reflect the state of these genes prior to activation and the speed with which they can be activated.

5. Other effects of heat shock on chromatin.

Although nucleosome structure and histone modification will be discussed further under Part II below, some observations on chromosomal proteins in relation to heat shock are presented here. Levinger and Varshavsky (32) have examined the protein content of nucleosomes containing the hsp70 gene in D. melanogaster. As with other transcribed genes, one out of two nucleosomes of the hsp70 gene contain ubiquitin-H2A while the content of ubiquitin-H2A is greatly reduced in the chromatin of non-transcribed satellite DNA. In the latter, nucleosomes are enriched in a non-histone chromosomal protein, D1. D1 is not present in the nucleosomes associated with the hsp70 genes or other transcribed genes. Again, the presence of ubiquitin-H2A and absence of D1 suggests that the hsp70 gene is maintained ready for transcription.

Heat shock also affects the synthesis of chromosomal proteins. In D. melanogaster, the synthesis of H2B is three-

fold induced while the synthesis of the other histones is decreased 2 to 10-fold (33). Normally, histone synthesis is coordinately regulated and it is not known whether the increase in H2B synthesis is because of transcriptional or translational activation. The effect of heat shock on histone modification will be discussed below.

C. Heat shock proteins.

1. Evolutionary conservation of heat shock proteins.

In D. melanogaster, where these polypeptides have been studied extensively, nine major hsps are observed, with the following approximate molecular weights: 83,000, 72,000, 70,000, 68,000, 34,000, 27,000, 26,000, 23,000 and 22,000 (1). Five polypeptides of molecular weights: 87,000, 70,000, 42,000, 32,000, and 30,000 are induced in trout RTG-2 cells, a fibroblast cell line, by heat treatment (36). If trout cells are exposed to sodium arsenite, polypeptides of 62,000 and 100,000 daltons are induced in addition to the five observed upon heat shock. The 70,000 dalton heat shock protein (hsp70) is most strongly induced in both trout and D. melanogaster. This protein is highly conserved in organisms ranging from E. coli to man (4). Antibodies to chick hsp70 cross-react with proteins of similar molecular weight in a large number of organisms (5) while peptide maps produced by V8 proteolysis of hsp70 from D. melanogaster,

chick and man have many peptides in common (37). Peptide maps of

the 83,000 dalton polypeptide (hsp83) from these same animals

show even greater similarities. Conservation is also observed

at the level of the DNA sequence; a D. melanogaster genomic

clone of hsp70 will hybridize with mouse heat shock mRNA (38) and

the predicted amino acid sequence of the yeast hsp70 gene is 72%

homologous with the D. melanogaster gene (39). Fig. 1 shows

part of the amino acid sequences of hsp70 from D. melanogaster,

yeast and trout. Non-homologous regions are boxed and it is quite

clear that hsp70 is highly conserved in these organisms. A

polypeptide of 30,000 daltons is also strongly induced in trout.

This protein probably corresponds to the small molecular weight

heat shock proteins of D. melanogaster, which are also highly

induced.

2. Isoelectric variants of the heat shock proteins.

Recently, two-dimensional gel electrophoresis of the D.

melanogaster heat shock proteins has revealed a response of

greater complexity than expected (40). Over 70 polypeptides in 14

different size classes are detected in both heat shocked salivary

glands and heat shocked D. melanogaster tissue culture cells.

The manner in which these polypeptides arise remains unclear. In

D. melanogaster, five genes code for the hsp70 polypeptide, but

Figure 1. Comparison of the predicted amino acid sequences of

hsp70 from trout, D. melanogaster and yeast. THS70.7 is

derived from a cDNA of trout hsp70 mRNA isolated by R. Kothary.

The D. melanogaster sequence is taken from Ingolia et al.

(39). The yeast sequence is from the YG100 gene, also of

Ingolia et al (39). Regions compared are amino acids number

128 to 406, based on the D. melanogaster hsp70 numbering.

Mismatches in the sequences have been boxed in. The single

dashes represent deleted amino acids relative to the compared

sequence. The solid line in the yeast sequence represents

information not yet available.

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                128
THS70.7  AE AYLG QKVSN AVI TVPAYFNDSQRQATKDAG VI AGLNVLRI INEPTAA SI AYG MDKG MSRE RNVLI FDLGGGTF
Dr. HS70 AE AYLG ESITD AVI TVPAYFNDSQRQATKDAG HI AGLNVLRI INEPTAA AL AYG LDKN LKGE RNVLI FDLGGGTF
YG100    AE SYLG AKVND AVV TVPAYFNDSQRQATKDAG TI AGLNVLRI INEPTAA AI AYG LDKK GKEE H-VLI FDLGGGTF


THS70.7  DVS ILTI EDG- IFEV KA TAGDTHLGGEDFDNRLV SHFVEE FKRK HKKDI SQN KRALRRLRTA CERAKRTL SSSSQ
Dr. HS70 DVS ILTI DEGSL FEVRS TAGDTHLGGEDFDNRLV THLAEE FKRK YKKDL RSNP RALRRLRTA AERAKRTL SSSTE
YG100    DVS LLFI EDG- IFEV KA TAGDTHLGGEDFDNRLV NHFIQE FKRK NKKDL STNQ RALRRLRTA CESQENFV SS-AQ


THS70.7  ASI EID SLFEGI DFYT SIT RARFEEM CSDLFRGTLE PVEK ALG DAKMDKAQIHDV VLVGGSTRIPKVQ RLLQDFF
Dr. HS70 ATI EID ALFEGQ DFYTKVS RARFEEL CANLFRNTLQ PVEK ALNDAKMDKGQIHDI VLVGGSTRIPKVQ SLLQEFF
YG100    TSV EID SKNEGI DFYT SIT RARFEEL CADLFRSTLD PVEK VLRDAKLDKSQVDEI VLVGGSTRI—————————————


                                                                           406
THS70.7  NGRELNKS INPDEAVG YGLAI QAAILSGDKS--ENVQDILLLDVAPLSLGIETAGG
Dr. HS70 HGKNLNLS INPDEAVA YGAAMQAAILSGDQS--GKIQDVLLVDVAPLSLGIETAGG
YG100    ——————————————————————————————————————————————GVAPLSLGIETA-G
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upon two-dimensional gel electrophoresis, 40 different isoelectric variants are observed. Post-translational modifications could explain some of these isoelectric variants. For instance, chick and hamster hsp70 and hsp87 are methylated at arginyl and lysyl residues (41,42), while the 87,000 dalton protein is ADP-ribosylated (43). Phosphorylation of the hsp70 protein of Dictyostelium discoideum has also been reported (44), but similar studies on the D. melanogaster hsp70 show no phosphorylation (45). If post-translational modification accounts for the isoelectric variants observed on two-dimensional gel electrophoresis, it seems unlikely that all of the modifications would be seen when mRNA isolated from heat shocked cells is translated in a heterologous system. In fact, all isoelectric variants are observed in gels of in vitro translations of heat shock mRNA from D. melanogaster (40). Unless post-translational modification is occurring in the in vitro translation system, individual transcripts must be present for each isoelectric variant. In support of this, Wadsworth (46) has isolated mRNA by hybridization to a genomic clone of the hsp70 gene from the 87C locus of D. melanogaster and translated the isolated mRNA in vitro. On one-dimensional SDS gel electrophoresis, four polypeptides of approximately 70,000 daltons were observed, which indicated that the cloned hsp70 gene had hybridized to different homologous transcripts. These polypeptides were also observed in vivo and partial proteolysis mapping revealed that the polypeptides are related. Deletions spanning both the 87A and 87C

heat shock loci, which are known to code for hsp70, result in
mutants which cannot produce the 70,000 dalton protein but which
still synthesize the three polypeptides of slightly larger
molecular weight (46). This means that the genes for these
polypeptides must reside outside of the known heat shock loci.

Three additional genes in D. melanogaster (48) and nine in
yeast (39) which are homologous with the hsp70 gene of D.
melanogaster have been identified recently. However, most of
these genes are expressed in cells at normal temperature and hence
cannot explain the many isoelectric variants observed in heat
shocked cells. Those genes homologous to hsp70 which are
expressed at normal temperature are called cognate genes (48).
Additional genes coding for heat shock proteins may still be found
outside of regions known to code for hsps, thereby accounting for
some of the isoelectric variants.

3. Subcellular localization of the heat shock proteins.

To gain further insight into the function of the hsps,
sub-cellular fractionation, electron microscope autoradiography
and immunofluorescent techniques have been used to determine the
localization of the hsps. Hsp83 of D. melanogaster is always
located in the cytoplasm and may be membrane bound (49,50).
While hsp70 and the small hsps of D. melanogaster are
distributed throughout the cell, a portion of these hsps is
concentrated in the nucleus during heat shock (49-53,125).

However, when the cells are shifted back to their normal growth temperature, the hsps leave the nucleus and enter the cytoplasm (45,53). Within the nucleus, hsp70 and hsp30 appear to be bound to the nuclear matrix. The nuclear matrix is functionally defined as the structure remaining after the extraction of lipids and histones and digestion by nucleases in a defined sequence (124). Various nuclear functions such as RNA transcription and DNA replication have been associated with the nuclear matrix (124). The nuclear hsps cannot be extracted from chromatin by 2M NaCl (50) and are present in nuclear scaffold preparations (54). Levinger and Varshavsky (50) therefore suggested that the hsps may protect chromatin structure during periods of stress, but this fails to account for the cytoplasmic subset of these hsps. Recently, Kloetzel and Bautz (55) irradiated cells with ultraviolet light and showed that the hsps are cross-linked to RNA. Hsps are found in both cytoplasmic and nuclear RNP particles, which may explain their observed localization. In addition, the small hsps of D. melanogaster can be isolated as an aggregate with the characteristics of RNP particles.

4. Heat shock and thermotolerance.

Because the heat shock response occurs in all organisms and is highly conserved, the function of the hsps must be important. Since a wide variety of compounds elicit the heat shock response, a protective role during times of stress has been proposed for the

heat shock proteins. Studies on thermotolerance in mammalian cell lines support the suggestion that hsps function in stress resistance. Thermotolerance develops in cells given a non-lethal heat, treatment before receiving a second, lethal heat treatment. The initial mild heat shock protects the cells from the second, severe treatment which would kill untreated cells. In hamster cells, thermotolerance has been correlated with heat shock protein synthesis: maximal thermotolerance only occurs with maximal hsp synthesis (56-60). In addition, arsenite, ethanol and recovery from anoxia, which all induce hsp synthesis also induce thermotolerance (61,62). A severe heat treatment before the protective heat treatment delays the acquisition of thermotolerance; at the same time, protein synthesis is repressed and recovery, which begins with hsp synthesis, is delayed. Thus, thermotolerance does not occur until the cell has synthesized a given quantity of hsps. Another example of thermotolerance occurs in D. melanogaster: phenocopy induction by heat treatment of embryos can be blocked by an earlier heat treatment (63). Phenocopy occurs when wild type D. melanogaster embryos develop into adult animals with phenotypes identical to those of known mutants after being stressed, for example, by heat treatment, during development. Presumably, the mutant phenotype results from the inactivation of a gene product required for normal development. Phenocopy protection correlates with heat shock protein synthesis as well. During thermotolerance, the cell continues protein synthesis throughout the second heat shock,

allowing expression of the functions required to prevent
phenocopy. Protein synthesis which occurs during thermotolerance
is a consequence of translational regulation and is correlated
with hsp synthesis (64). Control transcripts are stable in the
cell after the initial heat treatment, but are only translated
during recovery. A second, more severe heat treatment will not
block the recovered translation of control mRNAs. This means
that, after a mild heat shock, the translational apparatus becomes
more resistant to temperature elevation.

Recently, Lee et al. have identified adenylylated
nucleotides which accumulate during heat shock in E. coli and
S. typhimurium. Exposure of S. typhimurium to oxidizing
quinones also induces the adenylylated nucleotides which include
P^1,P^4-diadenosine-5'-tetraphosphate, P^1-(adenosine-5')-P^3
-(guanosine-5')-triphosphate and P^1-(adenosine-5')-P^3-
(guanosine-3'-diphosphate-5')-triphosphate (65). The adenylylated
nucleotides accumulate during heat shock, even in mutant cells
which do not make the heat shock proteins. This indicates that
the heat shock proteins are not responsible for the synthesis of
the adenylylated nucleotides. Lee et al. proposed that these
compounds are alarmones, or compounds which signal a specific
metabolic stress (66). Specifically, the adenylylated nucleotides
accumulate during oxidation stress and may trigger the heat shock
response. Heat shock is induced by many oxidizing agents, such
as hydrogen peroxide, menadione and diamide (1). Other compounds,
such as sodium arsenite react with sulfhydryls which, in the form

of glutathione, are known to be important in the maintenance of the cellular oxidation-reduction state. In line with this, levels of glutathione within the cell increase during thermotolerance (67). Thus, the heat shock phenomenon may be a response to oxidation stress and may be induced by alarmones in the form of adenylylated nucleotides.

5. Developmental regulation of the heat shock response.

Heat shock protein synthesis is also regulated developmentally. One of the first proteins synthesized by the developing mouse embryo is the 70 K hsp. Bensaude et al. (68) have shown that hsp70 is synthesized upon activation of the embryonic genome at the two cell stage. This is similar to recovery from heat shock in which synthesis of the heat shock proteins precedes transcription of other genes. Similarly, transcription from chromosomal site 67B in D. melanogaster is developmentally regulated (69). This region codes for two unidentified transcripts as well as for the small molecular weight hsps. All are expressed in normal development during the third instar and early pupal stages. One possibility is that products of these transcripts protect the animal from some stress during development. For example, anoxia could occur if the permeability of the pupal case limits oxygen diffusion. Alternatively, the developmentally regulated expression could be caused by some other factor. The third instar stage is

characterized by high titers of the hormone ecdysterone and the small heat shock proteins are induced in D. melanogaster tissue culture cells treated with ecdysterone (70).

The response of embryos to heat shock also varies depending upon the developmental stage of the embryo. Sea urchin embryos (71) can only induce hsps upon heat treatment after hatching while D. melanogaster (72) will only synthesize hsps after the blastoderm stage. In both cases, the embryo cannot respond to heat shock until it is transcriptionally active. Not surprisingly, the ability of the embryo to withstand heat treatment depends on its ability to synthesize heat shock proteins.

D. The response of RTG-2 cells to heat or sodium arsenite treatment.

Trout RTG-2 cells are a fibroblast cell line derived from mixed gonadal tissue of male and female rainbow trout (95). Kothary and Candido (36) have reported that heat shock occurs when RTG-2 cells are incubated at 27°C as opposed to 22°C, the normal growth temperature. As in other organisms, the response to heat shock varies depending on the length and severity of the stress. Five polypeptides of molecular weight 87,000, 70,000, 42,000, 32,000 and 30,000 may be induced by temperatures ranging from 27°C to 37°C: hsp87 and hsp42

are only observed below 29°C and incubation of the cells

above 34°C is lethal unless exposure time is short.

Synthesis of the hsps reaches its maximal levels after 5 hours

of incubation at 27°C and is maintained at that level for at

least 16 hours of treatment. Upon return to 22°C, heat shock

protein synthesis decreases non-coordinately. Synthesis of

hsp70 and hsp42 declines first, followed by a decline in the

synthesis of hsp87 and hsp30. Non-coordinate induction and

repression kinetics have been observed for the hsps of D.

melanogaster (6). The heat shock response of RTG-2 cells

differs from that of other organisms in that control protein

synthesis does not cease during heat shock.

A similar response to the heat shock response can be induced

in RTG-2 cells by treatment with sodium arsenite, which reacts

with sulfhydryl groups. Kothary and Candido (36) have shown that

5 proteins of identical molecular weight to the heat shock

proteins are induced by $NaAsO_2$ treatment. In addition,

polypeptides of 62,000 and 100,000 daltons are synthesized after

arsenite treatment. The polypeptides induced by sodium arsenite

treatment do not show identical induction kinetics. Rather, each

hsp is optimally induced and repressed with different kinetics

after removal of the arsenite. Unlike heat shock, arsenite

treatment of RTG-2 cells results in the repression of non-heat

shock protein synthesis. As in other organisms the block in

control protein synthesis is probably at the level of translation

since mRNA from cells treated with sodium arsenite can program

the synthesis of control proteins in the in vitro rabbit
reticulocyte system. Hence, sodium arsenite elicits a response
in RTG-2 cells which is similar, but not identical to the heat
shock response.

Part II. Histone acetylation and heat shock.

A. Histone acetylation.

The DNA of all eukaryotes is packaged into nucleoprotein

particles called nucleosomes (73). Besides DNA, each nucleosome

contains five basic proteins called histones. Histones H2A,

H2B, H3 and H4 form the core particle and are referred to as

the "core histones", while H1 is attached to the linker DNA

between nucleosomes. DNA replication and transcription seem to

occur on DNA which is packaged in nucleosomes. As part of the

process of gene activation, it has been proposed that post-

translational modification of histones may alter nucleosome

structure in such a way as to permit transcription (74). Histones

can be post-translationally modified in a number of ways,

including methylation, phosphorylation, ADP-ribosylation and

acetylation (74). Some evidence exists that histone acetylation

may be involved in gene activation.

Active genes are sensitive to digestion with DNAase I (75).

If nuclei are digested briefly with DNAase I, a procedure that

should release nucleosomes carrying transcribed sequences, the

resulting released nucleosomes contain hyperacetylated histones

(76,77). This suggests that nucleosomes from active genes have

hyperacetylated histones, but the nucleosomes released by DNAase I

digestion are not necessarily homogeneous. Two or more nucleosome

populations could be released by DNAase I and the nucleosomes

containing transcribed sequences are not necessarily identical to those containing hyperacetylated histones.

Histones may be acetylated in two ways. Histones H1, H2A and H4 are acetylated on the α-amino group of the amino terminal seryl residue shortly after synthesis. This modification occurs in the cytoplasm and is irreversible. In addition, the ϵ-amino groups of specific lysyl residues in the core histones may be acetylated reversibly in the nucleus (78,79). H2B, H3 and H4 contain four sites of acetylation in their N-terminal regions (residues 1-30), whereas H2A contains only a single lysyl residue which is subject to this modification. Acetylation neutralizes the basic charge of the histone molecule by +1 for each residue so modified. In a population of histone molecules, a given site is usually found in the modified and unmodified states. Histones H_2B, H_3 and H_4 can therefore exist in multi-acetylated forms with charges ranging from +5 to −1 in their N-terminal residues. It has been suggested that neutralization of the positive charge by acetylation could reduce the ionic interactions between the histones and the DNA, thereby leading to an altered nucleosome conformation which permits transcription (73,74).

Heat shock affects histone modifications. Acetylation of the histones is reduced during heat shock (34) while the methylation patterns change in a complex way (35). Methylation of H2B increases during heat shock while methylation of H3 decreases. Sodium arsenite causes methylation of H2B as well, but does not

affect H3 methylation. The significance of these alterations is not known, but histone modifications may affect the transcriptional changes that occur during heat shock.

B. The effect of sodium butyrate on histone acetylation.

Studies of histone acetylation have been greatly facilitated by the use of sodium butyrate. By inhibiting the histone deacetylase, sodium butyrate causes massive hyperacetylation of the histones (80-82). The term "hyperacetylation" here refers to increased steady-state levels of acetylation at the normal in vivo sites of this modification. Pulse-label studies of butyrate-treated cells have shown that two populations of acetylated histones exist (83): most of the histones are acetylated and deacetylated slowly, with a half-life of three hours, whereas a small subset of the histones is rapidly acetylated and deacetylated with a half-life of three minutes. The significance of these two populations remains a matter of speculation. Although histone hyperacetylation caused by sodium butyrate is reversible, prolonged treatment can inhibit the histone acetylase, resulting in unacetylated histones (84).

If histone acetylation is involved in gene activation, then treatment with sodium butyrate might be expected to activate repressed genes. Two-dimensional gel electrophoresis of proteins from butyrate treated and untreated cells has yielded conflicting results. In Friend erythroleukemic cells, a number of

polypeptides are present in butyrate treated cells that are not present in untreated cells (85). This is not true for Chinese hamster ovary cells (86), where butyrate causes no change in protein synthetic patterns. These contradictory results may be explained by the fact that butyrate treatment causes differentiation of the Friend erythroleukemic cells into mature haemoglobin producing cells (87). No changes in cell morphology or differentiated state are observed for the Chinese hamster cells upon butyrate treatment and this may explain the absence of new polypeptide synthesis in these cells.

In addition to the induction of haemoglobin in erythroleukemic cells, sodium butyrate has a number of effects on cultured cells. Among these are the induction of certain enzymes (84-86) and the inhibition of cell division (87). On the other hand, estrogen induction of ovalbumin in chick oviduct (88) and the corticosteroid induction of the enzyme tyrosine transaminase (89) in hamster cells are blocked in the presence of butyrate. Induction of five other proteins in hamster cells by glucocorticoids is also inhibited if butyrate is present (90). In the case of ovalbumin, butyrate has been shown to decrease the level of transcription. This effect may be mediated via the inhibition of histone deacetylase, since analogues of butyrate such as propionate and isobutyrate which are less effective at inhibiting ovalbumin induction are also less effective at causing the hyperacetylation of the histones.

Part III. The present investigation.

Although the heat shock proteins are synthesized in all

organisms during times of stress, little is known about the actual

functions of these proteins. This thesis is mainly concerned with

characterization of the heat shock proteins of trout RTG-2 cells

by two-dimensional gel electrophoresis. As in D. melanogaster,

each trout hsp detected by one-dimensional analysis separates

into several isoelectric variants upon two-dimensional gel

electrophoresis. Since the pattern of charge variants resembles

the pattern expected for proteins which are post-translationally

modified, cells were incubated with ^{32}P-ortho-phosphate to see

if the hsps were phosphorylated. No evidence for either

phosphorylation of the hsps or for changes in the phosphorylation

of other proteins was obtained. Messenger RNA was isolated from

heat shocked cells and translated in the in vitro rabbit

reticulocyte system. Most of the isoelectric variants could be

detected in in vitro translations, suggesting that either post-

translational modifications are occurring in the cell-free system,

or that different transcripts encode the isoelectric variants.

Heat shock mRNA was also hybridized to a cDNA clone coding for a

portion of the 70 K heat shock protein. The mRNA isolated by this

procedure was translated in vitro. All 70 K isoelectric variants

were present, which again suggests that multiple transcripts encode

the 70 K proteins.

A minor portion of this thesis is concerned with induction of

the heat shock proteins. This was studied using an antibody to chick hsp70 which cross-reacts with the trout hsp70. A protein of identical molecular weight to the heat shock 70 K protein which cross-reacts with the antibody to chick hsp70 exists at low levels in cells which have not been shocked. However, a rapid and massive increase in the amount of hsp70 could be demonstrated in stressed cells using the chick anti-hsp antibody.

The effect of histone acetylation on the heat shock response was also examined using sodium butyrate. Sodium butyrate was used at concentrations that cause a high degree of histone hyperacetylation, but synthesis of the heat shock proteins was neither induced nor blocked. Therefore, although heat shock causes some deacetylation of the histones in some systems (33), the state of histone acetylation does not appear to affect the heat shock response in RTG-2 cells.

Leaf 28 omitted in numbering.

Experimental Procedures

I. Materials.

All chemicals were obtained commercially and were of the highest purity or reagent grade. Special reagents were obtained as follows: RTG-2 cells were obtained from the American Type Culture Collection; Earle's salts, foetal bovine sera, nonessential amino acids, penicillin/streptomycin and the GIBCO Selectamine kit were all obtained from GIBCO; RNAase A (E.C.3.1.4.22) from BRL, micrococcal nuclease (E.C.3.1.4.7) from Sigma, DNAase I (E.C.3.1.4.6) from Boehringer Mannheim, BSA from Calbiochem, TEMED, acrylamide, bis-acrylamide and ammonium persulphate all from Bio-Rad Laboratories, ultrapure Urea from Schwartz/Mann, ^{35}S-methionine, ^{32}P-orthophosphate and ^{125}I-sodium iodide all from New England Nuclear, EnHance from New England Nuclear, Cronex intensifying screen from Dupont, unlabelled goat antirabbit IgG from Miles Laboratories, Whatman 541 filter paper from Fisher, Sephadex-G-50 from Pharmacia, guanidinium hydrochloride from Schwartz/Mann, Rabbit reticulocyte _in vitro_ translation kit from New England Nuclear.

II. Methods

A. Cell line and culture conditions.

RTG-2 cells are a fibroblast-like line derived from mixed gonadal tissue of male and female rainbow trout, Salmo gairdnerii (95). Cells were grown at $22^{\circ}C$ in disposable polystyrene flasks in Eagle's minimum essential medium containing Earle's salts, non-essential amino acids, 100 U/ml of penicillin-streptomycin and 10% fetal calf serum.

B. Induction with sodium arsenite, incubation at high temperature and treatment with sodium butyrate.

1. Sodium arsenite induction.

For induction with sodium arsenite, the medium was made 50 uM in $NaAsO_2$ and the cultures were incubated at $22^{\circ}C$, the normal growth temperature, for various times. To allow the cells to recover, the medium containing $NaAsO_2$ was removed and replaced with medium lacking arsenite. Recovery was for 2 hours. It has been found in most systems that the heat shock polypeptides are synthesized at a maximal rate after a recovery period following exposure of the cells to the inducing stimulus.

2. Induction by incubation at 27°C.

Heat treatment was accomplished by immersing the flask of cells in a water bath maintained at 27°C for the desired length of time. The flask was then removed and placed at 22°C for 2 hours to allow cell recovery prior to labelling.

3. Sodium butyrate induction.

For sodium butyrate treatment, 5 M sodium butyrate (pH 7.5) was added to the medium to give the desired final concentration. Cells were labelled with ^{35}S-methionine in the presence of sodium butyrate to prevent any deacetylation of the histones during the labelling period. To study the effect of histone acetylation on the heat shock response, the medium was made 5mM in sodium butyrate for 24 hours. $NaAsO_2$ was either added initially and induction was for 24 hours or arsenite was added 21 hours after the butyrate and induction was for 3 hours.

Radio-isotope labelling conditions.

1. ^{35}S-methionine labelling.

Cells were labelled in medium lacking unlabelled methionine
(Gibco Selectamine kit) and containing 50 uCi/ml of
^{35}S-methionine (New England Nuclear, 1000 Ci/mmole). Labelling
was carried out at 22°C for 2 hours. Incorporation was
terminated by removing the labelling medium and washing the cells
with ice-cold saline-EDTA (137 mM NaCl, 0.5 mM Na_2EDTA,
2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM glucose).
Cells were removed from the flask with a gentle stream of
saline-EDTA from a Pasteur pipette and centrifuged briefly in a
bench-top centrifuge. Pellets were immediately frozen at -70°C.
Harvesting was performed as quickly as possible to avoid
inadvertant induction of heat shock proteins.

2. ^{32}P-ortho-phosphate labelling.

Cells were labelled in medium buffered at pH 7.3 by 5mM HEPES
instead of phosphate, and containing 1 mCi/ml (0.3 uM) of ^{32}P-
ortho-phosphate (New England Nuclear, 3000 Ci/mmole), for 4 hours.
Cells were either induced with 50 uM $NaAsO_2$ for 3 hours before
^{32}P-labelling or at the same time as labelling with ^{32}P.

D. Polyacrylamide gel electrophoresis.

1. Sample preparation.

For two dimensional gel electrophoresis, cell pellets were resuspended in either O'Farrell sample buffer (96) (9.5 M Urea, 2% NP-40, 2% ampholines, 5% 2-mercaptoethanol) or 10 mM Tris-HCl, pH 7.5, 5 mM EDTA and freeze-thawed rapidly five times. Those samples in 10 mM Tris-HCl, pH 7.5, 5 mM EDTA were further hand homogenized and incubated for 15 min. on ice with 50 ug/ml of RNAase A and 50 ug/ml of DNAase I. Solid urea was then added to a final concentration of 9 M, followed by an equal volume of O'Farrell sample buffer.

Samples for one dimensional SDS polyacrylamide gel electrophoresis were added to an equal volume of loading buffer (0.1 M Tris-HCl, pH 6.8, 2.0% SDS, 0.32 M 2-mercaptoethanol, 15% sucrose) and boiled for two min.

Lyophilized histones for acid-urea polyacrylamide gel electrophoresis were solubilized in loading buffer consisting of 2.5 M urea, 0.9 M acetic acid, 0.5 M 2-mercaptoethanol and 10% sucrose.

2. Two dimensional gel electrophoresis.

Equilibrium two dimensional gel electrophoresis was performed as described by O'Farrell (96). The pH range of the isoelectric

focussing gels was from pH 4.5 to pH 6.8 and was determined by slicing a gel without sample into 1 cm lengths. The slices were incubated in 2 ml of degassed distilled water for 20 min before determining the pH. Electrophoresis on the isoelectric focussing gels was carried out for 17 hours at 400 V. The second dimension SDS gel consisted of a 5 to 22% exponential acrylamide gradient (0.08 cm x 19 cm x 14 cm) and electrophoresis was performed for 15 hours at 80 V. To obtain better resolution of the high molecular weight region, 8% acrylamide gels were used as the second dimension. Electrophoresis of these gels was carried out for 5 hours at 200 V. Non-equilibrium pH gradient gel electrophoresis was performed as described by O'Farrell et al. (97) using pH 3 - 10 ampholines. The first dimension was subjected to electrophoresis for 4 hours at 400 V and resulted in a pH gradient extending from pH 4.0 to pH 9.5. The second dimension consisted of an exponential acrylamide gradient gel as described above.

3. One dimensional SDS acrylamide gel electrophoresis.

One dimensional slab gel electrophoresis was performed using a modification (98) of the discontinuous buffer system of Laemmli (99). The separating gels (0.08 cm x 7.5 cm x 10 cm) contained 10% acrylamide (acrylamide:bisacrylamide ratio of 30:0.8,w/w), 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.03% TEMED, and 0.1% ammonium persulphate. The stacking gel contained 3.0% acrylamide (acrylamide: bisacrylamide ratio of 30:0.8,w/w), 0.125 M Tris-HCl, pH 6.8, 0.1%

SDS, 0.05% TEMED and 0.1% ammonium persulphate. Electrophoresis

was performed in 0.05 M Tris-HCl, pH about 8.3, 0.38 M glycine and

0.1% SDS for 2 hours at 120 V.

4. Acid-urea polyacrylamide gel electrophoresis.

Histones were separated on acid-urea gels by a modification

(100) of the procedure of Panyim and Chalkley (101). The

separating gels (0.08 cm x 7.5 cm x 10.0 cm) contained 15%

acrylamide (acrylamide:bisacrylamide ratio of 30:0.8,w/w), 2.5 M

urea, 0.9 M acetic acid, 0.5% TEMED and 0.125% ammonium persulphate.

Before the stacking gel was poured, the separating gel was prerun

for 2 hours at 200 V at 4°C in running buffer of 0.9 M acetic

acid. The stacking gel was composed of 4.5% acrylamide (acrylamide:

bisacrylamide ratio of 30:0.8, w/w), 2.5 M urea, 0.9 M acetic acid,

0.375 M potassium acetate, pH 4.0, 1% TEMED and 0.125% ammonium

persulphate. Electrophoresis of these gels was in running buffer of

0.9 M acetic acid at 100 V for 6 hours at 4°C.

5. Staining and autoradiography of the gels after
electrophoresis.

All gels were stained with 0.25% Coomassie blue in methanol:

glacial acetic acid:water (2:1:5,v/v) and destained in methanol:

acetic acid:water (2:1:5,v/v). At this point, one dimensional

gels were scanned on a Beckman DU-8 spectrophotometer.

Alternatively, densitometric tracings were obtained from
autoradiograms. Gels containing ^{35}S-labelled proteins were
treated with EnHance (New England Nuclear) as described by the
manufacturer. Dried gels were fluorographed at -70°C using
Kodak X-OMAT AR film. Gels containing ^{32}P-labelled proteins
were dried without pretreatment and autoradiographed at -70°C
using a Dupont Cronex intensifying screen.

E. Isolation of RNA.

Cells were grown to confluence in roller bottles, induced
with arsenite and scraped off the bottle in saline-EDTA. RNA was
isolated from the resulting cells as described (102). Briefly,
the cells were collected by centrifugation and homogenized on ice
in 6 M guanidinium chloride (Gu·HCl), 20 mM sodium acetate, 0.1 M
2-mercaptoethanol, pH 5.0. The homogenate was centrifuged at 4°C
for 15 min at 12,000 x g. Crude RNA was precipitated from the
supernatant with 0.5 volumes of 95% ethanol (-20°C). The
precipitate was pelleted by centrifugation at 12,000 x g for 15 min
at -10°C, resuspended in 7.5 M Gu·HCl, 25 mM sodium citrate, 50
mM 2-mercaptoethanol, pH 7.0 and reprecipitated as before. After
washing with 95% ethanol, the resulting pellet was dried and
extracted three times with distilled water. The resulting RNA
was precipitated with ethanol and redissolved in distilled water

at a concentration of 2 mg/ml. All glassware was treated with 0.1%
diethylpyrocarbonate and baked before use. Solutions were prepared
in distilled water which was treated with 0.1% diethylpyrocarbonate
and autoclaved.

Polyadenylated RNA (poly A$^+$ RNA) was prepared by
chromatography through an oligo-dT cellulose column (Collaborative
Research Inc., Type 2) using the procedure of Aviv and Leder
(103). Oligo-dT cellulose (0.1 g) was suspended in NETS buffer
(10 mM Tris-HCl, pH 7.5, 1 mM Na$_2$EDTA , 0.25 M NaCl, 0.5% SDS)
and loaded into a 1.0 ml syringe plugged with glass wool. Total
cellular RNA was diluted with NETS buffer and heated at 90°C for
5 min. After rapid cooling on ice, the RNA was loaded onto the
column and recirculated for 1/2 hour. The column was eluted with
ETS buffer (10 mM Tris-HCl, pH 7.5, 1 mM Na$_2$EDTA, 0.5% SDS).
Carrier E. coli tRNA was added to the poly A$^+$ RNA to a final
concentration of 100 ug/ml and the RNA was precipitated with
ethanol at -20°C.

F. Hybridization Selection.

An M13 clone containing 690 base pairs of the coding region
of a trout hsp70 cDNA was obtained from R. Kothary. The
bacteriophage were grown on E. coli JM101 for 8 hours and the
cells pelleted by centrifugation at 12,000 x g for 10 min. The
supernatant was made 3.7% in polyethylene glycol and 0.5 M in
NaCl, incubated for 15 min at room temperature and centrifuged

at 12,000 x g for 10 min. The resulting pellet was dissolved in 20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 1 mM EDTA. The solution was extracted once with phenol and twice with phenol:$CHCl_3$ (1:1). The DNA was precipitated with ethanol, redissolved, and reprecipitated again with ethanol. The pellet, consisting of the plus strand of the M13 recombinant DNA and containing the region complementary to part of the hsp70 mRNA, was resuspended in 400 ul of sterile diethylpyrocarbonate-treated water.

Ten ug of the plus strand was bound to nitrocellulose (Schleicher and Schuell, B-6) using a millipore sintered glass filtration unit as described by Young et al. (104). The filter was first washed with about 50 ml of 2xSSC. The DNA was diluted to 10 ml in 3xSSC and passed through the filter at approximately 0.75 ml/min. The filter was baked for 2 hours before hybridization to 10 ug of poly A^+ RNA as described by Tilghman et al. (105). The filter was incubated in hybridization buffer (50% formamide, 0.75 M NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM Na_2EDTA) containing 10 ug of poly A^+ RNA for 20 hours at 43°C. After washing with hybridization buffer at 43°C, bound RNA was released by incubating the filter in 90% formamide, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA at 45°C for one hour. Carrier E. coli tRNA was added before the RNA was precipitated with ethanol and lyophilized to remove any traces of formamide.

G. In vitro translation.

RNA was translated in a rabbit reticulocyte system (New England Nuclear) using ^{35}S-methionine as the label (106). Two ug of total RNA in 1 ul was translated in the rabbit reticulocyte lysate containing magnesium, spermidine, creatine phosphate, dithiothreitol and guanosine triphosphate as described by the manufacturer. After translation, 50 ug/ml of RNAase was added, a 5 ul aliquot of the translation mix was mixed with 5 ul of Laemmli sample buffer (99) for one dimensional gel electrophoresis, and the rest of the sample was precipitated with 6 volumes of 95% ethanol. The pellets were resuspended in O'Farrell sample buffer and separated on isoelectric focussing gels as described above.

H. Protein Blotting.

Cyanogen bromide-activated-paper was prepared as described by Clarke et al. (108). Proteins were separated on a 10% polyacrylamide SDS gel as described above. After electrophoresis, the gel was washed 3 times for 5 min each in 0.1 M sodium phosphate, pH 6.5 containing 0.1% SDS, and 3 times in 20 mM sodium phosphate, pH 6.5 without SDS. Proteins were transferred electrophoretically to CNBr-activated paper in 20 mM sodium

phosphate, pH 6.8 using a Bio-Rad electroblot apparatus. Transfer was for 4 hours at 1 A or 15 hours at 100 mA. After transfer, the CNBr-activated paper was incubated 4 times for 15 min each in quench buffer (0.1 M Tris-HCl, pH 8.0, 1% glycine, 0.1% BSA, 10% ethanolamine) to block any remaining reactive sites. The paper was then incubated at 37°C for 1 hour in 5 ml of Buffer A (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 5 mM Na_2EDTA, 0.1% BSA and 0.05% NP-40) containing 10 ul of the rabbit antibody to chick hsp70. After washing 4 times for 15 min in Buffer B (50 mM Tris-HCl, pH 7.4, 1.0 M NaCl, 5 mM Na_2EDTA, 0.1% BSA and 0.4% (w/v) sarcosyl) the paper was incubated in 5 ml of Buffer A containing 1×10^6 dpm/ml of ^{125}I-labelled goat anti-rabbit IgG. The paper was again washed in Buffer B and fluorographed using a Cronex screen.

I. Iodination of goat anti-rabbit IgG.

Goat anti-rabbit IgG was labelled by the chloramine T method of Greenwood et al. (109). Goat anti-rabbit IgG (1 mg/ml) was incubated with 1 mCi/ml of Na^{125}I (New England Nuclear, 17.4 Ci/mg) and 400 ug/ml chloramine T in PBS for 10 min. Then 3 mg/ml of Na_2SO_5 and 200 ug of BSA were added and the sample was chromatographed through a Sephadex G-50 column (1.5 cm x 20 cm) in 50 mM sodium phosphate, pH 7.4 plus 0.1% BSA. The totally excluded fractions were pooled.

J. Extraction of histones.

Histones were isolated at 4°C by a modification of the procedure of Marushige et al. (110). Cell pellets were homogenized in TMKS with 0.5% Triton-X-100 in a glass-teflon homogenizer. The sample was centrifuged at 3000 x g for 10 min and the crude nuclear pellet was washed once in TMKS. The pellet was then homogenized in 10 mM Tris-HCl, pH 7.4, and centrifuged at 12,000 x g for 15 min. The resulting chromatin pellet was extracted with 0.4 N H_2SO_4 for 20 min on ice and centrifuged for 20 min at 12,000 x g. The supernatant was added to four volumes of 95% ethanol (-20°C) and the histones were allowed to precipitate overnight at -20°C. All buffers contained 1 mM PMSF, 10 mM 2-mercaptoethanol and 10mM sodium butyrate.

RESULTS

Part I. Characterization of the heat shock proteins of RTG-2
    cells.

A. Two-dimensional gel electrophoresis of heat shock proteins.

Sodium arsenite was chosen to induce the heat shock proteins
in RTG-2 cells. Although the effect of $NaAsO_2$ is not identical
to the effect of elevated temperature (36,45), it is more
straightforward and more precise to use a chemical inducer than to
increase the temperature of the cells as a period of equilibration
must occur after the shift before the culture temperature reaches
that of the environment. Initially, an arsenite treatment
protocol was chosen which caused a strong induction of all heat
shock proteins as detected by one-dimensional gel electrophoretic
analysis (36). Cells were treated with 50 uM $NaAsO_2$ for 3 hours
and allowed to recover for 2 hours prior to labelling with [35]S-
methionine. Fig. 2 shows a representative fluorogram of the
protein pattern obtained from cells treated in this way. The
isoelectric focussing gel consisted of a pH gradient extending
from pH 4.5 to pH 6.8, while the second dimension was a 5% to 20%
polyacrylamide gradient. All two dimensional-gel electrophoresis
was performed under these conditions unless otherwise specified.
Clearly, the heat shock response is of much greater complexity

Figure 2. Two-dimensional fluorograms of proteins from RTG-2

cells. The first dimension was a pH gradient ranging

from pH 4.5 to pH 6.8 and the second dimension was a 5%

to 20% polyacrylamide gradient. The left end is the

acidic end. Molecular weight markers were:

phosphorylase b (94,000), BSA (67,000), ovalbumin

(43,000), soybean trypsin inhibitor (20,100) and ∝-

lactalbumin (14,400). Marker positions are indicated on

the left side of the gel. a) cells were untreated. b)

cells were treated with 50 uM NaAsO$_2$ for 3 hours and

allowed to recover in medium without NaAsO$_2$ for 2

hours before labelling with ^{35}S-methionine as

described in the text. Heat shock proteins are

numbered and the protein labelled A was tentatively

identified as actin. Exposure times were for 10 days and

500,000 cpm were loaded in a) and 300,000 cpm were loaded

in b).
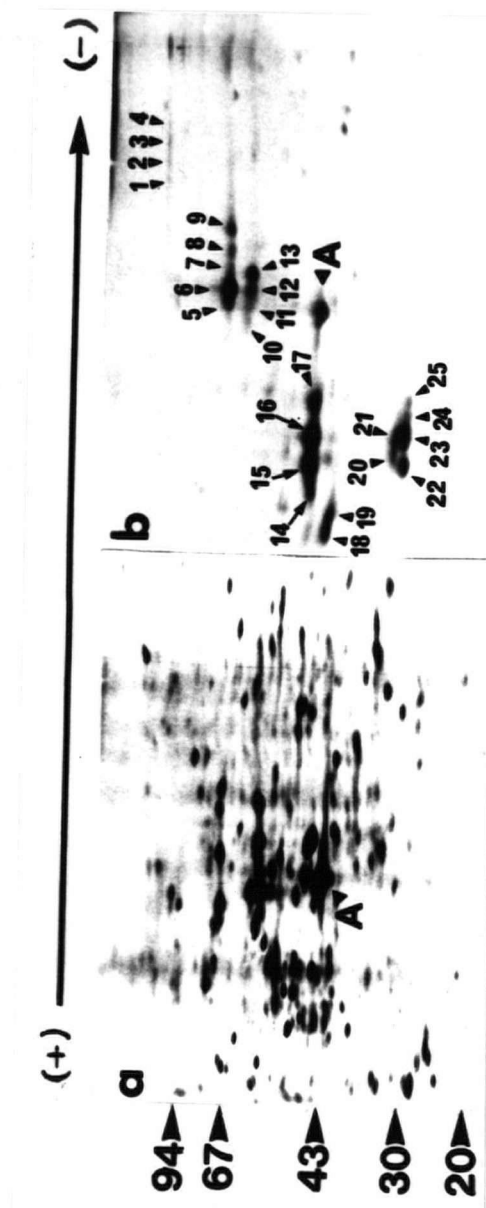
than is revealed by one-dimensional SDS gel electrophoresis alone.
In all, over 20 polypeptides are detected which are not present in
control cells or are present at low levels. These results are
summarized in Table I. Five size classes of polypeptides are
synthesized, each corresponding to a polypeptide band observed on
a one-dimensional gel, and each consisting of at least four
isoelectric variants. However, no polypeptides corresponding to
the hsp of 87,000 daltons are present on the two-dimensional
fluorogram.

To obtain better separation of the region of the gel where
hsp87 should be located, 8% polyacrylamide gels were used as the
second dimension. Examples of the separations obtained on 8%
polyacrylamide gels are shown in Fig. 3. The lower percentage of
polyacrylamide expands the region of the gel where an 87,000
dalton polypeptide should run as shown by the increased separation
of the 67,000 and 94,000 molecular weight markers. Many
polypeptides are apparent on the gel of the arsenite treated
sample (Fig. 3b) which are not obvious on the gel of the untreated
sample (Fig. 3a). However, the control gel in Fig. 3a is
underexposed and most of the polypeptides in the arsenite treated
sample can be faintly detected in the control sample. To ensure
that none of these polypeptides corresponded to hsp87, the
molecular weights of polypeptides in the arsenite sample migrating
between the 67,000 dalton marker and the 94,000 dalton marker were
determined. This was done by measuring the mobility from the
origin for each polypeptide. No polypeptides had molecular

Table 1. Molecular weights and isoelectric points (IEP) of heat shock proteins in Trout RTG-2 cells.

Size Class	Protein	IEP	M_r	Treatment			
				50µM NaAsO$_2$[1] for 3 h	50µM NaAsO$_2$ for 24 h	27°C for[1] 24 h	in vitro[2] translation
~100K	1		ND[3]	+	+	−	+
	2		ND	+	+	−	+
	3		ND	+	+	−	+
	4		ND	+	+	−	+
70K	5	5.91	67,000	+	+	+	+
	6	5.97	67,000	+	+	+	+
	7	5.98	69,800	+	+	+	+
	8	5.98	71,100	+	+	+	+
	9	6.06	70,000	+	+	+	+
60K	10	5.67	59,000	+	+	+	−
	11	5.80	60,000	+	+	+	−
	12	5.82	62,000	+	+	+	−
	13	5.89	62,000	+	+	+	−
40K	14	5.13	43,000	+	+	−	−
	15	5.23	44,000	+	+	−	−
	16	5.39	44,000	+	+	−	−
	17	5.61	44,000	+	+	−	−
	18	4.77	40,000	+	+	−	−
	19	4.93	40,000	+	+	−	−
30K	20	5.27	31,000	+	+	+	+
	21	5.46	31,000	+	+	+	+
	22	5.29	29,000	+	+	+	+
	23	5.41	28,000	+	+	+	+
	24	5.55	29,000	+	+	+	+
	25	5.59	29,000	+	+	+	+

1) All treatments were as described in Materials and Methods.
2) mRNA was isolated from cells treated with 50 µM NaAsO$_2$ for 6 h and translated in vitro as described in the text.
3) Not determined.
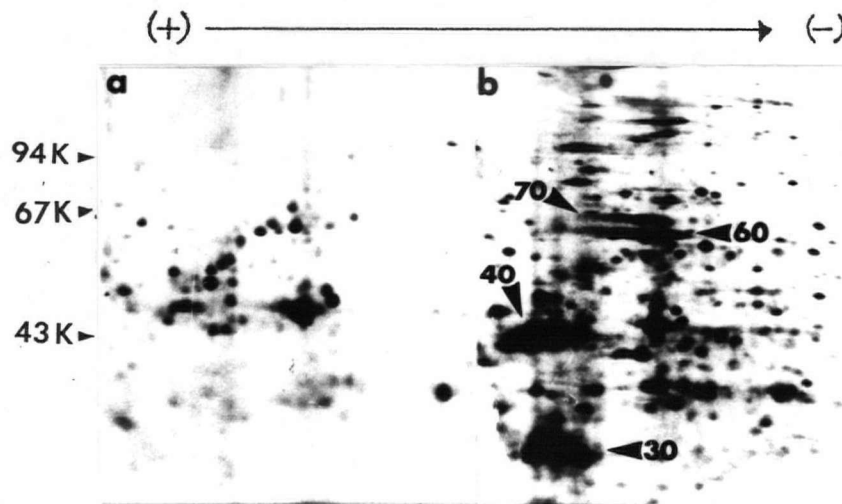
Figure 3. Two-dimensional fluorograms of proteins separated on 8% polyacrylamide gels in the second dimension. Cells were induced and labelled as described in the text. a) proteins from untreated cells. The gel contained 100,000 cpm and was exposed for 21 days. b) proteins from cells treated with 50 uM NaAsO$_2$ for 3 hours and allowed to recover for 2 hours. The gel contained 500,000 cpm and was exposed for 14 days. Molecular weight markers are indicated along the left side of the gel.

weights of 87,000.

Since the first dimension gel had a limited pH gradient of pH 4.5 to pH 6.8, it was possible that the 87K hsp might be too basic for these gels. Non-equilibrium pH gradient gel electrophoresis was performed to establish whether hsp87 has a basic isoelectric point. These gels had pH gradients ranging from pH 3 to pH 10. Fig. 4 shows that, although the other hsps are present, hsp87 is clearly not present in the basic region of the gel. The analysis is more difficult because most cellular proteins have acidic rather than basic isoelectric points causing a high density of spots in the acidic region of the gel. However, careful examination reveals that, as in the equilibrium pH gradient gels, no polypeptides of 87,000 daltons are present in the heat or arsenite treated sample which are not present in the control sample.

Similar patterns were observed for fluorograms derived from cells treated with arsenite for varying times or from cells incubated at 27°C, as shown in Fig. 5. It may be noted from Fig. 5 that heat treatment did not inhibit normal protein synthesis to the same extent as arsenite treatment. As expected, there are no 62,000 or 100,000 dalton polypeptides in the heat treated sample, since treatment at 27°C does not induce these polypeptides (36). Heat shock polypeptides 14 to 17 (the 42,000 dalton polypeptides) are also not detected in gels from heat-treated samples. However, the presence of these proteins is somewhat variable. Polypeptides 14 to 17 are not

Figure 4. Fluorograms of proteins from heat shocked cells

separated by non-equilibrium pH gradient gel electrophoresis.

Cells were induced and labelled as described in the text. a)

proteins from control cells. The gel contained 700,000 cpm

and was exposed for 2 days. b) proteins from cells

incubated at $27^{\circ}C$ for 4 hours. The gel contained 100,000

cpm and was exposed for 15 days. c) proteins from cells

treated with 50 uM $NaAsO_2$ for 24 hours. The gel contained

25,000 cpm and was exposed for 45 days. d) proteins from

cells treated with 50 uM $NaAsO_2$ for 3 hours and allowed to

recover for 2 hours. The gel contained 110,000 cpm and was

exposed for 17 days. Molecular weight markers are indicated

on the left side of the gel.
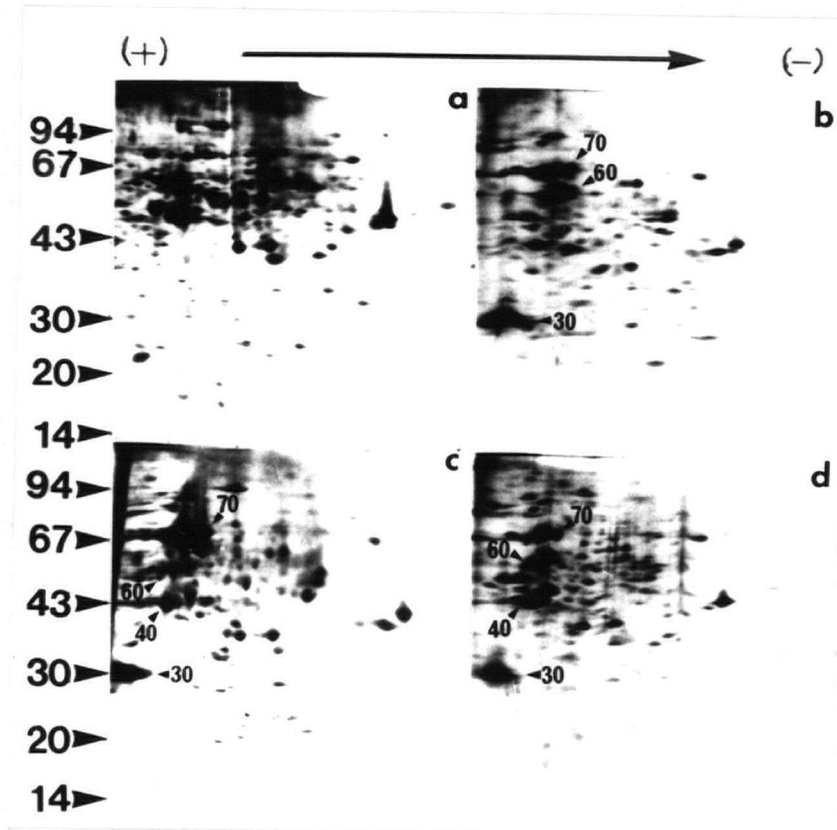
Figure 5. Heat shock proteins from cells treated with either
NaAsO$_2$ or heat. Cells were induced and labelled as
described in the text. a) proteins from untreated cells.
The gel contained 300,000 cpm and was exposed for 10 days.
b) proteins from cells treated with 50 uM NaAsO$_2$ for 3
hours. The gel contained 300,000 cpm and was exposed for 7
days. c) proteins from cells treated with 50 uM NaAsO$_2$
for 24 hours. The gel contained 120,000 cpm and was
exposed for 27 days. d) proteins from cells incubated at
27°C for 24 hours. The gel contained 100,000 cpm and
was exposed for 21 days. Molecular weight markers are
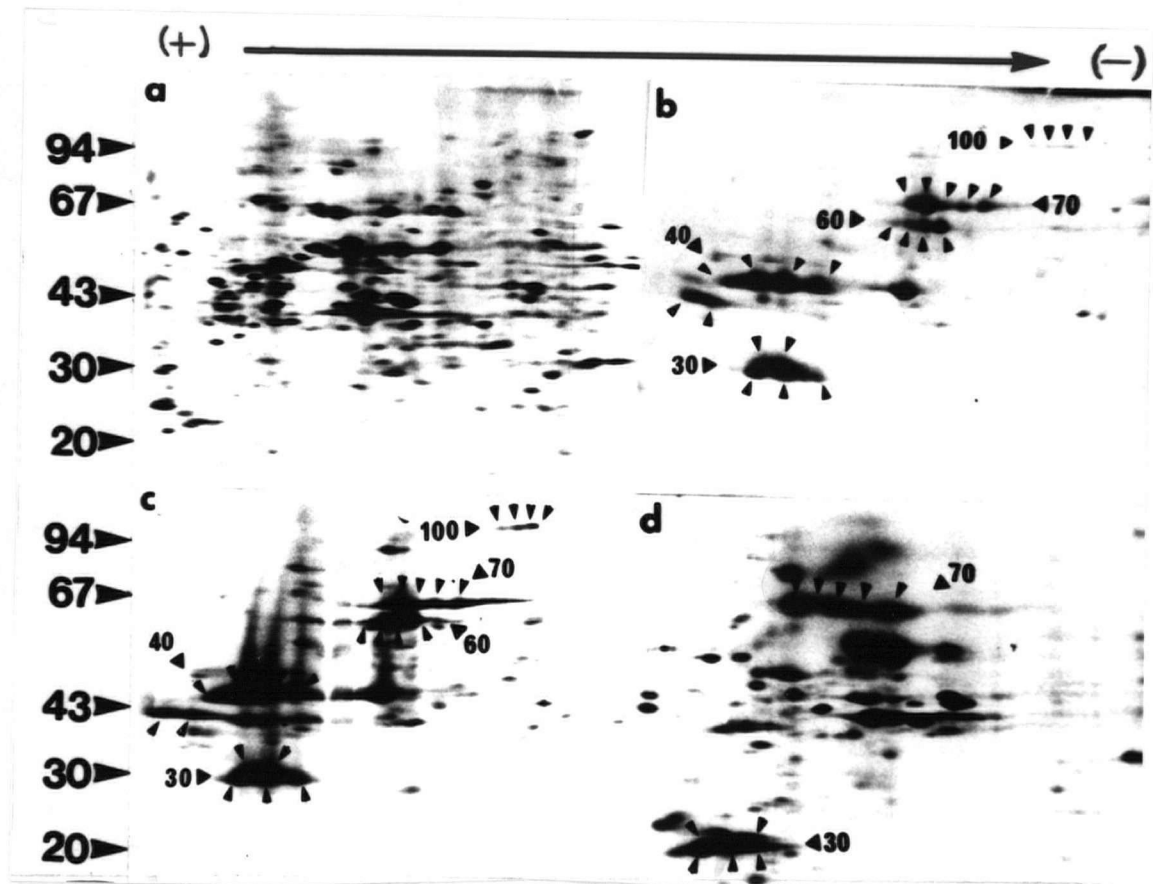indicated on the left side of the gel.

present in <u>in vitro</u> translations of heat shock mRNA, nor are
they present in samples that have been incubated on ice for 15
minutes in 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$. Representative
gels of these samples are shown in Fig. 6. The other treatment
protocols resulted in the synthesis of the expected hsp size
classes, each composed of several isoelectric variants.

B. Post-translational modifications of the heat shock proteins.

1. The effect of sodium arsenite treatment on phosphorylation of the hsps.

The isoelectric variants separate on the two-dimensional gels
in a manner expected for polypeptides which are post-
translationally modified. One commonly occurring post-
translational modification is phosphorylation. To determine if
the hsps are phosphorylated cells were incubated in phosphate-
free medium with ^{32}P-orthophosphate. Sodium arsenite was added
either 3 hours before the ^{32}P-orthophosphate or at the same time
as the ^{32}P-orthophosphate. This procedure should also detect
changes in the phosphorylation of other cellular proteins caused
by heat shock. Upon one-dimensional gel electrophoresis, as shown
in Fig. 7, no phosphorylation of the heat shock proteins was
detected and no changes in phosphorylated polypeptides were
observed in heat shocked cells under the conditions used. To
ensure that the phosphorylation of minor polypeptides was not

Figure 6. Two dimensional gels showing polypeptides 14 to 17
from cells treated in different ways. Cells were induced and
labelled as described in Methods. a) proteins from cells
treated with 50 uM NaAsO$_2$ for 3 hours. The gel contained
275,000 cpm and was exposed for 7 days. b) proteins from
cells incubated at 27° for 24 hours. The gel contained
100,000 cpm and was exposed for 25 days. c) proteins from
cells treated with 50 uM NaAsO$_2$ for 3 hours. The gel
contained 100,000 cpm and was exposed for 25 days. This
sample was suspended in 10 mM Tris-HCl, pH 7.4, 5 mM MgCl$_2$
and incubated on ice for 15 min; d) proteins resulting from
in vitro translation of mRNA isolated from cells treated
with 50 uM NaAsO$_2$ for 6 hours. The gel contained 116,000
cpm and was exposed for 28 days. The mRNA was isolated and
translated as described in the text. Each size class of the
hsps is identified. Molecular weight markers are indicated
on the left side for gels a and c.
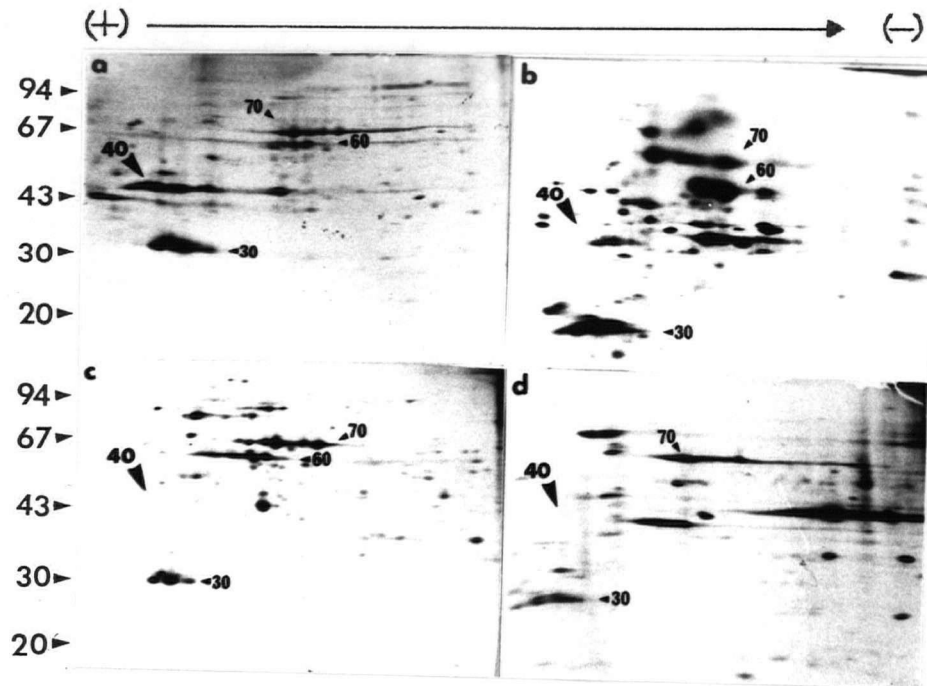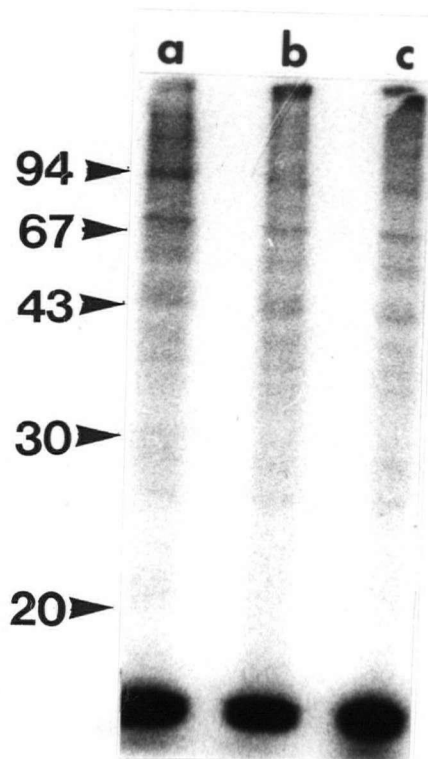
Figure 7. One dimensional analysis of phosphorylated polypeptides
from control and heat shocked cells. Cells were induced and
labelled as described in the text. a) proteins from non-heat
shocked cells; b) proteins from cells treated with 50 uM
$NaAsO_2$ for 4 hours while labelling with $^{32}P-$
orthophosphate; c) proteins from cells treated with 50 uM
$NaAsO_2$ for 4 hours before labelling with $^{32}P-$
orthophosphate for 4 hours. Molecular weight markers are
indicated on the left side of the gels.

masked by the presence of major phosphorylated proteins in both
heat shocked and control cells, two-dimensional gel
electrophoresis was performed also. Fig. 8 shows examples of two-
dimensional gels of phosphorylated polypeptides. None of the
phosphorylated polypeptides migrate in the positions expected for
heat shock proteins. Therefore, the phosphorylated polypeptides
are cellular proteins which were synthesized before arsenite
treatment and phosphorylated in the presence of $NaAsO_2$. Some
polypeptides are present in the control gel but not in the
arsenite gel. Different exposure times can account for some of
these polypeptides which can be detected faintly in the arsenite
sample. Others were not observed consistently in the various
unshocked samples. Therefore, under these labelling conditions,
arsenite treatment does not affect the phosphorylation of either
the heat shock proteins or cellular proteins.

2. In vitro translation of heat shock mRNA.

Another possible explanation for the charge heterogeneity of
the hsps would be the existence of several related but distinct
genes coding for each protein size class. This possibility is
supported by the fact that, in D. melanogaster, at least five
genes code for hsp70 (47). To determine whether different
transcripts exist for each hsp size class, RNA was isolated from
cells treated with 50 uM $NaAsO_2$ and translated in vitro in the
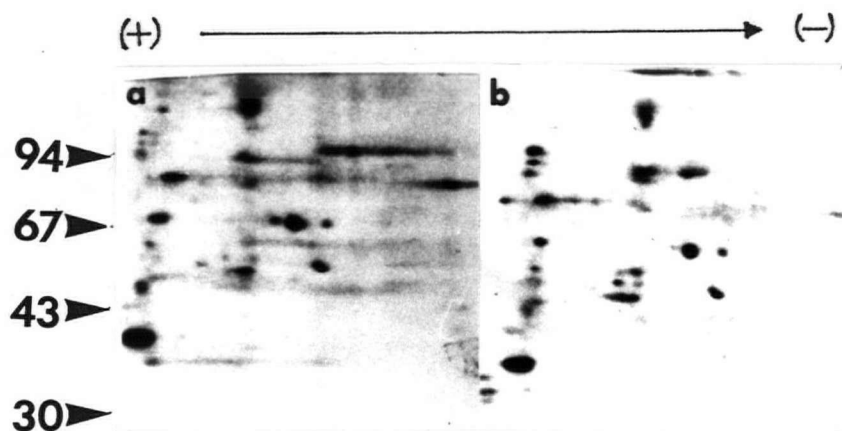
Figure 8. Two-dimensional analysis of phosphorylated

polypeptides. Cells were labelled as described in

Methods. a) proteins from untreated cells labelled for

4 hours with ^{32}P-orthophosphate. The gel contained

3×10^6 cpm and was exposed for 6 hours. b) proteins

from cells treated with 50 uM NaAsO$_2$ at the same

time as labelling with ^{32}P-orthophosphate for 4

hours. The gel contained 1×10^6 cpm and was exposed

for 3 hours. Molecular weight markers are indicated

on the left side of the gels.

rabbit reticulocyte system. In the simplest case, if the

reticulocyte system is incapable of the same post-translational

modification(s), isoelectric variants should only be observed if

variant mRNAs are present. Fluorograms obtained from in vitro

translations are presented in Fig. 9. Polypeptides 10 to 13 (the

62,0000 dalton hsps) and polypeptides 14 to 17 (the 42,000 dalton

hsps) are clearly not present on these fluorograms, as shown in

Table 1. The lack of polypeptides 14 to 17 is difficult to

explain, but as mentioned previously (Fig. 6), these proteins are

also absent from gels of samples which have been incubated in 10

mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ on ice for 15 min and from gels

of cells which have been incubated at 27°C. The small spots to

the right of hsp70 could represent incomplete translation

products from degraded hsp70 mRNA. All isoelectric variants are

present in the in vitro translation mix, meaning that either

post-translational modification is occurring in the translation

system or that more than one transcript exists for these

polypeptides.

3. Hybrid-selection of hsp70 mRNA.

A cDNA clone complementary to hsp70 mRNA of trout has

recently been isolated and used to investigate the number of

genes coding for this protein in trout (R. Kothary, personal

communication). A fragment of this cDNA was subcloned into M13

and the strand complementary to the hsp70 transcript was used
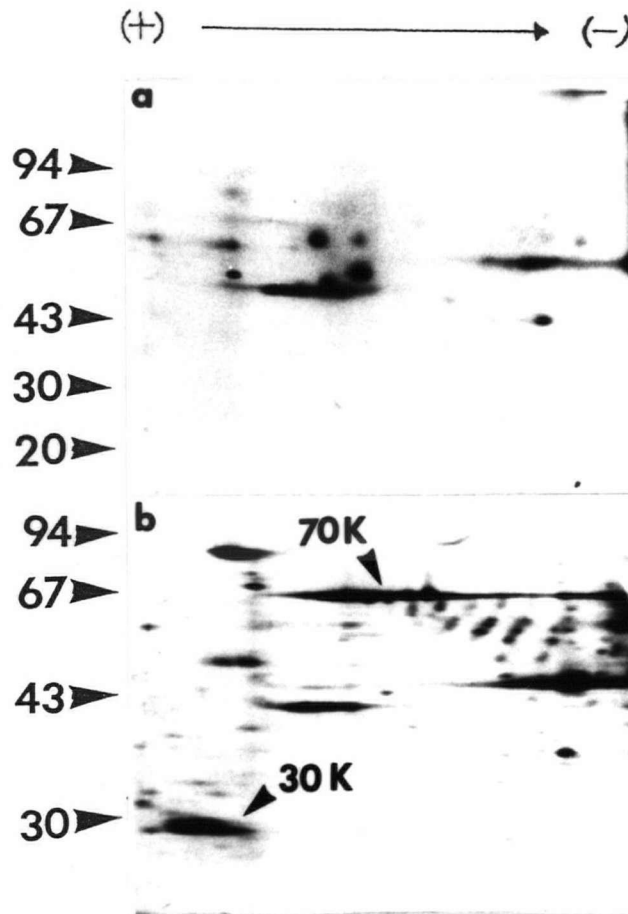
Figure 9. Fluorograms of proteins from in vitro translations of
mRNA isolated from untreated cells or from those treated with
NaAsO$_2$. Messenger RNA was isolated and translated from
cells treated as described in Methods. a) mRNA from control
cells. The gel contained 250,000 cpm and was exposed for 13
days. b) mRNA from cells treated with 50 uM NaAsO$_2$ for 6
hours. The gel contained 160,000 cpm and was exposed for 19
days. Heat shock proteins are labelled with arrows.
Molecular weight markers are indicated on the left side of
the gels.

in hybrid-selection experiments to determine whether the transcripts encoding the different hsp70 isoelectric variants hybridize to this sequence. Fig. 10 shows the M13 clone used in the hybrid-selection. Single stranded phage DNA was bound to nitrocellulose and annealed under stringent conditions to poly A$^+$ mRNA isolated from cells treated with arsenite. Bound RNA was eluted from the immobilized DNA and translated in vitro. The gel pattern from the in vitro translation mixture is shown in Fig. 11. A streak is observed corresponding to the 70 K variants. It should be noted that discrete spots are not usually observed for trout hsp70 in the in vitro translation system, as if the proteins aggregate in the IEF dimension under these conditions. Again, this result suggests either the presence of several transcripts or that post-translationai modification occurs in the in vitro translation system.

D. Induction of hsp70.

The induction of hsp70 after short arsenite treatments was studied using an antibody to chick hsp70 which was kindly supplied by Dr. M. J. Schlesinger (Department of Microbiology and Immunology, Washington University School of Medicine, St. Louis, Mo.). This antibody cross-reacts with trout hsp70 as shown in Fig. 12. Slight reaction is observed with proteins from cells which have not been shocked. This may mean that the cells are sufficiently stressed during harvesting to begin heat

Figure 10. Restriction map of the M13 clone used in the hybridization

selection of heat shock mRNA. A 690 base pair Pst I fragment from

the cDNA clone THS70.7 was subcloned into the Pst I site of M13

mp8 and generously supplied by R. Kothary. The trout clone

corresponds to the region coding for amino acids 128 to 348 of the

D. melanogaster hsp70 sequence and is represented by the open

box. The solid lines represent M13 sequence. The restriction

sites are: P, Pst I; T, Taq I and B, Bam HI.
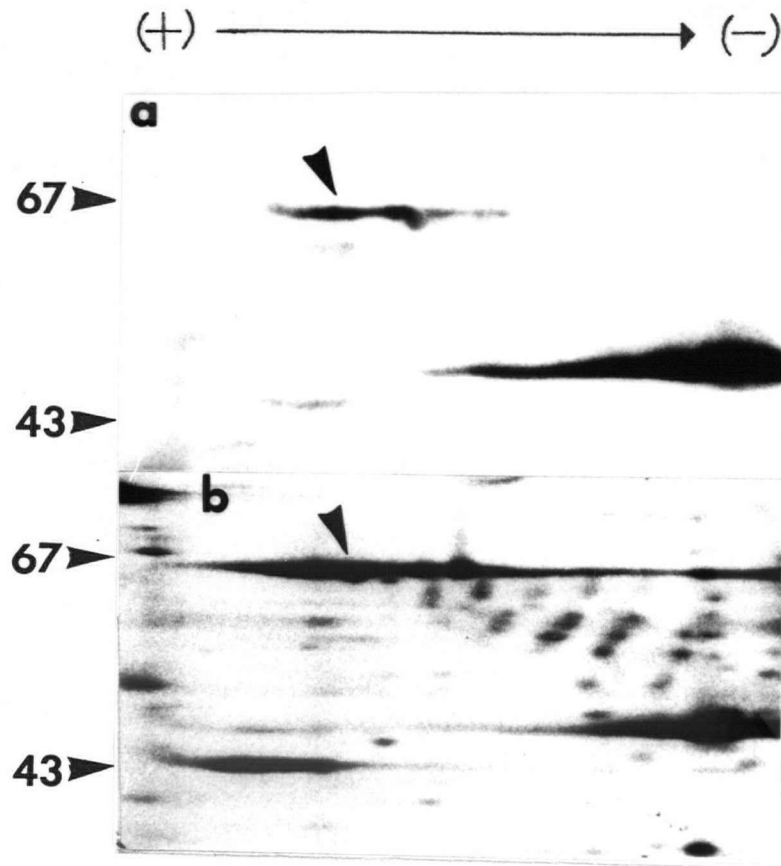
Figure 11. Fluorogram of proteins from in vitro translations of mRNA isolated by hybrid-selection to an hsp70 M13 clone. Poly A$^+$ RNA from cells treated with 50 uM NaAsO$_2$ for 5 hours was hybridized to an hsp70 M13 clone (Fig. 10) as described in Methods. Hybridization was for 16 hours at 43°C in 50% formamide. a) Two-dimensional fluorogram of selected mRNA. The gel contained 50,000 cpm and was exposed for 25 days. b) 70 K region of a fluorogram resulting from the translation of total mRNA from cells treated with 50 uM NaAsO$_2$ for 6 hours. The gel contained 160,000 cpm and was exposed for 19 days. The arrow indicates the hsp70 polypeptides. Molecular weight markers are indicated on the left side of the gels.
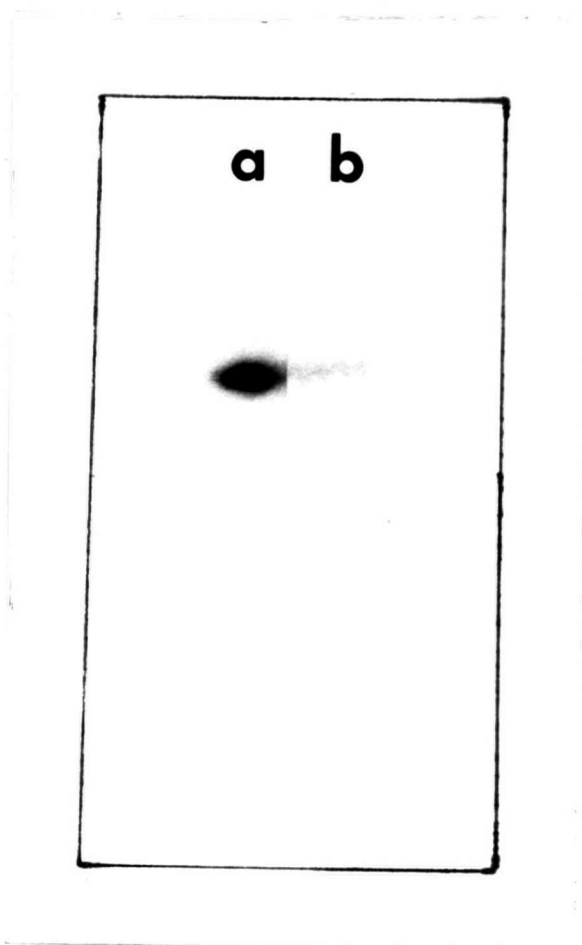
Figure 12. Cross-reaction of the antibody to chick hsp70 with

trout hsp70. Protein blotting was performed as described in

Methods. a) proteins from cells treated with 50 uM NaAsO$_2$

for 3 hours and allowed to recover for 2 hours. b) proteins

from untreated cells.

shock protein synthesis. To study hsp70 induction, cells were

treated with 50 uM NaAsO$_2$ for times ranging from 5 min to 2

hours before harvesting. Harvesting was performed as quickly as

possible to minimize induction of the heat shock proteins. As

can be seen in Fig. 13, lane a, the antibody still reacts with a

protein of about 70,000 daltons in unshocked cells. With

increasing times of arsenite treatment, the antibody reacts with

increasing amounts of the 70,000 dalton protein. An increase in

antibody binding is detectable after just 5 min of induction.
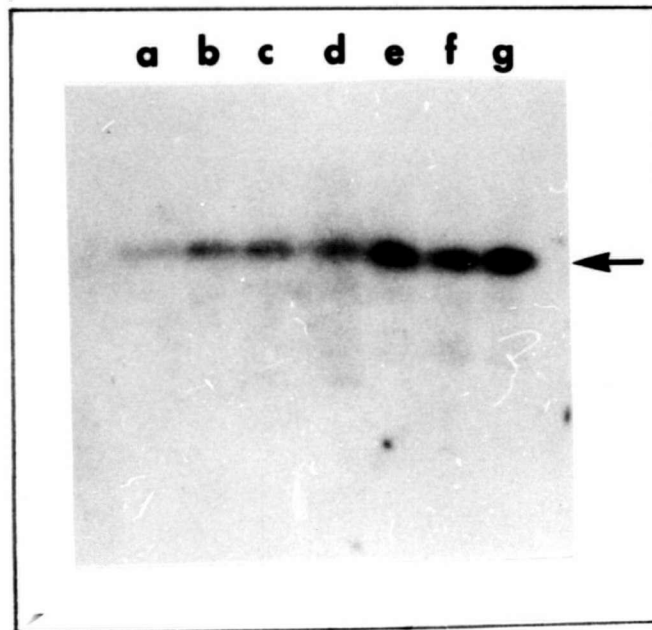
Figure 13. Induction of hsp70. Cells were induced for various
times and the proteins separated on one-dimensional SDS gels
and transferred to CNBr-activated paper as described in the
methods. a) proteins from unshocked cells; b) proteins from
cells treated with 50 uM NaAsO$_2$ for 5 min; c) 15 min; d) 30
min; e) one hour; f) 2 hours; and g) 3 hours. In all cases,
cells were allowed to recover for 2 hours before protein
extraction. The arrow indicates hsp70.

Part II. The effect of histone acetylation on the induction of

hsps by sodium arsenite.

A. Sodium butyrate induced histone acetylation.

RTG-2 cells were treated with varying concentrations of butyrate

for 24 hours to establish the best conditions to achieve maximum

acetylation. Histones were isolated from cells treated with different

concentrations of the fatty acid for 24 hours and separated on acid-

urea gels. These gels separate basic proteins according to their

charge. In the acid-urea system used here, only the acetylated

forms of H4 are completely resolved from the other histones.

However, the extent of histone acetylation can still be determined

using this gel system by monitoring the acetylation of H4. For

this reason, Fig. 14 and Fig. 15 show only the H4 region of the gel.

Fig. 14 shows the effect of treatment with different

concentrations of sodium butyrate on histone acetylation in RTG-2

cells. Comparison of the unacetylated and the monoacetylated forms

of H4 shows that acetylation is maximal after 24 hours of

treatment with 5 mM sodium butyrate. In the untreated sample, the

mono-acetylated form of H4 represents less than one-third of the

total H4 while 24 hours of butyrate treatment causes the

mono-acetylated form to increase until it almost equals the

acetylated form. A time course of butyrate treatment is shown in

Fig. 15. Cells were treated with 5 mM sodium butyrate for

different times and the histones isolated and separated on

Figure 14. The effect of different concentrations of sodium
butyrate on histone acetylation. Cells were induced with
different concentrations of sodium butyrate for 24 hours.
The histones were isolated as described in Methods, separated
on acid-urea gels, stained with Coomassie blue and the region
of the gel containing H4 was scanned at 545 nm with a
Beckman DU-8 spectrophotometer. A_o is the unacetylated form,
A_1 is the mono-acetylated form, A_2 is the di-acetylated
form and A_3 is the tri-acetylated form. The absorbance range
was 1.

A_0
A_1
A_2
A_3

50 mM

5 mM

3 mM

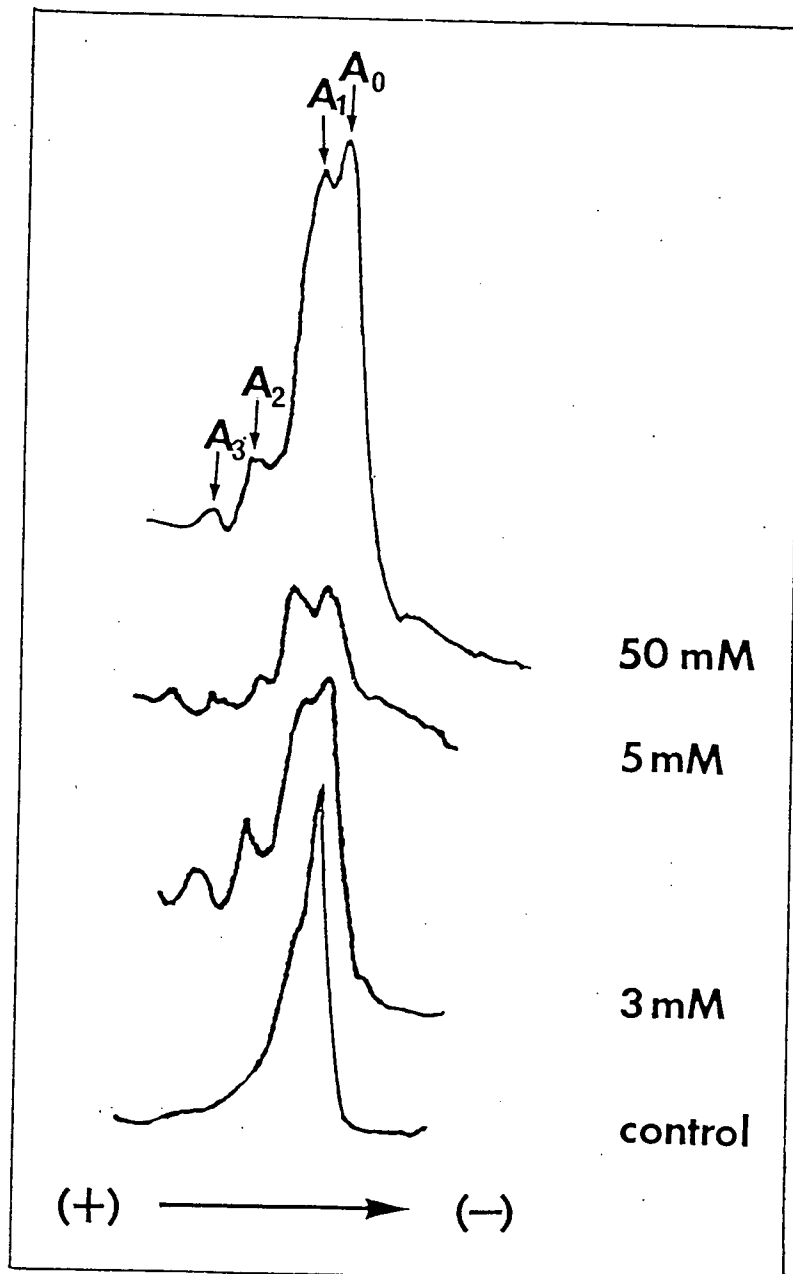control

(+) ⟶ (−)

Figure 15. Time course of histone acetylation induced by sodium
butyrate. Cells were induced with 5 mM sodium butyrate for
the indicated times. The histones were isolated as described
in the methods, separated on acid-urea gels and the region
of the gel containing H4 scanned at 545 nm. The acetylated
forms of the histones are labelled as in Fig.14. The
absorbance range is 1.
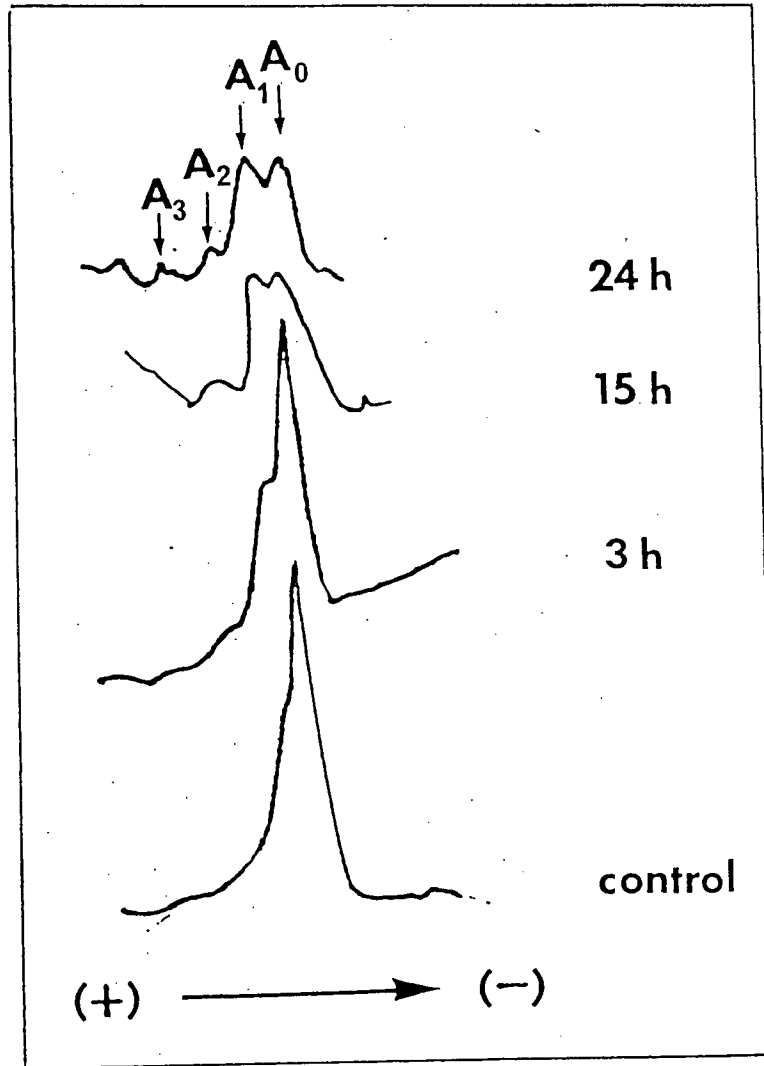
acid-urea gels. Histone acetylation was maximal after 15 hours of butyrate treatment. Since butyrate is known to block cell division at the G_1 stage, subsequent experiments were performed in 5 mM sodium butyrate for 24 hours or less to achieve maximal acetylation with minimal effects on cell division.

B. The effect of sodium butyrate on cellular protein synthesis.

To examine the effects of sodium butyrate on overall patterns of protein synthesis, cells were treated with 5 mM butyrate for either 3 hours or for 24 hours and then labelled with ^{35}S-methionine prior to two-dimensional gel electrophoresis. If acetylation increases transcriptional activity, then an increase in the synthesis of certain polypeptides might be expected to occur. There is no apparent difference in the protein synthetic patterns of the untreated sample and in the sample treated with butyrate for 24 hours (Fig. 16a and 16c). However, changes are observed in the protein synthetic pattern of the sample treated with sodium butyrate for 3 hours (Fig. 16b). Two polypeptides of 53,000 and 67,000 daltons increase in amount, while a third of 53,000 decreases. By 24 hours, the pattern has returned to that observed for control cells. Clearly, sodium butyrate is not causing drastic changes in the protein synthetic patterns of the RTG-2 cells.

Figure 16.   The effect of sodium butyrate on total cellular

protein synthesis as determined by two-dimensional gel

electrophoresis.   RTG-2 cells were treated with 5 mM butyrate

for 3 hours or 24 hours prior to labelling with ^{35}S-

methionine and separation of the total cellular proteins on

two-dimensional gels.   Portions of the gels in which

differential protein synthesis is observed are shown.   Panels

a, control; b, butyrate treatment for 3 hours; c, butyrate

treatment for 24 hours.   The arrowhead indicates the induced

proteins.   The large gel was treated with 5 mM butyrate for

three hours and molecular weight markers are indicated.
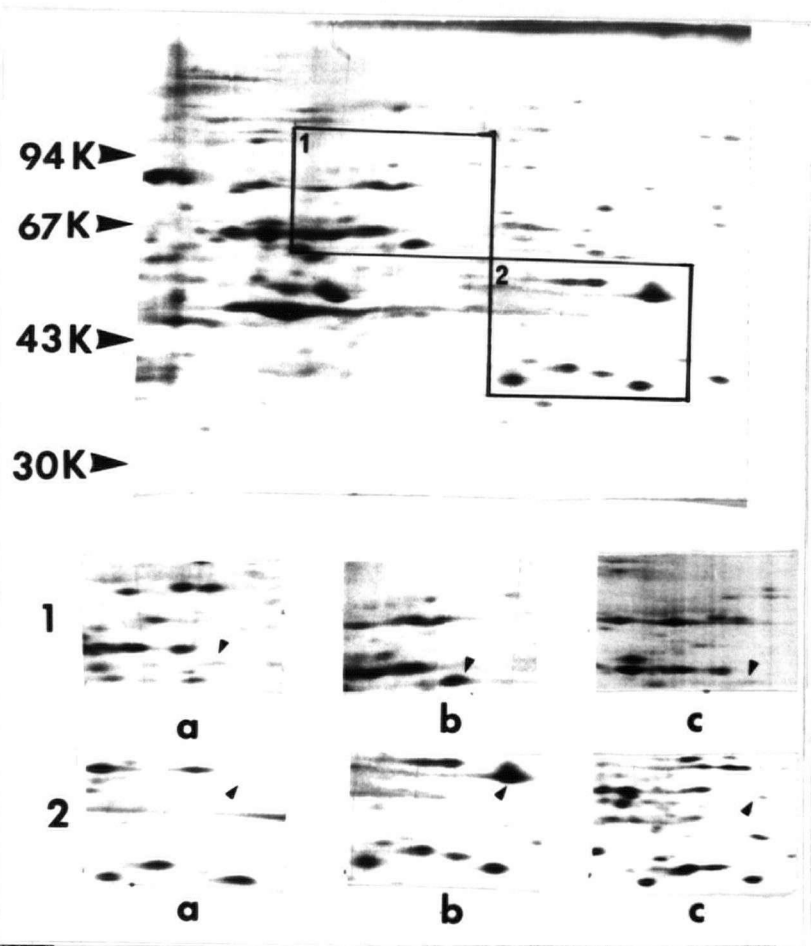
C. The effect of sodium butyrate on the response to sodium arsenite.

Since sodium butyrate can block the hormonal induction of certain enzymes (87,89), the effect of sodium butyrate on heat shock protein synthesis was examined. The effect of histone acetylation on the heat shock response was examined by treating cells with 5 mM butyrate for 24 hours before or during arsenite induction. The sodium arsenite was either added simultaneously with the butyrate (24 hour induction of the hsps), or for the final 3 hours of the butyrate treatment (3 hour induction of hsps). Despite the changes in acetylation caused by the different butyrate treatments, the heat shock proteins are still induced by sodium arsenite (Fig. 17).
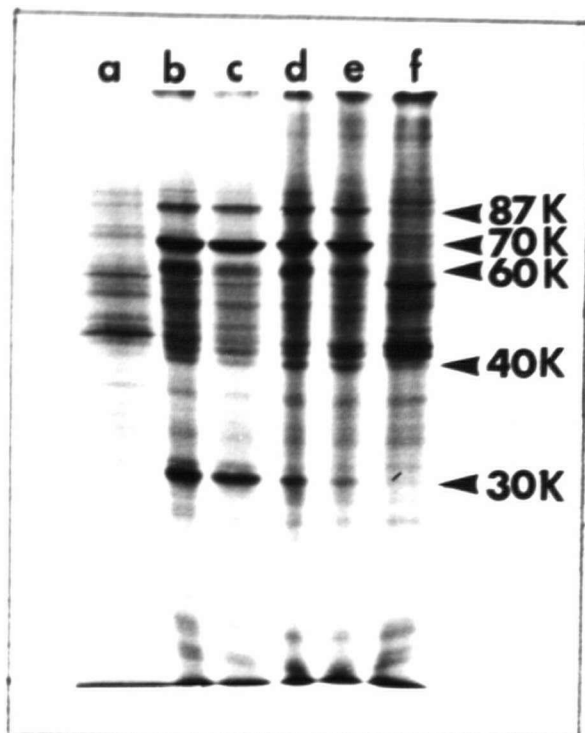
Figure 17. The effect of sodium butyrate on the induction of heat
shock proteins in RTG-2 cells by sodium arsenite. Cells were
treated with 5 mM sodium butyrate or 50 uM sodium arsenite or
both before labelling with ^{35}S-methionine and separation
of the proteins on SDS gels. Lanes a) sodium butyrate for 24
hours; b) sodium butyrate for 24 hours with sodium arsenite
added for the final 3 hours; c) sodium arsenite for 3 hours;
d) butyrate and arsenite for 24 hours; e) sodium arsenite
alone for 24 hours; f) untreated cells. Arrows indicate the
heat shock proteins.

Discussion

Part I. The response of RTG-2 cells to heat or sodium arsenite.

A. Two-dimensional gel electrophoresis of trout heat shock proteins.

Two-dimensional gel electrophoresis of trout RTG-2 cell heat shock proteins reveals a complex pattern. The five size classes of proteins separate into more than 20 isoelectric variants. Buzin and Petersen observed similar results in D. melanogaster (40). As in trout, the heat shock proteins separate into large numbers of isoelectric variants upon two-dimensional gel electrophoresis. In trout, the isoelectric points of the hsps range from pH 4.7 to pH 6.8 for hsp70. The D. melanogaster hsps have a greater range of isoelectric points: some of the isoelectric variants of the small hsps have isoelectric points of pH 8.0 to pH 10.0 (40). All of the trout polypeptides detected by one-dimensional gel electrophoresis are present on the two-dimensional gels except for the 87,000 dalton hsp. Hsp87 is not observed when the second dimension gel is an 8% gel, nor is it observed upon non-equilibrium pH gradient gel electrophoresis. The 8% gel should allow better separation of higher molecular weight polypeptides while the non-equilibrium pH gradient gel electrophoresis should allow separation of polypeptides with isoelectric points ranging from pH 4.5 to pH 10.0. The corresponding protein in D. melanogaster, hsp83, has an

)

isoelectric point of pH 5.33 (40). Thus, if the trout hsp87 is

at all similar to hsp83 in D. melanogaster, then it should be

detected in these gel systems. It is unlikely that the

isoelectric point of hsp87 is outside the range of the non-

equilibrium pH gradient gels. A plausible explanation is that

hsp87 might be undetected in these systems due to the presence

of extensive charge heterogeneity which spreads the label over

a large number of components. As hsp87 is induced in smaller

quantities than some of the other hsps (36), it may be difficult

to detect the presence of numerous charge variants by two-

dimensional gel electrophoresis.

Since carbamylation of amino groups in proteins by isocyanate

derived from urea can lead to charge heterogeneity, the possibility

that the isoelectric variants observed might be due to this reaction

must be considered. All precautions suggested by O'Farrell to

avoid this effect (96) were followed in these experiments. Samples

were either suspended in sample buffer and loaded on gels immediately

or they were frozen at -70°C. Sample buffer contained

protective ampholines and samples were not allowed to warm in the

presence of urea. Ultrapure urea solutions were either freshly made

each time or stored frozen in aliquots at -20°C. Finally,

isoelectric focussing gels were pre-run to remove isocyanate.

Furthermore, multiple spots are not observed on control gels, nor

for many of the non-hsp components of gels containing heat shock

proteins. Also, a high degree of charge complexity in hsps has been

observed in other systems (15,40,43,52). For these reasons, it can

be concluded that the observed heterogeneity is a characteristic feature of the hsps themselves.

In agreement with the data of Kothary and Candido, (36) the 100,000 dalton hsps and the 62,000 dalton hsps are not observed in heat shocked cells. However, surprisingly, the 42,000 dalton isoelectric variants are also not detected in heat shocked cells but are observed after a 24 hour arsenite treatment. The reverse was observed by Kothary and Candido (36): hsp42 was present in heat shocked cells, but not in cells which had been treated with arsenite for 24 hours. These hsps are not detectable in in vitro translations of mRNA isolated from arsenite-treated cells or in samples which have been incubated on ice for 15 minutes (Fig. 6). Thus, it appears as though these polypeptides are labile and that their presence or absence may be dependent on the preparation of the sample. This could explain the difference between the results presented here and those in reference 36. Another unexpected result is the absence of the 62,000 dalton isoeletric variants in the in vitro translation products of mRNA isolated from arsenite-treated cells. This suggests that these polypeptides may result from proteolytic degradation of larger polypeptides, perhaps the hsp70 variants.

B. The nature of the iso-electric variants of the heat shock proteins.

1. Post-translational modification of the heat shock proteins.

The nature of the heat shock isoelectric variants is unknown. No evidence for the phosphorylation of trout heat shock proteins was found, although phosphorylation of Dictyostelium discoideum hsp70 under similar conditions has been reported (44). Phosphorylation of hsp70 may be unique to D. discoideum however, as analogous experiments have not revealed phosphorylation of the D. melanogaster hsp70 (45). Phosphorylation may also be dependent on the agent employed to induce the heat shock proteins. Sodium arsenite may fail to affect steps of the phosphorylation process affected by heat or the two perturbants may affect different steps in the process. Vincent and Tanguay (45) have observed the phosphorylation of a cellular protein after heat treatment, but not after arsenite treatment. Further, the detection of hsp phosphorylation could be dependent on the labelling conditions. For example, the hsps would not be labelled under conditions used in the present study if the turnover of phosphate groups was very slow.

Other post-translational modifications of heat shock proteins have been shown to occur. Hsp70 and Hsp87 are methylated in chick and hamster cells (41,42) and hsp87 is ADP-ribosylated (43). These modifications may account for some of the charge

heterogeneity observed upon two-dimensional gel electrophoresis, but if the variants are caused predominantly by post-translational modification then similar modifications must occur in the cell-free rabbit reticulocyte system, for heat shock mRNA from both D. melanogaster (40) and trout results in multiple isoelectric variants upon translation in this system. Ovalbumin (113) and actin (114) are acetylated at their N-terminals in the in vitro rabbit reticulocyte system and other post-translational modifications may well occur in the cell-free system although they have not been reported to date.

2. Many genes code for heat shock proteins.

Alternatively, the heterogeneity of the hsps could be due to the existence of multiple amino acid sequence variants of these proteins. Such heterogeneity of amino acid sequence could be generated at the DNA level by the existence of several genes. In trout, the analysis of genomic DNA by Southern hybridization to an hsp70 cDNA clone reveals the existence of a small number (perhaps two or three) or closely homologous sequences, and several weakly hybridizing sequences (R. Kothary, personal communication). Five genes code for hsp70 in D. melanogaster, although recently three other genes with 70% homology to the hsp70 gene have been identified (48). A similar gene family consisting of 10 homologous genes exists in yeast (39). However, not all of these genes are expressed during heat shock. Some, the cognate genes, are expressed under normal conditions, and

hence cannot account for the charge variants observed upon heat shock.

3. Hybrid-selection of heat shock messenger RNA.

Since hybridization of heat shock mRNA to an hsp70 cDNA clone isolates mRNA which is translated into several charge variants, either different transcripts code for the different isoelectric variants, or post-translational modification is occurring in the in vitro translation system. Hybridization of heat shock mRNA from D. melanogaster to a genomic hsp70 gene isolates not only the transcript coding for the 70 K hsp, but also transcripts corresponding to minor hsps of 72,000, 73,000 and 75,000 daltons (46). Peptide mapping of these polypeptides shows that they are closely related. However, deletions spanning the 87C and 87A heat shock loci result in mutants which do not make hsp70, but do synthesize the three slightly larger polypeptides (47). The genes coding for hsp70 are known to exist at the heat shock loci 87A and 87C. Therefore, the genes coding for the minor proteins which are closely related to hsp70 must reside outside the known heat shock loci.

In soybean, over 20 polypeptides of 15,000 to 18,000 daltons are induced upon heat shock (115). A cDNA coding for one of the 16,000 dalton proteins has been isolated (116). These numerous polypeptides constitute a large gene family since many bands are present on Southern blots of soybean genomic DNA probed with the cDNA clone. The in vitro translation of the messenger RNA which hybridizes to the

16 K hsp cDNA clone results in a series of isoelectric variants similar
to those observed in this thesis. If the hybridization-selection is
performed at higher stringency, then some of the charge variants in the
in vitro translation disappear. In other words, the cDNA clone
coding for the 16,000 dalton hsp hybridizes to a number of transcripts
and the charge variants observed in this system are not caused by post-
translational modification in the in vitro translation system.
If the variation in sequence is small and the transcript large, as
would be expected for isoelectric variants of a 70,000 dalton
polypeptide, then it may be difficult to distinguish the different
transcripts by hybridization conditions. In the experiments
described here, although the stringency of hybridization was
high, increasing the stringency still further might result in the
selection of only one transcript.

4. Genetic polymorphism of the heat shock genes.

Some of the complexity of the hsps of D. melanogaster can also
be explained by the existence of genetic polymorphism in the fly
population. Sequencing of the small hsp genes from the Oregon R strain
has revealed a number of restriction site changes attributed to genetic
polymorphism (117). Since the RTG-2 cell line was derived from pooled
trout gonadal tissue (95), genetic polymorphism may also account for
some of the complexity of the trout hsps. It is likely that all of the
above factors contribute in some measure to the charge heterogeneity
seen in these proteins.

C. Induction of hsp70.

To study hsp70 induction, immunological techniques were employed because of their high sensitivity. An antibody to chick hsp70 which cross-reacts with trout hsp70 was used. This cross-reaction of the antibody to the chick protein with the trout protein is another example of the conservation of hsp70 structure in different species. In addition to strong reaction with hsp70 in heat shocked cells, slight but significant binding to a protein of 70,000 daltons is detectable in control cells. It is possible that hsp70 is constitutively present at low levels in control cells, or that the cells are sufficiently stressed during harvesting to begin making heat shock proteins. Similar observations have been reported in other systems (5,15,38). This cross-reaction could also have resulted if proteins similar to hsp70 are expressed in control cells (e.g. the cognate proteins), or, since the chick antibody is not a monoclonal antibody, from contamination by control proteins of the antigen used for immunization. The hsp70 used to innoculate the rabbits was isolated by Sepharose 6B and DEAE-cellulose chromatography (5). Purity of the hsp70 was assayed by one-dimensional gel electrophoresis. However, the two-dimensional gels presented in this thesis clearly show the presence of proteins of similar size

and charge as hsp70 in control cells. Such control proteins

which could result from the expression of the cognate genes, may

have contaminated the hsp70 preparation used in immunization.

Proteins coded for by the cognate genes may carry similar antigenic

determinants to those of hsp70 since the cognate genes of D.

melanogaster and yeast are 70% homologous to hsp70 at the amino

acid level (39,48). Recently, Lindquist et al. (118) have

isolated a monoclonal antibody to hsp70 which does not cross-react

with proteins from unshocked cells. Thus, although a number of

groups have reported the presence of hsp70 in control cells, the

protein detected in control cells is probably transcribed from a

cognate gene and is not identical to hsp70.

Although the antibody to chick hsp70 cross-reacts with

proteins in the control cells to a small degree, the induction

of hsp70 synthesis is still clearly detectable. A sharp

increase in antibody binding is detectable after 5 min of

treatment with 50 uM $NaAsO_2$ indicating the synthesis of hsp70

upon heat shock. However, the corresponding RNA transcript is

only detected by RNA dot-blot analysis after 15 min of treatment

(R. Kothary, personal communication). The disparity between the

appearance of the protein and the presence of the transcript may

reflect the sensitivity of the two techniques. Each transcript

can be translated many times to generate a large amount of

protein which is then detectable by immunological techniques.

In addition, more than one gene may code for the trout hsp70 and

transcripts from other genes may not hybridize to the hsp70 cDNA

clone under conditions of high stringency. Since the antibody is polyclonal, it will likely bind to several variant hsp70s. In D. melanogaster, the transcript is detectable 5 min after shift to 35°C (13) while the protein is detectable after 10 min of incubation at 35°C (18). These induction times are comparable to the results described in this thesis. However, as mentioned previously, comparisons between different organisms are not very meaningful because the induction of the hsps is affected by the severity of the stress.

Part II. The effect of histone acetylation on the response

to sodium arsenite.

A. The effect of sodium butyrate on trout cells.

Maximal levels of acetylation of RTG-2 histones are achieved

by the treatment of RTG-2 cells with 5 mM sodium butyrate for 15

hours. Further acetylation is not achieved by the use of higher

concentrations of butyrate or longer treatment times. Similar

results have been obtained for rat, mouse, hamster and Xenopus

cells (80). After 24 hours of butyrate treatment, no changes in

protein synthesis, as determined by two-dimensional gel

electrophoresis, are apparent. However, treatment for shorter

lengths of time from 3 hours to 6 hours results in an increase in

the amounts of two polypeptides and a decrease in a third. It is

possible that the level of acetylation achieved after 3 hours of

butyrate treatment is sufficient to affect the expression of these

proteins whereas further acetylation does not alter their

original pattern of expression. Since butyrate is known to change

the distribution of the cell population across the cell cycle, it

is more likely that these proteins are cell-cycle specific.

Sodium butyrate blocks mammalian cells in G_1 phase (91). In

chinese hamster ovary cells, butyrate has also been shown to have

little effect on protein synthesis (86), but in Friend

erythroleukemic cells, butyrate has been shown to induce a large

number of proteins (85). This difference may be explained by the

fact that butyrate causes differentiation of Friend

erythroleukemic cells into mature cells that produce haemoglobin

(87). Hence, a number of new polypeptides are synthesized in

Friend erythroleukemic cells whereas in chinese hamster cells and

RTG-2 cells, which are relatively unaffected by butyrate,

synthesis of only a few polypeptides is induced.

B. The effect of sodium butyrate on the response to sodium
   arsenite.

Hyperacetylation of the histones has no effect on the

induction of heat shock proteins by sodium arsenite. Cells were

either treated with butyrate before arsenite treatment or were

treated simultaneously with both butyrate and $NaAsO_2$. The heat

shock proteins were induced in all cases. Treatment with sodium

butyrate for these time-periods (greater than 3 hours) causes both

bulk histone acetylation and acetylation of the rapidly acetylated

subset of the histones. The extent of both types of histone

acetylation increases dramatically under these conditions. It is

difficult to imagine how the induction of the heat shock proteins

could remain unaffected if the state of histone acetylation is

important in the heat shock response. Arrigo (34) has reported

that heat shock causes deacetylation of histones in D.

melanogaster cultured cells. If this is true, then it appears

to be a consequence of the heat treatment rather than part of the

induction mechanism for hsps since hyperacetylation of the

histones does not block heat shock protein synthesis. The ability
of butyrate to block the activation of steroid-inducible genes
(92-94) does not appear to apply to all inducible genes. In
particular, it has no effect on the induction of heat shock
proteins in the system described here. This is perhaps not
surprising, since different control regions are probably involved
in these two classes of inducible genes, and they may act via
different mechanisms. The heat shock genes are activated virtually
immediately upon exposure of cells to an appropriate inducer,
whereas steroid induction typically requires several hours
(122,123). Thus, while the heat shock genes are primed for
immediate transcription, the activation of hormone-inducible genes
seems to require additional processes, at least one of which is
susceptible to inhibition by sodium butyrate.

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