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Christopher Leon Baszczynski

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THERMAL SHIFT-INDUCED GENE EXPRESSION AND REGULATION IN MAIZE (*ZEA MAYS* L.)

by

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Department of Plant Sciences

Submitted in partial fulfillment of the
requirements for the degree of
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ABSTRACT

Rapid elevation in the incubation temperature of maize seedlings leads to the new and(or) enhanced synthesis of a group of heat shock polypeptides (HSPs). The response is: a) rapid (HSP synthesis is detectable within 15 minutes following thermal shifts); b) reversible (the "pre-shift" pattern of polypeptide synthesis is re-established when seedlings are returned to the control temperature); and c) transitory (maintenance of seedlings at the elevated temperature leads to a gradual reduction of HSP production and the establishment of a new pattern of polypeptide synthesis). The synthesis of an apparently identical set of HSPs is noted in maize plumules, mesocotyls, radicles and young leaves.

In vitro translational analyses indicate that the HSPs represent the products from translation of polyadenylated messenger RNAs. Post-transcriptional and post-translational regulatory mechanisms may determine the final array of polypeptides which are produced.

In maize, changes in the synthesis of polypeptides and their mRNAs appear to represent a normal response not only to heat shock but to any thermal shift; the array of gene products and the degree to which they are synthesized are determined by several factors including: a) the initial growing temperature; b) the temperature shift increment; c) the temperature regime within which the shift is carried

out; d) the rate of increase of seedling temperature; and e) the duration of the temperature treatment.

While HSP synthesis in maize is analogous to the response observed in other species, maize HSPs exhibit different degrees of immunological relatedness to HSPs from other organisms. High molecular weight HSPs from maize, soybean and pea exhibit similar degrees of cross-reactivity with antibodies to maize 73-89 kD HSPs. Antibodies to maize 18 kD HSPs react strongly with maize 18 kD HSPs, to a lesser extent with soybean 18 kD HSPs, and very little with 17 kD HSPs from pea. Quail, mouse and tadpole HSPs do not react with antibodies to maize HSPs. Thus, while the synthesis of polypeptides in response to thermal shifts or stresses may be universal, differences exist in the classes of polypeptides which are synthesized, and in the degree of relatedness of polypeptides of apparently similar size from different species.

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TABLE OF CONTENTS

	Page
CERTIFICATE OF EXAMINATION	ii
ABSTRACT	iii
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
LIST OF TABLES	x
LIST OF FIGURES	xi
LIST OF APPENDICES	xiv
ABBREVIATIONS	xv
CHAPTER 1 - INTRODUCTION TO THE STUDY OF ENVIRONMENTAL STRESS RESPONSES	1
1.1 General Introduction	1
1.2 Temperature as a Biological Stress Factor	3
1.3 High Temperature Stress and Heat Shock	5
1.3.1 High Temperature Stress	5
1.3.2 Heat Shock in Animal Systems	9
1.3.3 Heat Shock in Plant and Fungal Systems	21
1.4 Maize as a Valuable System for the Study of Environmental Stress Responses	26
1.5 Proposed Research and Thesis Objectives	29
CHAPTER 2 - RESPONSE OF MAIZE TO HEAT SHOCK AND SYNTHESIS OF HEAT SHOCK PROTEINS	32
2.1 Introduction	32
2.2 Materials and Methods	33
2.2.1 Germination of Seeds and Growth of Seedlings	33
2.2.2 Temperature Treatments	33
2.2.3 Incorporation of Radioactive Precursors and Extraction of Polypeptides From Maize tissues	35
2.2.3.1 Labelling and Extraction of Polypeptides From Batch Tissue	35
2.2.3.2 Labelling and Extraction of Polypeptides From Tissues of Individual Seedlings	36
2.2.4 Determination of Protein Content and Incorporated Radioactivity of Tissue extracts	37
2.2.5 One-dimensional SDS-PAGE Separations of Polypeptides	37
2.2.6 Two-dimensional IEF-SDS PAGE separations of Polypeptides	39
2.2.7 Visualization of Electrophoretically Separated Polypeptides	40
2.2.7.1 Coomasie Brilliant Blue R-250 Staining	40
2.2.7.2 Fluorographic Detection of Newly Synthesized Polypeptides	40
2.2.7.3 Photography of Stained Gels and Fluorograms	41
2.3 Results	42
2.3.1 Label Incorporation into Proteins as a Function of Heat Shock Temperature	42

2.3.2 Comparison of One-dimensional SDS-PAGE Separated polypeptides From Heat-Shocked Seedlings..... 42
2.3.3 Comparison of Two-dimensional IEF-SDS-PAGE Separated Polypeptides Synthesized in Heat Shock Seedlings..... 47
2.3.4 Kinetic Properties of HSP Synthesis..... 51
2.3.4.1 Time of Initiation of Maize HSP Synthesis Following Heat Shock..... 51
2.3.4.2 Quantification of HSP Synthesis at Control and Heat Shock Temperatures..... 51
2.3.4.3 Recovery in Protein Synthetic Patterns Following Heat Shock..... 55
2.3.4.4 Synthesis of HSPs During Prolonged Incubation at Heat Shock Temperatures..... 59
2.3.5 Polypeptide Synthesis in Pooled Tissue From Several Seedlings Compared to Individual Seedlings..... 62
2.3.6 Comparison of Different Radioactive Amino Acid Precursors for Labelling Newly Synthesized Polypeptides in Maize..... 62
2.3.7 HSP Synthesis in Different Tissues of Maize... 67
2.4 Discussion..... 70

CHAPTER 3 - *IN VITRO* TRANSLATIONAL ANALYSIS OF RNA FROM CONTROL AND HEAT-SHOCKED SEEDLINGS..... 78
3.1 Introduction..... 78
3.2 Materials and Methods..... 79
3.2.1 Isolation of RNA From Maize Seedlings..... 79
3.2.2 Fractionation of RNA by Oligo(dT)-Cellulose Chromatography..... 80
3.2.3 *In Vitro* Translation of Maize RNA and the Determination of Incorporated Radioactivity into Newly Translated Products..... 81
3.2.3.1 Rabbit Reticulocyte Lysate System..... 81
3.2.3.2 Wheat Germ Extract System..... 82
3.2.4 Determination of Optimum Conditions for the *In Vitro* Translation of Maize Plumule RNA..... 83
3.2.5 One- and Two-dimensional PAGE Separations of the *In Vitro* Translated Products..... 84
3.3 Results..... 84
3.3.1 Establishment of Optimal Conditions for *In Vitro* Translation of Maize RNA..... 84
3.3.2 One-dimensional SDS-PAGE Analysis of the *In Vitro* Translation Products From Control and Heat-Shocked Seedlings..... 85
3.3.3 Two-dimensional IEF-SDS-PAGE Analysis of the *In Vitro* Translation Products From Control and Heat-Shocked Seedlings..... 89
3.3.3.1 Comparison of the Polypeptides Synthesized *In Vitro* and *In Vivo*..... 89
3.3.3.2 Comparison of the *In Vitro* Translations of Total RNA and Poly(A)+ mRNA..... 97
3.4 Discussion..... 97

CHAPTER 4 - POLYPEPTIDE SYNTHESIS IN MAIZE SEEDLINGS SUBJECTED TO A SERIES OF TEMPERATURE SHIFT REGIMES..... 108
4.1 Introduction..... 108
4.2 Materials and Methods..... 109
4.2.1 Growth of Seedlings..... 109
4.2.2 Temperature Treatments and Determination of Internal Seedling Temperatures..... 109
4.2.3 Polypeptide Extraction and Determination of Label Incorporation into Newly Synthesized Polypeptides..... 110
4.2.4 Isolation and In Vitro Translation of RNA from Seedlings Following Temperature Shifts or Heat Shock..... 111
4.2.5 One- and Two-dimensional PAGE Analysis of the Polypeptides Synthesized Under Different Temperature Shift Regimes..... 111
4.3 Results..... 112
4.3.1 Determination of Internal Seedling Temperatures Under Different Growth and Temperature Shift Conditions..... 112
4.3.2 Label Incorporation and One-dimensional SDS-PAGE Analysis of Polypeptides Synthesized at Different Growing Temperatures..... 116
4.3.3 Examination of Polypeptide Synthesis Following Different Temperature Shift Regimes.. 119
4.3.3.1 One-dimensional SDS-PAGE Comparison of Polypeptides Synthesized Following Shifts Within the Heat Shock Temperature Range.... 119
4.3.3.2 One-dimensional SDS-PAGE Comparison of Polypeptides Following a Range of Temperature Shifts..... 122
4.3.3.3 Two-dimensional IEF-SDS-PAGE Comparison of In Vivo and In Vitro Synthesized Polypeptides Following Temperature Shifts and Heat Shock..... 125
4.4 Discussion..... 131

CHAPTER 5 - IMMUNOCHEMICAL ANALYSIS OF THE HEAT SHOCK RESPONSE IN MAIZE..... 140
5.1 Introduction..... 140
5.2 Materials and Methods..... 141
5.2.1 Antigen Purification and Purity Monitoring..... 141
5.2.2 Injection of Rabbits, Boosting and Blood Collection..... 148
5.2.3 Characterization of Antisera..... 149
5.2.3.1 Identification of Reacting Polyclonal Antibodies..... 149
5.2.3.2 Identification of Maize HSP-specific Antibodies..... 153
5.3 Results..... 155
5.3.1 Purification of Maize Heat Shock Polypeptides.. 155
5.3.2 Characterization of Antisera..... 160

5.3.2.1 Identification of Reacting Polyclonal Antibodies..... 160
5.3.2.2 Identification of Maize-specific Antibodies..... 167
5.3.2.3 Immunological Detection of Time of Initiation of HSP Synthesis Following Thermal Shifts..... 171
5.3.3 Examination of the Immunological Relatedness of HSPs From Various Sources..... 175
5.3.3.1 Immunological Relatedness of 18 kD HSPs From Different Tissues of Maize..... 175
5.3.3.2 Immunological Relatedness of HSPs From Different Species..... 178
5.4 Discussion..... 186

CONCLUSIONS..... 193
APPENDICES..... 198
APPENDIX 1. Standard Curves Used For Determinations of Molecular Masses of Polypeptides Resolved by Polyacrylamide Gel Electrophoresis..... 200
APPENDIX 2. Standard Curves Used For Determinations of Isoelectric Points of Polypeptides Resolved by Isoelectric Focussing..... 202

BIBLIOGRAPHY..... 203
VITA..... 227

LIST OF TABLES

Table	Description	Page
I	Kinetics of maize HSP synthesis.	54
II	Quantification of polypeptide synthesis at at control and heat shock temperatures.	56
III	Optimal conditions for *in vitro* translation of maize messenger RNAs.	86
IV	Maize plumule growth rate at different temperatures.	115
V	Intensity of HSP synthesis as a function of temperature shift increment.	135
VI	Reactivity of antibodies to maize HSPs with polypeptides from different plant and animal species.	185

LIST OF FIGURES

Figure	Description	Page
1	Plot of 14C-leucine incorporation into proteins from maize plumules at 27C and following a one hour heat shock at various temperatures.	44
2	Coomasie blue-staining and fluorographic images of 1-D SDS-PAGE separations of polypeptides from maize plumules at 27C and following heat shock at various temperatures.	46
3	Fluorograms of 2-D IEF-SDS-PAGE separations of polypeptides from maize plumules at 27C and following heat shock at 35, 41 and 44C.	49
4	Fluorograms of 1-D SDS-PAGE separations of polypeptides isolated from maize plumules at various times following a shift from 27C to 41C.	53
5	Fluorograms of 1-D SDS-PAGE separations of polypeptides from maize plumules during recovery from a one hour heat shock at 41C or 44C.	58
6	Fluorogram of 1-D SDS-PAGE separations of polypeptides isolated from maize plumules at various times during continued high temperature treatment.	61
7	Fluorograms of 1-D SDS-PAGE separations of polypeptides extracted from the pooled tissues from 20 excised plumules, or from individual intact or excised plumules of control and heat-shocked seedlings.	64
8	Fluorogram of 2-D IEF-SDS-PAGE separations of polypeptides synthesized in maize plumules labelled with different radioactive amino acids under control and heat shock conditions.	66
9	Fluorogram of 2D IEF-SDS-PAGE separations of polypeptides synthesized in different tissues from control and heat-shocked seedlings.	69
10	Plot of 35S-methionine incorporation into newly synthesized translation products as a function of amount of RNA from control or heat-shocked plumules added to the rabbit reticulocyte or wheat germ cell-free translation systems.	88

11	Fluorograms of 1-D SDS-PAGE separations of products from *in vitro* translations of RNAs from control or heat-shocked plumules in the rabbit reticulocyte and wheat germ translation systems.	91
12	Fluorograms of 2-D IEF-SDS-PAGE separations of polypeptides synthesized *in vivo* and the products derived from *in vitro* translation of RNA from control and heat-shocked plumules.	94
I3	Comparison of isoelectric point determinations conducted on electrophoretically-separated and fluorographically revealed polypeptides synthesized *in vivo* or translated *in vitro*.	96
14	Fluorograms of 2-D IEF-SDS-PAGE separations of products derived from *in vitro* translation of total RNA or poly(A)+ mRNA from control and heat-shocked plumules.	99
15	Plot of incubator temperatures and changes in temperature inside intact plumules following thermal shifts.	114
16	Plot of 35S-methionine incorporation and fluorograms of polypeptides synthesized at different growing temperatures.	118
17	Fluorograms of 1-D SDS-PAGE separations of polypeptides synthesized at 27, 32 or 35C and following various upward temperature shifts.	121
18	Fluorograms of 1-D SDS-PAGE separations of polypeptides synthesized at 15, 20, 25 or 30C, and following 10C upward thermal shifts or shifts to a common heat shock temperature.	124
19	Fluorograms of 2-D IEF-SDS-PAGE separations of polypeptides synthesized *in vivo* or the products from *in vitro* translation of RNA from plumules at 15C or following a shift from 15C to 25C.	128
20	Fluorograms of 2-D IEF-SDS-PAGE separations of RNAs from plumules at 15C or following a shift from 15C to 25C for 0.5, 1, 3 or 6 hours.	130
21	Flowchart of the protocol used for isolation and purification of maize HSPs for use in polyclonal antibody production.	143
22	Fluorograms of 1-D SDS-PAGE separations of the purified preparations of the maize HSP classes.	157

23	Fluorograms of 2-D IEF-SDS-PAGE separations of the purified 73-89 kD and 18 kD HSP classes.	159
24	Ouchterlony assays for detection of reactivity of antibodies to maize HSPs with polypeptides from maize.	163
25	Immunoblots of 1-D SDS-PAGE-resolved polypeptides showing specificity of reaction of antibodies to maize HSPs with maize polypeptides.	165
26	Fluorograms of 1-D SDS-PAGE separations of the products immunoprecipitated from total polypeptide extracts using antibodies to maize HSPs.	170
27	Fluorogram and spectrophotometric scan of the same fluorogram showing the amount of 18 kD HSP immunoprecipitated from total polypeptide extracts of seedlings subjected to heat shock for various lengths of time.	173
28	Fluorograms of 1-D SDS-PAGE separations of total plypeptide extracts from different maize tissues and the products which were immunoprecipitated from the same tissues using antibodies to maize 18 kD HSPs.	177
29	Immunoblots of 1-D SDS-PAGE separations of polypeptides from maize, soybean and pea tissues which were reacted with antibodies to maize HSPs.	180
30	Fluorograms of 1-D SDS-PAGE separations of the products immunoprecipitated from total polypeptide extracts from maize, soybean and pea tissues using antibodies to maize HSPs.	184

LIST OF APPENDICES

Appendix		Page
APPENDIX I	Plots of relative mobilities of standard proteins used for molecular mass determinations of SDS-PAGE-resolved polypeptides.	200
APPENDIX II	Plots of pH value distributions in tube gels following isoelectric focussing in the presence of different ampholine mixtures.	202

ABBREVIATIONS

A260 or A280	absorbance at 260 or 280 nanometers
ANP	anaerobic protein or polypeptide
BBS	borate buffered saline
BSA	bovine serum albumin
°C or C	Celsius
CFA	complete Freund's adjuvant
Ci	Curie
cpm	counts per minute
cv.	cultivar
dT	deoxythymidilic
EDTA	ethylenediamine tetraacetic acid
GAR	goat anti-rabbit
GBq	gigabecquerel
hnRNP	heterogeneous nuclear ribonucleoprotein
HRP	horseradish peroxidase
HSP	heat shock protein or polypeptide
IEF	isoelectric focussing
IgG	gamma globulin
K+	potassium ion
kD	kilodalton
mA	milliampere
Mg++	magnesium ion
mL or uL	millilitre or microlitre
Mr	molecular mass

NaCl	sodium chloride
NCS	n-chlorosuccinimide
NP-40	Nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pI	isoelectric point
PMSF	phenylmethylsulfonylfluoride
poly(A)+	polyadenylated
poly(A)-	non-polyadenylated
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TBS	Tris buffered saline
Tris	tris-hydroxymethylaminomethane
1-D	one-dimensional
2-D	two-dimensional

CHAPTER 1

INTRODUCTION TO THE STUDY OF ENVIRONMENTAL STRESS RESPONSES

1.1 General Introduction

The concept of stress has broad applications and as such forms the basis for investigations in many disciplines. While the term "stress" can be defined in mechanics (an external force which imposes a state of strain onto a body) such exact physical terminology is not completely applicable to biological systems (Levitt, 1980). Furthermore, the medical concept of stress differs substantially from both the physical and biological concepts (Selye, 1973; Levitt, 1980).

In recent years, the term stress has been used in biological terms to describe "any environmental factor potentially unfavourable to living organisms" (Levitt, 1980), where the environment includes any natural and(or) artificial conditions which the organism may be forced to encounter. It should be emphasized that the term "unfavourable" presupposes that the organism will somehow respond to the imposed conditions. In order to study the

effects of stress, either the stress or the response (or both) should be measurable. This task may often be arduous, since biological systems are not governed strictly by physical laws. Present technology however facilitates the observation and, in many cases, the measurement of the responses of living organisms to environmental stress conditions.

When an organism is subjected to unfavourable environmental conditions, it can respond in one of several ways: a) it can move to an alternate location and thus alleviate the stress; b) it can remain under the present conditions and experience injury or death; or c) it can remain in its present location but undergo external and(or) internal changes which allow it to either tolerate or adapt to the adverse conditions. While all three may be broadly categorized as responses to stress, the third alternative requires that the organism deal with the stress condition, possibly in order to avoid injury or death. In many cases, this form of response involves changes in basic intracellular processes (eg., respiration, gene induction and regulation, protein synthesis, etc.) and so from a biological and genetical point of view, merits investigation. As will become clearer from the literature review which follows, a large variety of external stimuli or stresses lead to changes in gene activity which then directly or indirectly influence other cellular processes. Thus, studies in this area could provide some insights into

the genetic basis for stress response. In addition, an understanding of the molecular events accompanying the responses to stress (and the observed tolerance to some stress conditions), could provide a basis for the selection of superior strains capable of tolerating and possibly even thriving under conditions previously described as unfavourable. The implications of such selection in commercially or agronomically important species are obvious.

1.2 Temperature as a biological stress factor.

In terms of basic requirements for growth and development of organisms, temperature plays a critical role since it governs the rate of most metabolic processes. For example, oxygen consumption and respiration rates in animals increase approximately two- to three-fold following a 10C increase in temperature (Schmidt-Nielsen, 1975); plants exhibit a three-fold increase in transpiration rates following a 10C increase (Kimball, 1974); and the nuclear cycle in maize is approximately four times longer at 20C than at 35C (Verma, 1972; Verma and Lin, 1978). Since DNA, RNA, and protein syntheses occur during specific phases of the nuclear cycle, the rates of these processes will also be influenced by temperature. Enzyme reactions require the interaction of molecules. At higher temperatures, the movement of molecules is accelerated such that the probability of collision, and hence reaction, increases (Lehninger, 1975). The rate of virtually every cellular

process exhibits a dependence on temperature. In poikilotherms, this temperature influence is manifested rapidly in every cell of the organism and leads to a measurable alteration in not only the rates of intracellular processes but in the rate at which the whole organism functions. As the temperature decreases for example, most poikilothermic animals become more and more inactive (Schmidt-Nielsen, 1975). Thus a cellular response can induce a physiological effect at the whole organism level.

Scientific studies examining the effect of temperature stress were reported over a century ago (eg. Sachs (1864) and de Vries (1870); as in Levitt, 1980). Studies on the tolerance to temperature extremes have been most prevalent (Levitt, 1980). The potential stress conditions that an organism may encounter include high temperature conditions and low temperature conditions; the latter encompasses both chilling and freezing stress. The terms high and low are relative since, in nature, there are certain thermophilic bacteria which can grow at temperatures exceeding 90C (Brock, 1967; Brock and Darland, 1970), and certain aquatic species that survive well below the normal freezing point of water (Schmidt-Nielsen, 1975). In general, organisms may be classified on the basis of the temperature ranges in which they thrive (psychrophiles, 0 to 20C; mesophiles, 10 to 30C; thermophiles, 30 to 100C)(Levitt, 1980). Stress conditions represent deviations from the normal growing range for a particular organism.

1.3 High temperature stress and "heat shock".

1.3.1 High temperature stress.

While certain thermophilic micro-organisms can thrive under extremely high temperature conditions, few other organisms are capable of carrying out a complete life cycle at temperatures above approximately 50C (Brock, 1970; Schmidt-Nielsen, 1975). Certain dormant phases of an organism's life cycle however (eg., seeds, dry mosses, fly larvae), may survive hyperthermic conditions that would otherwise be lethal to the organism (Schneider-Orelli, 1910, Groves, 1917, and Noerr, 1974; in Levitt, 1980; Schmidt-Nielsen, 1975). The important component here appears to be moisture content since tissues in the dehydrated state can tolerate more severe temperature treatments than their hydrated counterparts (Levitt, 1980). Just (1877) demonstrated that dried seeds can survive boiling in water for several hours, as long as they don't imbibe water during this treatment (Levitt, 1980). The killing temperature for maize caryopses with a moisture content of 10% or less exceeded 80C, while seeds with a 75% moisture content were killed at 40C (Robbins and Petsch, 1932). Certain fly larvae can survive temperatures exceeding 100C if first dehydrated (Hinton, 1960).

Another critical factor which determines whether or not a high temperature is perceived as a stress is the exposure time, which varies inversely with the heat-killing temperature (Levitt, 1980). If a treatment is of a brief

enough duration, it may never be perceived as a stress and no changes in the organism would be detected. Presumably, each organism will be able to tolerate a specific length of time at a specific temperature. For example, while a temperature treatment of less than two minutes at 60C is sufficient to kill Tradescantia plants, treatment at 40C requires more than 12 hours before death ensues (Collander, 1924, in Levitt, 1980). Studies have also revealed that a gradual rise in temperature increases the upper limit for survival (Altschuler and Mascarenhas, 1982). This increased tolerance is most likely due to gradual acclimation to the increasing temperatures.

In studying cellular responses to high temperature stress, one must consider a) the physical and chemical properties of cells and the molecules present in these cells, and b) how these properties may be influenced by thermal energy changes. One must also be able to distinguish between a cellular response resulting from the primary stress (heat) and that which is due to a secondary manifestation of the primary stress (Levitt, 1980). The most common form of secondary heat-induced stress is drought or desiccation where water loss due to heat leads to intracellular changes. While this may not be a problem for most animals, the moisture content of plants is very susceptible to temperature fluctuations and injury due to desiccation often occurs following prolonged high temperature stress (Krans and Johnson, 1974). The increased

transpiration rates at elevated temperatures result in more rapid water loss from leaf surfaces (Levitt, 1980).

Primary high temperature stress (ie., where heat itself is the inducer of intracellular changes or injury) can directly or indirectly lead to alterations in normal cell function. In plants, high temperature treatments can lead to growth inhibition (Pulgar and Laude, 1974); this has been suggested as a possible protective mechanism to prevent serious high temperature injury (Levitt, 1980). Nutrient assimilation may also be reduced at these higher temperatures (Levitt, 1980). In both plants and animals, high temperature stress can lead to respiratory disturbances resulting in carbon dioxide or oxygen toxicity (Schmidt-Nielsen, 1975; Levitt, 1980). Denaturation of many proteins occurs above 45C (Schmidt-Nielsen, 1975), and although this may not lead to death of the organism, it may dramatically alter cell structure (if the protein is structural) or certain biochemical processes (if the protein is an enzyme). Alternatively, temperature-sensitive enzymes may become inactivated in the absence of denaturation, or heat-induced biochemical lesions may result in altered accumulation of intermediates necessary for growth (eg., vitamins, cofactors) (Kurtz, 1958; Schmidt-Nielsen, 1975; Levitt, 1980). Nucleic acids may also be denatured by heat (Peacocke and Walker, 1962) and proteins, nucleic acids and sugars can be degraded at higher temperatures due to increased activity of hydrolytic enzymes (Lehninger, 1975;

Levitt, 1980). In addition, lipids in membranes may undergo phase changes resulting in alterations in the permeability to ions and molecules; high temperatures increase this permeability and lead to extensive leakage of cellular metabolites (Bernstam and Arndt, 1973).

Many metabolic reactions within a cell are interdependent (as in biosynthetic pathways). The rate of these reactions may exhibit differential temperature optima such that high temperature treatments may lead to the depletion or accumulation of certain intermediates in a metabolic pathway (Schmidt-Nielsen, 1975). The processes of photosynthesis and respiration in plants are also interdependent. Respiration has a higher temperature optimum than photosynthesis such that at elevated temperatures (ie., above the temperature compensation point), plant reserves start to be depleted (Lundegardh, 1949, in Levitt, 1980). Synthetic and degradative pathways are generally interdependent for normal production of a cell component. High temperatures appear to increase the activity of hydrolytic enzymes to the point where the rate of degradation of a product exceeds the rate of its synthesis (Lehninger, 1975).

There is an overwhelming number of papers and monographs devoted to the physiological and ecological aspects of temperature stress in microbial, animal and plant systems. With the advent of improved biochemical, genetical and molecular techniques, it has also become possible to

examine more precisely the effects of temperature and other environmental stress conditions on intracellular molecular processes. As will be demonstrated, the refinement in resolution capabilities (with such techniques as electrophoresis, fluorography, recombinant DNA methods and monoclonal antibody production) has made it possible to detect both subtle and dramatic changes in certain metabolic processes, in the absence of any apparent change(s) at the whole organism level. Studies utilizing these improved techniques have led to the increased awareness that most intracellular processes are directly or indirectly under genetic control and that many environmental stress conditions, including high temperature stress, are capable of inducing gene activity (Adams and Rinne, 1982).

1.3.2 Heat shock in animal systems.

The exploitation of the heat shock phenomenon as a means of studying gene induction and regulation in response to environmental stresses developed from the observations of Ritossa (1962) that a brief and rapid shift in incubation temperature induces a change in the puffing pattern of the polytene chromosomes of Drosophila. These heat-inducible puffs (ranging in number from six to nine depending on the species) occur at specific sites on the chromosome, and may be detected within one minute of the temperature increase. The severity and duration of the heat shock influences the size of the puffs, which increase for approximately 30 to 40

minutes following a shift from 25C to 37C. With the induction of heat shock puffs, which requires RNA synthesis, there is a concomitant regression of pre-existing puffs (Ashburner and Bonner, 1979). It was later discovered that heat shock also led to the new and(or) enhanced synthesis of a characteristic set of polypeptides (Tissieres et al., 1974). These observations have led in turn to extensive investigations into the genetic and molecular events associated with the response to heat shock and other environmental stresses.

The heat shock response has now been documented in a large variety of organisms and as such, has been reviewed in several papers and monographs (Ashburner and Bonner, 1979; Adams and Rinne, 1982; Schlesinger et al., 1982; Tanguay, 1983; Atkinson and Walden, 1984). In all systems studied, a rapid increase in the incubation temperature (of sufficient magnitude) leads to a reduction in synthesis of pre-existing proteins, and the new and(or) enhanced synthesis of a small set of proteins, termed the heat shock proteins (HSPs). These HSPs have molecular masses distributed largely in two molecular weight ranges, one group consisting of 'high' molecular weight polypeptides (60 to 90 kilodaltons (kD)), and a second group of 'low' molecular weight polypeptides (15 to 30 kD) (Kelley and Schlesinger, 1978; Ashburner and Bonner, 1979; Atkinson, 1981; Tanguay and Vincent, 1981; Dean and Atkinson, 1983; Voellmy et al., 1983). Synthesis of HSPs appears to occur

in most tissues of the organisms which have been studied and while the polypeptides appear to be the same in some cases (Tissieres et al., 1974; Currie and White, 1981; Atkinson et al., 1983; Ketola-Pirie and Atkinson, 1983), differences in HSPs among tissues have been detected (Sondermeijer and Lubsen, 1978; Atkinson, 1981; Dean and Atkinson, 1983). In many cases, these differences appear to be the result of charge heterogeneity in some of the polypeptide classes.

While synthesis of the various HSP classes may commence at different times, they are detectable within the first eight to twelve minutes following thermal shift in Drosophila; maximum synthetic rates are achieved after approximately 60 minutes (Ashburner and Bonner, 1979; Lindquist, 1980). HSP synthesis also intensifies and by 60 minutes these proteins represent the major polypeptides being synthesized. In quail myoblasts, synthesis of the HSPs achieves a level comparable to the major structural protein, actin (Atkinson, 1981). In Drosophila, synthesis of HSPs accounts for greater than 50% of the total incorporated precursor (Ashburner and Bonner, 1979). Following six to eight hours of continuous high temperature treatment, the HSPs in Drosophila constitute approximately 10% of the cell's total protein (Moran et al., 1978).

In addition to being rapid, the synthesis of HSPs is also reversible; when cells, tissues, or organism are returned to the initial culture temperature, normal (control) patterns of polypeptide synthesis resume over the

course of several hours (Atkinson, 1981; Roccheri et al., 1981; Bienz and Gurdon, 1982; Ketola-Pirie and Atkinson, 1983).

It has now been clearly established that following heat shock, messenger RNAs are transcribed or made available for translation into the heat shock proteins. Evidence for this comes from in vitro translational analysis of isolated mRNAs (Mckenzie and Meselson, 1977; Kelley et al., 1980; Kruger and Benecke, 1981; Bienz and Gurdon, 1982; Ballinger and Pardue, 1983) and from direct analysis of the RNA or the corresponding cDNA probes (Findly and Pederson, 1981; DiDomenico et al., 1982; Zimmerman et al., 1983). The regulation of mRNA and protein synthesis during heat shock is complex and varies among different species, although it has been most extensively studied in Drosophila. The general observation is that some HSPs (and presumably their RNAs) are produced at low levels in the absence of heat shock and their synthesis is greatly enhanced following a temperature increase (Findly and Pederson, 1981; Tanguay, 1983; Velazquez et al., 1983). Analysis of nuclear RNA isolated prior to heat shock, labelled in vitro with 32P and assayed by hybridization to cDNA probes of the 70 kD and 26 kD heat shock genes, indicates the presence of heat shock mRNAs in nuclei of non-heat-shocked cells (Findly and Pederson, 1981). Low levels of the 70 kD heat shock protein of Drosophila have been detected in non-heat-shocked cells using monoclonal antibodies to this HSP (Velazquez et al.,

1983). Zimmerman and co-workers have recently shown that mRNAs for some HSPs accumulate during certain normal developmental stages in the absence of heat shock. This observation will be discussed later in terms of the possible function of the heat shock proteins.

Accompanying the change in the synthesis of polypeptides, is a shift in the distribution of polysomes; the shift involves a breakdown of pre-existing polysomes and the reappearance of new polysomes at a level approximately 30% of the original (McKenzie et al., 1975; Kruger and Benecke, 1981). In yeast, the mRNAs for control polypeptides disappear rapidly from the cells during heat shock and those mRNAs which are retained continue to be translated (Lindquist, 1981). In Drosophila on the other hand, the mRNAs for normal (control) polypeptides are not degraded during the heat shock, but are maintained in the cell and translated following return to control temperatures (Lindquist, 1980). Isolation and analysis of polysomal and post-polysomal poly(A)+ RNA has further revealed that the mRNAs synthesized before heat shock (ie., control messages), are present in polysomes during the heat shock. Thus, additional polysomes containing heat shock messages are assembled during the temperature shift (Kruger and Benecke, 1981). Translation of polysomal mRNAs from heat-shocked cells results in the synthesis of both control and heat shock polypeptides. However, in vivo, the control messages present in polysomes assembled during heat shock are not

translated efficiently, implying translational control of HSP synthesis in these Drosophila cells (Kruger and Benecke, 1981). Translation of the RNAs in homologous in vitro cell-free systems derived from either control or heat-shocked cells, has revealed that translational signals or factors are present in heat-shocked cells which direct the preferential translation of mRNAs for the heat shock proteins (Storti et al., 1980; Scott and Pardue, 1981; Kruger and Benecke, 1981). These signals or factors are most likely associated with the ribosomes or polysomes, as the addition of a 160,000xg supernatant fraction from the cytoplasm of heat-shocked cells does not alter the results obtained in the in vitro translations (Kruger and Benecke, 1981).

Further evidence that heat shock protein synthesis may be controlled at the translational level comes from experiments in which Xenopus oocytes were enucleated prior to heat shock. The levels of HSP synthesis observed were comparable to those found in oocytes containing nuclei (Bienz and Gurdon, 1982). It is conceivable however, that the HSPs are transcribed from the mitochondrial genome, or that enucleation induces a stress which leads to synthesis of these polypeptides. Many other physical and chemical agents are known to induce HSPs in a variety of organisms(see summary in Ashburner and Bonner, 1979; Tanguay, 1983).

While control of the heat shock response in Xenopus may

be largely translational, the response in Drosophila (and probably other species) is controlled at both the transcriptional and translational levels. In addition to the in vitro translational studies described previously, evidence for translational control in Drosophila is also supported by experiments which demonstrate that: a) addition of Actinomycin D to block RNA synthesis prior to temperature elevation, prevents HSP synthesis (transcriptional control) (Lindquist, 1980); b) elongation rates of polysomes containing control messages are specifically reduced while the elongation rates of those polysomes containing heat shock messages appear to be enhanced following heat shock (translational control) (Ballinger and Pardue, 1983); c) the rates of induction and intensification of messenger RNAs for the 70 kD and 26 kD HSPs of Drosophila are identical suggesting transcriptional co-regulation of the genes for these polypeptides (Findly and Pederson, 1981); and d) the synthesis of HSPs and the heat shock messages is self-regulated (transcriptional and post-transcriptional) (DiDomenico et al., 1982). In this latter study it was demonstrated that the intracellular level of heat shock proteins regulates the rate of synthesis of the mRNAs for these HSPs. If the production of functional HSPs is inhibited by addition of amino acid analogues or protein synthesis inhibitors, transcription of the heat shock mRNAs continues and these transcripts accumulate to high levels. Normally, a specific level of

HSPs must accumulate in these cells before the synthesis of heat shock messages returns to normal levels (DiDomenico et al., 1982). Thus, the level of HSPs regulates the rate and extent of mRNA transcription, which in turn regulates the further synthesis of HSPs. If the HSPs indeed have some biological function (which as yet has not been demonstrated), this method of regulation (essentially a feedback mechanism) would ensure that only the required levels of HSPs are synthesized and maintained in response to high temperature treatments. Based on these findings, Rensing et al. (1982) have generated a mathematical model for the regulation of the heat shock response in Drosophila.

In addition to transcriptional, post-transcriptional and translational regulation of the heat shock reponse, it is also possible that post-translational modifications (eg., methylation, acetylation, phosphorylation) may be operative, although these have not been studied extensively. It has been suggested that inconsisties in estimates of molecular masses of the HSPs may be attributable to some of these modifications (Ashburner and Bonner, 1979).

While the biochemical and molecular mechanisms involved in the heat shock response are becoming well understood, the role or function of the heat shock proteins remains obscure. The initial suggestion, that these HSPs provide protection against thermally-induced cellular injury, has been generally supported by experiments which indicate that organisms exposed to a sub-lethal high temperature are able

to survive a subsequent high temperature treatment which would otherwise be lethal (Mitchell et al., 1979; Loomis and Wheeler, 1980; Dean and Atkinson, 1983). An increased tolerance by pre-treatment at sub-lethal temperatures has also been demonstrated in several plant systems (see next section). Although not shown directly, the acquired thermotolerance following a brief exposure to sub-lethal temperatures appears to require the synthesis of heat shock proteins (Leeper et al., 1977; Landry et al., 1982a, 1982b; Dean and Atkinson, 1983).

Other studies which attempt to address the question of HSP function have focussed on normal cellular processes which appear to be perturbed following heat shock. Histones for example, have been found to undergo synthetic changes following heat treatment in Drosophila. A band which co-migrates with histone H2b exhibits enhanced synthesis while the other core histones undergo a reduction (10-fold in the case of H1) in synthesis (Sanders, 1981; Tanguay et al., 1983). The response of H2b to heat shock is under transcriptional control since Actinomycin D can inhibit the production of H2b as well as the other heat shock proteins. However, H2b is not induced with arsenite as are the HSPs, suggesting that its synthesis is not co-ordinated with the induction of the other HSPs (Tanguay et al., 1983). In addition to changes in synthesis, the post-translational modifications of histone are also altered by heat shock. H3 undergoes decreased methylation while H2b exhibits enhanced

methylation (Camato and Tanguay, 1982), and all four nucleosome core histones show a dramatic reduction in acetylation patterns (Arrigo, 1983) following a temperature increase.

An examination of the structure of heterogeneous nuclear RNA (hnRNA) in Drosophila before and after heat shock has revealed that many of the major heat shock proteins are associated with the poly(A)+ hnRNA as well as mRNP complexes, and appear to play a role as ribonucleoproteins (Kloetzel and Bautz, 1983). Some of the HSPs are also found associated with hnRNPs in low amounts in control cell cultures. This finding supports the observations of low levels of HSPs and their mRNAs in control cells when assessed by cDNA probes (Findly and Pederson, 1981) and monoclonal antibodies (Velazquez et al., 1983). Mayrand and Pederson (1983) have shown that, while hnRNA synthesis continues at high levels during heat shock in Drosophila, the transcripts do not assemble into complete hnRNP particles, presumably due to a reduction in the availability of RNP proteins. Since this assembly is required for normal processing of those mRNAs containing intervening sequences, Mayrand and Pederson (1983) propose that the heat shock mRNAs may utilize an alternate mechanism of nuclear RNA maturation. The majority of the heat shock genes, like the genes for the histones and the interferons, lack intervening sequences (Lifton, 1978, Corces et al., 1980, and Houghton et al., 1981; in Mayrand and Pederson,

1983). Newly made histone mRNAs enter the cytoplasm rapidly during heat shock (Spradling et al., 1977). Interferon production increases rapidly during the stress of viral infection or heat shock, presumably in the absence of hnRNP assembly (Petralli et al., 1965; in Mayrand and Pederson, 1983). Thus, the transcription and processing of mRNAs for the HSPs may take place by a mechanism which is not influenced by temperature. If these HSPs are important in the response to stress (eg., as interferon is for viral infection), this mechanism would ensure that the mRNAs are made available rapidly for translocation into, and translation in the cytoplasm (Mayrand and Pederson, 1983).

Minton and co-workers (1982) have used another approach to assess the role of heat shock proteins. They have examined the interaction of proteins in solution and have found that proteins which are relatively resistant to thermal denaturation or inactivation, will prevent other, thermally-unstable proteins from being denatured during high temperature treatments. By this mechanism, HSPs in the cell may non-specifically stabilize other stress-labile proteins, and allow them to maintain a more or less normal cellular function (Minton et al., 1982). This model has, as yet, not been tested using purified heat shock proteins.

Attempts at intracellular localization of HSPs have shown that following heat shock, some of these proteins are present in nuclear preparations (Mitchell and Lipps, 1975; Arrigo, 1980; Tanguay and Vincent, 1981), others are in the

cytoplasm, and some (for example the 70 kD HSP of Drosophila) are present in both fractions (Arrigo et al., 1980; Tanguay and Vincent, 1982). Translocation studies have revealed that many cellular proteins, including a prominent 45 kD cytoskeletal protein, migrate to the nucleus during high temperature treatment; some of these proteins may serve in the regulation of protein synthesis (Tanguay and Vincent, 1982). During recovery, there is a rapid shift of low molecular weight HSPs from the nucleus back to the cytoplasm (Tanguay and Vincent, 1982). There are however, no consistent patterns of cellular localization of the heat shock proteins among species, making it difficult to relate the cellular distribution of HSPs, to their function during high temperature stress (Tanguay, 1983). A recent study for example, has revealed that mRNAs for three of the seven HSPs in Drosophila (83 kD, 28 kD and 26 kD) accumulate in adult ovarian nurse cells and pass into the oocyte during normal development (ie., in the absence of heat shock) where they persist until blastoderm formation of the embryos. Furthermore, the mRNAs for these three HSPs do not accumulate in the embryos following a heat shock. It has been suggested that the proteins coded for by these mRNAs may be important in regulating gene expression during the stages of oogenesis and early embryo development; it is known that nuclear transcription is arrested during pre-blastoderm embryo formation, such that these HSPs may stabilize the existing control of gene expression until

transcription resumes during subsequent embryo development (Zimmerman et al., 1983). Bensaude and co-workers (1983) have recently shown that polypeptides identical to two of the mouse HSPs are synthesized during the early stages of normal mouse embryo development (in the absence of heat shock).

While the biochemical, genetical and molecular features of the response of animal systems to heat shock are becoming well understood, the role or function of these proteins remains obscure. Studies in plant systems (to be discussed presently) have added support to the concept of thermal protection or adaptation as one potential function of the HSPs. However, additional investigations are required before the process will be totally understood.

1.3.3 Heat shock in plant and fungal systems.

While the heat shock phenomenon in animals has been under investigation for over two decades, the initiation of studies in this area in plant and fungal systems have been much more recent. The first major report on heat shock protein synthesis in plants (soybean and tobacco cells) (Barnett et al., 1980) appeared in the literature approximately four months after the present study on corn was initiated. Since then, there has been considerable documentation from this laboratory and others, on the effects of heat shock in plants (and fungi). These reports have described changes in the patterns of protein synthesis

(Loomis and Wheeler,1980; Baszczynski et al., 1981; Key et al., 1981; Altschuler and Mascarenhas, 1982; Baszczynski et al., 1982a, 1982b; Cooper and Ho, 1983; Kapoor, 1983; Meyer and Chartier,1983; Silver et al., 1983), mRNA synthesis and distribution (Key et al., 1981; Schoffl and Key, 1982; Baszczynski et al., 1982c, 1983a; Kapoor, 1983), and other biochemical and physiological processes (Baszczynski et al., 1982d; Key et al., 1982; Scharf and Nover, 1982; Baszczynski, 1983; Baszczynski et al., 1983b; Bewley et al., 1983; Hadwiger and Wagoner, 1983; Key et al., 1983; Nover et al., 1983; Silver et al., 1983; Baszczynski et al., 1984; Walden et al., 1984).

When plants or plant cell cultures are subjected to a rapid increase in their growing or incubation temperature, they exhibit a rapid change in the pattern of newly synthesized polypeptides. The response to heat shock involves the new or enhanced synthesis of a group of heat shock proteins, analogous to the response in animal systems described in the preceeding section. The HSPs in both plants and fungi generally consist of a group of high molecular weight (70 to 100 kD) and a group of low molecular weight (15 to 30 kD) polypeptides (Barnett et al., 1980; Key et al., 1981; Cooper and Ho, 1983). Two-dimensional polyacrylamide gel electrophoretic separation and fluorographic analysis of polypeptides have shown that this low molecular weight HSP group represents a complex set of newly synthesized products (Key et al., 1981; Meyer and

Chartier, 1983; Silver et al., 1983). Following return to the initial growing temperature, a normal (control) polypeptide synthetic pattern resumes within several hours (Barnett et al., 1980; Key et al., 1981).

The heat shock proteins in plants and the fungi have been shown in several cases to be the translation products of mRNAs which are either newly transcribed, or which exhibit increased availability for translation following heat shock. This finding is based on both in vitro translational studies (Key et al., 1981; Kapoor, 1983) and analyses utilizing cloned cDNAs to the mRNAs for heat shock proteins (Schoffl and Key, 1982). The indication from the latter study is that a) most of the low molecular weight HSPs in soybean are the products of different gene sequences (while a few may represent chemical modifications of other, pre-existing polypeptides), and b) the poly(A)+ mRNAs for these HSPs become highly abundant in the cytoplasm following heat shock (implying increased transcription) (Schoffl and Key, 1982). While extensive studies have not been conducted, existing evidence suggests that transcriptional, translational and post-translational mechanisms of regulation for heat shock mRNA and polypeptide synthesis may be operative in plants (Key et al., 1981; Schoffl and Key, 1982; Feickert et al., 1983).

The role or function of heat shock proteins in plants also remains obscure. However, as a result of the suggested 'protective' function of these HSPs, studies have been

conducted to examine the influence of various temperature shift regimes on the subsequent tolerance of plants to high temperature treatments. It has been shown for example, that while both a rapid shift and a gradual shift from 25C to 40C lead to the synthesis of HSPs, the gradual shift allows continued protein synthesis when temperatures are subsequently raised above 45C. There also appears to be a protection of normal protein synthesis at these higher temperatures (Altschuler and Mascarenhas, 1982). Independent studies on the same system have confirmed these results (Key et al., 1984). This increased maintenance of normal levels of protein synthesis at higher temperatures is accompanied by the increased survival of soybean plants at elevated temperatures (Altschuler and Mascarenhas, 1982; Key et al., 1983). Based on these and other studies in plants, and those in the animal systems described earlier, there is strong indication that one of the functions of HSP synthesis is to provide thermal protection against otherwise lethal temperatures.

Changes in normal cellular processes following heat shock have been examined as a possible means of assessing HSP function in plants and fungi. Silver and co-workers (1983) have noted an increase in the phosphorylation of histones and non-histone nuclear proteins in the fungus Achlya, where they have suggested that chromatin condensation may represent the initial response to heat shock (Silver et al., 1983). Changes in the relative levels

of phosphorylation of several ribosomal proteins have also been observed following heat shock in tomato (Scharf and Nover, 1982).

Resistance to fungal infection in peas appears to require the synthesis and accumulation of a group of 20 or more 'resistance' proteins (Hadwiger and Wagoner, 1983). If pea tissue is heat-shocked prior to fungal infection, the mRNAs for these resistance proteins fail to accumulate in the cytoplasm and infection ensues. If the resistance response is initiated prior to the heat shock, normal fungal resistance is observed (Hadwiger and Wagoner, 1983). It is not clear however, whether the HSPs are directly involved in this process.

A few studies in plants have also shown differential localization of HSPs and changes in their intracellular distribution during temperature shifts. In soybean, the low molecular weight, 15 to 18 kD HSPs constitute a large proportion of the labelled proteins associated with the nuclear fraction during the heat shock treatment; the 70 and 90 kD HSPs are less abundant in the nucleus (Key et al., 1982). During a chase at 40C, the 15 to 18 kD HSPs remain associated with the nucleus, while during a similar chase at 28C, these HSPs become more uniformly distributed throughout the cell. However, when seedlings are returned to 40C following the chase at 28C, the 15 to 18 kD HSPs migrate back into the nucleus (Key et al., 1982). Utilizing autoradiography coupled with electron microscopy, Nover and

co-workers (1983) have recently shown that a high proportion of the labelled proteins synthesized following heat shock in tomato have been localized in the nucleoli and in granular structures in the cytoplasm. Mitochondria and proplastids exhibited little or no labelling. Approximately half of all the 17 kD HSP synthesized following heat shock in tomato cell cultures appears to accumulate in the 'heat shock granules'(hsg) (Nover et al., 1983). The function of these hsgs is not clear but they provide for a recompartmentalization of proteins within cells, a feature which has been suggested to be essential for the acquisition of thermotolerance (Velazquez et al., 1980; Panguay and Vincent, 1982; Nover et al., 1983).

The number of studies on heat shock in plants is increasing rapidly. While many aspects of the heat shock response appear to be common to plants, fungi and animals, there are many unique features which merit investigations in each kingdom.

1.4 Maize as a valuable system for the study of environmental stress response.

Maize is an important commercial and agronomic crop, and as such, continuous efforts are being made to improve yield. The ability of the plants to grow over a broad range of environmental conditions (including temperature extremes, varying moisture conditions, different soil types, agrichemical treatments) can influence the growth and

development of the plant and therefore the yield. While the biological, ultrastructural or physiological manifestations of many of the stress responses are apparent (Levitt, 1980; Crevecoeur et al., 1983; Stamp et al., 1983; Tal, 1983), the genetic and molecular bases for the observed phenotypic responses are not well understood. Thus, studies designed to ascertain the molecular events which lead to altered gene expression under adverse growing conditions, could prove valuable in the selection of plants or cultivars with increased tolerance to these conditions.

Certain stress factors have been shown to result in altered patterns of gene expression in corn. Following anaerobic treatment of maize roots for example, there is a selective synthesis of alcohol dehydrogenase (Hageman and Flesher, 1960; Sachs and Freeling, 1978) which has been shown to be essential for survival (Schwartz, 1969). In addition, the enhanced synthesis of a set of 20-25 'anaerobic proteins' (ANPs) has been observed in roots during anaerobiosis (Sachs et al., 1980). A concommitant decrease in the synthesis of normal (aerobic) polypeptides is also noted under these conditions. Similar patterns in ANP synthesis have been found in several tissues of maize including root, endosperm, scutellum, mesocotyl, coleoptile and anther wall (Okimoto et al., 1980). This response to oxygen deprivation involves changes in the ability of messenger RNAs from these tissues to be translated (Sachs et al., 1980).

Other stress factors that have been shown to result in changes in the expression of gene products in maize include drought conditions, which lead to alterations in water soluble proteins (Botha, 1979), and water stress, which results in minor quantitative changes in the types of polypeptides synthesized under the stress condition (Bewley et al., 1983). Studies have also revealed that auxin treatment of maize coleoptile sections rapidly alters the ability of certain mRNAs to be translated (Zurfluh and Guilfoyle, 1982). Maceration of maize leaf tissue prior to labelling and extraction of polypeptides gives rise to a wound response, as evidenced by changes in the patterns of newly synthesized polypeptides (Boothe et al., 1982). Results from preliminary experiments in this laboratory have shown also that certain agrichemicals may lead to alterations in gene product expression.

Many of these same stress factors have been found to induce changes in gene expression in other plant species. However, there are several reasons why maize is particularly suitable for studies in this area. Aside from its obvious commercial and agronomic importance, one of the principal factors contributing to the overall value of Zea mays L. as a system for biological investigations is its well-defined genetics. This feature allows one to produce and maintain genetically homogeneous populations. In this way, the contributions from population variability (which are prevalent in many studies in animal systems) can be either

controlled or eliminated. Continuing studies in this laboratory for example, demonstrate that different inbred lines of maize and their reciprocal hybrids exhibit differences in the gene products produced under otherwise identical conditions. Furthermore, the genotype of the plant influences to some extent, its subsequent responses to environmental stress conditions.

Other factors which make corn amenable to biological investigations include: a) continuous availability of large numbers of homozygous individuals; b) ease of growth and maintenance of plants; and c) the large array of genetic stocks including polyploids, aneuploids, and stocks carrying chromosomal aberrations or supernumerary chromosomes. In addition, the morphology and developmental biology of maize is well defined (Kiesselbach, 1949; Bonnett, 1953; Scandalios, 1982) and numerous biological, genetical, biochemical and molecular investigations characterizing the corn system have been documented.

1.5 Proposed research and thesis objectives.

The study of gene regulation requires the ability to examine different states of expression of one or more genes, either at the level of primary (messenger RNA) or secondary (protein) gene products, or ultimately at the level of the gene (DNA). While examination of different strains or developmental stages of a species can provide different states of gene expression, studies of this nature can be

greatly facilitated by the presence of an inducible system, one where an externally applied stimulus (physical, biological, chemical) can induce a particular change in the subsequent expression of one or more genes. If the application or duration of the stimulus can be controlled precisely (possible in many cases), its influence can be examined further in different tissues, genotypes and species, as well as at different developmental stages.

Perhaps the best example of an inducible genetic system in eucaryotes is the heat shock system described initially in Drosophila and later in many other animal systems. At the onset of the present study, the heat shock response had not been described in plant systems. In addition, the influence of temperature on gene expression during normal maize development has not been well documented. The present investigation therefore, is both important and timely.

The major objectives of this thesis are: a) to provide an in depth molecular genetical characterization of the heat shock response in maize; and b) to examine the protein and RNA synthetic changes associated with growth of maize at different temperatures, and following temperature shifts within the normal growing range of corn. These investigations are greatly facilitated by the extremely powerful techniques of: c) high resolution one- and two-dimensional polyacrylamide gel electrophoresis for polypeptide separation; d) fluorographic analysis for the detection of newly synthesized polypeptides; e) in vitro

translational analysis for the evaluation of cellular messenger RNA populations; and f) immunochemical methods for protein identification and the examination of structural relatedness. The use of a single inbred cultivar of maize for the majority of the investigations also provides stringent control of genotypic influences.

While some of the data reported below have been published (Baszczynski et al., 1982a, 1983a, 1984), they have not been discussed in the Introduction. I attempt below to integrate all my research and therefore have included (where appropriate and with citation) some of the published data. Collectively, the studies should yield a better understanding of the mechanism of gene expression and regulation in maize under normal temperature regimes, and following high temperature stress conditions such as heat shock.

CHAPTER 2

RESPONSE OF MAIZE TO HEAT SHOCK AND SYNTHESIS OF HEAT SHOCK PROTEINS

2.1 INTRODUCTION

The studies described below present the first in depth analysis of the response of maize to heat shock. One- and two-dimensional gel electrophoretic separations and fluorographic analysis are used to describe and characterize the temperature-dependent changes in the patterns of polypeptide synthesis which accompany heat shock. The times of appearance and rates of enhancement of the heat shock polypeptides, the rates of recovery following heat shock, and the influence of tissue source are examined. Collectively, these studies provide the initial characterization of this response, and form the basis for subsequent investigations into the role of temperature on gene expression during the growth and development of maize.

2.2 MATERIALS AND METHODS

2.2.1 Germination of seeds and growth of seedlings

The present studies were carried out using a single source of the inbred cultivar "Oh43" of Zea mays L. to minimize genetic variability. This material has been maintained at least twenty generations in the University of Western Ontario nursery and by all standard breeding criteria represents an isogenic line. Seeds were lightly dusted with the commercial anti-fungal powder "Vitaflo" (Uniroyal Chemical) and placed 1-2 cm apart between moistened filter papers in either nine or 15 cm Petri plates. The plates were covered with aluminum foil and the seeds were allowed to germinate in the dark at 27C (unless otherwise noted) until the plumules were 1-2 cm long (approximately five days). Radicles of these seedlings were approximately 5-10 cm long. For experiments requiring leaf tissue, seeds of Oh43 were treated with 'Vitaflo', they were positioned 3-5 cm apart in flats containing soil, and the flats were placed in an incubator preset to 25C. Fluorescent plus incandescent illumination was provided at an irradiance of 1000 micro-einsteins m^{-2} sec^{-1} with a 16 hour light/eight hour dark cycle and the soil was watered as necessary. Seedlings were allowed to grow until the third emerged leaf was approximately 15-20 cm long.

2.2.2 Temperature Treatments

When plumules were 1-2 cm long, the Petri plates

containing the seedlings were rapidly transferred from 27C to incubators preset at various temperatures ranging from 27 to 50C for one hour. These initial studies were conducted to determine the temperature range over which heat shock protein synthesis is elicited. Following one hour at 35, 38, 41, 44, 47, or 50C, the Petri plates were returned to 27C, the seedlings were incubated with radioactively-labelled amino acid precursors, and polypeptides were extracted as described in the next section.

A second group of seedlings grown at 27C were shifted to 41C for 15, 30, 45, 60 or 120 minutes prior to labelling at 27C, to provide an estimate of the time required for synthesis of each HSP class.

To measure the rates of recovery in polypeptide synthetic patterns following heat shock, seedlings grown at 27C were shifted to 41C or 44C for one hour and then returned to a 27C incubator. At various times (up to ten hours) seedlings were sampled, plumules were labelled and polypeptides were extracted.

To determine if HSP synthesis persists during prolonged heat shock, seedlings grown at 27C were shifted to and maintained for several hours at 41C. Plumules were labelled for the last two hours of every three hour sampling period and the polypeptides were extracted.

2.2.3 Incorporation of radioactive precursors and extraction of polypeptides from maize tissues.

2.2.3.1 Labelling and extraction of polypeptides from batch tissue.

Following temperature treatments, the terminal 0.5-1.0 cm tips of 20 plumules or the terminal 1-2 cm tips of 20 primary radicles were excised and placed in 1 mL of an aqueous solution containing 33.3 uCi/mL(1 Ci = 37 GBq) of L-[U-14C] leucine (New England Nuclear, 353.0 mCi/mmol). The labelling media containing plumules or radicles were transferred to incubators preset at either 27C or 41C and incubated for two hours with periodic agitation. At the end of the two hours, the tissues were washed quickly and thoroughly to remove excess label, and polypeptides were solubilized in an extraction buffer containing 200 mM Tris-HCl (pH 7.5), 5% SDS (Bio-Rad Laboratories), 7.5% 2-mercaptoethanol and 1 mM phenylmethylsulfonylfluoride (PMSF, Boehringer Mannheim) as described previously (Baszczynski and Hughes, 1981a; Hughes, Baszczynski and Ketola-Pirie, 1981). The tissue was homogenized in two volumes of extraction buffer in a porcelain mortar and pestle, transferred to a glass homogenizer fitted with a teflon pestle, and further homogenized for 60 seconds. The extracts were left on ice for 3-5 minutes; the latter homogenization step was repeated and the extracts were centrifuged in 15 mL Corex tubes at 7700xg for 20 minutes. The supernatants were decanted into 5 mL glass tubes, placed in a boiling water bath for one minute, and either used

directly or stored at -20C until needed.

2.2.3.2 Labelling and extraction of polypeptides from tissues of individual seedlings.

During the course of this investigation, a method was developed to permit the treatment, extraction and electrophoresis of polypeptides from tissues of individual, pedigreed, five-day-old seedlings. Following temperature treatments, single intact or excised plumules or primary radicles, excised mesocotyls, or 1 cm x 1 cm leaf sections were exposed to an aqueous solution of either L-[U-14C] leucine (33.3 uCi/mL; New England Nuclear, 353.0 mCi/mmol) or L-[35S] methionine (100uCi/mL; New England Nuclear, 1042 Ci/mmol) for two hours. In one experiment (see results), plumules were also labelled with either L-[U-14C]-valine (33.3 uCi/mL; New England Nuclear, 250 mCi/mmol) or, L-[U-14C]-lysine (33.3 uCi/mL; New England Nuclear, 300 mCi/mmol) to examine the difference in patterns obtained with different radio-labelled probes. Labelling of intact seedlings was achieved by immersing the terminal 0.5-1.0 cm of either plumule or radicle into the labelling solution in a 1.5 mL microfuge tube. The remainder of the seedling was covered with wetted filter paper to maintain high humidity. At the end of the labelling period, the seedlings were rinsed quickly and the plumule or radicle was excised and homogenized in 100 uL of extraction buffer using a ground glass micro-tissue grinder (Radnoti). The homogenate was transferred to a 400uL microfuge tube and centrifuged at

10,000 rpm for five minutes in a Beckman microfuge (Model B). Supernatants were transferred to a second 400 uL microfuge tube, placed in a boiling water bath for one minute, and used directly or frozen and stored at -20C until needed. Plumules, radicles, mesocotyls, or leaf sections which were excised prior to labelling were extracted in a similar manner.

2.2.4 Determination of protein content and incorporated radioactivity of tissue extracts.

Protein concentrations in the lysates were determined by the turbidimetric assay described by Gomings and Tack (1972), using bovine serum albumin (BSA) as a standard. Samples were prepared for radioactivity determinations (Mans and Novelli,1960) by spotting 5 uL aliquots of sample extract onto glass fibre filter disks (Whatman), soaking the filters in 10% trichloroacetic acid for 15 to 30 minutes, rinsing in two washes of each of 5% trichloroacetic acid, 3:1 ethanol:anhydrous ether, and ether, and drying for about one hour at 50C. The filters were transferred to scintillation vials, 10 mL of a cocktail containing 8g Omnifluor (New England Nuclear) per 1000 mL of "scintanalyzed" toluene (Fisher) was added, and incorporated radioactivity was measured using a Beckman LS-230 scintillation counter.

2.2.5 One-dimensional SDS-PAGE separations of polypeptides.

One-dimensional gel electrophoresis in the presence of

SDS was carried out according to the method described by Laemmli (1970) with modifications. The separating gel consisted of either a 3-15% or a 7.5-17.5% polyacrylamide linear gradient which was allowed to polymerize overnight to ensure gradient uniformity. The next day a 3% polyacrylamide stacking gel was poured on top of the separating gel and allowed to polymerize for 30 to 60 minutes. The tissue extracts were thawed, placed in a boiling water bath for one minute and samples consisting of either a constant amount of protein or a constant amount of radioactivity (25,000 acid-precipitable counts were used routinely) were loaded into preformed wells in the stacking gel. A 5 uL sample of standard proteins from a low molecular weight calibration kit (phosphorylase b, 94,000 daltons (d); albumin, 67,000 d; ovalbumin, 43,000 d; carbonic anhydrase, 30,000 d; trypsin inhibitor, 20,100 d; lactalbumin, 14,400 d; Pharmacia Fine Chemicals) was also added to one well for molecular mass (Mr) determination of separated polypeptides. Bromophenol blue was added either to each well or to the upper buffer reservoir as a tracking dye, and gels were electrophoresed at 5 mA per gel (constant current) overnight and then at 15 mA per gel until the dye front reached the bottom of the gel. At this point, gels consisting of a 3-15% polyacrylamide gradient were removed, and separated polypeptides were visualized as described in section 2.2.7. Gels consisting of a 7.5-17.5% polyacrylamide gradient were electrophoresed for a further

30 to 45 minutes at 15 mA per gel to increase polypeptide separation.

2.2.6 Two-dimensional IEF-SDS-PAGE separations of polypeptides.

Maize polypeptides were separated further by two-dimensional PAGE using the method of O'Farrell (1975) with some modifications. The IEF tube gels contained 2% ampholines (LKB Instruments Inc.) consisting of either a mixture of 80% pH range 5-8 and 20% pH range 3.5-10, or only the pH range 3.5-10 ampholines. To prepare samples for loading on the first dimension IEF gel, 5 mg of solid urea per 10 uL of lysate were added and completely dissolved by vortexing, and samples were mixed with one volume of a solution containing 9.5 M urea, 5% 2-mercaptoethanol and 1 mM PMSF. NP-40 was added to the mixture to give an NP-40:SDS ratio of eight:one, and finally ampholines (pH range 5-8) were added to a final concentration of 2%. Samples were loaded onto pre-run gels and the subsequent focussing schedule consisted of 16-18 hours at 400 volts followed by 1-1.5 hours at 800 volts (constant voltage). The IEF tube gels were extruded, equilibrated in two changes of extraction buffer for ten minutes each, and layed onto the second dimension slab gel which consisted of either a 3-15% or a 7.5-17.5% polyacrylamide linear gradient gel overlayed with a 3% stacking gel. 1% agar in 1% SDS was used to hold the tube gel in place. Running conditions for the second dimension gels were identical to those described

for one-dimensional slab gels. Routinely, 100,000 acid-precipitable counts of lysate were added to the first dimension IEF tube gels. The pH gradients established in the electrophoretically-focussed gels was determined by slicing up a gel and determining the pH of a water extract (Saleem and Atkinson, 1976) or by directly measuring the pH in the intact gels with a pH probe (Bio-Rad Gel Pro-pHiler, Bio-Rad Laboratories).

2.2.7 Visualization of electrophoretically-separated polypeptides.

2.2.7.1 Coomasie brilliant blue R-250 staining.

To visualize the separated polypeptides, the slab gels were removed from the gel moulds following electrophoresis, one corner was notched for identification, and the gels were placed in a 0.2% solution of Coomasie brilliant blue R-250 (Bio-Rad) in 50% methanol:10% acetic acid for 1-2 hours for simultaneous fixation and staining of proteins. Separate pre-fixing prior to staining did not yield any differences. Following staining, the gels were destained over several hours in 50% methanol:10% acetic acid. When most of the background was clear, the gels were transferred to 5% methanol:10% acetic acid for 1 hour and then to 7% acetic acid for storage prior to photography and/or fluorography.

2.2.7.2 Fluorographic detection of newly synthesized polypeptides.

Gels were prepared for fluorography as described by Bonner and Laskey (1974) and dried onto Whatman 3MM filter

paper with a Bio-Rad model 224 gel slab dryer. Fluorographs were prepared by apposing dried gels at -70C to Kodak RP-Royal X-Omat film which was preflashed to an optical density of 0.15 (Laskey and Mills, 1975). The exposed films were developed in Kodak X-ray Developer (five minutes at 21C) and fixed with Kodak X-ray Fixer.

To determine the relative amounts of incorporation of radioactivity into individual polypeptides, regions from dried gels corresponding to bands on the fluorogram were excised, hydrated, and incubated in 0.5 mL of 90% NCS (Amersham Corp.) for three hours at 55C (Liebermann et al, 1980). Radioactivity was determined by scintillation counting of solubilized samples in a cocktail containing 8 g of Omni-Fluor in 1000 mL of toluene and 500 mL of Triton X-100 (New England Nuclear). Blank regions of the gels were used to measure efficiency and background.

2.2.7.3 Photography of stained gels and fluorograms.

Stained gels were photographed on a light box (Graphiclite Model GL-10) using Kodak 2415 Technical Pan film in a 35mm camera equipped with a yellow filter, to maximize contrast and resolution. Fluorograms were photographed on the same set-up but without a filter on the camera. Films were developed for eight minutes at 21C in Kodak HC-110 Developer (diluted 8 mL in 450 mL of water) and fixed in Kodak Fixer. Following washing and drying, the negatives were printed onto Ilford Multigrade paper using

the Ilfospeed Developer and Fixer at manufacturer's recommended dilutions.

2.3 RESULTS

2.3.1 Label incorporation into proteins as a function of heat shock temperature.

The incorporation of 14C-leucine into proteins extracted from plumules which were subjected to a one hour temperature elevation is shown in Figure 1. The graph indicates a deflection point at approximately 44C. A one hour heat shock at any temperature below this point does not markedly alter the incorporation of amino acid precursor. Above 44C, increasing temperature results in a decrease in incorporation such that at 50C, total protein synthesis is reduced to about 15% of the control level.

2.3.2 Comparison of one-dimensional SDS-PAGE-separated polypeptides from heat-shocked seedlings.

The one-dimensional (1-D) electrophoretically-separated polypeptides from plumules subjected to a one hour incubation at 27, 30, 33, 35, 38 41 or 44C are shown in Figure 2. The Coomasie blue-stained gel in Figure 2A indicates excellent resolution of polypeptides with molecular masses between 10 and 100 kilodaltons (kD) and reveals no qualitative differences between samples exposed to the different temperatures. Since samples loaded onto gels contained a similar number of counts, the quantitative

Figure 1. Plot of the incorporation of acid-precipitable counts of 14C-leucine per mg of protein as a function of temperature treatment. The treatment consisted of a one hour incubation of maize plumules at each of the indicated temperatures followed by two hours of labelling at 27C. Values represent the mean of three independent measurements with standard errors not exceeding 7000 cpm/mg protein. Reproduced from Baszczynski et al., 1982a; Can. J. Biochem. 60:569-579.

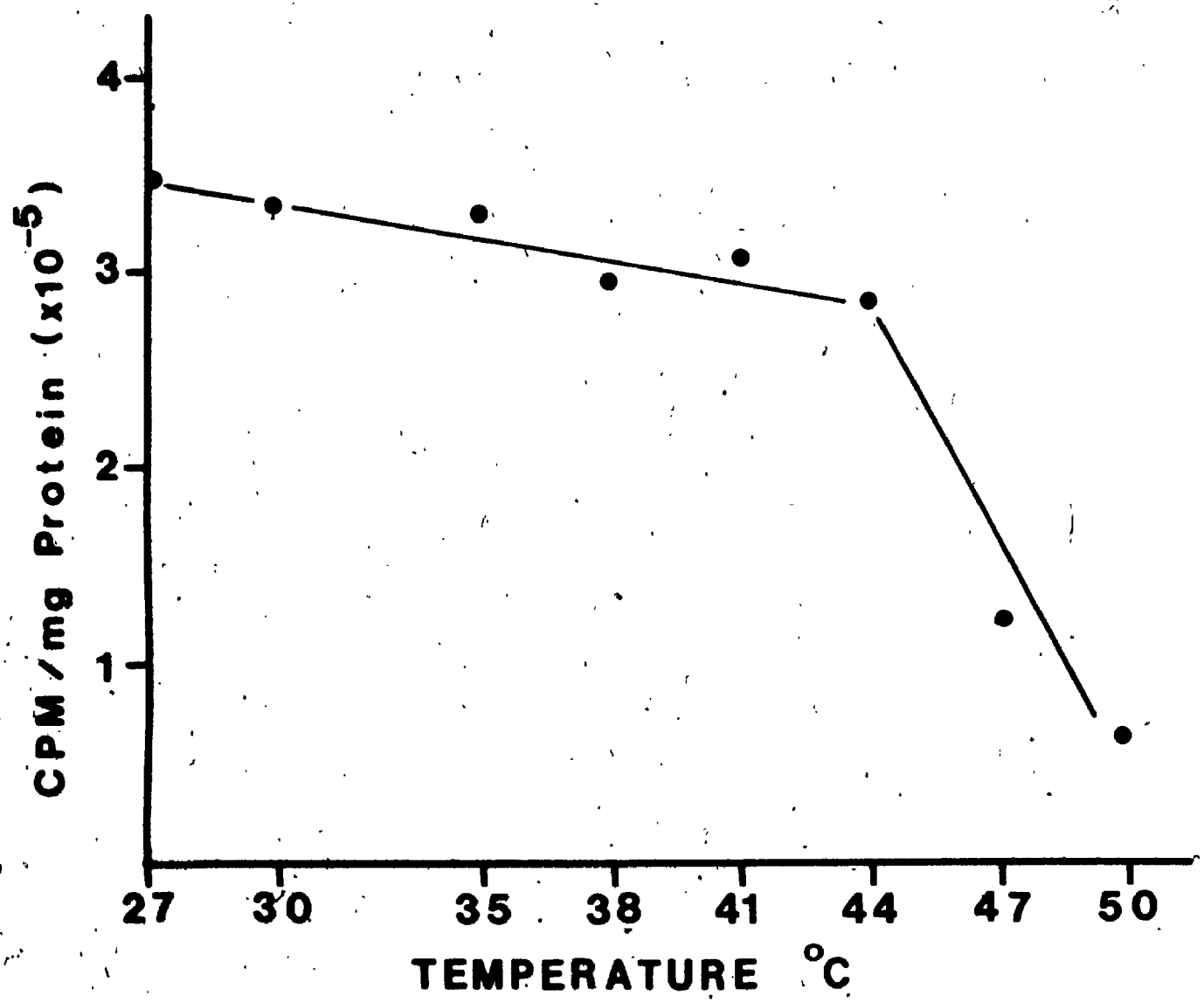
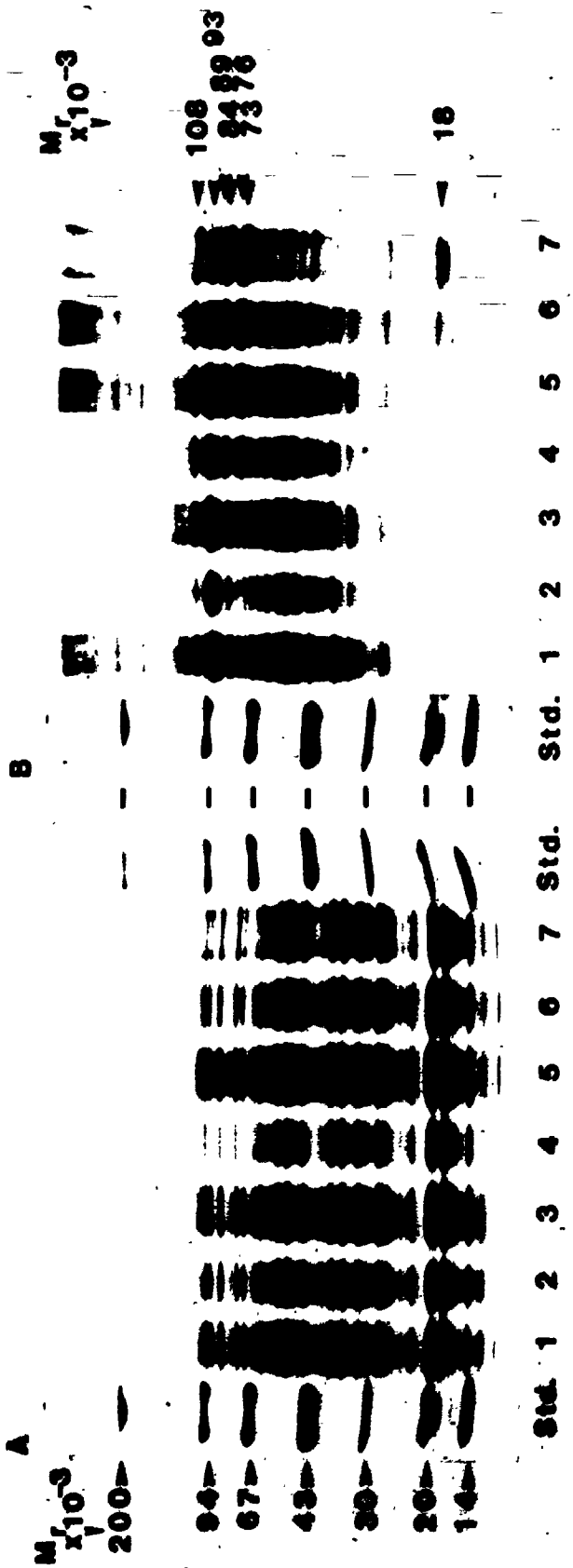

CPM/mg Protein (x10^{-5})
4
3
2
1
27
30
35
38
41
44
47
50
TEMPERATURE °C

Figure 2. SDS-polyacrylamide (3-15% gradient) gel electrophoretic (PAGE) separation of the polypeptides from maize plumules subjected to a one hour heat shock at temperatures ranging from 27C to 44C. A. Coomasie blue-stained gel. B. Fluorogram of newly synthesized polypeptides. Lanes 1 through 7 correspond to a one hour incubation at 27, 30, 33, 35, 38, 41 or 44C prior to labelling at 27C. Approximately 25,000 cpm of acid-precipitable lysate was applied to each well of the gel. Positions of co-electrophoresed molecular mass (Mr) marker proteins are indicated by arrows on the left while positions of the six prominent HSP classes as well as the major 93 kD control polypeptide are indicated on the right. Fluorogram was exposed for five days. Reproduced from Baszczynski, et al., 1982a; Can. J. Biochem. 60:569-579.


A
B
Std.
1
2
3
4
5
6
7
Std.
Std.
1
2
3
4
5
6
7
Std.
$M_r \times 10^{-3}$
200
94
67
43
30
20
14
$M_r \times 10^{-3}$
108
94
80
43
18

differences between lanes are attributable to varying amounts of total protein loaded. The reproducibility of polypeptide patterns between lanes indicates that the temperature treatments are not inducing protein degradation. Figure 2B represents the fluorographic image produced from the gel in Figure 2A and represents those polypeptides which were newly synthesized during the two hour labelling period following the temperature treatments. The array of polypeptides synthesized in five-day-old plumules at the control temperature (27C, lane 1) includes a prominent polypeptide with an Mr of 93 kD. A one hour heat shock at or above 35C results in a dramatic change in the types of polypeptides synthesized including the new or enhanced synthesis of six major polypeptide classes with Mrs of 108, 89, 84, 76, 73 and 18 kD. These "heat shock polypeptides" (HSPs) intensify with increasing temperatures, while other polypeptides show a reduction in label incorporation, especially above 41C. HSP synthesis is also detected above 44C, although total protein synthesis is reduced drastically.

2.3.3 Comparison of two-dimensional IEF-SDS-PAGE-separated polypeptides synthesized in heat-shocked seedlings.

The changes in polypeptide synthetic patterns following heat shock were further analyzed by two-dimensional (2D) electrophoresis, to establish whether the molecular weight HSP classes noted in 1-D gels represented more than one polypeptide (Figure 3). The major spot corresponding to the

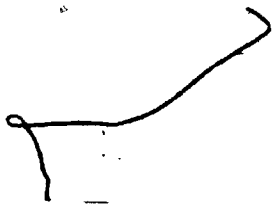
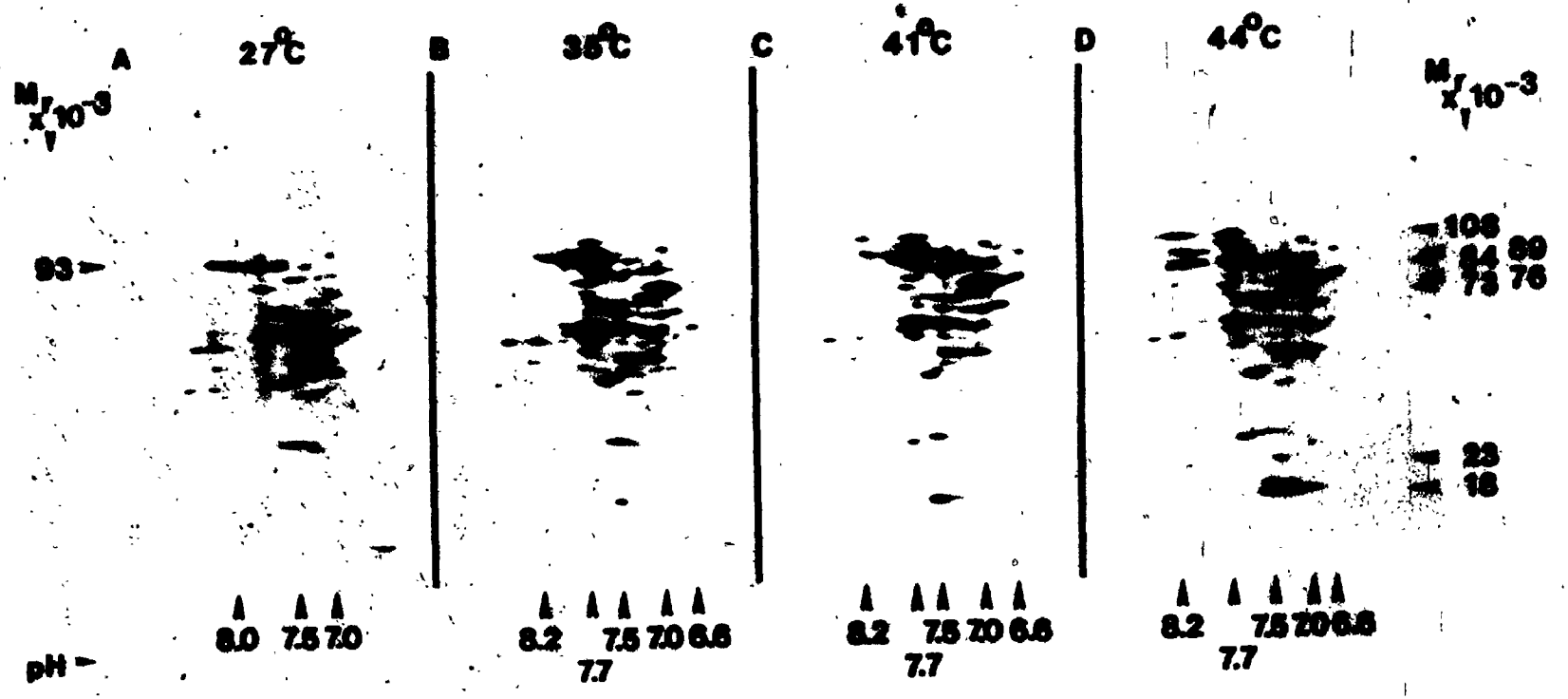


Figure 3. Fluorogram of two-dimensional (2-D) IEF-SDS-PAGE separations of the polypeptides synthesized in maize plumules at 27C (A) or following a one hour heat shock at 35C (B), 41C (C) or 44C (D). The first dimension IEF gel contained pH range 3.5-10 ampholines. The second dimension consisted of a 3-15% polyacrylamide linear gradient slab gel. Approximately 100,000 cpm of acid-precipitable lysate was loaded on each first dimension IEF gel. Positions of HSPs are indicated by arrows on the right while the prominent 93 kD polypeptide in control samples is indicated on the left. Approximate isoelectric points (pIs) are indicated at the bottom. Fluorograms were exposed for five days. Reproduced from Baszczynski et al., 1982a; Can. J. Biochem. 60:569-579.


A
27°C
B
35°C
C
41°C
D
44°C
$M_r \times 10^{-3}$
93
106
80
76
23
18
8.0 7.5 7.0
8.2 7.5 7.0 6.8
7.7
pH

93 kD prominent control polypeptide has an approximate isoelectric point (pI) of 8.0 (panel A). Following a one hour—heat shock at 35C or above, this major spot decreases in intensity accompanied by a temperature-dependent enhancement of new spots corresponding to the HSP Mr classes noted in 1-D gels. The 108 kD HSP is present as a single spot with an approximate pI of 7.7 in the 2-D fluorogram following heat shock at 35C (panel B). A second spot corresponding to this 108 kD HSP class becomes visible and intensifies dramatically following incubation at higher temperatures (panels C and D).

Each of the other Mr classes of HSPs also resolves into more than one isoelectric form when examined on fluorograms of 2-D gels. The 89 kD and 84 kD HSP classes are each resolved into a family of four to six polypeptides with pIs ranging between 7.0 and 7.7. Some of the spots corresponding to the 84 kD class are also noted in controls (panel A) and intensify following heat shock. The 73 kD class exhibits a major spot with a pI of 7.0 while the 18 kD class is resolved into a prominent spot having a pI of 7.5, and less distinct spots with pIs between 7.0 and 7.3. These latter spots are better resolved on gels in which a narrower range of ampholines is used in the first dimension IEF tube gels (shown later). The 2-D gels also revealed enhanced synthesis of an additional polypeptide (approximately 23 kD) following heat shock; this polypeptide was not detected in the previous 1-D gel.

In addition to the observed enhanced synthesis of a select set of polypeptides, there are also polypeptides which show reduced synthesis, and those which do not exhibit any apparent change in their relative rates of synthesis.

2.3.4 Kinetic properties of HSP synthesis.

2.3.4.1 Time of initiation of maize HSP synthesis following heat shock.

The fluorogram in Figure 4 displays the changes in synthesis of the HSPs following 0, 15, 30, 45, 60, or 120 minutes incubation at 41C. Estimates of the interval during which synthesis of each HSP class is first observed are provided in Table 1. The 84 kD HSP class is synthesized at low levels in the controls (Figure 4, lane 1). By 15 minutes at 41C, enhanced or novel synthesis of the 108, 84 and 73 kD classes of polypeptides are observed (lane 2), and the intensity of these increases for at least 120 minutes at 41C (lane 6). The 18 kD HSP class does not appear to be actively synthesized before 60 minutes at 41C. Note also the enhanced synthesis of a 23 kD polypeptide following a 120 minute incubation at 41C. The duration of the temperature treatment clearly affects the level of synthesis of the heat shock polypeptides.

2.3.4.2 Quantification of HSP synthesis at control and heat shock temperatures.

Estimates of the amount of 14C-leucine incorporated into each HSP class following a one hour heat shock at 41C

Figure 4. Fluorogram of a SDS-PAGE (3-15% gradient) separation of the polypeptides synthesized in maize plumules subjected to a temperature shift from 27C to 41C for the times indicated, followed by two hours of labelling at 27C. Positions of standard marker proteins and of the HSPs are indicated by arrows on the left and right respectively. Approximately 25,000 cpm of acid-precipitable lysate was loaded into each well of the gel. Fluorogram was exposed for five days. From Baszczynski et al., 1982a; Can. J. Biochem. 60:569-579.

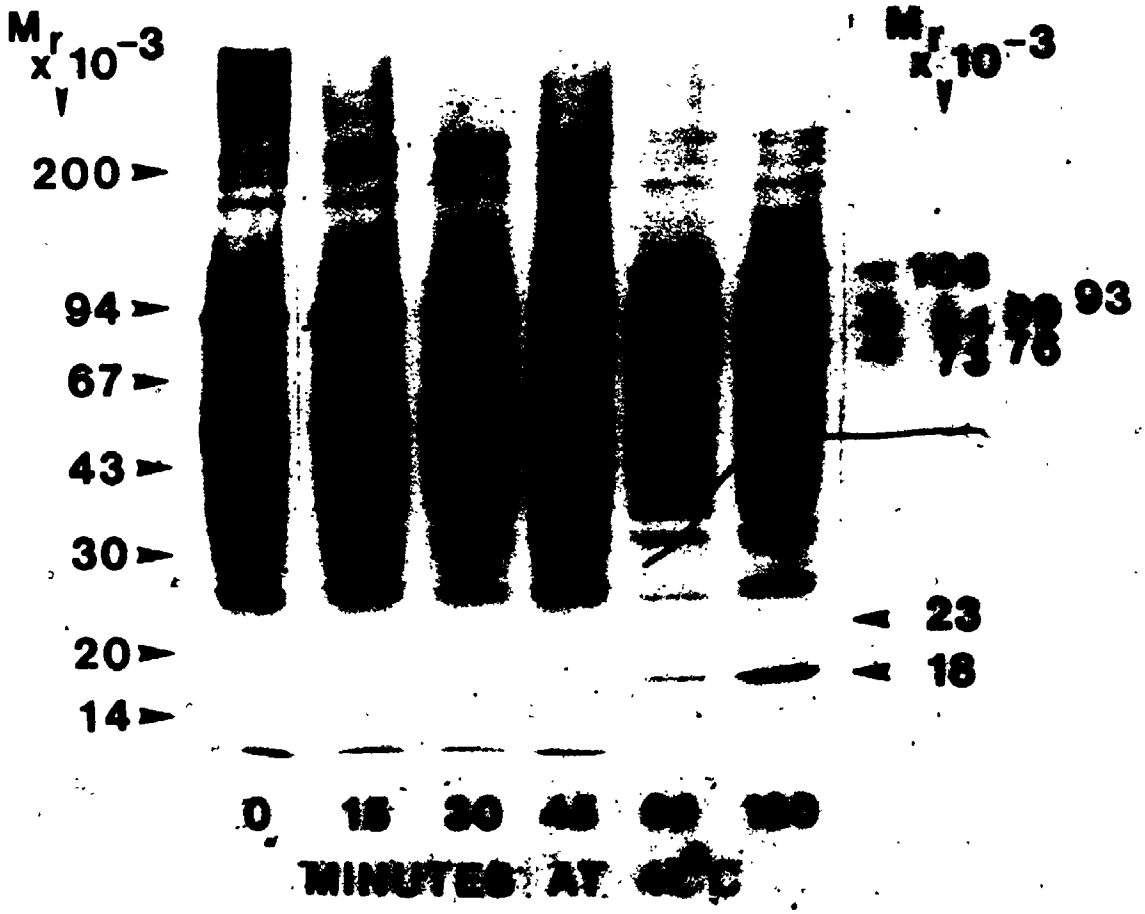

$M_r \times 10^{-3}$
200
94
67
43
30
20
14
$M_r \times 10^{-3}$
93
23
18
0
MINUTES AT

Table I. Time required for appearance of the HSP classes following heat shock and for disappearance of the HSPs during recovery or continued heat shock.

HSP class (kilodaltons)	Minutes required for appearance[a]	Minutes required for disappearance[b]	
		During recovery	During continued heat shock
108	0 - 15	120 - 240	180 - 360
89	45 - 60	30 - 60	540 - 720
84	0	60 - 120	540 - 720
76	30 - 45	60 - 120	720 - 1440
73	0 - 15	60 - 120	720 - 1440
18	45 - 60	30 - 60	180 - 360

a Minutes required for appearance represents an estimate of time interval during which enhanced synthesis of each HSP class becomes detectable. Seedlings were shifted from 27 to 41C for 15, 30, 45, 60 or 120 minutes and then labelled at 27C for two hours. Time zero is measured from when seedlings were first shifted to the 41C incubator.

b. Minutes required for disappearance represents an estimate of the time interval during which synthesis of an HSP class becomes undetectable or returns to the control levels.

and into identical regions of the control (27C) gel are presented in Table 2. In the control gel, the regions corresponding to the HSPs represent approximately 3% of the total incorporated counts. Following heat shock at 41C, these same regions account for about 16% of the incorporated radioactivity representing an approximate five-fold increase in the new or enhanced synthesis of polypeptides in these regions. While these estimates are approximate, they do suggest that HSP synthesis does not increase uniformly for all HSP classes. The 93 kD region of the gel shows a reduction in incorporated radioactivity following heat shock.

2.3.4.3 Recovery in protein synthetic patterns following heat shock.

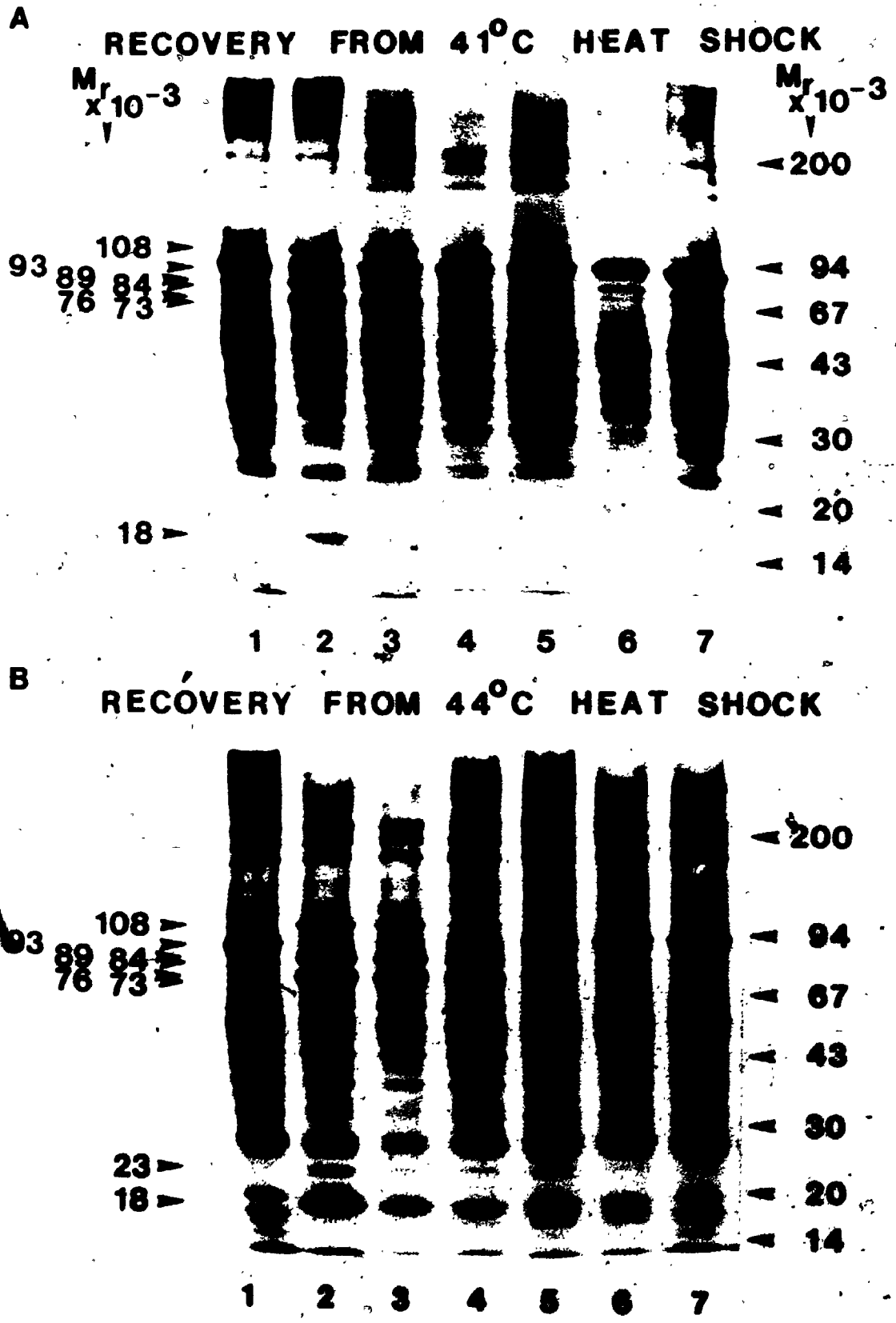
Fluorograms showing the changes in the patterns of polypeptide synthesis during recovery from a one hour heat shock at 41 or 44C are shown in Figures 5A and 5B, respectively. The results indicate that, while recovery is rapid and complete by eight hours at 27C following the heat shock, the rate of disappearance of each HSP class varies, and is also dependent on the heat shock temperature. Plumules subjected to one hour at 41C and placed at 27C for 30 minutes prior to labelling (Figure 5A, lane 3) exhibit both the enhanced synthesis of the 93 kD polypeptide and the decreased synthesis of the six HSP classes observed when plumules are labelled directly following the heat shock (Figure 5A, lane 2). Under the same conditions following

Table II. Quantification of select polypeptides synthesized at control and heat shock temperatures.

Temperature (C)	Polypeptide class (kilodaltons)	CPM incorporated	Percent of total incorporated counts
27	108	45.9	0.2
	93	927.5	3.7
	84-89	91.4	0.4
	73-76	631.1	2.5
	18	90.2	0.4
41	108	120.1	0.5
	93	636.6	2.6
	84-89	814.8	3.3
	73-76	2456.7	9.8
	18	543.5	2.2

In each case, excised tissue was placed at each of the indicated temperatures for one hour prior to labelling with 14C-leucine at 27C. The radioactivity incorporated into each protein (or accompanying region in control gel) is given and also expressed as a percentage of the total acid-precipitable radioactivity (25,000 counts) applied to the gel.

Figure 5. Fluorogram of the 1-D SDS-PAGE (3-15% gradient) separation of the polypeptides synthesized in maize plumules during recovery from a one hour heat shock at 41C (A) or 44C (B). Lane 1 in each case represents the 27C pattern of synthesis. Lane 2 represents extracts from plumules which were labelled for two hours at 27C directly following heat shock at the indicated temperatures. In lanes 3 through 7, seedlings were returned to 27C for 0.5, 1, 2, 4 or 6 hours, respectively, prior to labelling for two hours at 27C. HSPs and standard marker protein positions are indicated by arrows on the left and right, respectively. Approximately 25,000 cpm of acid-precipitable lysate was loaded into each well of the gel. Fluorograms were exposed for five days. Reproduced from Baszczynski et al., 1982a; Can. J. Biochem. 60:569-579.


A
RECOVERY FROM 41°C HEAT SHOCK
Mr x10-3
Mr x10-3
200
108
93
89
84
76
73
94
67
43
30
18
20
14
1 2 3 4 5 6 7
B
RECOVERY FROM 44°C HEAT SHOCK
200
108
93
89
84
76
73
94
67
43
30
23
18
20
14
1 2 3 4 5 6 7

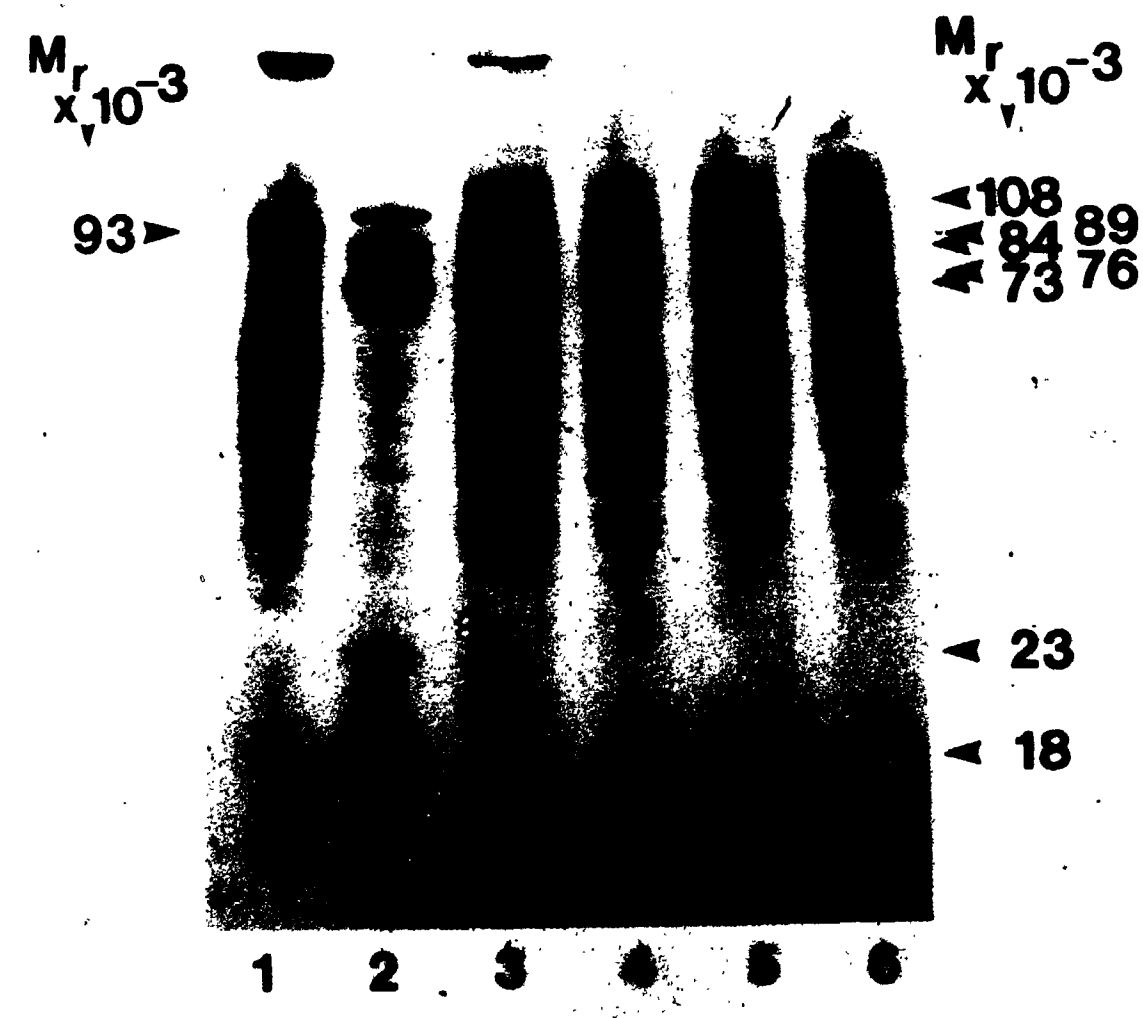
heat shock at 44C, the 93 kD polypeptide does not show increased synthesis until after approximately two hours of recovery at 27C (Figure 5B, lane 5).

The different HSP classes do not exhibit a uniform decrease in synthesis during recovery. Reduced synthesis is noted earlier in the recovery period for the 18 kD and 89 kD polypeptide classes than for the other HSPs. Synthesis of the 84 kD polypeptide persists beyond the eight hour recovery period and as mentioned earlier, this polypeptide is also apparently synthesized at low levels in the control samples (Figures 5A and 5B, lane 1; Figure 2, panel A). Estimates of the time intervals during which HSP synthesis becomes undetectable are summarized in Table 1.

2.3.4.4 Synthesis of HSPs during prolonged incubation at heat shock temperatures.

Seedlings shifted to and maintained at 41C for up to 24 hours were analyzed at three hour intervals to assess whether HSP synthesis continues during prolonged heat shock. As indicated in Table 1 and revealed in Figure 6, the synthesis of HSPs does not persist beyond 24 hours and the rate of disappearance is class specific for each of the six Mr classes of HSPs. There does not appear to be any correlation between the time or rates of appearance and disappearance of the heat shock polypeptides during either recovery or prolonged heat shock.

Figure 6. Fluorogram of a 1-D SDS-PAGE (7.5-17.5% gradient) separation of the polypeptides synthesized in maize plumules during continued incubation at a heat shock temperature (41C). Lane 1 corresponds to extracts from plumules labelled at 27C. Lanes 2 through 6 represent extracts from plumules shifted to 41C for 3, 6, 9, 12 or 24 hours and labelled at 41C for the last two hours of each incubation period. Positions of standard marker proteins are indicated by arrows on the left. Approximately 15,000 cpm of acid-precipitable lysate was loaded into each well of the gel. Fluorogram was exposed for eight days.


M_r x 10^{-3}
93
M_r x 10^{-3}
108
89
84
76
73
23
18
1
2
3
4
5
6

2.3.5 Polypeptide synthesis in pooled tissue from several seedlings compared to individual seedlings.

The patterns of newly synthesized polypeptides obtained from SDS-PAGE separations of extracts from 20 excised plumules or from individual excised or individual intact plumules are compared in Figure 7. While minor quantitative differences were observed, the patterns of polypeptide synthesis were qualitatively identical indicating that: a) the synthetic changes accompanying heat shock can be studied in individual seedlings as well as pooled tissue from a population of seedlings; and b) the newly synthesized polypeptides are not the result of tissue damage induced by excising the plumules.

2.3.6 Comparison of different radioactive amino acid precursors for labelling newly synthesized polypeptides in maize.

To examine the differences associated with using different radioactive amino acids for labelling newly synthesized proteins, plumules (from seedlings grown at 25C or following a one hour shift from 25C to 42C) were incubated in either 14C-leucine, 14C-valine, 14C-lysine, or 35S-methionine and polypeptides were extracted as described previously. Fluorograms of the two-dimensional electrophoretic separations of the polypeptides synthesized in the presence of these precursors are presented in Figure 8. While quantitative and qualitative diffetences are observed between the patterns of newly synthesized polypeptides in control plumules, polypeptides of all six

Figure 7. Fluorogram of the SDS-PAGE (3-15% gradient) separation of polypeptides synthesized in intact or excised plumules from seedlings grown at 27C or following a one hour shift from 27C to 41C. Lanes 1 and 4, extracts from 20 plumules excised prior to labelling; lanes 2 and 5, extracts from single plumules excised prior to labelling; lanes 3 and 6, extracts from single plumules which were intact during labelling. Positions of prominent 93 kD control polypeptide and the HSPs are indicated by arrows. Approximately 25,000 cpm of acid-precipitable lysate was applied to each well of the gel. Fluorograms were exposed for five days. Modified from Baszczynski et al., 1982a; Can. J. Biochem. 60:569-579.

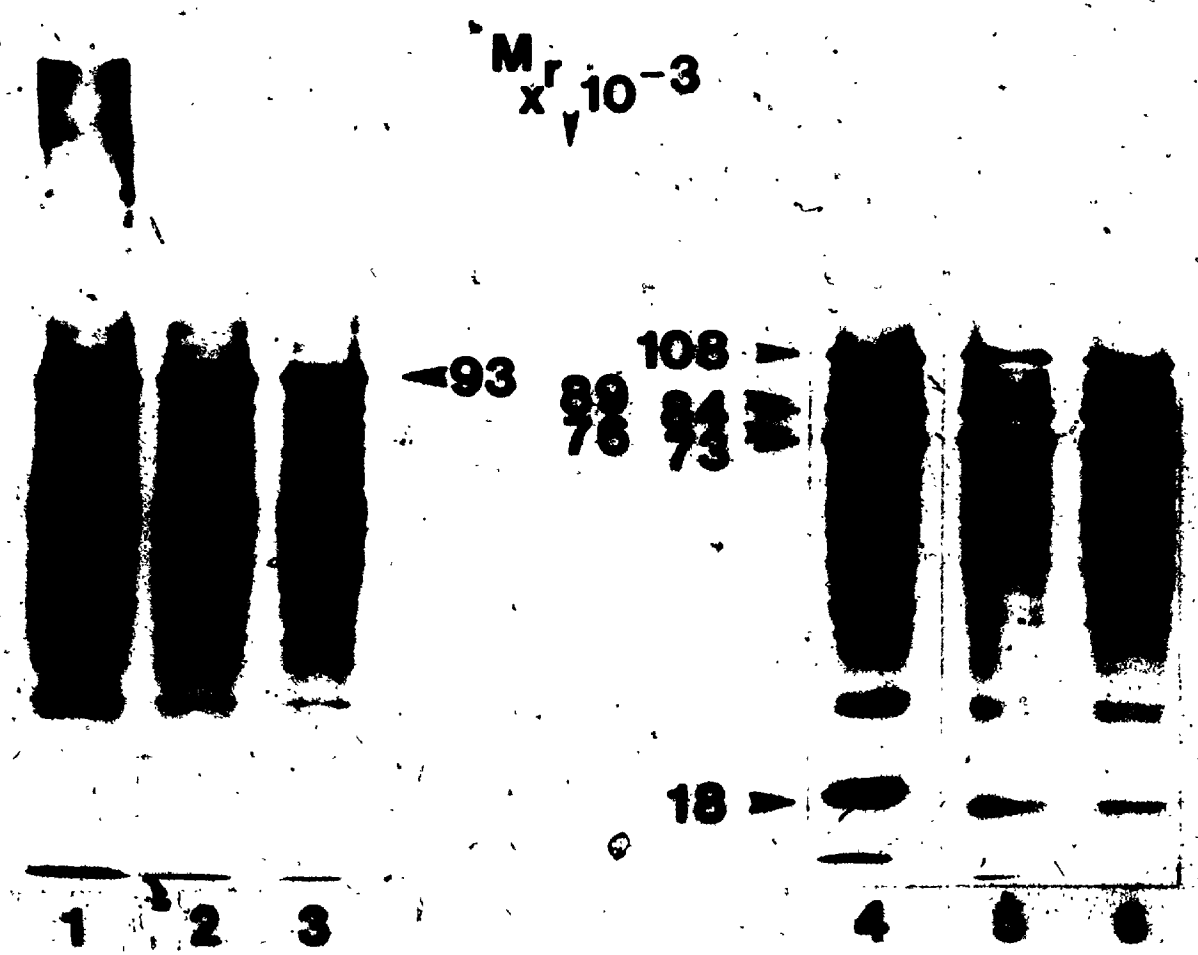
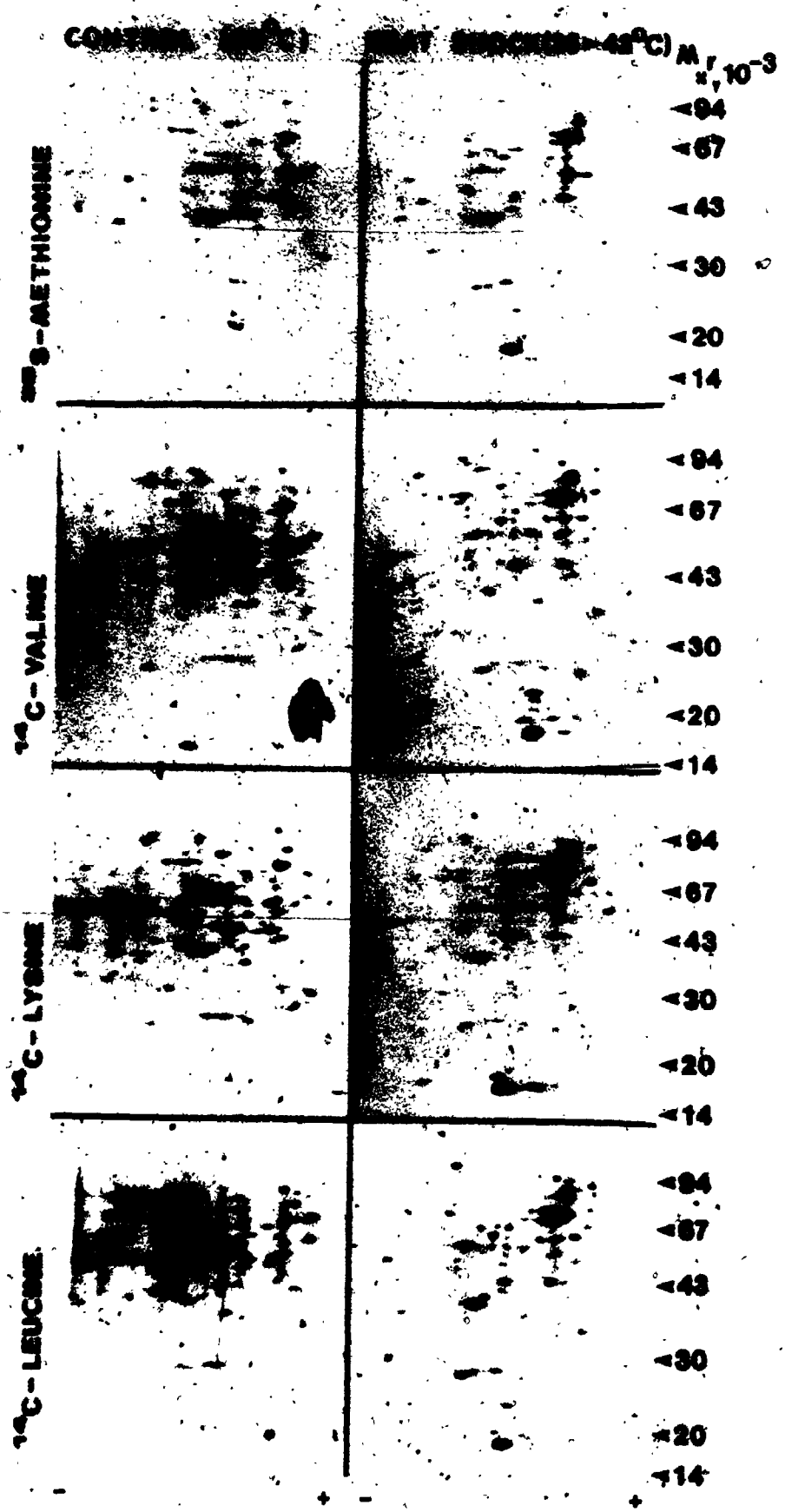

$M_r \times 10^{-3}$
93
108
18
1
2
3
4
5
6

Figure 8. Fluorograms of the 2-D IEF-SDS-PAGE separations of polypeptides synthesized in plumules at 25C or following a one-hour shift to 42C prior to labelling for two hours at 25C in the presence of 35S-methionine, 14C-valine, 14C-lysine or 14C-leucine. The first dimension IEF gel contained a mixture of 80% pH range 5-8 and 20% pH range 3.5-10 ampholines. The second dimension consisted of a 7.5-17.5% polyacrylamide gradient slab gel. Positions of standard marker proteins are indicated by arrows on the right. Approximately 40,000 cpm of acid-precipitable lysate was loaded onto each first dimension IEF gel. Fluorograms were exposed for twelve days.

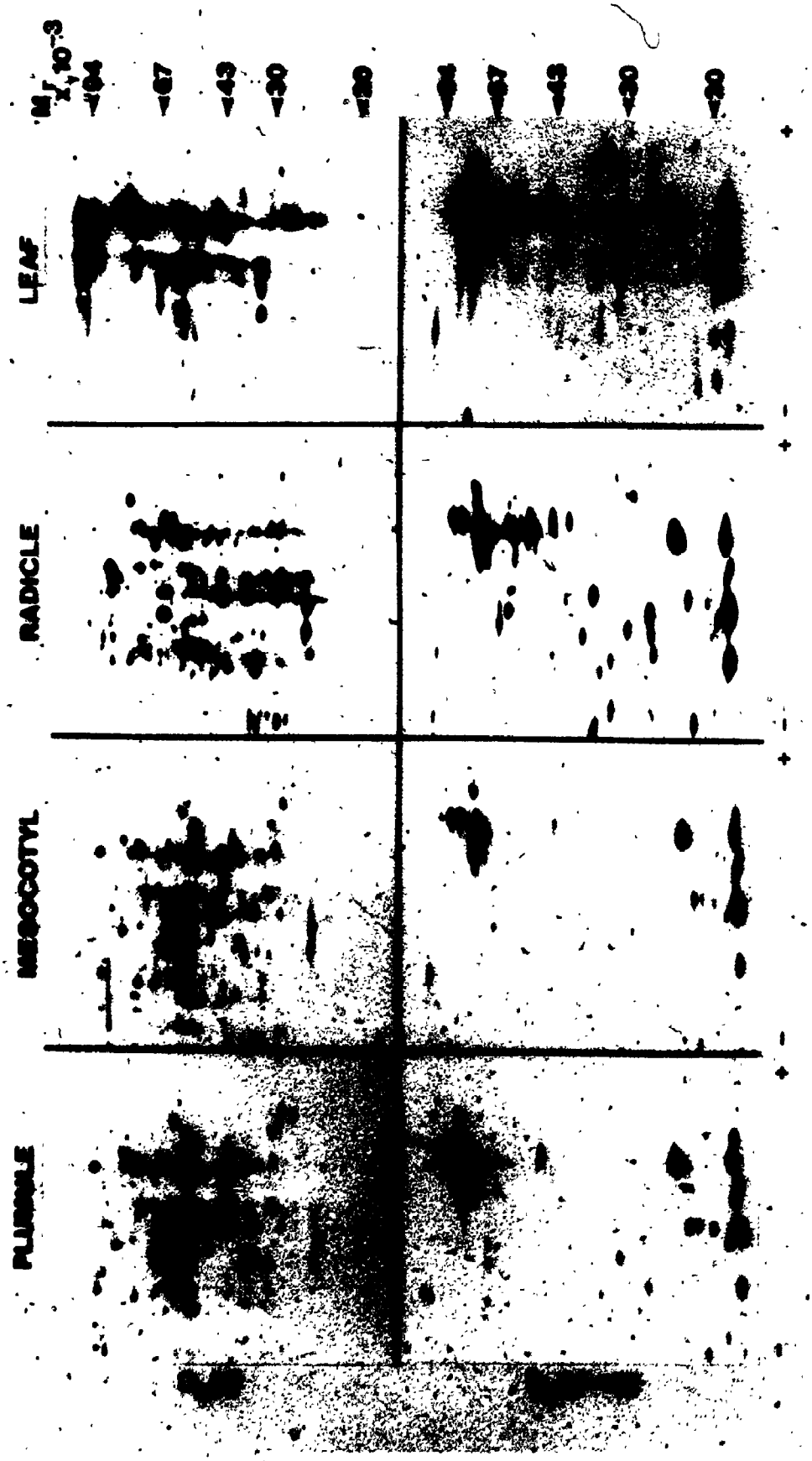

CONTROL (37°C)
HEAT SHOCK (30 min 43°C)
$M_r \times 10^{-3}$
94
67
43
30
20
14
^{35}S-METHIONINE
^{14}C-VALINE
^{14}C-LYSINE
^{14}C-LEUCINE
-
+
-
+

major HSP classes from heat-shocked plumules contain each of the four amino acids (although in different proportions). A label concentration dose study and a time course study were initially conducted to determine the optimum labelling time and label concentration of each precursor. The slight differences in temperature conditions from those used previously (ie., 25C vs 27C) represent initial attempts at examining whether different growing or temperature shift conditions might influence the patterns of polypeptide synthesis (see Chapter 4).

2.3.7 HSP synthesis in different tissues of maize.

The response of maize to heat shock was most extensively investigated using plumules of five-day-old seedlings. A very similar response to heat shock was also noted in several other maize tissues including primary radicles, mesocotyls, and young leaves (Figure 9). Although tissue-specific differences were observed, all tissues exhibited synthesis of the same six major molecular weight classes of the heat shock polypeptides described previously. These results also reveal that when seedlings are labelled at the heat shock temperature (42C), synthesis of HSPs is more intense, presumably due to the longer (three vs one hour) incubation at the high temperature. Note also that the 23 kD polypeptide which exhibited minor enhancement under some conditions in the previous gels, shows marked enhancement under the present conditions. The precise

Figure 9. Fluorograms of the 2-D IEF-SDS-PAGE separations of polypeptides synthesized in plumules, mesocotyls, radicles or young leaves at 30C or following a one hour shift to 42C prior to labelling at 42C for two hours. The ampholine mixture in the IEF gels and the gradients used in the second dimension slab gels were the same as in Figure 8. Positions of standard marker proteins are indicated by arrows on the right. Approximately 100,000 cpm of acid-precipitable lysate was loaded onto each IEF gel. Fluorogram was exposed for five days.


$M_r \times 10^{-3}$
LEAF
RADICLE
MESOCOTYL
PLUMULE

temperature conditions therefore appear to be important in determining the final array of polypeptides which are synthesized (see Chapter 4 for detailed study).

2.4 DISCUSSION

Maize seedlings subjected to a rapid upward temperature shift exhibit a dramatic change in the types of polypeptides which are synthesized. This response, which involves the novel or enhanced synthesis of a small group of "heat shock polypeptides", is apparently analogous to that found in Drosophila (Ashburner and Bonner, 1979) and other animal (Kelley and Schlessinger, 1978; Bouche et al., 1979; Atkinson, 1981) and plant (Barnett et al., 1980; Key et al., 1981) systems. Some of the results obtained in the present study in maize have been published (Baszczynski et al., 1982a) and have been integrated here into a more extensive characterization of the protein synthetic changes accompanying the response of maize to heat shock.

The results from Figure 1 indicate that heat shock temperatures below 44C do not markedly alter protein synthetic rates. Above 44C, the rate of protein synthesis decreases rapidly with increasing temperature suggesting that, in maize, this temperature may represent an inflection point, above which temperature shifts are potentially lethal. This observation has been supported by field data which demonstrate that the survival of maize plants which

were subjected to a one hour heat shock above 42C was greatly reduced relative to those heat-shocked below this temperature (Baszczynski et al., 1982d). In 1864, Sachs estimated that only 10 minutes at 49-51C is lethal for maize (in Levitt, 1980). This temperature interval is within the 45-65C range which is considered to be the threshold for high temperature stress for most moderate thermophiles (Levitt, 1980). Similar curves for protein synthetic rates have also been obtained for soybean and certain other crop plants (Key et al., 1982). In all cases, the temperature inflection points are between 40 and 44C.

While differences were not observed in the Coomasie blue-stained gels of the electrophoretically-separated polypeptides, fluorographic analysis of these same gels revealed a temperature-dependent change in the patterns of newly synthesized polypeptides (Figure 2). The HSPs in maize exhibit a size distribution which is similar to other plant and fungal systems which have been studied (Barnett et al., 1980; Key et al., 1981; Kapoor, 1983). The present results have also been confirmed by a recent study in maize (Cooper and Ho, 1983). Although discrepencies exist in the molecular masses assigned to the HSPs (which may be due in part to different gel conditions, different tissues, or the use of different genotypes), the basic size distribution of HSPs is the same.

There is little or no change in the levels of synthesis of control polypeptides below 41C in maize (Figure 2B).

While some organisms exhibit a similar response (May and Rosenbaum, 1980), others show a depression of synthesis of most control polypeptides following heat shock (Tissieres et al., 1974; Ashburner and Bonner, 1979). Since a constant number of counts of acid-precipitable lysate were loaded into each well of the stacking gel, the reduced intensity of control polypeptides noted at 44C may be the result of the dramatically enhanced synthesis of HSPs, rather than the reduced synthesis of control polypeptides. It has been shown in Drosophila however, that during heat shock, mRNAs for control polypeptides are preferentially released from polysomes leading to a reduced level of synthesis of control polypeptides (Kruger and Benecke, 1981).

Two-dimensional analysis revealed a complex pattern of 18 or more spots corresponding to the six molecular weight classes of HSPs noted in 1-D gels. Under the present conditions of extraction and electrophoresis, these polypeptides focussed with isoelectric points ranging between 6.6 and 8.2. These values are somewhat more basic than those obtained in animal systems (Atkinson, 1981). The differences may be due in part to the extraction conditions since other studies have shown that different solubilization buffers lead to alterations in the migrations of polypeptides in IEF gels (Atkinson, personal communication; also see results in Chapter 3).

The observation that several spots may be present for one HSP class suggests that either: a) heat shock induces

the synthesis of families of polypeptides of similar molecular mass but differing in charge; or b) translational and/or post-translational modifications of one polypeptide in a molecular weight class leads to different charge variants of that single polypeptide. In vitro translational analysis (see next chapter) indicates that both situations may exist in maize. Recent studies utilizing cloned DNAs for the 18 kD HSPs in soybean have revealed that many of the polypeptides in this molecular weight class are the products of individual genes while some represent chemical modifications of other polypeptides (Schoffl and Key, 1982, 1983).

While enhanced synthesis of HSPs occurs following heat shock, some polypeptides exhibit reduced synthesis and others show no change. Since the total incorporation of amino acid precursors into proteins does not change dramatically at these temperatures (Figure 1), these differential levels of synthesis of the various polypeptide classes most likely represent a temperature-specific shift in the translational machinery of the cell to the selective translation of a small group of mRNAs. In Drosophila, a homologous in vitro translation system prepared from heat-shocked cells will preferentially translate those messages which are abundant in cells following heat shock (Kruger and Benecke, 1981). This finding implies that different intracellular translational conditions exist in control and heat-shocked cells.

Synthesis of HSPs in maize was noted as early as 15 minutes following a shift from 27C to 41C. Although shorter time intervals were not examined, synthesis of HSPs in Drosophila has been detected within the first 8-12 minutes of heat shock (Ashburner and Bonner, 1979; Lindquist, 1980). After approximately 60 minutes at 41C, the level of incorporation of radioactively-labelled precursor into polypeptides with Mrs corresponding to the maize HSPs exhibits a five-fold increase. This increase, although substantial, is considerably less than in Drosophila where the HSPs account for approximately 50% of the total incorporated counts (Ashburner and Bonner, 1979). However, unlike Drosophila, maize does not exhibit an almost complete cessation of synthesis of control polypeptides following heat shock; thus, it is likely that the absolute rate of synthesis of HSPs is considerably lower in maize than in Drosophila.

When maize seedlings are returned to a 27C incubator following a one hour heat shock at either 41C or 44C, there is a rapid and complete recovery in the polypeptide synthetic patterns within approximately eight hours. While induction of HSP synthesis occurs rapidly (within 15 minutes), recovery proceeds slowly and is clearly dependent on the heat shock temperature (Figure 5). Recent studies on recovery rates in Drosophila cells have generated similar observations and have revealed that the disappearance of at least one HSP (70 kD) is co-ordinated with the reappearance

of synthesis of normal (control) polypeptides (DiDomenico et al., 1982). If the seedlings which have been allowed to recover are then allowed to continue development in the nursery or greenhouse, they grow into normal corn plants. These findings, coupled with the observation that Coomasie blue-stained polypeptide patterns do not exhibit any changes following the temperature treatments, provide strong evidence that the HSPs are indeed newly synthesized products and not the result of heat-induced degradation. The identification of genes for the HSPs in many other systems (see Introduction) provides further support that the HSPs are not artefacts.

Seedlings which are maintained at 41-42C for extended periods of time (24 hours) do not show continued synthesis of the HSPs. Instead there is a gradual decrease in the synthesis of the HSPs and the establishment of a new, stable (in terms of hours) polypeptide synthetic pattern (see Figure 6). Cooper and Ho (1983) have substantiated this finding. As with recovery, the rate of disappearance of each HSP class is somewhat different. In other systems (Drosophila, soybean), HSP synthesis continues for as long as the high temperature is maintained (Moran et al., 1978; Key et al., 1981).

The data in Table 1 suggest that, in addition to molecular mass and isoelectric points, the HSPs may also be defined in terms of kinetic parameters. The rates of appearance and disappearance during either recovery or

continued heat shock are thus a characteristic property of each HSP class under a given set of temperature conditions.

An important development during the course of this investigation was a system which permitted the treatment, labelling and analysis of polypeptides from different tissues of a single 4-5 day old seedling. While a considerable amount of the work described in this chapter was conducted on extracts of pooled tissue from many seedlings, the polypeptides extracted from control or heat-shocked plumules of individual seedlings were identical to those obtained from the pooled extracts. Furthermore, excision prior to temperature treatment and labelling did not lead to a "wounding" response. The concern of wound protein production has also been expressed in other studies (Zurfluh and Guilfoyle, 1980; Currie and White, 1981; Theillet et al., 1982).

From a genetical point of view, the use of a single seedling system has several advantages. Since many different sources of a pedigreed variety (eg., Oh43) may exist, and since the control of the zygotic genotype is routine in maize, analysis of various tissues (Baszczynski and Hughes, 1981b; Hughes and Walden, 1981) by the single seedling method should facilitate the investigation of discrete allele control within populations. Furthermore, the extraction and electrophoretic separation of polypeptides from many individual seedlings will yield more information with reference to population variability than

analysis of extracts from pooled tissue.

The response to heat shock does not appear to be limited to plumules; other tissues including mesocotyls, radicles and young leaves (Figure 9) as well as embryos and mature leaves (J. Boothe, personal communication) also exhibit enhanced synthesis of the same HSP classes. In each case though, tissue-specific differences in patterns of polypeptide synthesis are also observed. A common response to heat shock by several tissues from a single organism has also been noted in other studies (Atkinson et al., 1983; Dean and Atkinson, 1983). The relative synthesis of the various HSP classes in these organisms (as in maize) appears to be tissue-dependent.

Fluorograms of 2-D PAGE-separated polypeptides which had been labelled with each of the four precursors (Figure 8) revealed that the HSPs contain all four amino acids. Since 35S-methionine yielded the highest incorporation, subsequent in vivo studies were conducted using the latter precursor. This change was also necessary since the in vitro translational studies outlined in the next chapter utilized 35S-methionine and direct comparisons between the polypeptides synthesized in vivo and in vitro required use of the same radioactively-labelled precursor.

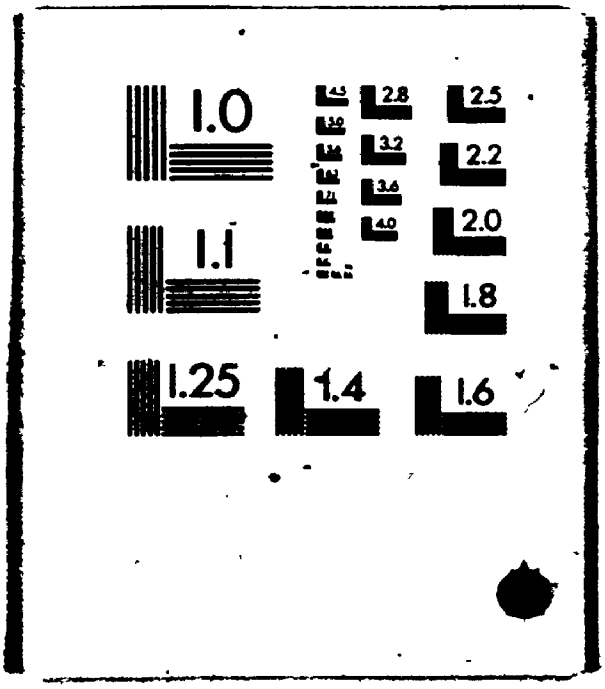
CHAPTER 3

IN VITRO TRANSLATIONAL ANALYSIS OF RNA FROM CONTROL AND HEAT-SHOCKED SEEDLINGS

3.1 INTRODUCTION

It has been implied, although shown in only a few systems, that the changes in polypeptide synthetic patterns following heat shock are the result of new and(or) enhanced gene activity. The rapid and dramatic changes in polypeptide synthetic patterns and the abundance of isoelectric variants of some of the HSP classes observed in maize, prompted an analysis of the synthesis and/or availability of mRNAs before, during and following heat shock. Isolation of RNA from control and heat shocked-seedlings, purification of mRNAs, and translation of these mRNAs *in vitro* demonstrates that the heat shock response in corn involves changes in the population of available RNAs. Moreover, differences in the *in vivo* and *in vitro* synthesis of HSPs suggest that the heat shock response in maize may also involve translational or post-translational regulatory mechanisms.

2



3.2 MATERIALS AND METHODS

3.2.1 Isolation of RNA from maize seedlings.

Seeds of Zea mays L. (cv. Oh43) were allowed to germinate in the dark at 27C for 4-5 days as described in the previous chapter. The intact seedlings were then either maintained at 27C or subjected to a one hour heat shock at 42C. At the end of the one hour incubation, plumules were excised, frozen in liquid nitrogen, and either extracted directly or stored at -70C until required. Total RNA was extracted by a modification of the perchlorate-isopropanol methods of Wilcockson (1975) and Lizardi and Engelberg (1979) as described by Sachs et al. (1980). The plumule tissue was first ground to a fine powder with porcelain mortars and pestles which had been previously chilled either in liquid nitrogen or on dried ice. Approximately 0.5 g of powdered tissue was mixed with 3 mL of solution A (50 mM Tris-HCl, pH 7.5 (Sigma), 5% SDS (Bio-Rad), 200 mM NaCl (Fisher), 15 mM EDTA (Sigma), and 0.07% proteinase K (Boehringer Mannheim, added just prior to extraction)), and the suspension was incubated at 35C for 15 minutes and at 55C for five minutes. The extract was centrifuged at 12,000xg for 15-20 minutes and 2 mL of solution B (3.5 M sodium perchlorate) was added to the supernatant, mixed thoroughly, and the mixture was incubated at 55C until the cloudy solution became clear. 2.5 mL of solution C (80% ethanol saturated with sodium perchlorate) was added to the

cleared solution, the tube was agitated on a vortex mixer immediately, and a further 17.5 mL of solution C was added to it. The mixture was incubated at 4C for one hour and centrifuged at 3000xg for 20 minutes at 4C, and the pellet was solubilized in 5 mL of solution D (25 mM Tris-HCl, pH 7.5, 5% SDS, 7.5 mM EDTA). Nucleic acids were precipitated from this solution by the addition of 20 mL of solution C (at 4C for one hour) and pelleted by centrifugation at 3000xg for 20 minutes (at 4C). The resulting pellet was dissolved in 7.5 mL of solution E (25 mM Tris-HCl, pH 7.5, 0.2% SDS, 1 mM EDTA). A second precipitation step was carried out by adding 12.5 mL of solution F (3 M sodium acetate, pH 6.0) and 0.6 volumes (4.5 mL) of isopropanol to the solution, and incubating it at -20C for at least four hours. The solution was centrifuged at 3000xg for 20 minutes at -10C. The pellet was suspended in 10 mL of 10% solution F in ethanol and placed at -20C for at least four hours. The sample was centrifuged at 3000xg for 20 minutes at -10C and the pellet was dried thoroughly by lyophilization. The RNA pellet was dissolved in 50-100 uL of sterile distilled water, the A260 and A280 were determined using a Gilford model 240 ultraviolet spectrophotometer, and the RNA solution was frozen and stored at -70C until required.

3.2.2 Fractionation of RNA by oligo(dT)-cellulose chromatography.

Total maize plumule RNA was separated into poly(A)+ and

poly(A)- RNA fractions as described by Aviv and Leder (1972). Approximately 20 A260 units of the aqueous RNA suspension in 0.5-1.0 mL of application buffer (0.5 M KCl, 0.01 M Tris-HCl, pH 7.5) was applied to a 1.0 g column of oligo(dT)-cellulose type 7 (PL Biochemicals, binding capacity 95.3 A260 units poly(A)/g), which had been previously equilibrated with application buffer. Column flow was monitored using a Pharmacia U.V. absorbance monitor and chart recorder. The column was eluted with application buffer and the non-absorbed material (poly(A)- RNA) was recycled over the column and eluted, and the poly(A)-⁺RNA fraction was collected. The material bound to the column (poly(A)+ RNA) was eluted first in 0.1 M KCl-0.01 M Tris-HCl, pH 7.5, and then with 0.01 M Tris-HCl, pH 7.5. All peaks were collected separately and the eluants were made 0.2 M with respect to NaCl, mixed with 2.5 volumes of cold 95% ethanol, and precipitated overnight at -20C. The precipitated RNA samples were centrifuged, and the pellets were lyophilized and solubilized in sterile water.

3.2.3 In vitro translation of maize RNA and the determination of incorporated radioactivity into newly translated products.

3.2.3.1 Rabbit reticulocyte lysate system.

Total RNA and poly(A)+ RNA from control and heat-shocked plumules were used to direct the *in vitro* synthesis of polypeptides in a heterologous *in vitro* translation kit (New England Nuclear), using 35S-methionine

as the labelling probe and the ingredients and methodology described in the kit. RNA concentrations used ranged from 0.5-20 ug for total RNA and 0.5-5 ug for poly(A)+ RNA. Translations were carried out in a 37C water bath for 60 minutes. At the end of the incubation, the reaction tubes were placed on ice to terminate the translation process, and two replicate 1.0 uL samples from each tube were spotted on 1 cm squares of Whatman No. 1 filter paper, dried, and placed in a boiling 10% trichloroacetic acid bath for 10 minutes. The filters were then washed twice with water, ethanol and acetone. After drying, the filters were placed in scintillation vials, 0.5 mL of NCS tissue solubilizer (Amersham) was added to the vials, and the vials were placed at 55C for 30 minutes. At the end of the incubation, each vial received 17 uL of glacial acetic acid and 10 mL of scintillation fluid (8 g Omnifluor (New England Nuclear) in 1000 mL of "scintanalyzed" toluene (Fisher)), and the samples were counted on a Beckman LS 230 scintillation counter. The remainder of the translation products were frozen and stored at -70C until required.

3.2.3.2 Wheat germ extract system.

Total RNA from both control and heat-shocked plumules were translated in a wheat germ in vitro translation system from Bethesda Research Laboratories (Bethesda, MD) using 35S-methionine as the labelling probe. RNA concentrations similar to those used in the rabbit reticulocyte system were

found to be suitable. Translation of the RNA was carried out at 25C for 60 minutes, the reaction was stopped, and the incorporated counts were determined as described earlier.

3.2.4 Determination of optimum conditions for the in vitro translation of maize plumule RNA.

The principal factors which influence the fidelity of translation of RNA in vitro (RNA purity, RNA concentration, potassium ion (K^+) concentration and magnesium ion (Mg^{++}) concentration) were each independently varied, to establish the most suitable combination of conditions. The absorbance of the sample provided an estimate of RNA purity; A260 to A280 ratios of 2.0 or higher were indicative of high RNA purity and low protein contamination. The other three components (RNA, K^+ and Mg^{++} concentrations) were analyzed by conducting three sets of translations. In the first set, RNA concentration was varied while K^+ and Mg^{++} concentrations were fixed. When the optimal RNA level was determined, it was kept constant in the second set of translations and K^+ concentration was varied. Finally, in the third set, a range of Mg^{++} concentrations was tested. The optimal amount in each case was the concentration which yielded both the highest incorporation of radio-labelled precursor into newly translated products, and the greatest number of translated polypeptides as monitored by one- and two-dimensional gel electrophoresis and fluorography. RNA concentrations ranging from 0.5 to 20 ug per 25 uL translation assay were examined. The K^+ and Mg^{++}

concentrations tested ranged from 50 to 200 mM and 32.5 to 50 mM, respectively.

3.2.5 One- and two-dimensional PAGE separations of the in vitro translated products.

The translation products were prepared for 1-D electrophoresis by mixing a sample of the translation products (consisting of approximately 25,000 acid-precipitable counts) with 1-2 volumes of an SDS buffer (described previously) and boiling for one minute. The samples were then loaded into wells of a stacking gel and electrophoresed as described previously. For 2-D electrophoresis, samples consisting of approximately 100,000 acid-precipitable counts were mixed with four volumes of a buffer containing 9.0 M urea, 5% 2-mercaptoethanol, 1 mM PMSF and 5% ampholines (pH range 3.5-10, LKB Instruments, Inc.) prior to loading on the first dimension IEF gels. The second dimension consisted of a 3-15% or a 7.5-17.5% polyacrylamide slab gel overlayed with a 3% polyacrylamide stacking gel. All other electrophoretic conditions were as described previously.

3.3 RESULTS

3.3.1 Establishment of optimal conditions for in vitro translation of maize RNA.

The components of the two in vitro translation systems used in these studies initially were varied to determine the

optimal conditions for maximum translation of all size classes of maize RNA. The rabbit reticulocyte system yielded effective translations with a variety of RNA and K^+ concentrations; higher Mg^{++} concentrations than those recommended in the kit, resulted in increased levels of 35S-methionine incorporation into the newly translated products. The wheat germ system exhibited a very strong dependence on high potassium concentrations (>135 mM) for translation of polypeptides with molecular masses greater than 70 kD. While effective translations with either system were dependent on the amount of RNA added to the translation mixture, optimal RNA levels were very similar in both systems. Table 3 summarizes the conditions found to be most suitable for the in vitro translation of RNAs from control and heat shocked seedlings in the rabbit reticulocyte and wheat germ systems. Under these conditions, the reticulocyte lysate system always stimulated the incorporation of four to six times more radioactivity (cpm per ug RNA) into the newly translated products than did the wheat germ system (Figure 10).

3.3.2 One-dimensional SDS-PAGE analysis of the in vitro translation products from control and heat-shocked seedlings.

To assess the changes in the RNA populations before and after heat shock, the in vitro translation products were separated by 1-D gel electrophoresis and the newly translated products were visualized by fluorography. Figure

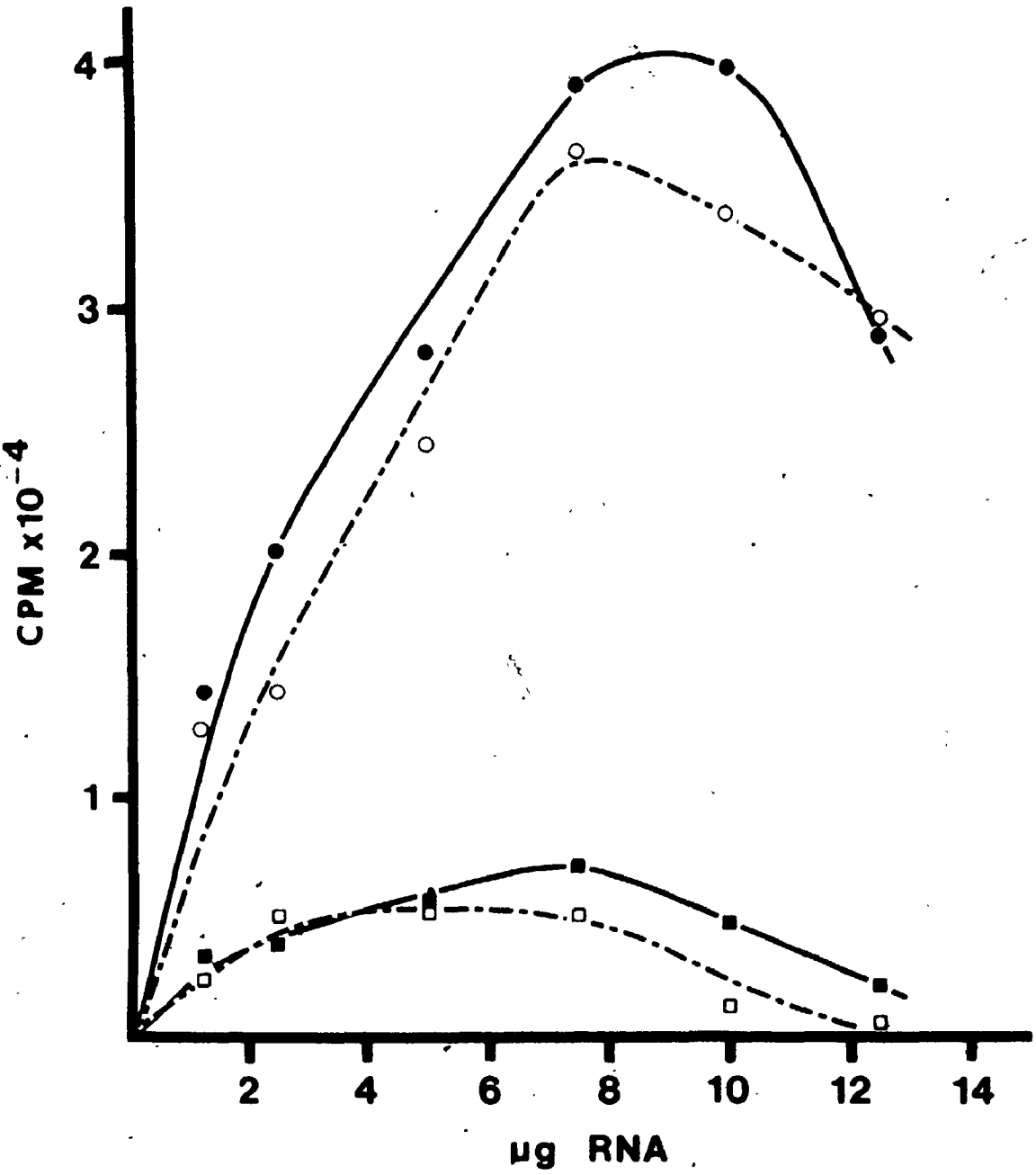
Table III. Concentrations of RNA, K+, and Mg++ required for optimal translation of maize RNAs in the rabbit reticulocyte and wheat germ extract *in vitro* translation systems.

Component	Reticulocyte Lysate		Wheat germ	
	Control[a]	Heat Shock[b]	Control[a]	Heat Shock[b]
ug RNA/25uL	6-10	8-13	6-8	5-8
[K+] mM	80-140	80-140	130-150	130-150
[Mg++] mM	30-50	30-50	30-50	30-50

a Translation conditions were assessed when RNA from control (27C grown) plumules was used to direct the *in vitro* synthesis of polypeptides in the corresponding translation systems.

b Translation conditions were assessed when RNA from heat-shocked (27C to 41C) plumules was used to direct the *in vitro* synthesis of polypeptides in the corresponding translation systems.

Figure 10. Comparison of the relative incorporation of 35S-methionine (cpm) into newly synthesized, acid-precipitable, translation products as a function of the amount of total RNA from control or heat-shocked plumules added to either the rabbit reticulocyte or the wheat germ *in vitro* translation systems. Control RNA in reticulocyte system (●); heat shock RNA in reticulocyte system (○); control RNA in wheat germ system (■); heat shock RNA in wheat germ system (□). Values represent mean cpm from three independent determinations with standard errors for any point not exceeding 150 cpm. Reproduced from Baszczynski *et al.*, 1983a; Can. J. Biochem. Cell Biol. 61:395-403.


4
3
2
1
CPM x10⁻⁴
2
4
6
8
10
12
14
µg RNA

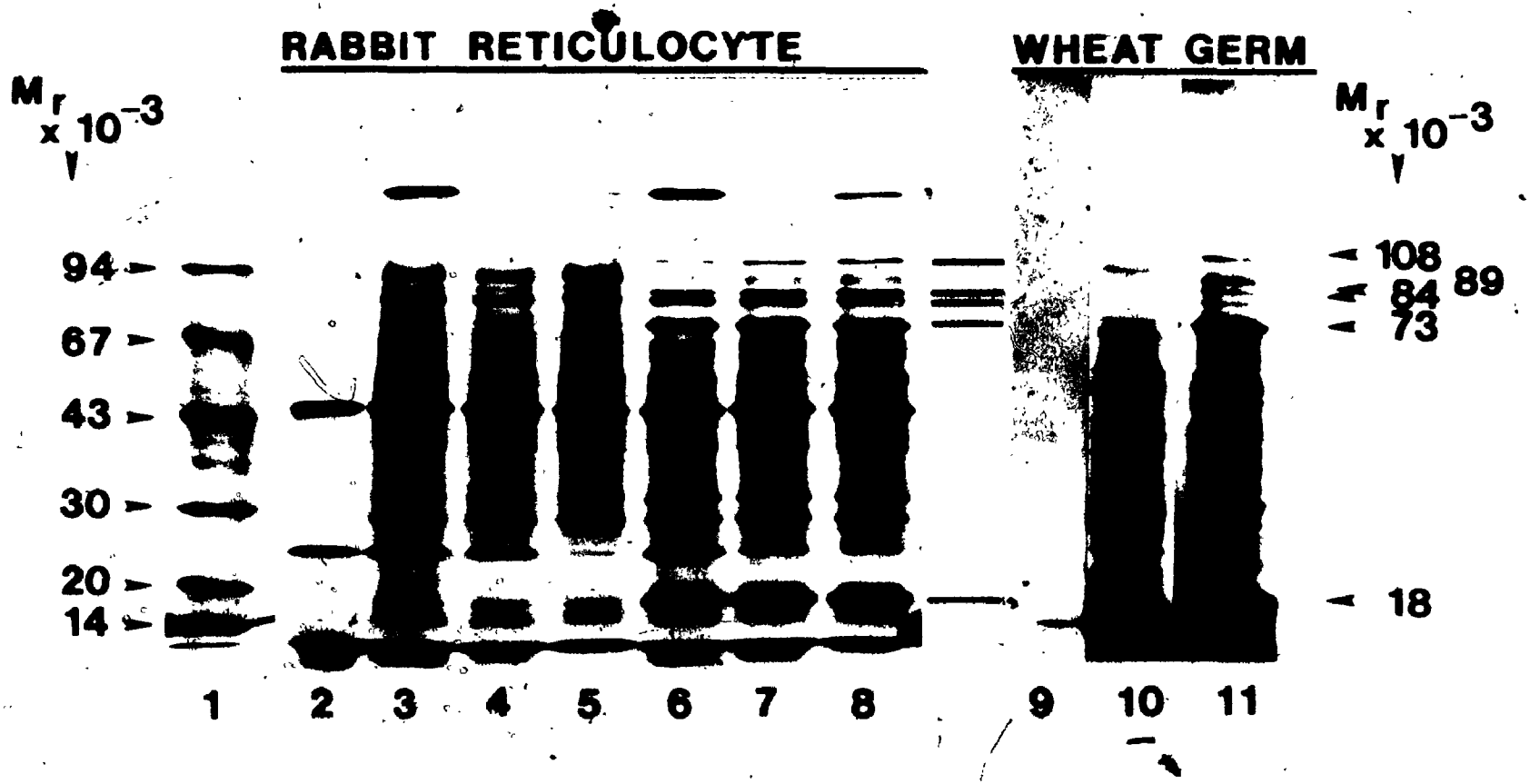
11 compares the products synthesized in both the rabbit reticulocyte (lanes 2-8) and the wheat germ (lanes 9-11) systems. Lanes 2 and 9 contain the products from translations carried out in the absence of any exogenously added RNA. Lanes 3-5 and 6-8 represent translations in which increasing amounts of RNA from control and heat-shocked plumules, respectively, were added. While minor differences exist in the products derived from the two systems, translations of the RNA from heat-shocked plumules in either system (lanes 6-8 and 11) result in new and(or) enhanced synthesis of the same group of polypeptides. These polypeptides, with Mrs of 108, 89, 84, 73, and 18 kD are low or absent in controls and intensify following heat shock; they correspond precisely to five of the six HSP classes observed *in vivo*. The 76 kD HSP class is apparently not translated *in vitro*.

3.3.3 Two-dimensional IEF-SDS-PAGE analysis of the in vitro translation products from control and heat-shocked seedlings.

3.3.3.1 Comparison of the polypeptides synthesized in vitro and in vivo.

The products obtained from the *in vitro* translation of RNAs from control and heat-shocked seedlings and the *in vivo* synthesized polypeptides were resolved by 2-D gel electrophoresis (Figure 12). The polypeptides synthesized *in vivo* (Figure 12A) and *in vitro* (Figure 12B) from control plumule RNA, exhibit some quantitative and qualitative differences. Following heat shock, new or enhanced

Figure 11. Fluorogram of a 1-D SDS-PAGE (3-15% gradient) separation of the products obtained from the *in vitro* translation of maize plumule RNAs in the rabbit reticulocyte (lanes 2-8) and in the wheat germ (lanes 9-11) translation systems. Lane 1, stained standards (see Materials and Methods); lanes 2 and 9, no added RNA; lanes 3, 4, 5, and 10, translations in the presence of 1.25, 2.5, 5, and 5 ug, respectively, of control RNA; lanes 6, 7, 8, and 11, translations in the presence of 1.25, 2.5, 5, and 5 ug, respectively, of heat shock RNA. Arrowheads on the left and right indicate the Mrs and the positions of standard and heat shock polypeptides, respectively. Dashes between control and heat shock lanes in wheat germ translations connect corresponding polypeptides. Approximately 25,000 cpm of acid-precipitable lysate was loaded in each well of the gel. Fluorograms were exposed for five days. Reproduced from Baszczynski *et al.*, 1983a; Can. J. Biochem. Cell Biol. 61:395-403.


RABBIT RETICULOCYTE
WHEAT GERM
M_r x 10^{-3}
94
67
43
30
20
14
1
2
3
4
5
6
7
8
9
10
11
M_r x 10^{-3}
108
89
84
73
18

synthesis of a similar set of heat shock polypeptides is observed among the products synthesized in vivo and in vitro. As noted in the 1-D gels, the 76 kD HSP is absent among the in vitro translation products. The 76 kD polypeptide has a slightly higher mobility and is more acidic than the prominent 73 kD HSP.

The number of isoelectric variants of some of the HSP classes differs among the in vivo and in vitro translated products. For example, additional spots corresponding to the 18 kD HSP class are noted in vitro. There are fewer isoelectric variants of the high molecular weight HSPs in vitro than there are in vivo. This implies that translational and(or) post-translational changes, which alter the charge forms of the polypeptides, may be occurring in vivo.

The polypeptides obtained from in vitro translations uniformly migrate with more acidic pIs than their in vivo counterparts (Figure 12). As mentioned earlier, these apparent discrepencies in pI values are partially due to different solubilization conditions which impart uniform alterations in the net charge of the polypeptides. When the in vitro translation products are mixed with an equal volume of the SDS buffer used to solubilize in vivo synthesized polypeptides, the electrophoretically separated products migrate with identical isoelectric points (Figure 13).

Figure 12. Fluorographic comparison of 2-D IEF-SDS-PAGE separations of the polypeptides synthesized *in vivo* (A and B) and the products derived from an *in vitro* translation of total RNA (C and D), from control (A and C) or heat-shocked (B and D) maize plumules. Arrowheads in B and D point to the position of the 76 kD HSP which is synthesized *in vivo* but apparently not *in vitro*. The IEF gel contained pH range 3.5-10 ampholines and the second dimension consisted of a 3-15% gradient gel. Relative molecular masses of the prominent control polypeptide and the HSPs are indicated by arrows on the left and right, respectively. Approximately 100,000 cpm of acid-precipitable translation products was loaded onto each IEF gel. Fluorograms were exposed for five days. Reproduced from Baszczynski *et al.*, 1983a; Can. J. Biochem. Cell Biol. 61:395-403.

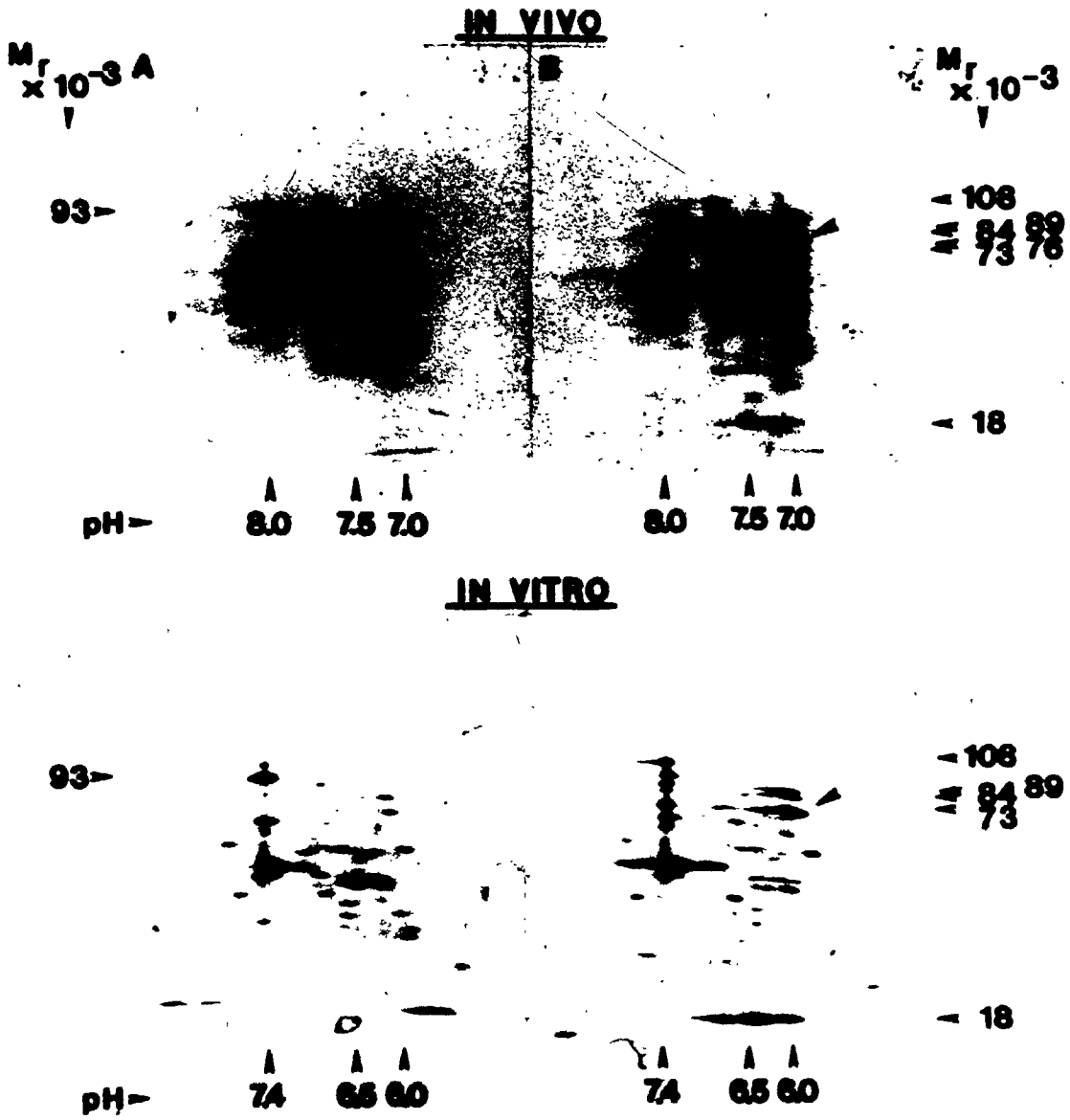
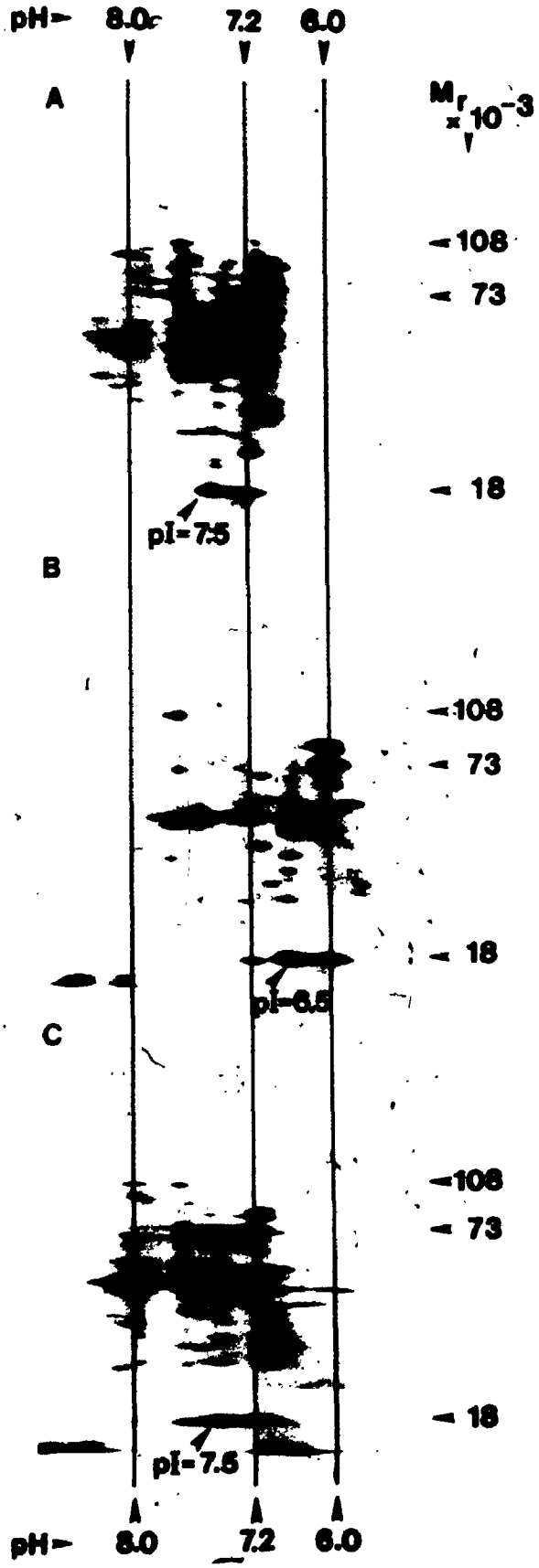

IN VIVO
$M_r \times 10^{-3}$ A
$M_r \times 10^{-3}$
93
106
84
73
89
76
18
pH
8.0
7.5
7.0
8.0
7.5
7.0
IN VITRO
93
106
84
73
89
18
pH
7.4
6.5
6.0
7.4
6.5
6.0

Figure 13. Comparison of isoelectric point (pI) determinations conducted on electrophoretically separated and fluorographically revealed polypeptides in the presence or absence of SDS. A. Polypeptides synthesized *in vivo* in heat-shocked plumules (as in Figure 12B); B. products from an *in vitro* translation of total RNA from heat-shocked plumules (prepared as described for translation products in Materials and Methods); C. products from an *in vitro* translation of total RNA, after mixing with an equal volume of SDS extraction buffer and then prepared as in A. Positions of reference HSPs are indicated by arrows on the right. Regions of identical pH value in the three gels are connected by vertical lines. pI values indicated by arrowheads in each figure refer to the same prominent 18 kD HSP. Each gel was loaded with approximately 100,000 cpm of acid-precipitable translation mixture. Fluorograms were exposed for five days. Reproduced from Baszczynski *et al.*, 1983a; Can. J. Biochem. Cell Biol. 61:395-403.


pH→ 8.0 7.2 6.0
A
M_r x 10^{-3}
108
73
18
pI=7.5
B
108
73
18
pI=6.5
C
108
73
18
pI=7.5
pH→ 8.0 7.2 6.0

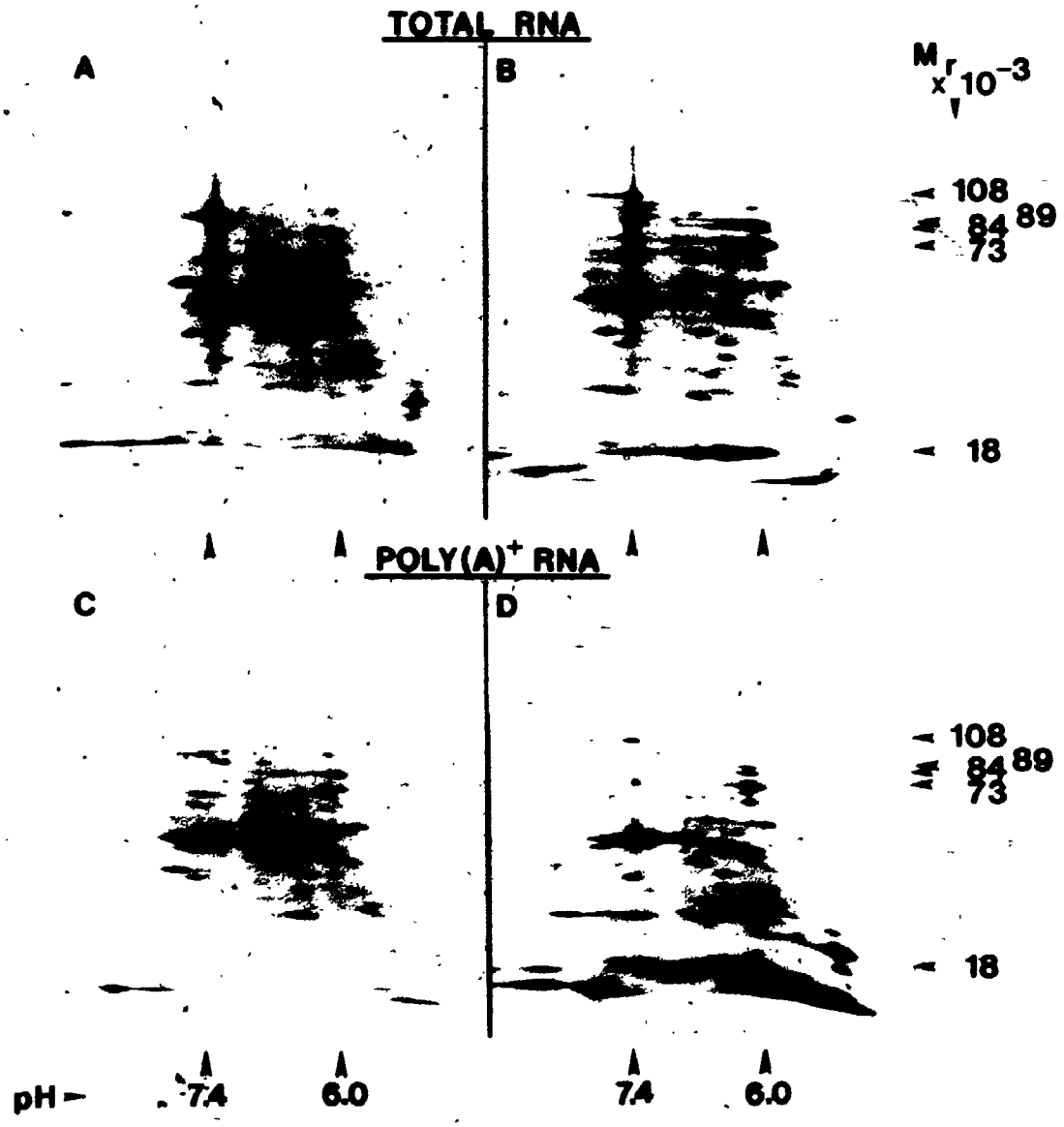
3.3.3.2 Comparison of the in vitro translations of total RNA and poly(A)+ mRNA.

The 2-D electrophoretic separations of polypeptides synthesized *in vitro* from total RNA and poly(A)+ mRNA of control and heat-shocked plumules are presented in Figure 14. In general, the poly(A)+ fraction separated by oligo(dT)-cellulose chromatography represented less than 10% of the total RNA added to the column. Due to its purity however, very small amounts (0.5-1.0 ug) were sufficient to stimulate incorporation of radioactivity into the *in vitro* synthesized products. While quantitative differences exist, the majority of the polypeptides translated *in vitro* from total RNA are the same as those synthesized from poly(A)+ mRNA. These syntheses of high molecular weight polypeptides are apparently reduced in translations of poly(A)+ mRNA and the resolution of low molecular weight polypeptides is increased. These results indicate that the heat shock polypeptides are translated from polyadenylated mRNAs (Figure 14).

3.4 DISCUSSION

Following incubation at elevated temperatures for brief periods of time, new or enhanced synthesis of a series of heat shock proteins is induced as described in the previous chapters. It has been demonstrated in only a few systems that the alterations in polypeptide synthesis following heat

Figure 14. Fluorographic comparison of 2-D IEF-SDS-PAGE separations of the products from *in vitro* translations of control total RNA (A), heat shock total RNA (B), control poly(A)+ mRNA (C), and heat shock poly(A)+ mRNA (D) in the rabbit reticulocyte translation system. Mrs of the HSPs are indicated on the right and two reference pH values are shown by arrows under each figure. Each IEF gel was loaded with approximately 100,000 cpm of acid-precipitable translation mixture. Fluorograms were exposed for five days. Reproduced from Baszczynski *et al.*, 1983a; Can. J. Biochem. Cell Biol. 61:395-403.


TOTAL RNA
A
B
$M_r \times 10^{-3}$
108
84
89
73
18
POLY(A)+ RNA
C
D
108
84
89
73
18
pH
7.4
6.0
7.4
6.0

shock are the result of changes in transcription (Ashburner and Bonner, 1979; Kelley et al., 1980; Key et al., 1981; Kruger and Benecke, 1981; Schoffl and Key, 1982). In this study (Baszczynski et al., 1983a), fluorographic analysis of the 1-D and 2-D electrophoretically-separated products obtained from the in vitro translations of corn plumule RNA were used to examine the changes in RNA populations associated with the heat shock response in maize.

It was necessary initially to establish the optimal concentrations of the components and conditions which can influence the fidelity of RNA translations, since different conditions can markedly affect the results (Merrifield, 1979). As summarized in Table 3, similar RNA concentrations were suitable in both systems, but the wheat germ system exhibited a strong dependence on high K^+ concentrations (>135 mM) for effective translation of RNAs for the high molecular weight polypeptides. Kapoor (1983) has also found that effective translation of Neurospora RNA in the wheat germ system requires increased K^+ concentrations. While the wheat germ may represent a more homologous system for translating corn mRNAs (based on phylogenetic relatedness), the rabbit reticulocyte system proved to be more effective and practical (the total radioactivity incorporated into newly translated products was consistently higher than (Figure 10), and the resolution and reproducibility of the electrophoretically separated polypeptides was superior to (Figure 11) the wheat germ system). For these reasons,

subsequent *in vitro* translations were conducted utilizing the rabbit reticulocyte system.

The results in Figure 11 (lanes 3-5) indicate that the *in vitro* translation systems used in this study translate exogenously added maize RNA into polypeptides ranging in size from 15 to 100 kilodaltons. The polypeptides synthesized under equivalent temperature conditions *in vivo* are in this same size range. Lanes 2 and 9 (blank lanes), which represent translations in the absence of exogenously added RNA, suggest that there is little or no endogenous RNA in the wheat germ system. Of the two prominent bands noted in the reticulocyte lysate blank lane (lane 2), the lower band (approximately 22 kD) represents the product from translation of an endogenous RNA species. This RNA is presumed to be insensitive to the nuclease treatment used to remove endogenous RNAs from the system (Pelham and Jackson, 1976). Evidence for this being an endogenous RNA comes from the observation that, the intensity of this band decreases as the amount of exogenously added maize RNA increases (compare progression in lanes 3-5 and also in lanes 6-8). The second band (at about 45 kD) may represent an artifact generated by some ribosome-independent process such as the binding of 35S-methionine to a component of the translation kit (Pelham and Jackson, 1976). It does not appear to exhibit changes in intensity with increasing amounts of exogenously added RNA.

In comparing the two *in vitro* translation systems, some

differences were noted in the products obtained from each system (compare control patterns in lanes 3-5 with lane 10). The polypeptide patterns from translations in the rabbit reticulocyte system were consistently more reproducible in these studies. Analysis of the products obtained from translations of RNA from heat-shocked plumules revealed enhanced synthesis of five molecular weight classes of polypeptides (lanes 6-8 and 11). This observation implies that following heat shock in maize, there is a change in either the availability of certain mRNAs or in their ability to be translated. The numerous studies in Drosophila indicate that new or enhanced transcription of mRNAs occurs during heat shock (Ashburner and Bonner, 1979). While the presence of low levels of mRNAs for the HSPs has been demonstrated in non-heat-shocked tissues of Drosophila (Findly and Pederson, 1981; Velazquez et al., 1983), there is little evidence that the mRNAs persist or accumulate in these tissues. The study of Zimmerman and co-workers (1983) showed that mRNAs for the 83 kD, 28 kD, and 26 kD HSPs of Drosophila accumulated in the adult ovarian nurse cells and passed into the oocyte during normal development. It was suggested that this accumulation may be important in regulating gene expression during oogenesis, rather than in increasing the ability of these tissues to respond to heat shock (Zimmerman et al., 1983). Xenopus oocytes which were enucleated or treated with amanitin prior to heat shock still exhibited HSP synthesis, apparently in the absence of

any new RNA transcription. It has been suggested that preformed mRNAs for the HSPs are present in the cytoplasm of these oocytes (Bienz and Gurdon, 1982).

Closer examination of the *in vitro* translated products by 2-D gel electrophoresis (Figure 12) reveals several important features. While many of the polypeptides present in the *in vivo* synthetic patterns are also present among the *in vitro* translated products, some qualitative and quantitative differences exist. These differences may be attributable to: a) differential distribution of the RNA species isolated from plumules relative to those available for translation within plumule tissues; b) differential relative rates of translation of maize RNAs *in vivo* and in the heterologous *in vitro* system (Ballinger and Pardue, 1983); and/or c) translational or post-translational mechanisms such as chemical modification, amino acid substitution, etc., which may alter the mobilities of the polypeptides. Quantitative differences are most likely due to a) or b) above while qualitative differences are probably attributable to size or charge modifications (Scharf and Nover, 1982; Silver *et al.*, 1983).

The apparent absence of translation of a 76 kD HSP *in vitro* suggests that either: a) the mRNAs for the 76 kD HSP are unstable in the *in vitro* system; b) the mRNAs for this HSP class is present but in an untranslatable form; or c) the 76 kD HSP class is not coded for by a unique gene but represents an *in vivo* modification of another heat

shock-induced polypeptide. In the absence of cDNA probes, it is not possible to screen for the presence of a specific gene or its mRNA product. The development of a homologous *in vitro* translation system derived from maize germ might also facilitate discrimination between some of these possibilities. Attempts at production of such a system (F.A. Burr, personal communication) have not been completely successful. The impact of a truly homologous system on the fidelity of RNA translations was elegantly demonstrated in the work of Kruger and Benecke (1981) which showed that a translation system derived from heat-shocked *Drosophila* cells preferentially translated mRNAs for the HSPs. Messenger RNAs for control polypeptides were translated at reduced levels. The reverse situation was noted with a translation system derived from control (25C-grown) cells (Kruger and Benècke, 1981).

The rabbit reticulocyte *in vitro* translation system used in these studies is designed to translate faithfully exogenously added mRNAs without post-translational modifications of the translated products (Pelham and Jackson, 1976). Each *in vitro* synthesized polypeptide therefore, should represent an individual gene product. Examination of the distribution of polypeptides on the basis of isoelectric points reveals the presence of fewer charge variants of the high molecular weight HSPs among the *in vitro* translated products. This observation is especially pronounced at higher temperatures (not shown) and strongly

suggests that at least some of the isoelectric forms noted *in vivo* represent charge modifications of a single polypeptide. Additional spots corresponding to the 18 kD HSP class were frequently observed in different proportions *in vitro*, suggesting differential relative rates of synthesis or levels of processing of these polypeptides *in vivo* and *in vitro*. The studies in soybean have revealed a complex pattern of spots on 2-D gel separations of the 18 kD HSPs (Key *et al.*, 1981). Recent experiments utilizing cDNAs to these heat shock RNAs have shown clearly that while several of the 18 kD HSPs are coded for by unique genes, some of the low molecular weight HSPs represent post-translational modifications of other polypeptides (Schoffl and Key, 1982, 1983).

In addition to the relative changes in the spots observed on 2-D gels, a total and uniform shift in the range of isoelectric points of the polypeptides was also noted in gels of the *in vitro* translated products. The pI value for each polypeptide was approximately 0.6-1.0 pH units lower than the value of its molecular weight analogue *in vivo* (Figure 12). The results in Figure 13 indicate that the pIs of proteins or polypeptides are influenced by the buffer in which samples are prepared. The presence of SDS in the lysate (*in vivo*) yielded pI values for all polypeptides which were more basic than those obtained in the absence of SDS (*in vitro*). Although SDS-protein interactions are unstable in the presence of the non-ionic detergent NP-40,

(used in preparation of samples for loading onto IEF gels)(O'Farrell, 1975), SDS binds to protein with a high binding constant (Ray et al., 1966) such that the NP-40 may not be removing all the SDS. The presence of this tightly associated SDS could account for the more basic pI values obtained for the electrophoretically-separated, in vivo synthesized products. Other buffers have since been shown to also influence the migration of polypeptides in the IEF dimension; in some cases, the pI of a single polypeptide only may be markedly altered (Burr G. Atkinson, personal communication).

A comparison of the products obtained from in vitro translations of total and purified poly(A)+ mRNA (Figure 14) yielded qualitatively similar results. However, translations of poly(A)+ mRNA resulted in enhanced synthesis of low molecular weight polypeptides and the apparent decreased synthesis of high molecular weight polypeptides (including the HSPs). A similar distribution of high and low molecular weight products following in vitro translation of poly(A) + mRNAs have been found in soybean (Key et al., 1981). These differences may reflect the breakdown of larger mRNAs or differential recovery of certain mRNAs following oligo(dT)-cellulose chromatography, but the results establish that the 108, 89, 84, 73 and 18 kD HSP classes are translated from polyadenylated messenger RNAs. Translational and/or post-translational regulatory mechanisms, however, are most likely involved in determining

the final form(s) of these HSPs.

The experiments and analyses in this and the preceeding chapters establish that heat shock in maize induces rapid and reversible changes in the spectrum of polypeptides synthesized. The new and/or enhanced synthesis of heat shock polypeptides is the direct result of an increased transcription or availability of poly(A)+ mRNAs for these HSPs. The variability in rates of appearance, intensification and disappearance of the HSPs, and the differences observed between various temperature treatments suggest that regulation of the molecular events involved in the response is under strict, temperature-dependent control. A critical examination of the influence of different growing temperatures on the subsequent response to temperature shifts is the subject of the next chapter.

CHAPTER 4

POLYPEPTIDE SYNTHESIS IN MAIZE SEEDLINGS SUBJECTED TO A SERIES OF TEMPERATURE SHIFT REGIMES

4.1 INTRODUCTION

The studies described in the preceeding chapters demonstrated that rapid temperature shifts to elevated incubation temperatures (heat shock), lead to dramatic changes in the synthesis of polypeptides and the polyadenylated mRNA transcripts which code for these polypeptides (HSPs). In addition, some interesting observations (Chapter 2) suggested that the precise temperature conditions used for growth and treatment of seedlings might influence the patterns of gene expression. While most of the animal systems in which the heat shock response has been examined have a limited "control" growing temperature (eg., 35-38C for mammalian systems), maize as well as other plant systems will grow and develop "normally" over a broad temperature range (approximately 15C to 35C). This offers the unique opportunity to examine: a) the polypeptide synthetic patterns produced at different growing temperatures; b) the influence of 'growing' temperature on

the subsequent response to heat shock; and c) the polypeptide synthetic changes associated with various temperature shifts which do not exceed the normal growing range for maize (eg., a shift from 20C to 30C). The present investigation demonstrates that the initial growing temperature clearly influences the polypeptide synthetic patterns at each growing temperature and following temperature shifts and heat shock.

4.2 MATERIALS AND METHODS

4.2.1 Growth of seedlings

Seeds of Zea mays L. (cv. Oh43) were germinated in the dark for 4-15 days in incubators preset at temperatures ranging from 15-35C. To minimize developmental differences due to growing temperature, all material used in subsequent experiments was chosen at a morphologically uniform stage (plumules were approximately 1.5 cm long, radicles were approximately 7 cm long).

4.2.2 Temperature treatments and determination of internal seedling temperatures.

Intact seedlings were subjected to a series of temperature shifts over several temperature increments (as noted in Figure legends). In order to measure the actual temperatures within intact seedlings during these temperature treatments, a miniature thermo-couple probe was inserted directly into the plumule of a single seedling and

kept there for the duration of the experiment. Additional probes were inserted into each incubator to obtain simultaneous measurements of temperature changes due to experimental manipulations (eg., opening incubator doors). The probes were connected to a digital recorder (Model CR5, Campbell Scientific, Inc.) and temperature readings were generated and recorded at 20 second intervals. The data were plotted and rates of change in temperature were determined.

4.2.3 Polypeptide extraction and determination of label incorporation into newly synthesized polypeptides.

Following the temperature treatments, intact plumules were labelled for two hours (at either the growing temperature or at the temperature to which they were shifted) with either 100 uCi/mL of 35S-methionine or 33.3 uCi/mL of 14C-leucine as described previously.

To determine the incorporation of radio-labelled amino acid precursor as a function of growing temperature, five replicates of plumules (10 plumules per replicate) were labelled as above and the polypeptides were extracted as before. The incorporated radioactivity and total extracted protein of each replicate were measured as described previously and the mean cpm per ug of protein was determined for seedlings grown at 15, 20, 25, 30 or 35C.

For electrophoretic analysis, individual intact plumules were labelled, polypeptides were extracted and incorporated radioactivity was determined as before.

4.2.4 Isolation and in vitro translation of RNA from seedlings following temperature shifts or heat shock.

Seedlings grown at various temperatures were subjected either to a 10C upward temperature shift or to a heat shock temperature (42C) for various lengths of time as indicated in Figure legends. Total RNA was then isolated as described in the preceeding chapter and used to direct the synthesis of polypeptides in a rabbit reticulocyte *in vitro* translation system with 35S-methionine as the radioactively-labelled precursor.

4.2.5 One- and two-dimensional PAGE analysis of the polypeptides synthesized under different temperature shift regimes.

Lysates from *in vivo* or *in vitro* synthesized polypeptides were diluted with appropriate buffers where necessary (see previous chapters) and were loaded into preformed wells of SDS polyacrylamide slab gels for 1-D separations, or onto the first dimension IEF tube gels for 2-D separations. Gel concentrations and electrophoretic conditions were exactly as described in preceeding chapters except that the IEF gels contained a mixture of ampholines (80% pH range 5-8, 20% pH range 3.5-10, LKB). Specific conditions are indicated in Figure legends.

4.3 RESULTS

4.3.1 Determination of internal seedling temperatures under different growth and temperature shift conditions.

Figure 15 presents a plot of the temperature inside incubators or inside intact plumules as a function of time, for a series of temperature shift regimes (see Figure legends for particulars). Since recordings were made every 20 seconds, very small or rapid fluctuations in temperature could easily be detected. Data indicate that the incubator temperatures remained very steady over the several days of seedling growth (not shown) and during the temperature treatments (Figure 15). The measurements made inside intact plumules following transfer from one incubator to another show that: a) the temperature increases or decreases exponentially with time following the shifts; b) there are no significant fluctuations during the course of the temperature change; c) the internal seedling temperature reaches the incubator temperature after approximately 20 minutes; and d) the rate of change from one temperature to another appears to be the same in both the upward and downward directions. Table 4 summarizes the number of days required for seedlings grown at each of the different temperatures to produce 1.5 cm long plumules.

Figure 15. Plot of preset temperatures inside incubators (solid lines) or changes in temperature inside intact plumules following thermal shifts (squares and triangles) as a function of time. Although readings were obtained every 20 seconds, only every other measurement is plotted for clarity.

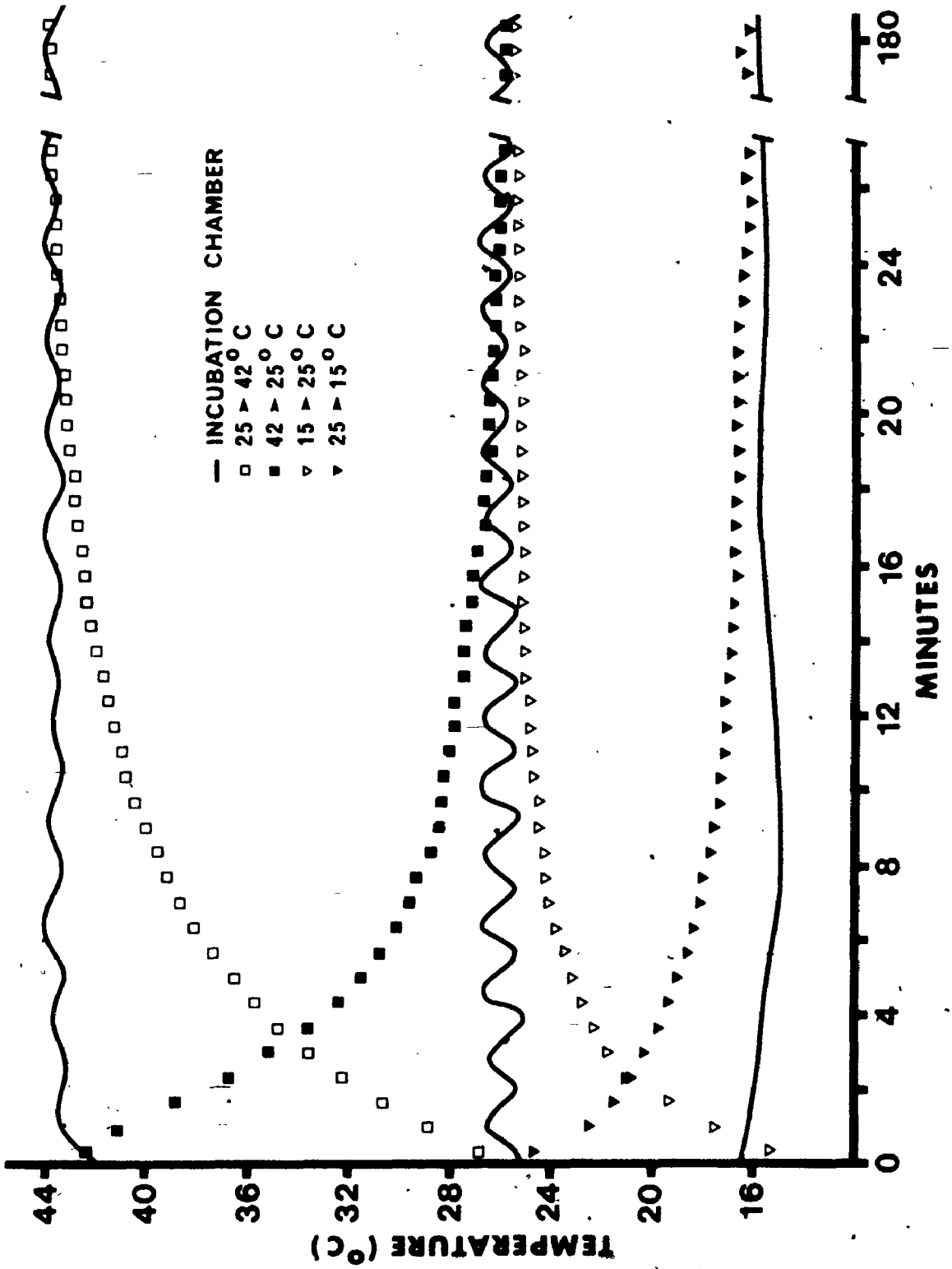

INCUBATION CHAMBER
25 ▸ 42° C
42 ▸ 25° C
15 ▸ 25° C
25 ▸ 15° C
TEMPERATURE (°C)
44
40
36
32
28
24
20
16
0
4
8
12
16
20
24
180
MINUTES

Table IV. Time required for Oh43 seedlings grown at different temperatures to produce 1.5 cm long plumules.

Germination and Growth Temperature (C)	Days for Plumules to Grow 1.5 cm [a]
15 ± 1.5	17 ± 1.5
20 ± 1	11 ± 1
25 ± 1	7 ± 1
30 ± 1	5 ± 0.5
35 ± 1.5	4 ± 0.5

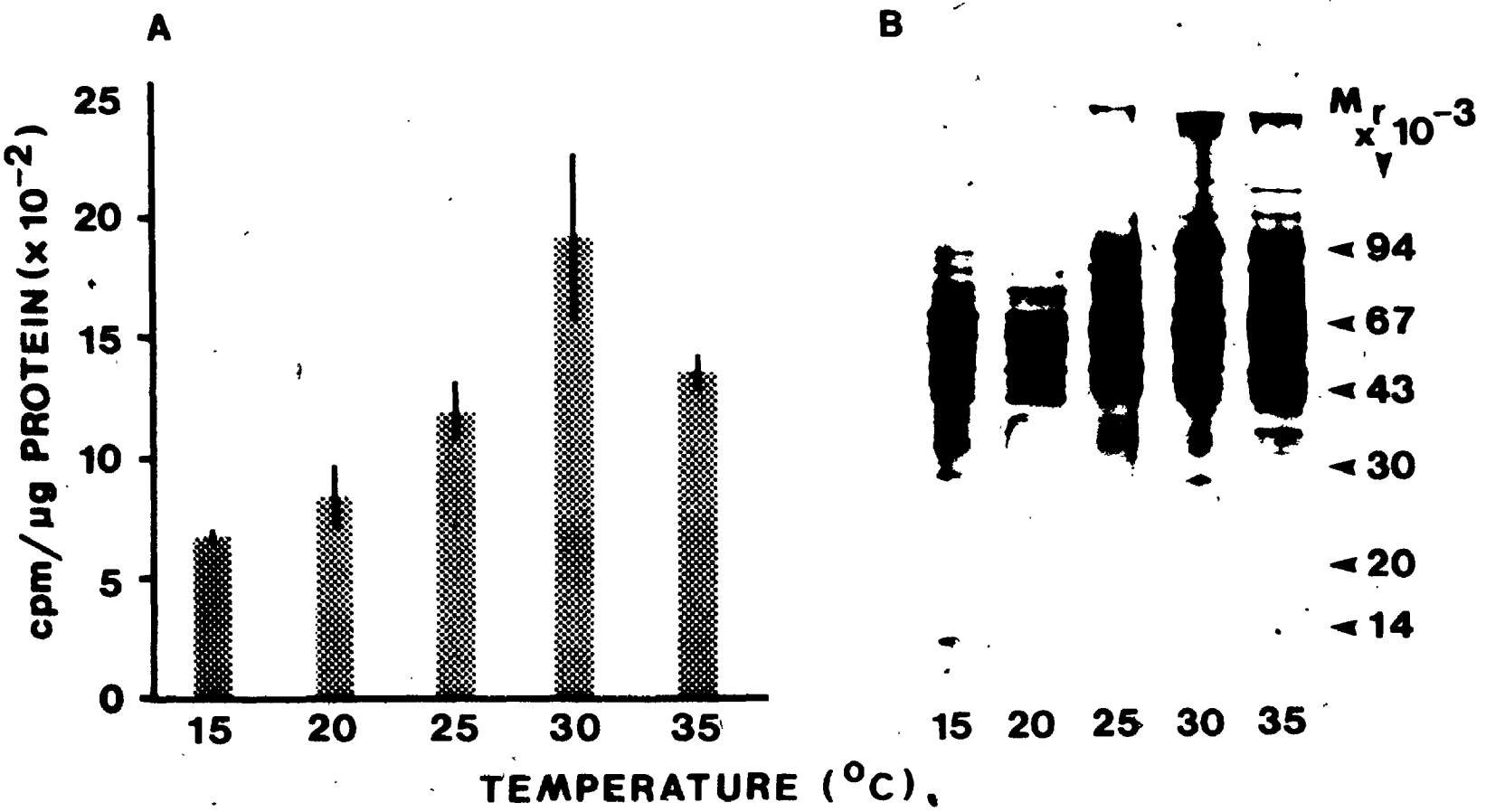
a Since seeds may imbibe water at different rates, the values represent the mean number of days for plumules to grow 1.5 cm.

4.3.2 Label incorporation and one-dimensional SDS-PAGE analysis of polypeptides synthesized at different growing temperatures.

The incorporation of radioactively-labelled amino acid precursors into newly synthesized polypeptides was determined for plumules from seedlings grown at 15, 20, 25, 30 or 35C. The mean level of incorporation (cpm per ug of protein) and standard errors have been plotted in Figure 16A. The incorporation of 35S-methionine into acid-insoluble material from plumules of seedlings grown at 30C is approximately three times higher than for those grown at 15C. Seedlings grown at 35C exhibit less incorporation than those grown at 30C suggesting that the maximum rate of incorporation of precursor into this tissue occurs at approximately 30C.

The polypeptides isolated from plumules of seedlings grown at 15, 20, 25, 30, or 35C were resolved by 1-D SDS-PAGE and subjected to fluorographic analysis (Figure 16B). Many of the polypeptides synthesized at the different temperatures are common; in addition, growth at the various temperatures leads to specific differences in the types of polypeptides which are synthesized. These results indicate that a control polypeptide synthetic pattern must be defined in terms of the temperature at which seedlings are grown since the response to subsequent temperature shifts may be influenced by the initial growing temperature (see next section).

Figure 16. Label incorporation and polypeptide synthesis as a function of growing temperature. 16A. Plot of cpm of 35S-methionine incorporation into acid-precipitable lysate from plumules of maize seedlings grown at 15, 20, 25, 30 or 35C. Vertical black bars represent standard errors of the mean. 16B. Fluorogram of the one-dimensional SDS-PAGE (7.5-17.5% gradient) separations of the newly synthesized polypeptides from plumules of the seedlings grown at 15, 20, 25, 30 or 35C. Positions of relative Mr marker proteins are indicated by arrows on the right. Approximately 20,000 cpm of acid-precipitable lysate was added to each well of the gel. Fluorograms were exposed for five days.


A
B
cpm/µg PROTEIN (x 10⁻²)
25
20
15
10
5
0
15
20
25
30
35
TEMPERATURE (°C)
Mr x 10⁻³
94
67
43
30
20
14
15 20 25 30 35

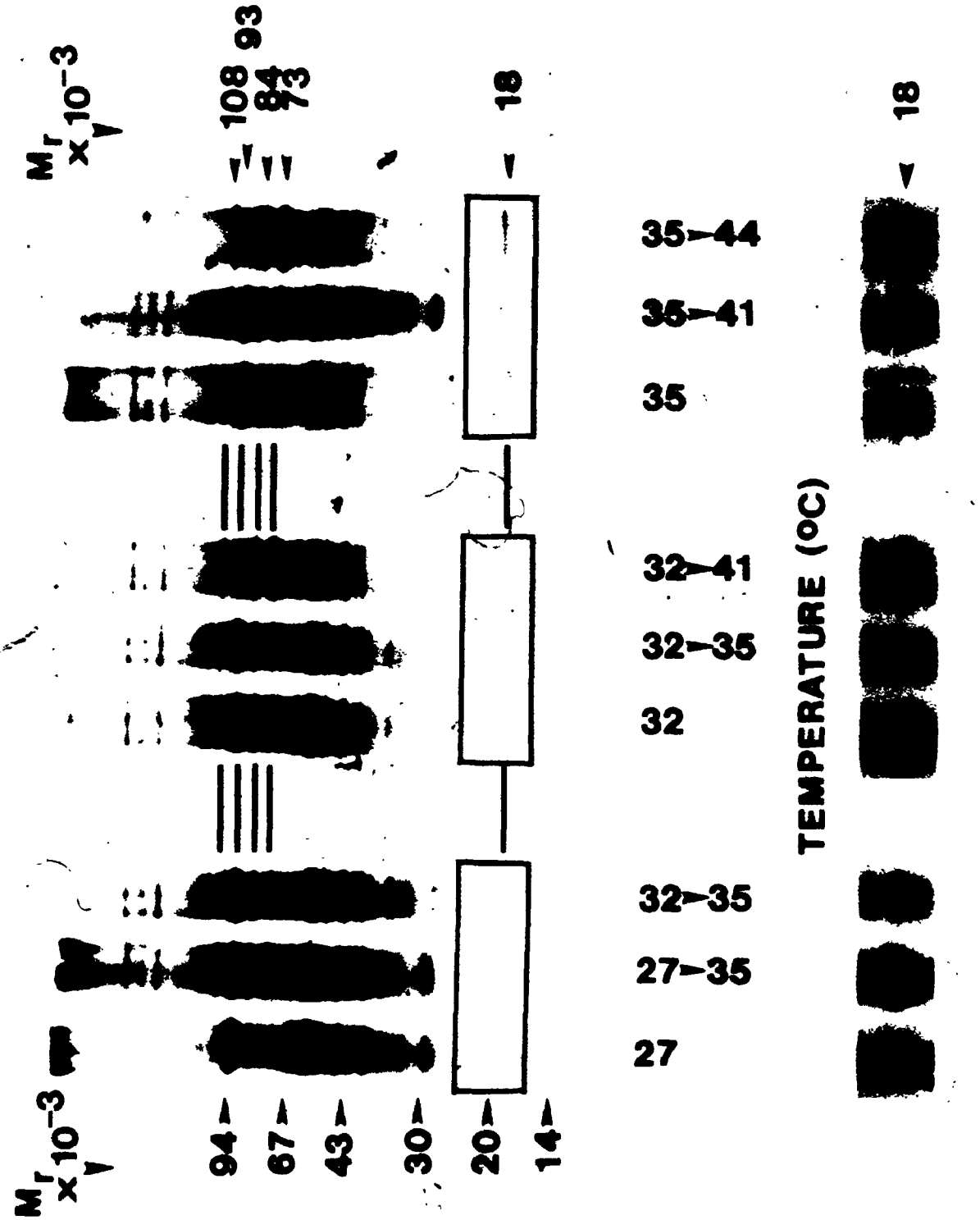
4.3.3 Examination of polypeptide synthesis following different temperature shift regimes.

4.3.3.1 One-dimensional SDS-PAGE comparison of polypeptides synthesized following shifts within the heat shock temperature range.

In the original heat shock studies (Chapter 2), seedlings grown at 27C exhibited a change in synthetic patterns when shifted to 35C or above. To determine whether 35C represents a critical temperature required for the synthesis of HSPs, seedlings grown at 27, 32 or 35C were subjected to various upward temperature shifts (Figure 17). As demonstrated in Chapter 2, a one hour shift from 27C to 35C leads to the enhanced synthesis of the six HSP classes (Figure 17, lane 2). However, when seedlings grown at 32C (Figure 17, lane 4) are shifted to 35C for one hour (Figure 17, lanes 3 and 5), there is no appreciable increase in synthesis of these HSPs. It may also be noted that the synthetic patterns from seedlings grown at 27C or at 32C exhibit some differences; these differences are also present following the shifts to 35C suggesting that the initial growing temperature may influence the polypeptide synthetic changes induced following the thermal shifts. Seedlings grown at 35C (Figure 17, lane 7) do not show synthesis of the HSPs but yield a synthetic pattern which generally resembles that of the 27C or 32C grown seedlings (data from Figure 16B substantiate this).

Temperature shifts from 27C to 35C (Figure 17, lane 2) and from 35C to 41C (Figure 17, lane 8) result in

Figure 17. Fluorograms of the 1-D SDS-PAGE (3-15% gradient) separation of polypeptides synthesized at 27, 32 or 35C, and following one hour temperature shifts over the increments indicated under each lane. Regions of the fluorograms outlined in grey rectangles have been overexposed and reproduced below each lane to emphasize the 15-25 kD region of the gels. Positions of Mr marker proteins are indicated by arrows on the left. Positions of reference HSPs and the prominent 93 kD control (27C) polypeptide are indicated by arrows on the left. Approximately 25,000 cpm of acid-precipitable lysate was added to each well of the gels. Fluorograms were exposed for five days. Overexposed regions were exposed for ten days.

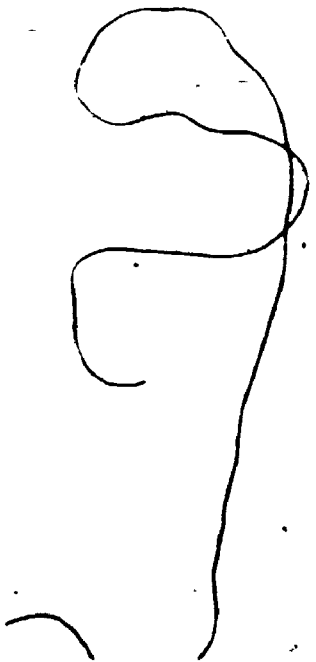

M_r x 10^{-3}
108
93
84
73
18
18
35–44
35–41
35
32–41
32–35
32
32–35
27–35
27
TEMPERATURE (°C)
M_r x 10^{-3}
94
67
43
30
20
14

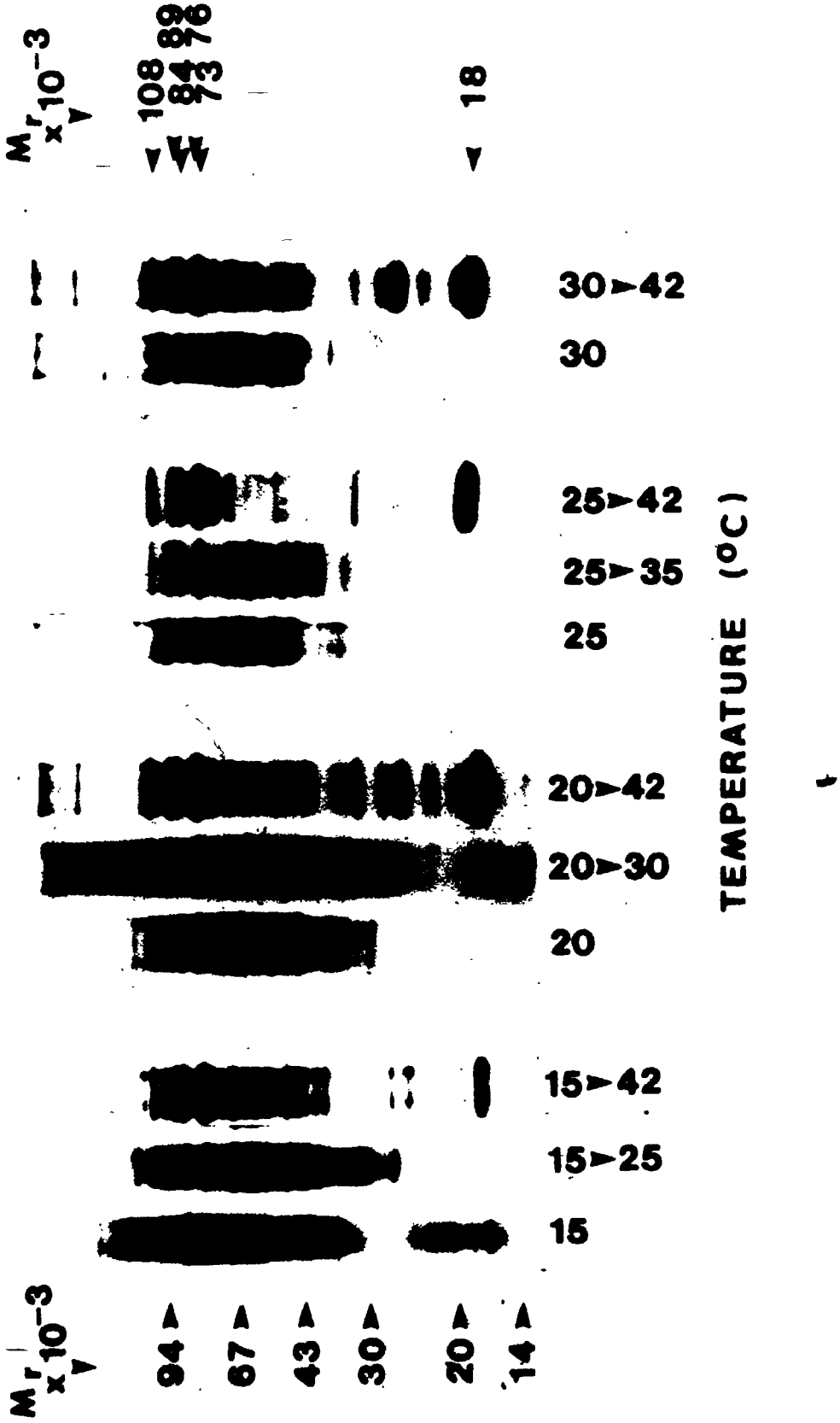
polypeptide synthetic patterns which are very similar in terms of the relative intensities of all bands (including the HSP bands). This suggests that the degree to which enhanced synthesis of some polypeptide classes and depressed synthesis of other classes occurs also is dependent on the initial or pre-shift temperature, and on the temperature increment over which the seedlings are shifted.

4.3.3.2 One-dimensional SDS-PAGE comparison of polypeptide synthesis following a range of temperature shifts.

To examine more closely the influence of growing temperature on the response of seedlings to subsequent temperature shifts, maize seedlings were grown at 15, 20, 25, 30 or 35C and subjected to either a 10C upward temperature shift or to a heat shock temperature (42C) for one hour. Fluorographic analysis (Figure 18) reveals that each growing temperature yields a unique synthetic pattern (as was also shown in Figure 16) and that a one hour heat shock at 42C leads to enhanced synthesis of the same six molecular weight HSP classes. Additional bands also exhibit enhanced synthesis following heat shock; these are dependent on the initial growing temperature. For example, a polypeptide with an approximate molecular weight of 31 kD is strongly enhanced following a heat shock from 20C to 42C, is less intense following a heat shock from 25C to 42C or from 30C to 42C, and is not present following a heat shock from 15C to 42C. Similarly, a 23 kD polypeptide intensifies strongly following a shift from 30C to 42C but shows much

Figure 18. Fluorograms of the 1-D SDS-PAGE (7.5-17.5 gradient) separations of the polypeptides synthesized in plumules of maize seedlings grown at 15, 20, 25 or 30C, and following either 10C upward temperature shifts, or shifts to a common heat shock temperature (42C) as indicated under each lane. Positions of relative Mr marker proteins are indicated by arrows on left while the positions of the six major HSP classes are indicated on the right. Approximately 25,000 cpm of acid-precipitable lysate was applied to each well of the gels. Fluorograms were exposed for five days.




M_r x 10^{-3}
108
89
84
76
73
18
30→42
30
25→42
25→35
25
20→42
20→30
20
15→42
15→25
15
TEMPERATURE (°C)
M_r x 10^{-3}
94
67
43
30
20
14

less enhancement following a shift to 42C from 15, 20 or 25C. These results confirm that the final array of heat shock polypeptides is markedly influenced by the initial (pre-shift) temperature.

Examination of lanes 2, 5, and 8 (Figure 18) reveals that a 10C upward temperature shift also leads to the enhanced synthesis of some of the high molecular weight HSP classes (compare especially lanes 5 and 6). This observation strongly suggests that enhanced synthesis of these polypeptides may not necessarily be the result of heat shock, but may reflect a synthetic change in response to temperature shifts in general. The synthesis of the low molecular weight 18 kD HSP class is not observed following these 10C upward temperature shifts, suggesting that it may represent a "heat-shock-specific" class of polypeptides. The 18 kD HSP has not been detected even in extensively over-exposed fluorograms of polypeptide patterns from plumules shifted from 15C to 25C, or from 20C to 30C.

4.3.3.3 Two-dimensional IEF-SDS-PAGE comparison of in vivo and in vitro synthesized polypeptides following temperature shifts and heat shock.

Although new or enhanced synthesis of some of the HSP classes is noted following temperature shifts within the normal growing range for maize (previous section), the 18 kD class is not detected following these shifts and the entire response is much less dramatic than that observed following heat shock. To assess whether differences exist in the

levels of available mRNAs for these polypeptides following the two temperature shift strategies (ie., 15-25C versus 25-42C), total plumule RNA was isolated and translated *in vitro* in a rabbit reticulocyte system as described previously. A comparison is presented in Figure 19 of the polypeptides synthesized *in vivo* and the products obtained from the *in vitro* translation of RNA isolated from plumules following the 15-25C temperature shifts. The results from fluorographic analysis of the 2-D gel separations indicate that while a 15-25C shift leads to slight enhancement in the synthesis of high molecular weight HSPs *in vivo*, all HSP classes exhibit substantially higher levels of synthesis when one examines the products derived from the *in vitro* translation of mRNAs isolated from seedlings following the same shift (15-25C). Most striking is the 18 kD class which is not detected following the 15-25C shift *in vivo*, but is prominent in electrophoretic separations of the *in vitro* translated products. Similar results have been obtained following shifts from 15-25C and 20-30C. This finding implies that the mRNAs for this 18 kD class of polypeptides as well as the other HSPs are present following these lower temperature shifts, but that these RNAs are not translated as efficiently *in vivo*.

The changes in availability of the mRNAs for these polypeptides as a function of time following temperature shift were examined as shown in Figure 20. The results reveal that all molecular weight classes of the HSPs can be

Figure 19. Fluorograms of the 2-D IEF-SDS-PAGE separations comparing the polypeptides synthesized *in vivo* in plumules of maize seedlings grown at 15C or following a 15-25C shift, with the products obtained from the *in vitro* translation of RNAs isolated from plumules of seedlings subjected to the same temperature conditions. The IEF gels contained a mixture of 80% pH range 5-8 and 20% pH range 3.5-10 ampholines. The second dimension consisted of a 7.5-17.5% linear gradient gel. Positions of reference HSPs are indicated by arrows on the left. Arrows on fluorograms point to expected positions of the 108 kD (top), 73-76 kD (middle) and 18 kD (bottom) HSPs in each gel. Approximately 100,000 cpm of acid-precipitable lysate was applied to each IEF gel. Fluorograms were exposed for five days.

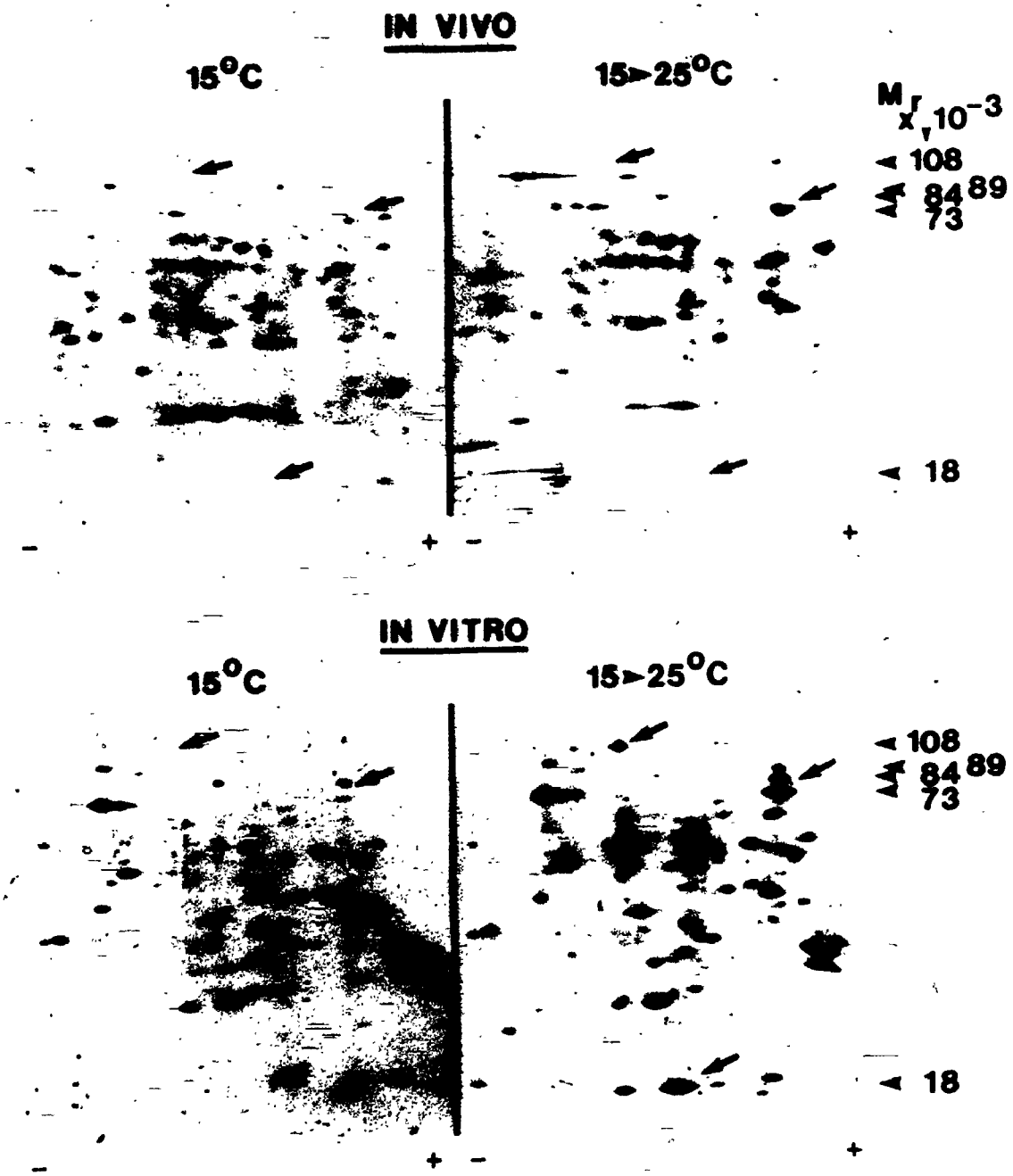
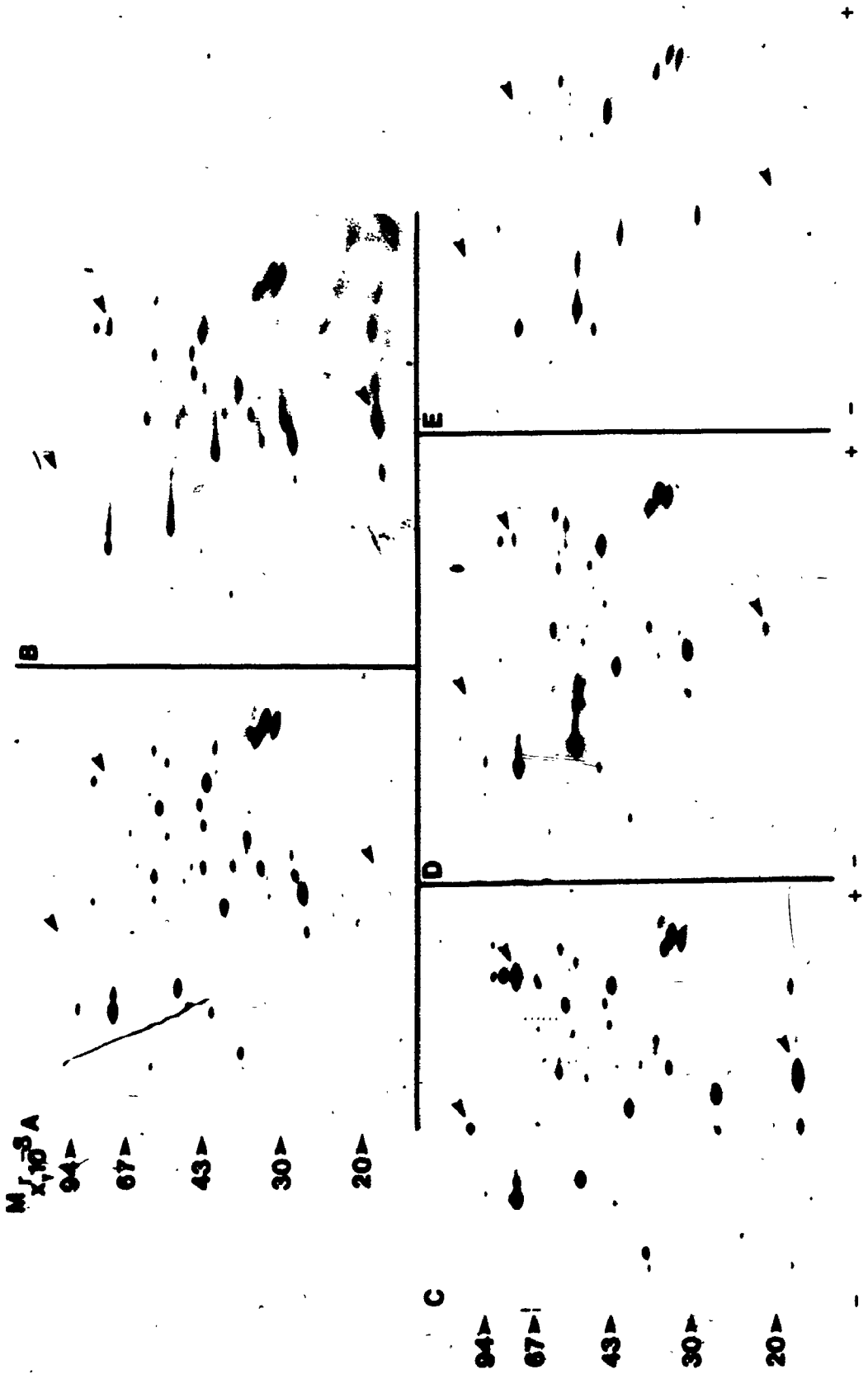

IN VIVO
15°C
15→25°C
$M_r \times 10^{-3}$
108
84
89
73
18
IN VITRO
15°C
15→25°C
108
84
89
73
18

Figure 20. Fluorograms of the 2-D IEF-SDS-PAGE separations of the products obtained from the *in vitro* translation of RNAs isolated from plumules of maize seedlings grown at 15C (A), or following a shift from 15C to 25C for 0.5 (B), 1 (C), 3 (D) or 6 (E) hours. Gel conditions were exactly as described for Figure 19. Positions of relative Mr marker proteins are indicated by arrows on the left. Arrows on the fluorograms point to corresponding expected positions of the 108 kD (top), 73-76 kD (middle) and 18 kD (bottom) HSP classes. Approximately 100,000 cpm of acid-precipitable lysate was applied to each IEF gel. Fluorograms were exposed for five days.


E
B
D
C
$M_r \times 10^{-3}$
94
67
43
30
20
94
67
43
30
20

detected among the *in vitro* translated products by 30 minutes following a shift from 15C to 25C. These intensify to a maximum at approximately 60 minutes and then exhibit a reduction in intensity over the next five hours at 25C, presumably due to a decrease in the population of translatable mRNAs for these polypeptides.

4.4 DISCUSSION

While the response to high temperature shock has been examined in many organisms, few studies have focussed on the broader aspect of thermal shift-induced changes in gene expression (where the temperature shift may represent a change that does not exceed the normal growing temperature range of the organism). The heat shock studies have revealed that the extent to which HSP synthesis occurs is clearly dependent on the temperature to which the organisms are shifted; generally, higher temperatures (to a limit) yield more intense synthesis of HSPs (Barnett *et al.*, 1980; Fink and Zeuthen, 1980; Lindquist, 1980; Tanguay and Vincent, 1981; Baszczynski *et al.*, 1982a; Ketola-Pirie and Atkinson, 1983). In addition, Lindquist (1980) has shown that the syntheses of the HSPs in *Drosophila* vary independently of each other at different temperatures. Studies in two systems (*Tetrahymena* and *Rana*) have also revealed that downward temperature shifts (cold shock) lead to induction of novel gene activity (Fink and Zeuthen, 1980;

Ketola-Pirie and Atkinson, 1983). These observations suggest that alterations in the patterns of polypeptide synthesis are dependent on the temperature increment over which the organism is shifted, and that changes in gene expression may represent a general response to thermal shifts. This latter hypothesis has been difficult to test due to the restricted normal growing temperature range of most of the animal systems studied. Maize on the other hand (like many plants) can grow and develop normally at temperatures ranging from less than 15C to greater than 35C and thus provides an ideal system for assessing the impact of growing temperature and different temperature shift regimes on gene expression.

The rate of growth and development of maize seedlings is clearly dependent on the growing temperature (Table 4). This is not surprising since the time to complete one nuclear cycle in maize (cv. Seneca 60) varies from 16.5 hours at 20C to 4.4 hours at 35C (Verma, 1972). However, when the plumules are 1-2 cm long, they are morphologically indistinguishable. This difference in growth rate is also reflected in the differences in levels of incorporation of radio-labelled amino acid precursor into protein (Figure 16A). The incorporation is approximately three times higher at 30C than at 15C and the growth rate is approximately three times faster at 30C than at 15C (Table 4). It is somewhat surprising that the incorporation rate is lower at 35C than at 30C; this may however reflect an increased rate

of turnover such that the net rate of label incorporation appears to be lower.

Examination of the patterns of polypeptide synthesis from seedlings grown at 15, 20, 25, 30 or 35C reveals that many similarities exist (especially in the polypeptides of molecular masses ranging from 40 to 70 kD), although each growing temperature yields a unique characteristic pattern of newly synthesized polypeptides (Figure 16B). A prominent 93 kD polypeptide is actively synthesized when seedlings are grown at 25, 30 or 35C but not when grown at 15 or 20C. Similarly, a 70 kD polypeptide is more pronounced in plumules from seedlings grown at 15C than from seedlings grown at the four other temperatures. These results indicate that a difference of 5 to 10C degrees in growing temperature is sufficient to yield a different "control" pattern of synthesis. This enables an examination of the changes in polypeptide synthesis during the transition between two temperatures for which the starting and final polypeptide spectra are defined. Such a study would determine for example, whether a shift from 20C to 30C leads to a gradual transition from the 20C pattern to the 30C pattern, or whether the shift leads to the production of a unique set of transition proteins (analogous to those found during the response of maize to anaerobiosis (Sachs et al., 1980)) prior to establishing the 30C synthetic pattern.

Before studying the synthetic changes following temperature shifts within the 15 to 30C temperature range, a

series of temperature shifts in the heat shock range were conducted to establish whether there was a threshold temperature below which HSP synthesis did not occur. The results in Figure 17 indicate that HSP synthesis is not necessarily induced by a shift to an absolute temperature (ie., 35C), but rather by a shift which exceeds a certain temperature increment (eg. 8C for seedlings grown at 27C). Moreover, the relative intensities of synthesis of the HSPs are dependent on both the temperature increment over which the seedlings are shifted and the absolute temperature in which the shift occurs (see Table 5).

Examination of the influence of temperature increment on HSP synthesis in Drosophila has revealed that all HSPs do not intensify uniformly with increasing temperature. A shift to 33C from the control temperature of 23C leads to markedly increased synthesis of the 82 kD HSP. The synthesis of this polypeptide increases to a much lesser extent (approximately 50%) when the Drosophila cell cultures are shifted to 38C. On the other hand the 70 kD HSP intensifies slightly following a shift to 33C but exhibits dramatic enhancement of synthesis when shifted to 37C or 38C. Synthesis of the low molecular weight Drosophila HSPs is most intense following a shift to 35C or 37C (Lindquist, 1980). The rate and sequence in which synthesis of HSPs becomes depressed in subsequent recoveries is also dependent on the interval over which the cells were shifted (DiDomenico et al., 1982). Other studies have also shown

Table V. Relative intensity of HSP synthesis as a function of temperature increment.

Temperature or Temperature Shift (C)	ΔC	Relative Intensity of HSP Synthesis
27	0	-
32	0	-
35	0	-
32-35	3	+/-
35-41	6	++
27-35	8	++
32-41	9	+++
35-44	9	++++

++++ HSP synthesis very intense
- HSP synthesis undetectable
+/- HSP synthesis just detectable

that the temperature increment is important in determining the extent to which HSP synthesis occurs (Atkinson, 1981; Key et al., 1981; Baszczynski et al., 1982a; Kapoor, 1983).

The investigations which revealed apparent differences in the levels of synthesis of HSPs within the heat shock range for maize were subsequently extended in order to examine the response to temperature shifts over a broader range of temperatures. The results indicate that different growing temperatures yield unique polypeptide synthetic patterns; more importantly, the initial or "pre-shift" temperature markedly influences the final array of polypeptides produced in response to various temperature shifts (Figure 18). It is also apparent from Figure 18 that a variety of temperature shifts (many within the normal growing range for maize) give rise to the enhanced synthesis of some of the high molecular weight HSPs. The low molecular weight, 18 kD class of maize HSPs, on the other hand, does not exhibit enhanced synthesis following these temperature shifts. This is observed both in 1-D and 2-D gel electrophoretic separations. These findings suggest that the high molecular weight HSPs may represent a more general class of polypeptides synthesized following a range of temperature shifts; from previous observations, this synthesis is most likely dependent on the increment over which the shift is made and the absolute temperature range within which the shift is carried out. Thus a 10C shift

from 20C to 30C results in more intense synthesis of these polypeptides than a 10C shift from 15C to 25C (Figure 18, lanes 5 and 2, respectively).

This latter aspect of the present investigation is therefore unique since no other studies have examined the impact of different growing temperatures on the changes in polypeptide synthesis following subsequent thermal shifts. Furthermore, these findings reveal that changes in polypeptide synthesis occur as a general response to thermal shifts, and suggest that this response may represent a transitional alteration in gene expression. The earlier observation that maintenance of maize seedlings at a heat shock temperature does not lead to continued HSP synthesis (see Table 1) but rather to a gradual disappearance of HSPs and an establishment of a new, stable 'control-like' pattern of polypeptide synthesis, supports the hypothesis that this alteration in gene expression may be a transitional response necessary for 'acclimatization' of the seedlings to the new temperature.

Examination of the products from <u>in vitro</u> translations of RNA isolated from plumules following various temperature shifts further supports this hypothesis. The results from this study revealed that the mRNAs for all classes of HSPs (including the 18 kD class) were available for translation by 30 minutes following a shift from 15C to 25C but were no longer abundant at six hours following the shift to 25C. Thus both the polypeptides and their RNAs exhibit a

transitional enhancement of synthesis. The relative levels of synthesis of these polypeptides *in vitro* were similar following a one hour shift either from 15C to 25C or from 25C to 42C, whereas *in vivo*, synthesis of HSPs was much less intense following the 15C to 25C shift.

DiDomenico and co-workers (1982) have shown that the level of the 70 kD HSP in *Drosophila* cells regulates the degree of accumulation of the mRNAs for this HSP. Following the accumulation of a specific quantity of the 70 kD HSP, further transcription is repressed and mRNAs are destabilized such that additional 70 kD HSP production is prevented. The system thus regulates itself. If the production of functional 70 kD HSP is blocked by growing cells in the presence of the amino acid analogue canavanine (which substitutes for arginine), transcription of mRNAs for this HSP continues resulting in the accumulation of these mRNAs to very high levels. When the block is removed, HSP resumes and a certain quantity of functional 70 kD HSP must accumulate before mRNA levels return to normal (DiDomenico *et al.*, 1982). The transitory nature of mRNA and polypeptide synthesis observed in maize may represent a similar regulatory mechanism whereby increased transcription following thermal shifts leads to synthesis of temperature shift polypeptides which in turn repress further mRNA transcription and subsequent polypeptide production. Acclimatization of the seedlings to the new temperature then leads to a gradual disappearance of these temperature shift

polypeptides and their mRNAs and to the establishment of a pattern of polypeptide synthesis specific to the new temperature.

CHAPTER 5

IMMUNOCHEMICAL ANALYSIS OF THE HEAT SHOCK RESPONSE IN MAIZE

5.1 INTRODUCTION

Antibodies prepared against specific antigens provide a sensitive assay system for the detection and discrimination of that antigen. Since the antibodies recognize specific three-dimensional molecular configurations, similarities in the molecular structure of antigens from different sources may be ascertained. Based on the apparent universal occurrence of heat shock proteins, there has been speculation that certain HSPs (most notably the 70kD HSP) may be related in different species and among different tissues of a single organism. While the use of antibodies (polyclonal or monoclonal) may not contribute to an understanding of the functional similarities between HSPs from different sources, they may identify homologous structural domains in these molecules. This section describes the purification of individual molecular weight classes of the maize HSPs, the production of polyclonal antibodies, and the immunochemical characterization of the

maize HSPs.

5.2 MATERIALS AND METHODS

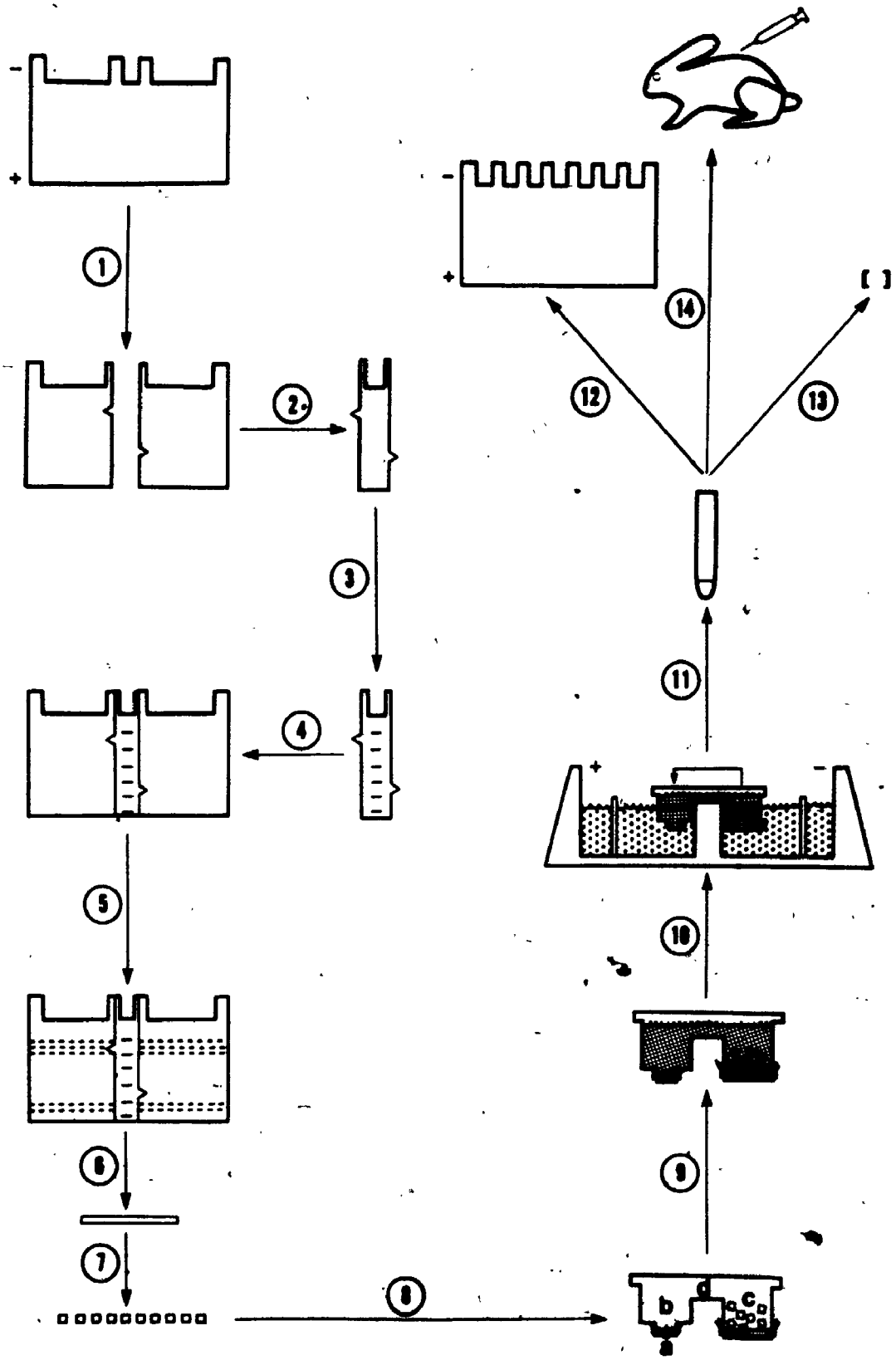
5.2.1 Antigen purification and purity monitoring.

Seedlings of Oh43 were germinated at 25, 27 or 30C as described previously. The Petri dishes containing the intact 5-6 day old seedlings were subsequently transferred for three hours to an incubator preset to 42C. After one hour, 15 seedlings were removed, the plumules (1-1.5 cm) were rapidly excised and placed into 1.0 mL of an aqueous solution containing 200 uCi/mL of 35S-methionine (New England Nuclear, 1042 Ci/mmol) which had also been pre-incubated at 42C, and the labelling medium containing the plumules was returned to the 42C incubator. The latter manipulations were conducted in the dark and as rapidly as possible to minimize temperature fluctuations. At the end of the three hour incubation period, the excised plumules were removed from the labelling medium and rinsed thoroughly. The plumules from the remaining unlabelled seedlings were excised, the labelled and unlabelled plumules were pooled, and polypeptides were extracted as described in Chapter 2.

Samples of the extracted proteins were loaded into preformed wells of a one-dimensional SDS polyacrylamide slab gradient (7.5-17.5%) gel overlayed with a 3% stacking gel. As shown schematically in the flow chart in Figure 21, the

Figure 21. Flowchart of the protocol used for isolation of maize HSPs for antibody production.

1. Total polypeptide extracts in large wells and standard marker proteins in center well are separated by SDS-PAGE.
2. Gel slice containing resolved marker proteins is excised and removed from slab gel.
3. Gel slice is rapidly stained to permit visualization of resolved marker proteins.
4. Stained slice is re-positioned in slab gel.
5. Regions of slab gel corresponding to positions of desired polypeptides are identified and cut out.
6. Slices are carefully removed from gel.
7. Slices are cut up into small pieces.
8. Gel pieces are transferred to concentration cup chamber c.
9. Buffers (see Materials and Methods) are added to chambers a, b, c and d of the concentration cup.
10. Polypeptides are electroeluted from gel pieces and concentrated into chamber a.
11. Concentrated protein sample is collected.
12. Purity is ascertained by re-electrophoresis and fluorography.
13. Concentration of purified protein is determined.
14. Pure protein samples are used for antibody production in rabbits.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2
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5
6
7
8
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10
11
12
13
14
[ ]
b
c

two outside wells were approximately 5 cm wide by 1-1.5 cm deep to facilitate the loading of larger volumes of sample extract. The centre well was 1 cm wide by 1-1.5 cm deep and was used for loading either a small aliquot of the total extract, or standard Mr marker proteins. Approximately 250-300 uL of sample containing 0.75-1.5 mg of total extracted protein were loaded into each of the larger wells of the gel. Six to eight gels were routinely loaded and run per day for four to five days to get sufficient purified protein for immunization of rabbits. The gels were electrophoresed as described previously.

At the end of the run, the gel moulds were removed from the electrophoresis tanks, the glass plates were pried apart such that the gel remained on one of the plates, and the centre lane was sliced from the gel as shown in the schematic diagram. Notches were inserted during the slicing of the centre lane such that the strip could be removed and later re-inserted in the correct orientation.

The centre gel slice was removed, placed in a fresh solution of 25% methanol: 10% acetic acid containing 0.2% Coomasie brilliant blue R-250,and agitated vigorously for 20-30 minutes. The remainder of the slab gel was wrapped in Saran wrap and placed at 4C to minimize protein diffusion. After 20-30 minutes in fresh stain, the dark blue bands could usually be detected against the lighter blue background. Occasionally, a brief destaining period (20-30 minutes) in fresh 25% methanol:10% acetic acid was required

to allow detection of the bands. The time involved in staining and destaining was kept to a minimum in order to decrease the extent of diffusion of proteins in the slab gels. The proportion of methanol in the staining and destaining solutions was found to be important since higher levels of methanol led to shrinkage of the gel slices, while lower amounts resulted in expansion of the slices. These latter distortions made re-alignment of the slab gel and the gel slice difficult and band isolation questionable.

When the bands in the gel slices were visible, the slice was rinsed in distilled water and placed back into its slot in the slab gel. Sections from the slab gels corresponding to the regions identified previously in the gel slice as containing the various HSP molecular weight classes were cut out. The sections were transferred to a clean glass plate, chopped up into small pieces (approximately 2x3x3 mm), and transferred into the concentration cups of an ISCO Model 1750 Sample Concentrator (ISCO). Each double-chambered cup contained the gel pieces corresponding to one molecular weight class of hsps from one slab gel; eight to twelve cups were routinely used. As illustrated in the schematic in Figure 21, the cups consisted of a large chamber into which the gel pieces were placed and a small 200 uL chamber into which proteins were concentrated. Dialysis membrane, placed on the bottom of each chamber, permitted ion flow through the solution in the cup. The small chamber was filled with 200 uL of cup buffer

(0.057 M Tris-acetate, pH 8.6) containing 2% sucrose, and the remainder of the cup was filled with cup buffer such that the contents of the concentration chamber were not disturbed. The cups were positioned in a buffer tank containing Tris-acetate buffer (pH 8.6) and the proteins were electro-eluted from the gel pieces for 4-5 hours at 1-1.5 watts per cup. All manipulations were conducted using gloves to eliminate protein contamination. At the end of the concentration period, the buffer was carefully removed from the cups such that the concentration chambers remained undisturbed. The concentrated protein solutions were then removed from the small chambers using a 1 mL syringe fitted with a 23 gauge needle to which was attached a small piece of Tygon tubing (to prevent puncturing of the dialysis membrane). The concentrated protein solutions (total volume of 200 uL) were transferred to clean 3 mL glass tubes and made 2 mM with respect to phenylmethylsulfonylfluoride (PMSF). The samples from each cup initially were kept separate until the protein purity and concentration could be determined. The concentrated samples were frozen and stored at -70C until required.

The protein concentration of each sample was determined by the method of Lowry (1951) using bovine serum albumin (prepared in cup buffer with sucrose) as a standard. Small aliquots of each of the concentrated samples were electrophoresed on one-dimensional slab gels and the separated polypeptides were analyzed by fluorography. The

presence of a single band in the case of the 18 kD HSP class, and a doublet of bands in the case of either the 73-76 kD or the 84-89 kD HSP classes, were indicative of high purity. Those samples which did not exhibit this degree of purity were pooled, re-electrophoresed, reconcentrated and checked for increased purification. Those samples within each molecular weight class which exhibited the greatest purity and highest protein concentration were pooled.

Since the total volume of the sample had to be reduced prior to injection, alum precipitates of each of the pooled samples were prepared as described by Lazarides and Hubbard (1976). One-ninth volume of an aqueous 10% aluminum chloride solution was added dropwise with continuous vortexing to the protein sample. The pH of the mixture was then adjusted to 7.4 with 1.0 M NaOH and the solution was allowed to stand at room temperature for 30 minutes. The slurry was diluted with one-third volume of phosphate buffered saline (PBS), pH 7.4, and centrifuged at 300xg for 10 minutes. The pellet was washed twice in PBS and finally resuspended in a small amount of PBS to bring the total volume to approximately 500-750 uL.

Each alum precipitate was mixed subsequently (1:1,v/v) with complete Freund's adjuvant (CFA, Gibco) in 10 mL Bijou bottles. The CFA was added in 4-5 small aliquots and the mixture was vortexed vigorously between each addition. After the final addition of CFA, the mixture was further

vortexed until the resulting emulsion remained aggregated when a droplet was placed on water. The emulsion (approximately 1-1.5 mL) was transferred into a 3 mL syringe fitted with a 1.5 inch long, 20 gauge needle.

5.2.2 Injection of rabbits, boosting and blood collection.

Six month old New Zealand white female rabbits were housed in wire cages, and food (Purina rabbit chow) and water were supplied ad libitum. The rabbits were allowed to acclimatize to their new environment for six to eight weeks prior to being used for immunological studies. Before injecting the rabbits, a sample of blood was collected and used for the preparation of pre-immune serum. A small incision was made in the central artery of the ear and approximately 10-15 mL of whole blood was collected in 40 mL glass Corex tubes. The blood was placed at 37C for 30 minutes and then at 4C for 18-24 hours to permit clotting. The blood samples were then centrifuged at 1000xg for 20 minutes and the sera were transferred to 15 mL conical tubes and centrifuged at 1000xg for 8 minutes to remove contaminating red blood cells. The sera were incubated for 30 minutes at 56C to inactivate complement and then stored at -70C until required.

The rabbits were injected intradermally or subcutaneously in the back of the neck in 5-6 spots with the prepared antigens. Two rabbits were injected with the 18 kD HSPs, one with the 73-76 kD HSPs, one with the 84-89 kD

HSPs, and one with the total high molecular weight group (73-89 kD) of HSPs. Eight weeks later the rabbits were boosted (with antigens prepared in the same way) via a deep muscular injection in the hind legs. The rabbits were boosted a second time, four to six weeks later, and then bled 10 to 14 days after that. The blood was collected from the marginal ear vein with the aid of an ear bleeding apparatus (Bellco) and mild vacuum. The whole blood (approximately 35-40 mL) was allowed to clot and the immune sera were prepared as described previously for pre-immune sera.

5.2.3 Characterization of antisera.

5.2.3.1 Identification of reacting polyclonal antibodies.

The presence of reacting antibodies in the sera was determined by ring tests, Ouchterlony double diffusion tests and immunoblot analyses. For the ring test analysis, a 50 uL aliquot of each serum (including pre-immune sera) was placed in a 500 uL glass test tube. These sera were then carefully overlayed with a 50 uL aliquot of each of the purified antigens. The tubes were allowed to sit at room temperature for 60-120 minutes, after which the interfaces between the antigens and antisera were examined for the presence of a white precipitate (indicating positive reaction). Ouchterlony plates were prepared by pouring 3.0 mL of melted 1% Noble agar in 20 mM borate buffered saline (BBS), pH 8.4, onto standard glass microscope slides. Wells

were cut out using a standard gel hole puncher. The centre well in each case was filled with 10 uL of one of the antisera and the surrounding six wells contained the various purified antigens arranged such that a) each antigen-antibody combination could be tested, and b) cross-reactivity between antigens could be detected (see Figure legends and Results section for particulars). The gels were placed in a humid chamber and proteins were allowed to diffuse for 24 hours at room temperature. The Ouchterlony plates were then immersed in cold BBS and placed at 4C for 24 hours. The BBS was replaced every 12-24 hours for four days, to remove unreacted proteins from the gel. The plates were removed from the BBS solution, inverted onto Whatman No. 1 filter paper and allowed to dry for 24 hours. When dry, the filter paper was peeled off, the gel surface was lightly wiped with water to remove residual filter paper, and the plates were stained in 2% Coomasie brilliant blue R-250 in BBS. Excess stain was removed by rinsing in BBS and the plates were allowed to air dry prior to photographing (as described previously for gels).

As a further test for the presence of reacting antibodies, the sera were utilized for immunoblot analysis. Total polypeptide extracts from control and heat-shocked plumules were subjected to one-dimensional PAGE as described previously. At the end of the electrophoretic run, the slab gels were removed from the gel moulds and prepared for electro-blotting as outlined in the Trans-Blot Cell

operating instructions (Bio-Rad Laboratories). A sponge pad was pre-saturated in transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM Glycine in 20% methanol) and placed on a plastic hinged holder. A piece of wet Whatman No. 1 filter paper was placed on the sponge, and the slab gel was carefully layed on the filter paper to avoid trapped air bubbles. A sheet of pre-soaked nitrocellulose membrane (Bio-Rad Laboratories) or alternatively a sheet of Gene Screen membrane (New England Nuclear) was placed on the gel surface, again avoiding trapped air bubbles. Another piece of filter paper was placed on the membrane and a second soaked sponge was placed on top to complete the sandwich. The plastic holder was closed and the entire sandwich was placed in the Trans-Blot Cell such that the membrane was anodal to the gel. Up to four gels could be transferred at one time using this set-up. The cell was filled with transfer buffer, a cooling coil was inserted into the cell, and the proteins were allowed to electro-transfer from the gel onto the nitrocellulose for 3.5 hours at 60 volts (0.22 amperes).

At the end of the electrophoretic transfer, the nitrocellulose sheets were carefully removed, air dried and used for immunological assays. One side of each membrane sheet (onto which Mr marker proteins had been transferred from the gels) was carefully cut off, stained in Amido black (4 ug/ml) in 50% methanol:10% acetic acid for 2 to 4 minutes, and destained in 90% methanol:2% acetic acid until

the background cleared. This strip served as a reference for molecular mass determinations of the polypeptides identified by subsequent immunological assays.

The antisera prepared from the blood of rabbits immunized with the maize heat shock polypeptide preparations were tested for the presence of HSP-specific antibodies using the Bio-Rad Immun-Blot (GAR-HRP) Assay kit (Bio-Rad Laboratories). The nitrocellulose (or Gene Screen) membranes were immersed in Tris-buffered saline (TBS, containing 20 mM Tris-HCl, pH 7.5, 500 mM sodium chloride) for 10 minutes. The membranes were then immersed in a blocking solution (3% gelatin in TBS) for 60 minutes and gently agitated. This treatment blocks all the remaining sites on the membrane and minimizes non-specific binding of the antibodies to the membranes. Following the blocking step, the membranes were transferred to a tray containing first antibody solution. This consisted of a 1:100 dilution of an antiserum preparation in antibody buffer (1% gelatin in TBS). The membranes were incubated in this solution overnight with gentle agitation.

The next day, the membranes were removed from the first antibody solution, washed twice in TBS for 10 minutes and transferred to second antibody solution (consisting of a 1:3000 dilution of the goat anti-rabbit IgG-horseradish peroxidase conjugate supplied in the assay kit). The membranes were again incubated overnight with gentle agitation. Following two 10 minute washes in TTBS (TBS

containing 0.05% Tween-20), the membranes were immersed in HRP colour development solution. The latter was prepared by dissolving 60 mg of HRP colour development reagent into 20 mL of cold methanol, and adding this solution to 100 mL of TBS containing 60 uL of cold 30% hydrogen peroxide (stabilized). The colour solution was prepared immediately prior to use. Development of the colour reaction was monitored during the next 45 minutes and the reaction was stopped when coloured bands were visible and background staining was low or absent. Termination of the reaction was accomplished by immersing the membranes in two 10 minute changes of distilled water. The membranes were photographed while still wet to enhance the colour of the bands, and then air dried for storage.

5.2.3.2 Identification of maize HSP-specific antibodies.

While the characterizations described in the preceding section potentially would detect the presence of antibodies specific to any polypeptides in the HSP molecular weight ranges, they could not establish conclusively that the antibodies are specific to the maize HSPs.. To identify the presence of maize HSP-specific antibodies, the sera were tested using an immunoprecipitation assay described by Kelley and Schlessinger (1982) and Atkinson and co-workers (1983). Control and heat-shocked maize seedlings were labelled as described previously, and extracted in two volumes of RIPA buffer (50 mM Tris-HCl, pH 7.2, 0.15 M

sodium chloride, 1.0% sodium deoxycholate, 1.0% Triton X-100, 0.1% SDS). Approximately 30 uL of protein extract (containing 100,000 cpm) was mixed with an equal volume of antiserum, and incubated at room temperature for 20 minutes. In each case, a sample of polypeptides from both control and heat-shocked seedlings was assayed to permit detection of HSPs in control samples. After the 20 minute incubation, 100 uL of a preparation of Staphylococcus aureus cells (Pansorbin, Calbiochem-Behring Corp.; 7.1% w/v; binding capacity: 2.0 mg of human IgG per mL of cell suspension) was added, and the mixture was incubated at room temperature for a further 20 minutes. The samples were centrifuged for 3 minutes in a Beckman microfuge (Model B) and the pellets were washed three times in RIPA buffer. After the final centrifugation, the pellets were suspended in 50 uL of extraction buffer (200 mM Tris-HCl, pH 7.5, 5% SDS, 7.5% 2-mercaptoethanol, 1mM PMSF) and placed in a boiling water bath for 1 minute. The mixture was centrifuged for 2 minutes in the microfuge and the supernatants were subjected to slab gel electrophoresis. Electrophoretic conditions, gel staining and fluorographic analysis were exactly as described previously. Spectrophotometric scans of fluorograms (where shown) were carried out using a Shimadzu Graphicord UV-250 recording spectrophotometer fitted with a GSC-3 gel scanner and connected to a Shimadzu PR-1 graphic printer. The measurements were made at the maximum absorbance wavelength for the blue film base (635 nm) and

gave an indication of the relative intensities of bands on the fluorograms.

5.3 Results

5.3.1 Purification of maize heat shock polypeptides.

The purity of each electro-eluted and concentrated protein sample was checked by 1-D and 2-D PAGE separation and fluorographic visualization. As shown in Figure 22 (panel A), the 18 kD HSPs were obtained in extremely high purity by this method. Each lane represents an independent isolation of the corresponding polypeptide classes. While the high molecular weight HSPs could be isolated in high purity as a group (panel D), it was more difficult to separate these into the two groups consisting of the 73-76 kD HSPs (panel B) and the 84-89 kD HSPs (panel C). Thus, although initial attempts at separating these classes were made, subsequent isolations involved purifying these as a high molecular weight HSP group (73-89 kD). Figure 23 provides the comparison of the 2-D PAGE separations of the two groups of purified HSPs (ie., the 73-89 kD and the 18 kD groups). Although the isolated HSP groups consisted of more than one polypeptide (ie., four in the case of the 18 kD HSPs), there was no evidence of contamination by any other polypeptides. Furthermore, the 18 kD polypeptides were not detected in comparable polypeptide preparations from non-heat-shocked seedlings, although some of the high

Figure 22. Fluorograms of the 1-D SDS-PAGE separations of the purified preparations of the 18 kD (A), 73-76 kD (B), 84-89 kD (C) and 73-89 kD (D) HSP classes. Each lane corresponds to an independent isolation of the polypeptide class. Molecular masses of the purified HSPs are indicated by arrows on the right. Each band represents the incorporation of approximately 200 cpm of 35S-methionine. Fluorograms were exposed for approximately six days.

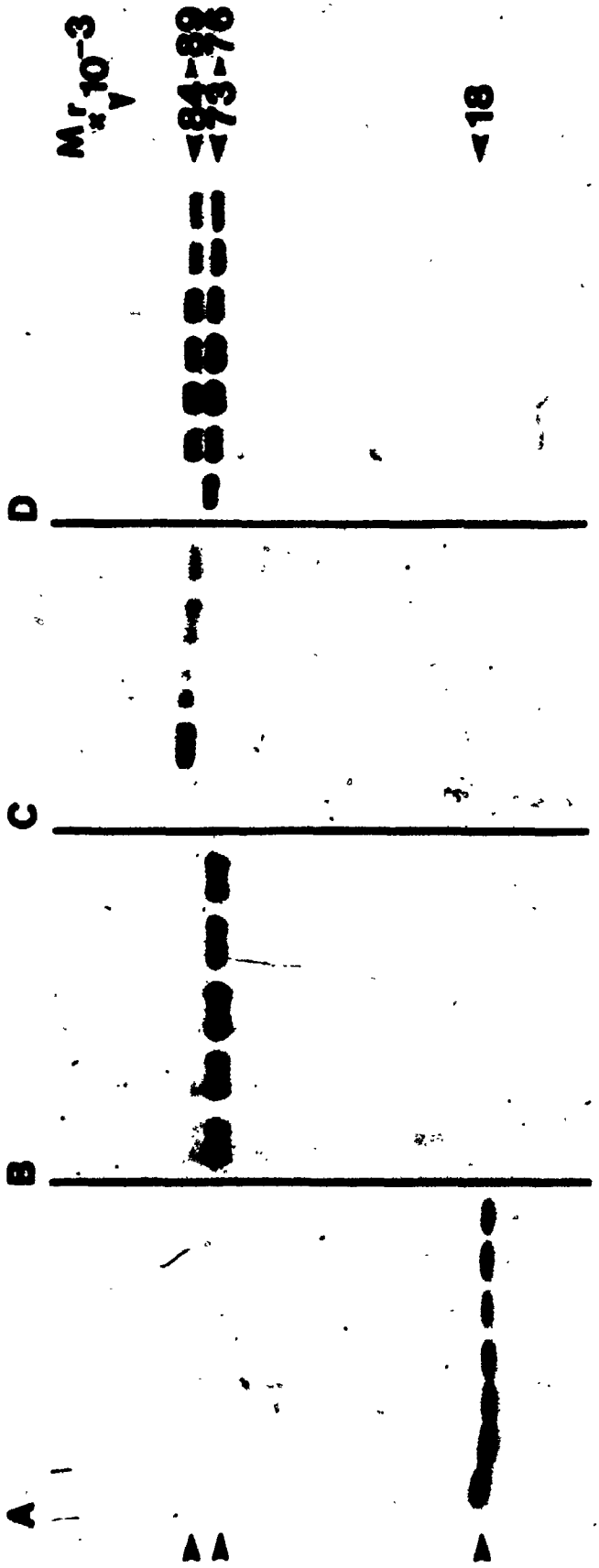
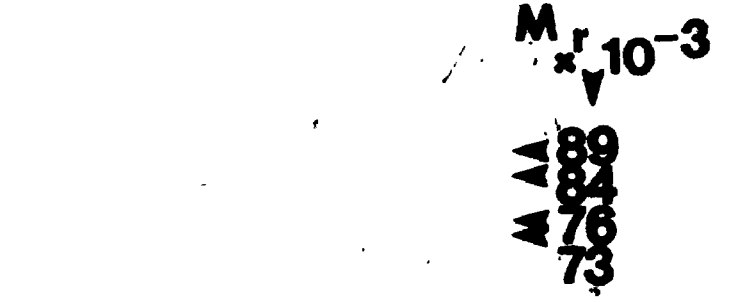

$M_r \times 10^{-3}$
94-92
43-92
18
D
C
B
A

Figure 23. Fluorograms of the 2-D IEF-SDS-PAGE separations of purified preparations of the 73-89 kD and the 18 kD HSP classes. Molecular masses of the purified HSPs are indicated by arrows on the right. Approximately 1500 cpm of the purified 73-89 kD HSPs and approximately 1000 cpm of the purified 18 kD HSPs were loaded on the IEF gels. Fluorograms were exposed for seven days.

PURIFIED 73-89kD HSPs

PURIFIED 18kD HSPs



molecular weight HSPs (ie., the 84 kD and the 73 kD) were present in trace amounts in non-heat-shocked seedlings. The concentration of purified protein recovered by the electro-elution method was routinely between 0.7 and 1.6 ug/uL.

Based on the above criteria of purity, these polypeptides were used for preparation of antisera as described in Materials and Methods.

5.3.2 Characterization of antisera.

5.3.2.1 Identification of reacting polyclonal antibodies.

Initial ring tests indicated that each of the immune sera contained precipitating antibodies. Tests in which preimmune sera were overlayed with the antigens yielded no precipitin rings. Similarly, when immune sera were overlayed with non-specific antigens or buffer, no rings were observed. The immune sera therefore yielded precipitin reactions with only those antigens against which they were prepared. The one exception was a preparation of antisera to the high molecular weight HSPs which exhibited slight precipitation when tested against the [illegible] kD HSP antigen. The method used for the purification of the HSP classes eliminated any chance of contamination of antigen preparations from one class with that of the other such that this slight precipitin reaction may indicate the presence of antibodies directed against a site which is common to the low and high molecular weight HSPs.

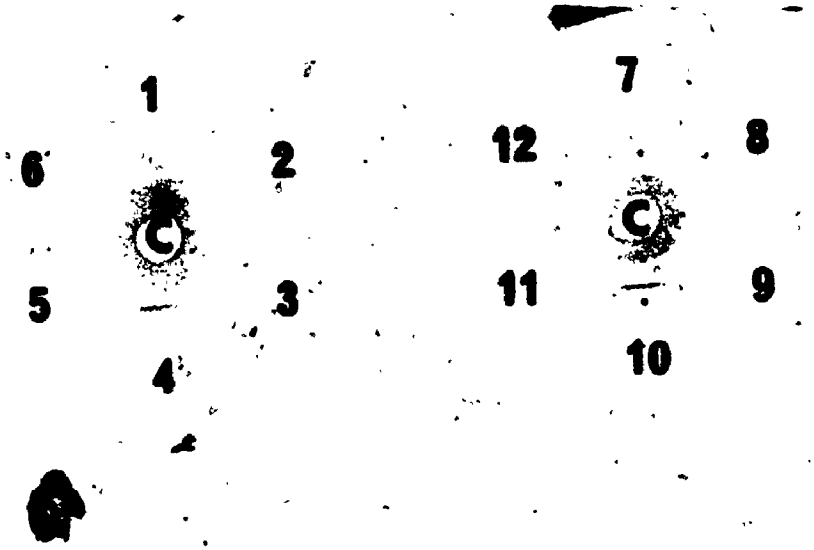
The specificity of the antisera was also analyzed by Ouchterlony tests. As shown in Figure 24A, antisera against the 18 kD HSPs yielded a precipitin arc only when tested against either total polypeptide extract from heat-shocked plumules (Figure 24A, well 4 and 10) or a purified preparation of 18 kD HSPs (Figure 24A, well 1). Antisera against the 73-89 kD HSPs yielded precipitin arcs when tested against a total polypeptide extract from heat-shocked plumules or a purified preparation of the high molecular weight HSPs (Figure 24B, wells 1 and 4, respectively). This preparation of antiserum against the high molecular weight HSPs exhibited a faint precipitin arc when tested against the purified 18 kD HSPs (Figure 24B, well 5) (as was also observed in the ring test) and also when tested against a total polypeptide extract from control plumules (Figure 24B, well 2). This latter finding confirms the observation that some of the high molecular weight HSPs are synthesized in non-heat-shocked tissues.

Immunoblot analysis was also utilized to identify the presence of antibodies to the maize HSPs in the sera. The bands observed in the photograph (Figure 25) of the antiserum-treated nitrocellulose blots of SDS-PAGE separations of total polypeptide extracts represent the specific polypeptides which bound antibodies in sufficient quantities to be detectable by the goat anti-rabbit/horseradish peroxidase (GAR-HRP) assay system (detection sensitivity: less than 100 picograms of purified

Figure 24. Ouchterlony double diffusion assays showing reactions of antisera to maize HSPs with polypeptide preparations from non-heat-shocked and heat-shocked plumules.

Well	Sample
A)	
1	Purified 18 kD HSPs
2,8	Blank
3,9	Purified 73-89 kD HSPs
4,10	Total lysate from heat-shocked plumules
5,7,11	Total lysate from non-heat-shocked plumules
6,12	Extraction buffer
C	Anti-18 kD HSP antiserum
B)	
1	Purified 73-89 kD HSPs
2	Total lysate from non-heat-shocked plumules
3	Blank
4	Total lysate from heat-shocked plumules
5	Purified 18 kD HSPs
6	Extraction buffer
C	Anti-73-89 kD HSP antiserum

A



B

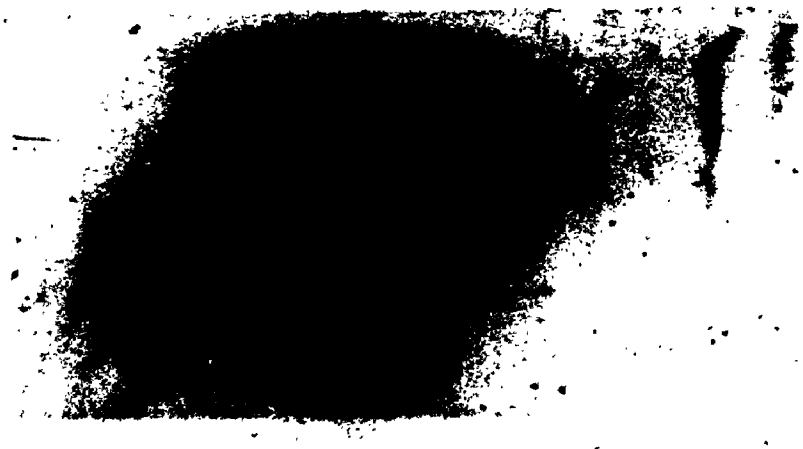
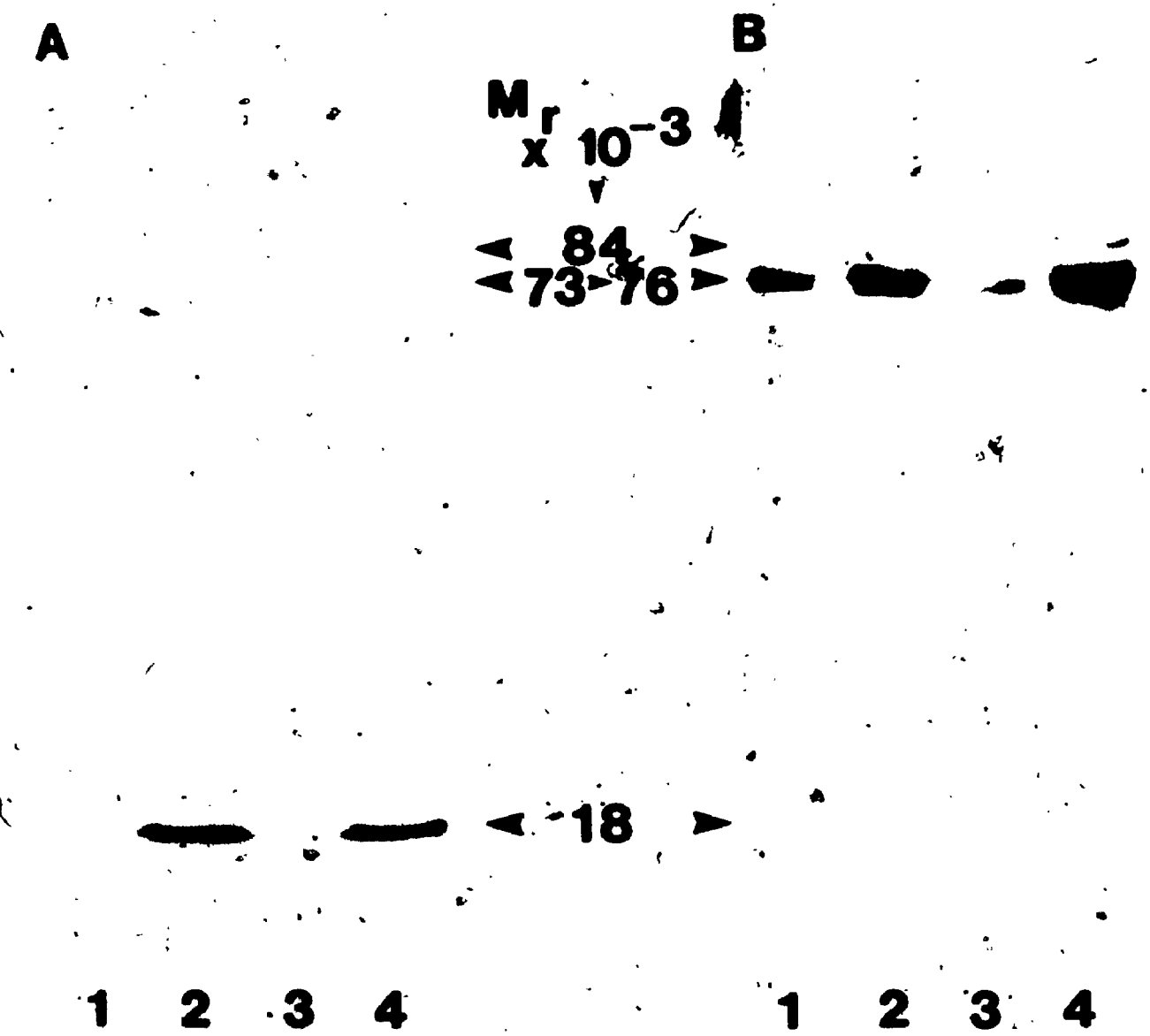


Figure 25. Immunoblots of 1-D SDS-PAGE-resolved polypeptides from maize plumules which were electro-transferred to nitrocellulose membrane and reacted with antisera to either the 18 kD HSPs (A) or the 73-89 kD HSPs (B) of maize plumules. Horseradish peroxidase conjugated goat anti-rabbit (HRP-GAR) antibodies were used to visualize positive reactions. Lanes 1 and 3, polypeptides from non-heat-shocked plumules; lanes 2 and 4, polypeptides from heat-shocked plumules. Positions of HSP reference polypeptides are indicated by arrows in the middle of the figure.


A
B
M_r x 10^{-3}
84
73
76
18
1 2 3 4
1 2 3 4

protein; Bio-Rad Laboratories). The results (Figure 25A) indicate that antibodies prepared against the 18 kD HSPs react with a single class of polypeptides having a molecular weight of 18 kD. A positive reaction is observed in the lane corresponding to the separation of polypeptides from heat-shocked plumules, but also to a small extent in the lane which represents polypeptides from control plumules. While this latter observation initially suggests that the low molecular weight HSPs are present in low amounts in non-heat-shocked plumules, other evidence (to be presented) indicates that the 18 kD HSPs are probably not present in non-heat-shocked tissues. This positive reaction may be due to slight contamination of the serum by trace amounts of antibodies directed against low molecular weight polypeptides which migrate in the region of the 18 kD HSPs. While these 16-20 kD polypeptides are detected in Coomasie blue-stained gels, they are absent in fluorograms of these same gels as well as in autoradiograms of the nitrocellulose blots used for the immunoblot analysis above. Autoradiograms of the nitrocellulose blots further reveal the presence of a radioactively-labelled polypeptide at the 18 kD position in the lane corresponding to the heat-shocked sample; no band is detected at the same position in lanes corresponding to control samples (even after long exposures of the autoradiograms).

The antisera prepared against the 73-89 kD HSPs yielded positive reactions on nitrocellulose blots of

SDS-PAGE-separated polypeptides from plumules of either control or heat-shocked seedlings (Figure 25B). This is to be expected based on: a) the observed synthesis of some of the high molecular weight HSPs in non-heat-shocked tissues; and b) the presence of polypeptides in this molecular weight range which appear in relatively equal proportions in the Coomassie blue-stained SDS-PAGE separations of polypeptides from either control or heat-shocked plumules.

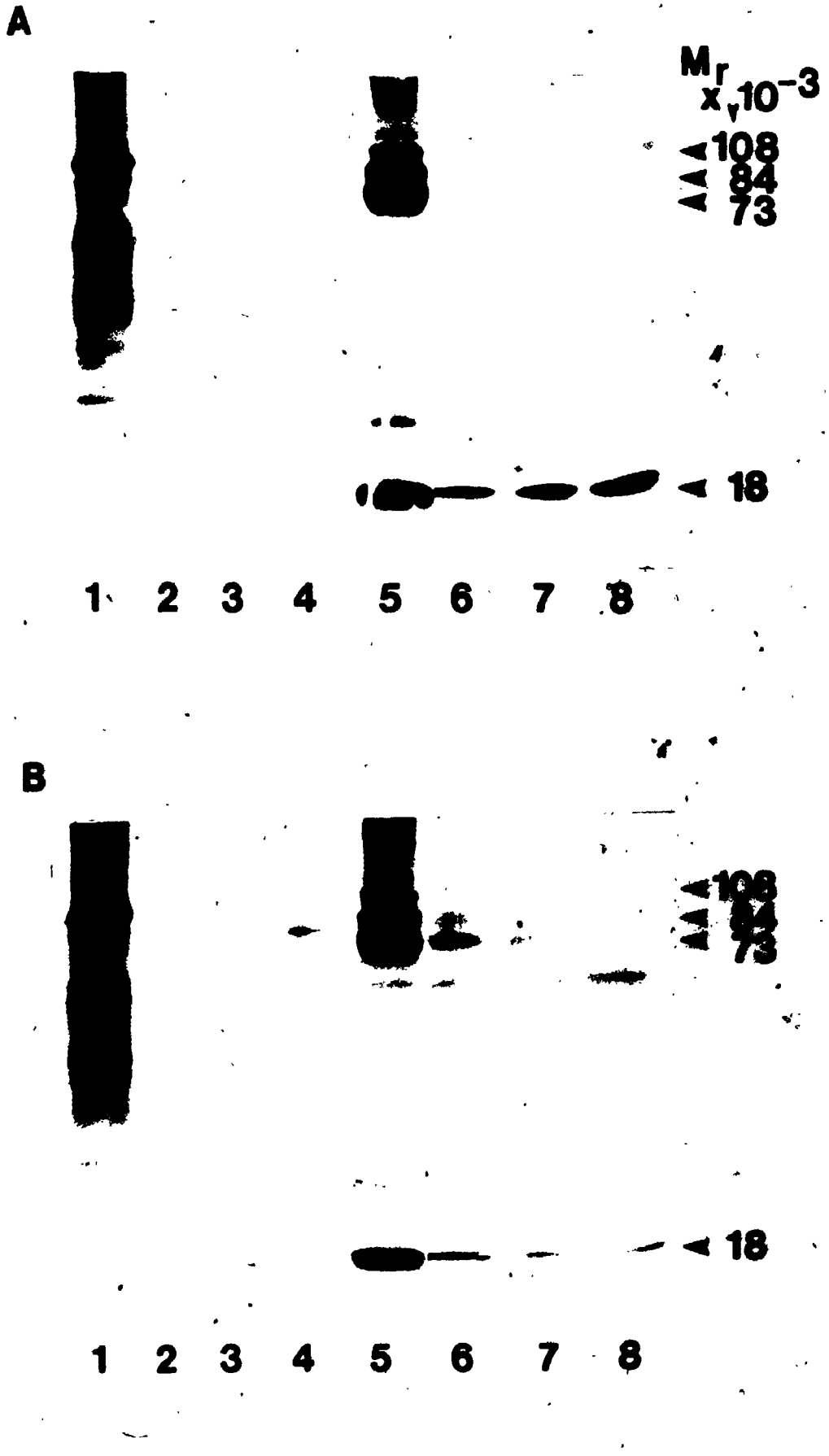
5.3.2.2 Identification of maize-specific antibodies.

From the studies outlined in the preceding sections, it is clear that the antisera contain precipitating antibodies which react with polypeptides having the same molecular weights as the maize HSPs. In order to clearly establish the presence of maize HSP-specific antibodies, polypeptide extracts were reacted with the serum preparations and the immunoprecipitated products were electrophoretically resolved (as described in Materials and Methods). Only those polypeptides which form a stable complex with antibodies are collected in the immunoprecipitates and thus should be the only products resolved on the gels. Taking advantage of the fact that HSP synthesis is low or absent in non-heat-shocked seedlings and greatly enhanced following heat shock, fluorographic analysis of the immunoprecipitated, SDS-PAGE-separated products should establish unambiguously whether HSP-specific antibodies are present in the sera.

The data in Figure 26A reveal the absence of any detectable radioactively-labelled polypeptide bands in the lanes corresponding to 1-D SDS-PAGE separations of the immunoprecipitated products from lysates of non-heat-shocked maize plumules which were reacted with antiserum prepared against the 18 kD HSPs. In comparable lanes corresponding to products obtained from immunoprecipitations of lysates from heat-shocked plumules, a single radioactively-labelled polypeptide band is observed. This polypeptide co-migrates precisely with the 18 kD HSP band observed in SDS-PAGE separations of total polypeptide extracts from heat-shocked seedlings indicating that the serum contains antibodies capable of forming a precipitable complex specifically with the 18 kD maize HSPs. Since the HSPs are immunologically undetectable in non-heat-shocked plumules, the observed synthesis of 18 kD HSPs following heat shock most likely represents de novo synthesis of this polypeptide class.

SDS-PAGE separations and fluorographic analysis of the immunoprecipitated products from total polypeptide lysates reacted with antisera prepared against the 73-89 kD HSPs (Figure 26B) indicates that the sera contain antibodies which react with polypeptides that co-migrate with the high molecular weight maize HSPs. Radioactively-labelled bands are observed in lanes corresponding to samples from both control and heat-shocked plumules. These results indicate that the observed synthesis of at least some of the high molecular weight HSPs represents the enhanced synthesis of

Figure 26. Fluorograms of 1-D SDS-PAGE separations of total polypeptide lysates from control (lane 1) and heat-shocked (lane 5) maize plumules, and of the products which were immunoprecipitated from these total lysates with antisera to either the 18 kD HSPs (A) or the 73-89 kD HSPs (B) of plumules. Lanes 2, 3 and 4 in each case represent the immunoprecipitated products from total lysates of control plumules containing 30,000, 50,000 and 100,000 counts respectively, of acid-precipitable material. Lanes 6, 7 and 8 represent the corresponding immunoprecipitates from heat-shocked plumules. Wells 1 and 5 were loaded with approximately 30,000 counts of acid-precipitable lysate. Positions of HSP reference polypeptides are indicated by arrows on the right. Fluorograms were exposed for four days.


A
M_r x 10^{-3}
108
84
73
18
1 2 3 4 5 6 7 8
B
108
84
73
18
1 2 3 4 5 6 7 8

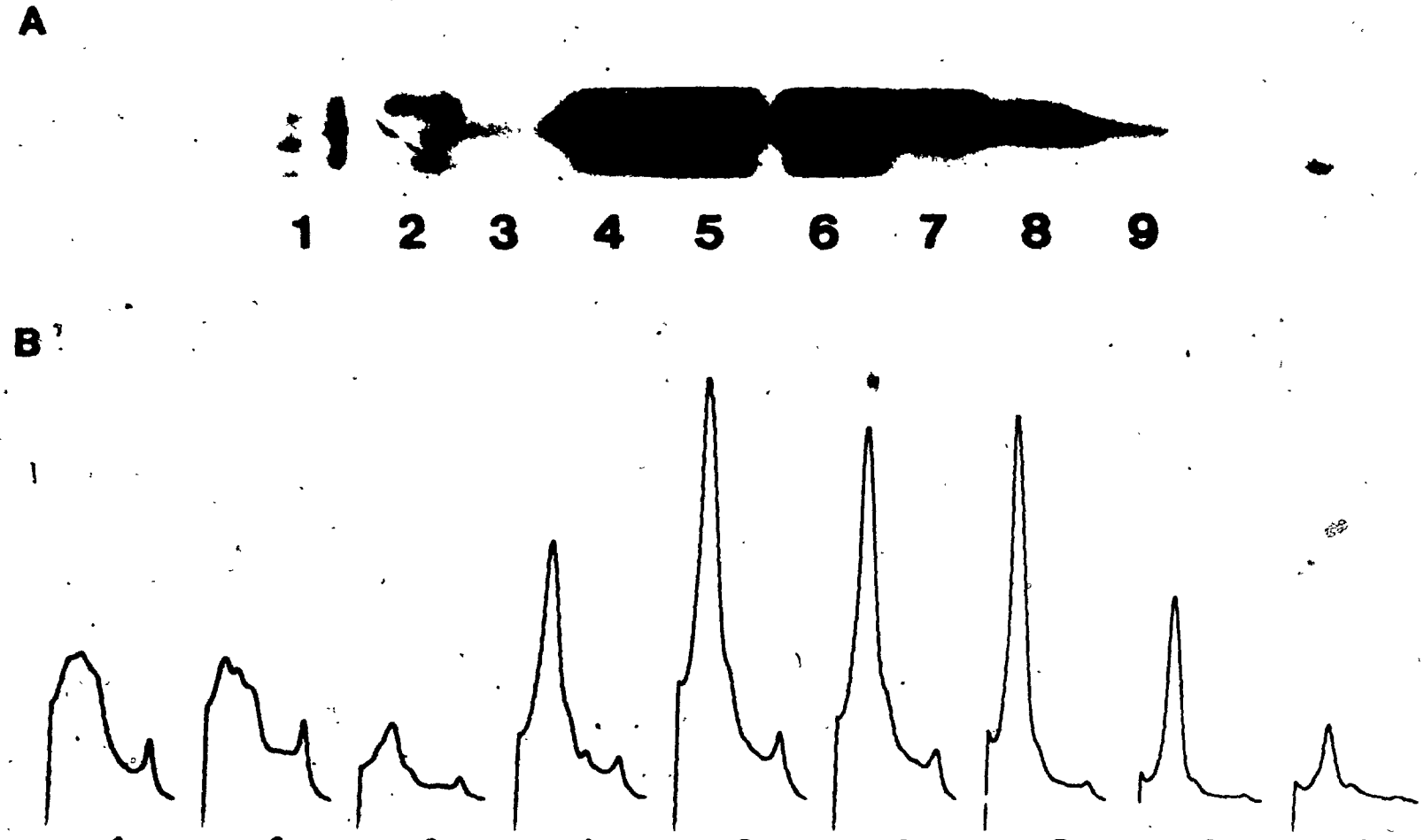
polypeptides which are normally synthesized (albeit at low levels) in non-heat-shocked plumules. In addition there is apparently some reaction of the antibodies against the 73-89 kD HSPs with the 18 kD HSP class as evidenced by the presence of a radioactively-labelled band at a position corresponding to the expected position of the 18 kD HSP class. As indicated previously, the HSP purification method utilized eliminates any possibility of cross-contamination of these two HSP molecular weight groups (ie., the 73-89 kD and the 18 kD groups), such that this serum preparation evidently contains some antibodies which recognize binding sites common to both HSP groups.

5.3.2.3 Immunological detection of time of initiation of HSP synthesis following thermal shifts.

It was previously shown that synthesis of the 18 kD HSP class was not detectable by fluorography before approximately 60 minutes following a shift from 27C to 41C (see Figure 4). Due to the increased sensitivity of immunochemical methods over other means of protein detection, experiments were conducted to determine if antibodies to the 18 kD HSPs could detect synthesis of this 18 kD HSP class before 60 minutes. Since the immunoprecipitation method can selectively remove the 18 kD polypeptides, it was possible to enrich for this HSP class by conducting the immunoprecipitations on larger samples of total polypeptide extracts. Figure 27A presents fluorograms of 1-D SDS-PAGE separations of the products

Figure 27. (A). Fluorogram of the 1-D SDS-PAGE separations of the immunoprecipitated products from total lysates obtained from maize plumules following a shift from 27C to 41C. Only the section of the fluorogram corresponding to the 14-23 kD region of the gel is shown. Lanes 1 through 9 correspond to the products of this molecular size which were immunoprecipitated from plumules after 0, 10, 20, 30, 40, 60, 120, 180 or 240 minutes at 41C. Each sample used for immunoprecipitations initially contained approximately 450,000 counts of acid-precipitable lysate. Fluorogram was exposed for two days.

(B). Corresponding spectrophotometric scans (635 nm) of each of the lanes in the fluorogram in (A). The plot from left to right in (B) corresponds to a top to bottom scan of the fluorogram in (A).


A
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8 9

immunoprecipitated from approximately 100uL of total lysates obtained from plumules which were subjected to a shift from 27C to 42C for 0, 10, 20, 30, 40, 60, 120, 180 and 240 minutes prior to labelling for one hour at 27C. Corresponding spectrophotometric scans (635 nm) of the 18 kD region in each lane of the fluorogram are presented in Figure 27B. The results illustrate that synthesis of the 18 kD HSP class begins as early as 20-30 minutes following the thermal shift. Maximal synthesis occurs between 40-60 minutes after which there is a gradual decline in the intensity of this polypeptide class. The triplet of bands observed in lanes 1 and 2 most likely represent the recovery of products other than the 18 kD HSPs which either bound to the antibodies weakly, or were carried along during the immunoprecipitations. Alternatively, they may in fact indicate the presence of trace amounts of the 18 kD HSPs in non-heat-shocked plumules. More specific probes such as cDNAs may distinguish between these possibilities. The observation that 18 kD HSPs are synthesized as early as 30 minutes or less is in agreement with <u>in vitro</u> translation studies which show that mRNAs for the HSPs are present in these cells within 30 minutes following temperature shifts; the RNAs reach a maximal level at approximately 60 minutes and then gradually decrease over the next six hours (see Figure 20).

5.3.3 Examination of the immunological relatedness of HSPs from various sources.

5.3.3.1 Immunological relatedness of 18 kD HSPs from different tissues of maize.

Earlier studies (see Figure 9) indicated that following a shift from 30C to 42C, plumules, radicles, mesocotyls and young leaves from maize seedlings exhibit new and(or) enhanced synthesis of a similar set of HSPs. In order to examine whether the 18 kD HSPs from different tissue sources are immunologically related, total extracts of polypeptides from each tissue were subjected to the immunoprecipitation procedure outlined above. Fluorograms of 1-D SDS-PAGE separations of total polypeptide lysates (top) as well as the products which were immunoprecipitated from these total lysates using antiserum produced against the 18 kD HSPs (bottom) are presented in Figure 28. The 18 kD HSP class is absent in total lysates from control (30C) samples and shows pronounced synthesis following heat shock (30-42C). No bands are detected in the immunoprecipitates from tissues of control seedlings while a single prominent 18 kD polypeptide band is observed in lanes corresponding to immunoprecipitates from each of the tissues from heat-shocked seedlings. Since the samples of each tissue used in these immunoprecipitation experiments contained the same number of acid-precipitable counts, intensities of the bands represent the relative relatedness of HSPs from different tissues. The 18 kD HSP band in lane 7 was consistently less intense than in lanes 5, 6 and 8. It

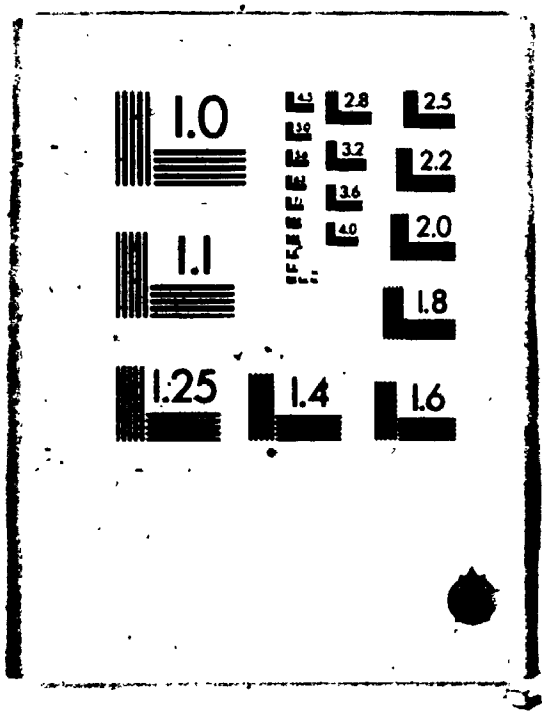
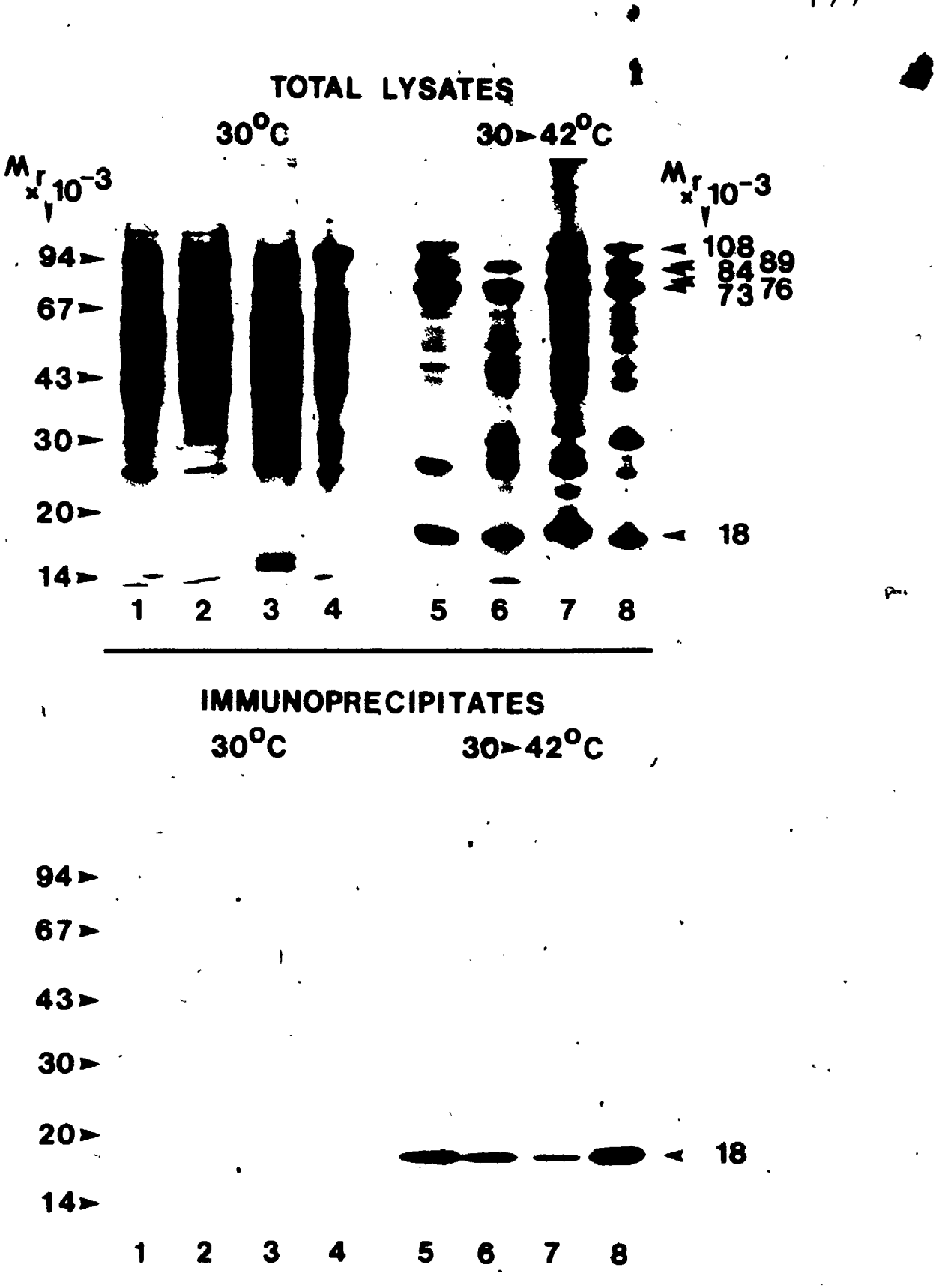


Figure 28. Fluorograms of the 1-D SDS-PAGE separations of the polypeptides obtained from plumules (lane 1 and 5), mesocotyls (lane 2 and 6), radicles (lane 3 and 7) or young leaves (lane 4 and 8) from maize seedlings grown at 30C or following a shift from 30-42C. The electrophoretic separations of total polypeptide lysates from each tissue are shown in the top of the figure while the products obtained by immunoprecipitation of these same lysates with antiserum prepared against the 18 kD HSPs from maize plumules are shown in the lower part of the figure. Approximately 100,000 counts of acid-precipitable material from each of the total lysates was utilized for the immunoprecipitations. Approximately 25,000 acid-precipitable counts of total lysate were loaded in each well of the gel in the upper part of the figure. Positions of standard marker proteins are indicated by arrows on the left while positions of reference HSPs are indicated by arrows on the right. Fluorograms were exposed for five days.

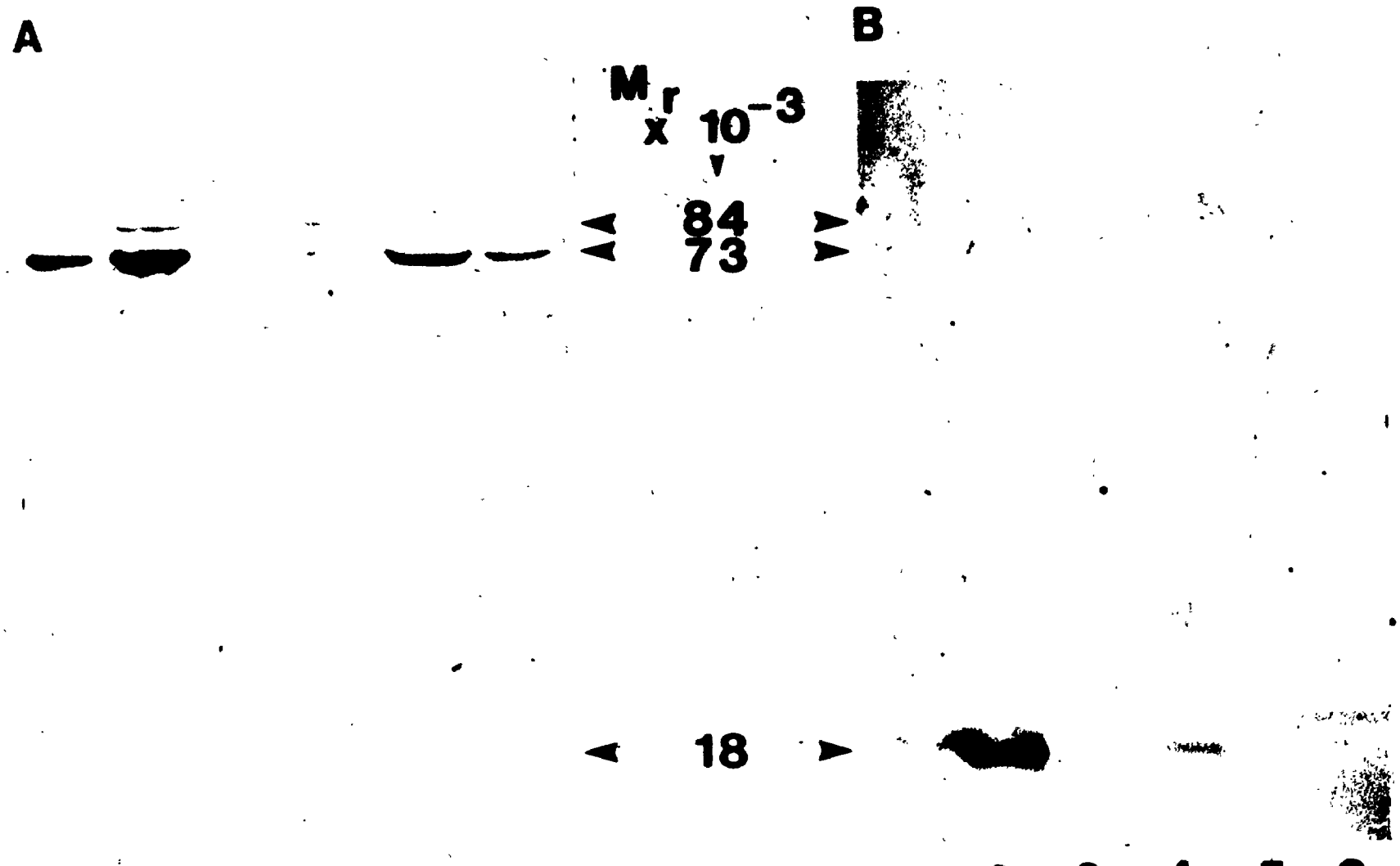

TOTAL LYSATES
30°C
30►42°C
Mr x 10-3
94►
67►
43►
30►
20►
14►
1 2 3 4 5 6 7 8
Mr x 10-3
108
84
73
89
76
18
IMMUNOPRECIPITATES
30°C
30►42°C
94►
67►
43►
30►
20►
14►
1 2 3 4 5 6 7 8
18

contains the SDS-PAGE-resolved immunoprecipitates from radicle tissue. This observation suggests that in terms of the ability of the antibodies to recognize binding sites, the 18 kD HSPs from plumules may be more similar structurally to the 18 kD HSPs from young leaves and mesocotyls than to the 18 kD HSPs from radicles. The establishment of whether these HSPs represent products from identical gene sequences in the different tissues awaits the availability of appropriate cDNA clones.

5.3.3.2 Immunological relatedness of HSPs from different species.

In order to examine whether the HSPs observed in maize might be structurally related to HSPs of similar molecular mass described in other plant and animal systems, immunological tests were carried out in which antibodies to maize HSPs were reacted with polypeptide extracts from control and heat-shocked tissues of several species. The results from immunoblot analysis of 1-D SDS-PAGE-separated polypeptides from maize (cv. Oh43) plumules, soybean (cv. Linderine) hypocotyls, and pea (cv. Dwarf Wando) epicotyls are compared in Figure 29. All three species have been shown to exhibit synthesis of HSPs of similar molecular mass (Key et al., 1981; Altschuler and Mascarenhas, 1982; Baszczynski et al., 1982a; Hadwiger and Wagoner, 1983). Antibodies prepared against the maize 73-89 kD HSPs were capable of reacting specifically with polypeptides in the 73-89 kD molecular weight range in all three species.

Figure 29: Immunoblots of 1-D SDS-PAGE-resolved polypeptides from maize plumules (lanes 1 and 2), soybean hypocotyls (lanes 3 and 4) and pea epicotyls (lanes 5 and 6) which were electro-transferred to nitrocellulose membrane and reacted with antisera to either the 73-89 kD HSPs (A) or the 18 kD HSPs (B) of maize plumules. Lanes 1, 3 and 5 in each case represent samples from non-heat-shocked tissues while lanes 2, 4 and 6 represent the corresponding samples from heat-shocked tissues from these three plant species. Positions of HSP reference polypeptides of maize are indicated by arrows in the middle of the figure.


A
B
M_r x 10^{-3}
84
73
18
1 2 3 4 5 6
1 2 3 4 5 6

Positive reactions with polypeptides from both control (Figure 29A, lanes 1, 3 and 5) and heat-shocked (Figure 29A, lanes 2, 4 and 6) tissues were obtained supporting the contention that synthesis of these high molecular weight HSPs occurs in non-heat-shocked seedlings and becomes enhanced following heat shock. Although all three species showed positive reactions, the degree to which the soybean and pea HSPs reacted with antibodies to maize HSPs varied. This observation suggests that while the high molecular weight HSPs from the three plant species may be structurally similar in terms of ability to react with maize anti-HSP antibodies, the relative levels of synthesis of the different classes of the high molecular weight HSPs may be species-specific.

A parallel experiment utilizing antibodies to the maize 18 kD HSPs was conducted to assess the relatedness of the low molecular weight HSPs from different plant species. Immunoblot analysis of polypeptides from non-heat-shocked tissues from the three plant species revealed little or no detectable reaction with the antibodies to maize 18 kD HSPs (Figure 29B, lanes 1, 3 and 5). The extent of reaction of polypeptides from heat-shocked tissues of each of the three species with the maize antibodies was different, even though all three species make heat shock polypeptides of approximately 18 kD (Figure 29B, lanes 2, 4 and 6). Polypeptides extracted from heat-shocked pea epicotyls showed virtually no reaction with antibodies to maize 18 kD

HSPs; polypeptides from heat-shocked soybean hypocotyls exhibited a positive reaction at 30-50% of the level of reaction noted for polypeptides from heat-shocked maize plumules treated with the same antibodies. Since similar amounts of total protein were loaded in each lane, the results suggest that either the maize 18 kD HSPs are more similar to soybean than to pea low molecular weight HSPs, or that the amount of low molecular weight HSPs relative to other polypeptides is higher in maize and soybean than in peas.

Immunoprecipitations of polypeptides from total protein extracts from the three plant species (Figure 30) further support the above findings. A single polypeptide band at the 18 kD position (see Figure 30A) is noted for all three species on fluorograms of gels in which a constant number of counts of acid-precipitable lysate were utilized in the immunoprecipitations. This band is most intense for maize, less intense for soybean and very faint for pea. There is little or no cross-reactivity of the antibodies to the 18 kD maize HSPs with high molecular weight HSPs from any of the three plant species.

Antibodies to the maize HSPs were also tested against polypeptide extracts from control and heat-shocked tissues from a few readily accessible animal species, previously shown to produce heat shock polypeptides. The results are summarized in Table 6, along with those for other organisms also tested with antibodies to maize HSPs. While all

Figure 30. Fluorogram of the 1-D SDS-PAGE separations of the products which were immunoprecipitated from total polypeptide lysates obtained from maize plumules (lanes 1 and 4), pea epicotyls (lanes 2 and 5) or soybean hypocotyls (lanes 3 and 6). Lanes 1-3 represent samples from 25C-grown seedlings while lanes 4-6 represent the corresponding samples from heat-shocked (25-42C) seedlings. Positions of standard molecular mass marker proteins are indicated by arrows on the left and the expected position of the 18 kD HSP of maize is indicated on the right. Approximately 100,000 counts of acid-precipitable material from each total lysate was utilized for immunoprecipitations. Fluorograms were exposed for six days.

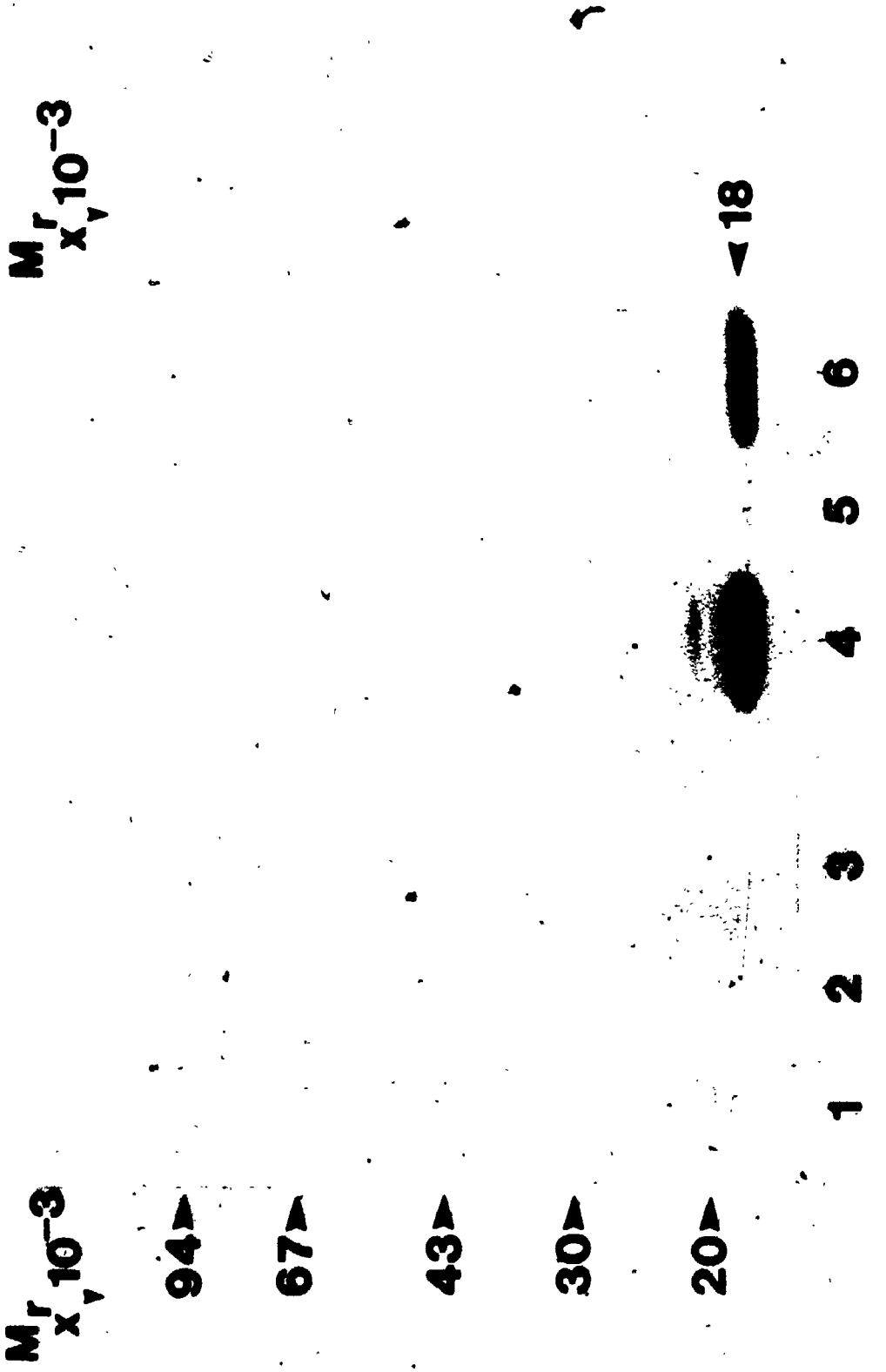

M_r x 10^{-3}
94
67
43
30
20
M_r x 10^{-3}
18
1
2
3
4
5
6

Table VI. Reactivity of antibodies to maize HSPs with polypeptides from heat-shocked tissues from different plant and animal species.

Organism	Molecular weights	Source	Reactivity with antibodies to: 73-89 kD HSPs	18 kD HSPs
Maize:			[a]	
Plumule	108,89,84,76,73, 23,18	Baszczynski et al., 1982a;	++++	++++
Radicle	same as above	Baszczynski et al., 1984	++	++
Mesocotyl	same as above	(in press)	+++	+++
Leaf	same as above		++++	++++
Soybean:				
Hypocotyls	92,84,70,68,15-27	Key et al., 1984 (in press)	+++	++
Pea:				
Pods	70-85, 10-25	Hadwiger and Wagoner, 1983		
Epicotyls	70-90, 17	Baszczynski, this thesis	+++	+/-
Quail:			[b]	
Red Blood Cells	90,70,26	Atkinson and Dean, 1984 (in press)	-	-
Mouse:				
Spleen Cells	110,105,90,70, 65,26	Rodenhiser et al., (submitted)	-	-
Tadpole:				
Hind Limb Epidermis	65,25	Ketola-Pirie and Atkinson, 1983	-	-

a plus signs represent degree of reactivity relative to maize plumules (++++);
b minus signs indicate no reaction.

systems studied produced high molecular weight HSPs in the 65-90 kD range, polypeptides from the animal systems tested did not react with maize antibodies when assayed by any of the immunological tests described above. The possible significance of this differential response between these species will be discussed later.

5.4 Discussion

The availability of antibodies to specific proteins or their polypeptide constituents provides a means of probing for the presence of that protein or polypeptide in cells. Protein antigens present at levels too low to be detectable by most staining methods (ie., nanograms or less) can be measured by various immunochemical methods (Eisen, 1980). The purification of maize HSPs and the preparation of antibodies to these polypeptides were undertaken in order to: a) provide an additional means of evaluating the levels of synthesis of the HSPs under different temperature conditions; and b) to permit an examination of the relatedness of the various HSP classes with each other, and among different maize tissues or different species.

While the methods of preparation of polyclonal antibodies in rabbits is generally straightforward (Kabat, 1976; Eisen, 1980), production of antibodies to the maize HSPs was more tedious due to the fact that: a) the HSPs are present in extremely small quantities (especially the 18 kD

HSPs which are apparently not synthesized in non-heat-shocked cells); b) these HSPs can only be visualized by examining newly synthesized polypeptides, making purity monitoring a time-consuming process; and c) some polypeptides which appear in the Coomassie blue-stained gel patterns have mobilities very similar to those of the HSPs. The strategy therefore was: a) to establish initially the presence of precipitating antibodies in the sera (ring tests and Ouchterlony plates); b) to identify antibodies specific to polypeptides in the molecular weight range of the maize HSPs (eg., 73-89 kD or 18 kD) (immunoblot analysis); and finally c) to identify antibodies capable of binding specifically to the maize HSPs (immunoprecipitations and fluorographic analysis of the SDS-PAGE separations of the immunoprecipitated products).

In spite of the potential value of antibodies to the study of HSP synthesis, only a few reports have described the production of antibodies to heat shock proteins. Polyclonal antibodies to the major chicken HSPs (Kelley and Schlesinger, 1982) and monoclonal antibodies to the major Drosophila 70 kD HSP (Velazquez et al., 1983; Velazquez and Lindquist, 1984) have revealed that the heat shock proteins in these organisms are synthesized at a basal level in non-heat-shocked cells and intensify following thermal shifts. Monoclonal antibodies to soybean (J.L. Key, personal communication) and quail (B.G. Atkinson, personal communication) HSPs are being developed presently.

The various immunological assays utilized in the present study demonstrate that the high molecular weight HSPs are present in non-heat-shocked plumules and intensify following heat shock. However, immunoprecipitations of polypeptide extracts with antibodies to the 18 kD plumule HSPs did not reveal the presence of 18 kD HSPs in this tissue in the absence of thermal shifts. Thus, the 18 kD polypeptides are either not synthesized at preshift temperatures, or they are synthesized at levels which are below the limit of sensitivity of this assay. In chicken embryo fibroblasts, the low molecular 24 kD HSP was not detected in non-heat-shocked tissues using the same immunoprecipitation technique (Kelley and Schlesinger, 1982).

The antiserum prepared against the 18 kD maize HSPs was capable of binding the 18 kD HSPs from total polypeptide extracts obtained from plumules as early as 30 minutes following the shift from 27C to 42C (Figure 27). It is conceivable that more sensitive detection methods might demonstrate the presence of the 18 kD HSP class even earlier than shown in this study. From the measurements of the interval during which the internal temperature of plumules reaches that of the incubator following temperature shifts (about 20 minutes; see Figure 15), it appears that synthesis of HSPs and most likely their mRNAs may be initiated even before the seedling has "experienced" the full impact of the temperature to which it has been shifted.

Thus, the initiation of synthesis of some of these polypeptides begins "in transition" from one temperature to another. The transitory nature of the response to temperature shifts is in agreement with results shown previously which demonstrated the gradual decline in both the synthesis of heat shock polypeptides (Figure 6 and 27) and in the availability of mRNAs for these polypeptides (Figure 20) during continuous incubation at the shift temperature.

Several studies have examined HSP synthesis in different tissues of the same organism (Tissieres et al., 1974; Sondermeijer and Lubsen, 1978; Currie and White, 1981; Baszczynski et al., 1982b; Atkinson et al., 1983; Dean and Atkinson, 1983; Ketola-Pirie and Atkinson, 1983; Baszczynski et al., 1984). While many HSPs appear to be common to all the tissues studied in any one species, some HSPs appear to be specific to certain tissues. This latter observation may be due however to an inability to detect low levels of synthesis of HSPs in those tissues in which they appear to be absent. Furthermore, most workers have assumed that polypeptides of similar molecular mass and(or) charge in different tissues of a single organism, are in fact the products from expression of the same gene sequence. This assumption is testable by several methods including the use of immunochemical methods or cDNA probes. Kelley and Schlesinger (1982) have demonstrated for example that HSPs from embryo fibroblasts and from heart, musle, liver, eye,

brain and carcass tissue all react with antibodies to the two major high molecular weight HSPs from chicken embryo fibroblasts. Polypeptides from a number of tissues which do not appear to synthesize the 24 kD HSP did not react with antibodies to the 24 kD HSPs (Kelley and Schlesinger, 1982). My work reveals that all maize tissues which were probed with the antibodies to plumule HSPs (Figure 30) exhibited the presence of polypeptides capable of cross-reacting with the antibodies produced in plumules. Thus, there appears to be correspondence between the presence in a tissue of an HSP of a specific molecular mass, and its ability to react with antibodies prepared to an HSP of the same molecular mass in a different tissue. These results, although indirect, strongly suggest a similarity in the structure of HSPs in various tissues of an organism.

The numerous studies on heat shock protein synthesis have revealed that a polypeptide of approximately 70 kD exhibiting enhanced synthesis following heat shock, occurs in virtually every organism that has been examined. A number of approaches have been used to ascertain whether the HSPs in different species are in fact the same. Proteolytic digests of the 68-70 kD and 84-85 kD HSPs from chick embryo fibroblasts, Drosophila embryonic cells and human lymphoblastoid cells have generated peptide maps which exhibit a considerable amount of similarity in the sizes of fragments obtained (Voellmy et al., 1983). In recent studies by Shah and co-workers (1984) and Sinibaldi and

Dietrich (1984), cloned genes to the 70 kD HSPs of Drosophila have been utilized for hybrid selection of homologous sequences from the maize genome. The deduced amino acid sequences based on partial nucleotide sequence reveal that approximately 80% of the amino acid sequences for the 68-76 kD HSPs of Drosophila, yeast and maize may be homologous (Shah et al., 1984).

Immunochemical studies have further revealed that antibodies to the 70 kD HSP of chicken embryo fibroblasts are capable of reacting with polypeptides of approximately the same molecular mass in a large array of organisms including Saccharomyces, Dictyostelium, Caenorhabditis, Drosophila, Xenopus, mouse cells, human cells and maize seedling roots (Kelley and Schlesinger, 1982). In the present study on the other hand, cross-reactivity of antibodies to maize HSPs was detected only with HSPs from other plant species and not with HSPs from the animal systems examined. This suggests that either: a) the level of HSPs in these animal cells is too low to react in sufficient quantities as to be detectable with the maize antibodies; or b) the antisera contain antibodies that recognize specific determinants which are abundant on HSPs from plant cells but either low or absent on HSPs from animal cells. Since polyclonal antisera, by definition, contain a heterogeneous array of antibody molecules to a variety of antigenic determinants on the same molecule (Eisen, 1980), it is not surprising that different antibody

preparations (eg., the antibodies to chicken instead of maize HSPs) may exhibit differences in their ability to cross-react with similar antigens from various sources.

It appears therefore that there is some similarity in the structural properties of selected HSPs from different sources. Whether this structural similarity is related to a common function of these HSPs in the different organisms (eg., thermotolerance) has yet to be established.

Conclusions

Temperature constitutes an important parameter in the overall biological function of living organisms and perturbations of this parameter can lead to disruptions of cellular homeostasis. As a result, a variety of changes may be elicited as a response to thermal fluctuations. Conditions such as high temperature stress or heat shock have been shown to induce dramatic alterations in gene expression at the polypeptide, RNA and DNA levels (see literature review in Introduction). For this reason, the "heat shock phenomenon" has been exploited for various studies of gene and gene product induction and regulation.

I have attempted to provide insight into the influence of thermal shifts on the synthesis of gene products in maize. The data which I present in the second and third chapters constitute the first major description and characterization of the response of maize to "heat shock". These chapters provide the groundwork for subsequent investigations into the general impact of temperature shifts on RNA and polypeptide synthesis; the results from these studies allow me, in the fourth chapter, to develop the concept that alterations in gene product expression most likely occur as a general response to any thermal shift and represent a normal biological response to changes in the environmental temperature conditions. Finally, the production of antibodies to the maize HSPs (Chapter 5)

allows me to confirm immunologically several of my previous observations, and more importantly, to examine the relatedness of HSPs from different maize tissues as well as from a few different plant and animal species. Collectively, a number of interesting and important features regarding the impact of temperature and thermal shifts on gene expression in maize emerge from these studies:

1. Shifts to temperatures above approximately 40C (heat shock) lead to a dramatic alteration in the pattern of polypeptide synthesis. This involves the enhanced and, in some cases, new synthesis of a group of heat shock polypeptides (HSPs) which are analogous but not identical to those observed in other organisms.

2. The HSPs represent products resulting from the translation of polyadenylated mRNAs which become available in the cytoplasm following heat shock as a result of either: a) new or increased transcription; or b) modifications in pre-existing HSP-RNAs which make the RNAs available for translation following heat shock.

3. All tissues of maize which have been studied appear to have the capacity to respond to heat shock in a similar manner (ie., with the synthesis of a similar set of polypeptides). This common response in different tissues suggests that if these HSPs have a biological function (eg., their proposed role in increasing

thermotolerance), at least some of the HSPs most likely serve a similar role in all tissues in which they are synthesized. Immunological studies utilizing antibodies prepared to the maize plumule HSPs (Chapter 5) confirm that the 18 kD HSPs from several maize tissues are immunochemically (and presumably structurally) related.

4. Different growing temperatures lead to different "control" patterns of polypeptide synthesis and influence the subsequent response to thermal shifts. This observation is important since it indicates that the changes observed following one temperature shift regime may yield results which are quite different from those observed following another regime. Maize seedlings grown at different temperatures synthesize a similar set of HSPs when shifted to a heat shock temperature; in addition, unique synthetic differences specific to seedlings grown at each temperature are also noted.

5. Changes in gene expression may occur following any temperature shift which is of sufficient magnitude. The term "magnitude" as used here, refers to the sum of a number of factors, the interaction of which determines the array of gene products and the extent to which they are synthesized. These factors in the minimum include: a) the initial growing or "pre-shift" temperature; b) the temperature increment over which the tissues are

shifted; c) the temperature regime in which the shift is carried out (eg., 15-25C vs. 30-42C); d) the rate at which the temperature changes from the initial to the final temperature; and e) the duration of the temperature shift. Recent studies by Walden and co-workers indicate that the genotype also is important in controlling the types of polypeptides which are synthesized under different temperature conditions.

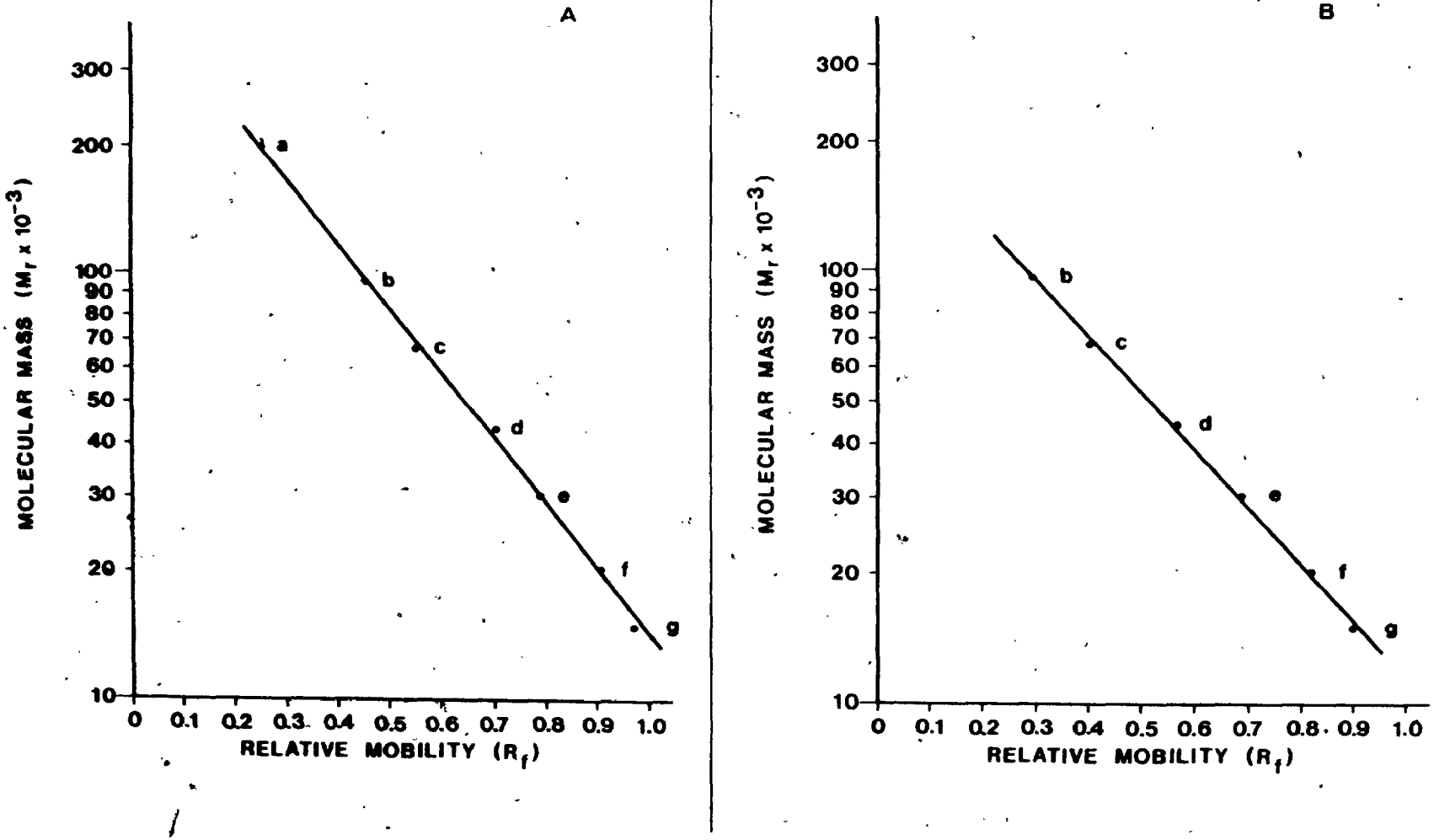
6. While new and(or) enhanced polypeptide synthesis may represent a universal response to heat shock, and most likely to any temperature shift, the actual polypeptides which are synthesized are not necessarily identical in size or structure in all species. In maize, as in other plants, the array of low molecular weight polypeptides appear to be much more complex than in animal systems. The polypeptides of approximately 70 kD which are suggested to be common to most organisms are immunologically related in at least soybean, pea and maize. On the other hand, antibodies to the maize high molecular weight HSPs do not show any reactivity with 65-70 kD HSPs from quail, mouse and tadpole, under the same conditions. Thus, there is considerable variability in the degree of relatedness in different species of similar size polypeptides produced under similar stress conditions. However, until a funotion for the different HSPs is unambiguously established, it

is difficult to draw any conclusions about the possible relatedness of these polypeptides in the different species.

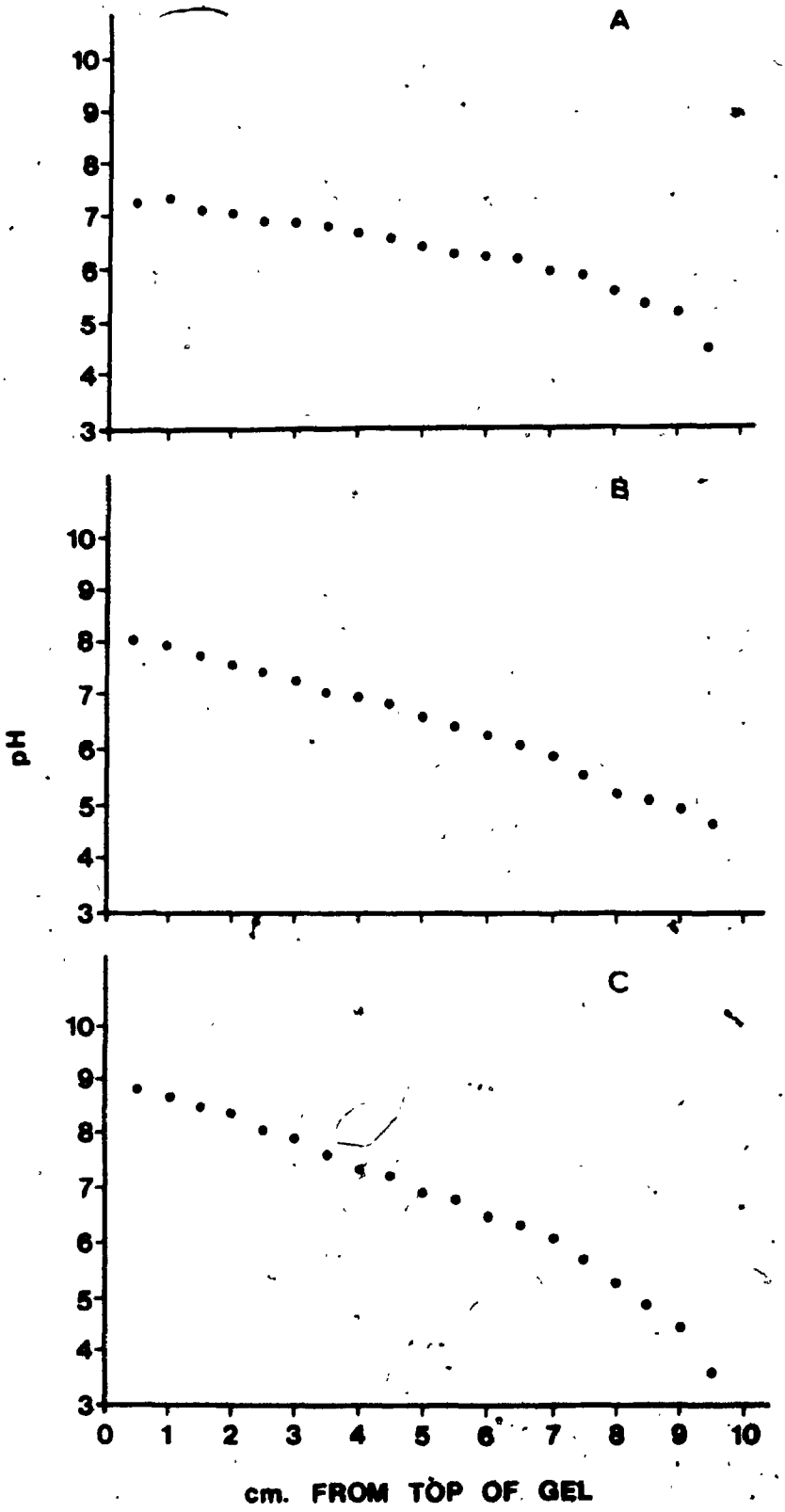
The studies described in this thesis have contributed in several areas towards an understanding of the events involved in the normal response of maize to thermal shifts and in the response to potential stress conditions such as heat shock. It would appear that the concept of "heat shock", at least in plants, and probably in animals, should be expanded or modified to accomodate the general notion of "temperature shift", since my work suggests that gene expression induced by heat shock (and probably cold shock or stress) represents the extreme case of a more general response to thermal changes. If alterations in gene activity are important in allowing the organism to accomodate or adapt to rapid temperature changes, then it is biologically and evolutionarily feasible that a mechanism should have developed which is capable of "sensing" and "responding" to both major and minor perturbations in the thermal conditions of the organism's environment.

APPENDICES

Appendix 1. Plots of relative mobilities of standard molecular mass marker proteins in either 3-15% linear gradient (A) or 7.5-17.5% linear gradient (B) SDS-polyacrylamide slab gels. Standard proteins included a) myosin, 200 kilodaltons (kD); and proteins from a low molecular weight calibration kit from Pharmacia Fine Chemicals consisting of: b) phosphorylase b, 94 kD; c) albumin, 67 kD; d) ovalbumin, 43 kD; e) carbonic anhydrase, 30 kD; f) trypsin inhibitor, 20.1 kD; and g) lactalbumin, 14.4 kD. Values for relative mobilities represent means of at least five determinations with standard errors for any point not exceeding 0.01.


A
B
MOLECULAR MASS ($M_r \times 10^{-3}$)
300
200
100
90
80
70
60
50
40
30
20
10
a
b
c
d
e
f
g
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
RELATIVE MOBILITY (R_f)

Appendix 2. Plots of pH values in focussed IEF tube gels as a function of distance along the length of the tube gel. Ampholines in the IEF gels consisted of either only pH range 5-8 ampholines (A), only pH range 3.5-10 ampholines (C), or a mixture of 20% pH range 3.5-10 and 80% pH range 5-8 ampholines (B). The points represent the mean values for determinations made on at least four IEF gels. Standard errors about the mean for any point did not exceed 0.04 pH units.


A
B
C
pH
10
9
8
7
6
5
4
3
0 1 2 3 4 5 6 7 8 9 10
cm. FROM TOP OF GEL

Bibliography

Adams, C. and Rinne, R.W. (1982). Stress protein formation: Gene expression and environmental interaction with evolutionary significance. Int. Rev. Cytol. 79: 305-315.

Altschuler, M. and Mascarenhas, J.P. (1982). Heat shock proteins and effects of heat shock in plants. Plant Mol. Biol. 1: 103-115.

Arrigo, A.-P. (1980). Investigation of the function of the heat shock proteins in Drosophila melanogaster tissue culture cells. Mol. Gen. Genet. 178: 517-524.

Arrigo, A.-P., Fakan, S. and Tissieres, A. (1980). Localization of the heat shock-induced proteins in Drosophila tissue culture cells. Devel. Biol. 78: 86-103.

Arrigo, A.-P. (1983). Acetylation and methylation patterns of core histones are modified after heat or arsenite treatment of Drosophila tissue culture cells. Nucl. Acids Res. 11: 1389-1404.

Ashburner, M. and Bonner, J.J. (1979). The induction of gene activity in Drosophila by heat shock. Cell 17: 241-254.

Atkinson, B.G. (1981). Synthesis of heat-shock proteins by cells undergoing myogenesis. J. Cell Biol. 89: 666-673.

Atkinson, B.G., Cunningham, T., Dean, R.L. and Somerville, M. (1983). Comparison of the effects of heat shock and metal-ion stress on gene expression in cells undergoing myogenesis. Can. J. Biochem. Cell Biol. 61: 404-413.

Atkinson, B.G. and Walden, D.B., eds. (1984). Changes in Gene Expression in Response to Environmental Stress, Academic Press, New York (in press).

Atkinson, B.G. and Dean, R.L. (1984). The effects of stress on the gene expression of amphibian, avian and mammalian blood cells, in Changes in Gene Expression in Response to Environmental Stress, B.G. Atkinson and D.B. Walden, eds., Academic Press, New York (in press).

Aviv, H. and Leder, P. (1972). Purification of biologically active globin messenger RNA by chromatography on oligothymidilic acid-cellulose. Proc. Natl. Acad. Sci. (USA) 69: 1408-1412.

Ballinger, D.G. and Pardue, M.L. (1983). The control of protein synthesis during heat shock in Drosophila cells involves altered polypeptide elongation rates. Cell 33: 103-114.

Barnett, T., Altschuler, M., McDaniel, C.N. and Mascarenhas, J.P. (1980). Heat shock induced proteins in plant cells. Devel. Genet. 1: 331-340.

Baszczynski, C.L. and Hughes, W.G. (1981a). Tissue-specific electrophoretic differences in polypeptides from maize cultivars. Maize Genet. Coop. Newslett. 55:116-117.

Baszczynski, C.L. and Hughes, W.G. (1981b). Comparison of maize polypeptides from various sources. 147th Natl. Amer. Assoc. for the Advancement of Sci. annual meeting, Toronto, Ont., Canada, Jan. 3-8 (abstr. 432).

Baszczynski, C.L., Walden, D.B. and Atkinson, B.G. (1981). Effect of heat shock on gene expression in maize (Zea mays L.). J. Cell Biol. 91 (Part 2): 368 (abstr. 21032).

Baszczynski, C.L., Walden, D.B. and Atkinson, B.G. (1982a). Regulation of gene expression in corn (Zea mays L.) by heat shock. Can. J. Biochem. 60: 569-579.

Baszczynski, C.L., Boothe, J.G., Walden, D.B. and Atkinson, B.G. (1982b). Studies on the heat shock response in different tissues and genotypes of maize. Maize Genet. Coop. Newslett. 56: 112-113.

Baszczynski, C.L., Walden, D.B. and Atkinson, B.G. (1982c). An in vitro analysis of the heat shock response in maize (Zea mays L.). Can. J. Genet. Cytol. 24:618 (abstr.)

Baszczynski, C.L., Walden, D.B. and Atkinson, B.G. (1982d). Analysis of the heat shock response in maize. Maize Genet. Coop. Newslett. 56: 111-112.

Baszczynski, C.L. (1983). Temperature-dependence of the heat shock response. Maize Genet. Coop. Newslett. 57: 160.

Baszczynski, C.L., Walden, D.B. and Atkinson, B.G. (1983a). Regulation of gene expression in corn (Zea mays L.) by heat shock. II. In vitro analysis of RNAs from heat-shocked seedlings. Can. J. Biochem. Cell Biol. 61: 395-403.

Baszczynski, C.L., Boothe, J.G., Atkinson, B.G. and Walden, D.B. (1983b). Polypeptide synthesis following upward and downward temperature shifts. Maize Genet. Coop. Newslett. 57: 161-162.

Baszczynski, C.L., Walden, D.B. and Atkinson, B.G. (1984). Maize Genome response to thermal shifts, in Changes in Gene Expression in Response to Environmental Stress, B.G. Atkinson and D.B. Walden, eds., Academic Press, New York (in press).

Bernstam, V.A. and Arndt, S. (1973). Effects of supraoptimal temperatures on the myxomycete Physarum polycephalum. I. Protoplasmic streaming, respiration and leakage of protoplasmic substances. Arch. Mikrobiol. 92: 251-261.

Bensaude, O., Babinet, C., Morange, M. and Jacob, F. (1983). Heat shock proteins, first major products of zygotic gene activity in mouse embryo. Nature 305: 331-333.

Bewley, J.D., Larsen, K.M. and Papp, J.E.T. (1983). Water-stress-induced changes in the pattern of protein synthesis in maize seedling mesocotyls: A comparison with the effects of heat shock. J. Exp. Bot. 34: 1126-1133.

Bienz, M. and Gurdon, J.B. (1982). The heat shock response in Xenopus oocytes is controlled at the translational level. Cell 29: 811-819.

Bonner, W.M. and Laskey, R.A. (1974). A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46: 83-88.

Bonnett, O.T. (1953). Developmental morphology of the vegetative and floral shoots of maize. Agricultural Experiment Station, University of Illinois, Bulletin 568.

Boothe, J.G., Walden, D.B. and Atkinson, B.G. (1982). A possible injury response observed in polypeptide patterns from treated maize leaf tissue. Maize Genet. Coop. Newslett. 56: 113-114.

Botha, F.C. (1979). The effect of drought conditions on water soluble proteins of two maize lines. Z. Pflanzenphysiol. 95: 371-375.

Bouche, G., Amabric, F., Cainzergues-Ferrer, M. and Zalta, J.P. (1979). Effects of heat shock on gene expression and subcellular protein distribution in Chinese hamster ovary cells. Nucl. Acids Res. 7: 1739-1747.

Brock, T.D. (1967). Life at high temperatures. Science 158: 1012-1019.

Brock, T.D. (1970). High temperature systems. Ann. Rev. Ecol. Systm. 1: 191-220.

Brock, T.D. and Darland, G.K. (1970). Limits of microbial existence: Temperature and pH. Science 169: 1316-1318.

Camato, R. and Tanguay, R.M. (1982). Changes in the methylation pattern of core histones during heat shock in Drosophila cells. EMBO J. 1: 1529-1532.

Comings, D.E. and Tack, L.O. (1972). Similarities in the cytoplasmic proteins of different organs examined by SDS gel electrophoresis. Exptl. Cell Res. 75: 73-78.

Cooper, P. and Ho, T.-H.D. (1983). Heat shock proteins in maize. Plant Physiol. 71: 215-222.

Crevecoeur, M., Deltour, R. and Bronchart, R. (1983). Effects of subminimal temperature on physiology and ultrastructure of Zea mays embryo during germination. Can. J. Bot. 61: 1117-1125.

Currie, R.W. and White, F.P. (1981). Trauma-induced protein in rat tissues: A physiological role for a "heat shock" protein? Science 214: 72-73.

Dean, R.L. and Atkinson, B.G. (1983). The acquisition of thermal tolerance in larvae of Calpodes ethlius (Lepidoptera) and the in situ and in vitro synthesis of heat-shock proteins. Can. J. Biochem. Cell Biol. 61: 472-479.

DiDomenico, B.J., Bugaisky, G.E. and Lindquist, S. (1982). The heat shock response is self-regulated at both the transcriptional and posttranscriptional levels. Cell 31: 593-603.

Eisen, H.N. (1980). Immunology, second edition, Harper and Row Publishers, Inc., Hagerstown, Maryland.

Feickert, D.C., Cooper, P. and Ho, T.-H.D. (1983). In vitro translation of mRNA coding for heat shock proteins in maize. Plant Physiol. Suppl., 72: 31 (abstr. 171).

Findly, R.C. and Pederson, T. (1981). Regulated transcription of the genes for actin and heat-shock proteins in cultured Drosophila cells. J. Cell Biol. 88: 323-328.

Fink, K. and Zeuthen, E. (1980). Heat shock proteins in Tetrahymena studied under growth conditions. Exptl. Cell Res. 128: 23-30.

Hadwiger, L.A. and Wagoner, W. (1983). Effect of heat shock on the mRNA-directed disease resistance response of peas. Plant Physiol. 72: 553-556.

Hageman, R.H. and Flesher, D. (1960). The effect of anaerobic environment on the activity of alcohol dehydrogenase and other enzymes of corn seedlings. Arch. Biochem. Biophys. 87: 203-209.

Hinton, H.E. (1960). A fly larva that tolerates dehydration and temperatures of -270C to 102C. Nature (London) 188: 336-337.

Hughes, W.G. and Walden, D.B. (1981). Electrophoretic study of maize pollen polypeptides. 147th Natl. Amer. Assoc. for the Advancement of Sci. annual meeting, Toronto, Ont., Canada, Jan. 3-8 (abstr. 431).

Hughes, W.G., Baszczynski, C.L. and Ketola-Pirie, C. (1981). Improved conditions for the extraction of maize polypeptides. Maize Genet. Coop. Newslett. 55: 116-117.

Kabat, E.A. (1976). Structural concepts in Immunology and Immunochemistry, second edition, Holt, Rhinehart and Winston, New York.

Kapoor, M. (1983). A study of the heat-shock response in Neurospora crassa. Int. J. Biochem. 15: 639-649.

Kelley, P.M. and Schlesinger, M.J. (1978). The effect of amino acid analogues and heat shock on gene expression in chicken embryo fibroblasts. Cell 15: 1277-1286.

Kelley, P.M., Aliperti, G. and Schlesinger, M.J. (1980). In vitro synthesis of heat-shock proteins by mRNAs from chicken embryo fibroblasts. J. Biol.Chem. 255: 3230-3233.

Kelley, P.M. and Schlesinger, M.J. (1982). Antibodies to two major chicken heat shock proteins cross-react with similar proteins in widely divergent species. Mol. Cell. Biol. 2: 267-274.

Ketola-Pirie, C.A. and Atkinson, B.G. (1983). Cold- and heat-shock induction of new gene expression in cultured amphibian cells. Can. J. Biochem. Cell Biol. 61: 462-471.

Key, J.L., Lin, C.Y. and Chen, Y.M. (1981). Heat shock proteins of higher plants. Proc. Natl. Acad. Sci. (USA) 78: 3526-3530.

Key, J.L., Lin, C.Y., Ceglarz, E. and Schoffl, F. (1982). The heat shock response in plants. Physiological considerations, in Heat Shock: From Bacteria to Man. M. Schlesinger, M. Ashburner and A. Tissieres, eds., p. 329, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Key, J.L., Lin, C.Y., Ceglarz, E. and Schoffl, F. (1983). The heat shock response in soybean seedlings. In, Nato Advanced Studies Workshop on Genome Organization and Expression in Plants, L. Dure, ed., p. 25, Plenum Press.

Key, J.L., Kimpel, J., Vierling, E., Lin, C.Y., Nagao, R.T., Czarnecka, E. and Schoffl, F. (1984). Physiological and Molecular analyses of the heat shock response in plants, in Changes in Gene Expression in Response to Environmental Stress., B.G. Atkinson and D.B. Walden, eds., Academic Press, New York (in press).

Kiesselbach, T.A. (1949). The structure and reproduction of corn. Agricultural Experiment Station, Univ. of Nebraska College of Agriculture, research Bulletin 161.

Kimball, J.W. (1974). Biology, third edition, Addison-Wesley Publishing Company, Reading, Massachusetts.

Kloetzel, P.-M. and Bautz, E.K.F. (1983). Heat-shock proteins are associated with hnRNA in Drosophila melanogaster tissue culture cells. EMBO J. 2: 705-710.

Krans, J.V. and Johnson, G.V. (1974). Some effects of subirrigation on bentgrass during heat stress in the field. Agron. J. 66: 526-530.

Kruger, C. and Benecke, B.-J. (1981). In vitro translation of Drosophila heat-shock and non-heat-shock mRNAs in heterologous and homologous cell-free systems. Cell 23: 595-603.

Kurtz, E.B.,Jr. (1958). Chemical basis for adaptation in plants. Understanding of heat tolerance in plants may permit improved yields in arid and semiarid regions. Science 128: 1115-1117.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature 227: 680-685.

Landry, J., Chretien, P., Bernier, D., Nicole, L.M., Marceau, N. and Tanguay, R.M. (1982a). Thermotolerance and heat shock proteins induced by hyperthermia in rat liver cells. Int. J. Radiat. Oncol. Biol. Phys. 8: 59-62.

Landry, J., Bernier, D., Chretien, P., Nicole, L.M., Tanguay, R.M. and Marceau, N. (1982b). Synthesis and degradation of heat shock proteins during development and decay of thermotolerance. Cancer Res. 42: 2457-2461.

Laskey, R.A. and Mills, A.D. (1975). Quantitative film detection of 3H and 14C in polyacrylamide gels by fluorography. Eur. J. Biochem. 56: 335-341.

Lazarides, E. and Hubbard, B.D. (1976). Immunological characterization of the 100A filaments from muscle cells. Proc. Natl. Acad. Sci. (USA) 73: 4344-4348.

Leeper, D.B., Karamuz, J.E. and Henle, K.J. (1977). Effect of inhibition of macromolecular synthesis on the induction of thermotolerance. Proc. Am. Assoc. Cancer Res. 18: 139.

Lehninger, A.L. (1975). Biochemistry, second edition, Worth Publishers, Inc., New York.

Levitt, J. (1980). Responses of Plants to Environmental Stresses. Volume 1. Chilling, Freezing and High Temperature Stresses. second edition, T.T. Kozlowski, ed., Academic Press, Inc., New York.

Liebermann, D., Hoffman-Liebermann, B. and Sachs, L. (1980). Molecular dissection of differentiation in normal and leukemic myoblasts: separately programmed pathways of gene expression. Devel. Biol. 79: 46-63.

Lindquist, S. (1980). Varying patterns of protein synthesis in Drosophila during heat shock: Implications for regulation. Devel. Biol. 77: 463-479.

Lindquist, S. (1981). Regulation of protein synthesis during heat shock. Nature 293: 311-314.

Lizardi, P.M. and Engelberg, A. (1979). Rapid isolation of RNA using proteinase K and sodium perchlorate. Anal. Biochem. 98: 116-122.

Loomis, W.F. and Wheeler, S. (1980). Heat shock response of Dictyostelium. Devel. Biol. 79: 399-408.

Lowry, D.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Mans, R.J. and Novelli, D.G. (1960). A convenient, rapid and sensitive method for measuring the incorporation of radioactive amino acids into protein. Biochem. Biophys. Res. Commun. 3: 540-548.

May, G.S. and Rosenbaum, J.L. (1980). Induction and synthesis of heat shock proteins in Chlamydomonas reinhardtii. J. Cell Biol. 87 (Part 2): 272a (abstr. R 2110).

Mayrand, S. and Pederson, T. (1983). Heat shock alters ribonucleoprotein assembly in Drosophila cells. Mol. Cell. Biol. 3: 161-171.

McKenzie, S.L., Henikoff, S. and Meselson, M. (1975). Localization of RNA from heat-induced polysomes at puff sites in Drosophila melanogaster. Proc. Natl. Acad. Sci. (USA) 72: 1117-1121.

McKenzie, S.L. and Meselson, M. (1977). Translation in vitro of Drosophila heat-shock messages. J. Mol. Biol. 117: 279-283.

Merrifield, P.A. (1979). Protein synthesis during thyroid hormone-induced degeneration of tadpole tail muscle. Ph.D. Thesis, University of Western Ontario, London, Ontario, Canada.

Meyer, Y. and Chartier, Y. (1983). Long-lived and short-lived heat-shock proteins in tobacco mesophyll protoplasts. Plant Physiol. 72: 26-32.

Minton, K.W., Karmin, P., Hahn, G. and Minton, A.P. (1982). Nonspecific stabilization of stress-susceptible proteins by stress-resistant proteins: A model for the biological role of heat shock proteins. Proc. Natl. Acad. Sci. (USA) 79: 7107-7111.

Mitchell, H.K. and Lipps, L.S. (1975). Rapidly labeled proteins on the salivary gland chromosomes of Drosophila melanogaster. Biochem. Genet. 13: 585-603.

Mitchell, H.K., Moller, G., Petersen, N.S. and Lipps-Sarmiento, L. (1979). Specific protection from phenocopy induction by heat shock. Devel. Genet. 1: 181-192.

Moran, L., Mirault, M.-E., Arrigo, A.P., Goldschmidt-Clermont, M. and Tissieres, A. (1978). Heat shock of Drosophila melanogaster induces the synthesis of new messenger RNAs and proteins. Phil. Trans. Roy. Soc. London B. 283: 391-406.

Nover, L., Scharf, K.-D. and Neumann, D. (1983). Formation of cytoplasmic heat shock granules in tomato cell cultures and leaves. Mol. Cell. Biol. 3: 1648-1655.

O'Farrell, P.H. (1975). High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250: 4007-4021.

Okimoto, R., Sachs, M.M., Porter, E.K. and Freeling, M. (1980). Patterns of polypeptide synthesis in various maize organs under anaerobiosis. Planta 150: 89-94.

Peacocke, A.R. and Walker, I.O. (1962). The thermal denaturation of sodium deoxyribonuclease. II. Kinetics. J. Mol. Biol. 5: 560-563.

Pelham, H.R.B. and Jackson, R.J. (1976). An efficient mRNA-dependent translation system from reticulocyte lysate. Eur. J. Biochem. 67: 247-256.

Pulgar, C.E. and Laude, H.M. (1974). Regrowth of alfalfa after heat stress. Crop Sci. 14: 28-30.

Ray, A., Reynolds, J.A., Polet, H. and Steinhardt, J. (1966). Binding of large organic anions and neutral molecules by native bovine serum albumin. Biochem. 5: 2606-2616.

Rensing, L., Olomski, R. and Drescher, K. (1982). Kinetics and models of the Drosophila heat-shock system. Biosystems 15: 341-356.

Ritossa, F. (1962). A new puffing pattern induced by heat shock and DNP in Drosophila. Experientia 18: 571-573.

Robbins, W.J. and Petsch, K.F. (1932). Moisture content and high temperature in relation to the germination of corn and wheat grains. Bot. Gaz. 93: 85-92.

Roccheri, M.C., DiBernardo, M.G. and Guidice, G. (1981). Synthesis of heat-shock proteins in developing sea urchins. Devel. Biol. 83: 173-177.

Rodenhiser, D.I., Jung, J.H. and Atkinson, B.G. (submitted). The effect of heat shock on gene expression in isolated mammalian (human and mouse) white blood cells; A comparison eith the in situ responses.

Sachs, M.M. and Freeling, M. (1978). Selective synthesis of alcohol dehydrogenase during anaerobic treatment of maize. Mol. Gen. Genet. 161: 111-115.

Sachs, M.M., Freeling, M. and Okimoto, R. (1980). The anaerobic proteins of maize. Cell 20:761-767.

Saleem, M. and Atkinson, B.G. (1976). Isoelectric points and molecular weights of salt-extractable ribosomal proteins. Can. J. Biochem. 54: 1029-1033.

Sanders, M.M. (1981). Identification of histone H2b as a heat-shock protein in Drosophila. J. Cell Biol. 91: 579-583.

Scandalios, J.G. (1982). Developmental genetics of maize. Ann. Rev. Genet. 16: 85-112.

Scharf, K.-D. and Nover, L. (1982). Heat-shock-induced alterations of ribosomal protein phosphorylation in plant cell cultures. Cell 30: 427-437.

Schlesinger, M.J., Ashburner, M. and Tissieres, A., eds. (1982). Heat Shock: From Bacteria to Man, Cold Spring Harbor Laboratory, Cold Spring Harbor.

Schmidt-Nielsen, K. (1975). Animal Physiology: Adaptation and Environment, Cambridge University Press, London.

Schoffl, F. and Key, J.L. (1982). An analysis of mRNAs for a group of heat shock proteins of soybean using cloned cDNAs. J. Mol. App. Genet. 1: 301-314.

Schoffl, F. and Key, J.L. (1983). Identification of a multigene family for small heat shock proteins in soybean and physical characterization of one individual gene coding region. Plant Mol. Biol. 2: 269-278.

Schwartz, D. (1969). An example of gene fixation resulting from selective advantage in suboptimal conditions. Amer. Nat. 103: 479-481.

Scott, M.P. and Pardue, M.L. (1981). Translational control in lysates of Drosophila melanogaster cells. Proc. Natl. Acad. Sci. (USA) 78: 3353-3357.

Selye, H. (1973). The evolution of the stress concept. Amer. Sci. 61: 692-699.

Shah, D.M., Rochester, D.E., Krivi, G.G., Hironaka, C.M. Mozer, T.J., Fraley, R.T. and Tiemeier, D.C. (1984). Isolation and characterization of a maize gene encoding major heat-shock protein, Hsp70. J. Cellul. Biochem. suppl. 8B: 244 (abstr. 1477).

Silver, J.C., Andrews, D.R. and Pekkala, D. (1983). Effect of heat shock on synthesis and phosphorylation of nuclear and cytoplasmic proteins in the fungus Achlya. Can. J. Biochem. Cell Biol. 61: 447-455.

Sinibaldi, R.M. and Dietrich, P.S. (1984). Zea mays DNA sequence homology with Drosophila 83 and 70 kilodalton heat shock genes. J. Cellul. Biochem. suppl. 8B: 245 (abstr. 1478).

Sondermeijer, P.J.A. and Lubsen, N.H. (1978). Heat shock peptides in Drosophila hydei and their in vitro synthesis. Eur. J. Biochem. 88: 331-339.

Spradling, A., Pardue, M.L. and Penman, S. (1977). Messenger RNA in heat-shocked Drosophila cells. J. Mol. Biol. 109: 559-587.

Stamp, P. Geisler, G. and Thiaporn, R. (1983). Adaptation to sub- and supraoptimal temperatures of inbred maize lines differeing in origin with regard to seedling development and photosynthetic traits. Physiol. Plant. 58: 62-68.

Storti, R.V., Scott, M.P., Rich, A. and Pardue, M.L. (1980). Translational control of protein synthesis in response to heat shock in D. melanogaster. Cell 22: 825-834.

Tal, M. (1983). Selection for stress tolerance, in Handbook of Plant Cell Culture, eds., D.A. Evans, W.R. Sharp, P.V. Ammirato and Y. Yamada, Macmillan Publishing Co., New York.

Tanguay, R.M. and Vincent, M. (1981). Biosynthesis and characterization of heat shock proteins in Chironomus tentans salivary glands. Can. J. Biochem. 59: 67-73.

Tanguay, R.M. and Vincent, M. (1982). Intracellular translocation of cellular and heat shock induced proteins upon heat shock in Drosophila Kc cells. Can. J. Biochem. 60: 306-315.

Tanguay, R.M. (1983). Genetic regulation during heat shock and function of heat shock proteins: a review. Can. J. Biochem. Cell Biol. 61: 387-394.

Tanguay, R.M., Camato, R., Lettre, F. and Vincent, M. (1983). Expression of histone genes during heat shock and in arsenite treated Drosophila Kc cells. Can. J. Biochem. Cell Biol. 61: 414-420.

Theillet, C., Delpeyroux, F., Fiszman, M., Reigner, P. and Esnault, R. (1982). Influence of the excision shock on the protein metabolism of Vicia faba L. meristematic root cells. Planta 155: 478-485.

Tissieres, A., Mitchell, H.K. and Tracy, U.M. (1974). Protein synthesis in salivary glands of D. melanogaster. Relation to chromosome puffs. J. Mol. Biol. 84: 389-398.

Velazquez, J.M., DiDomenico, B.J. and Lindquist, S. (1980). Intracellular localization of heat shock proteins in Drosophila. Cell 20: 679-689.

Velazquez, J.M., Sonoda, S., Bugaisky, G. and Lindquist, S. (1983). Is the major Drosophila heat shock protein present in cells that have not been heat shocked? J.Cell Biol. 96: 286-290.

Velazquez, J.M. and Lindquist, S. (1984). hsp70: Nuclear consentration during environmental stress and cytoplasmic storage during recovery. Cell 36: 655-662.

Verma, R.S. (1972). Nuclear cycle in maize (Zea mays) root tips. Ph.D. Thesis, University of Western Ontario, London, Ontario, Canada.

Verma, R.S. and Lin, M.S. (1978). Chemically induced alterations of the nuclear cycle and chromosomes in root meristem cells of maize. J. Hered. 69: 285-294.

Voellmy, R., Bromley, P. and Kocher, H.P. (1983). Structural similarities between corresponding heat-shock proteins from different eucaryotic cells. J. Biol. Chem. 258: 3516-3522.

Walden, D.B., Baszczynski, C.L., Boothe, J.G. and Atkinson, B.G. (1984). Thermal regulation of gene expression in maize. Can. J. Biochem. Cell Biol. (submitted).

Wilcockson, J. (1975). The differential precipitation of nucleic acids and proteins from aqueous solutions by ethanol. Anal. Biochem. 66: 64-68.

Zimmerman, J.L., Petri, W. and Meselson, M. (1983). Accumulation of a specific subset of D. melanogaster heat shock mRNAs in normal development without heat shock. Cell 32: 1161-1170.

Zurfluh, L.L. and Guilfoyle, T.J. (1980). Auxin-induced changes in the patterns of protein synthesis in soybean hypocotyl. Proc. Natl. Acad. Sci. (USA) 77: 357-361.

Zurfluh, L.L. and Guilfoyle, T.J. (1982). Auxin-induced changes in the population of translatable messenger RNA in elongating maize coleoptile sections. Planta 156: 525-527.

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