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INDUCTION OF RESISTANCE TO ULTRAVIOLET LIGHT IN *ESCHERICHIA COLI* BY HEAT SHOCK

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Submitted to the School of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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ABBREVIATIONS

AppppAp	P1,P4-diadenosine-5'-tetraphosphate
ApppA	P3-diadenosine-5'-triphosphate
AppppG	P1-(adenosine-5')P4-(guanosine-5')-tetraphosphate
ApppG	P1-(adenosine-5')-P3-(guanosine-5')-triphosphate
ApppGpp	P1-(adenosine-5')-P3-(guanosine-3'-diphosphate-5')-triphosphate [Adenylated nucleotides, dinucleoside polyphosphates]
DNA	deoxyribonucleic acid
Genetic markers:	
ara	arabinose
gal	galactose
his	L-histidine
kdgK	ketodeoxygluconokinase
lac	lactose
leu	L-leucine
met	L-methionine
mgl	methyl-galactoside
mtl	maltose
rac	prophage rac
rfbD	TDP-rhamnose synthetase
rpsL	streptomycin resistant
supE	supressor of amber (UAG) mutations
thi	thiamine
thr	L-threonine
thy	thymine
tonA	outer membrane protein receptor for T1,T5
trp	L-tryptophan
tsx	receptor for phage T6
xyl	xylose
hsp	heat shock protein
HTP	High Temperature Production
K	10^3, when used with relative molecular masses
PAGE	Polyacrylamide Gel Electrophoresis
RNA	ribonucleic acid
SDS	sodium dodecyle sulfate
Tris	2-amino-2-hydroxymethyl propane-1,3-diol
uv	ultraviolet

GENETIC NOTATION

Throughout this manuscript products of Escherichia coli (E.coli) genes are designated by the name of the gene, non-underlined, with its first letter capitalized, e.g.DnaK is the protein product of the dnaK gene.

1. INTRODUCTION

Living systems are under selective pressure to regulate a vast array of biochemical reactions during frequently changing surroundings. They have evolved continuously, in a hazardous environment that contains toxic chemicals and exposes them to radiation, by developing a hierarchy of self defence mechanisms. Data are now rather compelling for the existence of a common response to stress in living beings, since all organisms so far tested respond to changes in their local environment by the rapid, preferential synthesis and accumulation of a set of proteins termed heat shock or stress proteins or hsps [1]. This stress can be either a sudden increase in temperature [2-4], treatment with chemicals [4], bacteriophage infection [5,6], or exposure to DNA damaging agents [7]. Any one stress yields a characteristic pattern of hsps that may overlap with that induced by other stresses.

This section will review our basic understanding of both heat shock response and DNA repair mechanisms and outline the objectives of the work described in the remainder of the thesis.

1.1. Studies on The Heat Shock Response

Man has long studied the effects of heat on himself and other living things, but studies of the heat shock response began in 1962, with the publication of a little noticed paper describing a new set of puffs on the salivary glands of chromosomes of a fruit-fly, *Drosophila buscukii*; these puffs were induced by heat, dinitrophenyl phosphate or sodium salicylate [8]. Recently, it has been realized that its components are among the most conserved genetic elements known at present, involving recognizable homology across the boundaries of the procaryotic, eucaryotic and archaebacterial kingdoms [3].

Bacteria have a broader temperature range for normal growth than do most higher organisms and the heat shock response of *Escherichia coli* (*E.coli*) can be seen over a wide range of growth temperatures. There appears to be no threshold temperature that must be achieved to evoke the heat shock response. A shift from 30°C to 43°C causes induction of accelerated synthesis of all cell proteins. Shifts from 28°C to 43°C-47°C, which result in restricted rates of growth, bring about a more pronounced heat shock induction, because of a diminished synthesis of general cellular proteins. On the other hand, shifts to temperatures that do not permit balanced growth even in a very rich medium (47°C and above)

bring about a nearly exclusive synthesis of hsps and this synthesis appears to continue as long as the (now dead) cells can make proteins [4].These hsps are also known as High Temperature Production proteins (HTP proteins).

1.1.1. Definition of the HTP Regulon

Many of the genes of *E.coli* are organized as functional units called regulons, in which unlinked and individually controlled genes can be coordinately controlled by a common regulatory gene [9]. In other words one or more operons are under the control of a regulatory gene. Most of the regulons of *E.coli* are responsive to environmental stress, nutrient limitation, or damage caused by toxic chemicals or physical agents.

Evidence now indicates that among the many proteins induced by high temperature in *E.coli* is a set of 17 whose genes constitutes a regulon designated as the HTP regulon [10]. Work in two laboratories has established that a nonsense mutation in strain K165 that is in a gene called *htp*R [10] or *hin* [11] or *rho*H [12], and maps near min 75 on the *E.coli* chromosome, eliminates the temperature response for HTP protein (or hsp) synthesis. This effect is followed by the death and lysis of the mutant cells after an hour of growth at 42°C. On the other hand the synthesis of hsps is

increased within one min after a shift from 30°C to a higher growth temperature (43°C) in wild type E.coli [4]. Maximum rates of heat shock protein synthesis are reached within 5-10 min, and by about 30 min after the shift synthesis of hsps has declined to a new level characteristic of the higher temperature [4]. The increased synthesis of hsps is caused by increased transcription initiation from heat shock gene promoters which are under the control of the product of htpR gene, σ^{32} [11]. The demonstration that heat shock genes have promoters recognized by $E\sigma^{32}$, where E stands for RNA polymerase core enzyme having subunits $\alpha_2\beta\beta'$, led to the idea that the heat shock response is controlled by factors that affect the amount or the activity of $E\sigma^{32}$ [13-16]. Some evidence suggests that increase in the amount of σ^{32} may account for the induction of heat shock mRNA synthesis [13-18]. Consistent with the idea, Tilly et al. have shown that rpoH mRNA levels increase with kinetics similar to heat shock gene mRNA levels but they did not distinguish individual mRNA levels [19].

Recently, Erickson et al. have examined the transcription of the rpoH gene during steady state conditions and after shifts to higher temperatures [20]. They confirmed previous findings [11-17] that the product of rpoH gene σ^{32}, directs RNA polymerase to initiate transcription from heat shock

promoters at all temperatures. The transcription of heat shock genes is increased when the cells are exposed to high temperature because of increased transcription initiation by σ^{32} RNA polymerase. They found that rpoH is the only gene in its transcription unit. There are at least three rpoH promoters. Two of them were recognized by the most abundant form of RNA polymerase, $E\sigma^{70}$ and were active at both low and high growth temperatures. Transcripts from third promoters were present only after shift to lethal temperatures (50°C). It is not known what form of RNA polymerase recognizes the third promoter. The level of rpoH mRNA increased five fold by 8 minutes after shift to higher temperatures, but the synthesis of rpoH mRNA increased by less than two folds.

Fujita et al. have also found that rpoH is transcribed from three promoters, two major P1, P2 and one minor P2* [21]. The transcription from P2 promoter enhanced transiently upon heat shock while not from the P1. P1 might play a role in supplying a basal level of σ^{32} necessary at lower temperature. Alternatively it could be regulated under certain stress conditions other than heat shock. The level of P2* varied from experiment to experiment, however, it also increased after heat shock. Their finding supports the concept that each of the multiple promoters within a single

gene or operon has a different regulatory role [22].

Increase in σ^{32} mRNA reflects either a direct control of the stability of the rpoH mRNA or indirect stabilization due to increased translation of rpoH mRNAs at high temperature [15,19-21]. Grossman et al. have shown that σ^{32} is an unstable protein with a half life of approx. 4 min so changes in its rate of synthesis will rapidly result in changes in its intracellular concentration. Simply an increase in the synthesis of σ^{32} was sufficient to cause an increase in the synthesis of hsps at low or high temperature. No other inducing signal was required and the expression of the heat shock genes during steady-state growth seemed to be limited by the amount of σ^{32} in the cell. They also found that the synthesis of σ^{32} initially increased and then declined after 10 min upon a temperature shiftup. Its synthesis was repressed at some step after transcription initiation perhaps at the level of translation or of mRNA stability. This post transcriptional repression of σ^{32} synthesis, coupled with the fact that its half life is 4 min., could explain the decline in hsp synthesis, following a peak of the heat shock response. It is possible that some regulator(s) of the heat shock response also play some role, because it is still not clear by which process extracellular stress is sensed and

transduced into an intracellular signal.

1.1.2. Proteins and Genes of the HTP Regulon

The identification of the hsps as a part of the heat shock response has depended primarily on the analysis of two dimensional electrophoresis gels before and after temperature shift [23-25]. Seven of the seventeen HTP polypeptides [Table 1] have been identified in detail. These seven extensively characterized genes are: groEL [26], groES [27], dnaK [28], dnaJ [29], rpoD [14], lysU [25] and lon [30].

It is now known that the groE operon encodes two proteins, GroES and EL (15 and 65K respectively), but, in the earlier literature, mutations mapping to the two genes were not distinguishable as such [27,31]. GroES and GroEL proteins interact functionally in such a way that mutations in groEL can suppress multiple phenotypic effects of mutations in groES. Also mutations in either gene yield similar phenotypic effects on phage assembly [27]. In vitro, the two proteins cosediment in the presence of ATP, and purified GroES protein inhibits the weak ATPase activity of the GroEL protein. The GroE proteins are essential for the morphogenesis of many (perhaps all) coliphages [32]. Though details differ from virus to virus, a defect in the normal

Table.1: The heat shock proteins of *Escherichia coli*.

No.	α-Num. designation[a]	Mol. wt[b].	Name	Gene[c]	Possible function
1.	B25.3	25,300	-	*htp*A	-
2.	B56.5	62,883	GroEL	*mop*A(94) (*gro*EL)[d]	Morphology of coliphage (weak ATPase activity); some role in RNA and DNA synth.(?)
3.	B66.0	69,121	DnaK	*dna*K (0.5)	Phage DNA replication (weak ATPase activity); Modulation of heat shock response; necessary for RNA and DNA synthesis
4.	B83.0	70,263	Sigma	*rpo*D	Promoter recog.; subunit of RNA polymerase.
5.	C14.7	14,700	-	*htp*E	-
6.	C15.4	10,670	GroES	*mop*B(94) (*gro*ES)[e]	Morphology of coliphage; role in RNA and/or DNA synth.(?)
7.	C62.5	62,500	-	*htp*G	-
8.	D33.4	33,400	-	*htp*H	-
9.	D48.5	48,500	-	*htp*I	-
10.	D60.5	60,500	lysyl-tRNA synthase (II)	*lys*U (93.5)	Charging of tRNA; synthesis of alarmones(?)
11.	F10.1	10,100	-	*htp*K	-
12.	F21.5	21,500	-	*htp*L	-
13.	F84.1	84,100	-	*htp*M	-
14.	G13.5	13,500	-	*htp*N	-
15.	G21.0	21,000	-	*htp*O	-
16.	H94.0	94,000	Lon	*lon*(10)	ATP-dependent protease.
17.	H26.5	26,500	DnaJ	*dna*J(0.5)	Some role in RNA and DNA synth.

a) Nomenclature described by Pederson and Neidhardt (1978) *Cell* 14,179-190.b) Mol.wt.determined by PAGE, except for no. 2,3,4 and 6, which were determined from DNA sequence. c) The no. in parentheses refer to gene position as shown by Bachmann.B.(1983) *Micro.Biol.Rev*.47,180-230.d) *gro*EL: for *mop*A.and e) *gro*ES: for *mop* B.
Table reproduced from ref. no. 4.

processing of virion protein is a common feature of infected groE mutants. Tail assembly is blocked in T5 [33] and head assembly in T4 [34,35] and lambda phage [36-38]. GroEL is reported to assciate with the phage lambda B protein which forms part of the head-tail connector of the virion. The groE gene is induced along with certain other heat shock genes during early stages of phage lambda infection [5,6].

The cellular functions of GroES and GroEL proteins in uninfected cells is unknown. They are believed to be essential for growth because some mutants selected for an inability to support phage growth possess a temperature sensitive growth phenotype. At high temperatures, they account for an astounding 15% of total cellular protein [24,39]. Even at normal temperatures, they are among the most abundant proteins in the cell. Mutations have effects like altering permeability, causing filamentation and disrupting normal patterns of DNA, RNA and protein synthesis at non-permissive temperatures [40-42].

E.coli dnaK was originally identified as a host gene required for replication of bacteriophage lambda [43,44]. It appears to interact with both the lambda O and P proteins, the former stimulating and latter inhibiting its

ATPase activity [45]. Its function in the uninfected cell cannot yet be specified, although it is essential for cell growth. Purified DnaK protein has weak ATPase activity and is capable of autophosphorylation at a threonine residue [28]. When mutants of dnaK are placed at restrictive temperatures, RNA synthesis appears to stop before DNA synthesis [46]. This is the second most abundant heat shock protein and its gene is perhaps one of the most conserved elements in biology. DnaK has been thought to be a modulator of the heat shock response, being necessary for the eventual damping of the induction during continued incubation at high temperature [4]. Grossman et al. have observed recently, the continued synthesis of σ^{32} and hsps in the dnaK756 mutant [15]. The mutation caused a defect in the shut-off phase of the heat shock response. Earlier, they had suggested that a role of DnaK in modulating the heat shock response might be to modify σ^{32}, perhaps by phosphorylation of σ^{32} [13]. But they have been unable to detect phosphorylation of σ^{32} [15]. Their experiments suggest that DnaK, either directly or indirectly, acts to repress translation of rpoH mRNA. Induction of the heat shock response and a corresponding increase in the expression of dnaK would cause the translational repression of rpoH mRNA. The resulting decrease in σ^{32} synthesis would lead to a rapid decline in the intracellular levels of σ^{32}

and a decrease in the rate of synthesis of hsps. The dnaK756 mutation partially stabilizes σ^{32}, as well as some other unstable proteins [15]. The role of dnaK and other hsp genes in protein turnover needs further investigation. However, Ramsay has demonstrated that although the DnaK protein is produced in large amounts in response to thermal stress, it is not required for the development of heat resistance [47]. A mutation in the gene had little or no effect on induced thermotolerance.

The product of the dnaJ gene is like the DnaK protein. It is necessary in the same way for phage DNA replication and is essential for cell growth in uninfected cells [29]. It binds with no apparent specificity to both single and double stranded DNA. This HTP protein has the most basic isoelectric point among the known hsps and is the only E.coli HTP protein reported to be largely or entirely associated with the cell envelope.

The majority of cellular transcription requires the predominant, or primary sigma (σ) factor [48,49]. In E.coli, the primary σ factor is referred to as σ^{70}. This 70K protein (σ^{70}) is the product of the rpoD gene. Its synthesis is enhanced by heat shock. In the eubacteria, the core RNA polymerase contains four subunits with the stoichiometry

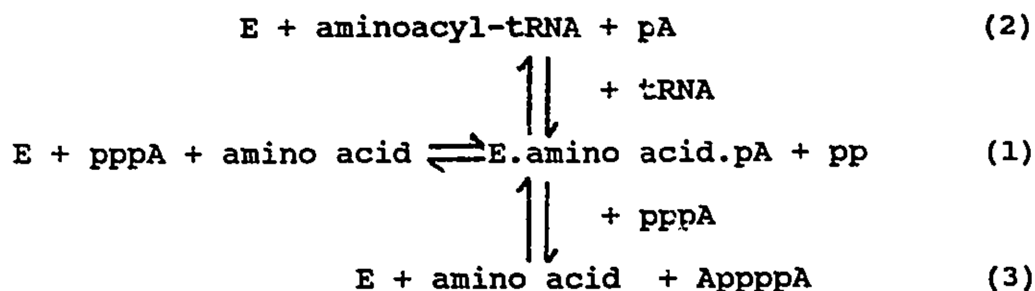
$\alpha_2\beta\beta'$. This core enzyme interacts with individual specific proteins, known as σ factors, to form a holoenzyme with the minimal composition, $\alpha_2\beta\beta'\sigma$. Only the holoenzyme can initiate transcription accurately at promotor sequences [49]. Following transcription initiation, the σ factor is released and the core enzyme elongates an RNA chain [6]. Therefore, the σ subunit is necessary for selective DNA binding and may well play a role in the regulation of transcription initiation in E.coli.

The products of rpoD and rpoH (σ^{70} and σ^{32}), compete for RNA polymerase. The $E\sigma^{32}$ with composition $\alpha_2\beta\beta'\sigma^{32}$ is unable to recognize the promoters which are usually transcribed by $E\sigma^{70}$. Instead it transcribes the promoters for hsps. Besides the strict difference in the promotor selectivity, both enzymes share similar properties in some other respects such as molecular composition, formation of active initiation complexes, sensitivity to salt concentration and temperature dependence. This suggests that the same mechanism operates in the initiation step of transcription by $E\sigma^{32}$ and $E\sigma^{70}$. It is noteworthy that a mutant that produces increased amounts of σ^{70} synthesizes reduced amounts of heat shock proteins [50]. Conversely, when cellular amounts of σ^{70} are reduced by inefficient suppression of an rpoD amber mutation, larger amounts of

hsps are synthesized [16,48,51]. Various genetic observations suggest functional interactions between the two σ factors *in vivo* [11,17,51]. Fujita *et al* have shown recently that σ^{32} could be replaced *in vitro* by the addition of excess σ^{70} indicating that two factors are interchangeable on the same core enzyme [16]. This supports the view that the spectrum of gene expression in *E. coli* is under a dynamic control by the exchange of σ factors of RNA polymerase, as originally suggested for the gram positive bacterium, *Bacillus subtilis* [52]. However, the mechanism(s) by which the exchange of these factors is facilitated upon heat shock remains unsolved.

The product of *lys*U gene is an isospecies of Lysyl-tRNA synthetase (LysS) and is heat inducible. Under most growth conditions, only the product of the *lys*S gene is made. Lysyl-tRNA synthetase catalyses the activation of lysine and its attachment to tRNA for protein synthesis [25]. This enzyme is reported to be adept at synthesizing various phosphorylated derivatives of adenosine, ApppA, AppppG, ApppA, ApppG and ApppGpp [53]. These adenylated nucleotides are synthesized *in vitro* and, presumably, *in vivo* by a side reaction of amino acyl-t-RNA synthetases that result in the adenylation of ATP, ppGpp, GTP, GDP and ADP [54]. In 1966, Zamecnik *et al*. found AppppA synthesized in an *in vitro*

system consisting of ATP,Mg^{2+}, L-lysine and purified E. coli Lysyl-tRNA synthetase [55]. The mechanism of its formation was determined to be as shown schematically:



Reaction (2) is strongly preferred under *in vitro* conditions. However, at low tRNA concentrations or in the presence of micromolecular concentrations of Zn^{2+} salts, some aa-tRNA synthetases (E) preferentially catalyse reaction (3). It is likely that AppppA and related adenylated nucleotides are produced by reaction (3) *in vivo*.

Recently these small molecules have been found to accumulate in *Salmonella typhimurium* and *E.coli* after a heat shock [54]. Other stress conditions, such as treatment with ethanol or hydrogen peroxide, cadmium, etc., which are known to induce hsps also induce these nucleotides. The intracellular concentration of the nucleotides increases five to ten fold within 5 min after temperature shift and continues to increase for at least an additional 15 min.

Initially it was proposed that they might serve as ligands to activate htpR and thus would function as specific "alarmones" [53]. The term alarmone has been proposed by Stephens et al. to denote a low molecular weight intracellular compound that is synthesized in response to a specific metabolic stress and acts homeostatically to increase the probability of a cell's survival [56]. However some of the treatments tested, including exposure to nalidixic acid, which induce hsps do not induce these alarmones [57]. An htpR mutant, which is unable to induce synthesis of hsps, still accumulates adenylated nucleotides, indicating that their accumulation is not due to synthesis of hsps [54]. Moreover, the time course of the induction of these dinucleotides (5-15 min) is slower than that of the hsps (1-5 min). Recently, Plateau et al. did experiments with E.coli strains overproducing dinucleotide tetraphosphate hydrolase [58]. This enzyme reduces the concentration of the dinucleotides by ten-fold in the cell. They showed that the reduced levels of dinucleotides neither changed the pattern of induction of hsps nor reduced the protection against the hydrogen peroxide stress as compared to control cultures having higher cell concentration of these nucleotides [58].

Another HTP protein, the Lon protease, is the product of lon or capR gene and is an ATP-dependent enzyme. It initiates a major route by which E.coli mutants defective in lon, exhibit an abnormal SOS response (see p.27). Successful development of the latter apparently depends on the ability of Lon to degrade SulA protein, a cell division inhibitor [59]. This inhibitor accumulates in the abnormal SOS response [60]. Also, lon mutants accumulate a large quantity of mucopolysaccharides and have decreased ability to lysogenize phage lambda.

The seven well-studied HTP genes are not linked to the putative regulatory gene, htpR; groEL and groES map at 93.5, dnak and dnaJ at 0.5, rpoD at 67, lysU at 93 and lon at 10 min. The other ten hsps or HTP proteins have neither been identified nor have their genes been mapped.

To summarize, the proteins identified so far appeared to be involved in the major macromolecular processes of the cell: DNA replication (DnaK and J), RNA synthesis (sigma factor; RpoH and RpoD), protein synthesis (LysU), protein processing or assembly (GroES and L) and protein degradation (Lon protease).

1.1.3. Physiological Role of the Procaryotic Heat Shock Response

Evidence for the function of the heat shock response is indirect and incomplete.It comes mainly from experiments performed to study the survival of normal cells under the conditions which induce hsps (e.g., temperature shift up, viral infection, or treatment with ethanol, ultraviolet radiation, nalidixic acid, etc.) and of mutants defective in HTP function. The following are the various cellular effects that accompany the induction of these stress proteins which suggest possible functions for the heat shock response.

1.1.3.1. First of all, no direct evidence is available to support the idea that DNA damage can be the cause of loss of viability at lethal temperatures. Extended exposure to 50°C leads to an initial unfolding of the bacterial chromosome, as judged by its decreased sedimentation constant, following which its sedimentation increases greatly as a result of association of a large amount of protein with the nucleoid [61]. A large amount of the nucleoid - associated protein is GroEL [61]. At growth permissive temperatures, DNA strand breakage occurs, but efficient repair processes might mask any outward signs of damage [62]. Temperature shift up is known to have an effect

on DNA winding: for each degree rise in temperature the bond angle between adjacent bases decreases by 0.012°C [60]. This is proposed to be one of the probable reasons for changes in rates of synthesis of proteins following temperature shift [23]. Second, protein denaturation could also take place, but that is possible only upon prolonged exposure to lethal temperatures [62].

1.1.3.2. The heat shock genes are subject to independent systems of activation by stress. HTP proteins or hsps which are induced by ethanol treatment also are under control of the htpR regulon. However the kinetics of induction of proteins by ethanol seems to be different in the sense that the hsps are induced more slowly as compared to their induction by temperature shift-up [60]. Under normal intracellular conditions (pH 7.7, 0.2 M KCl), the interactions of many proteins with DNA would be exquisitely sensitive to small changes in ionic strength or pH so that such perturbations of protein-DNA interactions might affect DNA stability [63]. Ethanol has been shown to affect transmembrane transport in bacteria and might also alter intracellular pH by this means. The effect of ethanol would thus be consistent with the notion that the principal inducer of the heat shock response is a particular perturbation of the intracellular ionic balance, conceivably

an alteration of the intracellular pH [64]. Moreover, ethanol also affects the translation process [63,64]. Effects have been noted on the process of transmembrane translocation as well [65]. Given these diverse effects, each of which can be expected to generate many secondary changes in cell structure and function, it is not helpful at present to speculate on which ones are the most pertinent for induction of the heat shock response.

If normal E.coli cells are subjected to heat shock (30°C to 42°C) prior to being shifted to a higher lethal temperature of 55°C, death occurs more slowly than if a direct shift from 30°C to 55°C is made. This phenomenon is known as thermotolerance and is also observed after stress treatments (e.g., exposure to ethanol) which induce hsps. The converse is also true, i.e., heat shock induces tolerance to ethanol and other forms of stresses [51]. Experiments with the htpR 165 mutant support the view that the heat shock response confers thermotolerance and ethanol -tolerance: the mutant dies and lyses even at 37°C [4]. This suggests that the concentration of HtpR at this temperature must be below the threshold value necessary to keep the cell viable. Either it is absolutely necessary for viability that the abundance of HTP proteins be elevated above a certain critical concentration, or one of the HTP

proteins not ordinarily made at low temperatures is essential, or there is some function of HtpR other than its role as the positive effector of the HTP response.

1.1.3.3. Some of the heat shock gene products (GroEL and DnaK and J) are synthesized in increased amounts upon infection of the cell with lambda phage, while host DNA, RNA and protein synthesis decreases sharply [5,6]. This induction is <u>htp</u>R$^+$ dependent. Also, the <u>htp</u>R gene is necessary for growth of lambda at higher temperatures. HTP production could be a response of the host to damage inflicted by the infection and at the same time be favorable for lambda growth. Several stages of the phage growth cycle have been shown to depend on <u>htp</u>R gene function. These include prophage excision, head assembly and processing or assembly of tail fibers.

1.1.3.4. Many of the hsps are induced upon ultraviolet (uv) irradiation of the cells. This hsp induction is <u>htp</u>R-dependent and requires stronger doses (100 $J.m^{-2}$) than does induction of the SOS response (discussed later) [7]. Nalidixic acid, an inhibitor of DNA gyrase, is another SOS response inducer which is also known to induce some of the stress proteins [7] and is much more effective than uv. Maximum induction is reached within 10-15 min of

nalidixic acid treatment, while the peak with uv treatment is 15-20 min. Other inhibitors of DNA gyrase are comuromycin and chlorobiocin, which also cause a similar induction of stress proteins. Two decades ago, it was noticed by Bridges et al. that 50-52°C treatment causes the same changes in DNA sedimentation constant as does radiation damage [66]. It has therefore been suggested that the intracellular signal for hsps induction by heat and SOS response inducers might be a damage to the same cellular component, i.e. DNA [57].

As discussed before, one of the heat shock proteins, Lon protease, does play an important role in the recovery from the SOS state by degrading the inhibitor of the cell division,SulA. This raises the question of the cross resistance induced by high temperature and DNA damaging agents, as well as other treatments. Nevertheless, a definite role of heat shock in response to radiation-induced damage or to inhibitors of DNA replication, has not yet been uncovered. It is known that HtpR is essential for growth and survival at all temperatures, but the nature of its precise function is unknown. It is possible that an HtpR-dependent process is responsible for conferring increased resistance to the deleterious effects of further stress such as high temperature and ethanol.

1.2. Studies on DNA Repair Mechanisms

DNA is a highly reactive chemical species and therefore is the target of numerous physical and chemical treatments. Heat causes deamination of bases and base lose by glycosylic hydrolysis; ultraviolet (uv) radiation produces pyrimidine dimers and 6-4 photoproducts; ionizing radiation results in ring opening, base fragmentation and single and double strand breaks. Chemical agents that modify DNA, range from activated oxygen species to metals, alkylating agents and polycyclic aromatic hydrocarbons. It is therefore quite remarkable that DNA is functionally more stable than the two other cellular macromolecules, RNA and protein; e.g., a 3.4 kilobase fragment containing an Alu sequence was recently cloned from a 2400 year old mummy [67]. This stability can be attributed to the double helical structure, which carries the information in duplicate.

Equally important for functional stability of DNA are the various repair mechanisms. These are molecular mechanisms of different complexities that seem to undo modifications or damage to DNA and maintain cellular survival as well as genetic integrity. The study of these repair systems is most advanced in bacteria, particularly in E.coli, but similar processes are unknown to exist in eucaryotes from yeast to human cells [48, 68]. Their importance is confirmed by the

existence of several human genetic diseases apparently due to inadequate DNA repair and evidence that cancer arises from mutations, including the demonstrated mutagenicity of most known carcinogens [48].

E.coli has three largely error free pathways of DNA repair [48, 68]:

(i) Photoreactivation mediated by DNA photolyase (a product of the phr gene), which breaks the cyclobutane ring of a pyrimidine dimer; (ii) excision repair; and (iii) recombinational or post-replication repair. The last two are critical for the survival of cells whose DNA is damaged, as these pathways remove modified (incorrect) nucleotides and rely on the redundant information in the complementary strand to restore the duplex. In base-excision repair the base is first removed by a glycosylase, then the abasic sugar is removed by apurinic/apyrimidinic (AP) endonucleases and DNA polymerase. Abasic sugars (AP sites) could be generated by direct action of damaging agents. In nucleotide excision repair, modified bases are removed in the form of an oligonucleotide. The single strand gaps so generated are filled in by polymerases and ligases. This type of repair appears to be particularly important in conferring radiation resistance in bacterial cells [69]. Excision repair in E.coli has been well characterized at the molecular level.

The genes for the nucleotide excision repair proper are *uvr*A, *uvr*B and *uvr*C [70]. Mutations in any of the three *uvr* genes make cells extremely sensitive to uv as well as to mitomycin C, nitrous acid, psoralen and many other genotoxic agents. The *uvr* genes are not essential for viability of *E.coli*, as deletion mutants of all the three genes (i.e., *uvr*A$^-$, *uvr*B$^-$ and *uvr*C$^-$) have been isolated [71]. In the third repair mechanism, when the polymerase encounters certain nucleotide adducts, such as pyrimidine dimers, it stops replicating and reinitiates about 1000 base pairs beyond the adduct, thus generating a single stranded gap that contains a modified nucleotide. This discontinuity or postreplication gap is filled in by the RecA protein, which transfers the corresponding portion of the sister duplex into the gap. This model for recombinational repair, which was formulated for *E.coli* two decades ago [72], remains essentially unchanged.

1.2.1. Regulation of DNA Repair

Bacteria have evolved complex and efficient systems for responding to the vicissitudes of their lives. As mentioned before, they manage to maintain the complex coordination of DNA replication and cell growth and division even under genotoxic stresses. Many of these adjustments are carried out by changing the pattern of gene expression to

enable the cell to repair DNA before irreversible reactions take place. The following are the well understood inducible regulatory networks which are known to operate at the levels of DNA repair (or protection) proteins according to the need, i.e., inducible repair pathways that enable cells to display increased resistance to the deleterious effects of chemical mutagens and radiation.

1.2.1.1. The SOS Response

The global network induced by treatments with DNA damaging agents (typically uv) that allow repair of the resulting damage caused, has been called SOS repair [73]. The principle involved is that survival with some loss of information is better than no survival at all. It is a bypass system that allows DNA chain growth across damaged segments at the cost of fidelity of replication. It is an error prone process; even though intact DNA strands are formed, the strands are often defective [74]. SOS repair is thought to invoke a relaxation of the editing system in order to allow polymerization to proceed across a thymine dimer (uv damage) despite the distortion of the helix. Recently, it has been suggested that damaged DNA induces an error prone replication system that has less proofreading activity than the normal replication system. This lower-fidelity system causes all newly made DNA strands to have a

higher than normal number of mispaired bases so that most progeny are mutants. The SOS repair system is thought to be a major cause of uv induced mutagenesis. Now the question is why should the SOS system normally be shut down, and also what is the mechanism by which it is switched on? Clearly, if cells have evolved an efficient editing function for maintaining high fidelity in replication, it is reasonable that they should have a means of making all error-prone replicative processes inactive. A negatively controlled regulon comprising about 20 genes is responsible for this response. This regulon includes a LexA protein, which is the repressor of the SOS genes, including lexA itself and the recA gene. RecA is induced in greater amounts upon the DNA damage. A small amount of it is converted to a protease. This causes the proteolytic cleavage of LexA protein. Inactivation of LexA protein turns on the uvrA, uvrB, uvrD (but not uvrC) genes, responsible for excision repair; the umuDC genes, responsible for the mutagenic damage bypass mechanism; sulA and B, involved in cell division; and a number of din (damage inducible) genes, whose function is unknown, along with lexA and recA, the regulators themselves. The SOS response in this way increases the cells' survival by inducing excision (uvr genes), recombinational (recA genes) and mutagenic (umuDC, recA genes) repair and by inhibiting cell division (sulA gene) to allow more time for

repair to occur. Upon the recovery of cells from DNA damage, the activating signal disappears, autodigestion slows down, LexA accumulates and the status quo ante is re-established, where there is a low level of constitutive expression of all SOS genes. The repressors of lambdoid phages are also cleaved by RecA protein during the SOS response resulting in phage induction and the escape of phage from the dying cell.

As discussed above, SOS repair is mutagenic. When SOS repair activity is induced by treatments such as uv radiation, the mutations caused by the radiation includes some that are not due to error prone repair of uv damage, but due to the mutator activity of the SOS repair system in undamaged portions of the DNA [75]. SOS inducing agents could be divided into two classes with respect to their mutagenic potency: (1) potent mutagens like uv radiation, which not only induces SOS repair activity, but also produces numerous sites in DNA requiring its error-prone action; (2) weak mutagens, which induce SOS repair activity by arresting DNA replication, but do not otherwise damage the genetic material. Thymine starvation and nalidixic acid arrest DNA replication, but do not introduce any structural damage requiring SOS repair activity, except perhaps at the replication fork(s)[75]. Long periods of thymine starvation cause high mutation levels. A weak mutagenic effect is seen

after starvation in the WP2thyA$^-$ mutant of E coli B/r which is found to be absent in lexA and recA mutants [76]. These findings confirm the conclusion that uv radiation and thymine starvation share a common pathway of mutagenesis [77]. Moreover, these treatments enhance uv mutability at low fluences as does thermal treatment of tif-1 strains of E. coliB/r, a temperature sensitive mutant [78]. Treatment with either nalidixic acid or ionizing radiation causes effects similar to that of thymine starvation, i.e., pretreatment with these agents enhances uv mutability at low doses. They appear to be inducers of SOS repair and virtually non-mutagenic in lex mutants [78].

1.2.1.2. The Adaptive Response

The term adaptive response is now reserved solely for the phenomena resulting from the activation of the Ada regulon, which is responsible mainly for the prevention of mutation and killing by alkylating agents [79]. Alkylating agents, of which methylating agents appear to be widespread environmental mutagens, act through covalent modification of the cellular genome to generate miscoding base derivatives and lesions that block DNA replication. All oxygens and nitrogens in DNA can be modified by methylating agents, except for the nitrogens forming a glycosyl bond with deoxyribose, oxygens in phosphodiester bonds and the

exocyclic amino groups, resulting in 14 different types of primary lesion. However, the most relevant adducts are O^6-methylguanine, which is a miscoding base and 3-methyladenine, which is a cell killing lesion. The main function of the adaptive response is the repair of these two harmful base derivatives. To remove 3-methyladenine, the base-sugar bond is cleaved by a DNA-glycosylase to release the altered base residue in free form and generate a repairable apurinic/apyrimidinic (AP) site [80]. In contrast O^6-methylguanine, which is also the intracellular signal for induction of Ada protein, is corrected by direct reversal of damage. This is a function accomplished by the regulatory Ada protein which transfers particular methyl groups to one of its own cysteine residue in a self-methylation reaction. The methylated protein binds tightly to a specific DNA sequence in the response and thereby facilitates the initiation of transcription. The protein is not regenerated and undergoes suicide inactivation as a consequence of the DNA repair event. Recently sites of initiation of transcription of the ada and alkA genes have been determined. It has been found that the methylated Ada protein is an activator of the ada and alkA genes [75]. Other genes alkB and aidB are also controlled by the promoter of the ada gene, the functions of which are not clear [83-85].

1.2.1.3. Response to Oxidative Stress

E.coli cells exposed to 60μM hydrogen peroxide for 10 min. become resistant to killing by 10mM hydrogen peroxide: this adaptation is independent of lexA, recA and adaA, but is inhibited by chloramphenicol, indicating a requirement for protein synthesis [79]. This adaptation has several regulatory components and is accompanied by the production of adenylated dinucleotide (alarmones) and about 30 proteins. One component comprises the OxyR regulon, which is a positive regulator of nine proteins that are induced upon challenge with hydrogen peroxide. Four of these proteins are: catalase/peroxidase, Mn-superoxide dismutase, glutathione reductase and NAD(P)H-dependent alkyl hydroperoxide reductase; the others have not been identified. Similarly, neither the molecular mechanism of induction nor the identity of the remaining twenty one oxidative stress proteins is known. In addition it has recently been found that endonuclease IV is induced by superoxide-generating oxidants, but not by hydrogen peroxide. It is not affected by oxyR mutations [86]. Thus, it seems that there are at least three regulons that respond to oxidative stress: the OxyR regulon, the hydrogen peroxide-induced proteins (independent of the OxyR regulon) and finally the proteins induced by superoxide radicals.

In the following section we will see that there is

considerable overlap between the various stress response networks, the SOS response, adaptation, the response to oxidative damage, the heat shock response and the ethanol stress response etc, indicative of a concerted cellular effort to protect and repair DNA.

1.2.2. Interactions in the Inducible Systems

In addition to the adaptive response, alkylating agents also induce the SOS response, but the response is initiated by a different alkylation product. The induction of the SOS response by alkylaton damage is enhanced in the strains which are deficient in 3-methyladenine DNA glycosylase activity (alkA⁻ and tag⁻). The persisting 3-methyladenine residues in DNA of such strains are presumably the SOS inducing because they block DNA replication [87]. Conversely, an observed inhibition of SOS induction in cells induced for the adaptive response to alkylating agents [88] might be due to enhanced levels of glycosylase.

The adaptive response, the SOS response and the heat shock response in E.coli, which are all induced by environmental stress, have quite different mechanisms of positive regulation. The activated RecA protein enhances proteolytic cleavage of the repressor of the SOS genes. The HtpR protein is an alternative sigma factor that stimulates selective

transcription of the heat shock genes. Neither of these mechanisms bear any resemblance to the covalent modification of the Ada protein, that triggers the adaptive response to alkylating agents. On the other hand, a high concentration of DNA damaging agents such as nalidixic acid induces proteins of the heat shock response as well as inducing the SOS systems [7]. Also Lon, the protease responsible for the proteolysis of the lethal SOS induced, SulA, is a heat shock protein.

The evidence that these regulons are related highlights the need to learn about their induction. One can envision each regulon as a unique responder to a particular stress. By this interpretation, the simultaneous response of two regulons would indicate that an environmental agent had inflicted stress on several cellular systems. A more complex alternative would suggest that some regulons could share inducers and some proteins could belong to more than one regulon. Sorting out these relations will require considerably more information than is now available.

1.2.3. Response to stress of microorganisms adapted to extreme environments

Microorganisms are found in diverse living conditions and some of them are constantly under stress. A lot of work has

been done on the effect of stresses on the microorganisms not usually exposed to hostile environments [2-4]. It is hard to define " hostile " or " extreme " living conditions. Generally, a culture well aerated at 1 atmosphere in sera or growth medium at approx. neutral pH and temperature near that of our bodies is considered to be in " normal " living conditions. But microorganisms have long been known to exist in such extreme conditions as hot springs, some of which may also be very acid; salty natural lakes and salterns; very acid streams, such as mine effluents, which may also contain toxic heavy metals; on dry rock surfaces in deserts; and at sea depths where pressures may be very high [89].

These organisms have acquired additional means to protect themselves from the lethal or otherwise detrimental effects of the environment and to permit them to withstand stringent conditions, e.g., Halobacterium cutirubrum, high salt; Thermus aquaticus, high temperature; Micrococcus radiodurans (Dienococcus radiodurans), high uv; etc. Not much is known about the response of these microorganisms, living in harsh conditions, to further chemical or physical stress. These organisms are good tools for the study of the various mechanisms responsible for their adaptation to living conditions which are otherwise considered to be hostile.

The repair of ultraviolet induced damage to DNA by the various repair processes has been studied the most, and is of particular interest in the case of the halophilic bacteria. These bacteria, in their natural environment of salterns and salt lakes [69] are exposed to intense sunlight including a relatively high dose of ultraviolet radiation. Previous work in this laboratory has shown that extremely halophilic bacteria are very resistant to ultraviolet light compared with E.coli [90-92]. All the extreme halophiles studied by Sharma et al [92] i.e, Halobacterium cutirubrum, Halobacterium salinarium and Halobacterium halobium, were active in photoreactivation. H.cutirubrum in particular could be photoreactivated 100% from any measurable level of survival.

Recently Daniels et al. have shown that when the members of the genus Halobacterium were subjected to heat stress or osmotic shock, there is rapid increase in the synthesis of a few hsp proteins [93]. Both heat shock and osmotic shock are likely to be natural stresses for halophilic bacteria. The observation that certain chemicals and environmental stresses other than heat shock induce hsps, and that synthesis and turnover of hsps are influenced by the metabolic state of the cell [2,94], point towards a more central role for these proteins in cellular homeostasis. The

major hsps of H.volcanii also have a function under normal growth conditions since most of them are synthesized at detectable levels in the absence of stress [94]. The apparent induction of some hsps by another environmental stress, the salt shock, may be an indication that each hsp protein has a unique function and that various stresses preferentially induce the synthesis of only a subset of the stress inducible proteins. Two members of hsp family (DnaK and GroEL) are known to be induced by uv irradiation in E.coli [7]. It is not known if the synthesis of these proteins is enhanced upon uv irradiation in halobacteria. It is possible that these bacteria have more efficient DNA repair mechanisms developed in them constitutively as a way of adaptation to their natural living environment, i.e., high salt (as high as 4M NaCl) and intense sunlight [69].

1.3. Objectives of the Project

Based on the information discussed above, the following general hypothesis was formulated. Exposure to physical or chemical stresses, e.g., heat shock, osmotic shock etc., leads to an increased ability to repair the damage to DNA caused by subsequent stress treatments and thus to a greater resistance to a second, potentially mutagenic, challenge.

The present study was initiated to investigate the response of heat-stressed *E.coli* cells to a further challenge by uv irradiation. The specific aims of the research in this thesis were to investigate:

i) The effect of heat shock on uv survival of *E.coli*.

ii) The possibility of involvement of genes of known DNA repair pathways in the above phenomenon.

iii) The similarity between the response of the heat shocked bacteria to challenge by either uv irradiation or a lethal temperature.

2. EXPERIMENTAL

2.1. MATERIALS

Materials were purchased from the following suppliers:

Standard laboratory glassware and disposable supplies: Fisher Scientific or Canlab, Ottawa, Ontario.

Chemicals and Difco growth media components: BDH, Ontario.

Phast system electrophoresis gels and chemicals: Pharmacia (Canada) Inc. Dorval, Quebec, Canada.

Chloramphenicol was a gift from Dr. D. Johnson,Department of Biology, University of Ottawa.

2.2. BACTERIAL STRAINS

The bacterial strains used in these studies and their sources were as follows. Table 1 shows their detailed genetic constitutions.

2.2.1. Escherichia coliB,ATCC 11303, also known as E.coli B wild type, Luria strain. Source: American Type Culture Collection, Rockland, Maryland, US.

2.2.2. Escherichia coli JE1011 (NRC2071), a mutant of E.coli K12 originally isolated by Hirota (personal communication from Dr. B. J. Bachmann, E.coli Genetic Stock Center, Yale University). Source: National Research Council, Culture Collection, Ottawa, Ontario.

2.2.3. Escherichia coli AB1157 (CGSE1157) and its derivatives were obtained through the courtesy of Dr. B. J.Bachmann, E.coli Genetic Stock Center,Yale University.Source: E.coli Genetic Stock Center, Yale University, New Haven, USA, (CGSE).
These bacteria are all derivatives of E.coli K12.

a. E.coli AB1157 (CGSE1157); parent strain.
b. E.coli AB1899 (CGSE1899); lon 1 mutant.
c. E.coli AB2463 (CGSE2463); rec A13 mutant.

d. *E.coli* AB2494 (CGSE2494); *lex* A1 mutant.

e. *E.coli* AB1886 (CGSE1886); *uvr* A6 mutant.

f. *E.coli* AB1885 (CGSE1885); *uvr* B5 mutant.

g. *E.coli* AB1884 (CGSE1884); *uvr* C34 mutant.

All strains were stored at -20°C as stock cultures in PY medium (next section; 2.3.1.3) to which an equal volume of 25% (w/v) glycerol had been added after overnight growth.

TABLE.2: Genetic constitution of the various *Escherichia coli* strains used in the present study.

Strain no.	Genetic markers
ATCC11303	Wild type *E.coli*B
JE1011 (NRC2071)	F^-, *thr*-1,*leu*B6,*his*-215,*trp*-84,*thi*-1,*ara*-14, *lac*Y1,*gal*T23,*xyl*-5,*mtl*-1,*azi*-16,*rpsl*-263, *ton*A61,*thyA710*,lambda$^-$, An *E.coli* K12 derivative.
AB1157 (CGSE1157)	F^-,*thr*-1,*ara*-14,*leu*B6,Δ(*gpt-pro*A)62,*lac*Y1, *tsx*-33,*sup*E44,*gal*K2,*his*G4,*rfb*D1,*mgl*-51, *rps*L31,*kdg*K51,*xyl*-5,*mtl*-1,*arg*E3,*thi*-1,rac$^-$, lambda$^-$, An *E.coli* K12 derivative and parent of following mutants.
AB1899 (CGSE1899)	F^-,*thr*-1,*ara*-14,*leu*B6,Δ(*gpt-pro*A)62,*lac*Y1, *tsx*-33,*sup*E44,*gal*K2,*his*G4,*rfb*D1,*mgl*-51, *rps*L31,*kdg*K51,*xyl*-5,*mtl*-1,*arg*E3,*thi*-1,rac$^-$, lambda$^-$,*lon*-1.
AB2463 (CGSE2462)	F^-,*thr*-1,*ara*-14,*leu*B6,Δ(*gpt-pro*A)62,*lac*Y1, *tsx*-33,*sup*E44,*gal*K2,*his*G4,*rfb*D1,*mgl*-51, *rps*L31,*kdg*K51,*xyl*-5,*mtl*-1,*arg*E3,*thi*-1,rac$^-$, lambda$^-$,*rec*A13.
AB2494* (CGSE2494)	F^-,*thr*-1,*ara*-14,*leu*B6,Δ(*gpt-pro*A)62,*lac*Y1, *tsx*-33,*sup*E44,*gal*K2,*his*G4,*rfb*D1,*mgl*-51, *rps*L31,*kdg*K51,*xyl*-5,*mtl*-1,*met*B1,*thi*-1,rac$^-$, lambda$^-$,*lex*A1.
AB1886 (CGSE1886)	F^-,*thr*-1,*ara*-14,*leu*B6,Δ(*gpt-pro*A)62,*lac*Y1, *tsx*-33,*sup*E44,*gal*K2,*his*G4,*rfb*D1,*mgl*-51, *rps*L31,*kdg*K51,*xyl*-5,*mtl*-1,*arg*E3,*thi*-1,rac$^-$, lambda$^-$,*uvr*A6.
AB1885 (CGSE1885)	F^-,*thr*-1,*ara*-14,*leu*B6,Δ(*gpt-pro*A)62,*lac*Y1, *tsx*-33,*sup*E44,*gal*K2,*his*G4,*rfb*D1,*mgl*-51, *rps*L31,*kdg*K51,*xyl*-5,*mtl*-1,*arg*E3,*thi*-1,rac$^-$, lambda$^-$,*uvr*B5.
AB1884 (CGSE1884)	F^-,*thr*-1,*ara*-14,*leu*B6,Δ(*gpt-pro*A)62,*lac*Y1, *tsx*-33,*sup*E44,*gal*K2,*his*G4,*rfb*D1,*mgl*-51, *rps*L31,*kdg*K51,*xyl*-5,*mtl*-1,*arg*E3,*thi*-1,rac$^-$, lambda$^-$,*uvr*C34.

* : *E.coli* AB2494 is not only a *lex* mutant but also differs from its parent, *E.coli* AB1157, in two other genetic markers. It is *arg*$^+$ and *met*$^-$. This difference might be associated to the close proximity of these markers.

2.3. METHODS

2.3.1. Growth of bacterial cultures

2.3.1.1. Growth medium

E.coli. cultures were grown in peptone-yeast extract (PY) medium as described by Fitt *et al*. [91]. The medium contained, per litre:

	$g.L^{-1}$
Peptone	10.00
Yeast extract	10.00
Sodium chloride	5.00
Disodium hydrogen phosphate heptahydrate	0.40

The pH was adjusted to 7.4 with 1N NaOH and the medium was autoclaved for 20 minutes at 121°C.

Solid medium consisted of PY medium supplemented with 2%(w/v) agar. It was poured into sterile, plastic petri dishes (approx. 10 ml per dish) and used for viable cell counts. Colonies were counted after overnight incubation at 37°C.

2.3.1.2. Non-nutrient dilution buffer

A non-nutrient buffer [95] was used for dilution before plating. It had the following composition per litre:

	$g.L^{-1}$
Disodium hydrogen phosphate heptahydrate	13.20
Potassium dihydrogen phosphate	3.00
Magnesium sulphate heptahydrate	0.20
Sodium chloride	4.00

The salts were added one at a time in the order shown above and the solution was autoclaved for 20 minutes at 121°C under pressure. The final pH of the buffer was 7.0.

2.3.1.3. Culture conditions

Working stock cultures on solid PY medium were prepared as follows. Liquid cultures were grown overnight in 125 ml culture flasks (Bellco Glass, Vineland, N.J, USA) fitted with Morton closures. Flasks containing sterilized medium (20 ml) were inoculated from stock glycerol cultures (See section 2.2.3) kept at -20°C and incubated at 30°C in a New Brunswick Scientific Model G25 gyratory shaker-incubator at 250 rpm. After overnight incubation, the cultures were streaked on an agar dish with the help of a sterilized loop and kept overnight in an incubator at 37°C. This starter culture dish was stored at 4°C in the refrigerator and was used for one week. For each experiment, a fresh liquid culture was started by picking a colony from a stock dish by loop and growing it overnight in PY medium at 30°C (or at the indicated temperature). In the morning, the

experimental culture was prepared by transferring a 20% (v/v) inoculum of the overnight culture into fresh medium.

2.3.2. Heat shock

Cultures were grown at 30°C to an A_{660nm} of about 1.0 (early-exponential phase, 10^9 cells.ml^{-1}) and were then shifted to the appropriate higher temperature (42°C in the case of *E. coli* B and *E.coli* JE1011) for heat shock. The final heat shock temperature was reached within 30 seconds as confirmed in separate experiments. Samples were withdrawn after 15 min, 30 min and 45 min of incubation at the higher temperature. The effect of the uv irradiation on the cells was determined immediately after the heat shock treatment. Control cultures were grown continuously at either 30°C or at the heat shock temperature (if the bacteria grew at the higher temperature) and their sensitivity to uv light was measured as discussed below.

In order to study the effect of heat shock in the absence of protein synthesis and growth, experiments were performed on *E.coli* JE1011 in the presence of chloramphenicol and in the non-nutrient medium. A stock solution of chloramphenicol (50 mg.ml^{-1} in ethanol, 0.2 ml) was added to a 20 ml culture immediately before the shift to 42°C . The final concentration of chloramphenicol was 500 μg.ml^{-1} of medium.

Samples were withdrawn at zero time, and after 15 min, 30 min and 45 min of heat shock. For the second treatment, 20 ml of a 30°C culture were centrifuged at 3000xgmax for 5 min and the cells were resuspended to an A_{660nm} of 1.0 in the dilution buffer prior to the heat shock.

2.3.3. Ultraviolet irradiation

Samples of the bacterial cultures, grown continuously at one temperature (controls) or after heat shock (tests) were diluted to about 1 x 10^6 cells per ml with dilution buffer and irradiated with a germicidal lamp (254 nm) in uncovered, sterile, glass Petri dishes (10 cm in diameter). Doses were measured with a Black-ray ultraviolet meter. Survival was determined by plating appropriate further dilutions on the solid medium. All experiments were performed at least twice.

2.3.4. Heat challenge

Control or heat shocked cultures at an A_{660nm} of 1.0 (10^9 cells.ml^{-1}) were transferred to a water bath shaker adjusted to the lethal, challenge temperature, 52°C or 55°C as appropriate, for 15 min. Separate experiments were done to check the time taken by the culture medium to reach the final temperature.It was found that the final temperature was reached within 30 seconds. The survival was calculated

from measurements of the number of viable cells in the culture before and after challenge at the lethal temperature.

2.3.5. Electrophoretic studies

The electrophoretic studies were performed using the Pharmacia LKB Phast System on extracts prepared by the method of Laemmli [96] from cells subjected to either (i) heat stress or (ii) nutritional stress (thiamine deprivation) and from appropriate control cells.

2.3.5.1. Heat stress

A fresh culture of the appropriate strain grown in PY medium at 30°C to an A_{660nm} of 1.0 was used. A control sample (20 ml) was withdrawn and the remainder of the culture was subjected to heat shock for the desired time as described above.

2.3.5.2. Nutritional stress

A starter culture of the strain under test was grown overnight in M9 medium [95] supplemented with the amino acids at 30°C. 50 $mg.L^{-1}$ of the amino acids: L-histidine, L-leucine, L-threonine, L-arginine, L-proline and 2 $mg.L^{-1}$ of thiamine was added to the medium as described by Fitt and Sharma [97]. This was used to inoculate a fresh culture,

which was grown to an A_{660}nm of 1.0. A control sample (20 ml) was withdrawn and the rest of the culture was centrifuged at 4000xg for 5 min. The cells were resuspended to an A_{660nm} of 1.0 in the above defined M9 medium lacking thiamine and incubation was continued at 30°C shaker incubator with vigorous shaking. Samples (10 ml) were taken out after 1.5 h, 3 h and 24 h and processed at once.

2.3.5.3. Preparation of extracts

In each case, the control or test sample was centrifuged at 3000xg for 8 min. The pellet was resuspended in 0.3 to 0.6 ml of sample buffer. The latter consisted of 0.625 M Tris-HCl buffer, pH 6.8 ; 2% (w/v) Sodium dodecyl sulphate (SDS); 10% (v/v) glycerol ; 5% (v/v) 2 - mercaptoethanol ; and 0.001% (w/v) bromophenol blue. The suspension was immersed in boiling water (100°C) for 3 min to denature proteolytic enzymes [98] and centrifuged for 10 min at 12000xg in a microcentrifuge.

Protein estimation was done by the standard BioRad modification of Bradford's method [99]. The appropriate SDS concentration (0.1% SDS) was used in the protein standard solution. All the sets of samples of the cell extracts were then adjusted with sample buffer to the same protein concentration (between 0.75 and 1.5 $\mu g.\mu l^{-1}$).

2.3.6. SDS-polyacrylamide gel electrophoresis

SDS-PAGE electrophoresis was performed using the automated Pharmacia PhastGel Electrophoresis System according to the manufacturer's instructions. The separations were done on PhastGel gradient, 10-15%, pre-cast polyacrylamide gels, using the appropriate SDS-Buffer strips. Samples (0.75μg-1.5μg of protein) were applied with the PhastGel applicator. Low molecular weight standards (Pharmacia electrophoresis calibration kit) were prepared according to the instructions with the kit and run simultaneously on each gel. Soybean trypsin inhibitor (M_R, 20 K; 0.4μg, μl^{-1}) was included as internal standard in each sample and also run separately in one lane. The internal standard appeared in a region of the gel where no other protein bands were visible after staining.

The gels were stained and destained according to the manufacturer's instructions with PhastGel Blue R, a Coomassie Blue R350 stain. 10%(v/v) Acetic acid - 30%(v/v) methanol was used as destainer. Once the destaining procedure was complete, the gels were kept for two days in a Petri dish containing a 1:1 mixture of the destainer and a preservative solution containing 10% (v/v) acetic acid-5% (v/v) glycerol in order to clear the background of the gels. The gels were then dried in air for an hour hanging in

a fumehood and finally stored in plastic wrap. The R_F values of the protein standards and their molecular weights (relative molecular masses, M_R) were used to establish a standard curve of log M_R verses R_F for each experiment. The R_F value was defined as the migration distance of the protein relative to the fastest migrating component of bromophenol blue. The M_Rs of the proteins detected in the samples were determined by interpolation.

2.4 STATISTICAL ANALYSIS OF THE RESULTS:

All the experiments were repeated at least twice and the average value was plotted. The standard deviation of the four values of the viable counts determined at each dilution was calculated and found to be less than or equal to the size of the symbols used in the figures.

3. RESULTS

3.1. Experiments with E.coli B and JE1011

3.1.1. Effect of heat shock on resistance to uv-irradiation

The effect of heat shock at 42°C on the ultraviolet (uv) resistance of Escherichia coliB and JE1011 revealed a difference between the strains (Figs.1 and 2).

The results in Fig.1 show that E.coliB grown at 30°C became more sensitive to uv light when transferred to 42°C. The effect was similar whether the exposure to higher temperature was for 45 minutes or the cells were grown continuously at 42°C overnight.

The temperature of 42°C was chosen for heat shock so as to maximize its effect without inhibition of the growth of either of the two strains. E.coliB and JE1011 both grew faster at 42°C than at 30°C or 37°C. Microscopic examination of the liquid cultures (before plating) did not reveal any significant change in the number of filamentous forms present before or after heat shock or growth at 42°C.

In contrast, the resistance of E.coli JE1011 to uv light rose significantly after 45 min at 42°C (Fig.2) as compared

Fig. 1

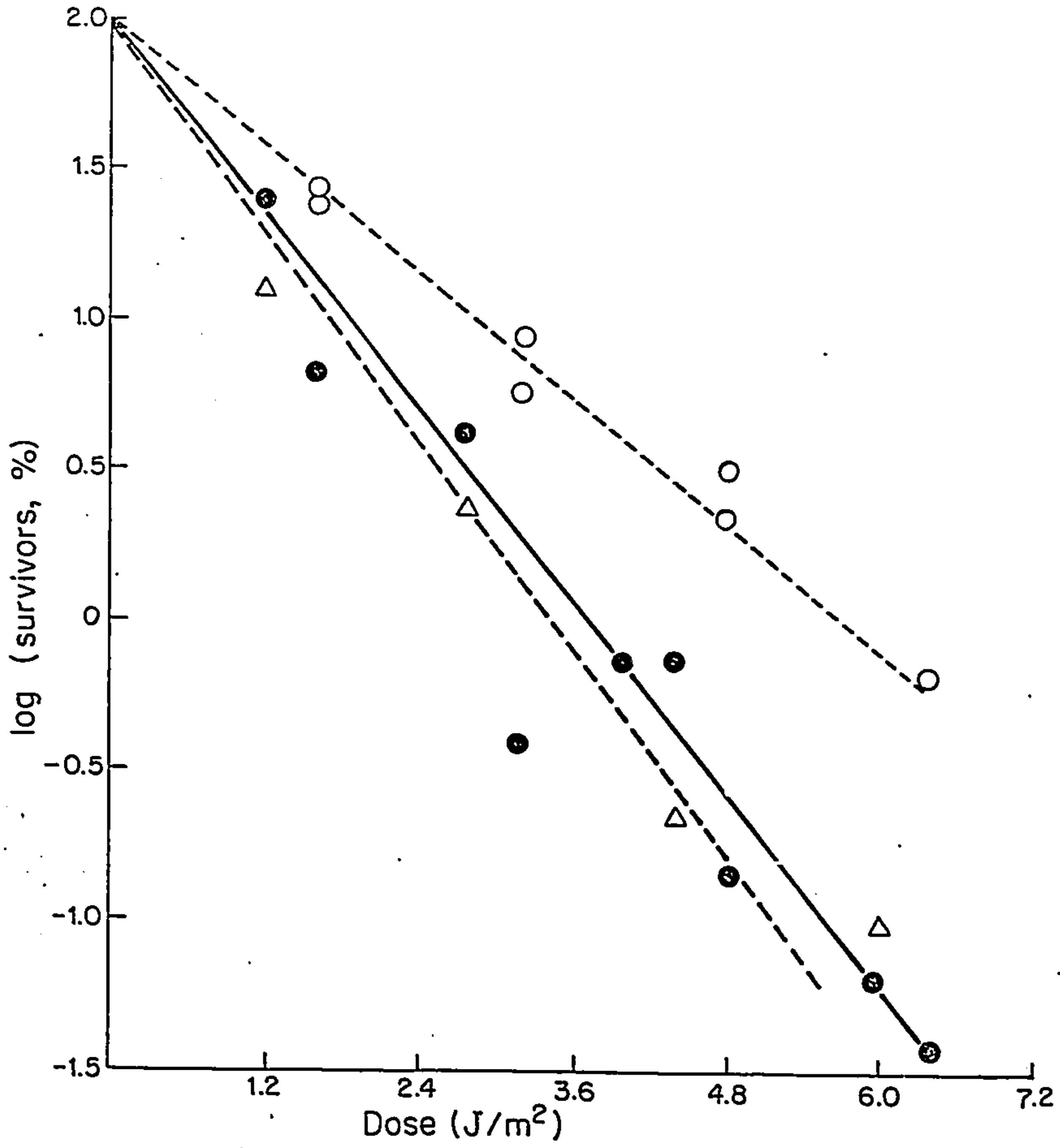


Fig.1 Comparision of the effect of heat shock continuous growth (>50 generations at 42°C) at 42°C on the resistance of *E.coli*B to uv irradiation: 30°C control, ○ ; continuous growth at 42°C, Δ ; heat shock for 45 min at 42°C, ●.

Fig. 2

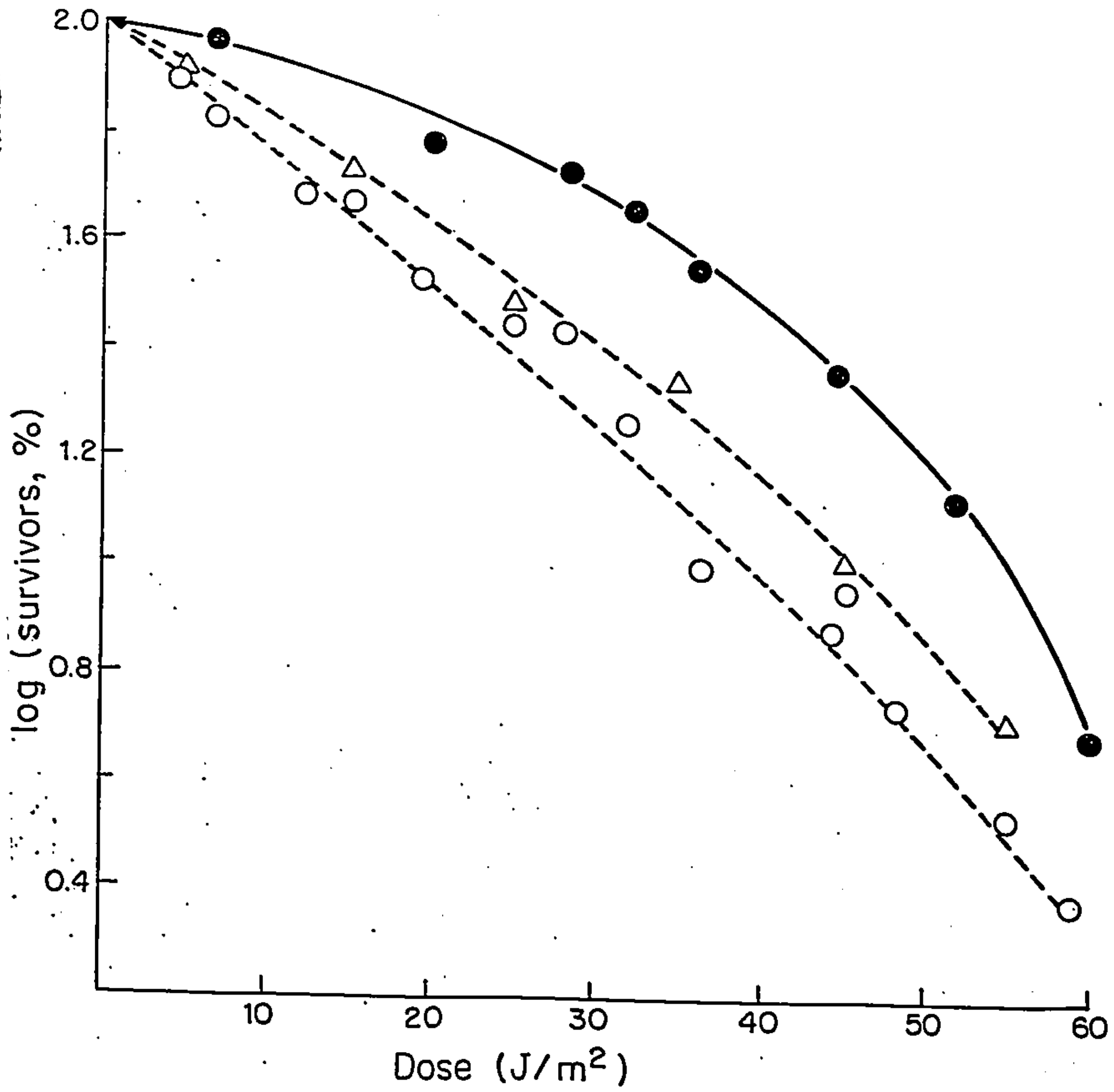


Fig.2. Comparison of the effect of heat shock or continuous growth (>50 generations) at 42°C on the resistance of E.coli JE1011 to uv irradiation: 30°C control, ○ ; continuous growth at 42°C, Δ ; heat shock for 45 min at 42°C, ● .

with that of cells grown continuously at 30°C. Further, when E.coli JE1011 was passaged at 42°C for at least fifty generations (42°C control, Fig.2) it was more resistant than after growth at 30°C, but less resistant than after 45 minutes of transient heat shock.

The resistance of the cells increased with duration of heat shock to a maximum after 45 min (Fig.3). The effect of longer exposure at 42°C was not studied because the cells entered stationary phase after 50-60 minutes at 42°C in the conditions of these experiments.

The maximum increase in resistance was 3.5-,2.5-and 2.2-fold at 35, 45 and 60 $J.m^{-2}$ respectively, so that it was similar over a wide range of doses. It should be emphasized that in all cases the cell suspensions were diluted to approx. $1x10^{6}$ $cells.ml^{-1}$ before irradiation to eliminate artifacts due to an increase in cell concentration during growth at 42°C. The failure of E.coliB to show any apparent increase in the resistance despite its rapid growth at 42°C proves that these precautions were effective.

3.1.2. Effect of growth and/or protein synthesis on the development of uv resistance

Experiments were performed to find whether the development

Fig. 3

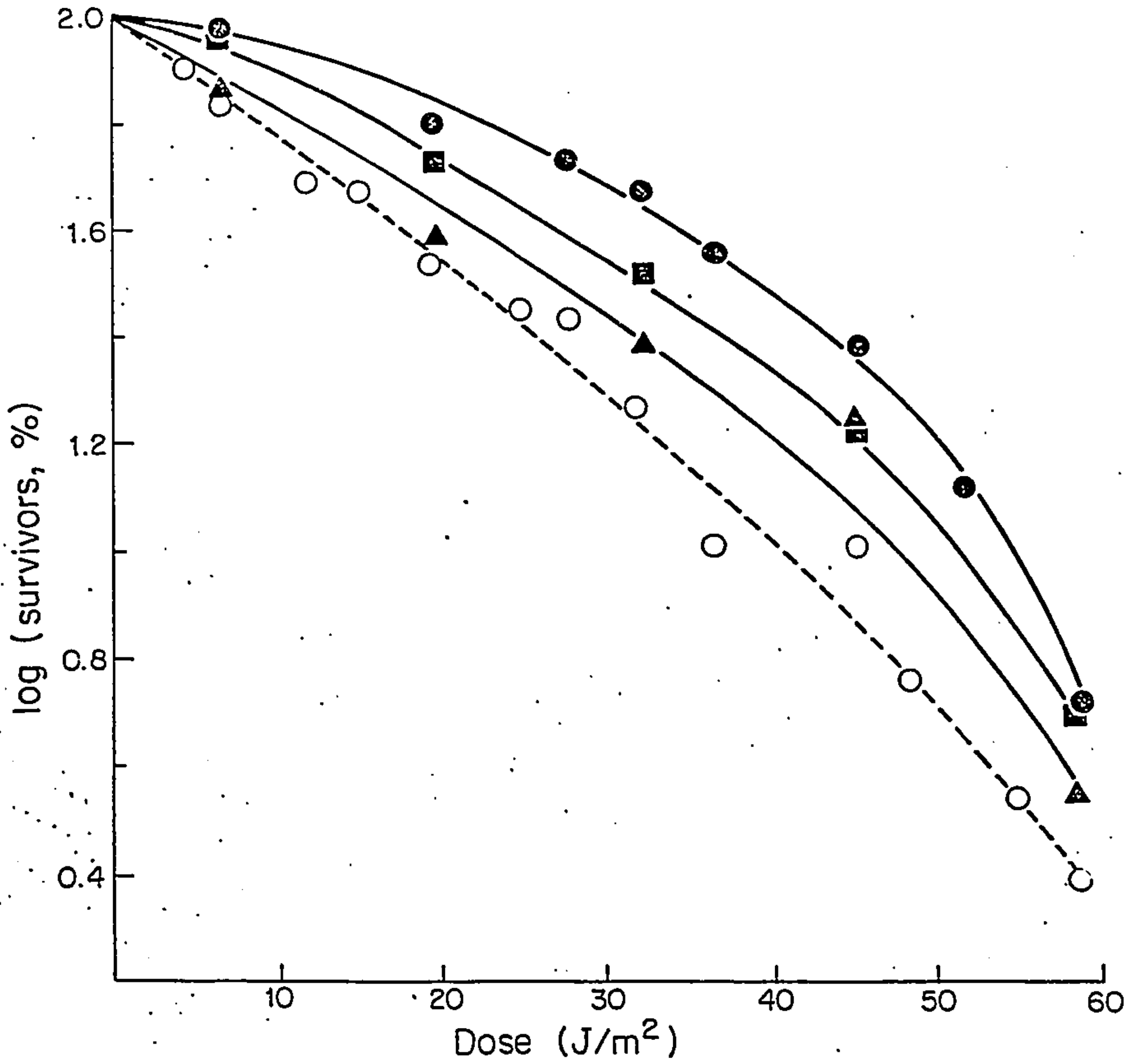


Fig.3. Effect of heat shock at 42°C on the resistance of *E.coli* JE1011 to uv irradiation. 30°C control, ○; 42°C for 15 min , ▲; 42°C for 30 min , ■; 42°C for 45 min , ●.

of increased resistance in E. coli JE1011 as a result of heat shock was dependent on growth or protein synthesis. Where E.coli JE1011 cells were subjected to heat shock either in growth medium supplemented with chloramphenicol or in non-nutrient dilution buffer, the development of resistance to the radiation was partially or completely inhibited (Fig.4). Chloramphenicol did not completely stop growth of the culture for about 15 minutes, but still the resistance to uv light was significantly lower than that of cells subjected to heat shock in the absence of the antibiotic. On the other hand, the surviving fraction of uv irradiated cells (following a dose of 35 $J.m^{-2}$) with and without heat shock in non-nutrient medium was almost the same as in the 30°C control.

These experiments show clearly that growth and/or protein synthesis were required for the development of the uv resistance in the E.coli JE1011.

3.1.3. Effect of heat shock on survival after heat challenge

The effect of heat shock on the ability of two strains of bacteria to survive at the lethal temperature of 52°C was then studied (Fig.5). The thermal resistance of E.coli JE1011 grown at 30°C was unaffected by exposure to 42°C for

Fig. 4

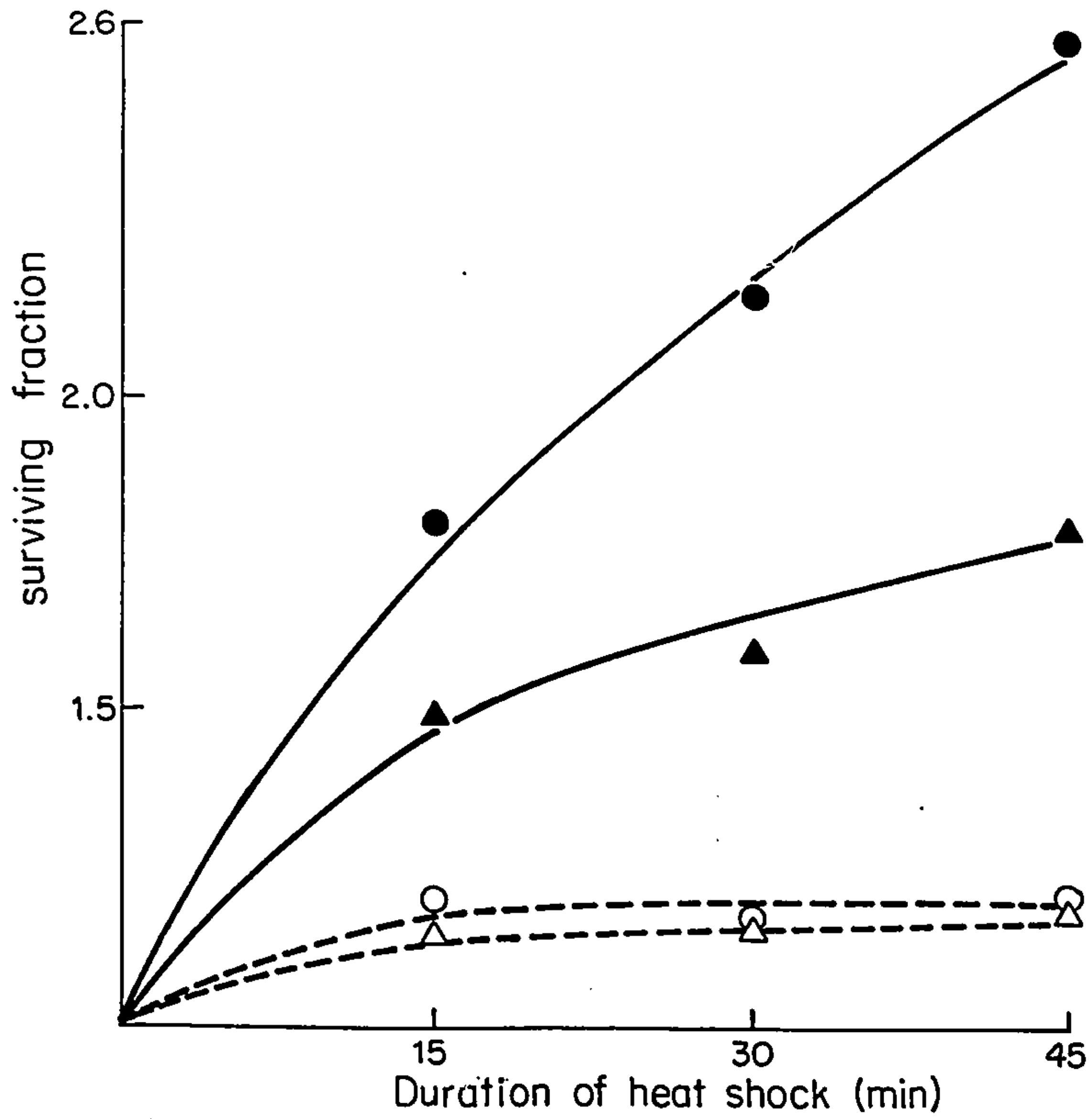


Fig.4. Requirement for growth and/or protein synthesis for the development of uv resistance in E.coli JE1011 as a result of heat shock at 42°C. Cells grown at 30°C were either maintained at 30°C in the presence of 500μg.ml^{-1} chloramphenicol (△) or subjected to heat shock at 42°C for the indicated time (i) in growth medium without antibiotic (●), (ii) in growth medium with 500μg.ml^{-1} chloramphenicol (▲), or (iii) after resuspension in non nutrient dilution buffer without antibiotic (○). Samples were then irradiated with uv light (35 J.m^{-2}). Survival was measured and expressed as a fraction of the survivors from the original control culture (30°C) at zero time after similar irradiation.

Fig. 5

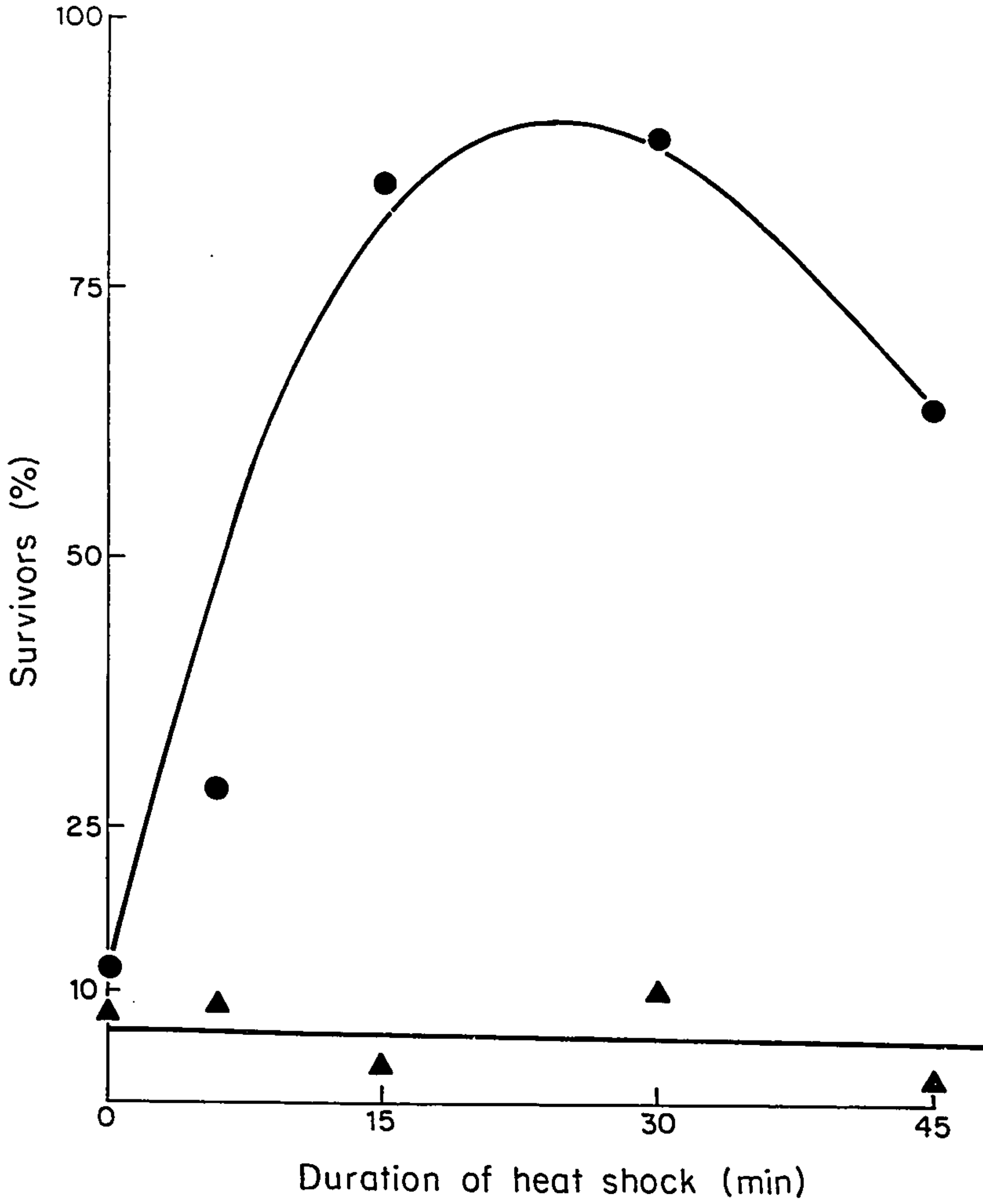


Fig.5. Effect of heat shock at 42°C as a function of time on the survival of *E.coli*B (●) and *E.coli* JE1011 (▲) after subsequent exposure to 52°C for 15 min.

upto 45 minutes, whereas there was a sharp rise in the survival of E.coliB, which appeared to peak after 15-30 minutes at 42°C and then declined.

3.2. Experiments on E. coli AB1157 and its mutants

The difference in the effect of heat shock on E.coliB and JE1011 might arise from the lon⁻ character of E.coliB. However since lon mutants of E.coli JE1011 are not available, the possibility that the gene plays some key role in the thermal induction of resistance to uv light could not be studied with this strain. Further experiments were done with E.coli AB1157, a well characterized K12 derivative (see Experimental, section 2.2.3), whose lon mutant as well as a variety of DNA repair mutants are available.

3.2.1. Effect of growth temperature on uv survival of E.coli AB1157

A series of experiments were undertaken to see the effect of various growth temperatures (25°C, 30°C, 37°C, 42°C, 45°C) on uv survival in E.coli AB1157 (Fig.6). It was found that uv resistance increased steadily with growth temperatures. However, when it was grown at 30°C and subjected to heat shock at 42°C for 45 minutes, the uv resistance remained identical to that of cells grown continuously at the high temperature.

Fig. 6

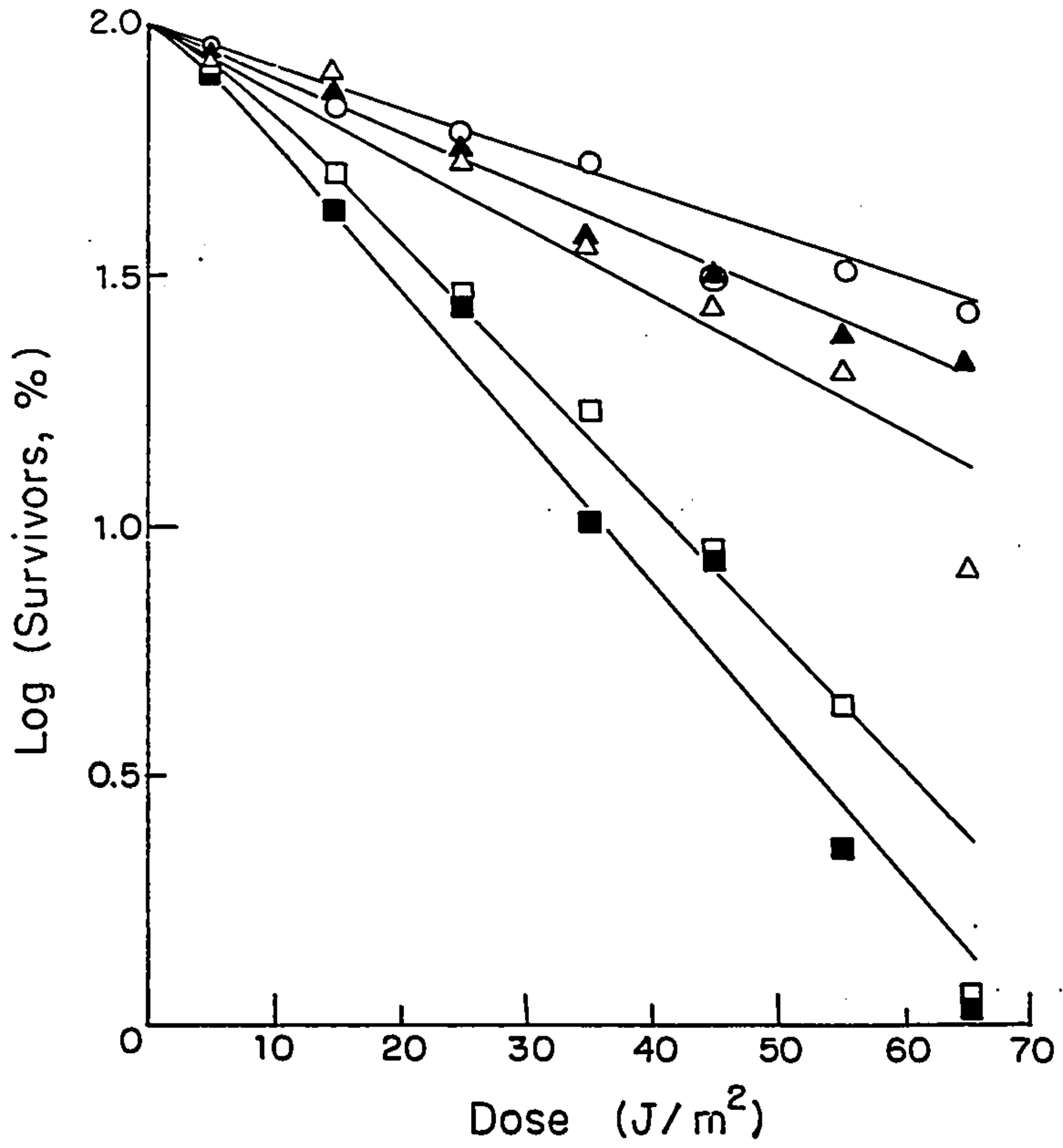


Fig.6. Effect of growth temperatures on resistance of *E.coli* AB1157 to uv light. Growth temperature: 25°C, ■; 30°C, □; 37°C, △ ; 42°C, ▲; 45°C, ○.

3.2.2. Effect of heat shock (48°C/45 min) on development of uv resistance in E.coli JE1011 and AB1157

Although heat shock treatment at 42°C had induced uv resistance in case of E.coli JE1011 Fig.2,it failed to do so with E.coli AB1157. More severe heat shock was therefore used and it was found that at 48°C a significant increase in the uv resistance of E.coli AB1157 took place after 45 minutes (Fig.7). A similar effect was observed with the E.coli JE1011, which developed a resistance after 45 minutes at 48°C (Fig,7) that was greater than that observed after exposure to 42°C for the same time.

The cells were able to divide slowly during the heat shock treatment for 45 minutes, but died after longer exposure (50 min at 48°C). Neither of the strains grew continuously at 48°C when the culture was started from a small inoculum.

3.2.3. Effect of the lon mutation on thermal induction of uv resistance

The effect of similar treatment was then examined, i.e, heat shock at 48°C for 45 minutes after growth at 30°C, on the uv survival of E.coli AB1899, the lon mutant of AB1157. The cultures were always diluted to approx. $10^6.ml^{-1}$ cells before they were irradiated with the indicated doses. The results in Fig.8 show that the mutation prevents the heat

Fig. 7

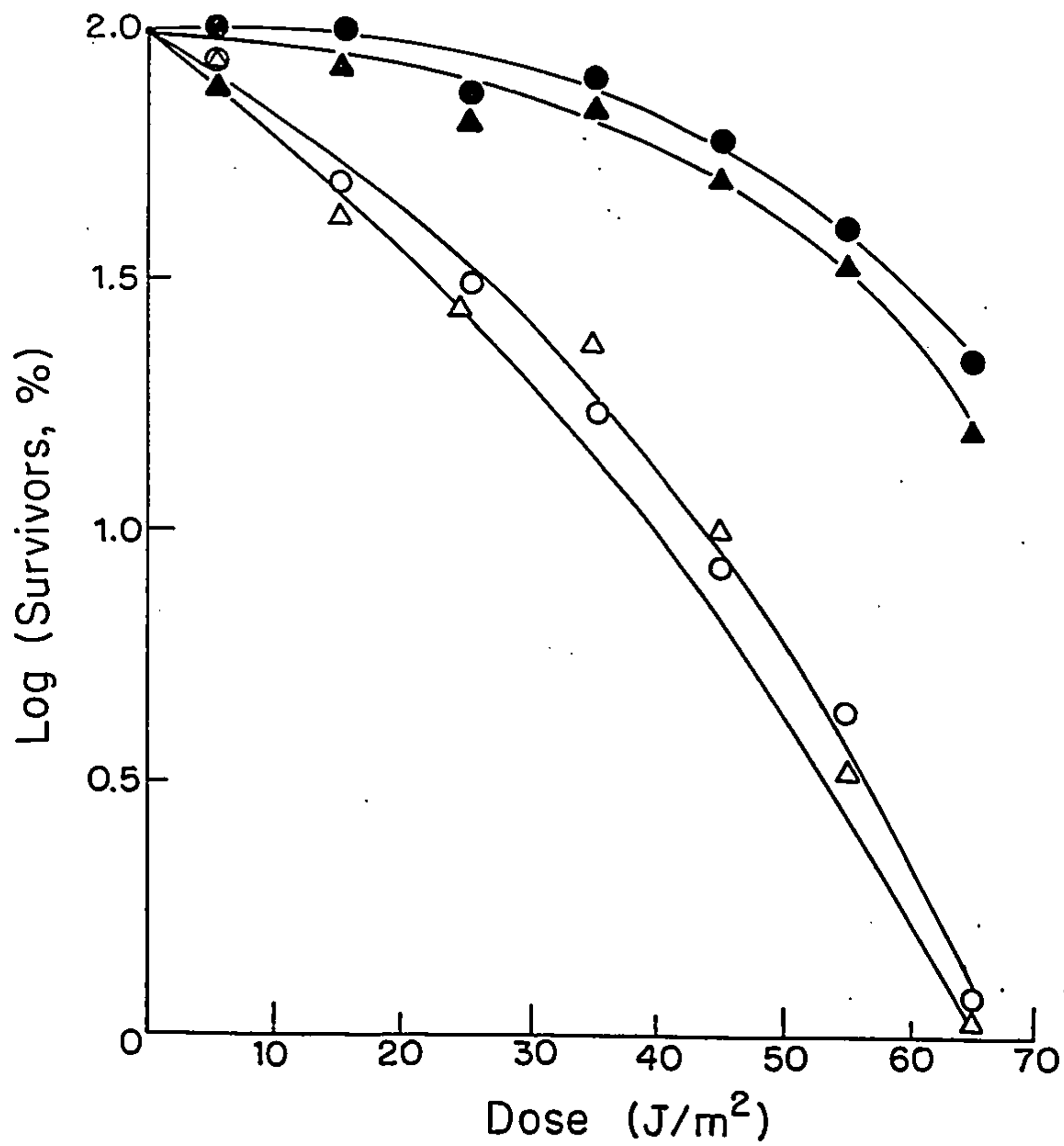


Fig.7. Effect of heat shock (48°C) on the resistance to uv of *E.coli* AB1157 and *E.coli* JE1011 grown at 30°C: AB1157, 30°C control, ○ ; AB1157, 45 min/48°C, ● ; JE1011, 30°C control, △ ; JE1011, 45 min/48°C, ▲.

Fig. 8

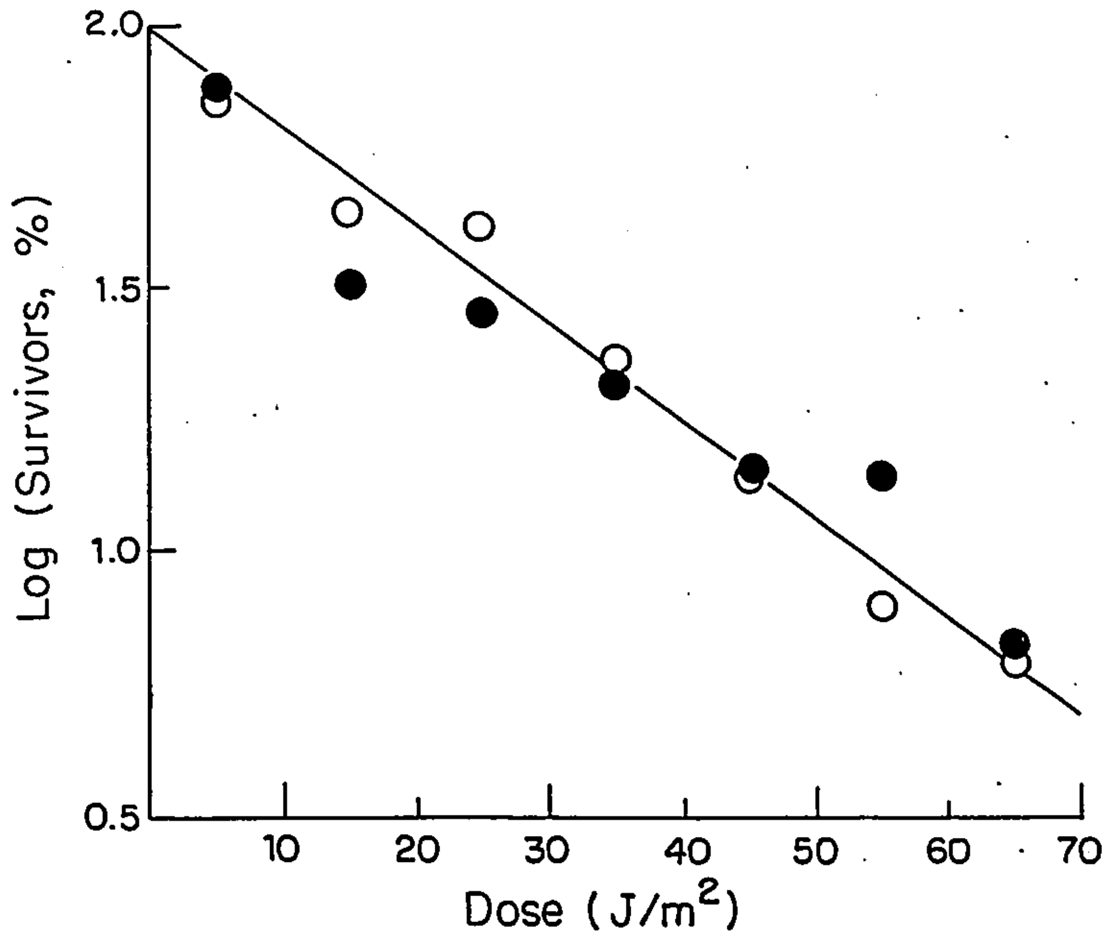


Fig.8. Effect of heat shock on uv resistance of the *lon* mutant of *E.coli* AB1157 (*E.coli* AB1899). Control (30°C), ○; heat shock (45 min/48°C), ●.

induced development of the uv resistance, indicating that the gene is essential for the process.

3.2.4. Effect of mutations in DNA repair genes on the thermal induction of uv resistance

A similar result was obtained with E.coli AB2463, the rec A13 mutant of E.coli AB1157, when it was subjected to the uv treatment. The sensitivities of the control and heat shocked cells to uv light were identical (Fig.9). A similar result was observed with E.coli AB1886, the uvr A6 mutant of E.coli AB1157, with which the uv resistance of control and heat shocked cells was similar (Fig.10a). Finally, E.coli AB1885, the corresponding uvr B5 mutant, showed very little difference in uv sensitivity after heat shock relative to the control cells, (Fig.10b).

It therefore appears that the products of the rec A and uvrA genes are absolutely required for the thermal induction of the uv resistance in these bacteria and the uvrB gene product is also necessary. The slight increase in the resistance in the case of the uvrB mutant may be an artifact due to the very small uv doses necessary with these sensitive cells.

Fig. 9

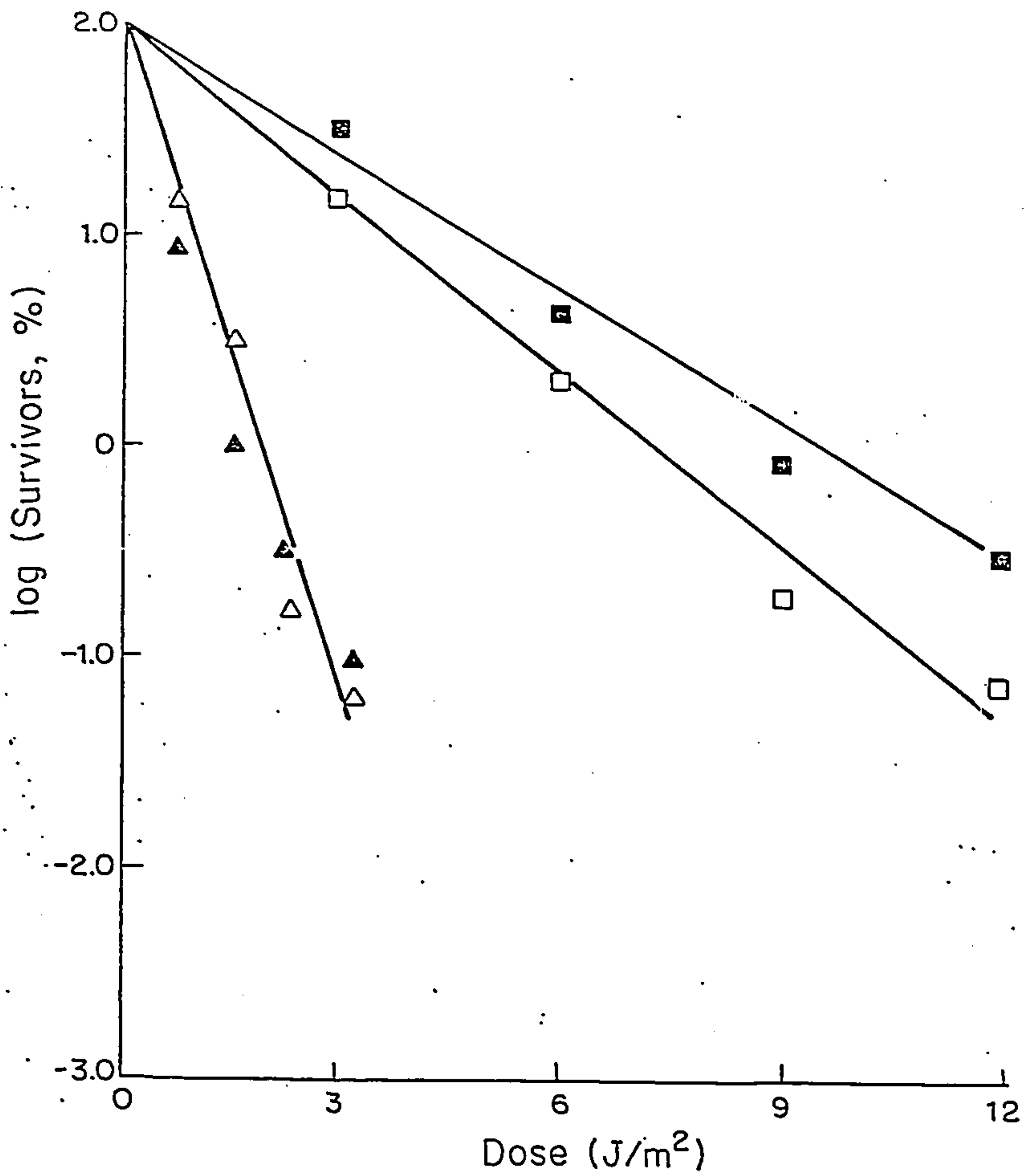


Fig.9. Effect of heat shock on the uv survival of the *rec* A (AB2463) and *lex* A (AB2494) mutants of *E.coli* AB1157. AB2463, 30°C control,△ ; AB2463, 48°C/45min, ▲; AB2494, 30°C control,□ ; AB2494, 48°C/45min, ■.

Fig. 10

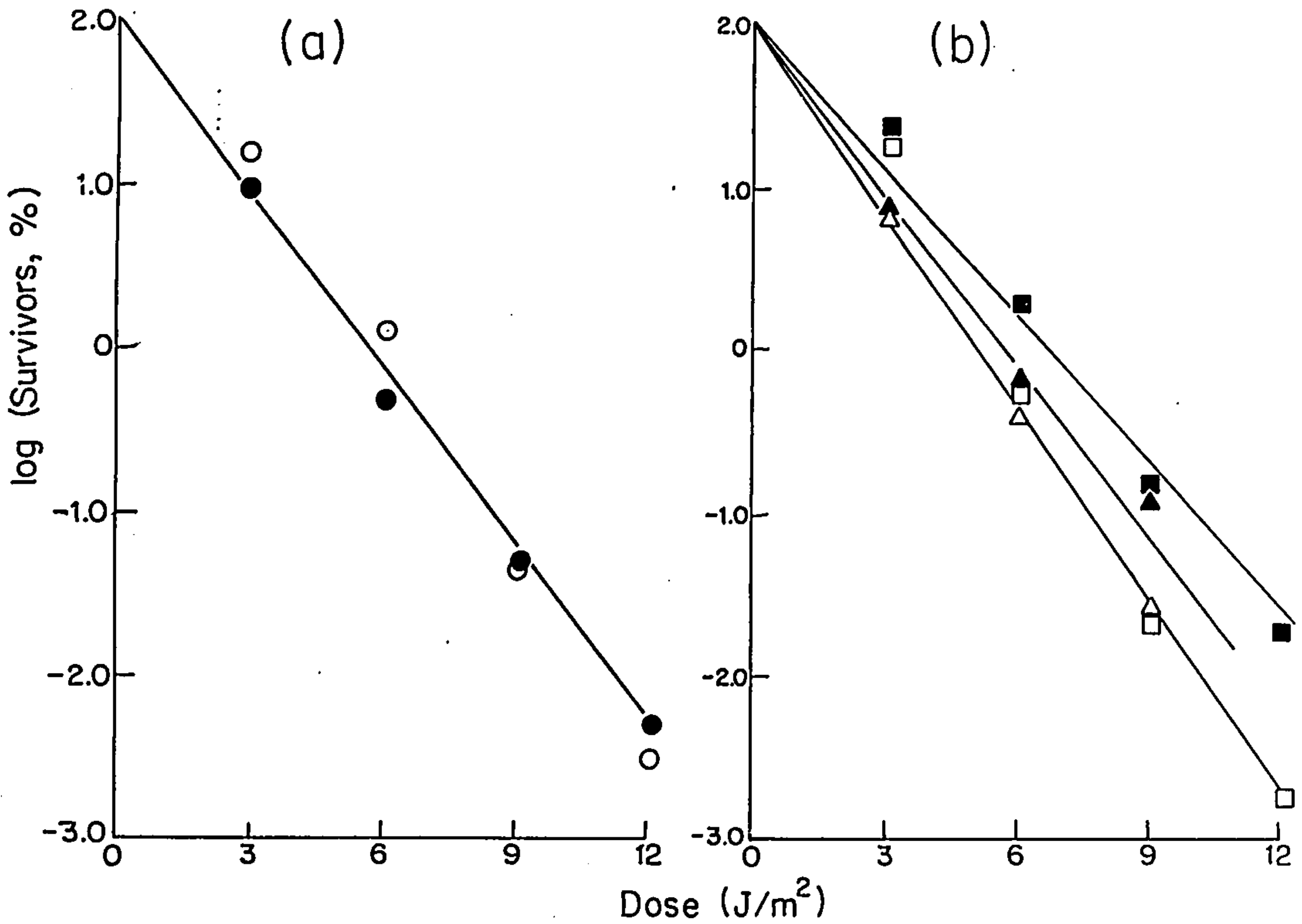


Fig.10. Effect of heat shock on the uv survival of the *uvr* A (AB1886), *uvr* B (AB1885) and *uvr* C (AB1884) mutants of *E.coli* AB1157. (a) AB1886, 30°C control, ○ ; AB1886, 48°C/45min, ● ; (b) AB1885, 30°C control, △ ; AB1885, 48°C/45min, ▲; AB1884, 30°C control, □ ; AB1884, 48°C/45min, ■.

The lex A1 and uvr C34 mutants, E.coli AB2494 and AB1884, respectively, gave less clear results (Fig.9 and Fig.10b). In each case, heat shock produced a definite increase in the uv resistance, but this was significantly less than with the parental strain. Thus when the survival in the heat shocked cultures was compared with that in the control (30°C) cultures at the uv doses that gave 5% survival in the controls, the resistance of E.coli AB1157 increased between nine and ten fold, whereas the corresponding increases were two fold for both the mutants.

3.2.5. Effect of heat shock (48°C/45 min) on the thermal resistance of E.coli JE1011, AB1157 and its mutants

In order to investigate the effects of 48°C heat shock on the thermal resistance of E.coli AB1157 (and its mutants), experiments were done similar to those with E.coli B and E.coli JE1011. It was found that a challenge temperature of 55°C was necessary instead of 52°C (used for E.coli B and JE1011) because E.coli AB1157 was very resistant at the lower temperature. Thus, a challenge for 15 minutes at 55°C was given after heat shock for 45 minutes at 48°C to this parent strain and its mutants.

Fig.11 shows that E.coli JE1011, E.coli AB1157 and its lon mutant AB1899, became more heat resistant. Previously, it was found (Fig.5) that no resistance to a challenge temperature of 52°C developed in E.coli JE1011 after 42°C shock and this remained true when the heat shock was at 48°C. However resistance did develop to a 55°C challenge (Fig.11). But it should be noted that E.coli JE1011 was very sensitive at this temperature,so that survival still remained below that observed when the challenge temperature was 52°C. In contrast, E.coli AB1157 and AB1899 became highly resistant to the 55°C challenge (Fig.11). In all the three cases, thermal resistance was maximum after 15-30 minutes of exposure to 48°C and in two cases (AB1157 and JE1011) fell after 45 minutes.

Finally, the effect of 48°C heat shock on the development of thermal resistance to a lethal temperature (55°C) was tested on the DNA repair mutants of E.coli AB1157. Fig.12 shows clearly that none of the mutations, i.e, rec, lex and uvrA, B and C, prevented the development of thermal resistance as a result of the heat shock.

Fig. 11

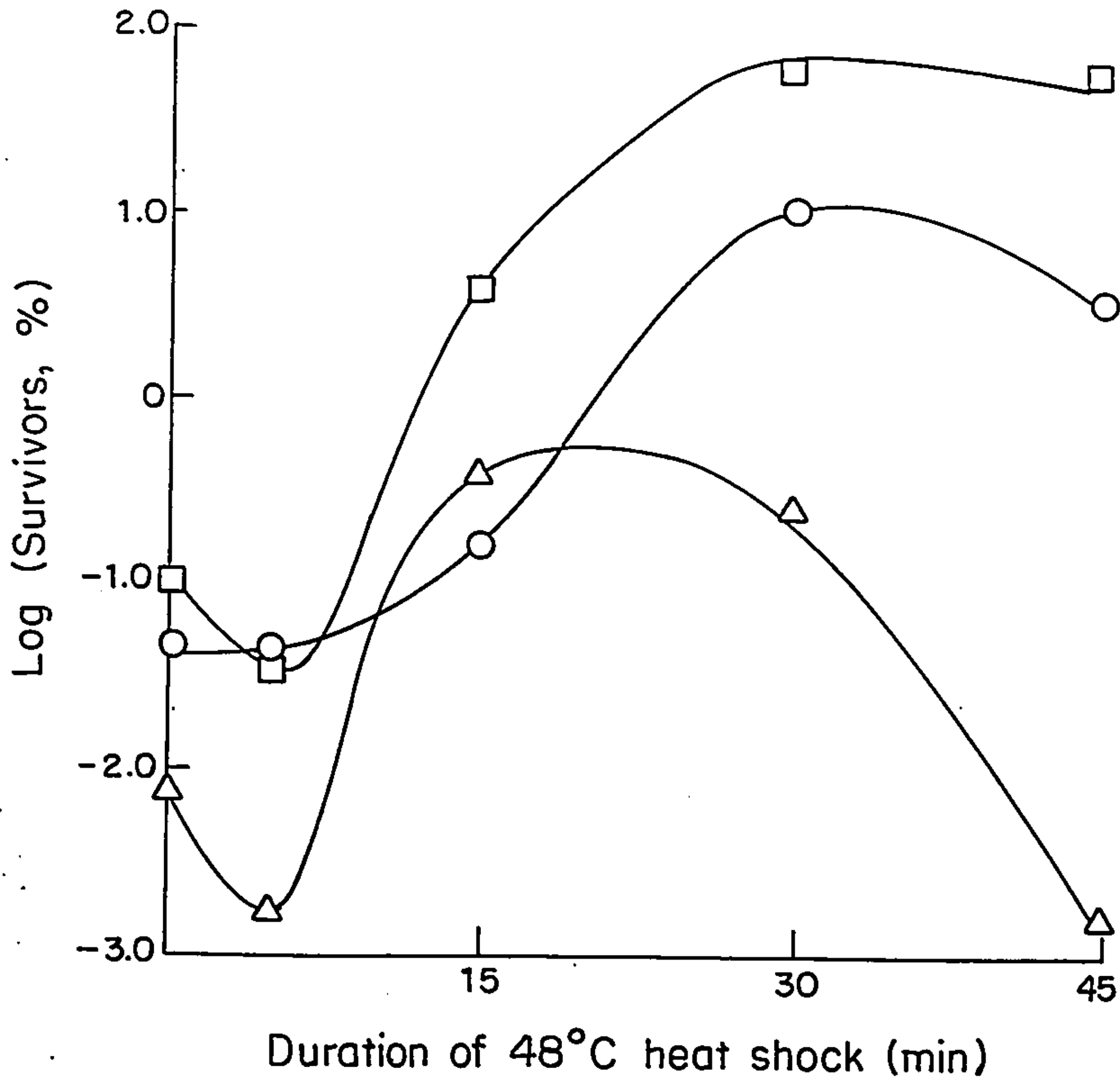


Fig.11. Effect of heat shock at 48°C for the indicated times on the survival of *E.coli* AB1157 (○), *E.coli* 1899 (□) and *E.coli* JE1011 (△) after subsequent exposure to 55°C for 15 min.

Fig. 12

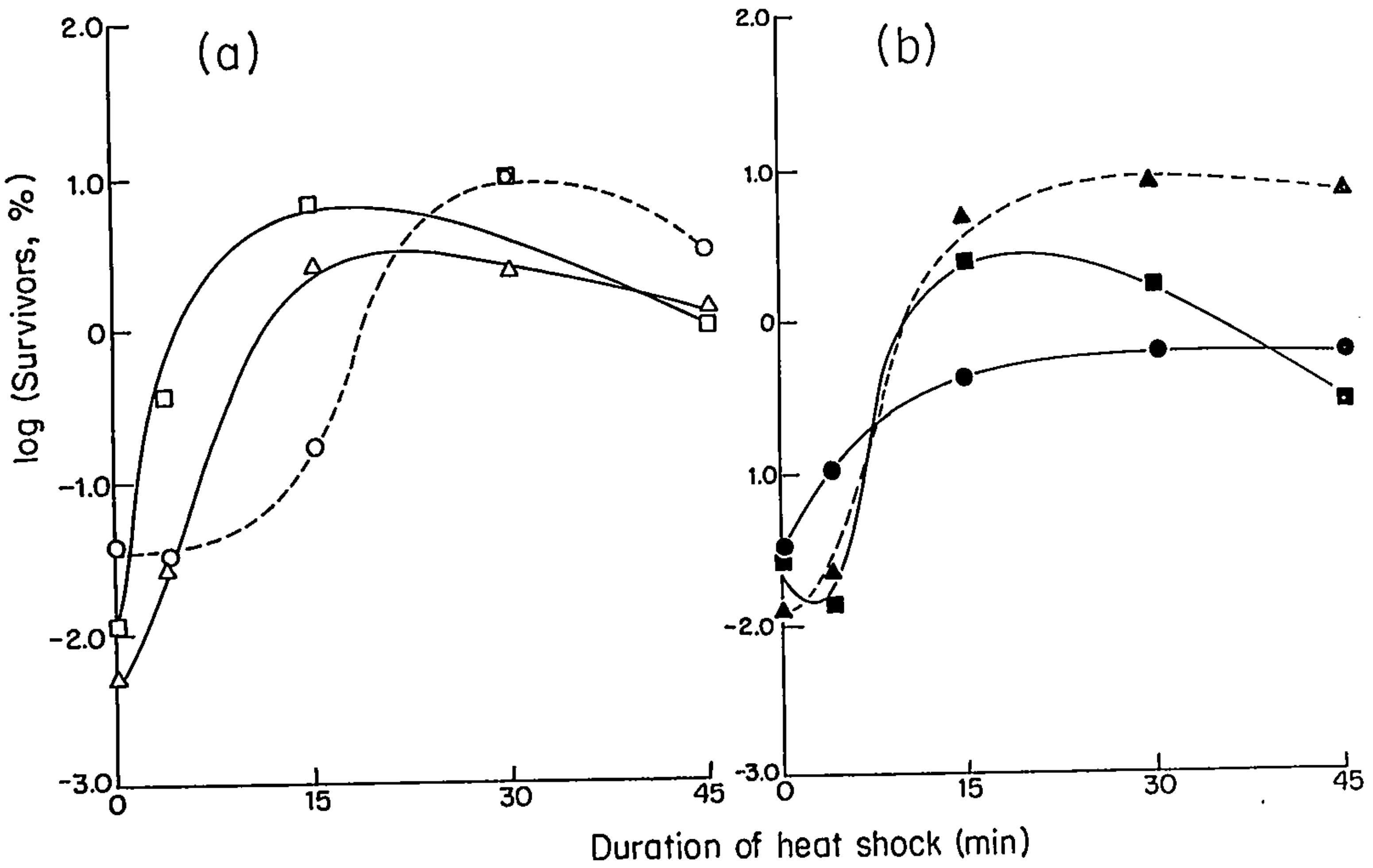


Fig.12. Induction of thermotolerance (55°C/15 min) in *E.coli* 1157 and its mutants by heat shock at 48°C for the indicated times of cultures previously growing at 30°C (see text): (a) AB1157, ○ ; AB2463, △ ; AB2494, □ ; (b) AB1884, ■; AB1885, ▲; AB1886, ●.

3.3. Resolution of proteins of bacterial cell extracts by SDS-polyacrylamide gel electrophoresis

3.3.1. Electrophoretic studies after heat shock

Fig.13 (a,b and c) shows the patterns of bands obtained after electrophoresis of the total protein in extracts of E.coli JE1011, AB1157 and its mutants. Two lanes in each gel contained protein standards. In one lane only a sample of the internal standard (Soybean trypsin inhibitor, 20K) was run. The other protein standard lane contained a mixture of proteins of known molecular weight. The mixed standards were: phosphorylase b, 94K; bovine serum albumin, 67K; ovalbumin, 43K; carbonic anhydrase, 30K; soybean trypsin inhibitor, 20K; and lactalbumin, 14K. The same amount of protein was applied in each lane of any one gel. The total amount of protein in the samples varied from one gel to another in the range 0.75 to 1.5μg.ml^{-1}.

Fig 13a shows the protein patterns of the extracts of control (30°C) and heat shocked cultures of E.coli JE1011 and AB1157. Three bands (band A, B and C) come up more strongly in the case of 48°C/ 45 min heat shocked culture extracts of both the bacteria as compared to their 30°C controls. 42°C heat shocked JE1011 (45 minutes) also shows these three intense bands. In contrast, after 10 minutes of

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Fig. 13

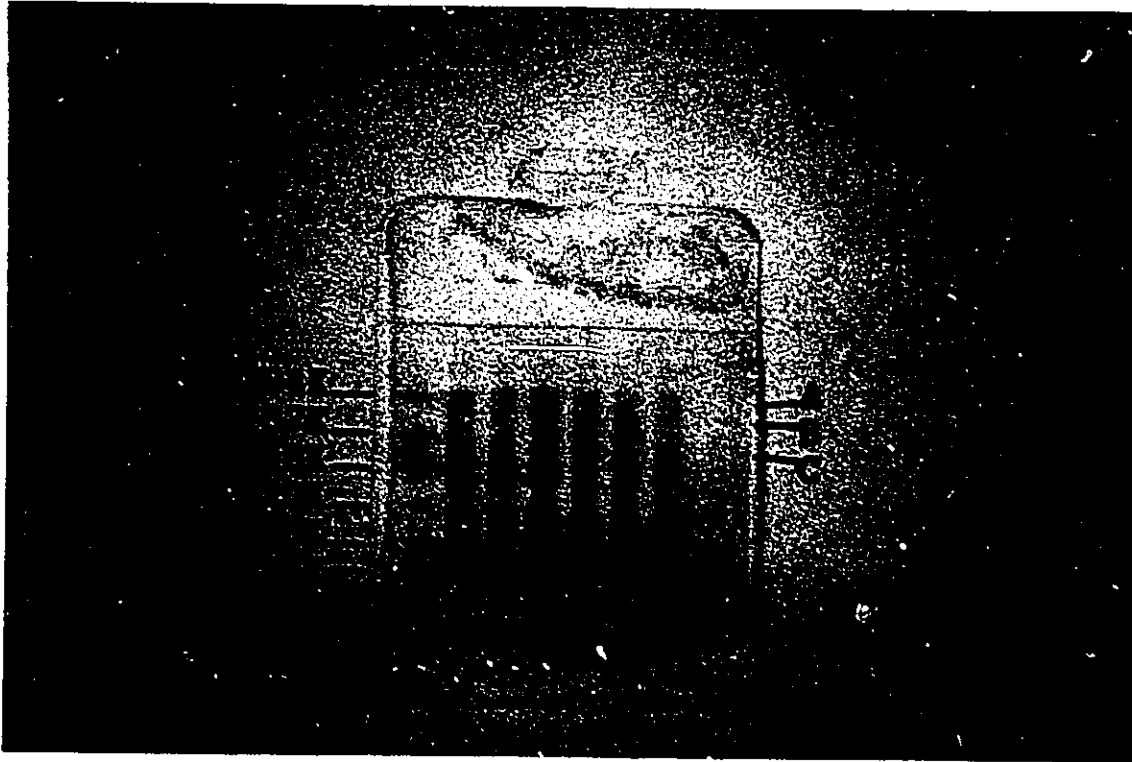

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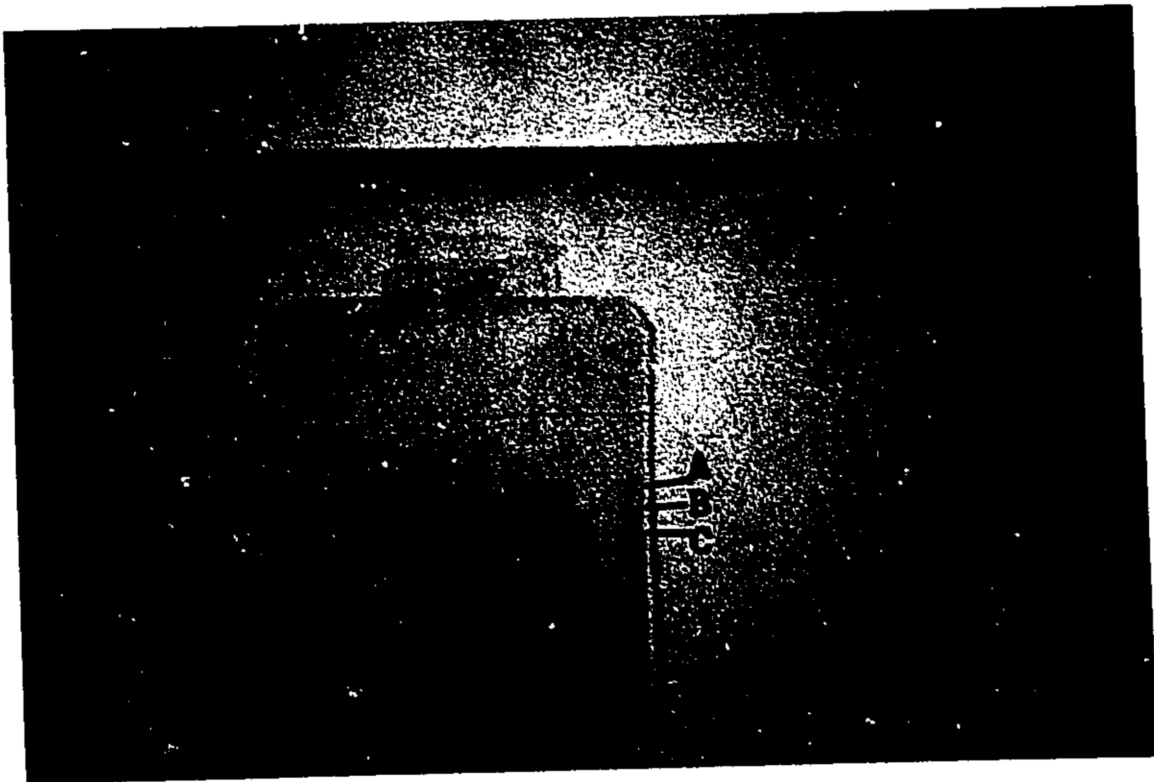
Fig.13. Analysis by SDS-PAGE of the protein present in <u>E.coli</u> JE1011, AB1157 and its mutants before and after heat shock. Cell extracts were prepared according to the method of Laemmli [96]. Equal amounts of protein were applied to each lane in any one gel (0.75-1.5μg.μl^{-1}). Molecular weight standards were run in lane 1 and lane 8 in each gel. One lane contained a mixture of the standards (Pharmacia calibration kit) and another contained the internal standard (0.4 μg,μl^{-1}). Lanes 2 to 7 contained the experimental samples:

	lane	std/E.coli	treatment
(a)	1	mol.wt.std.mix	-
	2	JE1011	30°C control
	3	JE1011	42°C/10 min
	4	JE1011	42°C/45 min
	5	JE1011	48°C/45 min
	6	AB1157	48°C/45 min
	7	AB1157	30°C control
	8	int.std.	-
(b)	1	int.std	-
	2	AB1886(uvrA$^-$)	30°C control
	3	AB1886(uvrA$^-$)	48°C/45 min
	4	AB1885(uvrB$^-$)	30°C control
	5	AB1885(uvrB$^-$)	48°C/45 min
	6	AB1884(uvrC$^-$)	30°C control
	7	AB1884(uvrC$^-$)	48°C/45 min
	8	mol.wt.std.mix.	-

cont...

Fig. 13



(c)	1	mol.wt.std.mix.	-
	2	AB1899(lon$^-$)	30°C control
	3	AB1899(lon$^-$)	48°C/45 min
	4	AB2463(rec$^-$)	30°C control
	5	AB2463(rec$^-$)	48°C/45 min
	6	AB2494(lex$^-$)	30°C control
	7	AB2494(lex$^-$)	48°C/ 45 min
	8	int.std.	-

A, B, and C arrows indicate three prominent protein bands which appear in all the heat shocked cell extracts.

heat shock the protein pattern of JE1011 was similar to that of its control.

In all the cell extracts of mutants of *E.coli* AB1157, except that of *lex* mutant, the three bands (A, B and C) stain more heavily after heat shock, as compared to their respective 30°C control (Fig.13b and c). The *lon*, *rec*, *uvr* A, B and C mutants behaved just like their parent. In the case of *lex* mutant, these bands can still be seen in the heat shocked sample, but they are much less intense than with the other cells.

The analysis was done visually. Laser densitometry of the gels, confirmed the results qualitatively, however densitometry in the conditions attempted did not give any additional, quantitative information. The relative molecular masses (M_R) of these proteins were found to be 89K, 78K and 63K (Fig.14).

3.3.2.Electrophoretic studies after nutritional stress

The protein pattern of *E.coli* AB1157 and its *lon* mutant (*E.coli* AB1899) was studied with and without nutritional stress, i.e., thiamine deprivation (Fig.15a,b), since this treatment has been shown by Fitt and Sharma to have an effect similar to that of heat shock on the development of

Fig. 14

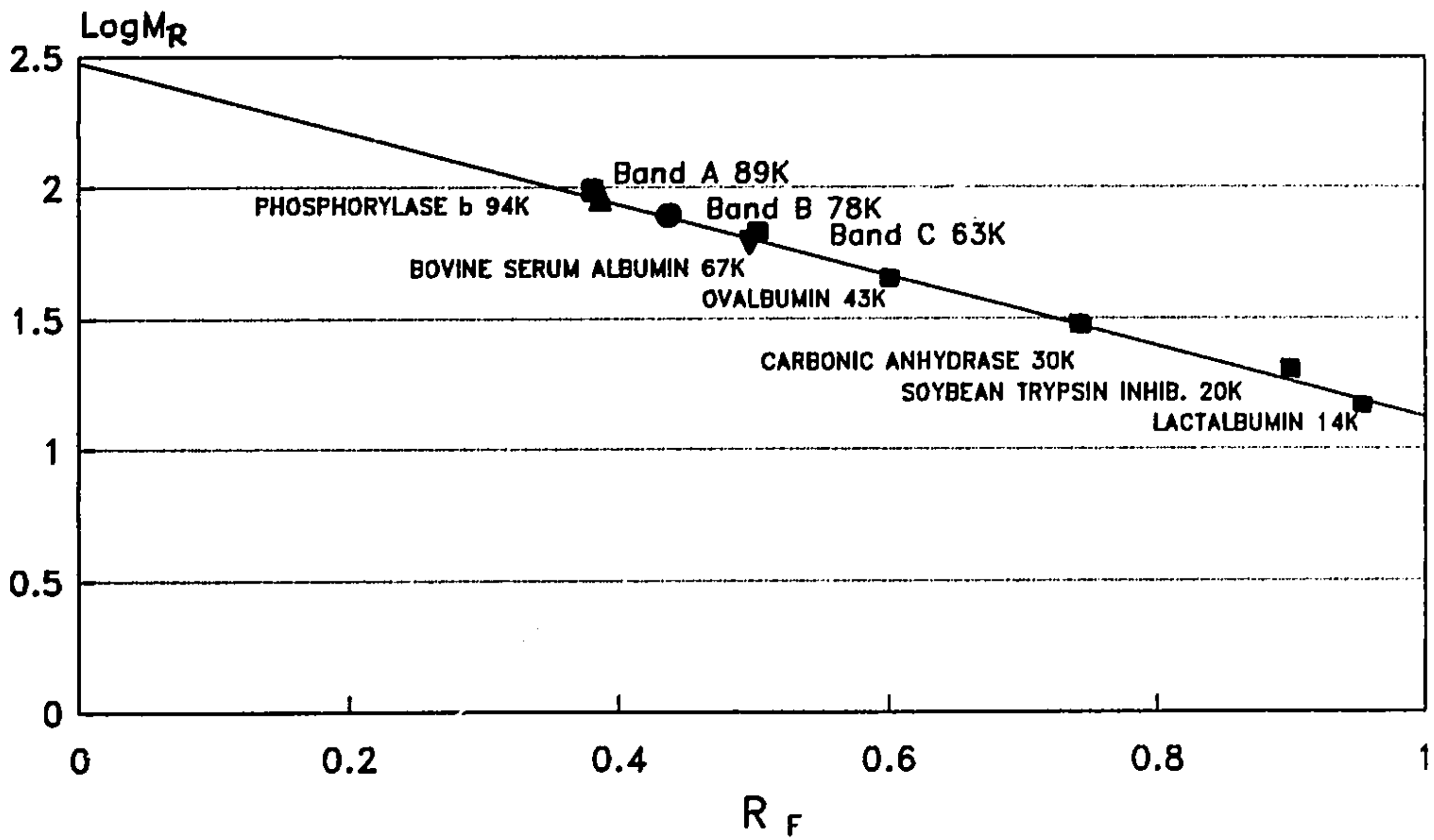


Fig.14. The graph shows the linear relationship between log of the relative molecular masses (log M_R) and relative mobility (R_F) of the protein standards. The M_R values of the proteins, as indicated on the Pharmacia Electrophoresis calibration kit for low molecular weight proteins, are: phosphorylase b, 94K; bovine serum albumin, 67K; ovalbumin, 43K; carbonic anhydrase, 30K; soybean trypsin inhibitor, 20K; lactalbumin, 14K. Plotted R_F values are mean of R_F values obtained for various protein standards (■) as well as three proteins in prominent bands (A, ▲; B, ●; and C, ▼) in ten independent experiments. The standard deviations for all the R_F values plotted were between ±0.01 to ±0.02 and represent actual symbols. The best line was fitted by the method of least squares. Three proteins in prominent bands A, B and C were found to have relative molecular mass of 89K, 78K and 63K respectively.

Fig. 15

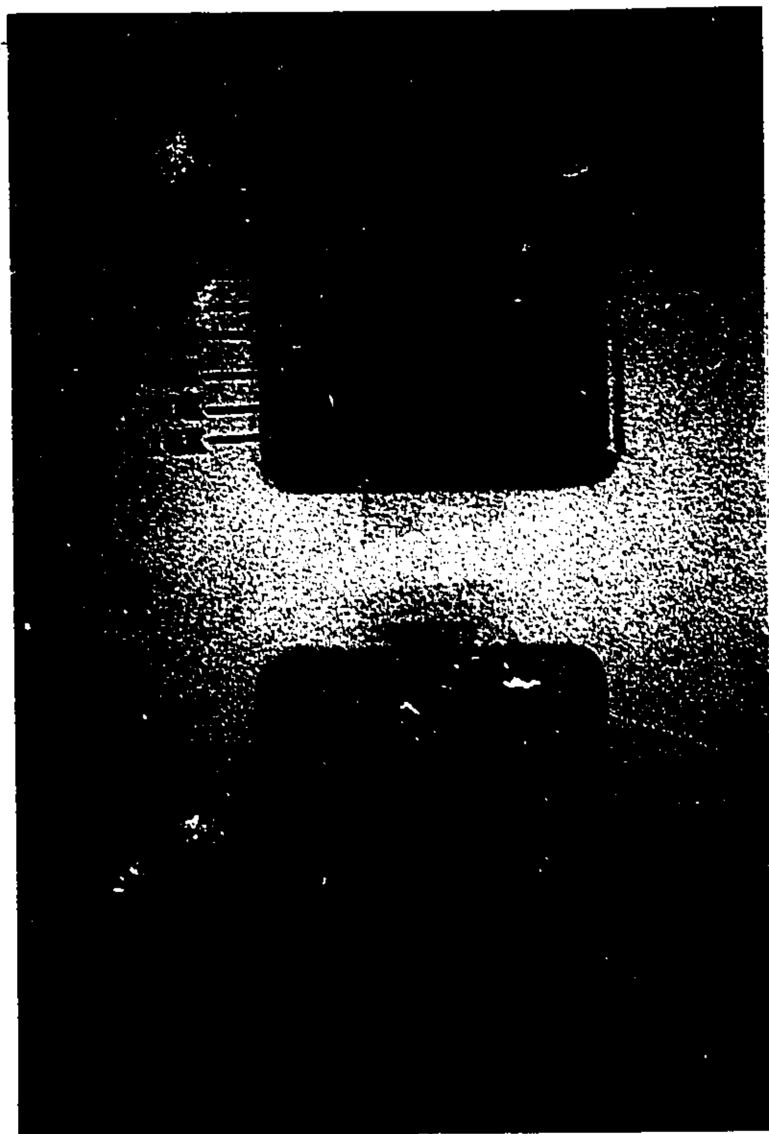


Fig.15. Analysis by SDS-PAGE of the protein present in E.coli AB1157 and AB1899 (lon mutant) grown in complete defined medium (controls) and in the absence of thiamine (1.5h, 3h and 24h tests). The cell extracts were prepared by the method of Laemmli [96]. Equal amount of protein was applied to each lane in any one gel (0.75-1.5μg.μl^{-1}). Molecular weight standards are indicated in lane 1 and 8 on both the gels. Lane 2 to 7 contained experimental samples:

	lane	std./E.coli	treatment
(a)	1	mol.wt.std.mix.	-
	2	AB1157	30°C control
	3	AB1157	1.5h test
	4	AB1157	3h test
	5	AB1157	24h test
	6	AB1899	24h test
	7	AB1899	30°C control
	8	int.std.	-
(b)	1	int.std.	-
	2	AB1157	30°C control
	3	AB1899	30°C control
	4	AB1899	1.5h test
	5	AB1899	3h test
	6	AB1899	24h test
	7	AB1157	24h test
	8	mol.wt.std.mix.	-

uv resistance in these bacteria [97], No difference in the protein bands was found between 30°C control cultures grown in complete defined medium or upon thiamine deprivation (for 1.5h, 3h and 24h), unlike the results of the heat shock experiments. The results were confirmed by laser densitometry.

4. DISCUSSION

The results in Fig.2 show that the heat shock at 42°C leads to a significant increase in the resistance of E.coli JE1011, previously grown at 30°C, to short wavelength (mainly 254nm) ultraviolet light. In contrast, wild type E.coli B not only failed to develop any resistance to the uv light after the same treatment, but became more sensitive to the radiation. Such an effect has not been observed previously in bacteria.

A similar effect to that seen with E.coli JE1011 was observed in yeast by Mitchel and Morrison [100]. They showed that when exponentially growing, diploid, wild-type Saccharomyces cerevisiae cells were subjected to a sudden rise in temperature, they responded by increasing their resistance to the lethal effects of uv light. Earlier, they had reported that heat shock induces increase in heat and ionizing radiation resistance [101].

Recently, Verbenko et al. reported that when wild type E.coli cells were exposed to gamma radiation after a 15°C to 43°C heat shock, they became resistant to radiation damage [102]. This effect was absent in htpR mutants, defective in the induction of the hsps. Hence they suggested that the

observed thermoinduced radioresistance involves hsps. However, details of this work are not readily available.

The process of thermal induction of uv resistance in E.coli (observed here) has many features in common with the classical heat shock response. Its development is time dependent; it is evident at a lower level in cells grown continuously at the higher temperature; and it requires protein synthesis and/or cell growth. However, the resistance develops more slowly than the usual heat shock response, since it increases for at least 45 min, whereas the classical heat shock response in E.coli or Salmonella typhimurium is known to peak more rapidly, after about 5 min [2]. A similar, fairly slow development of uv resistance took place in yeast [100]. The kinetics are closer to those reported for the accumulation of dinucleotide polyphosphates in bacteria after heat shock [2,54]. It is not clear why these compounds are overproduced during the various stress conditions, so it will be interesting to investigate their relationship to thermoinduced uv resistance in E.coli.

A possible explanation for the observed difference in the results of the initial work done on the E.coli JE1011 and E.coliB was given in our (Pardasani and Fitt) recently published paper [102]. We suggest that the effect might be

absent in E.coliB owing to its lon$^-$ character. Also, resistance of E.coliB to heat, increased about six fold, whereas that of E.coli JE1011 was unaffected. It is particularly interesting that the treatment that induced uv resistance in the latter, failed to enhance its thermal resistance. This suggested that the process which induces uv resistance is separable from the one that induces heat resistance after the heat shock treatment [103].

In order to prove that this phenomenon is not specific for the E.coli JE1011, further experiments were performed with a similar K12 derivative, AB1157. The latter differed from the former in two markers. E.coli AB1157 was arg$^-$ and pro$^-$ instead of trp$^-$ and thy$^-$. Moreover, its lon mutant as well as various mutants of DNA repair genes were available. Hence, it was possible to compare the effects observed in E.coli JE1011 and E.coliB with those seen using E.coli AB1157 and its lon mutant.

The experiments described in Fig.6 and 7 show that an effect essentially similar to that reported above with E.coli JE1011 is observed with the E.coli AB1157. However, a more severe heat shock is necessary in this case.

Similar experiments on the lon mutant of E.coli AB1157

(Fig.8) provided strong support for the previous suggestion that the lon$^-$ character of E.coliB was responsible for its failure to develop uv resistance after heat shock. Heat treatment failed to change uv resistance of E.coli AB1899, although, its capacity to develop thermal resistance was unimpaired or even greater than that of the parental strain (Fig.11). The results show clearly that the ability of the lon mutant to develop thermal resistance without increased uv resistance is similar to that of the E.coliB and confirms that the two effects must arise from the induction of two different systems [104].

In a separate study in this laboratory, it was shown that the thermal induction of uv resistance in E.coli JE1011 depends on an error-free DNA repair process [97]. A 45 min heat shock at 42°C to the cells caused a simultaneous reduction in the uv induced frequency of mutation to prototrophy of two nutritional markers, leucine and threonine. In contrast, induction of SOS repair by thymine starvation, caused the expected increase in mutation frequency upon uv irradiation. Thiamine deprivation produced an effect similar to the heat shock leading to an increased uv resistance and a large decrease in the radiation induced mutation frequency. It was interesting to note that two very different types of stresses were able to induce an error-

free DNA repair pathway in these cells, which was clearly distinguishable from the SOS response.

Further, Fitt and Sharma compared the mutation frequency of E.coli JE1011 and AB1157 after heat shock (48°C/45 min), for leucine and histidine markers respectively. In both cases, the heat shock caused an increase in the resistance to uv and a fall in the uv-induced mutation frequency, similar to the one observed in E.coli JE1011 after 45 min heat shock at 42°C [97]. It therefore seems clear that comparable, error-free DNA repair systems are induced in both the strains by these treatments.

Thus, the results with the lon mutant (E.coli AB1899) showed that this mutation prevents the heat induced development of the uv resistance and indicates that this gene is essential to the process. It is interesting to speculate on the possible role of the lon gene product in the induction of the error-free DNA repair pathway that is presumably responsible for the increase in radiation resistance and fall in uv induced mutation frequency in these experiments.

Gottesmann and others have shown (9,59,73,104) that a role of the Lon protease is to degrade the sulA gene product, which is an inhibitor of cell division. Synthesis of the

latter is induced by DNA damage, so the lon mutants which do not destroy the inhibitor, are more sensitive to uv irradiation than lon+ cells. The results described above show that the lon mutant of E.coli AB1157 is about equally sensitive to uv as its parent, under the conditions of our experiments. On the other hand it differs from AB1157 in not showing any increase in the resistance to the radiation after the heat shock. It could be argued that this arises through an inability of the mutant to degrade the sulA product, but this seems unlikely. If the DNA repair process were induced by the heat shock in E.coli AB1899 to an extent comparable to that in the parent strain prior to uv irradiation, then treated cells should be better able to remove the damage responsible for sulA induction than the control cells and thus still be more resistant than the latter to uv. It therefore seems likely that the effect of the lon mutation is a direct one and that the Lon protease is needed for induction of the repair pathway. A possibility that merits further study is that this enzyme might degrade a repressor controlling the error-free repair system. This would be analogous to the role of the Rec A protein in the induction of SOS repair, in which it is activated by DNA damage to a form able to catalyse proteolysis of the Lex A protein, the repressor of the SOS genes.

The results in Fig.9 and 10 show that the inducible system that is being studied here is dependent on a number of genes that are essential for classical error-free DNA repair [48,68], but which also form part of the SOS system [73,105]. Both the recA and the uvrA genes are clearly essential (Fig.9 and 10b). The two corresponding mutants of E.coli AB1157 showed no change at all in the uv resistance after a heat shock (48°C/45 min) that had a large effect on the parent strain (Fig.7).

The requirement for proficiency in recombination is in agreement with the results obtained with Saccharomyces cerevisiae by Mitchel and Morrison [100] who reported that heat shock caused an increase in the uv resistance in wild type yeast that was not observed in a mutant deficient in recombinational repair. They also monitored the changes in the heat shock induced uv resistance in excision repair mutants. The yeast cells defective in excision repair were able to increase their uv resistance in response to heat shock, although some differences between the induction of resistance in wild type (MJ67) and mutants (D7.3) were observed. It was suggested that these differences may be due to different metabolic rates at the elevated temperatures

and to different inherent protein synthetic capacities, since the two strains have different genetic backgrounds. The apparent complete lack of inducibility of the excision repair-proficient, but recombination repair-deficient strain (MS32), strongly suggests that the excision repair pathway is not inducible by heat shock and that thermal induction of uv resistance depends absolutely on recombinational repair.

However, this is not true for either the uvrA or uvrB mutants of E.coli AB1157 (Fig.9 and 10a). Although some effect was observed with the uvrB mutant, it was small enough that it is unlikely to be significant, so this gene also appears to be necessary. In contrast to the uvrA and uvrB mutants, E.coli AB1884, the uvrC mutant, showed a significant increase in uv resistance following heat shock, even though this was much less than with the parent strain. Two explanations might account for this result. First, the mutation may be leaky and some induction may occur, so that the level of active UvrABC endonuclease in the heat shocked cells becomes sufficient to allow a partial development of radiation resistance. Alternatively an active UvrABC endonuclease may not be essential in the repair process

concerned. It may be noted that the UvrAB complex has DNA binding capacity and has endonuclease activity while UvrC is required for maximum activity [48,68]. Both _in vitro_ [106,107] and _in vivo_ [108,109], the _uvr_A and _uvr_B genes have been shown to be under the control of the cellular SOS system, which responds to DNA damage or the inhibition of DNA replication. Foster and Strike [110] have recently shown that the _uvr_C gene is regulated separately, which supports the suggestion [105] that the UvrAB complex has additional functions that are not related to the activity of the intact UvrABC endonuclease.

The result with the _lex_A1 mutant of _E.coli_ AB1157 was unexpected. The LexA protein is the general repressor of the SOS genes [48,68,97,111]. Earlier results of Fitt and Sharma [97] suggested that SOS repair can not be responsible for the thermal induction of uv resistance. Hence, it was expected that this mutation would have no effect on the induction of error free repair. The mutant used in the present study [AB2494, _lex_A1] is also referred to as _lex_A (Ind$^-$) [48,68,111]. It produces a repressor that is not degraded by the activated RecA protease, so that the SOS genes can not be induced [48]. It can be seen (Fig.10b) that the mutant developed much less resistance to uv light than the parent strain as a result of the heat shock, even though

there was still a definite increase similar to that obtained with uvrC mutant. At least two explanations seem possible. First, the mutation might prevent complete induction of the error-free pathway, because the latter shares several genes with the SOS network, which might nevertheless be selectively induced by heat shock or thiamine deprivation without triggering the error-free system. Alternatively, the abnormal rate of degradation of DNA in the uv irradiated lex mutants, which is known to be ten times than that in the wild type cells [70], may swamp the capacity of the heat inducible system to restore viability to the levels observed in the parent strain. In any event, the significant increase observed in this case is in agreement with earlier evidence that the SOS system can not be involved [97]. These findings on the study of the effect of mutations in DNA repair genes on the thermal induction of error-free repair in E.coli have been incorporated into a paper accepted for publication [112].

Finally, none of the mutations examined in these studies affected the ability of the strains to become more heat resistant as a result of heat shock, whatever their effect on the inducibility of uv resistance. This proves once again that the two effects are independent [112].

Recently, VanBogelen _et al_. [113] have shown that induction of the heat shock response and thermotolerance are two distinct inducible states of _E.coli_. Thermotolerance is a state of resistance to thermal killing and results from some unknown cellular process that occurs outside the σ^{32}-related heat shock response. The process leading to thermal protection is triggered by such agents as moderate heat, ethanol, $CdCl_2$ and prolonged treatment with hydrogen peroxide. The second state, heat shock, is brought about by a σ^{32}-dependent induction of seventeen or so proteins and this state is necessary for cellular adjustment to growth at elevated temperatures. Cell division could be one of the processes requiring the heat shock response for adaptation to growth upon increase in temperature [114].

VanBogelen _et al_. could induce the HtpR regulon by addition of isopropyl thio-β-D-galactoside (IPTG) to _E.coli_ cells containing multiple copies of the _htp_R gene under the control of an IPTG-inducible promoter [113]. The levels of all but three hsps increased upon 15 min IPTG treatment at 28°C. But induction of HTP regulon by this means did not provide protection against the challenge temperature of 50°C. On the other hand, treatment with cadmium chloride (600 μM for 15 min) and hydrogen peroxide (70 $\mu g.ml^{-1}$ for 60 min), which weakly induce three or four of the seventeen

hsps, led to the same degree of protection to the challenge temperature as did the heat shock (28°C to 42°C) or ethanol treatment (10%) for 10 min. The poorest inductions by IPTG relative to heat were those of proteins C14.7, D60.5, and G13.5 (Table 1, section 1.1.2). These proteins were also not induced by hydrogen peroxide treatment for 60 min, yet thermotolerance developed; two of them (C14.7 and G13.5) were induced by puromycin treatment and third by nalidixic acid, neither of which could produce thermotolerance. The differences in the induction of hsps by heat and IPTG seem irrelevant to thermotolerance.

Clearly, the results indicated that thermotolerance can develop in response to chemical treatments that induce fewer than half of the hsps, and virtually complete induction of the heat shock regulon could occur without the concomitant development of any thermotolerance.

The proteins coded for by the _dna_K and _gro_EL genes are the two major heat shock proteins of _E_._coli_. Both rise to much higher levels after heat shock. These two proteins constitute 1.4% and 1.6% respectively, of total cell protein at 37°C [20]. Minton _et_ _al_. have suggested that such proteins act non specifically to stabilize labile proteins against heat denaturation [115]. Thermotolerance, in this

view, would be due to "stockpiling" of these stabilizing proteins during heat shock, enhancing survival during subsequent incubation at the lethal temperature. This concept is supported by others [2]. However, recent results of Ramsay [47] argue against this simple model, since he shows a mutation in dnaK does not prevent the induction of thermotolerance. The dnaK756 mutant used in his work is temperature sensitive for growth at 43.5°C and can not support the growth of phage lambda [43]. In addition, the mutant fails to regulate the heat shock response correctly [27], displays an increased rate of heat inactivation [47] and the mutant protein has reduced ATPase activity as compared to native DnaK protein [45]. The data of Ramsay suggest that none of the functions affected by the mutation are involved in thermotolerance and the induced thermotolerance proceeds by a pathway that does not involve dnaK. Thus, although DnaK protein does have a protective effect during heat inactivation, this is a separate phenomena from the protection afforded by prior heat shock.

The interpretation that heat shock and thermotolerance are separable, though related, phenomena has been reached by some [47,116] and is not far different, from the views expressed by Lindquist [2]. The above findings indicate that one or more unknown process(s) outside the heat shock

response is (are) essential to the development of thermotolerance. If the heat shock proteins are at best only indirectly concerned with the development of thermotolerance, then the seventeen-protein bacterial heat shock response has function(s) other than protection against the lethal effects of high temperature. This highlights the need for further investigation. It can be speculated that there could be an overlap between the heat shock response and thermally induced uv-resistance reported in this thesis, since both the processes are separable from the phenomenon for development of thermotolerance. The groEL and dnaK genes are also induced by DNA damage [7], but their role in DNA repair is not known. It is possible that they might be involved in the inducible, error free DNA repair process, but further work will be needed to establish this point.

Preliminary comparative electrophoretic studies were done on protein extracts of E.coli JE1011, and on E.coli AB1157 and its mutants, in an attempt to identify protein(s) whose concentration might be altered in heat shocked cells compared with the control bacteria. The simplest approach, i.e., separation of unlabeled proteins by one dimensional SDS Page Electrophoresis and visual examination of protein bands after Coomassie Blue staining, was followed, in order

to see if any obvious changes were detectable. Three proteins with relative molecular weights: 89($\pm$1.04)K, 78($\pm$1.03)K and 63($\pm$1.05)K appeared to be more prominent in protein extracts from heat shocked cells of all the E.coli strains tested, except that of lexA mutant in which they were present, but fainter. The fact that the three bands all appeared in each of the uvrA, uvrB and uvrC mutant as well as in recA, lon and even in the lex mutants does not seem to exclude the possibility that they play a role in the process leading to uv resistance. This will be particularly the case if the process responsible for thermal induction of uv resistance involves several genes, some playing regulatory and some playing a direct role. However, the fact that they also appear in the cell extracts of E.coli JE1011 at 42°C (45 min heat shock) when no thermal resistance is induced does seem to exclude their playing a major role in that process. Their absence in cell extracts of E.coli JE1011 subjected to heat shock for 10 min at 42°C, suggests that these are the type of proteins not observed after a short heat shock.

The molecular weights of these three proteins (89K, 79K and 66K) were compared with the known molecular weights of hsps (table 1; section 1.1.2). It was found that their molecular weights did not match with any of those of known

hsps. Since the experiments were quite reproducible and the standard deviation was low (±1K for 10 experiments) it is possible that these proteins are different from hsps. Two dimensional electrophoresis of radiolabeled proteins of various E.coli cells, with and without heat shock, can give precise information about the change in the protein pattern before and after the treatment, so it would be of interest to undertake such studies in these cases.

Experiments by Fitt and Sharma, in this laboratory have shown that thiamine deprivation induces uv resistance as well as a decrease in mutation frequency in E.coli AB1157 and its lon mutant (Fitt and Sharma, unpublished data). Fig.15a and b show that thiamine deprivation for 1.5h, 3h and 24h had no visible effects on the protein patterns of the E.coli AB1157 and E.coli AB1899 as compared to their respective controls in complete medium. The three prominant bands which were observed in the heat shocked extracts were not distinguishable in the extracts of the thiamine-deprived cells. These results suggest that the effects of nutritional stress are not related to the appearance of these proteins, secondly, that heat and nutritional stress might induce different systems, both responsible for uv resistance. Alternatively, the three proteins concerned may not be related to the induction of error-free DNA repair.

Further work of Fitt and Sharma has shown that the development of uv resistance is slower in the lon, rec and also in the lex (DM49) mutant as compared to the parent strain (unpublished observation). This indicates that the process responsible for the induction of uv resistance after thiamine deprivation is partially dependent on these two genes and that they may be required for the rapid development of the resistance. A decrease in the uv induced mutation frequency in the parent and the lon mutant confirm the error free nature of the phenomena. The mutation frequency did not change in rec mutant, obviously because of suppressed SOS response in the rec mutants. The lex mutant (DM49) of E.coli AB1157 also gave results similar to rec and lon mutants. The lex (AB2494) could not give clear results because of slow growth in the defined medium used in the studies. Another interesting observation was that uvrA, the excision repair mutant of E.coli AB1157 did not show induction of the uv resistance after the nutritional stress. Also the mutation frequency was not decreased. The fall in the mutation frequency in the parent cells must be due to reduction in the signal for SOS repair, i.e., a fall in number of dimers. The experiments indicate that the thiamine deprivation induces an error-free pathway dependent upon the excision repair system only. This pathway seems to be responsible for the induction of uv resistance and decrease

in mutation frequency after the nutritional stress. There was no induction of thermotolerance to lethal (55°C) temperature after the nutritional stress, again suggesting that the two phenomenon of uv resistance and thermotolerance are not related.

Earlier results with E.coli JE1011 (Fig.2) showed that the effect of heat shock is not due to damage to DNA, because a high level of induction of uv resistance was achieved upon shift to 42°C from 30°C [103]. The cells grew faster at 42°C as compared to that at 30°C, showing that no major disturbances of DNA metabolism occured at this temperature. Heat shock at 48°C on the other hand, stopped growth 15 min after the shift. Thiamine deprivation, a nutritional stress, did not cause loss in the viability of the cells for at least 24h after they were transferred to thiamine deficient medium, while at least two cell doubling occurred in the first 1.5h, presumably before the intracellular pools of the vitamin were exhausted [97]. Both, thiamine deprivation and heat shock, induced increase in resistance, while a decrease in the uv induced mutation frequency was observed [97]. Fitt and Sharma proposed that both the stress treatments, neither of which is known to damage the DNA, can induce the same error free repair system.

As discussed above, there are many similarities between the process of induction of uv resistance after heat shock and thiamine deprivation in the E.coli AB1157. Both the treatments are non-DNA damaging. The most marked similarity is the dependence upon the excision repair pathway. The response in the lon, the rec and the lex mutants is also the same so long as short term treatments are compared. The uv resistance is induced after longer thiamine deprivation in both the mutants, while longer heat shock could not be tried at 48°C because the cells enter the stationary phase, the growth stops and cell viability starts to fall after 50 min. The slow development of uv resistance in the three mutants after thiamine deprivation can not be explained at this stage and requires further investigation.

If the two phenomena induce the same process, they are at least partially-dependent on the Lon protease and RecA protein. Further, both the induction of uv resistance by either stress seems to be independent of the process responsible for the induction of thermotolerance.

The studies described in the present thesis open the field for further investigation.

It would be interesting to observe this phenomenon of

induction of uv resistance after the stress in excision repair-deficient mutants infected with plasmids causing overproduction of the Uvr-endonuclease. In other words, experiments with uvr mutants in which the inducibility of excision repair system can be achieved by infection with a plasmid that is carrying an expression vector for the repair enzyme. If the restoration of the excision repair function is accompanied by the induction of uv resistance, then it would be a direct proof of the involvement of the excision repair system in the phenomenon observed.

Another possibility could be that the process observed here is dependent on the entirely new pathway having genes and their protein products overlapping with the well known heat shock response (groEL and dnaK), excision repair (uvrA,B and C) and the SOS response (lexA).

Investigation of thermally-induced uv resistance in an htpR mutant might provide additional information on the role of this regulon in the observed phenomena. If the mutant cells have the capacity to acquire the uv resistance after heat shock then it would indicate that the classical heat shock response is not involved in this effect. On the other hand, the loss of capacity of induction of uv resistance due to mutation would indicate that one or more protein(s) of the

heat shock response are playing some role in the process. Then it would become necessary to investigate the effect of individual mutations in the genes of HTP regulon such as *dna*K, *gro*EL etc, in the induction of thermally-induced uv resistance.

Minton *et al*. suggested that non-specific stabilization of stress susceptible proteins by stress resistant proteins specially hsps might occur [115]. It is known that the *dna*K mutants are defective in shut-down of the heat shock response and overproduced hsps [15]. As mentioned before, this protein is also induced by uv and nalidixic acid, both being DNA damaging treatments. However, no role for DnaK is yet known in DNA repair [7]. It will be interesting to study the phenomena of thermal induction of uv resistance in the *dna*K mutants overproducing hsps. It is possible that one or more hsps might stabilize the proteins of error-free pathway.

As mentioned before, the kinetics of induction of the uv resistance matches with the kinetics of induction of dinucleoside polyphosphates. Further investigation of changes in their levels before and after heat shock at 48°C for 45 min in *E.coli* AB1157 might support the suggestion that these molecules play some significant role in the

phenomena. In particular, Plateau et.al. [58] used E.coli strain SC122 transformed with the plasmid pUC1247 carrying apaH, the gene coding the E.coli ApppppN hydrolase, to investigate the putative role of the ApppN nucleotides in triggering the heat shock response. In these strains, ApppN concentration was decreased by a factor of about 10, as compared to the wild type cells, due to overproduction of the hydrolase. The same strains were also used to examine the role of ApppN in protection against oxidative damage by hydrogen peroxide. Their studies indicated that the heat shock response in E.coli is not dependent on a high intracellular concentration of diadenosine tetraphosphate. In addition, their high levels are also unnecessary for adaptation to hydrogen peroxide stress. The transformed E.coli cells, overproducing polynucleotide hydrolases could be used to investigate the role of dinucleoside polyphosphates in the thermal induction of uv resistance, since a marked reduction in the effect reported in this thesis should be observed if these compounds play an important part in initiation of the synthesis of the relevant proteins. This might also facilitate identification of the latter.

It is possible that several other non DNA damaging, physical or chemical, stresses also induce resistance to uv or even

other mutagenic agents. An effort to investigate effect of such stresses, e.g., osmotic shock, ultrasound, exposure to toxic non-mutagenic chemicals, etc. would indicate clearly if this is a general response of cells for protection against further damage, unlike the SOS and adaptive responses. This further highlights the need to search for the extent to which this repair pathway is active in organisms which are adapted to extreme living conditions such as high salt concentrations and high temperatures. These findings could be useful in developing methods for use in a study of the relationship between the adaptation to environmental stress and resistance to DNA damage.

To conclude, the induction of the uv resistance after heat stress, a non DNA damaging treatment, suggests presence of a DNA repair pathway in bacteria that has not been observed before [103]. The phenomenon has been found to be dependent upon several genes, namely, *lon*, *rec*A, *uvr*A and B and at least partially on *uvr*C and *lex*A [104,112]. It was also observed that the induction of uv resistance and thermotolerance are two independent processes. These data together with those of Fitt and Sharma (97 and unpublished observations) suggest that the process induced to higher levels by heat stress is the well-known excision repair pathway. The work described here provides a basis for the

study of this phenomenon at the molecular level and for detailed examination of induction of DNA repair by other stresses that do not damage DNA or interfere directly with its replication.

5. SUMMARY

5.1. Heat shock induces resistance to uv radiation in *E.coli* JE1011 and AB1157.

5.2. The phenomenon was not observed in *E.coli*B, ATCC11303, which is *lon*$^-$ in character.

5.3. Further experiments on the mutants of *E.coli* AB1157 suggest that *lon*, *rec*, *uvr*A and B mutations abolish the effect completely, while the *uvr*C and *lex* mutations restrict its full development.

5.4. The heat shock used (42°C/45 min) induced thermotolerance to a challenge by the lethal temperature of 52°C in *E.coli*B, but not in *E.coli* JE1011. On the other hand, higher heat shock at 48°C for 45 min induced thermotolerance to a 55°C challenge in *E.coli* AB1157 as well as in JE1011, suggesting that the two phenomena, thermo-induced uv resistance and thermotolerance, are not related.

5.5. Thermotolerance was induced in *lon*, *rec*, *uvr*A,B,C and *lex* mutants of *E.coli* AB1157, indicating that these genes are not involved in the process and confirming that heat tolerance and uv resistance develop separately.

5.6. Resolution of the unlabelled proteins of the cell extracts of the control (30°C) and heat shocked cultures of *E.coli* JE1011, *E.coli* AB1157 and its mutants, by SDS PAGE showed induction of three proteins with molecular weights:

89K, 78K and 63K. The results did not give definite proof for involvement of these proteins in thermally-induced uv resistance, but did exclude the possibility of their playing any role in thermotolerance.

5.7. Analysis of proteins of cell extracts of E.coli AB1157 and AB1899, the lon mutant, before and after thiamine deprivation for 1.5h, 3h and 24h did not show any obvious difference. The three proteins (section 5.6) seen after heat shock were not observed after this nutritional stress, which is also known to induce uv resistance in the E.coli [97]. This suggests that either (i) the two stresses induce separate pathways responsible for the uv resistance, which is unlikely, or (ii) the proteins are not involved in the phenomenon.

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