Effects of Inhibitors of Protein, RNA and DNA Synthesis on Heat-injured *Salmonella typhimurium* LT2

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SUMMARY

The role of protein, RNA and DNA synthesis in the repair of thermal injury in *Salmonella typhimurium* was investigated. Thermal injury was assessed by the 'minimal medium recovery' system: after heat treatment, higher viable counts are obtained on minimal-medium agar than on complex-medium agar, and the ability of heated bacteria to form colonies on complex-medium agar is recovered when they are incubated in liquid minimal medium. This recovery is inhibited by rifampin and chloramphenicol, but not by nalidixic acid. In addition, rifampin causes a loss in viability. Alkaline sedimentation analyses of radioactively labelled DNA showed that hydroxyurea and rifampin, unlike chloramphenicol and nalidixic acid, cause DNA breaks in heated bacteria. The results indicate that rifampin is lethal to heated bacteria and that chloramphenicol, though not lethal, prevents repair of thermal damage.

INTRODUCTION

We have previously reported (Gomez *et al.*, 1973) that, following heat treatment, *Salmonella typhimurium* LT2 shows a lower survival on complex-medium agar than on minimal-medium agar. This phenomenon, known as minimal medium recovery (MMR), is analogous to the results found in ultraviolet light irradiated *rec⁻ Escherichia coli* (Ganesan & Smith, 1968). The toxic effect of complex media on heat-treated *S. typhimurium* is believed to be due to its ability to induce DNA breaks in heated bacteria (Gomez & Sinskey, 1973). We have suggested that heat treatment causes a primary lesion in DNA or its metabolic and catabolic enzyme systems (Gomez & Sinskey, 1973). Incubation in complex media converts this primary lesion into *in vivo* DNA breaks or alkali-labile bonds, and these events are accompanied by a loss of viability.

Differential plating on minimal versus complex medium can therefore be used as a measurement or indicator of thermal injury (Gomez *et al.*, 1973). A repair process for this type of injury has been observed (Gomez & Sinskey, 1973; Wilson & Davies, 1976).

When heated cultures are incubated in liquid minimal medium, the viable counts on complex media increase until they are equal to those on minimal media. This repair process occurs in the absence of growth. In fact, exponential growth does not start until the viable counts on minimal medium and complex medium are the same, i.e. until the heat injury has been repaired. In addition, when repair has been completed, complex media no longer induce DNA breaks.

To study the mechanisms of thermal injury and repair, inhibitors have been used to probe the site of injury and to determine the metabolic requirements of repair. Iandolo & Ordal (1966) reported that the recovery (regain of salt tolerance) of thermally injured *Staphylococcus aureus* was not inhibited or affected by penicillin, cycloserine, 2,4-dinitrophenol or

chloramphenicol. However, actinomycin D completely inhibited repair. Likewise, Tomlins & Ordal (1971) showed that rifamycin, an inhibitor of RNA synthesis, blocked repair of *S. typhimurium* after heat treatment. With *S. typhimurium*, ATP and protein synthesis were also required for repair, as determined by the inhibitory effects of 2,4-dinitrophenol and chloramphenicol.

In this paper, we report the effect of inhibitors of protein, RNA, and DNA synthesis on MMR of heated *S. typhimurium* LT2, and their relationship to DNA breaks.

METHODS

Bacteria. Strain DB21, derived from *S. typhimurium* LT2 and cured of known prophages (Botstein, 1968), was provided by D. Botstein of the Department of Biology, Massachusetts Institute of Technology, and used throughout these studies.

Media, culture and enumeration procedures. *Salmonella typhimurium* DB21 was grown at 37 °C to the exponential phase in a glucose/mineral-salts medium (M-9; Gomez *et al.*, 1973), to a density of 1.0×10^8 to 3.0×10^8 viable bacteria ml⁻¹. All cultures were shaken (150 rev. min⁻¹) in 250 ml Erlenmeyer flasks.

For radioactive labelling, M-9 medium contained (per ml) 250 µg 2'-deoxyadenosine (Boyce & Setlow, 1962) and either 5 to 10 µCi [*methyl*-³H]thymidine (6.7 Ci mm⁻¹, New England Nuclear) or 5 µCi [*methyl*-¹⁴C]thymidine (51.5 mCi mm⁻¹, New England Nuclear). The labelled bacteria were harvested by centrifuging (4150 g) for 5 min at 37 °C, washed once, centrifuged again, and resuspended in M-9 medium containing (per ml) 20 µg non-radioactive thymidine and 250 µg 2'-deoxyadenosine. The bacteria were incubated for 30 min at 37 °C to chase the intracellular radioactivity into polymeric DNA.

Both unlabelled and labelled bacteria were harvested by centrifuging, washed, and resuspended in distilled water at 37 °C, to a final concentration of 3×10^8 to 9×10^8 viable bacteria ml⁻¹.

Viability was determined by spreading the surface of agar plates with 0.1 ml of appropriate dilutions in 0.067 M-sodium phosphate buffer (pH 7.0). The media used for enumeration were M-9 and TSY [trypticase soy agar (BBL) enriched with 0.5 % yeast extract (Difco)]. TSY and M-9 plates were incubated at 37 °C for 24 and 48 h, respectively, and visible colonies were counted.

Heat treatment. The ³H-labelled and unlabelled *S. typhimurium* LT2 were heated to 50 °C for 15 min. Control cultures were held at 37 °C for 15 min.

Post-heat treatment of unlabelled bacteria. Portions (19 ml) of M-9 medium, with or without metabolic inhibitors, were inoculated with heat-treated bacterial suspension (1 ml) and incubated at 37 °C. At various times during incubation, the number of viable bacteria was determined. Metabolic inhibitors were used at the following concentrations: chloramphenicol (Sigma), 100 µg ml⁻¹; hydroxyurea (Sigma), 0.1 M; rifampin (Calbiochem), 40 µg ml⁻¹; nalidixic acid (Sigma), 20 µg ml⁻¹. To remove the inhibitors, 3 ml samples were filtered through Millipore membranes (HA 0.45 µm; 24 mm diam.), washed twice with 5 ml 0.067 M-phosphate buffer (pH 7.0) and resuspended in 3 ml phosphate buffer.

Post-heat treatment of labelled bacteria. Portions (19 ml) of M-9 medium, with or without metabolic inhibitors at the concentrations listed above, were inoculated with heat-treated ³H-labelled bacterial suspension (1 ml) and incubated at 37 °C. After 15 min, the bacteria were harvested by centrifuging and resuspended in 1 ml distilled water. These suspensions were then subjected to alkaline sedimentation analysis.

In a different experiment, portions (10 ml) of M-9 medium containing chloramphenicol

Fig. 1. Post-heat treatment incubation in M-9 medium with inhibitors. Bacteria in water were held at 50 °C for 15 min. The culture was then diluted (1:20) into M-9 medium at 37 °C containing: (a) nalidixic acid, 20 $\mu\text{g ml}^{-1}$; (b) rifampin, 40 $\mu\text{g ml}^{-1}$; (c) chloramphenicol, 100 $\mu\text{g ml}^{-1}$. At various times, the cultures were plated on M-9 agar ($\blacktriangle$) and TSY agar ($\bullet$).

(100 $\mu\text{g ml}^{-1}$) were inoculated with heat-treated ^3H -labelled bacterial suspension (1 ml). After different times of incubation at 37 °C, 10 ml double-strength TSY broth was added and the cultures were incubated for a further 15 min at 37 °C. The bacteria were harvested and resuspended in distilled water, as described in the previous section. The bacterial suspensions were subjected to alkaline sedimentation analysis.

Alkaline sedimentation analyses. These procedures have been described previously (Gomez & Sinskey, 1973). The amount of bacteria deposited on each gradient ranged from 3×10^7 to 9×10^7 , giving an estimated 0.5 to 1.5 μg DNA per gradient. A value of 1.6×10^{-8} μg DNA/cell was adopted (Davies & Sinskey, 1973).

RESULTS

Effect of metabolic inhibitors on minimal medium recovery

Nalidixic acid (NA) did not inhibit repair of thermal injury (Fig. 1a). When rifampin (RIF) was incorporated into M-9 medium (Fig. 1b), the M-9 plate counts decreased and the TSY plate counts increased and approached the M-9 plate counts. Thus there was loss of viability and some bacteria underwent repair. In fact, in the presence of RIF the viability of heated bacteria decreased by more than 90 %.

Chloramphenicol (CAP) did not affect the viability of heated bacteria (Fig. 1c). The TSY plate counts increased following incubation in liquid medium, but did not reach the same

Fig. 2

Fig. 3

Fig. 2. Alkaline sedimentation of ^3H -labelled DNA (●) from bacteria incubated in M-9 medium containing 0.1 M-hydroxyurea (M-9/HU). (a) Control bacteria (not exposed to 50°C) incubated in M-9/HU for 15 min at 37°C . (b) Heated bacteria (exposed to 50°C for 15 min) incubated in M-9/HU for 15 min at 37°C . (c) Heated bacteria (exposed to 50°C for 15 min) incubated in M-9/HU for 240 min at 37°C . All gradients contained ^{14}C -labelled DNA (○) from bacteria which were neither heated nor incubated in M-9/HU.

Fig. 3. Alkaline sedimentation of ^3H -labelled DNA (●) from bacteria incubated in M-9 medium containing rifampin ($40\ \mu\text{g ml}^{-1}$) (M-9/RIF). (a) Control bacteria (not exposed to 50°C) incubated in M-9/RIF for 15 min at 37°C . (b) Heated bacteria (exposed to 50°C for 15 min) incubated in M-9/RIF for 15 min at 37°C . (c) Heated bacteria (exposed to 50°C for 15 min) incubated in M-9/RIF for 240 min at 37°C . All gradients contained ^{14}C -labelled DNA (○) as in Fig. 2 legend.

value as the M-9 plate counts. The difference between the M-9 and TSY plate counts was still evident after 21 h. This could reflect inhibition of repair of most of the bacteria.

Effect of inhibitors on DNA molecular weight

Previously (Gomez & Sinskey, 1973) we showed that incubation in M-9 medium does not affect the DNA weight-average molecular weight of heated bacteria. However, DNA molecular weight was reduced when hydroxyurea (HU) or RIF was incorporated into M-9 medium (Figs 2*b* and 3*b*). In the presence of HU, the reduction of DNA molecular weight was still evident after 4 h incubation (Fig. 2*c*), indicating that DNA breaks caused by HU were not repaired. After 4 h, there appeared to be a slight repair of the DNA breaks caused by RIF, but this was not always observed and we do not consider the amount of repair was

Fig. 4

Fig. 5

Fig. 4. Alkaline sedimentation of ^3H -labelled DNA (●) from bacteria incubated in M-9 medium containing nalidixic acid ($20\text{ }\mu\text{g ml}^{-1}$) (M-9/NA). (a) Control bacteria (not exposed to $50\text{ }^\circ\text{C}$) incubated in M-9/NA for 15 min at $37\text{ }^\circ\text{C}$. (b) Heated bacteria (exposed to $50\text{ }^\circ\text{C}$ for 15 min) incubated in M-9/NA for 15 min at $37\text{ }^\circ\text{C}$. All gradients contained ^{14}C -labelled DNA (○) as in Fig. 2 legend.

Fig. 5. Alkaline sedimentation of ^3H -labelled DNA (●) from bacteria incubated in M-9 medium containing chloramphenicol ($100\text{ }\mu\text{g ml}^{-1}$) (M-9/CAP). (a) Control bacteria (not exposed to $50\text{ }^\circ\text{C}$) incubated in M-9/CAP for 15 min at $37\text{ }^\circ\text{C}$. (b) Heated bacteria (exposed to $50\text{ }^\circ\text{C}$ for 15 min) incubated in M-9/CAP for 15 min at $37\text{ }^\circ\text{C}$. All gradients contained ^{14}C -labelled DNA (○) as in Fig. 2 legend.

significant. The DNA molecular weight of control bacteria (not heated) was not affected by either HU or RIF (Figs 2*a* and 3*a*).

Incubation of heated bacteria in M-9 medium containing NA or CAP (Figs 4*b* and 5*b*) did not affect the DNA sedimentation patterns. This was expected since neither CAP nor NA affected the viability of heated bacteria.

DNA degradation

The conversion of acid-insoluble radioactivity to acid-soluble material during the various treatments can be estimated from Table 1. The amounts of radioactivity recovered from each set of gradients (e.g. Fig. 2 compared with Fig. 5) varied because different initial concentrations of [^3H]thymidine were used in each experiment. The values for Figs 2*b*, 3*b*, 4*b* and 5*b*, which correspond to 15 min exposures of heated cells to the different inhibitors, indicate that little or no DNA solubilization occurred. These amounts of solubilization are

Table 1. *Radioactivity recovered from alkaline sedimentation gradients*

Solubilization in heated bacteria is expressed as a percentage of the solubilization in control bacteria.

Treatment	$10^{-4} \times {}^3\text{H}$ -radioactivity (c.p.m.)	Solubilization (%)
Fig. 2(a)	9.67	0
2(b)	9.75	0
2(c)	7.77	19.7
Fig. 3(a)	12.45	0
3(b)	12.03	3.4
3(c)	9.92	20.3
Fig. 4(a)	12.42	0
4(b)	12.32	0.8
Fig. 5(a)	6.04	0
5(b)	5.81	3.8

Fig. 6. Alkaline sedimentation of ${}^3\text{H}$ -labelled DNA (●) from bacteria incubated in M-9 medium with or without chloramphenicol ($100 \mu\text{g ml}^{-1}$) (M-9/CAP) and shifted to TSY. (a) Heated bacteria (exposed to 50°C for 15 min) incubated in M-9 for 300 min at 37°C and then shifted to TSY for 15 min at 37°C . (b) Heated bacteria (exposed to 50°C for 15 min) incubated in M-9/CAP for 300 min at 37°C and then shifted to TSY for 15 min at 37°C . All gradients contained ${}^{14}\text{C}$ -labelled DNA (○) as in Fig. 2 legend.

not enough to account for the reduction in molecular weight observed in Figs 2*b* and 3*b*. Thus, the original molecular-weight reductions must be due to internal DNA scissions rather than to massive exonucleolytic activity. After 4 h exposure of heated bacteria to HU (Fig. 2*c*) and RIF (Fig. 3*c*), 19.7 % and 20.3 %, respectively, of acid-insoluble [${}^3\text{H}$]thymidine had been lost. During this period there was little or no repair of DNA breaks.

Effect of CAP on the sensitivity of heated bacteria to TSY-induced DNA breaks

One way of establishing the degree of repair of thermal injury is to determine the extent to which DNA in heated bacteria is sensitive to TSY-induced breaks (Gomez & Sinskey, 1973). If bacteria are exposed to TSY broth immediately after heat treatment, their DNA molecular weight is reduced. However, when bacteria are incubated in M-9 before shifting to TSY, the reduction of DNA molecular weight is less, and is eliminated after 5 h incubation in M-9 medium.

This offered a means of testing the significance of the inhibitory effect of CAP on the repair of thermal injury (Fig. 1c). When heated bacteria were incubated in M-9 containing CAP ($100 \mu\text{g ml}^{-1}$) for 300 min and then shifted to TSY, the reduction of DNA molecular weight still occurred (Fig. 6b). This is in sharp contrast to the results for bacteria incubated in M-9 medium without CAP (Fig. 6a).

DNA solubilization cannot be estimated in Fig. 6 because the experiments were performed with bacterial suspensions that had been labelled with different concentrations of [^3H]thymidine. However, prolonged incubation of heated bacteria in M-9/CAP results in approximately 4 % solubilization with no observable DNA molecular-weight reduction (unpublished results). We have previously reported (Gomez & Sinskey, 1973) that incubation of heat-damaged bacteria in TSY for 15 min causes negligible DNA degradation. Therefore, we doubt that the molecular-weight reduction observed in Fig. 6b is due to exonucleolytic activity.

DISCUSSION

The effect of RIF on the viability of heated bacteria is similar to that previously observed for HU and TSY (Gomez *et al.*, 1973). Exposure of heated bacteria to RIF causes loss in viability. In addition, both RIF and HU, as well as TSY (Gomez & Sinskey, 1973), cause a reduction in molecular weight.

Why do RIF and HU induce DNA breaks and loss of viability? RNA is degraded during heat treatment and resynthesized during repair in *S. typhimurium* (Tomlins & Ordal, 1971). We also know that endonuclease I is competitively inhibited by RNA *in vitro* (Goebel & Helinski, 1970). Thus, it is possible that inhibition of RNA synthesis by rifampin (Wehrli & Staehelin, 1971) may result in the activation of endonuclease I. The possibility that rifampin acts by inhibiting a repair process involving RNA synthesis cannot be discarded. However, the hypothesized inhibition would automatically result in DNA strand breakage and cell inactivation.

HU has been reported to act by inhibiting the ribonucleotide diphosphate reductase system, thus depleting the cell of DNA precursors (Krakoff, Brown & Reichard, 1968). Sinha & Snustad (1972) found that HU did not induce detectable single- or double-strand breaks in the DNA of *E. coli*. Thus our finding that HU induces DNA strand breaks in heated bacteria, leads us to believe that the repair of thermal damage, or at least maintenance of viability, requires DNA synthesis. However, the NA experiments show that inhibition of DNA synthesis *per se* is not enough to cause DNA breakage in heated bacteria. NA permitted repair of injured cells and did not cause any DNA strand breakage, although it is known to inhibit semiconservative DNA replication (Bourguignon, Levitt & Sternglanz, 1973). Thus, the lethal effect of HU on heated bacteria appears to be due to the inhibition of precursors of DNA synthesis involved in repair. However, DNA synthesis during repair of thermal injury has not been demonstrated by the classical techniques of [^3H]thymidine uptake into heated bacteria (Tomlins & Ordal, 1971).

The small amounts of DNA degradation during the 15 min treatments strengthen our argument that the observed reduction in DNA molecular weight is due to DNA strand breakage rather than to massive exonucleolytic activity.

Inhibition of the repair of approximately 80 % of the heated bacteria by CAP indicates a requirement for protein synthesis during repair. However, some bacteria can repair in the presence of CAP. Analysis of these results suggests three possibilities. (i) There is a distribution of damaged cells in which most (80 %) require *de novo* protein synthesis to replace heat-inactivated enzymes required for repair; but in some (20 %), the proteins required for repair were not lost during heating. (ii) There are two distinct types of damage, one which requires protein synthesis for repair and one which does not. Thus, in some cells, only a type of damage which can be repaired in the presence of CAP occurred. There is evidence that systems involved in the repair of ultraviolet light lesions can be of two types: growth-medium dependent (sensitive to CAP) and independent (insensitive to CAP) (Ganesan & Smith, 1972; Youngs, Van der Schueren & Smith, 1974). (iii) CAP does not penetrate, or does not act, until some bacteria have repaired.

The appearance of anomalously sedimenting DNA in all control profiles has been observed previously (Davies, Sinskey & Botstein, 1973; Gomez & Sinskey, 1973). The nature and biological significance, if any, of DNA sedimenting close to the bottom of the gradient has not been elucidated. Control experiments (not shown) demonstrated that this material was not dependent on the amount of bacteria deposited on the gradients. We have decreased, at discrete intervals, the amount of bacteria on the gradients, reducing the DNA concentration to a value 500 times smaller than that used in these experiments. To achieve the necessary specific activity of radioactivity in DNA, the amount of [*methyl*-³H]thymidine in the growth medium was increased to 200 $\mu\text{Ci ml}^{-1}$. The amount of DNA from control bacteria found to the left of the main peak was not affected by the dilution: it ranged from 15 to 30 %, with a mean value of 24 % and a standard deviation of 6 %. Throughout the range of cell dilutions there was no obvious trend to increase or decrease the amount of DNA to the left of the main peak, although the resolution in this area did increase so that two peaks were observed. Regardless of the nature and biological significance of this DNA, it in no way detracts from the qualitative aspects of our work. It should also be noted, without further speculation, that heated bacteria, treated with HU (Fig. 2) or TSY (Fig. 6) do not yield sedimentation profiles with fast-sedimenting DNA.

In conclusion, RIF, like HU (Gomez *et al.*, 1973), is toxic to heated cells. The functions which these compounds reportedly block, RNA synthesis by RIF (Wehrli & Staehelin, 1971) and DNA synthesis by HU (Krakoff *et al.*, 1968), are required to maintain viability and DNA integrity after heat treatment. CAP or protein synthesis inhibition is not lethal to heated bacteria, but prevents the repair of heat damage in most of them.

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