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Action of Hydrogen Peroxide on Growth Inhibition of *Salmonella typhimurium*

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SUMMARY

The effect of hydrogen peroxide on the growth of *Salmonella typhimurium* LT2 in a mineral glucose medium was investigated. The H_2O_2 produced a lag period, the duration of which increased as the concentration of H_2O_2 in the medium was increased from 1 to 60 μg./ml. Growth subsequent to the lag period proceeded at the normal growth rate at concentrations of H_2O_2 as high as 30 μg./ml. Storage of H_2O_2 in the sterile growth medium resulted in a disappearance of H_2O_2 with a half-life of about 48 hr. The disappearance of H_2O_2 because of reaction with glucose resulted in proportionate decreases in the growth inhibitory action of the medium. *Salmonella typhimurium* destroyed H_2O_2 rapidly (half-time = 60 min.)—an effect attributed largely to bacterial catalase. The catalatic activity of the bacteria was decreased or eliminated by boiling or by treatment with cyanide. The growth of the bacteria in H_2O_2 resulted in the development of H_2O_2 resistance. By subculturing the wild type LT2 in medium containing successively higher H_2O_2 concentration, a resistant strain designated LT2P was isolated which grew in the presence of H_2O_2 concentrations that were completely inhibitory to the wild type.

The experimental results support the generally accepted conclusion that bacterial catalase acts in protecting the organism from H_2O_2. It is pointed out that in biological media H_2O_2 readily forms adducts with many compounds, including carbonyls, amino acids and thymine. Consequently, the inhibitory effects of H_2O_2, especially in studies involving auxotrophs, may be partly or wholly due to the peroxide adduct rather than 'free' H_2O_2.

INTRODUCTION

In studies on the biological effects of irradiated media, we utilized the growth response of *Salmonella typhimurium* strain LT2 as one measure for the detection of radiolytically generated toxic products (Schubert, Watson & White, 1967; Schubert & Watson, 1969; Schubert, Watson, & Baecker, 1969). Since hydrogen peroxide (H_2O_2) is one of the primary molecular products produced by the action of ionizing radiation in aqueous solutions (Baxendale, 1964), we conducted a series of experiments to define the effect of chemically added H_2O_2 in our microbiological assay test system.

Most microbiological investigations have utilized survival (colony forming ability) for evaluating the toxic effects of H_2O_2. One of the few investigations dealing with the inhibitory effect of H_2O_2 on the exponential phase of growth was reported by Campbell & Dimmick (1966). These investigators, using *Serratia marcescens*, compared survival with growth after exposure to very high concentrations of H_2O_2 (30 mg./ml.).

Survival was not affected until after about 4 min. of contact with the solution, whereas the growth response changed immediately after contact. The latter changes were manifested over 40 hr by changes in the duration of the lag phase, in the growth rate and in maximal growth.

Frey & Pollard (1966) demonstrated that considerable periods of lag followed by normal growth resulted when exponentially growing bacteria of an *Escherichia coli* autotroph were inoculated into irradiated media. The duration of the lag period was dependent on the amount of H_2O_2 produced in the medium by radiolysis. The inhibitory action of the irradiated medium or of chemically added H_2O_2 could be eliminated by addition of catalase. The susceptibility of bacteria to H_2O_2 generally correlates with the catalase content of the organisms (McLeod & Gordon, 1923; Molland, 1947; Amin & Olson, 1968). However, no consistent correlation is obtained between sensitivity to ionizing radiation and catalase content (Engel & Adler, 1961; Adler & Clayton, 1962; Adler, 1963).

METHODS

Bacterial strain. Salmonella typhimurium strain LT2 was obtained from Professor E. Englesberg. The culture was maintained on nutrient agar (Difco) slants enriched with nutrient broth (Difco) to give an 0·8 % (w/v) solution.

Media. Bacteria were grown in a mineral glucose medium (pH 7·0) of the following percentage (w/v) composition: KH_2PO_4, 0·3; K_2HPO_4, 0·7; $(NH_4)_2SO_4$, 0·1; $MgSO_4.7H_2O$, 0·1 and glucose, 0·05, as sole source of carbon and energy (Englesberg, 1959). The carbon source and salts were sterilized separately by Millipore filtration and added as concentrated solutions. Desired concentrations of H_2O_2 were prepared by adding appropriate volumes of freshly prepared solutions of reagent grade H_2O_2 (non-stabilized) in distilled water to the above medium immediately before the addition of the bacteria.

Growth measurements. Glucose-grown organisms, harvested during the exponential phase of growth by centrifugation at 3° and washed once in saline, were inoculated into 18 × 150 mm. growth tubes containing the desired defined medium (final volume, 5 ml.) at a concentration of $5{\cdot}7 \times 10^7$ bacteria/ml. (Englesberg, 1959). The tubes were incubated at 37° on a rotary shaker and the turbidity was measured at designated intervals using a Fisher electrophotometer (427 mμ filter; blank, sterile medium) modified to accept the growth tubes (Englesberg, 1959). Growth measurements, based upon the average of duplicate tubes, were recorded in Fisher units (1 Fisher unit = $1{\cdot}14 \times 10^7$ bacteria/ml. = 5·8 μg. dry weight of bacteria/ml.). The logarithms to the base 2 of the Fisher units have been presented to facilitate the comparisons of growth rates.

Hydrogen peroxide determination. Hydrogen peroxide was measured by the titanium sulphate method (Egerton *et al.* 1954). Analyses were made after the removal of the organisms by centrifugation for 10 min. at 10,000 *g* at 3°.

Catalase. Stock solutions of catalase were prepared before use from beef liver catalase powder (Sigma Chemical Company, Inc., St. Louis, Missouri). Three μg./ml. of the catalase preparation in mineral glucose medium decomposed 24·5 μg. H_2O_2/ml. in 15 min. at pH 7·0 and 37° (see below).

RESULTS

Effect of H_2O_2 on the growth of Salmonella typhimurium. The effect of various concentrations of H_2O_2 on the growth of the bacteria in mineral glucose (0·05 %) medium is shown in Fig. 1. The exponential growth rate in the absence of H_2O_2 was 1·18 divisions per hr. The duration of the lag period increased as the H_2O_2 concentration was increased to 60 μg./ml. The growth rate subsequent to the lag period showed little change up to an H_2O_2 concentration of 30 μg./ml. By 22 hr the same degree of maximal growth on glucose was reached at all H_2O_2 concentrations tested.

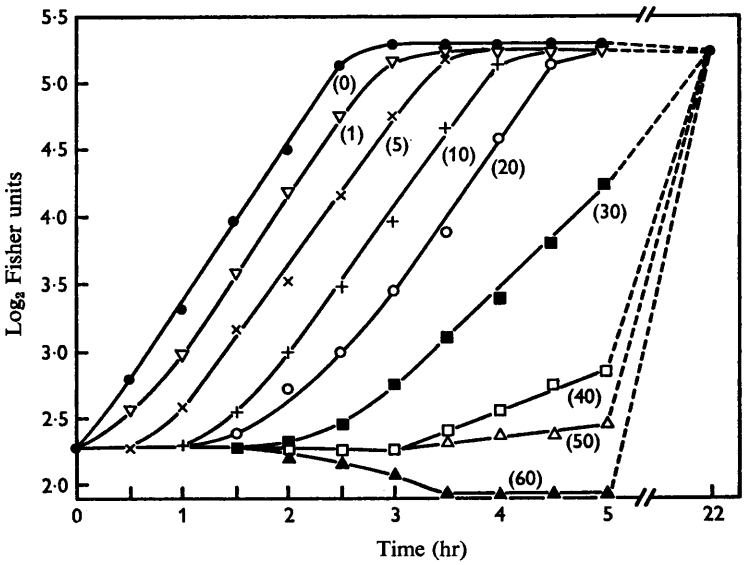

Fig. 1. Effect of increasing concentrations of hydrogen peroxide on the growth of *S. typhimurium* in a mineral glucose (0·05 %) medium. Numbers in parentheses represent the μg. H_2O_2/ml. added to the medium prior to inoculation with the cells. Inoculum: $5·7 \times 10^7$ organisms/ml.

Effect of storage time on the inhibitory action of hydrogen peroxide. We tested the effect of dark storage at 37° in sterile mineral glucose medium (pH 7·0) on the inhibitory action of H_2O_2. Figure 2 summarizes the results obtained when growth measurements were performed daily over a 5-day period with stored medium whose initial H_2O_2 concentration was 60 μg./ml. (These results are also representative of those obtained at lower peroxide concentrations.) A progressive loss in the inhibitory action of the stored solution occurred and after 5 days the growth response of the inoculated bacteria was indistinguishable from that of controls grown in the absence of H_2O_2. No bacterial contamination occurred in the stored media.

Moody (1963) showed that H_2O_2 reacts with glucose during incubation at low temperatures. The observed loss in inhibitory action of H_2O_2 in our stored medium corresponded to the decrease in H_2O_2 levels resulting from an interaction with glucose. Figure 3 shows representative results obtained in stored media with an initial H_2O_2 concentration of 20 μg./ml. Little change in the H_2O_2 concentration occurred over a 165-hr period in the stored growth medium in which glucose was omitted, while the

H_2O_2 disappeared with a half-life of approximately 48 hr in the medium containing glucose. These results are in agreement with our earlier findings (Schubert *et al.* 1969).

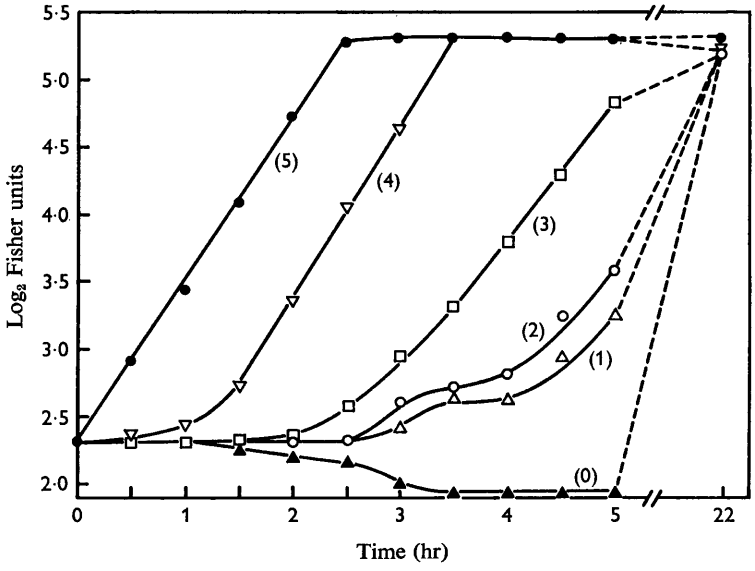

Fig. 2. Effect of storage time at 37° in mineral glucose (0·05 %) medium (pH 7·0) on the inhibitory action of hydrogen peroxide. Initial H_2O_2 concentration (o): 60 μg./ml. Numbers in parentheses represent the days of storage. Inoculum: $5{\cdot}7 \times 10^7$ organisms/ml.

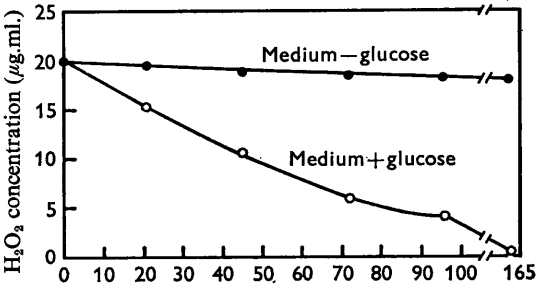

Fig. 3

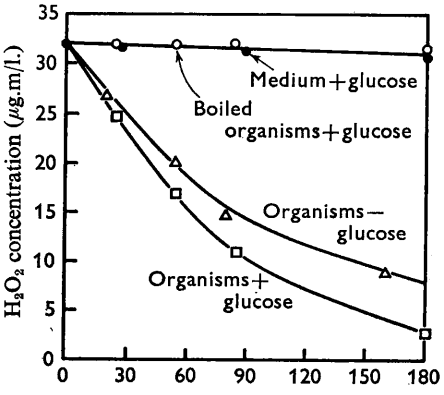

Fig. 4

Fig. 3. Effect of glucose (0·05 %) on the disappearance of hydrogen peroxide from growth medium (pH 7·0) at 37°. Initial H_2O_2 concentration: 20 μg./ml. Hydrogen peroxide was measured by the titanium sulphate method.

Fig. 4. Effect of *S. typhimurium* in the presence and absence of glucose on hydrogen peroxide disappearance. H_2O_2 concentration: 32 μg./ml. Inactivated organisms were boiled for 15 min. in distilled water and were resuspended in saline to a turbidity equivalent to $5{\cdot}7 \times 10^7$ bacteria/ml. Hydrogen peroxide was measured by the titanium sulphate method following the removal of the organisms by centrifugation.

Destruction of hydrogen peroxide by Salmonella typhimurium. The effect of *S. typhimurium* on the destruction of H_2O_2 was investigated. The bacteria were incubated in mineral medium containing 32 μg. H_2O_2/ml. in the presence and absence of glucose. Bacteria inactivated by boiling for 15 min. were similarly treated in glucose-containing medium. Hydrogen peroxide disappearance was measured at designated times over a 3-hr period (Fig. 4).

The growth response of the bacteria in the medium containing glucose was similar to that shown in Fig. 1 for the 30 μg. H_2O_2/ml. level in which the lag phase persisted for about 2 hr before exponential growth resumed. The data shown in Fig. 4 therefore describes the rate of destruction of H_2O_2 during the lag phase. The half-time of H_2O_2 disappearance was about 1 hr. At the point where the lag phase ended about 80% of the H_2O_2 originally in the medium had disappeared. H_2O_2 destruction by the organisms in the absence of glucose was appreciable but occurred at a lower rate than that obtained with the 'growing' organisms in glucose-containing medium. The disappearance of H_2O_2 from glucose medium in the presence of organisms inactivated by boiling did not differ significantly from the rate of loss obtained over 3 hr in the mineral glucose medium alone. We assume, as have most investigators, that the catalatic activity of the bacteria is largely due to catalase. We have not attempted to measure the catalase activity following degradation of the organisms (Weibull & Hammarberg, 1963; Frey & Pollard, 1966) because we believe that the catalatic activity of the intact viable organism is more meaningful and less likely to lead to contradictory results.

The effect on H_2O_2 disappearance following chemical inhibition of catalase was tested with potassium cyanide (KCN), a known catalase inhibitor (Nicholls & Schonbaum, 1963). Increasing amounts of KCN were added to sterile growth medium and the destruction of H_2O_2 by 3 μg. purified beef liver catalase/ml. ($1{\cdot}2 \times 10^{-8}$M)

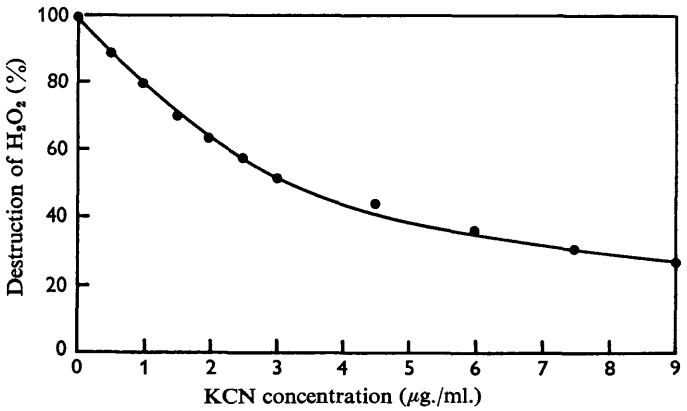

Fig. 5. Effect of potassium cyanide on the destruction of hydrogen peroxide by catalase. Four ml. volumes of complete mineral glucose medium (pH 7·0) containing 3 μg. catalase/ml. in the presence and absence of various concentrations of KCN were incubated for 15 min. at 37°. Two ml. of H_2O_2 containing medium were then added to each to give a total volume of 6 ml. The medium was incubated for 15 min. at 37° and was assayed for H_2O_2 by the titanium sulphate method. Initial H_2O_2 concentration: 32 μg./ml. Under the above conditions, 24·5 μg. were destroyed by 3 μg. catalase/ml. in the absence of KCN. Values plotted below are expressed as a percentage of the latter value.

was measured. The destruction of H_2O_2 decreased as the KCN content of the system increased (Fig. 5). However, even in the presence of a large molar excess (approximately 10^4) of KCN, complete inactivation of the catalase was not obtained under the conditions of this test.

The growth and H_2O_2 disappearance of KCN-treated organisms in the absence of glucose is shown in Fig. 6. Growth in mineral glucose medium containing 20 μg. KCN/ml. was inhibited for at least 4 hr (Fig. 6A) but by 22 hr reached approximately one half the level of maximal growth attained by the glucose and H_2O_2 (34 μg./ml.) controls. A drop in the turbidity of the suspension treated with KCN + H_2O_2 in the absence of glucose occurred during a 4-hr period (Fig. 6A). Hydrogen peroxide disappearance by KCN-treated organisms in the absence of glucose was markedly decreased (Fig. 6B). These results are compatible with an inactivation of the bacterial catalase by the inhibitor.

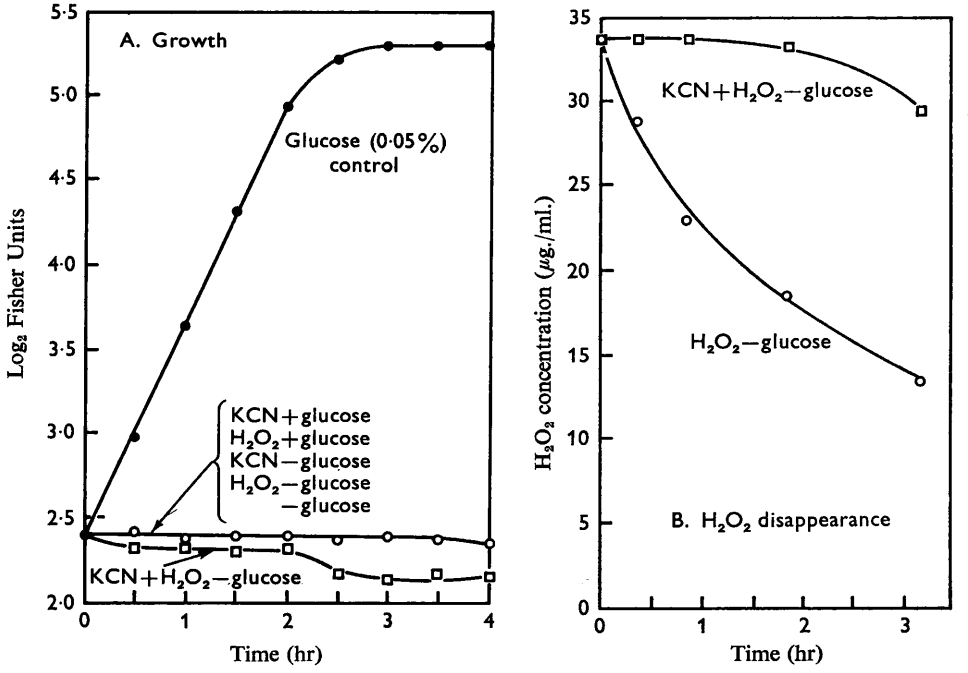

Fig. 6. Effect of a 15 min. pretreatment (37°) with potassium cyanide (20 μg./ml.) on growth (A) and hydrogen peroxide disappearance (B) by *S. typhimurium* in mineral medium in the *absence* of glucose. Initial H_2O_2 concentration: 34 μg./ml. Hydrogen peroxide was measured by the titanium sulphate method following the removal of the cells by centrifugation. Inoculum: $5{\cdot}7 \times 10^7$ organisms/ml.

Development of hydrogen peroxide resistance. The effect of a previous exposure to H_2O_2 on the ability of the organisms to respond to a subsequent H_2O_2 exposure was tested. Figure 7 shows the growth response obtained when 20 μg. H_2O_2/ml. were added to organisms growing in the presence and absence of 20 μg. H_2O_2/ml. The H_2O_2 was added when the cell-mass had doubled in the respective media. The response of the organisms growing previously in the absence of H_2O_2 was characterized by a

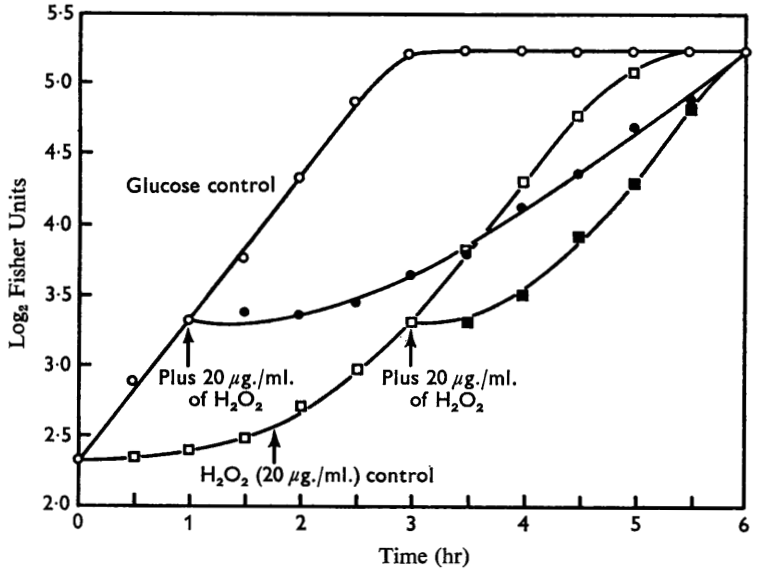

Fig. 7. Effect of adding 20 μg. hydrogen peroxide/ml. on the growth of *S. typhimurium* in mineral glucose (0·05 %) medium and in mineral glucose medium containing 20 μg./ml. of hydrogen peroxide. The H_2O_2 was added to each growth tube in a total volume of 0·2 ml. when growth had doubled in the respective media. Two tenths (0·2) ml. of distilled water was added to the control tubes at the same time. Inoculum: $5·7 \times 10^7$ organisms/ml.

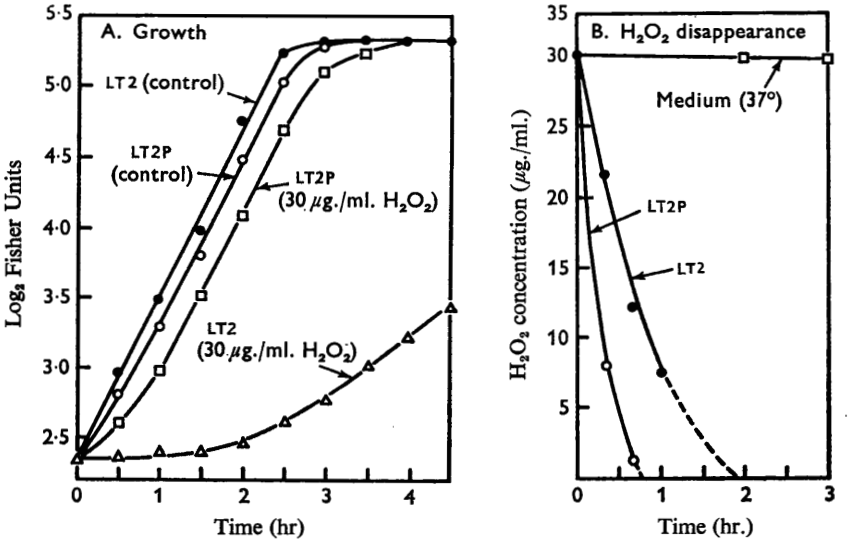

Fig. 8. Growth in mineral glucose (0·05 %) medium in the presence and absence of 30 μg. H_2O_2/ml. (A) and hydrogen peroxide disappearance (B) by the wild type (LT2) and peroxide resistant (LT2P) strains. Hydrogen peroxide was measured by the titanium sulphate method following the removal of the cells by centrifugation. Inoculum: $5·7 \times 10^7$ organisms/ml.

more prolonged lag phase and by an inhibition in the growth rate, as compared to the response of the organisms previously exposed to H_2O_2. The results suggest that the concentration of catalase in the organisms was raised following growth in the presence of H_2O_2.

To determine if growth in the presence of H_2O_2 resulted in the selection of an H_2O_2 resistant strain, wild type LT2 were sub-cultured daily into mineral glucose medium containing successively higher H_2O_2 concentrations. Following twelve such transfers, a strain achieving maximal growth within 22 hr in mineral glucose medium containing 600 μg. H_2O_2/ml. was isolated. This strain, designated LT2P, retained the ability to grow in the presence of H_2O_2 concentrations that were completely inhibitory to the wild type following eight successive transfers in the absence of H_2O_2. Figure 8 shows the growth (Fig. 8A) and H_2O_2 disappearance (Fig. 8B) of the LT2 and LT2P strains in the presence of an H_2O_2 concentration (30 μg./ml.) permitting the growth of the wild type. The growth rate of the LT2P strain in the absence of H_2O_2 was slightly lower than the normal LT2 response and only a slight inhibition in growth is produced by 30 μg. H_2O_2/ml. (Fig. 8A). The resistant strain removed H_2O_2 at a rate about 3·5 times greater than did the wild type (Fig. 8B).

DISCUSSION

The inhibitory action of H_2O_2 on the growth of *Salmonella typhimurium* manifests itself primarily in the production of a lag phase, the duration of which is proportional to the concentration of H_2O_2. No effect on the maximal growth reached at 22 hr in the glucose medium occurred at H_2O_2 concentrations to 60 μg./ml., nor were significant changes observed in the growth rate subsequent to the lag until 30 μg. H_2O_2/ml. was reached (Fig. 1). These results, obtained with an inoculum consisting of $5{\cdot}7 \times 10^7$ exponential phase bacteria/ml. are qualitatively similar to the response shown by *Serratia marcescens* (Campbell & Dimmick, 1966) and *Escherichia coli* (Frey & Pollard, 1966) to H_2O_2 under different experimental conditions.

Our results support the generally accepted conclusion that the catalatic activity of the organism plays an important role in protection against H_2O_2 (Fig. 4, 6). In studies reported elsewhere (Schubert & Watson, 1969), it was shown that the addition of catalase to H_2O_2 containing medium before inoculation with *Salmonella typhimurium* eliminated the lag phase completely. The addition of catalase at increasing times after inoculation, however, had a decreasing influence on the duration of the lag produced at the H_2O_2 concentration tested. When H_2O_2 was in contact with the organisms for about 45 min. before catalase was added, no effect on the subsequent duration of the lag phase was observed. Figure 4 shows that approximately 60% of the initial H_2O_2 in the medium was still available at this time. The mechanism by which H_2O_2 exerts this effect on the growth capability of the bacterial cell has been attributed to a type of repairable injury which affects cell division (Campbell & Dimmick, 1966).

The H_2O_2-resistant strain of *Salmonella typhimurium* isolated following repeated contact with H_2O_2 (Fig. 8) is of potential usefulness for evaluating the presence of toxic products other than H_2O_2 in irradiated media containing high H_2O_2 levels. Although we have isolated a number of strains which show an apparent increase in resistance to H_2O_2 when haemin is added to the growth meduim (Beljanski, 1955), we

have adopted the use of the former strain for this purpose since no supplementation of mineral glucose medium is required. In previous work we have shown that H_2O_2 interacts with organic molecules present in the growth medium. The loss of toxicity of stored growth medium containing H_2O_2 (Fig. 2), resulting from a glucose/H_2O_2 interaction (Fig. 3), is one example of this effect. Of greater significance, however, is the fact that H_2O_2 has been shown to react with carbonyl compounds, e.g. glyoxal, glycolaldehyde (Schubert *et al.* 1967) and histidine (Schubert *et al.* 1969) to form adducts which are more toxic to the growth of *S. typhimurium* than the individual compounds alone. These adducts may be readily decomposed by catalase (Weitzel, Buddecke & Schneider, 1961; Schubert *et al.* 1969). Similar increases in the toxic and mutagenic properties of various organic molecules following treatment with H_2O_2 have been shown in other biological systems (Wyss, Stone & Clark, 1947; Weitzel *et al.* 1961). In the report by Frey & Pollard (1966) a thymine-requiring auxotroph was used to study the effect of added H_2O_2. In unpublished work we have found that thymine reacts with H_2O_2 to form an adduct which is more toxic than H_2O_2 itself. We would suggest, therefore, that the use of auxotrophs as test systems for evaluating the biological effects of H_2O_2 or of irradiated solutions should be supplemented by studies with the corresponding wild-type strains.

The results of the present study are relevant to the effects produced by irradiated solutions on this microbiological test system. It should be emphasized, however, that we do not feel that H_2O_2 itself is the primary cause of biological damage, since it is highly unlikely that H_2O_2 ever exists free in a biological milieu (J. Schubert, to be published). It is our opinion that H_2O_2 adducts formed by interaction with various degradation products produced during irradiation are responsible for the antibacterial properties of irradiated media.

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