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Adaptation of a Soil Bacterium to Hydrazine Propellants

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The use of hydrazine (Hz) and its derivatives, 1-methylhydrazine, (MMH) and 1,1-dimethylhydrazine (UDMH), as missile propellants, fuels for aircraft emergency power supplies, and as reagents in chemical synthesis, required that the toxicity and environmental hazards of these compounds be determined. CLARK et al. (1968) presented a detailed discussion of the current information concerning Hz toxicity. More recent research was reviewed by BACK et al. (1978).

The information available on the toxic effects of hydrazine-like compounds to bacteria is sparse. STIEFEL et al. (1977) described an enzymatic system for breakdown and metabolism of Hz in nitrogen fixing bacteria. LONDON (1979) described the relative toxicity of Hz, MMH, and UDMH to a soil microbe, reporting that these compounds cause a concentration dependent increase in the lag time of this organism. He suggested several possible mechanisms for this phenomenon: (1) a bacteriocidal effect which significantly reduced the inoculum concentration; (2) since the culture ultimately resumed a normal growth pattern, the degradation of the fuels to non-toxic substances, or to an ineffective concentration; (3) an adaptive mechanism that enables the organism to overcome the inhibitory effects.

Three possible adaptive mechanisms by which bacteria may survive inhibitory environments are: (1) environmental selection of preexisting resistant variants in a population; (2) mutation to resistant variants; and (3) induction of specific enzyme systems which will counteract the effects of the toxic entity.

To determine if an inductive, selective, or mutation mechanism is involved in resistance to hydrazine fuel toxicity, we studied the effects of Hz, MMH, and UDMH on the growth kinetics of Enterobacter cloacea cultures that had been previously exposed to each of the fuels.

MATERIALS AND METHODS

All materials and procedures were essentially the same as those described by LONDON (1979).

Organism: The organism used in this study, designated D-31, was isolated initially from a top soil sample from southwestern Ohio and identified as Enterobacter cloacea.

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Maintenance of Stock Cultures: D-31 was maintained in the lyophilized state and on slants of Tryptic Soy Broth (Difco) solidified with 1.5% Bacto-Agar (Difco).

Growth Conditions: The medium used (SMS) was of the following composition in deionized water (g/L): KH_2PO_4, 0.950; K_2HPO_4, 2.440; $(NH_4)_2SO_4$, 1.000; $MgSO_4 \cdot 7H_2O$, 0.200; $FeCl_3 \cdot 6H_2O$, 0.001; and Glucose, 2.000.

This medium was prepared without glucose and was distributed into 500 mℓ Bellco Nephelo Culture flasks (side arm flasks) in 100 mℓ aliquots. The flasks were autoclaved for 15 minutes at 15 psi and 121°C. After cooling, 0.4 mℓ of 0.5 g/mℓ solution of glucose (sterilized with membrane filters of 0.22 μm porosity) were added aseptically to each flask. The flasks were incubated at room temperature for three days before use to insure sterility.

The 2.0 g/L concentration of glucose (rate limiting nutrient) was determined by LONDON (1979) to be optimal for this organism for these experimental conditions.

Method of Exposure: Since the fuels studied are water soluble, they were added directly to the medium with sterile microliter syringes to obtain the desired concentration. Hz, MMH, and UDMH (95%) were obtained from MCB Reagents, Cincinnati, Ohio, and stored under N_2. Before use, each bottle of fuel was tested for sterility. Viable organisms were not isolated at any time from any of the fuel samples.

Because the three fuels are basic, the medium was adjusted to its original pH of 7.1 with a sterile 10% (v/v) solution of HCl when required. The stability of Hz, MMH, and UDMH in this medium has been described previously (LONDON 1979).

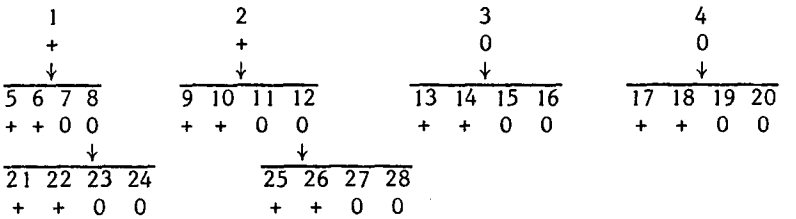
Propellant Concentration: We selected one concentration of each fuel that was shown to increase significantly the lag time but would eventually permit the culture to grow to maximum yield. The concentrations selected were 10 ppm for Hz, 20 ppm for MMH, and 50 ppm for UDMH (all v/v).

Growth Curve Determination: To follow culture growth, duplicate side-arm flasks with SMS and fuel were inoculated with 0.1 mℓ of a culture grown in SMS that was late in log phase (12-14 hours and approximately 40% transmittance). Prior to inoculation, the culture was adjusted to 60% transmittance (%T) with SMS using a Coleman Jr. Model 6D spectrophotometer (wave length = 570 nm). This adjustment was accomplished by aseptic transfer of a portion of the culture to a separate side-arm flask so that the original culture could be further maintained until stationary growth was obtained. The cultures were incubated at room temperature (21°C ± 2°) with shaking on an Eberbach 6000 reciprocating shaker at a rate of 100 cycles per minute.

Turbidity readings were taken at 2 hour intervals for 50 hours or until the culture reached a maximal growth of 25-30%T. The turbidity values were plotted versus time to yield a growth curve.

Determination of Lag Time: After the growth curves were prepared for each culture, five points were selected on each curve during the time the culture was clearly in the log phase of growth. A line was drawn through these points using a computer assisted least squares analysis. The projected time on the linear regression line which corresponded to 100%T was used as the experimental lag time (ELT). All slopes were similar and the correlation coefficients derived from the regression analysis were greater than 0.97. Note that the ELT is not the actual lag time of the culture but is a calculated indicator used to represent that time more concisely and to minimize any bias in experimental design or data manipulation. For other methods of lag time determinations, see WILSON and MILES (1964).

Inoculum Transfer Conditions: Each experimental condition was established in duplicate according to the following inoculum history flow-chart.



Where 1, 2, 3, etc = flask number

+ = fuel added before inoculation; 10 ppm Hz, 20 ppm MMH, 50 ppm UDMH.

0 = no fuel added.

↓ = transfer of 0.1 mℓ of culture after growth to approximately 40%T and adjustment to 60%T.

The transfer history of each culture will be designated by a series of "+"s and "0"s. For example, the series Hz: + → 0 → + represents a transfer history in which a culture was initially exposed to Hz, then transferred and grown in the absence of Hz, then transferred and grown again in the presence of Hz. A growth curve with this designation would be the curve obtained from the last culture in this series that was grown with Hz present.

RESULTS

Initial Propellant Effects on D-31 Growth: The growth curves and ELTs of D-31 when grown in SMS with and without added propellants are shown in Fig 1. Although the propellants caused a significant increase in the ELT, the cultures eventually assumed a normal growth pattern. Fig 1 also shows that 20 ppm MMH and 50 ppm UDMH caused a significant reduction in final growth yield.

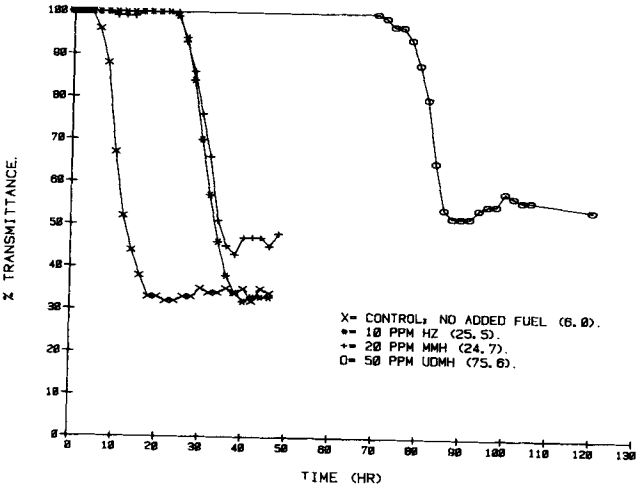


Fig 1. The effect of a selected concentration of hydrazine (Hz), 1-methylhydrazine (MMH), or 1,1-dimethylhydrazine (UDMH) on the growth of D-31. The numbers in parenthesis refer to the ELT of the culture. Some readings at 100%T have been omitted for clarity.

In each experiment the control flasks grew as expected, i.e., cultures with the history + → 0, displayed growth curves and ELT's that were similar to the original cultures that grew without added fuel. Cultures with the history 0 → + had growth curves and ELT's that were similar to the cultures initially grown with fuel present.

Effect of Previous Exposure to 10 PPM Hydrazine on Growth of D-31 in 10 PPM Hydrazine: A culture with the transfer history Hz: + → + displayed an ELT that was considerably shorter than the culture 0 → + and approached the ELT of the control. A comparison of these curves is shown in Fig 2.

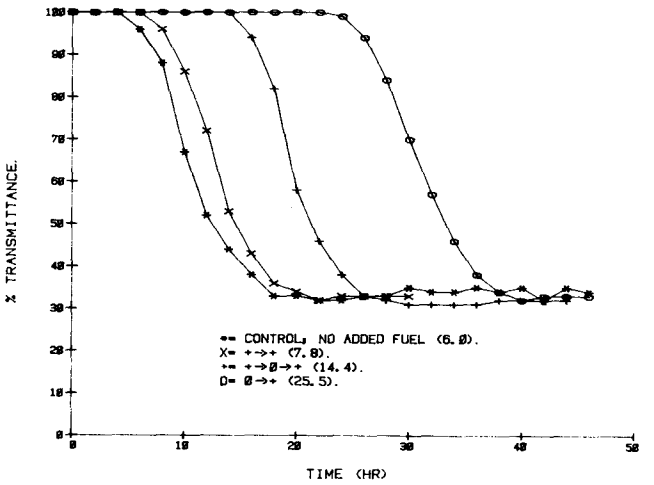


Fig 2. The effect of culture history on the growth response of D-31 to 10 ppm hydrazine. Refer to the text for explanation of the key. Numbers in parenthesis are the ELT's of the growth curves.

The ELT of the culture with prior Hz exposure (Hz: + → +) was 7.8 hours, while the control culture without prior Hz exposure (Hz: 0 → +) had an ELT of 25.5 hours. A culture with the history Hz: + → 0 → + had a higher ELT than that observed with prior exposure culture (Hz: + → +). A comparison of these curves is also shown in Fig 2. A culture with the history of Hz: + → 0 → 0 → +, had a growth curve and ELT identical to the culture described above (Hz: + → 0 → +). A culture that had the history Hz: + → 0 → 0 → 0 → 0 → + also showed a growth curve and ELT that was similar to the culture Hz: + → 0 → +. However, a culture with the history Hz: + → 0 → 0 → 0 → 0 → 0 → + → + displayed a growth curve and ELT similar to the culture Hz: + → +.

Effect of Prior Exposure to 20 PPM MMH on Growth of D-31 in 20 PPM MMH: When a culture with the transfer history MMH: + → + was established, it had a lower ELT than that observed in the initial exposure culture (MMH: 0 → +). A comparison of these growth curves is presented in Fig 3. The culture that was initially exposed had an ELT of 24.7 hours while the culture with the transfer history MMH: + → + had an ELT of 19.1 hours, the ELT being reduced by 30% (corrected for control ELT). This is in contrast to the 90% reduction observed with Hz exposure (Hz: + → + compared with

Hz: 0 → +). A culture with the transfer history MMH: + → 0 → + displayed a growth curve and ELT similar to that observed with the prior-exposure culture (MMH: + → +). A culture with three repetitive growth periods in SMS with 20 ppm MMH (MMH: + → + → +) also had a growth curve and ELT similar to the prior exposure culture (MMH: + → +). These curves and ELT's are also presented in Fig 3. Note that the final growth yields of the prior-exposure cultures (MMH: + → +; + → 0 → +; and + → + → +) were approximately equal to that of the control culture.

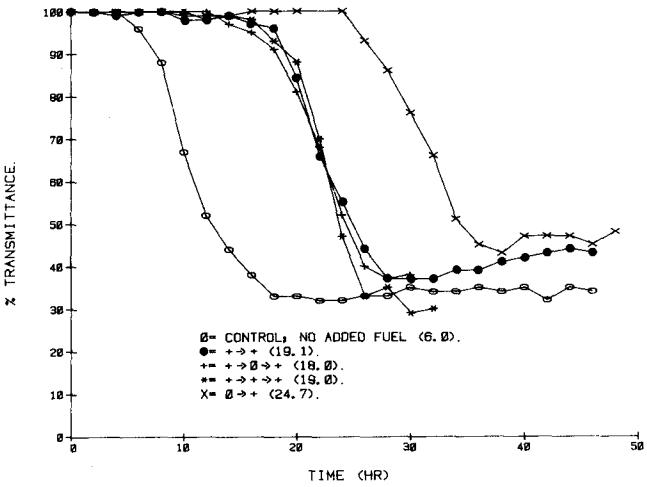


Fig 3. The effect of culture history on the growth response D-31 to 20 ppm monomethylhydrazine. Numbers in parenthesis are the ELT's of the growth curves.

Effect of Previous Exposure to 50 PPM UDMH on Growth of D-31 in 50 PPM UDMH: Growth of D-31 in SMS with a transfer history UDMH: + → + resulted in a 55% reduction in the ELT over that observed with the initial exposure culture (UDMH: 0 → +). A comparison of these growth curves is shown in Fig 4. The growth curve of a culture with the transfer history UDMH + → 0 → + is also shown in Fig 4. This culture had an ELT and growth curve that were identical to that observed with the prior-exposure culture (UDMH: + → +). However, the final growth yield was approximately equal to the control culture. Note the increase in final growth yield in both cultures with prior UDMH experience as compared with that of the initial exposure culture.

When D-31 is grown in SMS with 50 ppm UDMH for three consecutive growth periods (UDMH: + → + → +), the growth curve and ELT were the same as that observed with the prior exposure culture (UDMH: + → +; growth curve not presented).

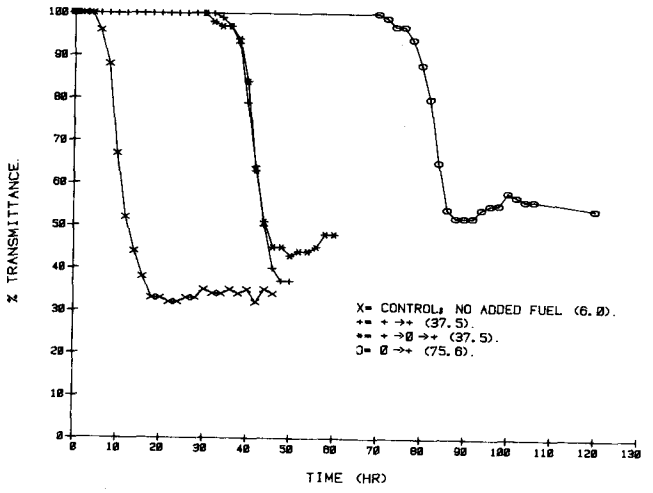


Fig 4. The effect of culture history on the growth response of D-31 to 50 ppm, 1,1-dimethylhydrazine. The numbers in parenthesis are the ELT's of the growth curves. Some readings at 100%T have been omitted for clarity.

DISCUSSION

Three factors determine the lag time in a culture under these experimental conditions. First, the inoculum concentration will influence the length of lag phase (SHIDA et al. 1974). When the number of organisms in the inoculum increases, the lag time decreases. Second, the source of the inoculum can affect the lag time, i.e. if the inoculum is from a log phase culture, the new culture may not exhibit a lag time, depending on growth conditions. However, if the inoculum is obtained from a lag or stationary phase culture, the new culture will display a lengthened lag time. Third, the method of detection of culture growth may not be sensitive enough to detect the changes in cell concentration that occur early in culture growth and therefore indicate a longer lag time than the actual lag time. For a more comprehensive review of the factors that influence lag time and growth kinetics, see HINSHELWOOD (1946). We suggest that the mechanism of adaptation of 10 ppm Hz is, in part, an inductive process, in which normally latent metabolic pathways or systems are expressed, with Hz as the inducer. These induced pathways bypass or counteract the inhibitory effects of Hz, allowing the organism to continue growing. When the inducer (Hz) was removed the gene no longer was expressed and the organism lost only a

portion of its resistance. However, when D-31 with prior Hz experience was grown for two growth periods in SMS without fuel, then transferred to SMS with Hz (Hz: + → 0 → 0 → +), the culture retained the same degree of resistance as the culture with the transfer history Hz: + → 0 → +. Thus, we suggested that the mechanism of adaptation to 10 ppm Hz also involves a selective process in which only the Hz resistant variants of the inoculum were able to grow. Once these variants were "selected out", they maintained their characteristic ELT when subsequently grown in 10 ppm Hz (Hz: + → 0 → 0 → 0 → 0 → 0 → +).

The mechanism of adaptation to 20 ppm MMH and 50 ppm UDMH appears to be selective only, rather than both selective and inductive. When D-31 with prior MMH or UDMH exposure was grown in SMS without added fuel and then transferred to SMS with fuel, the organism retained the initial level of resistance. Only the MMH and UDMH resistant variants were permitted to grow i.e., they were selected out. Thus, the increased lag time could be attributable to the relatively few resistant variants in the original inoculum, thereby extending the time before visible growth could be obtained. Another possibility is that the MMH and UDMH caused a mutation to resistant forms which were then selected out.

The three fuels may exert both bacteriostatic and bacteriocidal effects, as mentioned earlier, resulting in an extension in the ELT. This possibility and others are being examined further by studying the death rate of D-31 in a non-growing medium (SMS without glucose) with one of the three fuels present.

The increase in final growth yield when D-31 adapted to 20 ppm MMH and 50 ppm to UDMH (see Figs 3 and 4) could be important in understanding these adaptive mechanisms. Hz again appears to be different from MMH and UDMH by not affecting final yields. Several possible mechanisms to account for these observations are also currently being investigated.

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