GENETIC VARIATION AND ITS INFLUENCE ON PROTECTION BY L-CYSTEINE AGAINST GAMMA RADIATION

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Four closely related strains of *Escherichia coli* have been used in these studies. The effect on the survival of bacteria after treatment with different bacteriostatic and bactericidal agents viz: - radiation, H_2O_2, penicillin and heat was studied. An attempt has been made to correlate genetic constitution of the cells to H_2O_2 sensitivty, radiation response variability and protetion by the—SH containing amino acid. It has been shown that the surviving fraction of these strains if prviously treated with the protective agent shows a range in protecrion ratio depending upon the strain chosen, under similar experimental conditions.

Introduction

Probably the first publication on the effect of radiation on bacteria was published by Downes and Blunt in 1877. Minch in 1896 was the first worker to study the bactericidal effect of X-rays. Despite the fact that numerous workers made efforts in this field, our knowledge and understanding about the effect of irradiation on biological systems is still fragmentary. However, the advancement of bacteriological techniques and choice of suitable microorganisms for such experiments has, comparatively, given us rapid knowledge specially in the past few years.

In this connection, the problem of chemical protection against radiation injury is one of the most important problems of radiobiology today. Biologists are trying to get a satisfactory approach to this problem from different angles, and bacteria are one of the best tools used in experiments of this kind. Out of the long list of bacteria used in this connection *Escherichia coli* has been given preference, because much is known about the biochemistry of this organism together with its simple nutritional requirements. The best explored strains of this organism are B and B/r, originally isolated by Witkin[1] At the same time there are certain difficulties to handle these organisms for such studies, which are beyond the control of worker concerned e.g., radiation response variability of a cell population[2,3] alteration of sensitivity due to relatively minor changes in the experimental procedure,[4] physico-chemical environment of the cell,[3–9] phase state,[10] etc. Hence there is a wide range of data available on similar experiments by different workers using strains labelled as B and B/r.[2,3]

The present investigations were undertaken to find out whether or not probable genetic variations among closely related strains of the *Escherichia coli* species have some influence on a population's behaviour towards various microbiologically important bacteriostatic and bactericidal agents. In addition to this, special attention has been given to the question whether such a difference may influence the recovery of cells in the presence of the well known-SH containing protective agent, L-cysteine. It has been reported earlier by Stapleton[13] and others that this amino acid gives a dose reduction factor of 3.2 against X-rays. So far it has not been reported whether or not this DRF value is a "constant factor" for different bacteria and other cells.

Material and Methods

The organisms were maintained on nutrient agar slants (Difco). A loopfull of 18-hour grown culture from the slant was inoculated into 20 ml. of sterile peptone-water (1 percent) at pH 7.0. The culture was transferred to a shaking incubator at 37°C. Full growth at 20 hours, at this temperature was used for further studies.

The liquid culture was synchronized before full growth for all the experiments mentioned here. The synchronization method was adapted from the temperature-shock technique of Zeuthen.[11] After the final synchronization, the culture was examined microscopically for purity, synchronization, and clumping. If less than 90 percent of the cell population was non synchronized, the shock steps were repeated until at least 90 percent of the cells were in the same phase of growth as judged by microscopic observation. At this stage the optical density at 650 mμ on a DU Beckman Spectrophotometer Model 2400 ranged from 0.130–0.350 depending upon the strain chosen, but was fairly constant for a particular strain (±0.005).

The cells were centrifuged at 1000 xg. for 15 minutes at+2°C. in Spinco ultracentrifuge, washed thrice with 10 ml. of sterile distilled water and then resuspended in 5 ml. of sterile physiological saline. This stock culture was then diluted so as to give 400-500 cells per ml. The dilution was carried out in 9 ml. cold physiological saline

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blanks, the final dilution was made in 99 ml. saline blank in a conical flask. After dilution, the suspension was kept in the fridge until it maintained a temperature of 2-4°C. The cooled suspension was shaken well for 10 minutes with a magnetic stirrer. During stirring the temperature was not allowed to rise above +10°C. This was found necessary since a rapid lysis of cells occurred at higher temperature in such highly agitated condition.

From this well-shaken suspension, 4 ml. was transferred to a sterile pyrex tube (18×150 mm.) and 1 ml. of 0.01M L-cysteine solution in phosphate buffer at pH 6.8 (both seitz filtered) was added to the suspension just before irradiation. The final concentration of cysteine in the suspension thus being .002 M. The control contained 1 ml. of sterile buffer instead of cysteine.

The tubes were irradiated in a cobalt 60 γ-cell 220 (Canadian make). The radiation chamber (21×155 mm.) was capable of holding the samples at a constant height and distance from the source during irradiation when fitted with a specially constructed tube holder. The dose rate of γ-cell was 6.29×10^5 rads/hr as estimated by Fricke dosimetry.[23] (Cf. Miller)

After irradiation, 0.2 ml. aliquot of the irradiated suspension was transferred to a sterile petridish (95 mm. diameter), and after a constant interval of time (30 seconds) was pour plated by 15 ml. of melted plating medium at 45°C. (maintained in a water bath). The general formula used for keeping post-irradiation holding time constant was T=D−K+30 seconds where 'T'=total time elapsed between plating and total time of radiation; 'D'=time required to deliver a given dose and; 'K'=maximum radiation time required in a particular experiment. All the constituents of the plating medium were from Difco bacteriological grades.

Plating Medium.—Beef extract 0.3 percent; Peptone 0.5 percent; Glucose 0.5 percent; Bile Salt No. 3, 0.2 percent; Powdered agar 1.5 percent. pH 7.0±0.2

Addition of Bile Salt helps in inhibiting gram—positive contaminants, but has no noticeable effect on the survival of bacteria before or after treatment with the agents used. After solidification, the plates were transferred to a dark incubator at 37°C. in an inverted position. Colonies developed after 24 hours were taken as viable count. Practically no more colonies developed after 18 hours at this temperature.

Percentage of surviving fraction (with respect to a non-irradiated control) was calculated according to the formula:

$$\text{Number of colonies after a dose 'D'} = \frac{N_D}{N_O} \times 100$$

$$\text{Number of colonies in unirradiated control}$$

Mean Lethal Dose (MLD), LD_{50} and LD_{99} values were calculated from graphs and correspond to the 63, 50 and 99 percent inability of a cell population to form visible colonies under the above experimental conditions after the administration of a particular dose mentioned.

Protection ratio (PR) of cysteine was calculated from the equation $PR = \frac{N_P}{N_D}$ where N_P = number of colonies in the presence of protective agent and N_D=number of colonies in the unprotected control.

Dose Reduction Factor (DRF) was calculated as the ratio of γ-dose required to produce the indicated inactivation in the presence of cysteine to the dose required in the absence of the latter.

Results

Complete survival curves without cysteine for each strain are shown in Fig. 1.

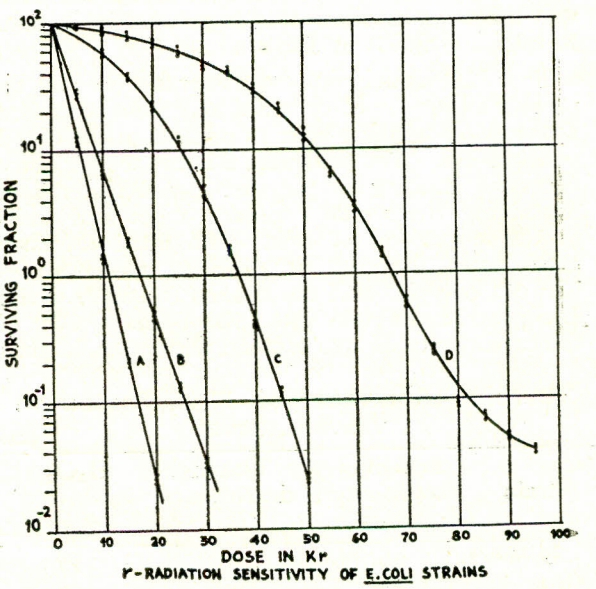


Fig. 1.—Showing relative sensitivity of *E. coli* strains to radiation. Dose in kiloroentgen on the abscissa; log percent surviving fraction on the ordinate. (Curve A strain B, Curve B strain 1899; Curve C strain B/r; Curve D strain 1157).

Dose reduction factor, protection ratio, MLD, LD_{50} and LD_{99} values have been shown in Table 1. The survival values were calculated from graphs (Fig. 2) showing survival with and without prior addition of L-cysteine. These values were experimentally confirmed within 5 percent personal error limits.

rod in a petri plate and immediately pour plated as usual. Complete absence of colonies after 24 hours at 37°C. was taken as Thermal Death Point (TDP) at the stated temperature (Table 2).

For hydrogen peroxide sensitivity tests one ml. aliquot of the suspension was kept shaken with 10

TABLE 1.—EFFECT OF L-CYSTEINE ON RADIATION SENSITIVITY OF *E. coli* STRAINS.

Strain	LD_{50} Kr		DRF value	MLD Kr		DRF value	LD_{4} Kr_{99}		LD_{99} Kr	PR Ranges
	a	b		a	b		a	b		
B	0.9	6.8	7.5	1.4	8.0	5.7	11.2	17.6	1.5	3.96–20.8
1899	1.25	7.75	6.2	2.2	9.75	4.4	17.5	27.0	1.5	2.88–19.1
B/r	8.0	29.5	3.6	10.8	35.0	3.2	37.7	77.0	2.1	2.3–109.25
1157	20.5	49.5	2.4	27.0	54.0	2.0	67.5	110.0	1.6	1.3–93.3

(a) Without prior addition of L-cysteine and (b) With prior addition of L-cysteine.

Penicillin sensitivity tests were carried out by serial dilution tube method. The suspension containing the known number of cells (usually 10^5/ml.) after incorporation of Seitz filtered penicillin 'G' units was incubated at 37 °C. for 24 hours. Visual absence of turbidity was confirmed by plating 1 ml. of aliquot of the suspension into nutrient agar (Difco) medium and optical density measurements in a DU Spectrophotometer. Absolute absence of colonies after incubination time was taken as measure of 100 percent inhibition. Results are shown in Table 2.

Thermal death point experiments were run by precision bore capillary tubes and were estimated as 100 percent killing after 15 minutes at the stated temperature. The cells were filled in the capillary tubes by reducing the pressure in a Buchner flask containing the sterile capillary tubes and well-shaken suspension of cells. The filled capillary tubes were dropped at different temperatures between 40-75°C. and were taken out with sterile forceps after different intervals of time. One of the capillary tubes was crushed with a sterile glass

TABLE 2.—SENSITIVITY OF *E. coli* STRAINS TO VARIOUS AGENTS.

Strain	Penicillin sensitivity units	Thermal death point °C.	H_2O_2 sensitivity %
B	46	58	27
B/r	62	65	52
1899	46	55	34
1157	82	60	58

ml. of H_2O_2 (0.6 percent total concentration) for 10 minutes. The initial concentration of cells was so adjusted to permit a thousand fold dilution before plating, to avoid the effect of residual H_2O_2 after plating. After 24-hour incubation, survival percentage was calculated by comparison with a non-treated control.

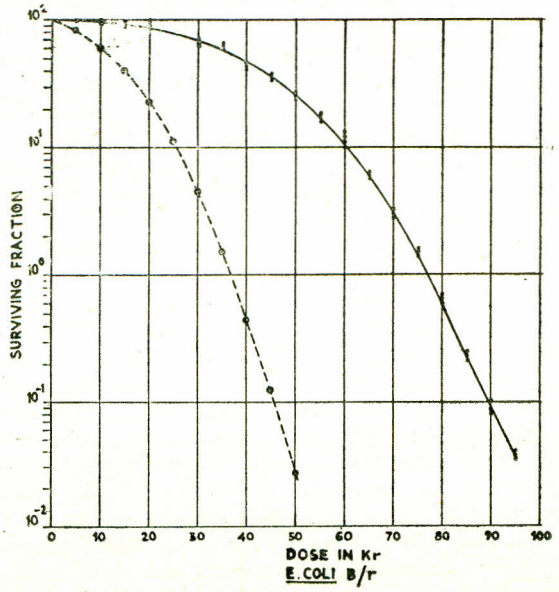
Discussion

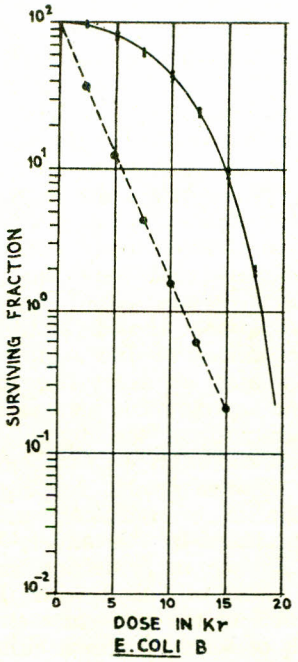
Results reported here indicate the importance of genetic makeup of a cell and its response to various agents deleterious to the *E. coli* strains. It will be noticed that there exist striking differences in the sensitivity to γ-radiation within the same species and under the same experimental conditions. As shown in Fig. 1 the sensitive strains show an exponential killing whereas the more resistant strains, i.e., B/r and 1157 show a sigmoidal curve. It has been shown [10,12,13] that sensitivity of *E. coli* to X-rays, can be varied to a great extent by inducing minor changes in the physiology of the cell. It has not been reported so far whether such a change in radiation response also influence protection afforded by various protective agents. Under our experimental conditions, the results clearly indicate that even without change in the environment or physiology of the cell, various degrees of protection can be achieved (Table 1).

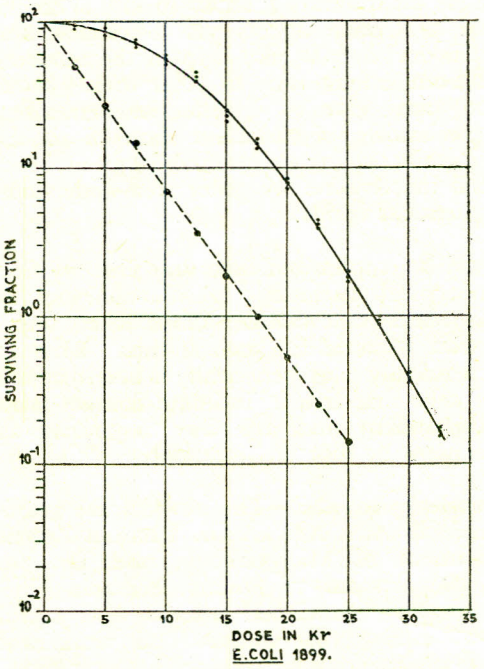
It had been suggested, for many sulfhydryl protective agents that protection afforded is mainly due to the capture of radiation-induced hydrogen peroxide radicals.[14] In such a case, however, cysteine protection should be independent of

strain chosen, so far as this '*indirect*' effect is concerned, since the same amount of H_2O_2 is to be expected at a particular dose under identical conditions.

Although there seems to be a good correlation between inactivation due to commercial H_2O_2







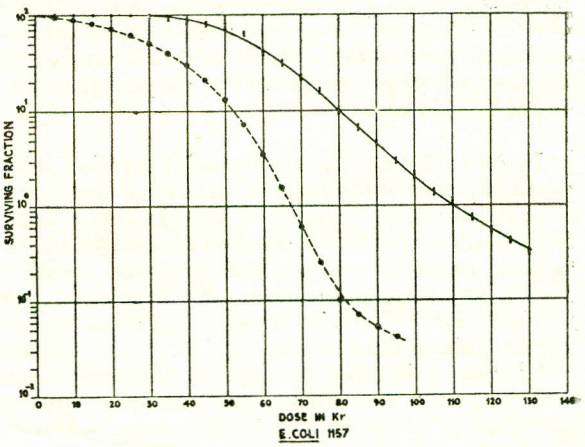


Fig. 2.—Showing L - cysteine protection at different doses.

Dose in kiloroentgen on the on the abscissa ; log percent surviving fraction on the ordinate.

•-----•-----• Without prior addition of L - cystein

•——•——• With prior addition of L - cysteine.

and radiation sensitivity of the cell, a similar relation is also seen towards penicillin sensitivity of the individual strains (Table 2). There seems to be no relation between sensitivity to the antibiotic, H_2O_2 or radiation and the temperature sensitivity of *E. coli*, possibly because of the wider difference in the mechanism of inactivation.

It will be seen (Fig. 1) that a multihit dose response is shown by strains 1157, and B/r. The shoulder of the curve is not formed for the strains B and 1899. The sensitive strains show the death rate peculiar to various bactericidal chemical agents. The cysteine protected and non-protected survival curves (Fig. 2) show that the latter forms a sigmoidal curve irrespective of the strain chosen. At the slope part of the curve, inactivation in the presence or absence of protective agent in the medium shows more or less an exponential killing. As we proceed to the end or toe part of the curve protection ratio varies more significantly, depending upon the general sensitivity of the strain under investigation. Hence, strain B which is most sensitive among the four strains shows a very low recovery after 15 Kr. The more resistant varieties show a progressive increase in protection ratio depending upon the overall resistance of the cell in question. If we examine the possible explanation of these results, keeping in view the basic mechanism of radiation protection and damage as proposed by Alexander,[15] it is possible that the difference in protection ratio obtained at different doses is linked with the degree of damage caused to the *pace-maker* enzyme system in the words of Krebs,[16] which either alters the course of chain reaction or forms toxic products deleterious to the cell. It is obvious then that the chemical production can be afforded only if the effect of absorbed energy on key enzymatic systems could be minimized.[15]

In other words, the degree of protection is dependent upon the extent of damage done due to irradiation and its repair by the protective agent. Sensitivity or resistance of a living cell should thus depend upon the natural shielding and ability to repair the damage caused. It is evident from our data that the strains B and 1899 show a lesser repair ability than strains B/r or 1157. Strain 1157, which is most resistant of the four strains chosen, seems to compete the radiation damage particularly at lower and higher doses. At relatively lower doses it shows a *threshold* dose response and at the toe part the repaprocess ris eems to be much more efficient than any of the other sensitive strains.

The importance of indirect effect of radiation due to inorganic and organic peroxides has been demonstrated in the catalase-negative *E. coli* H_7 strain[17] and other biologically important enzymes and chemical compounds.[18–20]. The present data on the effect of H_2O_2 on *E. coli* cells show fair agreement between a strain's sensitivity to γ-radiation and commercial H_2O_2. It will be noticed that about 1000-fold more H2O2 concentration is required to cause a measurable loss in viability than is normally produced by ordinary doses of radiation.[21] It was suggested,[17] therefore, that radiation plays at least three important roles for the *E. coli* H_7 strain: (a) It produces *prompt* inactivation, (b) It sensitises the cell to hydrogen peroxide (probably other agents), and (c) It produces hydrogen peroxide to which cells have been sensitised. This indirect effect has also been demonstrated in irradiated buffer not containing the *E. coli* cells. The buffer remained *toxic* for this strain even after three hours of irradiation. The importance of this radiation induced H_2O_2 has been demonstrated in almost all the living cells except seeds where virtual absence of water makes it highly improbable. Experiments done with virus and bacteriophages in this connection, show a dramatic desensitization upon dehydration.[18,22]

It will be an interesting study to find if multifunctional protective agents viz L-cysteine and other sulfhydryl agents may help in the recovery of a cell from damage due to other toxic agents. This study may give us a better understanding of the mechanism of radiation damage to the living cell while tracing the common steps involved in the cell inactivation by other well-understood physico-chemical agents.

It should be emphasized here that phenotypical and genotypical variations plus techniques of choice are important considerations when one is dealing with bacteria for such studies. Bacteria are biochemically and genetically a heterogenous group of cells that live in intimate contact with their environment and are very responsive to minor changes in that environment.

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from Becco Chemical Co. (50 percent saturation) and L-cysteine from L. Light and Co.

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