Response of γ-Irradiated Bacteria to Nutritional Substances Supplemented to the Postirradiation Culture Medium

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The response of γ-irradiated cells of bacteria to nutritional substances supplemented to the postirradiation plating medium considerably differed with species (or strains); the increase of the survival by the addition of nutritional substances was greater in the bacteria capable of growing without any growth factor (*e.g.*, *Escherichia coli* and *Serratia marcescens*) as compared with the organisms which require a variety of organic substances for the growth (*e.g.*, *Proteus morganii* and, particulary, spores of *Bacillus subtilis*). On the basis of these findings, the relationship between the processes involved in the "recovery" of bacterial cells inactivated by γ-radiation and the biosynthetic systems contained in the cells of each species (or strain) was discussed.

The evidence was also obtained with *Escherichia coli* and *Serratia marcescens* which indicated that the most of the irradiated cells of these organisms showing the induced nutritional requirements were not stable auxotrophic mutants.

INTRODUCTION

Several investigations have been reported which dealt with the effects of postirradiation culture media on the survival of microorganisms irradiated with ionizing radiations (*e.g.*, Stapleton *et al.* (1955), (1956)[1,2] made an extensive study on the "recovery" of bacteria from ionizing radiations by using *Escherichia coli*, and demonstrated that the ability of irradiated cells to form colonies was markedly influenced by the composition of postirradiation plating medium). Most of these investigations, however, have been made with *Escherichia coli*, which appears to be atypical one as a food microorganism, and little is still known about this problem with common microorganisms responsible for the spoilage of foods.

To extend such investigations to other species and strains of microorganisms, therefore, seems important not only from the radiobiological point of view but also from the viewpoint of food technology.

The work reported in the present paper was undertaken to elucidate the mechanism of this effect in a wide variety of bacteria including representative species of food microorganisms, and is primarily concerned with the response of γ-irradiated cells of *Bacillus subtilis*, *Proteus morganii*, *Serratia marcescens*, *Escherichia coli*, and an auxotrophic mutant of *Escherichia coli* to several nutritional substances added to the postirradiation media.

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MATERIALS AND METHODS

Organisms and Media

Bacillus subtilis (ATCC 6051)[3], *Proteus morganii* (NCTC 235), *Serratia marcescens* (AHU), two stains of *Escherichia coli* (IID ; 0-20[4]) and a vitamin B_{12}- and methionine-requiring strain of *Escherichia coli* (#215)[4] were used as test organisms. The last strain is an UV-induced mutant of *Escherichia coli* (0-20).

Stock cultures of these organisms were routinely maintained on slopes of broth agar media, and cells for irradiation studies were grown on nutrient broth which contained 5 g/L peptone, 5 g/L beef extract, and 1 g/L NaCl.

As basal media for the culture of cells after irradiation, minimum synthetic solid media which allowed each cell of non-irradiated test organisms plated to form a visible colony were used. These madia were used with and without added nutritional supplements. Nutritional substances employed as supplements were peptone (Daigo-eiyô-kagaku), beef extract (Kyokutô-seiyaku),

Basal medium for spores of *Bacillus subtilis*[3]

DL-Alanine	1780 mg
L-Glutamic acid	1470 mg
DL-Asparagine	2640 mg
Glucose	10 g
Mineral salts mixture*	100 ml
Agar	15 g
Distilled water	1000 ml
pH	7.2

* This contained the following amounts per L : K_2HPO_4, 30 g; KH_2PO_4, 10 g; NH_4Cl, 5 g; NH_4NO_3, 1 g; Na_2SO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 100 mg; $MnSO_4 \cdot 4H_2O$, 10 mg; $FeSO_4 \cdot 7H_2O$, 10 mg; $CaCl_2$, 5 mg; pH adjusted to 6.8-7.0[9].

Basal medium for *Proteus morganii*[5]

NaCl	2500 mg
K_2HPO_4	1000 mg
Na-glutamate	500 mg
DL-Asparagine	500 mg
DL-Methionine	50 mg
Nicotinic acid	20 mg
Ca-panthotenate	2 μg
Glucose	5 g
Agar	10 g
Distilled water	1000 ml
pH	7.2

Basal medium for *Serratia marcescens*[6]

$(NH_4)_2SO_4$	500 mg
NH_4Cl	500 mg
$MgSO_4 \cdot 7H_2O$	500 mg
KH_2PO_4	500 mg
Glucose	5 g
Agar	10 g
Distilled water	1000 ml
pH	7.0

Basal medium for *Escherichia coli* (IID; 0-20)[1]

KH_2PO_4	1 g
$(NH_4)_2HPO_4$	4 g
$MgSO_4 \cdot 7H_2O$	700 mg
Na-citrate·$11/2H_2O$	500 mg
Glucose	10 g
Agar	15 g
Distilled water	1000 ml
pH	6.8

Basal medium for *Escherichia coli* (#215)[1,4]

KH_2PO_4	1 g
$(NH_4)_2HPO_4$	4 g
$MgSO_4 \cdot 7H_2O$	700 mg
Na-citrate·$11/2H_2O$	500 mg
DL-Methionine	30 mg
Vitamin B_{12}	0.1 μg
Glucose	10 g
Agar	15 g
Distilled water	1000 ml
pH	6.8

yeast extract (Difco), and vitamin-free casamino acids (Difco). The composition of the basal medium for each organism employed was as described above respectively.

Preparation of Cells for Irradiation

In the cases of *Proteus morganii*, *Serratia marcescens*, and *Escherichia coli*, nutrient broth media inoculated were incubated without aeration at 37°C. After 24 hours' incubation these cultures were centrifuged and washed three times in M/15-phosphate buffer (pH 7.0) and finally resuspended in the buffer. In the case of spores of *Bacillus subtilis*, aerobic culture bottles containing nutrient broth agar were inoculated with a small amount of pre-culture and incubated for 4 days at 37°C. After the incubation under this condition spores were formed in most of the cells. Then the growth from each culture bottle was harvested with M/15-phosphate buffer (pH 7.0) using glass beads. After pasteurized for 20 minutes at 80°C, these cell suspensions were centrifuged, washed and resuspended as described above.

In any case the suspensions were filtered through filter paper (Toyo #2) to remove clumps of cells and then vigorously aerated at ice bath temperature for 20 minutes to maintain the oxygen concentration in the suspensions in constant level. Immediately after aeration the suspensions were poured into glass tubes (0.8 mm in thickness, and 10 mm in diameter) plugged with rubber stoppers.

These suspensions were then exposed for a given period of time to γ-rays in a uniform radiation field of the γ-ray irradiation facility equipped with 37 rod-shaped ^{60}Co (about 1,940 C in total)[10]. Radiation dose rates were constant throughout the experiments (216 kr/hour).

Plate Culture After Irradiation

Comparisons between different media were made by dispensing samples of

appropriate dilution from the same irradiated or control suspensions into sterilized petridishes with the basal or the supplemented medium and by incubating these plates at 37°C. Dilution was made to give a count of 100-200 colonies per plate. For counting *Bacillus subtilis* the three layer "sandwich" plate method described by Demain (1958)[3] was employed.

To express the activity of nutrient supplements the value "relative survival"[1] was used.

RESULTS AND DISCUSSION

Effects of Peptone and Beef Extract on the Growth of Irradiated Cells of *Escherichia coli*, *Serratia marcescens*, *Proteus morganii*, and of Irradiated Spores of *Bacillus subtilis*

Comparisons of the relative survivals of irradiated cells of *Escherichia coli*, *Serratia marcescens*, *Proteus morganii*, and of irradiated spores of *Bacillus subtilis* as functions of concentrations of peptone and beef extract in the post-irradiation plating media were illustrated in Figures 1 and 2. Among these organisms, spores of *Bacillus subtilis* were exposed to 1,200 kr and the others were exposed to 40 kr.

In the cases of *Escherichia coli*, *Serratia marcescens* and *Proteus morganii*, both peptone and beef extract supplemented to the postirradiation plating medium had, in general, a remarkable influence on the number of cells surviving γ irradiation ; the survivals were more or less greater on the supplemented media containing these nutritional substances than on the basal media, although the latter media were not limiting for non-irradiated cells of these organisms. These data suggest that peptone and beef extract supply, to a cetain extent, the

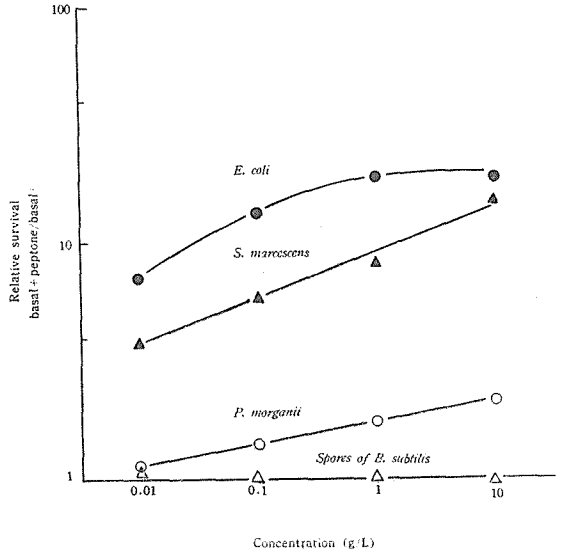

Fig. 1. Effect of peptone on survival of *Escherichia coli* (40 kr), *Proteus morganii* (40 kr), *Serratia marcescens* (40 kr) and spores of *Bacillus subtilis* (1200 kr).

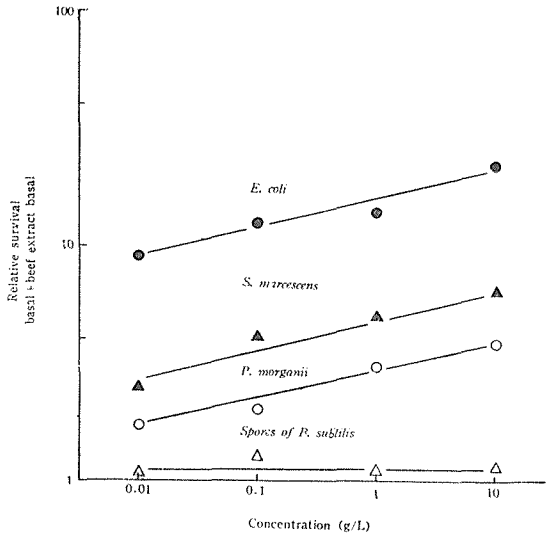

Fig. 2. Effect of beef extract on survival of *Escherichia coli* (40 kr), *Proteus morganii* (40 kr), *Serratia marcescens* (40 kr) and spores of *Bacillus subtilis* (1200 kr).

radiation-induced nutritional requirements for irradiated cells of these organisms. In the case of *Bacillus subtilis*, the addition of both peptone and beef extract failed to increase the survival on the postirradiation plating medium.

Relative survivals of irradiated cells of the non-spore-forming bacteria employed, with the exception of *Escherichia coli* grown on the medium containing peptone, increased with the concentrations of nutrient supplements in the post-irradiation plating media at concentration below 10 g/L. Irradiated cells of *Escherichia coli* grown on the peptone medium, on the other hand, did not show the increase of relative survival with the concentration of this supplement at the concentrations of higher than 1 g/L. The last mentioned phenomenon may partly be due to the action of a certain inhibiting factor associated with peptone, such as "peptone factor" which has been reported by Alper and Gillies (1958)[7].

The results obtained in this experiment also indicated that the response of the irradiated cells of bacteria to peptone or beef extract supplemented to the postirradiation plating media differed with species ; the increase of the survival by the addition of the nutritional substances was greater in the bacteria capable of growing without any growth factor (*e.g.*, *Escherichia coli* and *Serratia marcescens*) as compared with the organisms which require a variety of organic substances for their growth (*e.g.*, *Proteus morganii* and spores of *Bacillus mesentericus*). To ascertain whether irradiated vegetative cells of *Bacillus subtilis* show the similar behaviors, in this respect, to its irradiated spores is obviously of great importance in this comparative study of "chemical recovery" of bacteria ; further experiments concerning the response of irradiated vegetative cells of this organism to nutritional substances are now continued.

Response of Irradiated Cells of *Escherichia coli* to Yeast Extract and Vitamin-Free Casamino Acids Added to the Postirradiation Plating Media

Figure 3 represents data obtained in which the plate counts of irradiated

cells of *Escherichia coli* (40 kr) were performed on the basal media supplemented with yeast extract or vitamin-free casamino acids in various concentrations.

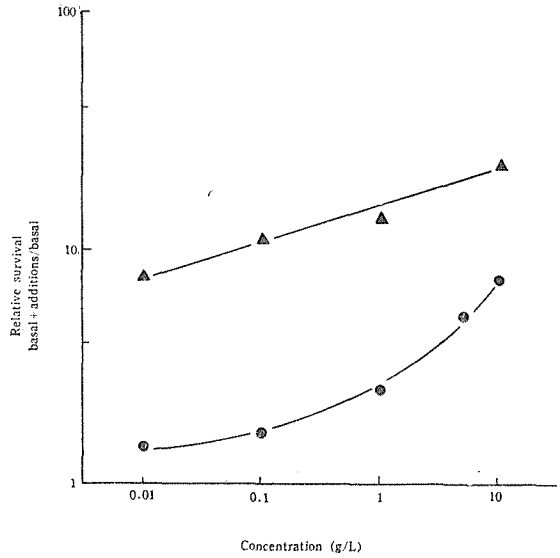

Fig. 3. Effect of yeast extract and casamino acids on survival of *Escherichia coli* (40 kr). ▲=yeast extract ; ●=casamino acids

These data revealed that the slope of the relative survival curve plotted as a function of the concentration of yeast extract closely resembled to those with peptone and yeast extract as were illustrated in Figures 1 and 2, and that the slope of the relative survival curve with casamino acids was different from those with peptone and beef extract. The activity of casamino acids for the recovery of irradiated cells of *Escherichia coli* was considerably lower than that of the other substances tested at concentrations below 0.1 g/L, although the former substance was never less active than the other substances at the concentrations higher than 1 g/L. This distinctive feature of the slope of the relative survival curve with casamino acids is probably associated with the multiplicity of the active substances contained in casamino acids.

Comparison of the activities of various nutrient substances for the "recovery" of irradiated cells of *Escherichia coli* was summarized in Table 1.

Table 1. Effect of various nutrient substances on survival of *Escherichia coli* (IID) (40 kr).

Concentration of additions (g/L)	Relative survival (basal+additions/basal)			
	Peptone	Beef extract	Yeast extract	Casamino acids
1.0	18.7	14.1	13.5	2.5
0.1	13.4	12.7	11.0	1.7

Effects of Peptone and Beef Extract on the Growth of Irradiated Cells of an Auxotrophic Mutant and Its Parent Strain of *Escherichia coli*

The response of a vitamin B_{12} and methionine requiring mutant, and its parent strain of *Escherichia coli* to peptone and beef extract added, in different concentrations, to the postirradiation plating media was shown in Tables 2 and 3.

Table 2. Effect of peptone on survival of *Escherichia coli* 0-20 and its auxotrophic mutant *Escherichia coli* #215* (50 kr).

Concentration of peptone (g/L)	Relative survival (basal+peptone/basal)	
	E. coli 0-20	*E. coli* #215
1.0	13.2×10^2	5.6×10^2
0.1	3.9×10^2	2.1×10^2

* This strain requires methionine and vitamin B_{12} for the growth.

Table 3. Effect of beef extract on survival of *Escherichia coli* 0-20 and *Escherichia coli* #215* (50 kr).

Concentration of beef extract (g/L)	Relative survival (basal+beef extract/basal)	
	E. coli 0-20	E. *coli* #215
1.0	13.6×10^2	3.6×10^2
0.1	6.7×10^2	0.6×10^2

* This requires methionine and vitamin B_{12} for the growth.

The data illustrated in these tables indicated that the irradiated cells of the wild strain gave higher relative survival than those of the auxotrophic mutant at all of the concentrations of supplements tested. The difference observed between the mutant and the wild strain in the response to added nutritional substances was almost similar to the difference between the species having different nutritional requirements, and may be due to the difference in the nutritional requirements between both strains ; irradiated cells of the strain having simple nutritional requirements were "recovered" more easily from the radiation damage than those of the strain which required additional growth factors besides the substances required by the former. It is also suggested from these data that vitamin B_{12} and methionine which are expected to be contained in peptone and beef extract may play a certain role in the "recovery" of irradiated cells of *Escherichia coli* by these nutritional supplements.

The relative survivals obtained in this experiment were always higher than those obtained with *Escherichia coli* IID (Figures 1, 2, 3 and Table 1). This may be attributable to the difference between the strains employed.

Effect of Postirradiation Temperature on the Survival of Irradiated Cells of *Escherichia coli* on the Basal and the Supplemented Media

Surviving fractions of irradiated cells of *Escherichia coli* IID as a function

of the postirradiation holding temperature on the basal media with and without added beef extract were compared. The data obtained were illustrated in Figure 4.

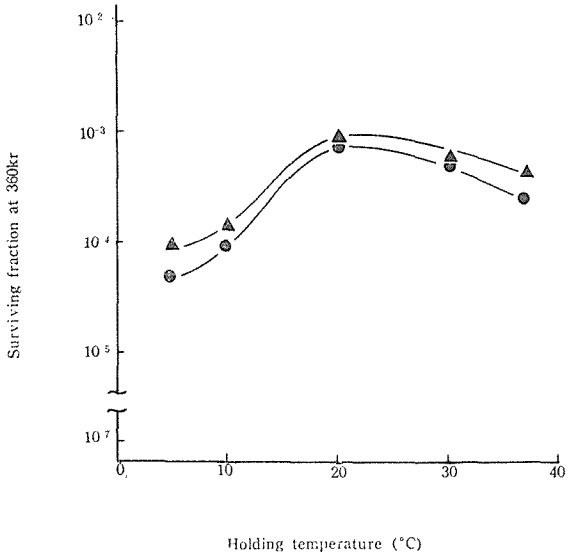

Fig. 4. Comparison of survival of *Escherichia coli* on basal medium and basal medium plus beef extract as a function of postirradiation holding temperature. (24 hrs)
▲=basal medium+beef extract (10g/L) ●=basal medium

Although the maximum value of survival was obtained on either medium at 20°C as was reported previously[8], the survival of irradiated cells was greater at the all postirradiation temperatures employed on the supplemented medium than on the basal medium without supplement. These data accorded fairly well with those reported by Stapleton *et al.* (1955)[1] with *Escherichia coli* strain B/r on synthetic media with and without added yeast extract.

It is assumed that the recovery of irradiated cells by the treatment at suboptimal temperature after irradiation was accelerated by active substance(s) contained in beef extract.

Possibility of the Occurrence of Auxotrophic Mutants by γ Irradiation

Since it seemed important in performing the present study to ascertain whether the occurrence of biochemical mutants induced by γ irradiation might be responsible for the differences between viable counts on basal media and those on supplemented media after irradiation, radiation-induced changes in nutritional requirements were examined with *Escherichia coli* IID and *Serratia marcescens* by the replica plating technique after Lederberg and Lederberg (1952)[9].

Replica plating procedure

Plates of complete media (basal media plus peptone) inoculated with irradiated cell suspensions (diluted to give a count of 100-200 colonies per plate) *Escherichia coli* IID and *Proteus morganii* (30-40 kr) were incubated for 24 hours at 37°C. Colonies which developed on the surface of the plates (initial plates) were transferred by replica plating

to the plates (replica) of minimum media (basal media) and also to the plates (second replica) of complete media (basal media plus peptone). After incubated at 37°C the situation and the number of colonies which developed on the surface of the both of these plates were compared with each other.

After repeated several times with both *Escherichia coli* and *Serratia marcescens*, these experiments gave the results which indicated that the number of colonies which developed on the reprica plates of basal media accorded exactly with those on the second replica plates of complete media; every colony on the plates of complete media (initial plates) was reproduced on the plates of basal media.

This result revealed that the most of the irradiated cells which showed the induced nutritional requirements were not stable auxotrophic mutants*.

In view of the results obtained by the above described experiments, it is suggested that the process involved in the "recovery" of bacterial cells inactivated by γ irradiation is closely related to the biosynthetic reactions in the cells. The difference between species (or strains) in the rate of "recovery" appears to be due to the nature of the biosynthetic systems contained in the cells of each species (or strains).

These results also suggest that the response of food microorganisms to postirradiation conditions should be studied more widely before the radiation sterilization procedure is applied to foods practically.

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REFERENCES

(1) G. E. Stapleton, A. J. Sbarra and A. Hollaender, *J. Bact.*, **70**, 7 (1955).
(2) A. Hollaender and G. E. Stapleton, "CIBA Foundation Symposium on Ionizing Radiations and Cell Metabolism", J. and A. Churchill Ltd., London, 120-130 (1956).
(3) A. L. Demain, *J. Bact.*, **75**, 517 (1958).
(4) R. Hayashi, H. Nakayama and H. Ikeda, *Bact. Japan*, **10**, 749 (1955).
(5) S. Matsui and E. Tomikawa, *Bact. Japan*, **10**, 459 (1955).
(6) W. J. Payne, *J. Bact.*, **72**, 834 (1956).
(7) T. Alper and N. E. Gillies, *J. gen. Microbiol.*, **18**, 461 (1958).
(8) H. Kadota, H. Miyoshi and H. Shibata, *B. J. S. S. F.*, **24** 1001 (1959).
(9) J. Lederberg and F. M. Lederberg, *J. Bact.*, **63**, 399 (1952).
(10) S. Shimizu, S. Tanaka and Y. Nakayama, This Bulletin, 37, 306 (1959).

* Although auxotrophic mutants must have occurred by the γ irradiation, the rate of occurrence of such mutants was too low to affect the surviving fractions obtained in the present and the preceding experiments[8].