SOME NUTRITIONAL ASPECTS OF BACTERIAL RECOVERY FROM IONIZING RADIATIONS[1]

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Partial reversal of damaging effects of ionizing radiations has been demonstrated by use of several techniques on a variety of types of living cells. Gray (1951), Allen *et al.* (1951), and others have reviewed the available literature concerning the nature of the recovery process. Henshaw (1940), Lamarque and Gros (1945), and others have presented evidence for the involvement of metabolism in repair processes leading to recovery. Stapleton *et al.* (1953) showed that the bacterium *Escherichia coli* strain B/r, cultured aerobically in nutrient broth and plated after X or γ irradiation on the same medium, gave maximal survival when incubated at a temperature well below that which is optimal for growth of this organism. The last authors suggested that the survival of irradiated *E. coli* at any temperature may be determined by the relative rates of synthetic and destructive processes in these cells. Since the radiation apparently induces an alteration in the optimal temperature for colony formation in these organisms, it seemed important to ascertain if the irradiated cell might also become more fastidious in its nutritional requirements. Since unirradiated *E. coli* can grow equally well on a simple synthetic medium composed of inorganic salts with a carbon source such as glucose and on natural materials such as nutrient broth, it was decided to investigate the comparative viability of irradiated bacteria plated on synthetic media of several types and on a complete medium such as nutrient agar or yeast extract agar. Cells cultured on nutrient broth gave a much smaller surviving fraction when plated on a simple synthetic medium than those plated on a complete medium. Moreover, additions of known organic compounds to a synthetic medium were conducive to recovery.

MATERIALS AND METHODS

Washed cell suspensions of *E. coli* strain B/r were used. For most of the work to be reported, the cells were grown in nutrient broth (Difco) under constant aeration at 37 C for 20 hours. The preparation of the samples for irradiation was essentially the same as reported by Stapleton *et al.* (1953). All suspensions were prepared in M/15 phosphate buffer (pH 6.8) and irradiated in oxygen saturated suspensions at ice-bath temperature. In some experiments other growth media were used as described in the text, but in all cases the treatments prior to irradiation were identical. Aliquots of the irradiated and control suspensions were surface plated by spreading on the various solid media.

RESULTS

Effect of temperature on survival of synthetic and complete media. Normal (unirradiated) *E. coli* strain B/r can form colonies almost equally well on medium I (table 1) as on a complete medium such as nutrient agar or yeast extract. Very little difference is noted in either size or number of colonies at the end of a 24 hour incubation period. X- and γ-irradiated cells, on the other hand, show a markedly decreased ability to express themselves on the synthetic medium (medium I) as compared to complete media (nutrient agar or medium I plus yeast extract). A significant difference in size and number of colonies developing on the two media is always noted after 24 hours' incubation. Moreover, incubation of those cells on medium I for up to 72 hours does not result in any increase in number of viable cells as measured by colony count. A comparison of the surviving fraction of irradiated cells as a function of the postirradiation incubation temperature on medium I with and without added yeast extract (20 mg/ml) is illustrated in figure 1. Although maximal survival was obtained on either medium at about 18 C, as previously reported (Stapleton *et al.*, 1953), the number of colony forming organisms is greater at all temperatures on the supplemented medium.

The obvious question arises as to whether the yeast extract is merely satisfying the require-

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TABLE 1

Composition of basal medium (medium I)

Components	Amount
KH_2PO_4	1 g
$(NH_4)_2HPO_4$	4 g
$MgSO_4$	0.7 g
Na citrate	0.5 g
Glucose	10 g
Distilled H_2O	1,000 ml
pH 6.8	

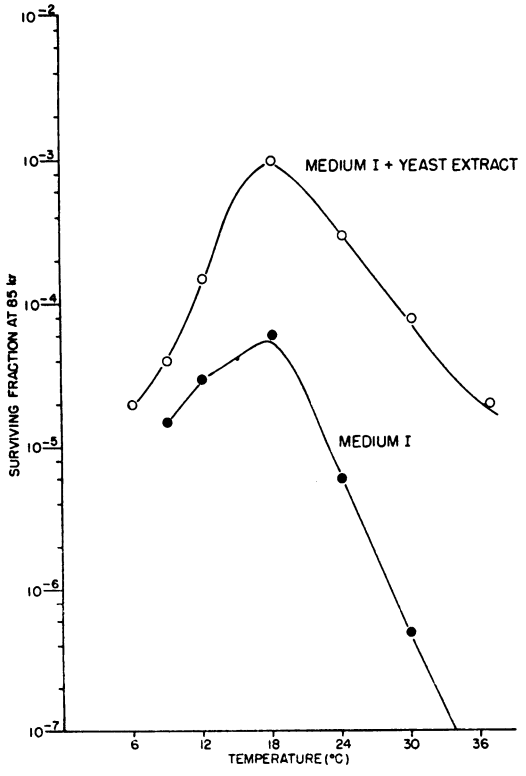

Figure 1. Comparison of viability on medium I and medium I plus yeast extract as a function of postirradiation incubation temperature.

ments for a collection of radiation induced auxotrophs. The evidence accumulated seems to indicate that this is not the solution to the problem of increased survival on the supplemented medium. Holding the irradiated suspension in the presence of added yeast extract at 37 C for a period as short as 30 minutes was sufficient to allow the cells to form equal numbers of colonies on nonsupplemented and supplemented media. Survival on unsupplemented medium I at 37 C was reduced to a few cells per milliliter by large doses of X or γ rays. A hundredfold more survivors were found on the supplemented medium I. Several hundred of the latter colonies were isolated and replated on both media, good growth being obtained for all isolates. These results would not be expected if stable auxotrophs were selected. On the basis of these experiments, we have assumed as a working hypothesis that some factor(s) in yeast extract may actually stimulate repair mechanisms in the irradiated cells. It is well to indicate that a relatively small fraction of the irradiated population shows this repair process. A plot of survival as a function of X-ray dose under the extreme conditions, i. e., on medium I at 37 C and on medium I plus yeast extract at 18 C, is shown in figure 2.

Assay system and attempted purification of recovery factors. Figure 1 shows that the difference in the surviving fraction of cells on medium I and medium I plus yeast extract becomes increasingly large at incubation temperatures above 18 C. To take advantage of the large difference at 37 C, a simple plating technique was devised

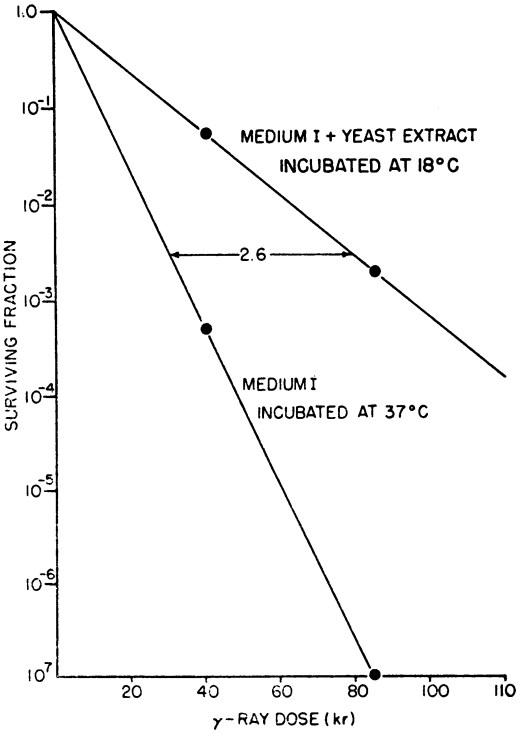

Figure 2. Surviving fraction of *Escherichia coli* B/r as a function of γ-ray dose.

which involved addition of known materials or isolated fractions from yeast extract or other extracts to medium I on plates. The ratio of the number of survivors, after a constant dose of X or γ rays, on medium I with and without additions, would be a measure of the activity of the added material. The surviving fraction on yeast extract is used as a standard set at 100. In routine assays, 0.05 ml aliquots of an irradiated suspension were surface plated by spreading on previously prepared solid medium I with and without additions. The effect of addition of various concentrations of yeast extract to medium I is shown in figure 3. The relative activity of unknown materials was determined by comparison with such a standard curve or with the activity obtained at the plateau level of this curve, about 20 mg of yeast extract.

A number of biological preparations were assayed as possible sources of recovery factor; among them were casein hydrolyzate (acid hydrolyzed, Nutritional Biochemical Co.), Casamino acids (Difco), N. Z. Case (Sheffield), beef extract (Difco), liver extract (Difco), peptone (Difco), Basamin (Anheuser-Busch), and several extracted liver fractions supplied by Armour and Company.[2] Although all of these preparations contained measurable activity, none surpassed yeast extract in activity per unit dry weight. Acid and alkaline hydrolysis of yeast extract resulted in little if any change in the activity. Autoclaving had no effect on activity as measured by our assay system. Heavy metal precipitation resulted in some loss of activity, but no measurable activity was found in the precipitate after removal of the heavy metal ions. The activity of yeast extract could be completely adsorbed on charcoal (Norit A) and could be eluted with ammoniacal ethanol at pH 8–10 with a resulting fivefold purification. The activity was found to be insoluble in fat solvents but could be purified about tenfold with step-wise precipitation of less soluble materials up through 90 per cent ethanol.

In a search for other sources of recovery factor, homogenates of several tissues of the rabbit were prepared in a glass homogenizer. Hot water extracts of the homogenates were made and assayed for activity; assays of the residues indicated that essentially all the activity was contained in the aqueous extract. The relative activities of extracts of the various tissues are illustrated in figure 4. It is clear from these limited data that spleen extracts contain more active material per unit dry weight than the other tissues assayed. Hematopoietic tissues other than spleen were not assayed. These results seemed sufficiently interesting to warrant further attempt at purification of the factors necessary for bacterial recovery. Fresh-frozen calf spleens were homogenized, and the hot water extracts prepared and assayed. As shown in figure 5, crude spleen extracts contained about three times as much activity as yeast extract per unit dry weight. The relative activities of the 90 per cent ethanol soluble and insoluble fractions are shown for comparison. Again, an additional tenfold purification was obtained, as was previously shown for yeast extract, when the spleen extract was extracted with a graded series of ethanol concentrations of 50–90 per cent. The 90 per cent ethanol soluble material was precipitated by addition of large volumes of cold acetone and dried under vacuum. This dried preparation retained its activity for several months. On the other hand, removal of ethanol from these extracts, followed by deep freezing at -20 C, resulted in extreme loss of activity within a period of 10–14 days.

Paper partition chromatography of the partially purified spleen extract was carried out with a descending solvent system of *n*-butanol-propionic acid-water. Samples of the dried material were dissolved in water and applied to large sheets of Whatman no. 1 paper (18½ by 22½ in), either in a thin line or in individual spots about one-half inch apart. The chromatograms were developed and, after drying, were cut into sections 1 cm wide corresponding to the R_f values of 0.0–1.0. Substances on these sections were eluted directly into tubes of hot basal medium (medium I) and assayed for activity as previously described. These assays revealed the presence of several components which, when assayed in various combinations, acted synergistically in promoting recovery of irradiated *E. coli*. Since a number of amino acids could be identified from the chromatograms, a mixture of pure amino acids (Nutritional Biochemical Corporation), corresponding to those identifiable

[2] The authors wish to thank Dr. J. B. Lesch of the Biochemistry section of the Armour Laboratories, Chicago, Illinois, for supplying the liver fractions used in these experiments.

on the chromatograms, was prepared and assayed. The amino acids were also assayed individually and in all possible combinations. A combination of four of them—glutamic acid, methionine, serine, and tryptophan—was as active as the whole group and as active as casein hydrolyzate. The activity of the amino acids was, however, less than 20 per cent of that obtainable with yeast or spleen extract. Other components of the partially purified spleen extracts were not identified, but active fractions always contained detectable amounts of material which showed absorption in the ultraviolet region, being maximal at about 260 mμ.

Recovery in chemically defined media. Simultaneously with the experiments on purification of recovery factors from spleen extracts, attempts were made to construct a chemically defined

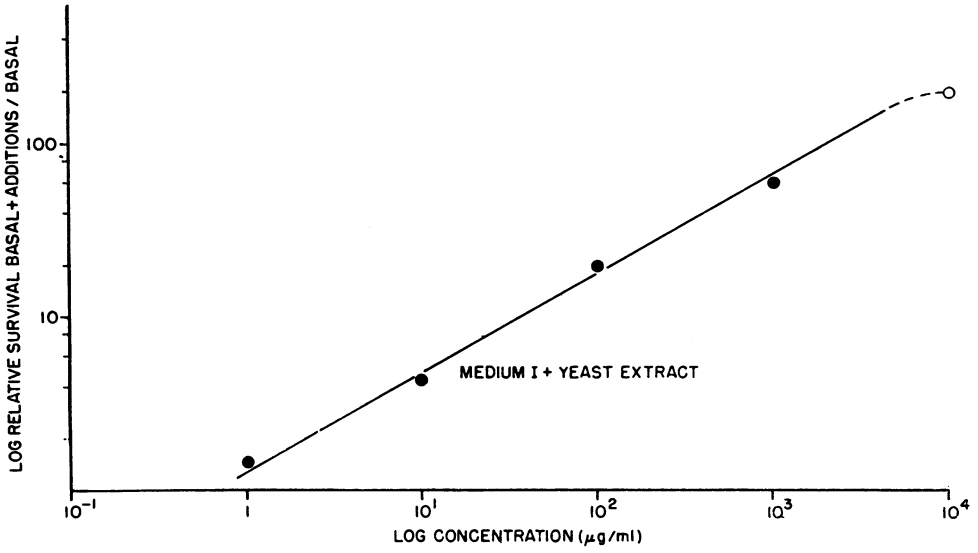

Figure 3. Activity of yeast extract; plate assays with incubation at 37 C.

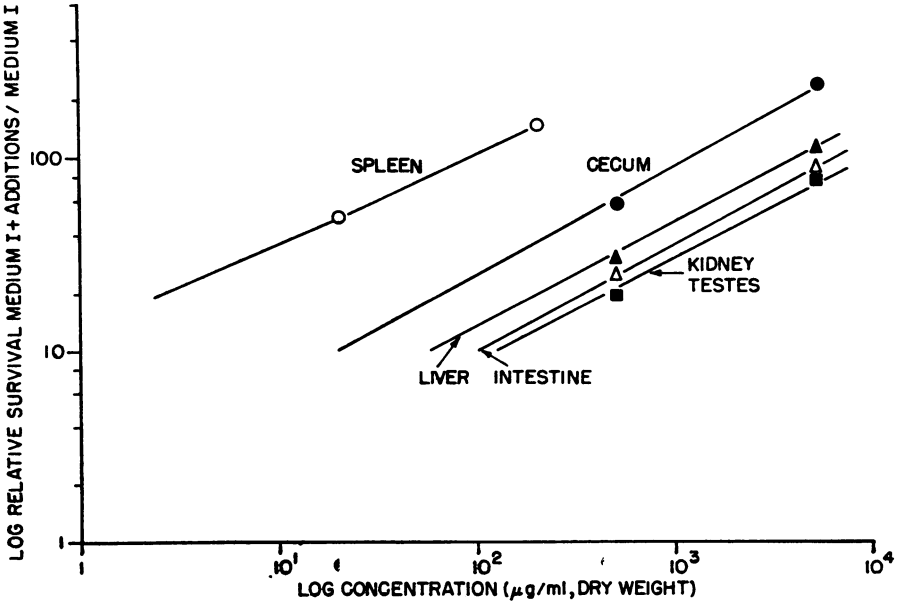

Figure 4. Relative activity of rabbit tissue homogenates; incubation at 37 C.

medium which would substitute for the extracts of natural materials in promoting recovery. A modified Lactobacillus assay medium (Ravel *et al.*, 1954) described in table 2 gave approximately the same activity as yeast or spleen extract. Deletion of the vitamin components from this medium resulted in no loss of activity. Omission of amino acids or the purine or pyrimidine components, however, effected a significant reduction of the activity of the medium. Guanine and uracil sufficed for the purine and pyrimidine requirements. Glutamic acid, glutamine, or aspartic acid filled the requirement for amino acids (table 3).

The simplest chemically defined medium which substitutes, under these experimental conditions, for extracts of natural materials is shown in table 4. The limited nitrogen concentration in this medium causes development of very small colonies on solid medium III. Addition of inorganic nitrogen as ammonium salts such as mono or dibasic phosphates, chloride, nitrate, molybdate, or sulfate to this solid medium resulted in inhibition of recovery. Ammonium tartrate, however, at concentrations below 0.5 mg per plate increased the size of the developing colonies without inhibition.

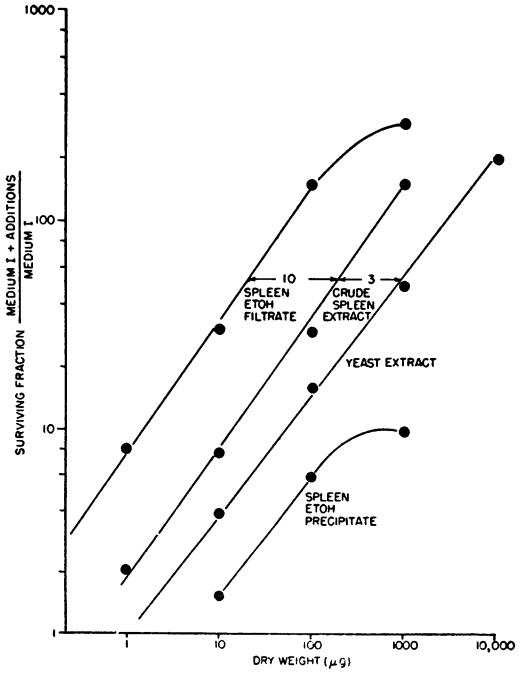

Figure 5. Relative activity of various purified spleen fractions as compared with yeast extract; incubation at 37 C.

TABLE 2

Composition of Lactobacillus assay medium

Components	Amount of Components in Stock Solution	Volume of Stock Solutions for 100 ml of Complete Medium
L-Amino acids*	40 mg	
DL-Amino acids†	80 mg	
Distilled H_2O	1,000 ml	97 ml
Inorganic salts A		
K_2HPO_4	25 g	
KH_2PO_4	25 g	
Distilled H_2O	250 ml	0.5 ml
Inorganic salts B		
$MgSO_4 \cdot 7H_2O$	10 g	
NaCl, $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$	0.5 g of each	
Distilled H_2O	250 ml	0.5 ml
Purine-pyrimidine supplement		
Adenine sulfate	100 mg	
Guanine hydrochloride	100 mg	
Uracil	100 mg	
Distilled H_2O	100 ml	1 ml
Vitamin supplement		
Pyridoxine hydrochloride	50 mg	
Inositol	15 mg	
Nicotinic acid	3 mg	
Riboflavin	3 mg	
Thiamin hydrochloride	3 mg	
p-Aminobenzoic acid	0.150 mg	
Folinic acid	0.150 mg	
Biotin	0.300 mg	
Ethanol 50%	30 ml	0.1 ml
Glucose		1 g
Agar		1.7 g

* Arginine hydrochloride, histidine hydrochloride, proline, glycine.

† Alanine, aspartic acid, cysteine, glutamic acid, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, valine.

The importance of salts A and B in the Lactobacillus assay medium is indicated by the finding, as shown in table 3, that the addition of glutamic

TABLE 3

Relative activity of various media compared to yeast extract

	Percentage
Medium I (table 1)	1
Medium I + 20 mg of yeast extract	100
Lactobacillus assay medium (table 2)	100
Medium II*	10
Medium II + 150 μg of glutamic acid + 110 μg of serine + 30 μg of methionine + 50 μg of tryptophan + 30 μg of guanine + 30 μg of uracil	100
Medium II + 150 μg of glutamic acid or 150 μg of glutamine or 150 μg of aspartic acid + 30 μg of guanine + 30 μg of uracil	100
Medium II + 150 μg of glutamic acid	20
Medium II + 150 μg of glutamic acid + 20 μg of vitamin mixture	20
Medium I + 150 μg of glutamic acid or 150 μg of glutamine or aspartic acid + 30 μg of guanine + 30 μg of uracil	20

* Salts A, 1 ml; salts B, 1 ml; glucose, 2 g; agar, 3.4 g; distilled H_2O, 200 ml; pH 6.8.

TABLE 4

Composition of medium III

Components	Amount
Glutamine	150 μg
Uracil	30 μg
Guanine	30 μg
Salts A*	1 ml
Salts B*	1 ml
Agar	3.4 g
Glucose	2.0 g
Distilled H_2O	200 ml
pH 6.8	

* See table 2.

acid plus guanine and uracil to medium I gave less than 20 per cent of the activity of these compounds in medium III. Elimination of salts A, the phosphates, from medium III reduces the activity of the medium by 80 per cent or more. Preliminary experiments indicate that Mn^{++} and Fe^{++} are not essential components of medium III.

The effect of growth conditions prior to irradiation on the recovery process. An investigation of the relation of the growth conditions prior to irradiation on the apparent induced fastidiousness of *E. coli* was considered essential in this work. Cells were grown on basal medium (medium I minus agar), nutrient broth, medium I plus added purines and pyrimidines, medium I plus added amino acids, and on medium III plus added inorganic ammonia as dibasic ammonium phosphate $(NH_4)_2HPO_4$. Excellent growth was obtained on these various media. The relative sensitivities of cells cultured on each medium prior to irradiation were determined. The surviving fractions of cells were ascertained on a variety of solid media, including the media on which the cells were originally cultured (table 5). The response of the cells to those factors found to be stimulatory for cells grown on nutrient broth was also investigated.

A comparison of the relative survival on medium I for cells cultured under the various conditions shows that cells cultured on nutrient broth are more sensitive than cells cultured under other conditions. It is of interest here that cells cultured on medium I prior to exposure to ionizing radiation show a greater survival when plated on this minimal medium than cells cultured on nutrient broth or on medium I plus amino acids or purines and pyrimidines. Moreover, the addition of these compounds to the basal growth medium results in a requirement for these compounds in the plating medium after irradiation. It is clear from table 5 that the cells cultured on the medium III are more resistant than those cultured on any of the other media used and that there is little response to materials added to the plating medium after irradiation. This is somewhat unexpected on the basis of the aforementioned effect observed when additions were made to medium I. Much more work will be required to determine the nature of the relative resistance of the cells cultured on medium III.

DISCUSSION

The results of previous experiments concerned with the effect of postirradiation incubation temperature (Stapleton *et al.*, 1953) suggested that the survival of irradiated bacteria is dependent on the relative rates of synthetic and destructive processes in the cell. It has been shown in the present work that fewer of these cells, cultured on nutrient broth prior to irradiation, are able to express themselves after exposure to ionizing radiation when plated on a simple inorganic salts-glucose medium. The effect of temperature on survival of irradiated cells on the minimal medium is much more pronounced

TABLE 5

Effect of culture medium on radiation sensitivity

Culture Medium	Surviving Fraction of Cells after 85 kr of γ Rays Plated on:				
	Medium I	Medium I + G + U	Medium I + AA	Medium III	Medium I + 20 mg of yeast extract
Medium I	2.2×10^{-6}	3.1×10^{-6}	4.0×10^{-6}	1.3×10^{-5}	1.3×10^{-5}
Medium I + G + U*	1.0×10^{-7}	1.0×10^{-6}	8.0×10^{-7}	3.0×10^{-6}	2.6×10^{-6}
Medium I + AA†	2.3×10^{-7}	2.9×10^{-6}	2.3×10^{-5}	5.0×10^{-5}	6.0×10^{-5}
Nutrient broth	3.0×10^{-8}	5.0×10^{-8}	8.0×10^{-7}	2.7×10^{-6}	3.5×10^{-6}
Medium III	1.0×10^{-4}			6.0×10^{-4}	3.0×10^{-4}

For medium I see table 1.
For medium III see table 4.
* G = guanine; U = uracil (50 μg each).
† AA = Difco casamino acids.
Surviving fractions are averages of three or more runs.
Incubation temp = 37 C.

than for cells plated on nutrient agar, yeast extract, or medium III. On the basis of this hypothesis we would postulate that the rate of destructive processes in irradiated cells plated on minimal medium is far greater than the rate of synthetic reactions. The nature of the stimulation afforded by the organic constituents of medium III then could be related to increased synthesis of vital systems within the cells plated on this medium or to reduction of the breakdown of vital systems in the irradiated cell. The induced requirement for a key amino acid, i. e., glutamic or aspartic acid or their amides, plus guanine and uracil—normal constituents of ribonucleic acid (RNA)—suggests that new protein synthesis, probably enzyme synthesis, is involved in bacterial recovery. The interrelation of RNA synthesis and protein synthesis is suggested by the recent experiments of Gale and Folkes (1954) on a subcellular fraction from Micrococcus. If protein synthesis is actually stimulated in irradiated cells by the addition of the amino acid or its derivative in conjunction with a purine and pyrimidine, this should be experimentally demonstrable. This will be a subject for future research. The active components in yeast extract or tissue extracts which stimulate recovery in irradiated bacteria have not been positively identified. The movement of the active components in these extracts on paper chromatograms compares favorably with the movement of the three known compounds which have been found partially to substitute for the extracts.

The information presented concerning the effect of cultural conditions on sensitivity to ionizing radiation and also the response to added factors is consistent with the concept that radiation may act on the cellular metabolism chiefly by interference with the synthesis of new enzyme within the cell, rather than by destruction of already existent systems. Some evidence for this concept has been presented by Billen and Lichstein (1952), who studied radiation induced inhibition of adaptive enzyme formation in *E. coli* (Texas).

Cells which have been cultured in a simple inorganic salts-glucose medium (medium I) may possess a large complement of enzymes, having had the task of performing *de novo* synthesis of all biologically important compounds as well as the necessary enzymes for formation of these compounds. On the other hand, cells cultured in a rich medium such as nutrient broth have had supplied to them numerous intermediates for synthetic reactions. When the latter cells are plated on a simple medium, they must, in order to express themselves, synthesize the enzyme systems essential for many synthetic reactions. Normal nonirradiated *E. coli* have the faculty for doing this, whereas ionizing radiations impair this function, as evidenced by the data presented in this paper.

It is of interest that addition of purines and pyrimidines or amino acids to the simple medium during growth of the organism prior to irradiation results in a lessened ability of *E. coli* to maintain itself after irradiation on the simple medium. As is shown in table 5, cells grown in medium I

plus purines and pyrimidines or amino acids require these compounds in the plating medium after irradiation.

A notable exception to this general finding is the case of cells cultured in the complete chemically defined medium (medium III). Such cells are strikingly more resistant than those cultured under any other condition studied and show almost complete independence of the plating medium after irradiation. The nature of the resistance of these cells has not been elucidated by these experiments and will be subject matter for future research on the general problem of recovery from the effects of ionizing radiation.

ACKNOWLEDGMENT

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SUMMARY

The B/r strain of *Escherichia coli* grown on a complete medium such as nutrient broth showed radiation induced requirements for nutritional factors.

Some evidence has been presented which indicates that the bacteria showing the nutritional requirements are not stable auxotrophic mutants.

Extracts of natural materials will supply the nutritional requirements. A chemically defined medium has been developed which will substitute for natural materials in bringing about recovery.

The type of media used to culture cells prior to irradiation determines to a great extent the sensitivity of the cell to ionizing radiation as well as the response of cells to added factors in the plating media after irradiation.

The data accumulated to date suggest that the recovery process investigated is related to the synthesis of new enzymes in the irradiated cell.

ADDENDUM

Since this paper was prepared, a publication has appeared by Cohen and Barner (1954) which concerns the role of "unbalanced growth" in ultraviolet inactivation of a thymineless strain of *E. coli*. The concept of unbalanced growth is not inconsistent with the data presented in this paper.

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