TESTS FOR GENETIC EFFECTS OF IRRADIATED OR CHEMICALLY TREATED MEDIA IN DROSOPHILA AND MICRO-ORGANISMS

by

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INTRODUCTION

In 1947, Stone *et al*. discovered that incubation in ultra-violet irradiated nutrient broth induced mutations in *Staphylococcus aureus* demonstrating that the biological action of radiation has a direct as well as an indirect component. After the initial burst of optimistic excitement when this discovery was thought by some as a step towards directed mutagenesis and to provide a tool for understanding the chemical nature of the gene, it only provided critics of the target theory an evidence against this mechanism of biological action of radiation. In recent years, however, interest in the indirect effect of radiation has been renewed by an entirely different development: prospects of large scale industrial use of radiation for treatment of food.

It is known that ionizing radiations in suitable doses can inhibit the breaking of dormancy of vegetables and can kill contaminating micro-organisms, parasites and infesting insects which may be present in food, without causing much rise in temperature of food. Raw food remains in the raw, uncooked state when it has been sterilized by irradiation, and frozen food can be sterilized while remaining frozen throughout the treatment. Also, because ionizing radiations possess high penetrating power food can be treated by it while packed and sealed in containers of such diverse materials as plastic, glass or metal. Subsequent contamination of packed and irradiated food is therefore most unlikely before the package is opened by the consumer. Irradiation can be applied to food by a process that operates without interruption; unlike products treated with heat, those sterilized

by irradiation do not have to undergo heating and cooling or any other discontinuous process. For preservation and extending the useful storage life of food ionizing radiations have therefore several advantages over the conventional method of heat treatment.

Increased availability of cheaper isotope and machine sources and several major advances in engineering and technology have made the industrial use of radiation for food preservation an economic feasibility. A mobile unit for irradiation of potatoes to inhibit sprouting has been in operation in Canada since 1962. Programmes for radiation treatment on commercial scale of several types of food for prolonging their useful storage life are already in operation or are at a very advanced stage of development in U.S.A. Similar programmes, though at a much smaller scale, are being developed in the United Kingdom and several other countries.

Studies on radiation chemistry of major food constituents like proteins, carbohydrates, fats and vitamins have shown that a large number of chemical alterations are produced in each component under the influence of ionizing radiation. The range of chemical substances formed in food that contains all the components together is obviously greater. Irradiated meat has been extensively studied and the array of compounds identified from it serves to illustrate this point. The following types of compounds have been identified: (1) water-soluble carbonyl-containing substances, probably from protein; (2) iso octane-soluble carbonyl-containing substances, mainly long chain aldehydes and ketones from plasmogens and other lipids; (3) volatile bases, mainly methylamine and ethylamine, from non protein nitrogenous substances; (4) volatile sulphur-containing substances, some of which are normally found in

meat but are present in greater amounts after irradiation, and (5) substances found only after irradiation, such as methyl mercaptan, isobutyl mercaptan and 3-methylthiopropionaldehyde. In addition, some changes are so complex that their nature is not yet known.

Now, if it is impossible to identify the nature of some chemical changes induced in food by irradiation, then the possibility exists that toxic and even mutagenic compounds are formed. And if they are formed, possible genetic hazards arising from consuming radiation sterilized food will have to be reckoned with and deserve investigating.

The present study was undertaken to gain information on the genetic effects of irradiated media in two systems:
(a) Bacteria: Ultraviolet light (UV) and X-ray irradiated media were tested for their lethal effects in Bacillus subtilis and UV, X-ray and Y-ray irradiated media were tested for phage induction, lethal and mutagenic effects in Escherichia coli. Since E. coli can grow in simple salt-glucose medium, identification of the compound of the medium responsible for generating a radiomimetic principle on irradiation was attempted. Experiments were also done to study the effect of such variables as dose rate, inoculum size and conditioning of bacteria by previous exposures to treated medium, on lethal effects of X-rayed medium. Experiments were also carried out to evaluate the lethal amd mutagenic effects of glyoxal in WP-2, a tryptophanless mutant of B/r strain of E. coli. Glyoxal is a radiodecomposition product of glucose and has been suggested by Berry et al. (1965) as the component responsible for observed lethal effects when mammalian cells are cultured in

irradiated growth medium. During the course of these studies it was found that spontaneous try^+ reversions occurred almost exclusively on the plate and rarely in the liquid growth medium. An attempt to test if this behaviour was common to other auxotrophic mutations in the genotype of WP-2 revealed a case of gene controlled mutational stability: Introduction of an additional requirement for adenine in WP-2 completely suppressed the revertability of try^- locus. A preliminary analysis of this interesting problem has been made.

(b) Drosophila melanogaster: The incidence of recessive lethal mutations in sex and 2nd Chromosome of Drosophila reared on irradiated food was determined. Following the report of Parkash (1965) in which he claimed strong mutagenic effect for irradiated DNA fed to Drosophila with food, the incidence of sex-linked recessive lethals was studied in flies fed on food mixed with irradiated DNA.

When testing the mutagenic effect of irradiated DNA one has to take account of the fact that feeding some samples of unirradiated DNA to Drosophila also produces 2nd Chromosome lethals (Gershenson, 1965; Mathew, 1965). Mutagenic effect of two samples of unirradiated DNA (Herring Sperm DNA; and a sample of DNA obtained from Dr. Gershenson) was therefore investigated.

It has also been claimed (Gershenson and Kiselyeva, 1958) that DNA specifically induces visible mutations affecting wings, a high proportion of which lies on the X-Chromosome. If confirmed, this will be a case of unprecedented mutagenic specificity by which a treatment produces a high frequency of visibles but no letal mutations on the X-Chromosome. In order to test this claim,

flies were reared on food to which a sample of unirradiated calf-thymus DNA kindly provided by Dr. Gershenson had been mixed. Recessive autosomal (2nd Chromosome) lethal and visible mutations were scored in the progeny of flies developing on this DNA treated food.

Review of Literature:

While the direct effects of radiation have been extensively investigated, comparatively little work has been done in the past to study the indirect biological effects of radiation. In recent years, however, increasing use of ionizing radiations for extending the useful storage life of food has caused concern about possible harmful effects on humans consuming irradiated food and this has necessitated the study of indirect effects. These studies can be broadly grouped into:

A. Feeding studies on laboratory animals: Many long-term tests with different animal species have been performed, particularly in the United States of America. These tests have given no indication of toxicity, carcinogenecity or histopathological change resulting from consuming irradiated food (McDowell and Raica, 1962). It is important to emphasize, however, that none of these experiments was designed to reveal induced genetic alterations.

B. Studies to test cellular and genetic effects: Experiments in which the effects on organisms of growth in irradiated substrate have been studied at cellular and genetic level are limited in number. They are moreover characterized by marked

differences in experimental variables such as chemical composition of the medium irradiated, type and dose of irradiation, test organism, and treatment condition. Therefore, critical comparisons between experiments of different workers are not possible.

For the purpose of present review these studies will be grouped into the following three categories on the basis of biological effect studied:
(1) Cytotoxic, (2) Cytological, and (3) genetic.

(1) Cytotoxic effects: The inhibitory effect on growth of Bacillus subtilis when plated on UV-irradiated nutrient agar was observed as early as 1935 (Blank and Arnold). Twelve years later Wyss et al.(1947) confirmed this observation for another species of bacteria. They showed that Staphylococcus aureus failed to grow when incubated in a UV-irradiated synthetic liquid medium consisting of mineral salts, sugar, amino acids and vitamins. The growth inhibitory effect was later found to arise from irradiation of glucose component.

Growth media irradiated with ionizing radiations have likewise been found to possess cytotoxic properties for a number of organisms. Molin and Ehrenberg (1964), for example, found that bacterial medium constituted with glucose irradiated with X-rays as water solution or in solid state had a strong anti-bacterial action on Pseudomonas sp 128. Evans (1947) reported deleterious effects of X-irradiated sea water on survival and activity of Arbacia eggs and sperms. Similarly, when E. coli strain 15 was incubated in X-ray irradiated minimal medium, synthesis of DNA,

MRNA, protein and inducible enzymes like β-galactosidase was inhibited resulting in suppression of growth of the incubated bacteria (Pollard et al., 1965). X-irradiated medium has also been found to damage chick embryo fibroblasts as evidenced by inhibition of spreading and growth of cells and their incapacity to synthesize Rous Sarcoma Virus. (Levison, 1966).

The growth inhibiting property of irradiated media, in all cases cited above, was attributed to radiation produced peroxides in the medium because the effect in each case could be completely negated by catalase. In support of this, the inhibitory effect of irradiated medium could be duplicated by adding hydrogen peroxide to unirradiated medium. On the other hand, there is also evidence to suggest that inhibitory products other than peroxides may be generated in the irradiated medium. Berry et al. (1965) for example demonstrated that the growth inhibitory principle, in irradiated carbohydrate solutions, responsible for lethality in mammalian in vitro cultures, could not be peroxide because it was stable to heat and catalase action. In the particular system investigated by the authors, the cytotoxic action was attributed to glyoxal, a radiation decomposition product of sugars.

A quite different mechanism by which irradiated medium can cause the killing of bacterial cells has been demonstrated by Šmarda and Čermák (1964). They found that incubation of lysogenic bacteria in uv-irradiated nutrient broth induces the formation of active bacteriophage resulting in lysis and cell death. Contrary to this, no induction of phage was obtained when Bugyaki et al. (1963) treated a lysogenic strain in minimal medium

to which irradiated glucose had been added. The negative results of Bugyaki et al. are not surprising, however, if it is considered that phage induction is strongly influenced by nutritional environment. Thus, Lwoff et al. (1950) have demonstrated that lysis occurs only when bacteria are grown in a rich medium both before and after exposure and none was found if minimal medium was used, even though this could support bacterial growth.

(2) Cytological effects: A part of the growth suppression and cytotoxic effects described in the preceding section, which cannot be studied in bacteria, may owe their origin to chromosomal aberrations. Higher plants provide a good system for testing this point. This approach to studying the indirect effects of ionizing radiation which was initiated by a group of Indian workers at New Delhi has been pursued in several laboratories. The main findings of the Indian group (Natarajan and Swaminathan, 1958; Natarajan 1960; Swaminathan et al., 1962; Chopra et al., 1963; Chopra and Swaminathan 1963;) can be summarized as follows:

(a) Treatment of germinating seeds of barley, Allium and Vicia faba in irradiated medium (for example White's medium or irradiated thymine solution) produced chromosome breaks in cells of root meristems.

(b) Cytological abnormalities such as chromosome breaks, lagging chromosomes at anaphase and micro-nuclei were found during mitoses in root tips of barley when embryoes excised from unirradiated seeds were grown on uncooked potato mash irradiated with 20-80 Krad

of X-rays. The frequency of micronuclei increased with increasing X-ray dose and the radiomimetic principle in irradiated tubers persisted for up to 8 months of storage at 4°C. (c) Treatment with irradiated fruit juices, likewise, produced chromosome breaks during root tip mitosis. No inter chromosomal changes were recorded.

Confirmation of the above findings came from the work of Moutschen and Matange (1965) and Holsten et al. (1965) who found that irradiated glucose solution produced chromosome breaks in barley and Vicia faba root tip and Tradescantia microspores. Chromosome breaks and interchanges have also been found in human lymphocytes cultured in irradiated medium TC 199 (Kesvan and Swaminathan, 1966) and medium to which irradiated sugar had been added (Shaw and Hayes, 1966).

Steward's group at the Cornell University in U.S.A. has tried to isolate the component in the irradiated medium responsible for producing chromosome breaks in Vicia and Tradescantia and for suppressing growth of carrot plant cells and tissue explants. They have concluded that the active principle is a radiation product of glucose. Attempts at further chemical characterization have not been successful so far though the indications are that formic acid or a salt of formic acid may be involved (Steward et al. 1967).

Kuzin in Russia has described an interesting experiment (Kuzin 1963) wherein irradiation of a shoot of Vicia plant produced radiomimetic compounds which could be translocated to other parts of the plant that had been carefully screened from radiation and caused growth inhibition and cytological

disturbances of cell division there. On the basis of chemical analysis of purified agent from irradiated tissue, Kuzin identified the biologically active fraction to be orthoquinoid in nature. In agreement with this he found that orthoquinones, obtained from enzymic oxidation of tyrosin, sharply reduced cell division and produced chromosome breaks in treated broad bean seedlings. Admittedly, a plant shoot is a far more complex substrate than sugar solution and consequently a greater array of radiation products in the former is to be expected. However, it is difficult to reconcile the differences between Steward's and Kuzin's identification as regards chemical constitution of the agent responsible for predominant biological effects.

(3) Genetic effects: The mutagenic effect of irradiated medium was first discovered by Stone et al. (1947) and to date theirs' are the only published reports of mutations induced by uv-irradiated medium in bacteria. Stone and co-workers found that streptomycin and penicillin resistant mutants occurred more frequently than in control when Staphylococcus areus was grown in UV-irradiated nutrient broth. The authors (Stone et al., 1948) later presented several lines of evidence to establish that the higher frequency of antibiotic resistant bacteria following incubation of the sensitive strain in irradiated broth was not due to selection of pre-existing mutants but was the result of induced additional mutations. The strongest evidence against selection was the observation that both forward and reverse mutations for ability to ferment mannitol were induced by treatment with irradiated broth.

Tests for mutagenicity of irradiated salts, glucose and other organic (consisting of amino acids and nucleic acid bases) components of a synthetic medium revealed that irradiation of salts alone was not mutagenic but irradiation of amino acids and purine and pyrimidine bases produced a significantly higher mutation rate than in the untreated controls. Mutagenic effect of irradiated glucose alone could not be determined because the treatment caused a very high lethality. From subsequent studies Wyss et al. (1947, 1948) attributed the mutagenicity of irradiated medium to organic (but not hydro) peroxides. The conclusion was based on the following observations.

(a) Incubation of bacteria in hydrogen peroxide treated nutrient broth or nutrient broth to which hydrogen peroxide treated amino acids and purine and pyrimidine bases had been added was mutagenic even though no free hydrogen peroxide could be detected at the time of inoculation of bacteria.

(b) Experiments in which bacteria were grown in sub-inhibitory concentrations of sodium azide (an enzyme poison which inhibits formation of catalase) showed a marked increase in the rate of mutations to penicillin and streptomycin resistance. The increased mutation rate was ascribed to intracellular accumulation of hydrogen peroxide.

(c) Treatment of washed bacteria in hydrogen peroxide was not mutagenic.

(d) Catalase treatment of irradiated and hydrogen peroxide treated broth abolished the mutagenic effect.

(e) The mutagenic effect of organic peroxides, produced inside the medium by irradiation or added from without was concluded to be a

specific one and not a result of growing bacteria in a medium with high oxidation-reduction potential. This was based on the observation that addition of several oxidizing and reducing agents to nutrient broth had no influence on mutation rates of bacteria incubated in it.

At the time these experiments were performed, the genic nature of bacterial mutations was not testable and therefore the experiments were repeated on a sexually reproducing fungus Neurospora (Wagner et al., 1950). The results obtained were in complete agreement with findings on bacteria with the only exception that hydrogen peroxide produced mutations in Neurospora even when no organic substance was present in the treatment medium. In contrast to these studies with bacteria and Neurospora, no mutagenic effect was found in exposures of Paramecium aurelia to either commercial or radiation produced hydrogen peroxide (Kimball, 1955; Kimball, Hearon and Gaither, 1955).

The mutagenic effect of feeding Drosophila on irradiated food has also been studied though the results obtained are conflicting. Swaminathan et al. (1963), for example, obtained a small but significant increase, over corresponding controls, in the frequency of sex-linked recessive lethal and visible mutations in the progeny of flies that had developed on irradiated food. A similar increase in the frequency of sex-linked recessive lethal, but not of visibles or dominant or mosaic lethals, was observed in experiments of Rinehart and Ratty (1965). Against this are the experiments of Reddi et al. (1965) who tested off-spring of flies raised on X-irradiated food for sex-linked recessive lethals, large deletions in the X-chromosome, sex-linked visibles and II/III translocations.

In spite of the large scale on which these experiments were done, no mutagenic effect of irradiated food could be detected. Similar negative results were obtained by Chopra (1965) in tests for the effect of irradiated food on dominant - and sex-linked recessive lethals and 2nd chromosome lethals. It thus appears that feeding Drosophila on irradiated food produces, at best, only a marginal effect which may under some circumstances be pushed beyond the borderline of statistical significance.

A dramatic increase in the frequency of sex-linked recessive and 2nd chromosome lethals in flies that had developed on food admixed with irradiated DNA was claimed by Parkash (1965a and b). However, none of several workers who have tried to repeat his experiments has found any basis to Parkash's claim because the results obtained have been entirely negative in all cases (Chopra, 1965; Khan and Alderson, 1965; Kaplan, 1966; Fahmy and Fahmy, 1966).

Mutagenic effect of calf-thymus DNA: The mutagenic effect of feeding Calf-thymus DNA to Drosophila larvae was first discovered by Gershenson (1939). He claimed that this treatment produced visible mutations. Muller (1941) and Rappaport (1940) repeated Gershenson's experiments but looked for sex-linked lethals because the latter are more frequent in mutagenic treatments with radiations and chemicals. No sex-linked lethals were found. The controversy was resolved, however, when Gershenson (1965) and Mathew (1965) simultaneously found that calf-thymus DNA affects mainly autosomes (2nd chromosome in their case) and produces hardly, if any, sex-linked lethals. Similar results were

obtained by Fahmy and Fahmy (1961) after injection of DNA into adult flies. The Fahmys also pointed out that the effect was not specific to DNA and injection of a number of other macromolecules could produce a similar effect. Gershenson and Kiselyeva (1958) have also claimed another type of mutagen specificity for calf-thymus DNA: that on feeding, DNA induces visibles (most of which affect the wings of the fly) but no lethal mutations on the X-chromosome. If confirmed, this type of specificity will be unprecedented and for which it is difficult to find a genetic explanation. However, Fahmy and Fahmy (1966) have not been able to verify Gershenson's observation.

MATERIAL and METHODS

A. BACTERIA.

Bacterial Strains

Bacillus Subtilis: No.23, a wild type strain, was used for studying the lethal effect of irradiated medium on B. subtilis. The strain was kindly provided by Mr. John Corran of this Institute.

Escherichia coli

gal 22 λ and gal 22 λs: Strains gal 22 , a K12 derivative lysogenic for phage λ , and gal 22 λs isogenic with gal 22 λ but sensitive to the phage, were obtained from the stock collection of Dr. O.J. Bishop of this Institute. They were used for studying the lethal effects of irradiated medium and to decide whether the bactericidal effect was caused by phage induction or by a direct effect of an antibacterial principle generated in the medium by radiation exposure. After it was established that irradiated medium did not induce λ phage, strain λs was employed in all subsequent studies on the lethal effects of irradiated medium.

gal 22 λs is sensitive to the antibiotic, streptomycin and, therefore, was also used for studying forward mutations to streptomycin resistance induced by treatment with irradiated medium.

WP-2 try⁻: is a tryptophan-requiring mutant of strain B/r of E. coli. WP-2 was used in the present study for studying the mutagenic effect of glyoxal. The culture used was obtained from Dr. B.A. Bridges of MRC Radiobiological Research Unit, Harwell.

AB 712: comes from Adelberg's collection and is auxotrophic for

three amino acids, viz., threonine, leucine and proline. The strain was used for scoring reverse mutations to independence of growth factor requirement after treatment with irradiated medium.

MEDIA

Nutrient broth and Nutrient agar: Nutrient broth was prepared by dissolving 13 gm. of 'Oxoid' Nutrient Broth per litre of distilled water. For preparing nutrient agar 1·5% w/v Davis New Zealand Agar was added to nutrient broth.

Spizizen's growth medium: was used for growth and treatment of B. Subtilis W23. A litre of liquid medium contained:

Spizizen's minimal salts (5x)	200 ml.
20% glucose	27 "
2% Casein hydrolysate	10 "
Mn^{++} (10^{-4}M)	10 "
Distilled water	to make 1 litre.

20% glucose and the remaining constituents were autoclaved separately for 20 minutes at 15 lbs. pressure per square inch and mixed together aseptically when cool.

The composition of Spizizen's minimal salts (5x) was as follows:

$(NH_4)_2SO_4$	10 grams.
K_2HPO_4	70 "
KH_2PO_4	30 "
$Na_3C_6H_5O_7.2H_2O$	5 "
$MgSO_4.7H_2O$	1 "
Distilled water	to make 1 litre

M_9 medium: M_9 medium, used for growing E. Coli gal 22λ and gal 22 λ s had the following composition:

M_9 salts (composition given separately)	100 ml.
4% glucose	50 "
$MgSO_4$ (0·1 M)	10 "
$CaCl_2$ (0·01 M)	10 "
Distilled water	to make 1000 ml.

Each solution was autoclaved separately at 15 lbs. pressure per square inch for 20 minutes and then mixed together aseptically.

M_9 salts

Na_2HPO_4	anhydrous	60 grams
KH_2PO_4	"	30 "
NaCl		5 "
NH_4Cl		10 "
Water		to make 1000 ml.

M medium of Haas and Doudney (1957): was employed for experiments with WP-2. A litre of M minimal medium contained:

M salts (composition given separately)	200 ml.
20% glucose	10 ml.
Water	to 1 litre.

M salts: were prepared as 5X concentration and contained per 1000 ml.

KH_2PO_4	19·5 grams.
K_2HPO_4	39·5 "
$Na_3C_6H_5O_7.2H_2O$	2·5 "
$MgSO_4.7H_2O$	0·5 "
$(NH4)_2SO_4$	5·0 "
Water	to make 1000 ml.

For growing WP-2, the minimal medium was supplemented with 6 μg per ml. tryptophan. 1·5% w/v David New Zealand agar was added to the minimal medium to obtain a satisfactory gel for M minimal agar plates. For mutation plates, minimal agar was supplemented with 0·75 or 1 μg per ml. tryptophan.

M salts, glucose and agar in water were sterilized separately by autoclaving for 20 minutes at 15 lbs. pressure per square inch and mixed together aseptically before pouring the plates. Each plate contained approximately 20 ml. of medium.

Brain Heart Infusion agar: was prepared by mixing together the following:

Oxoid Brain Heart Infusion	37 grams
Davis New Zealand agar	15 "
Distilled water	to make up 1000 ml.

The mixture was autoclaved for 25 minutes at 15 lbs. per square inch pressure.

Buffer: of the following composition was used as diluent for all *E. coli* experiments.

NH_2PO_4	3 grams
Na_2HPO_4 anhydrous	7 "
NaCl	4 "
$MgSO_4.7H_2O$	0·2 "
Water	to make 1000 ml.

Irradiation of the medium:

Ultraviolet (U.V): UV irradiation of the medium was done in 6 cm. diameter glass dishes. The volume of the medium in the dish was

kept to give a depth of 0·5 cm. The UV source was a Hanovia low pressure mercury germicidal tube giving 85% of its output at wavelength 2537Å in the UV zone. Irradiation was done at a distance of 25 cm. from the tube; the medium being constantly agitated.

X-rays: X-irradiation of the medium sterilized by autoclaving was done in tightly stoppered 20 ml. capacity MSE plastic centrifuge tubes. Very little air space was left in the tubes after stoppering so that the irradiation was essentially done under anaerobic conditions. The X-ray machine was operated at 140 KV, 5 MA without filters, giving a dose rate of 943 rad per minute.

γ-rays: γ ray irradiation was obtained from a 4000 Ci Co^{60} 'Hotspot' irradiation source. The standard dose rate was 17,321 rad per minute. Any variation in the dose rate used will be indicated in the relevant experiments. For study of the lethal effects, irradiation was done in screw cap $\frac{1}{2}$ oz. McCartney bottles without aeration. For mutation experiments larger volumes were irradiated in 100 ml. flasks.

Treatments in irradiated medium: For studying the lethal effects, nine ml. of irradiated medium in a test tube was seeded with 1 ml. of suspension of desired bacterial density and incubated in a shaker incubator maintained at 37°C. Viable counts were made at intervals by withdrawing a sample from the treatment mixture, diluting as necessary and plating.

The methods of treatment for the study of mutagenic effect will be described in the respective experiments.

Viable counts: Viable counts were made by spreading 0·1 ml. of suitable dilutions of the treatment mixture on each of 3 nutrient agar plates per determination. The dilution was so arranged as to give 100-200 colonies per plate. The average value based on the number of colonies appearing after 24 hour incubation at 37°C was taken for any one determination.

Procedure of preparing bacterial culture for treatment: A single colony from stock culture plate was suspended in 10 ml. of medium in a ½ oz. McCartney bottle and incubated overnight at 37°C. One ml. of overnight culture was seeded into 200 ml. of fresh medium to give a bacterial density of $5 \cdot 10^5$ to 10^6 per ml. and incubated with aeration at 37°C. This procedure gave an exponentially growing culture in 4 hours.

Isolation of auxotrophs in WP-2: For inducing additional nutritional requirements in WP-2, a culture grown in nutrient broth to stationary phase was washed free of broth and resuspended in minimal medium at a cell density of around 10^8 bacteria per ml. 3 ml. of the suspension was irradiated with a UV dose that gave a 0·1% survival. The irradiated bacteria were inoculated into 100 ml. sterile nutrient broth and incubated with aeration overnight at 37°C. The culture was washed free of broth in minimal medium and a suitable inoculum seeded into minimal medium supplemented with 6 µg per ml. tryptophan and containing 1000 international units per ml. of benzylpenicillin (Glaxo Ltd.). After 2 hour incubation at 37°C, the survivors were washed free of penicillin by repeated centrifugation and resuspended in minimal medium. A suitable dilution was plated on nutrient agar on which

both try$^-$ and try$^-$ combined with any other induced growth factor requirement yielded colonies. The auxotrophic mutants were finally identified by their failure to grow after replica plating to minimal medium enriched with tryptophan. The specific growth factor requirements isolated in this way were identified by streaking on tryptophan enriched minimal medium plates supplemented first with various combinations of pooled amino acids, vitamins or purine and pyrimidine bases and finally with the indicated individual growth factors.

Treatment of diauxotrophic strain try$^-$ ad$^-$ with mutagens: For studying the response of try$^-$ and ad$^-$ loci in di-auxotrophic strain try$^-$ ad$^-$ to mutagens, treatments were applied to stationary phase bacteria suspended in minimal medium minus glucose.

(i) UV: 3 ml. lots of suspension containing about 10^8 bacteria per ml. were exposed to UV irradiation from UV source already described. 0·1 ml. of the irradiated suspension was plated on suitably supplemented plates for scoring try$^+$ and ad$^+$ reversions.

All operations were performed in yellow light to avoid photo reactivation.

(ii) Ethyl methane sulphonate (EMS): Appropriate volumes of EMS (obtained from Eastman Organic Chemicals, Rochester, New York) were suspended in minimal medium minus glucose to give an end concentration of 0·1 M EMS. A concentrated suspension of bacteria was then added to give 10^8 bacteria per ml. The treatment was carried out at 37°C. The treatment was stopped by adding sufficient volume of 10% aqueous solution of sodium thio-sulphate to give a final concentration of 1% in the treatment mixture.

The suspension was washed three times in minimal medium and resuspended in the original volume of minimal medium. 0·1 ml. of the suspension was plated on suitably supplemented plates for scoring the two types of reversions.

B. DROSOPHILA

Stocks

Or-K

Oregon-K (Or-K) is a wild type stock of *Drosophila melanogaster*. This stock has been used in this laboratory for mutation experiments for a long time and has shown a spontaneous rate of about 0·3 per cent sex-linked lethals in periodic checks.

Muller-5

The Muller-5 (M-5) stock was used for scoring sex-linked recessive lethals. The X-chromosome of the stock carries the dominant Bar (B) and the recessive marker apricot (W^a) and has two inversions, the sc^{SI} inversion and inversion-S, the latter being included in the former. The inversions completely inhibit crossing over along the whole length of the X-chromosome. The formula for the stock can be written as follows:

$$Sc^{SI} \quad B \quad In\text{-}S \quad W^a \quad sc^8$$

Cy/BlL^2

This stock was used for screening recessive lethals on the 2nd chromosome. It is a balanced lethal stock with three dominant markers: Curly wing (Cy) in one of the 2nd chromosomes

and Bristle (Bl) and Lobe (L^2) in the other. Cy has two paracentric inversions, one in each arm, to prevent crossing over. Both chromosomes are lethal in homozygous conditions.

$\widehat{XX}$Cy/+

Heterozygous Curly virgin females with attached X; obtained from the cross $\widehat{XX}$♀♀X Cy/BlL^2 were used in the calf-thymus DNA feeding experiment for simultaneous scoring of visibles on the X-chromosome and 2nd chromosome lethals.

Treatment

(i) Irradiated medium: Two experiments were done to test the mutagenic effect of the irradiated food. In the first experiment, food consisting of 10% dried killed yeast, 10% sucrose and 3% agar in water was irradiated by the Radiation Research Laboratory, Wantage. A total dose of 1 Mrad of γ-rays to the food was delivered in a single exposure lasting 70 minutes from a 14000-c annular Cobalt-60 source. Twenty-four and 48 hour old heterozygous Curly (Cy/+) larvae from the cross Cy/BlL^2 x Or-K were transferred to the irradiated food about 36 hours after termination of the radiation exposure and the frequency of sex-linked and second chromosome (autosomal) recessive lethals was determined simultaneously in both treated males and females. Test of 2nd chromosome lethals was included because it has been shown that in certain treatments, feeding of calf-thymus DNA for example, produces specific and pronounced mutagenic effect on chromosome II of Drosophila while the X-chromosome remains largely refractory to the treatment. Since the treatment was given to heterozygous Curly larvae any

pre-existing lethal on the wild-type 2nd chromosome of one of the larvae would show up in all F_3 cultures. Such pre-existing lethals were excluded. In order to keep the experiment manageable and because of the known spontaneous mutation rate of the stock, controls were not included in this experiment.

In the second experiment maize meal-molasses food was used; the radiation dose and dose rate being the same as in the first experiment. Young Or-K females, mated 24 hours earlier, were transferred to the irradiated food for egg laying immediately on termination of radiation exposure, so that the developing flies would be exposed to the full effect of the treatment including that due to any transient radicals. Males which had developed on irradiated food were tested for dominant lethals and recessive sex-linked lethal mutations. Induced dominant lethality was investigated in view of chromosome breakage observed in plant material grown on irradiated medium. Similar breaks, if induced in Drosophila, will result in production of dominant lethals. Comparable controls were included in this experiment.

Scoring was done by the usual techniques, namely, hatchability for dominant lethals, Muller-5 test for sex-linked lethals and Cy/L test for second chromosome lethals.

(ii) Irradiated DNA: Herring sperm DNA (obtained from Koch-Light Laboratories Ltd., England) was exposed to 100,000 of γ-rays at the Radiation Research Laboratory, Wantage, and thoroughly mixed into maize meal molasses food at a concentration of 1·8% (the concentration used by Dr. Parkash, 1965). Oregon-K flies were allowed to feed and lay eggs on this food in half pint bottles.

After 3 days at 25°C, the parent flies were discarded and the eggs allowed to hatch and develop at 25°C. Food mixed with unirradiated DNA served as control. Males that had developed on DNA-treated food were tested for sex-linked recessive lethals according to Muller-5 method. In three replicates of the experiment no mutagenic effect was observed.

Dr. Parkash thought the difference between his results and mine to arise from difference in the composition of Drosophila food used. A fourth experiment was therefore done with food prepared according to the following formula provided by Dr. Parkash. Sugar, 7g; bran, 7g; maize meal, 6g; and agar 0·8g; cooked in 100 ml. water. The concentration of DNA, and ɣ-ray dose were the same as in the other 3 experiments.

Feeding of calf-thymus DNA: Calf-thymus DNA from a sample kindly provided by Dr. Gershenson was well mixed into maize meal molasses medium when cool at a concentration of 13%. Oregon-K flies were allowed to lay eggs and develop on this DNA-treated food at 25°C, the parent flies having been discarded after 3 days. The emerging males were individually mated to 3 virgin $\widehat{XX}$Cy/+ females. The F_1 males were scored for sex-linked visible mutations. A sample from Curly F_1 males was also tested for second chromosome recessive lethals by the usual Cy/L method.

RESULTS

A. BACTERIA

I. LETHAL EFFECT OF IRRADIATED MEDIUM

Bacillus subtilis: Incubation of a growing culture of Bacillus subtilis in nutrient broth irradiated with 226 Krad of X-ray or with UV for 3 hours at a distance of 25 cms. did not have an adverse effect on growth. However, nutrient broth is a complex growth medium, and it was considered likely that some of its components may protect the treated bacteria from the cytotoxic principle generated by irradiation. The effect on growth of X-irradiated (226 Krad) salt-glucose minimal medium was, therefore, next examined. A number of experiments were carried out, all of which gave similar results. Data from a representative experiment is given in Table 1 and Fig. 1. It was found that the number of viable, colony-forming, bacteria declined progressively with increasing period of incubation in the irradiated medium. The duration of treatment in the irradiated medium that gave the maximum decrease in the number of surviving bacteria depended on the number of bacteria initially seeded. With an initial inoculum of about 10^6 bacteria per ml., the lowest viable count was recorded after 8 hours of incubation. With smaller initial inocula, the minimum viability was reached more quickly. After the period of maximum decline, growth was resumed, first at a retarded rate and then at about the same rate as that of the untreated control.

Bacillus subtilis is known to harbour a defective phage which can be induced spontaneously as well as by treatment with several mutagens. It was therefore necessary to ascertain

Table 1. Effect on Survival of *B. subtilis* W23 incubated in X-irradiated minimal medium.

X-ray dose	% Survival after incubation in irradiated medium (Hours)							
	0	1	2	4	6	8	10	12
226 Krad	100	73·8	36·21	8·36	2·78	0·36	0·39	2·10
	viable counts per ml.							
CONTROL	$1{\cdot}3 \times 10^6$	$1{\cdot}6 \times 10^6$	$2{\cdot}7 \times 10^6$	$8{\cdot}5 \times 10^6$	$2{\cdot}8 \times 10^7$	$7{\cdot}7 \times 10^7$	$2{\cdot}2 \times 10^8$	$6{\cdot}2 \times 10^8$

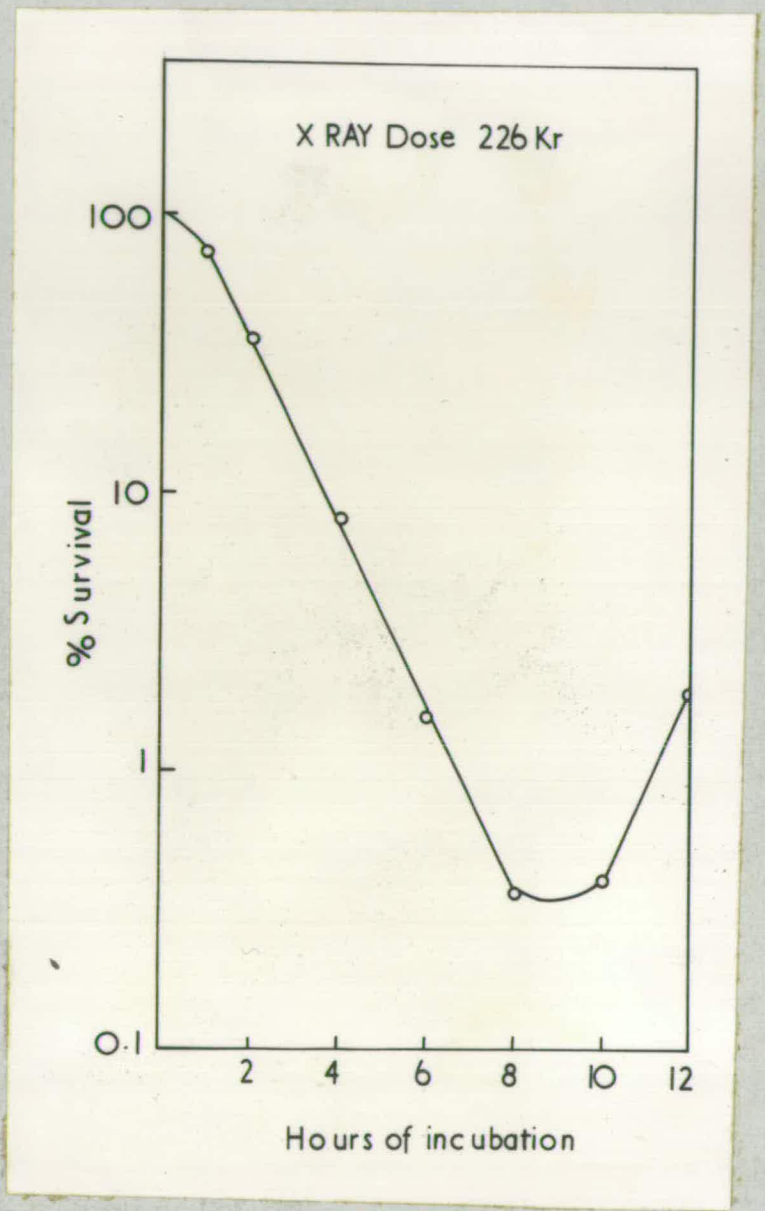

Fig. 1: Survival of Bacillus subtilis W-23 incubated in minimal medium irradiated with 226 Krad of X-rays.

whether the killing had been due to phage induction or to a direct effect of the irradiated medium. Experiments with B. subtilis could not yield a decisive answer between the two alternatives because a strain cured of the phage is not available in this bacterial species. In view of the complications arising from spontaneous phage induction resulting in lysis and cell death, further experiments were done with Escherichia coli.

Escherichia coli.

(a) EXPERIMENTS WITH X-IRRADIATED MINIMAL MEDIUM *

For experiments with X-irradiated medium, unless otherwise stated, M_9 medium was exposed to 226 Krad of X-rays at a dose rate of 943 rads per minute.

(i) Tests for phage induction by treatment with irradiated medium:

To test whether killing resulting from incubation in irradiated medium was due to phage induction or the effect of a bactericidal principle produced in irradiated medium, gal 22 λ and gal 22 λ s, two derivatives of E. coli K-12, one lysogenic for, and the other sensitive to, phage λ were incubated in X-irradiated M_9 medium. Killing, increasing with longer periods of incubation in the irradiated medium, was again observed (Table 2 and Fig. 2). The experiment was repeated twice with similar results. Table 2 and Fig. 2 are based on results of one experiment. It was observed that gal 22 λ was slightly, but insignificantly, more sensitive than gal 22 λ s to the action of irradiated medium. The survival curves for the two strains were, however, almost identical and it was clear that the bactericidal effect of the irradiated medium was not due to phage

* A preliminary report on the effect of X-irradiated medium in bacteria has been published in Microbial Genetics Bulletin 23 (1965).

induction. In order to confirm this conclusion, supernatant of the lysogenic strain after treatment with irradiated medium was titrated for phage assay. Supernatant of culture from the same original suspension grown in unirradiated medium served as control. The number of plaque forming units per ml. in the two series was not different showing that medium irradiated with 226 K rad does not induce phage

The survival of gal 22 λ s determined every hour following incubation in X-irradiated medium for up to 8 hours is given in Table 3 and Fig. 3.

(ii) Effect of X-irradiation of components of minimal medium:

In order to identify the component of the minimal medium responsible for its cytotoxic effect on irradiation, minimal medium or water and glucose components of the minimal medium were irradiated with 226 Krad of X-rays. Necessary unirradiated supplements were added subsequent to irradiation of the component in question. Irradiation of the salts component could not be included in these experiments because tube geometry of the X-ray apparatus did not allow simultaneous irradiation of 4 samples. The results are presented in Table 4 and Fig. 4.

It can be seen that incubation in medium whose water component had been irradiated produced only a suppression of growth and no decline in viable counts; growth at normal rate being resumed after a lapse of about 7 hours. Decline in the number of viable bacteria was steeper after incubation in medium whose glucose component had been irradiated compared with incubation in irradiated whole minimal medium. The decline, however, reached its

Table 2. The effect on survival of gal 22 λ and gal 22 λ s following incubation in X-irradiated medium.

Strain	% Survival after incubation in irradiated medium for hours			
	0	2	4	8
gal 22 λ	100	52·3	22·1	4·2
gal 22 λ s	100	55·8	24·6	5·2

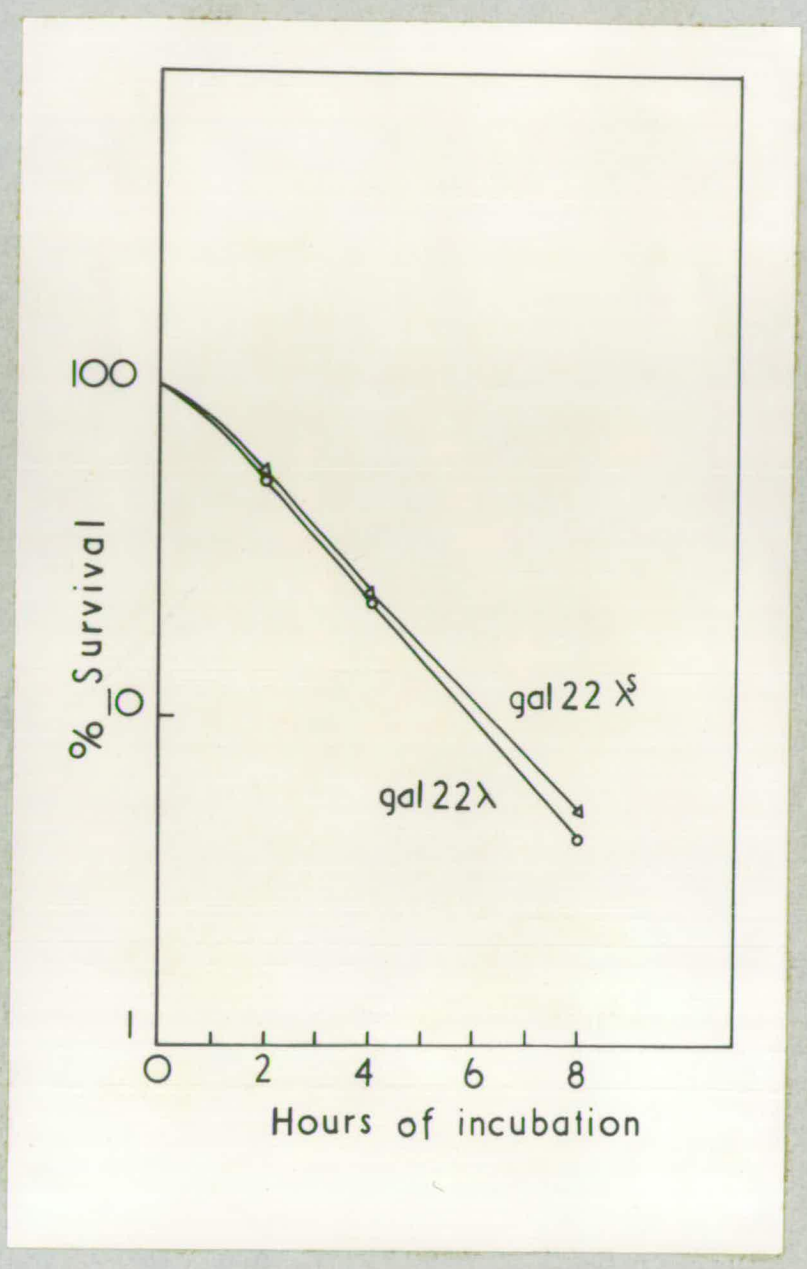

Fig. 2: Survival curves of gal 22λ and gal 22 λs following incubation in X-irradiated minimal medium.

Table 3. Survival of gal 22 λs after different durations of incubation in X-irradiated minimal medium

Duration of incubation in hours	Treatment	
	Irradiated medium % survival	Unirradiated medium viable counts/ml.
0	100	$4{\cdot}2 \times 10^6$
1	62·5	$5{\cdot}6 \times 10^6$
2	62·5	$1{\cdot}1 \times 10^7$
3	37·5	$1{\cdot}7 \times 10^7$
4	31·2	$3{\cdot}2 \times 10^7$
5	21·5	$5{\cdot}6 \times 10^7$
6	14·3	$1{\cdot}2 \times 10^8$
7	6·2	$1{\cdot}8 \times 10^8$
8	3·2	$3{\cdot}3 \times 10^8$

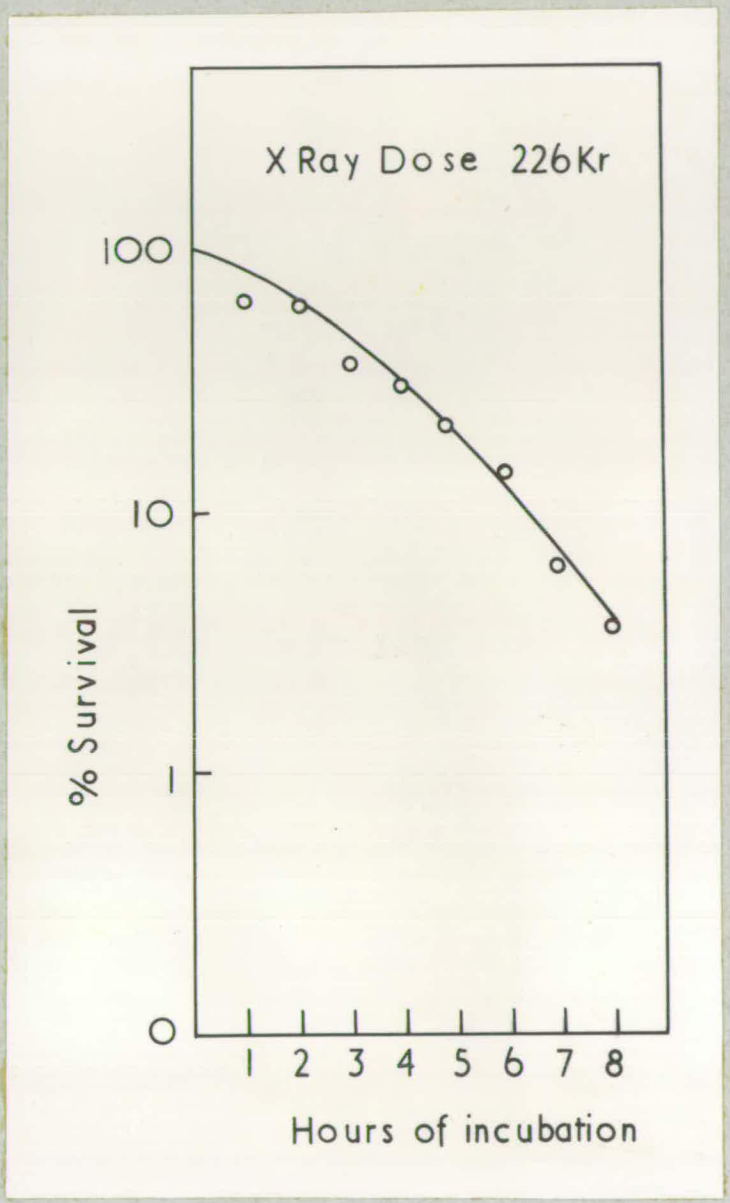

Fig. 3: Survival curve of E. coli incubated in X-irradiated minimal medium.

Table 4. Survival of gal 22 λ s incubated in X-irradiated minimal medium, or components of the minimal medium.

Treated component	% Survival after hours of incubation				
	0	2	4	6	8
Glucose	100	7·5	2·8	2·9	3·2
Minimal medium	100	40·0	34·1	9·4	1·6
Water	100	92·3	92·3	103·8	231·6
	viable counts per ml.				
Control	$3·0 \times 10^6$	$5·8 \times 10^6$	$1·6 \times 10^7$	$4·6 \times 10^7$	$1·4 \times 10^8$

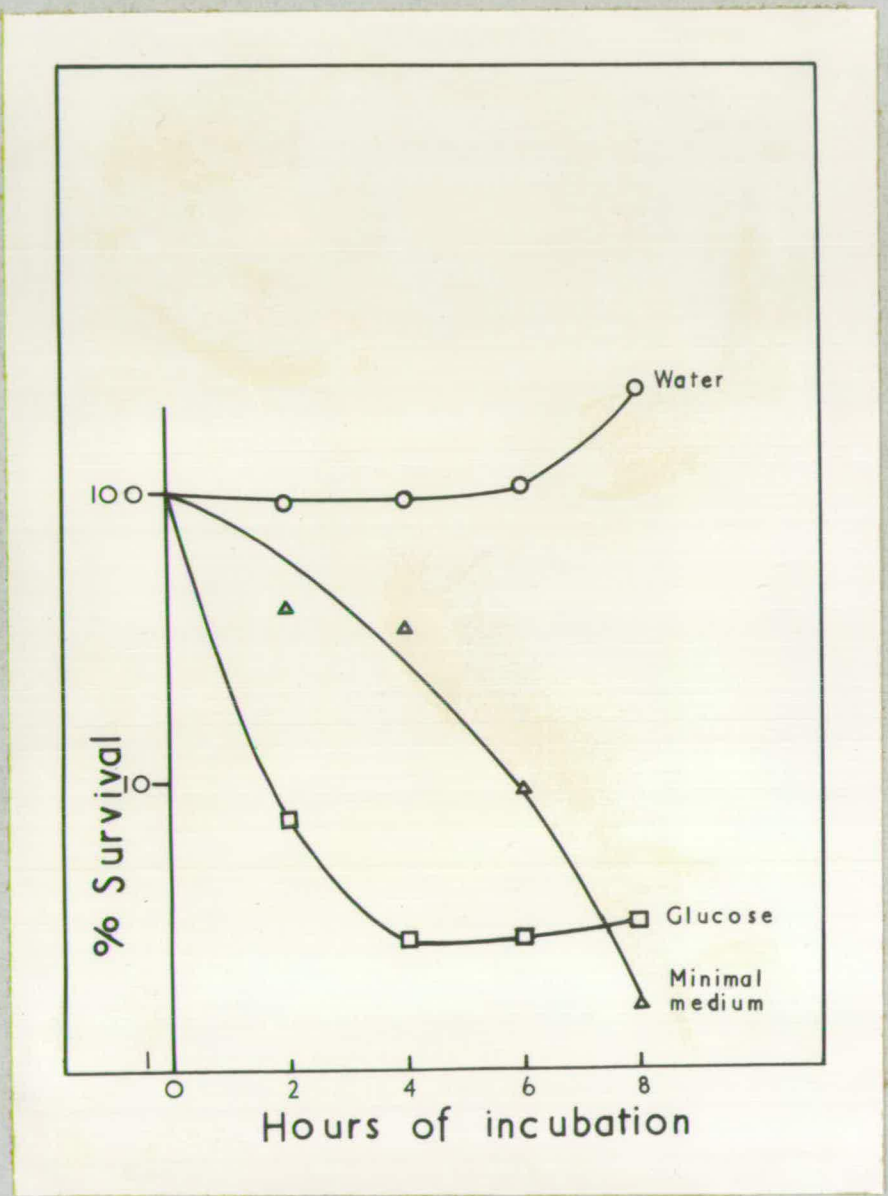

Fig. 4: Survival of E. coli incubated in X-irradiated minimal medium or in medium components of which had been X-irradiated.

maximum point after 4 hours' incubation in irradiated glucose while it continued up to 8 hours when incubation was done in irradiated minimal medium. This suggests that the bactericidal principle generated by X-rays in glucose solution may be qualitatively and/or quantitatively different from that produced in irradiated minimal medium. While the former is more potent and exhausted quickly from the medium, the latter has a longer stable life. Not enough is known about radiation chemistry of complex chemical solutions, however, to allow a definite conclusion on this point.

(iii) Effect of catalase, sensitivity to hydrogen peroxide and the response to irradiated medium:

It has been suggested by various workers (Frey and Pollard 1966 and others) that the lethality produced by irradiated medium is due to radiation induced peroxides in the medium. Frey and Pollard (1966) made determinations of peroxides produced in oxygenated water, glucose solution and minimal medium by a dose of $2 \cdot 7 \times 10^{4}$ rads of X-rays and found that the maximum peroxide content in their chemical system was $3 \cdot 5 \times 10^{-6}$ gm. per ml. Taking this value as a guide, experiments were conducted to see if commercial hydrogen peroxide added to the minimal medium simulates the effect of irradiated medium. It was also tested if catalase added to the irradiated medium prior to inoculating it with bacteria, removes the bactericidal effect of irradiated medium. Experiments with the following series were run to test these points.

Table 5. Effect of catalase on sensitivity to hydrogen peroxide and X-irradiated medium of gal 22 λ s.

Treatment	Viable counts/ml. following incubation in treated medium for hours				
	0	1	2	3	4
Control	$1{\cdot}4 \times 10^6$	$2{\cdot}1 \times 10^6$	$5{\cdot}6 \times 10^6$	$1{\cdot}4 \times 10^7$	$3{\cdot}8 \times 10^7$
Irradiated medium (226 Kr)	$1{\cdot}3 \times 10^6$	$2{\cdot}8 \times 10^5$	$1{\cdot}7 \times 10^5$	$2{\cdot}1 \times 10^5$	$1{\cdot}9 \times 10^5$
Irradiated medium (226 Kr) + Catalase	$2{\cdot}2 \times 10^6$	$3{\cdot}2 \times 10^6$	$8{\cdot}4 \times 10^6$	$1{\cdot}7 \times 10^7$	$3{\cdot}2 \times 10^7$
Hydrogen peroxide (6×10^{-6} g/ml.)	$1{\cdot}6 \times 10^6$	$1{\cdot}9 \times 10^5$	$1{\cdot}2 \times 10^5$	$9{\cdot}0 \times 10^4$	$7{\cdot}3 \times 10^4$
Hydrogen peroxide (6×10^{-6} g/ml.) + Catalase	$1{\cdot}1 \times 10^6$	$1{\cdot}2 \times 10^6$	$2{\cdot}7 \times 10^6$	$7{\cdot}4 \times 10^6$	$2{\cdot}2 \times 10^7$

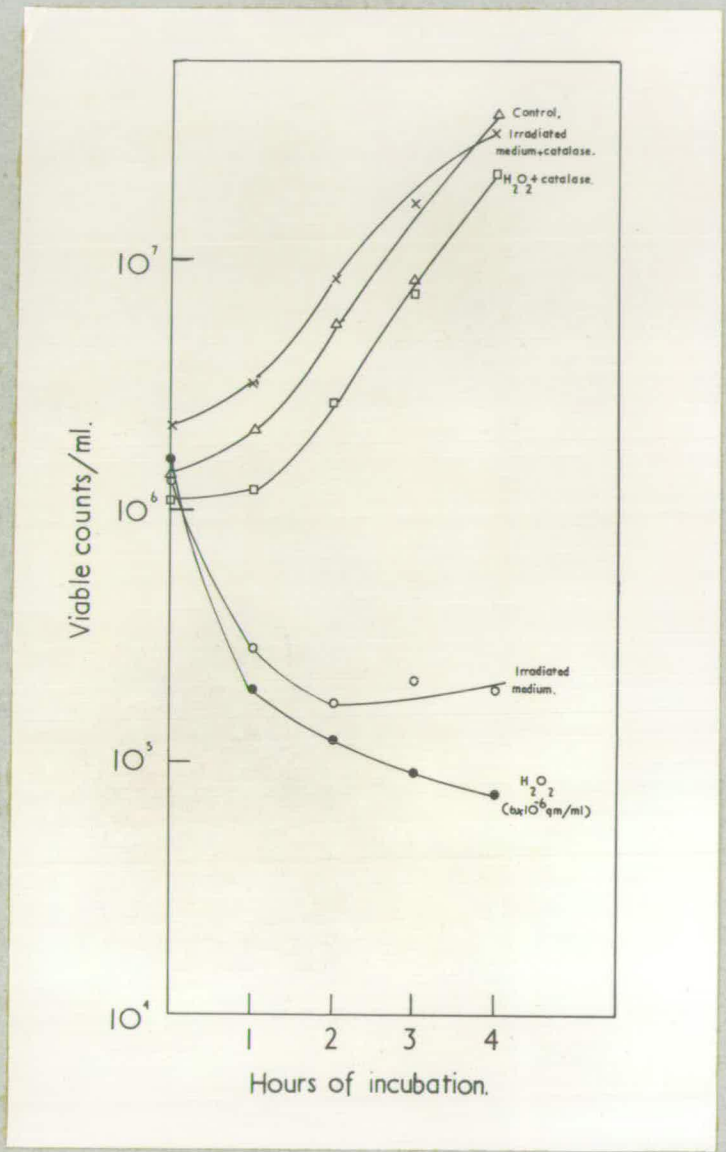

Fig. 5: The effect of catalase on sensitivity to hydrogen peroxide and X-irradiated minimal medium of E. coli.

(i) Un-irradiated M_9 medium

(ii) M_9 medium irradiated with 226 Krad of X-rays.

(iii) M_9 medium irradiated with 226 Krad of X-rays to which 5 μg per ml. catalase from bovine liver had been added 30 minutes prior to addition of bacteria.

(iv) M_9 medium containing 6×10^{-6} gm. per ml. commercial hydrogen peroxide.

(v) M_9 medium containing 6×10^{-6} gm. per ml. commercial hydrogen peroxide to which 5 μg per ml. catalase had been added 30 minutes prior to addition of bacteria.

Addition of 5 μg per ml. catalase to the medium was found to have no effect on the growth of bacteria and therefore a series with catalase alone was not included in the experiments outlined above. The experiment was repeated twice with identical results. Results of one experiment are given in Table 5 and Fig. 5. It was found that incubation in medium containing added hydrogen peroxide produced a pattern of lethality similar to that produced by irradiated medium. The quantitative difference in the effect produced by the two treatments is due to the fact that the concentration of H_2O_2 used in these experiments was approximately twice that which is expected to be generated by the dose of radiation used. Also, when bacteria were incubated in irradiated or hydrogen peroxide containing medium that had been treated with catalase prior to inoculation, the growth curves were superimposable on the control growth curve. These results suggest that radiation induced hydro- or organic peroxides constitute the bactericidal principle in the irradiated medium.

Table 6. The effect of inoculum size on survival of *E. coli* incubated in X-irradiated medium (200 Kr).

Inoculum size (Bacteria/ml.)	% Survival after hours of incubation in medium irradiated with 200 Kr.			
	0	1	3	4
$1{\cdot}6 \times 10^6$	100	48·00	20·40	20·80
$1{\cdot}6 \times 10^5$	100	19·37	13·75	7·50
$1{\cdot}6 \times 10^4$	100	10·83	9·16	5·83

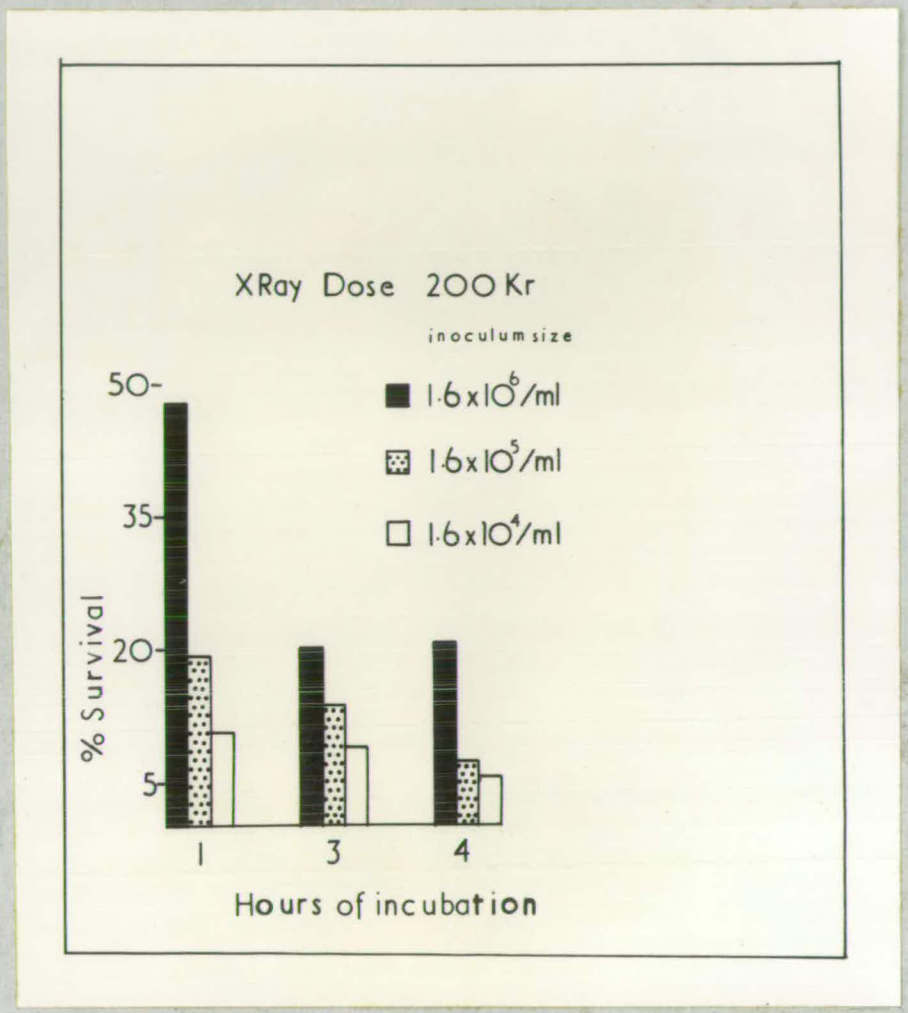

Fig. 6: The effect of inoculum size on survival of E. coli incubated in X-irradiated medium.

(iv) Effect of inoculum size on the bactericidal effect of Irradiated medium:

Exploratory experiments on the bactericidal effect of X-irradiated medium in which the various treatment conditions were varied had suggested that the magnitude of effect was greater when the number of bacteria incubated in the treated medium was small. To test the validity of this observation, an experiment was performed, wherein minimal medium irradiated with 200 Krad was split into 3 batches and each was seeded with bacteria from a log phase culture of gal 22 λ s at inoculum sizes differing by a factor of 10. The results are summarized in Table 6 and graphically represented in Fig. 6.

It is seen that the bacterial killing produced by irradiated medium is greater when smaller numbers are incubated into it, suggesting that the amount of bactericidal principle available per bacterium is an important factor in producing cell death. No mathematical relationship between the inoculum size and the bactericidal effect was, however, found.

(v) Resistance to irradiated medium of previously exposed bacteria:

Experiments were performed to see if bacteria surviving treatment in irradiated medium acquire resistance to a subsequent exposure. Bacteria from a log phase culture of E. coli gal 22 λ s were inoculated into medium irradiated with 200 Krad of X-rays at a cell density of $\sim 10^6$ bacteria per ml. and viable counts made at hourly intervals for 4 hours. Incubation in irradiated medium was continued for another 16 hours after which a sample from it was transferred into unirradiated medium to obtain a log phase

culture. A sample from the descendents of bacteria that had survived one exposure to irradiated medium was exposed to irradiated medium again and survival determined at one hour intervals. The same cycle of treatments was repeated a second time to see the response of cells that had been exposed to irradiated medium twice. The steps involved in these treatments are schematically outlined below:

Log phase culture of gal 22 λ s.

1st exposure — Medium irradiated with 200 Krad

Incubation at 37°C for 20 hours.
viable counts made for 1st 4 hours.
Inoculation into unirradiated medium to obtain log phase culture

2nd exposure — Medium irradiated with 200 Krad.

Incubation at 37°C for 20 hours.
viable counts made for first 4 hours.
Inoculation into unirradiated medium to obtain log phase culture.

3rd exposure — Medium irradiated with 200 Krad.

viable counts for 4 hours.

The results are given in Table 7 and Fig. 7. The results showed that bacteria that had received one exposure to irradiated medium acquired a significant resistance to a second exposure while those that had been exposed twice did not show any decline in viability when exposed to the treatment a third time. The resistance of previously exposed bacteria to subsequent treatment

Table 7. Sensitivity to irradiated medium of gal 22 λ s that had been previously exposed to irradiated medium.

Treatment	% Survival after hours of incubation in irradiated medium.				
	0	1	2	3	4
1st exposure to irradiated medium	100	6·00	5·42	5·42	6·85
2nd exposure to irradiated medium	100	29·39	25·15	29·39	30·30
	Viable counts per ml.				
3rd exposure to irradiated medium	$1·1 \times 10^7$	-	$4·4 \times 10^7$	$8·2 \times 10^7$	$2·0 \times 10^8$
CONTROL	$8·4 \times 10^6$	$1·05 \times 10^7$	$1·95 \times 10^7$	$3·35 \times 10^7$	$5·80 \times 10^7$

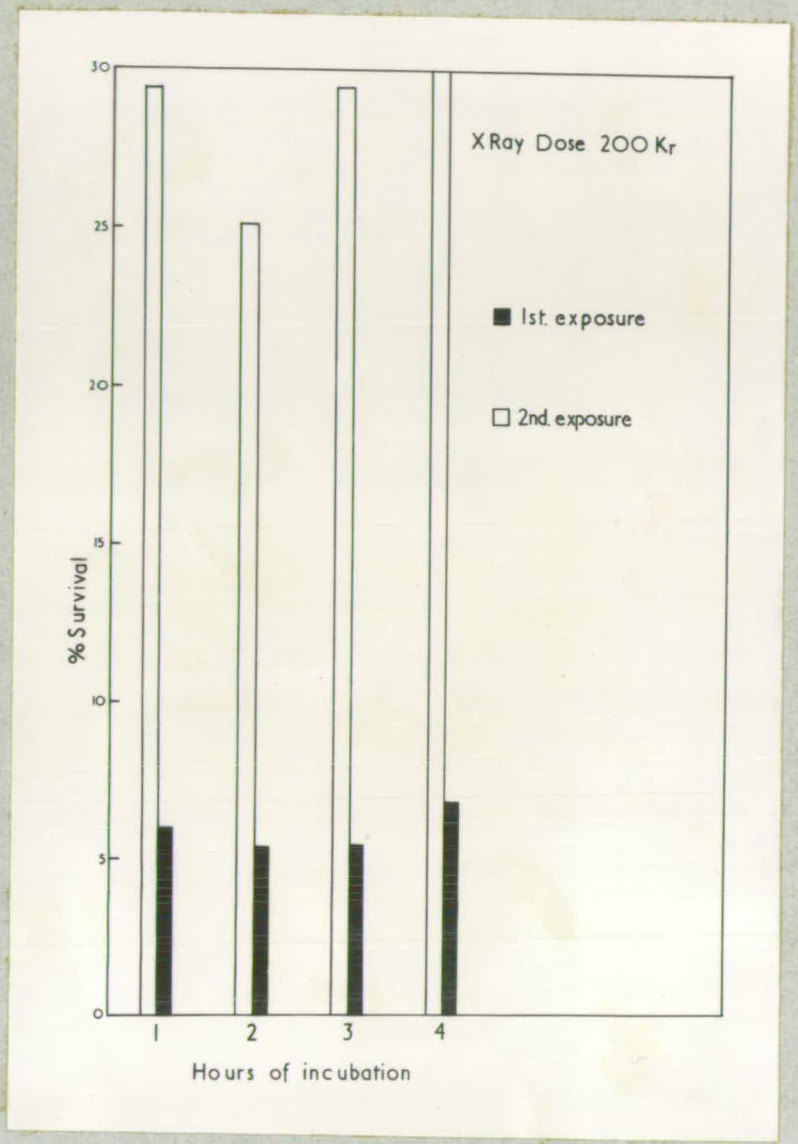

Fig. 7: The sensitivity to irradiated medium of E. coli that had been previously exposed to irradiated medium.

may be due to acquired tolerance or alternatively it may represent population of a genetically resistant strain derived from a mutant induced or selected by the treatment with irradiated medium. If the latter alternative is correct, radiation pasteurization of food can present a health hazard by providing means of induction and proliferation of harmful resistant strains in the absence of competition from the less resistant species that give indications of food spoilage. This point will be discussed further later.

(b) EXPERIMENTS WITH γ-RAY IRRADIATED MEDIUM

(i) Dose response curves:

Experiments using γ-ray irradiated medium were performed at the Wantage Research Laboratory of the U.K. Atomic Energy Authority. The radiation source used was a 4000 Ci/Co^{60} 'Hotspot', giving a dose rate of 17,321 rads per minute. Dose response curves for γ-ray dosages of 50, 100, 200, 500 Krad and 1 Mrad are presented in Fig. 8. The experiment was repeated twice with similar results. Table 8 and Fig. 8 are based on results of one of the two experiments.

It was found that for any given period of incubation in irradiated medium up to 4½ hours, the bactericidal effect increased with increasing γ-ray dose. Thus while 4 hour incubation in medium irradiated with 50 Krad reduced the survival to only 35%, the same period of incubation in medium exposed to 500 Krad reduced the survival to 0·08%. For each dose, with the exception of 500 Krad, the survival curves exhibited a shoulder

Table 8. Effect on survival of gal 22 λ s incubated in medium exposed to five dosages of Y-rays.

Y-ray dose to the medium (Kilo rads)	% Survival after incubation in irradiated medium for hours				
	0	1	2	3	4·5
50	100	100	64·3	42·1	31·5
100	100	57·5	7·8	2·0	1·6
200	100	50·5	2·2	0·9	0·36
500	100	8·5	1·6	0·12	0·07
1000	100	36·5	1·8	0·27	0·15
	Viable counts per ml.				
CONTROL	$1·7 \times 10^6$	$2·8 \times 10^6$	$4·3 \times 10^6$	$7·0 \times 10^6$	$1·5 \times 10^7$

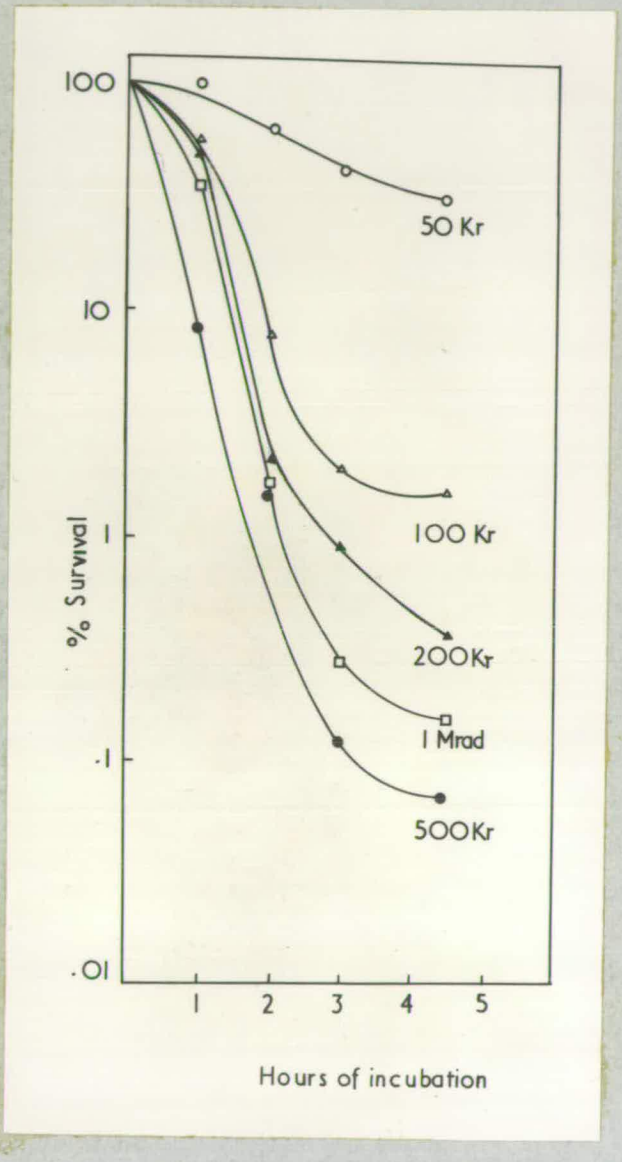

Fig. 8: The effect on survival of E. coli incubated in minimal medium exposed to five dosages of gamma rays.

for the first hour of incubation. This may correspond to the time taken by the bactericidal principle/s to reach the biologically important site within the bacterium. After the first hour, and up to 3 hours' incubation, the drop in survival was exponential. After 3 hours, a gradual levelling off of the survival sets in. The maximum bactericidal activity was exhibited by medium irradiated with 500 Krad. An increase beyond this dose decreased the cytotoxicity of irradiated medium suggesting that the bactericidal principle/s reach a saturation point at 500 Krad and increments of dose over 500 Krad serve only to inactivate it, thereby reducing its effectiveness.

(ii) Effect of dose rate on the bactericidal effect of irradiated medium.

A comparison of survival curves of bacteria incubated in X-irradiated and ϒ-ray irradiated medium showed the latter to be markedly steeper. Since an obvious difference between the two treatments, besides the energy values of the incident radiation, was the rate at which the radiation dose was delivered (943 rad per minute for X-rays; 17,321 rads per minute for ϒ-rays) it was considered likely that the production of bactericidal principle in the irradiated medium may be dose rate dependent. The possibility was examined by testing the bactericidal action of 200 Krad ϒ-irradiated medium when the dose was delivered either in 11 minutes 32 seconds (acutely) or in 19 hours (chronically). The results are presented in Table 9 and Fig. 9. It can be seen that the production of bactericidal principle in irradiated medium

Table 9. Survival of gal 22 λ s treated with medium irradiated with 200 Krad of γ-rays at two different dose rate

Dose rate	% Survival after incubation in medium for hours				
	0	1	2	3	4
943 rad per min.	100	83·7	75·1	27·5	2·8
17,321 rad per min.	100	54·2	19·9	2·8	0·36

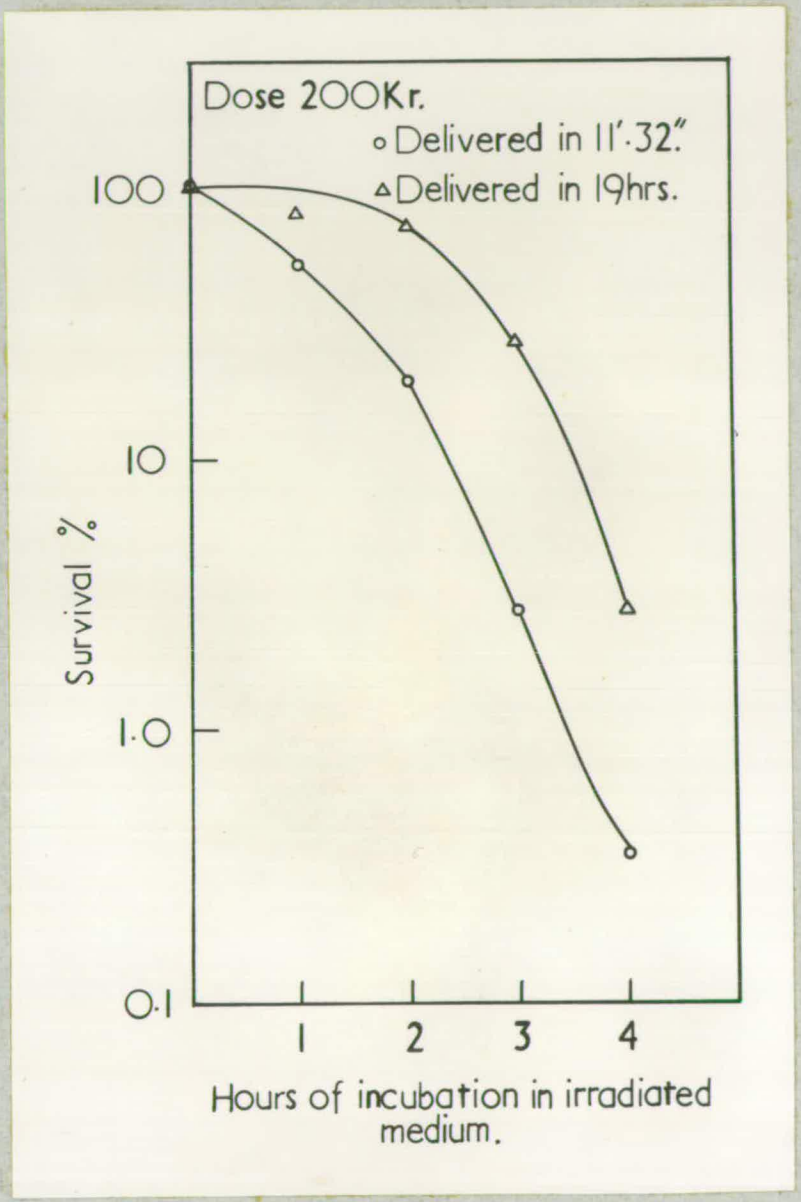

Fig. 9: Survival of E. coli treated with minimal medium irradiated with 200 Krad of gamma rays at two different dose rates.

is, in fact, dose rate dependent. A four hour incubation in irradiated medium, for example, produces 8 times as much killing when the dose is delivered acutely than when delivered chronically.

This observation has important bearing on the design of radiation sources meant for food sterilizing programmes. To obtain the maximum elimination of spoilage organisms from radiation preserved food, it will be of obvious advantage to have powerful sources that are capable of delivering the sterilizing dose in the shortest interval of time.

*(iii) Identification of the effective bactericidal component in γ-ray irradiated medium.

Results of the effect on survival of bacteria incubated in X-irradiated minimal medium or its components have already been described. The experiments were repeated using high dose rate irradiation from a Co^{60} γ-ray source. Irradiation of salts, which could not be done with X-rays, was included in these experiments. The composition of irradiated mixture and the unirradiated supplements added subsequent to the irradiation of component in question were as follows:

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* A preliminary report of this work has been published in Microbial Genetics Bulletin 25 (1966).

Component under consideration	Composition of Irradiated mixture	Unirradiated supplements added subsequent to irradiation
Water	9·75 ml. water	1·25 ml. M_9 salts; 1·25 ml. 1% glucose soln; 0·1 ml. each of 0·1M $MgSO_4$ and 0·01M CaCl
Glucose	1·25 ml. 1% glucose soln. + 8·5 ml. H_2O.	1·25 ml. M_9 salts; 1·25 ml. sterile water; 0·1 ml. each of 0·1 M $MgSO_4$ and 0·01 M CaCl.
Salts	1·25 ml. M_9 salts + 8·5 ml. H_2O.	1·25 ml. 1% glucose; 1·25 ml. sterile water; 0·1 ml. each of 0·1M $MgSO_4$ and 0·01 M CaCl.
Minimal medium	9·75 ml. M_9 medium	2·7 ml. sterile minimal medium.

The results are contained in Table 10 and Fig. 10. It is observed that the results are qualitatively similar to those obtained with X-irradiation of the medium. It was found that irradiation of only water produced a slight decrease in bacteria surviving treatment for the first two hours after which growth was resumed at about the normal rate. Irradiation of salt solution produced only a lag and no killing. The maximum cytotoxic effect was obtained by irradiation of glucose. When salt-glucose medium was irradiated, the effect was reduced. It thus appears that the salts when present in the irradiated mixture not only reduce the quantity of bactericidal principle produced but also react with radiation product/s of glucose solution responsible for cytotoxicity thereby reducing its effectiveness.

Table 10. Effect on survival of E. coli gal 22 λs incubated in irradiated medium or in medium components of which were irradiated with γ-rays.

Component irradiated	% Survival following incubation in irradiated medium for hours				
	0	1	2	3	5
Minimal medium	100	76·2	28·1	6·5	0·19
Glucose	100	53·6	2·8	0·20	0·05
Water	100	76·5	74·8	115·0	312·5
Salts	100	87·3	105·2	110·0	164·8
	Viable counts per ml.				
CONTROL	$1·2 \times 10^6$	$1·5 \times 10^6$	$2·6 \times 10^6$	$3·9 \times 10^6$	$1·3 \times 10^7$

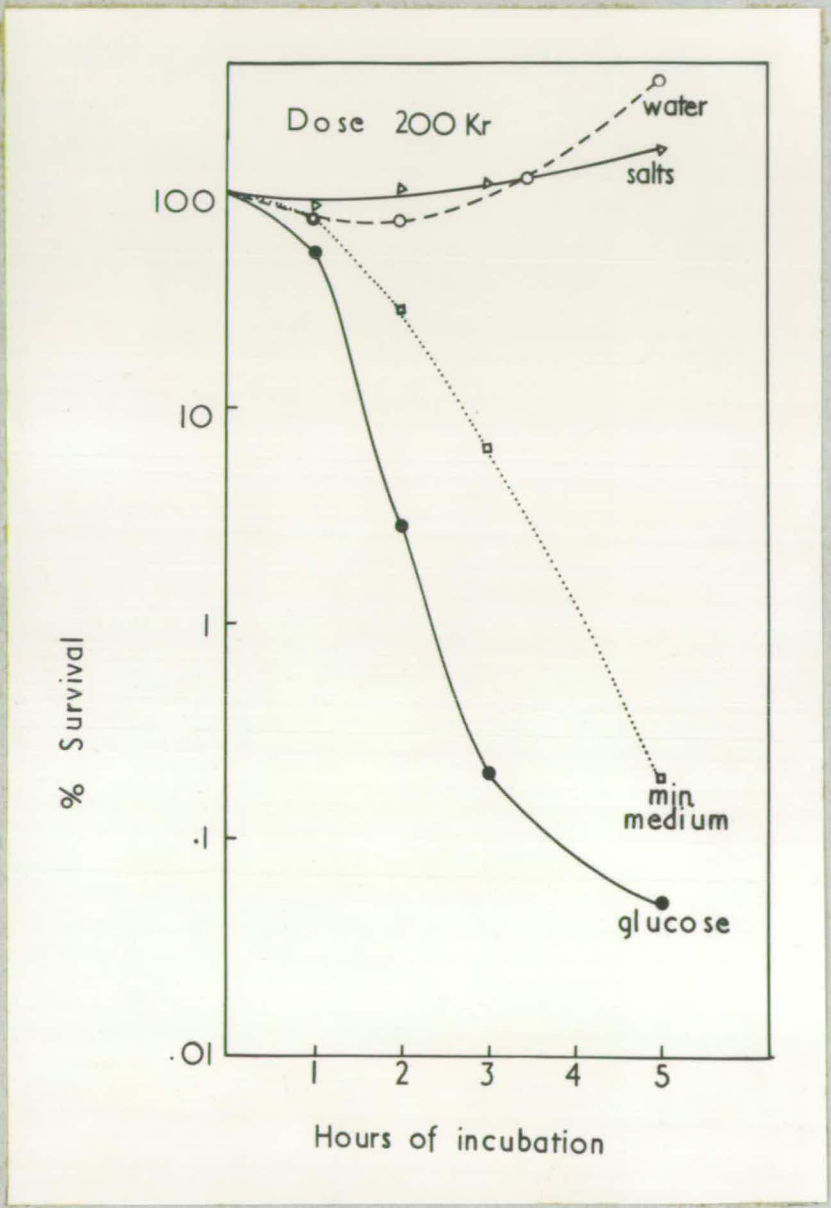

Fig. 10: The effect on survival of E. coli incubated in gamma ray irradiated medium or in medium components of which were irradiated with ɣ rays.

Table 11. The effect of post-irradiation storage at 4°C on the bactericidal activity of irradiated medium.

Day elapsing between irradiation and testing	% survival after incubation in medium irradiated with											
	100 Kr				200 Kr				500 Kr			
	Hours of incubation											
	1	2	3	4	1	2	3	4	1	2	3	4
0	59·2	7·8	2·0	1·5	50·3	2·2	0·9	0·3	8·5	1·6	0·12	0·07
30	86·6	> 100			82·8	67·1	45·7	28·5	81·3	23·7	18·6	5·4
64		> 100				> 100			64·8	62·5	> 100	

(iv) Stability of the bactericidal principle

To test the stability of bactericidal principle generated in the irradiated medium, the lethal effects of γ-ray irradiated medium were tested after 30 and 64 days of storage at 4°C. The results are summarized in Table 11. It was found that the bactericidal activity of irradiated medium decreased with storage and that the residual activity after storage depended upon the radiation dose originally delivered. After a month of storage, medium irradiated with 200 Krad and 500 Krad still produced a drop in survival of bacteria incubated in it and the decrease in survival increased with increasing period of incubation. The cytotoxic principle from 100 Krad irradiated medium, on the other hand was exhausted by the incubated bacteria in the first hour and growth was resumed afterwards. After 64 days of storage, there was no bactericidal activity left in the medium irradiated with 100 Krad and 200 Krad and a small amount of activity remaining in 500 Krad irradiated medium was exhausted within two hours.

(v) pH of the irradiated medium:

X-ray dose of 226 Krad did not change pH of the minimal medium used in the present study. Determinations made before commencement of the irradiation and immediately after it, showed the pH to remain constant at 6·9. The killing observed, thus, cannot be attributed to a change in this factor.

II. MUTAGENIC EFFECT OF IRRADIATED MEDIUM

The ability of irradiated medium to induce mutations was studied in two types of systems:

(a) Forward mutations from susceptibility to resistance to streptomycin in E. coli K-12.

(b) Reverse mutations to prototrophy in a triple auxotrophic strain (thr^-, leu^-, pro^-) in E. coli.

Reverse mutations:

Mutations to independence from requirement for three amino acids, threonine, leucine and proline following treatment in X-irradiated medium were studied in strain AB712. A culture of bacteria grown to log phase in nutrient broth was centrifuged and washed free of broth by three washings in minimal medium. The pellet after the third washing was suspended in minimal medium irradiated with 226 Krad of X-rays to give a cell density of about 10^8 bacteria per ml. and incubated with aeration at 37°C for two hours. The treated bacteria were centrifuged, washed in minimal medium and incubated at 37°C in nutrient broth for 3 hours to allow fixation and expression of induced revertants. The bacteria were then washed free of nutrient broth and resuspended in minimal medium at a cell density around 10^{10} per ml. For scoring reversions 0·1 ml. of the suspension was spread on the surface of each of suitably supplemented plates (containing 20μg per ml. of the required amino acids except the one for which reversions were being scored). Revertants were scored after 48 hour incubation at 37°C. Suitable controls, incubated in unirradiated medium but otherwise handled in the same way as the treated series

were kept for each experiment.

Because of the limit imposed on the volume of medium that could be X-rayed at one time, reversions to more than two markers could not be studied in one and the same experiment. Exploratory experiments showed that the effect of inoculum size, observed for the lethal effect of the irradiated medium, was operative also for the mutagenic effect. Experiments in which the irradiated medium was seeded with bacteria at densities greater than 5×10^8, gave inconsistent results making detection of a mutagenic effect more difficult.

Results of reversions induced by X-irradiated medium at the three loci thr^-, leu^- and pro^- in AB712 are given in Table 12. It can be seen that for leu^- and thr^- loci, irradiated medium has a weak mutagenic effect which can be demonstrated only by comparison with corresponding controls. The proline marker, however, did not respond to mutagenic effect of irradiated medium. In experiment 4 of Table 12, reversions to pro^+ and thr^+ were studied in the same treated suspension. It was found that while about 20 times more thr^+ revertants compared to control were obtained following treatment with X-rayed medium, there was no increase over control in the frequency of pro^+ revertants. The mutagenic response of pro^- relative to those of thr^- or leu^- to treatment with direct irradiation or with other mutagens is not known. However, the inability of irradiated medium to induce reversions at the pro^- locus suggests that different genes may respond quite differently to treatment with irradiated medium.

In experiments listed in Table 12, a post incubation for

Table 12. Reversions to thr^+, leu^+ and pro^+ induced by X-ray irradiated minimal medium in *E. coli* AB712.
(Experiments involving post treatment incubation in Nutrient Broth)

Expt. No.	Treatment	Survival %	Gene studied	Bacteria Screened	Reversions No.	Reversions/10^8 survivors
1	Control	100	leu	$8{\cdot}2 \times 10^{10}$	33	0·04
	X-ray	98·9	"	$8{\cdot}0 \times 10^{10}$	587	0·73
	Control	100	thr	$8{\cdot}2 \times 10^{10}$	46	0·05
	X-ray	98·9	"	$8{\cdot}0 \times 10^{10}$	511	0·63
2	Control	100	leu	$1{\cdot}8 \times 10^{11}$	21	0·01
	X-ray	86·04	"	$1{\cdot}02 \times 10^{11}$	602	0·59
	Control	100	thr	$1{\cdot}8 \times 10^{11}$	62	0·03
	X-ray	86·04	"	$1{\cdot}02 \times 10^{11}$	592	0·58
3	Control	100	pro	$1{\cdot}2 \times 10^{10}$	4	0·03
	X-ray	89·2	"	$3{\cdot}1 \times 10^{10}$	16	0·05
4	Control	100	pro	$5{\cdot}6 \times 10^{10}$	89	0·15
	X-ray	62·5	"	$2{\cdot}4 \times 10^{10}$	50	0·20
	Control	100	thr	$5{\cdot}6 \times 10^{10}$	78	0·13
	X-ray	62·5	thr	$2{\cdot}4 \times 10^{10}$	682	2·84

3 hours in nutrient broth was given before the treated bacteria were challenged with the selective media. Approximately 9 bacterial divisions will take place in this duration. If independence of a nutritional requirement were to confer a faster rate of growth on the prototrophs, a spurious increase in mutation rate could result from post-treatment incubation in nutrient broth. The following two experiments, however, ruled out the possibility of this factor being involved in the experiments described above.

(1) A reconstruction experiment was done by mixing known numbers of triple requirers with revertants for threonine and incubating the mixed population in nutrient broth for 3 hours. The relative proportion of the two types of bacteria was determined by plating washed suspension on suitably supplemented media. The results are given below.

Duration of incubation in nutrient broth (hours)	Viable bacteria of genotype		ratio $\frac{thr^-,leu^-,pro^-}{leu^-,pro^-,thr^+}$
	thr^-,leu^-,pro^-	leu^-,pro^-,thr^+	
0	$1{\cdot}6 \times 10^6$	$2{\cdot}1 \times 10^2$	7619
3	$1{\cdot}8 \times 10^9$	$2{\cdot}3 \times 10^5$	7826

These results showed that the rate of growth of two genotypes in a mixed population was not significantly different and therefore the increased frequency of reversions after treatment in irradiated medium was not due to faster rate of division of the prototrophs during the post treatment incubation in nutrient broth.

(2) In a second experiment, the step of intermediate growth in nutrient broth was dispensed with altogether. Fixation and expression of mutations was achieved by supplementing the minimal medium of mutation plates with limiting amounts (1 μg per ml.) of the amino acid for which reversions were being scored. The remaining nutritional requirements were fully supplemented by adding 20 μg per ml. of the required growth factor. This allowed a limited growth of the auxotrophs giving a background growth. Prototrophic revertants established themselves during this period, however, and grew into macro-colonies. Results of this experiment are given in Table 13.

Table 13. Reversions to thr^+ and leu^+ induced by X-rayed minimal medium.

Treatment	Survival %	Gene studied	Bacteria screened	No. Reversions	Reversions/10^8 survivors
Control	100	leu	4.9×10^9	1	0.02
X-rayed medium	58.4	"	4.0×10^9	29	0.72
Control	100	thr	4.9×10^9	11	0.22
X-rayed medium	58.4	"	4.0×10^9	87	2.17

An increase in mutation frequency over control, of the same order of magnitude as that found in experiments involving post-treatment growth in nutrient broth was again observed showing that a post-treatment growth in nutrient broth is not essential for induction of mutations by treatment with irradiated medium. It is also apparent that the increased incidence of mutations

following the treatment is a result of induction of new mutations and is not due to selection of pre-existing ones.

Forward mutations:

UV, X-ray and Y-ray irradiated medium was tested for its ability to induce forward mutations to streptomycin resistance in a streptomycin sensitive strain of E. coli K-12. Results of treatment with medium irradiated with the three types of radiation will be described separately.

Ultraviolet light (UV).

The source of radiation was a Hanovia low-pressure mercury discharge tube with 85% of its output comprising the band 2537Å in the UV spectrum. Irradiation of the medium was done in large 6 cm. diameter glass dishes, the depth of the medium in the irradiation dish being about 0·5 cm. A total dose of approximately $1{\cdot}8 \times 10^5$ ergs/mm.^{2} was delivered at a distance of 25 cm. from the tube in 3 hours to constantly agitated medium. At the end of UV exposure any loss of volume of the medium was made up by adding required volumes of sterile distilled water. The irradiated medium was inoculated with bacteria from an exponentially growing culture at bacterial densities of approximately 10^6 bacteria per ml. Treatment with irradiated medium lasted two hours in a shaker uncubator maintained at 37°C. 0·1 ml. of suitable dilutions of the treatment mixture were spread on the surface of nutrient agar plates at the start and termination of the treatment for viable counts to determine survival.

At the end of treatment approximately 10^7 viable bacteria

were retained on each of a required number of membrane filters and washed with two 20 ml. lots of sterile minimal medium. The membrane filters were allowed to sit on pre-warmed plates of Brain Heart Infusion agar for 5 hours at 37°C to allow expression of mutants after which they were transferred to nutrient agar plates containing 100 μg per ml. streptomycin and incubated at 37°C. Scoring for streptomycin resistant mutants was done after 48 hours' incubation.

Table 14 contains results obtained from treatment with UV irradiated medium. Results from the three experiments are consistent and show that treatment with UV irradiated medium brings about a significant increase over control in the frequency of mutations to streptomycin resistance. In experiment 3 of Table 14, the mutagenic effectiveness of UV irradiated minimal medium and nutrient broth were tested simultaneously in treatments of bacteria from the same suspension. While treatment with irradiated minimal medium gave a mutation rate similar to the other two experiments, incubation in irradiated nutrient broth gave a much higher mutation frequency. However, treatment in irradiated minimal medium decreased survival whereas irradiated nutrient broth permitted growth during the period of treatment. Valid comparison between the relative mutagenicity of the two media, therefore, cannot be made in the absence of information on the rate of growth of the streptomycin resistant mutants relative to the parent strain. The observation does suggest, nevertheless, that the lethal and mutagenic effects of the irradiated medium are independent events and that treatment that does not produce lethality can still be mutagenic.

Table 14. Mutations to streptomycin resistance in E. coli K-12 induced by medium irradiated with ultraviolet light.

Expt. No.	Medium	Treatment	Survival %	Bacteria screened	S^R mutants	S^R mutants/10^8 survivors
1	Minimal M_9	Control	100	$2{\cdot}8 \times 10^9$	12	0·42
		UV	58·75	$3{\cdot}0 \times 10^8$	32	10·66
2	Minimal M_9	Control	100	$5{\cdot}5 \times 10^8$	2	0·36
		UV	72·53	$2{\cdot}4 \times 10^8$	20	8·33
3	Minimal M_9	Control	100	$5{\cdot}7 \times 10^9$	18	0·31
		UV	81·0	$2{\cdot}8 \times 10^8$	34	12·14
	Nutrient Broth	Control	100	$3{\cdot}6 \times 10^9$	13	0·36
		UV	150	$1{\cdot}7 \times 10^8$	38	22·35

Table 15. Mutations to streptomycin resistance in *E. coli* K-12 induced by medium irradiated with 200 Krad of X-rays.

Experiment No.	Medium	Treatment	Survival %	Bacteria screened	S^R mutants	S^R mutants/10^8 survivors
1	Minimal M_9	Control	100	$6{\cdot}0 \times 10^8$	1	0·16
		X-ray		$5{\cdot}2 \times 10^8$	5	0·96
2	Minimal M_9	Control	100	$1{\cdot}2 \times 10^9$	0	0
		X-ray		$3{\cdot}9 \times 10^8$	4	1·02

X-ray:

Considerable difficulty was experienced in standardizing treatment conditions that will give consistent results with X-irradiated medium. The difficulty arose chiefly from the following two causes (1) only a limited volume of about 40 ml. of medium could be irradiated at any one time with the available X-ray apparatus and (2) the magnitude of the mutagenic effect was dependent on the number of bacteria per ml. seeded into the treated medium.

If the irradiated medium was seeded with many more than 5×10^7 bacteria per ml., the mutagenic effect became very small. The use of a low inoculum size, on the other hand, limited the number of treated bacteria that could be screened and consequently the number of mutants obtained was too low to give meaningful results.

Results obtained from treatment with X-irradiated medium of two experiments are given in Table 15. The initial inoculum size in the treatment mixture of the first experiment was $4{\cdot}8 \times 10^7$ and for the second experiment $3{\cdot}2 \times 10^7$ per ml. The rest of the screening procedure was the same as for treatment with UV irradiated medium. The frequency of streptomycin resistant mutant was small, but still showed a trend towards increase over corresponding control values.

γ-rays:

Experiments to test the effect of γ-ray irradiated medium were performed at the Wantage Research Laboratory, Wantage, where large volumes of medium could be treated in short times (dose rate

Table 16. Mutations to streptomycin resistance in E. coli K-12 induced by medium irradiated with 200 Krad γ-rays.

Experiment No.	Medium	Treatment	Survival	Bacteria screened	S^R mutants	S^R mutants/10^8 survivors
1	Minimal M_9	Control	100	1.7×10^8	3	1.76
		γ-rays 200 Krad	11.1	1.4×10^8	21	15.0
2	Minimal M_9	Control	100	2.2×10^9	8	0.36
		γ-rays 200 Krad	16.2	8.3×10^8	86	10.36

17321 rad per minute). Minimal medium was irradiated with 200 Krad and seeded with bacteria at densities close to 10^6 per ml. The treatment in irradiated medium extended over two hours. Subsequent screening procedure was the same as has already been described for UV irradiated medium. The results are given in Table 16. As can be seen, in both the experiments, the frequency of mutations to streptomycin resistance was significantly higher than in the control.

From the foregoing it is clear that when suitable screening systems are used, either for forward or for reverse mutations, the mutagenic effect of the irradiated medium can be demonstrated.

III. LETHAL AND MUTAGENIC EFFECTS OF GLYOXAL*

Berry, Hills and Trillwood (1965) have found that irradiated carbohydrate solutions are toxic to mammalian cells *in vitro*. They attributed the toxicity to glyoxal $(CHO)_2$ produced in these solutions from radiation decomposition of carbohydrates. Since glyoxal is a stable compound and is, therefore, likely to persist for considerable periods in carbohydrate rich foods preserved by radiation, the lethal and mutagenic effects of glyoxal were studied in *E. coli*.

(a) Lethal Effect:

In order to test if a correspondence, similar to that found for mammalian system, is obtained between toxic effects of a given radiation dose to the medium and of glyoxal concentration in the medium generated by this dose, the lethal effect of glyoxal was studied on the same organism (*E. coli* K-12) and under the same experimental conditions as prevailed during treatment with irradiated medium.

Glyoxal monohydrate (B.D.H.) was prepared as a stock solution containing 10 mg./ml. in distilled water and sterilized by autoclaving at 15 lbs. pressure for 20 minutes. Appropriate volumes of the stock solution were added to M_9 medium to get the desired glyoxal concentrations in the treatment medium. Viable counts were made at one hour intervals from commencement of the treatment, by spreading 0·1 ml. of a suitable dilution of the treatment mixture on the surface of nutrient agar plates. Dilutions of 10^{-2} to 10^{-5} at which viable counts are scored to

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* A preliminary report of this work has been published in Microbial Genetics Bulletin 25 (1966).

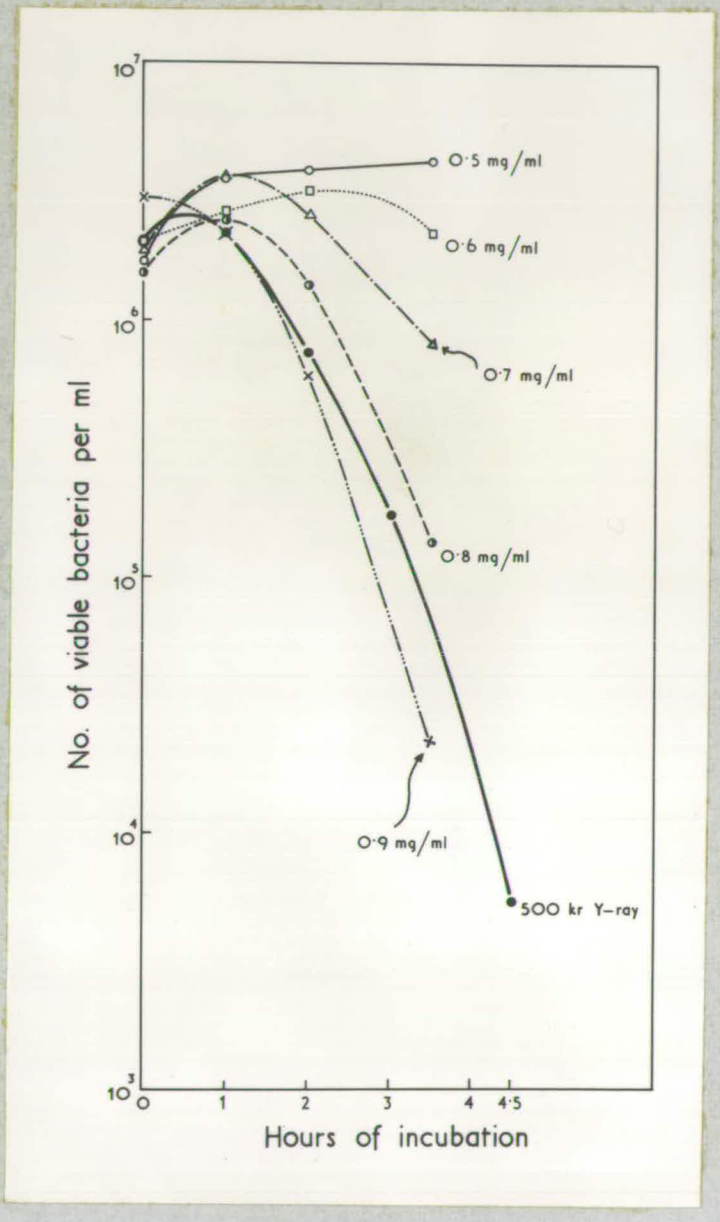

Fig. 11: The effect on survival of E. coli incubated in medium containing different concentrations of glyoxal.

obtain 100-200 colonies per plate effectively terminate the treatment by diluting out the glyoxal.

Five experiments, all giving reproducible results, were carried out using glyoxal concentrations ranging from 0·2 mg./ml. to 1 mg./ml. Results from a representative experiment are given in Fig. 11. It can be seen that glyoxal does not produce bacterial killing at concentrations up to 0·5 mg./ml. Above this level there is a progressive decrease in the number of colony forming bacteria with increasing glyoxal concentration. Also, the bactericidal effect increases with increasing duration of incubation in glyoxal containing medium.

In order to compare the bactericidal action of glyoxal and irradiated medium, the killing curve produced by irradiating medium with 500 Krad of γ-rays has been included in Fig. 11. It is seen that to simulate the level of lethality induced by 500 Krad to the medium, between 0·8 and 0·9 mg. per ml. glyoxal has to be incorporated into the medium. Berry, Hills and Trillwood (1965) have calculated the concentration of glyoxal in 1% solution of dextrose exposed to 2·5 Mrad of γ-rays to be 0·22 mg. per ml. It is thus apparent that the lethal effect of irradiated medium in the bacterial system cannot be ascribed to radiation produced glyoxal.

(b) Mutagenic effect:

To test the mutagenic effect, mutations from tryptophan requirement to tryptophan independence ($try^- \longrightarrow try^+$) in WP-2, a tryptophan requiring B/r strain of *E.coli*, were studied after glyoxal treatment.

(i) Mutations to try$^+$ in an untreated culture of E. coli WP-2 try$^-$

Spontaneous mutations to tryptophan independence in an untreated culture of WP-2 were found to show some peculiarities which merit consideration. Before going on to the mutagenic effect of glyoxal treatment, these will first be described.

When an exponential culture of WP-2 grown in minimal medium supplemented with 6 μg per ml. tryptophan was sampled for try$^+$ revertants by plating on minimal medium, the rate of background mutations was found to maintain itself at a low stable level of about 10^{-8}. If there is no selection against newly arising mutations one would normally expect an accumulation of revertants with growth. In several experiments, however, no such accumulation could be detected. Two possible explanations for this observation were considered.

(a) The prototrophs that have been growing in medium containing tryptophan are so conditioned that they fail to grow when plated on minimal medium. This possibility was tested by growing a population of try$^+$ revertants in tryptophan rich medium and plating a suitable dilution of it on three types of media for viable counts: (i) minimal medium, (ii) minimal medium supplemented with 1μg per ml. tryptophan, and (iii) nutrient agar. The viable counts on the three plating media were identical showing that neither the tryptophan content of liquid medium in which the revertants have been grown nor the richness of the plating medium has any influence on growth of the try$^+$ revertants.

(b) The possibility of an active selection against prototrophic revertants was next examined. 200 ml. of minimal medium

supplemented with 6 μg. per ml. tryptophan was inoculated with bacteria from an overnight culture at densities ranging from 2×10^6 to $2{\cdot}9 \times 10^8$ per ml. in different experiments. The frequency of try^+ revertants was determined every hour of subsequent growth up to 4 or 5 hours by spreading 0·1 ml. of the culture on a suitable number of minimal plates. Experiments in which cultures were started at low initial inocula, mutation rates were determined by inplating technique so that a sufficiently large bacterial population could be screened without having to use a very large number of plates. Results from two experiments are given in Tables 17 and 18. It is seen that with the growth of the culture, there was a progressive decrease in the frequency of try^+ revertants, demonstrating that the rate of mutations to try^+ in a growing batch culture of WP-2 is not proportional to growth rate; in fact there is a positive selection against the try^+ revertants.

To determine the stage at which the selection becomes operative, a reconstruction experiment was done in which known numbers of try^- and try^+ bacteria were mixed and grown at 37°C in minimal medium supplemented with 6 μg. per ml. tryptophan. Samples were plated at intervals on minimal agar or minimal agar + 20 μg. per ml. tryptophan to determine the number of try^+ bacteria relative to the total number (i.e., $try^- + try^+$). The data are given in Table 19. It was seen that the proportion of try^+ relative to the total number of bacteria remained constant during 3 hours of growth of the mixed population. Two repetitions of the experiment gave similar results. These experiments showed that the selection

Table 17. Frequency of try^+ revertants at intervals of growth in 6 μg/ml. tryptophan supplemented medium.

Duration of growth	Viable counts /ml.	Number of bacteria screened	Number of try^+ revertants	$try^+/10^8$
0	$2{\cdot}9 \times 10^8$	$3{\cdot}4 \times 10^9$	30	0·88
1	$6{\cdot}4 \times 10^8$	$9{\cdot}6 \times 10^9$	28	0·29
2	$9{\cdot}9 \times 10^8$	$1{\cdot}4 \times 10^{10}$	31	0·22
3	$1{\cdot}9 \times 10^9$	$2{\cdot}8 \times 10^{10}$	50	0·17
4	$2{\cdot}0 \times 10^9$	$3{\cdot}0 \times 10^{10}$	37	0·12

Table 18. Frequency of try^+ revertants at intervals of growth in 6 µg/ml. tryptophan supplemented medium.

Duration of growth	Viable counts per ml.	Number of bacteria screened	Number of try^+ revertants	$try^+/10^8$
0	$1{\cdot}3 \times 10^8$	$2{\cdot}2 \times 10^9$	18	0·81
1	$3{\cdot}4 \times 10^8$	$5{\cdot}1 \times 10^9$	19	0·37
2	$4{\cdot}8 \times 10^8$	$7{\cdot}2 \times 10^9$	16	0·22
3	$7{\cdot}2 \times 10^8$	$1{\cdot}0 \times 10^{10}$	21	0·21
4	$1{\cdot}3 \times 10^9$	$1{\cdot}9 \times 10^{10}$	17	0·087
5	$1{\cdot}3 \times 10^9$	$1{\cdot}8 \times 10^{10}$	23	0·12

Table 19. Number of try^+ bacteria relative to total number in a mixed population of try^- and try^+ bacteria grown in minimal medium supplemented with 6 μg/ml tryptophan.

Hours of growth	Total number of bacteria (try^- + try^+) per ml.	Number of try^+ bacteria per ml.	Ratio $\frac{try^+}{\text{Total bacteria}}$
0	$2{\cdot}87 \times 10^7$	$3{\cdot}30 \times 10^3$	$1{\cdot}1 \times 10^{-4}$
1	$4{\cdot}41 \times 10^7$	$4{\cdot}95 \times 10^3$	$1{\cdot}1 \times 10^{-4}$
2	$1{\cdot}29 \times 10^8$	$1{\cdot}30 \times 10^4$	$1{\cdot}0 \times 10^{-4}$
3	$3{\cdot}72 \times 10^8$	$3{\cdot}62 \times 10^4$	$0{\cdot}97 \times 10^{-4}$

against try$^+$ operates at the stage of nascent prototrophs while completed revertants are no longer selected against.

(ii) Spontaneous reversions to try$^+$ on medium supplemented with 1 μg per ml. tryptophan.

In mutation experiments designed to score prototrophic revertants from an auxotrophic strain, plating is routinely done on media supplemented with limiting amounts of required growth factors to allow for fixation and expression of induced mutants. When untreated WP-2 try$^-$ was plated on minimal medium enriched with 1 μg per ml. tryptophan, which allowed limited growth on the plates, the mutation rate became proportional to growth and new mutants arose on the plate. The selection against new prototrophs observed for growth in the liquid medium did not operate for mutations arising on the plate.

It was further observed that the average number of these plate mutants remained nearly constant and independent of plating density. This is borne out by results of one of several experiments, summarized in Table 20, that were performed to verify this observation. It is seen that when the number of bacteria spread on a plate is reduced by a factor of 10, the number of revertants is not reduced to 1/10th but remains nearly the same as for undiluted plating. Disproportionately higher mutation rates per plated bacteria are, therefore, obtained when the same suspension is plated at progressively higher dilutions. The reason for this "dilution effect" was investigated and it was found to be due to the fact that the final number of bacteria

Table 20. Number of try$^+$ revertants per plate on 1µg/ml. tryptophan enriched medium at four plating densities and their relationship to the population density attained after 24 hours' incubation.

Number of bacteria plated per plate	Number of bacteria attained per plate in 24 hours incubation at 37°C	Number of revertants observed (on 15 plates)	Number of revertants per plate	Number of revertants per 10^8 of final number attained
$1{\cdot}4 \times 10^8$	$1{\cdot}2 \times 10^{10}$	161	10·7	0·089
$1{\cdot}4 \times 10^7$	$1{\cdot}03 \times 10^{10}$	140	9·3	0·090
$1{\cdot}4 \times 10^6$	$8{\cdot}3 \times 10^9$	108	7·2	0·086
$1{\cdot}4 \times 10^5$	$8{\cdot}2 \times 10^9$	109	7·2	0·087

attained on a plate is almost constant over plating densities of 10^5 to about 5×10^8 bacteria per plate (column 2 of Table 20). The slight reduction in the number of revertants per plate as also in the number attained by bacteria on a plate at very low plating densities can be attributed to exhaustion of glucose from the plates before the lack of tryptophan becomes a limiting factor for growth.

(iii) Mutagenic effect of glyoxal in E. coli WP-2.

For studying the mutagenic effect of glyoxal, treatment was carried out, unless otherwise specified, in M medium of Haas and Doudney supplemented with 6 μg. per ml. tryptophan. Concentrations of 0·25 mg. per ml. to 1 mg. per ml. were prepared by adding required volumes of autoclaved sterilized stock solution containing 10 mg. per ml. glyoxal monohydrate in distilled water. In some experiments the treatment was done in M medium minus glucose. All treatments were given to bacteria in logrithmic phase of growth at 37°C.

At the end of treatment, cells were washed free of glyoxal by centrifugation and resuspended in minimal medium minus glucose. 0·1 ml. of the treated suspension was spread on the surface of each of minimal agar plates supplemented with 0·75 μg. tryptophan per ml. Reversions were scored after incubation for 48 hours at 37°C.

Results of 9 experiments when glyoxal treatment was given in tryptophan supplemented medium are given in Table 21. Mutation rates in Table 21 are given both as mutations per plate as well as per survivors. The corrected mutation rate per plate is obtained by subtracting the mean number of colonies per plate in the control

series from the mean number per plate of the corresponding treated series. Mutations per 10^8 survivors are arrived at by dividing the corrected number of mutations per plate by the number of live bacteria spread on a plate and then computing the number per 10^8 plated bacteria.

It was found that when the treatment was done in medium supplemented with tryptophan, at concentrations less than 0·5 mg. per ml., glyoxal produced no increase of mutation rate over control.

However, when concentrations higher than 0·5 mg. per ml. were used, in all paired comparisons glyoxal treatment gave a higher mutation rate than the corresponding control.

A perusal of Table 21 shows considerable variation between experiments in control mutation rates. This variation is due to the difference in plating densities. It will be recalled that for any particular culture, spontaneous reversions to try^+ per plate remain constant independent of the number of bacteria spread on a plate. For instance if, say, 10 reversions appear on a plate when 10^6 bacteria are spread on it, on an average 10 reversions per plate will still be obtained when plating densities are 10^5 or 10^7 per plate. Thus, if control mutation rates are expressed per bacteria plated, large variations can be obtained for even the same cell suspension when plated at different bacterial densities. When this factor is taken into consideration, variation of mutation rates between experiments falls within the range expected between different cultures.

Similarly, variation in mutation rates produced by the same glyoxal treatment in different experiments is also evident.

Table 21. Reversions to try$^+$ induced by glyoxal treatment in M medium supplemented with 6 μg. tryptophan per ml. in WP-2 try$^-$.

Expt. No.	Treatment		Survival %	Live bacteria per plate	Corrected mutations	
	Concentration mg./ml.	Duration of treatment (minutes)			per plate	per 10^8 survivors
(1)	(2)	(3)	(4)	(5)	(6)	(7)
1	0	0	100	$6{\cdot}05 \times 10^9$	7·1	0·11
	0·5	120	69·2	$1{\cdot}0 \times 10^9$	36·6	3·66
2	0	0	100	$9{\cdot}0 \times 10^9$	9·4	0·10
	0·5	120	97·3	$2{\cdot}2 \times 10^9$	32·3	1·46
3	0	0	100	$8{\cdot}3 \times 10^7$	7·0	8·43
	0·5	120	93·5	$7{\cdot}6 \times 10^7$	47·9	63·02
4	0	0	100	$6{\cdot}0 \times 10^6$	1·7	28·33
	1·0	60	0·9	$4{\cdot}1 \times 10^6$	3·7	90·24
5	0	0	100	$5{\cdot}8 \times 10^6$	6·8	117·24
	1·0	60	0·03	$2{\cdot}0 \times 10^6$	15·7	785·0
6	0	0	100	$2{\cdot}0 \times 10^7$	4·5	22·50
	1·0	90	3·3	$1{\cdot}4 \times 10^7$	16·0	114·28
7	0	0	100	$8{\cdot}4 \times 10^6$	7·2	85·71
	1·0	90	0·14	$7{\cdot}7 \times 10^6$	11·1	144·15
8	0	0	100	$6{\cdot}4 \times 10^6$	4·9	76·56
	1·0	90	0·04	$4{\cdot}3 \times 10^6$	20·8	483·72
9	0	0	100	$9{\cdot}2 \times 10^6$	7·0	76·08
	1·0	90	0·03	$2{\cdot}2 \times 10^6$	6·9	313·63

Table 22. Relative sensitivity to glyoxal treatment of try^- and two independently isolated try^+ revertants in mixed population.

Revertant No.	Duration of growth in 0·8 mg./ml. glyoxal medium	Number of viable bacteria per ml. of genotype			ratio $\frac{try^-}{try^+}$
		$try^+ + try^-$	try^-	try^+	
1	0	$1{\cdot}26 \times 10^8$	$4{\cdot}17 \times 10^6$	$1{\cdot}21 \times 10^8$	0·034
	1 hour	$7{\cdot}40 \times 10^6$	$2{\cdot}58 \times 10^5$	$7{\cdot}14 \times 10^6$	0·036
2	0	$8{\cdot}30 \times 10^7$	$6{\cdot}59 \times 10^4$	$8{\cdot}29 \times 10^7$	0·00079
	1½ hour	$5{\cdot}61 \times 10^5$	$8{\cdot}62 \times 10^2$	$5{\cdot}60 \times 10^5$	0·0015

Though it is difficult to offer a complete explanation for this observation, it seems likely that the background of dead cells influences the yield of mutations. This is suggested by the observation that experiments in which survival was low, the mutation rates were high. In mutation experiments, at very low survivals, the number of dead bacteria plated alongside the live bacteria becomes high. These dead bacteria on the mutation plates could provide material for feeding and allow fixation of mutants that would otherwise be lost.

Two other possibilities that could account for the higher mutation rates at low survivals were tested and excluded. These are:

(a) Glyoxal treatment preferentially selects for try^+ revertants. to test this possibility, reconstruction experiments were done in which try^- and spontaneous or glyoxal induced try^+ bacteria from growing cultures were mixed and the mixture treated with glyoxal. If glyoxal treatment really selects for try^+ bacteria, the ratio $\frac{try^-}{try^+}$ should be lower at the end of than at the beginning of glyoxal treatment. The results of such a test of two revertants is given in Table 22. The slightly better survival of try^- bacteria in the two experiments may be fortuitous. The results do show, however, that treatment of a mixed population of try^- and try^+ bacteria with glyoxal does not preferentially select for try^+ genotype.

(b) Glyoxal produces delayed mutations: At lower plating densities, the plated bacteria can undergo more residual divisions on the plate. If division dependent delayed mutations are produced by glyoxal treatment, higher mutation rates will be realized when

plating densities are low.

This was tested by plating the glyoxal treated suspension of bacteria at progressively greater dilutions. The results of one of the experiments in which plating densities differed by a factor of two are given below.

Glyoxal treatment	Survival %	Plating density per ml.	Corrected try^+ mutants / 10^6 survivors
1 mg./ml. for 1 hour	41·6	$6·0 \times 10^8$	0·125
		$3·0 \times 10^8$	0·110

The results do not suggest that delayed mutations are induced by glyoxal treatment.

In another series of experiments, glyoxal treatment was carried out in medium devoid of tryptophan and glucose. The object was to see if bacterial growth and division in the presence of glyoxal was necessary for the latter to be mutagenically effective. The results are summarized in Table 23. It was observed that, in general, lethality resulting from glyoxal treatment was significantly lower when the treatment was done in buffered salts (M medium lacking glucose and tryptophan) compared to treatment carried out in medium that supported bacterial growth. Glyoxal was found to be mutagenic, however, even when administered to bacteria that were not dividing during the treatment period. No correlation between lethal and mutagenic action of glyoxal was observed. For example, for 60 minute treatment in medium containing glyoxal, the per cent survival decreased from 96·6 to 5·0 as glyoxal concentration was increased from 0·25 to 1 mg. per

Table 23. Reversions to try^{+} induced by Glyoxal treatment in M medium minus glucose in WP-2 try^{-}

Experiment No.	Treatment Concentration mg./ml.	Treatment Duration Minutes	Survival %	Live cells plated/ml.	Corrected Mutations per plate	Corrected Mutations per 10^{8} survivors
1	1	60	5	$5{\cdot}2 \times 10^{9}$	77·6	14·9
	0·75	60	66·6	$5{\cdot}1 \times 10^{9}$	66·6	13·0
	0·5	60	80·0	$4{\cdot}6 \times 10^{9}$	49·3	10·7
	0·25	60	96·6	$2{\cdot}9 \times 10^{9}$	33·5	11·5
	0	0	100	$1{\cdot}5 \times 10^{9}$	6·7	4·46
2	1	30	83·7	$5{\cdot}2 \times 10^{9}$	29·4	5·65
	1	60	40·5	$4{\cdot}7 \times 10^{9}$	24·7	5·25
	1	90	7·8	$1{\cdot}0 \times 10^{9}$	16·5	16·5
	0	0	100	$3{\cdot}7 \times 10^{9}$	13·6	3·67

ml. (Experiment 1, Table 23). In spite of these changes in survival, however, no change in the corrected mutation rate was observed. A similar trend was observed when the duration of treatment was varied for the same glyoxal concentration. This suggests that killing resulting from glyoxal treatment represents physiological damage rather than lesions in the genetic material. Another observation was that glyoxal concentration of 0.25 mg. per ml. which never gave a mutagenic effect in growth medium, was effective when the treatment was done in buffered salts.

The relative effectiveness of the two treatment conditions (treatment in buffered salts and treatment in growth medium) was, therefore, next examined by treating bacteria from the same suspension in the two types of media. The results for three glyoxal concentrations are summarized in Table 24. It was again observed that, 0·25 mg. per ml. glyoxal that is not mutagenic in treatment in growth medium is weakly mutagenic for treatment in buffered salts. At higher concentrations, i.e., 0·5 and 1 mg. per ml., however, the corrected mutation rates under the two sets of conditions are not different.

The lack of knowledge about the pathway of glyoxal mutagenesis does not permit a definitive explanation for these observations. However, for UV irradiation, repair of induced mutational damage is known to be better in medium containing glucose compared to that in medium lacking glucose (Witkin, 1959). If a similar situation is obtained for glyoxal mutations, the absence of mutagenic effect of low glyoxal concentration treatment in growth medium compared to a small mutagenic effect for treatment in buffered salts could

Table 24. Comparison of the mutagenic effect of glyoxal in treatments carried out in growth medium and minimal salt solution.

Experiment No.	Treating medium.	Glyoxal conc. Duration of treatment 60 min.	Survival %	Try^+ mutations per plate	Corrected mutations per 10^8 survivors
1	Minimal salt soln.	0·25 mg. per ml.	100	23·1	3·85
	Growth medium	"	100	16·8	0
		Control	100	17·7	
2	Minimal salt soln.	0·5 mg. per ml.	97·3	26·0	12·46
	Growth medium	"	90·8	27·0	13·64
		Control	100	16·9	-
3	Minimal salt soln.	1 mg. per ml.	6·3	21·3	9·1
	Growth medium	"	0·3	23·8	8·8
		Control	100	18·2	-

be due to repair of the premutational lesions in the former.

It should be stressed, however, that the present finding of mutagenicity of glyoxal does not in any way threaten the use of ionizing radiations in practical food irradiation programme because the concentrations at which glyoxal is mutagenic are not likely to be generated in irradiated food of even high sugar content.

IV. A CASE OF GENE-CONTROLLED MUTATIONAL STABILITY IN WP-2*

In the course of study of mutagenic activity of glyoxal in WP-2 try$^-$, it was found that in an untreated liquid culture of WP-2, selection against prototrophs maintains a very low level of background try$^+$ mutants (about 10^{-8}). However, when a sample of the same suspension was plated on limiting amounts of tryphophan in the plates, selection against try$^+$ reversions did not operate and mutations readily arose on the plates (for details see page 68). This was an unusual situation and it was of interest to see if other growth factor requirements in the genotype of WP-2 also show a similar response. A mutation for adenine requirement introduced into this strain showed that the unusual behaviour of try$^-$ does not represent reversion pattern of all auxotrophic mutants in WP-2. However, it lead to another interesting observation: in the diauxotrophic strain try$^-$ locus was rendered stable both to spontaneous and to mutagen induced reversions. A preliminary study of this gene controlled mutational stability will now be described.

A mutation to adenine requirement was induced in WP-2 by UV irradiation followed by penicillin screening. Tests of this doubly auxotrophic strain showed the pattern of ad$^+$ revertants to be completely different from that found for try$^+$ reversions: while almost all try$^+$ revertants arise on the plates, almost all ad$^+$ revertants arise in the culture. This has been concluded from the observation that the same average number of colonies per plate was obtained when a suspension of try$^-$ ad$^-$ bacteria was plated on tryptophan (10 µg. per ml). supplemented plates with or without the

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* Result of this work has been published in Mutation Research 4: 382-384 (1967).

addition of limiting amounts (1 μg. per ml.) of adenine. Also, the dilution effect observed for tryptophan revertants was absent for adenine reversions and there was a very good proportionality between the number of bacteria plated and ad^+ revertants obtained. The reversion rate to ad^+ varied from 3·7 per 10^8 to 19·3 per 10^8 in different experiments.

An unexpected striking observation was the complete suppression of try^+ reversions in the doubly requiring strain. Five experiments were carried out, each involving 7·5 to 8·5 x 10^9 plated bacteria. While the frequencies of adenine reversion ranged from 3·7 to 19·3 per 10^8, no try^+ revertants at all were obtained.

The following possibilities that might account for the stability of try^- were considered and tested:

(1) Adenine supplementation of the mutation plates, which had to be done for scoring try^+ mutations, interferes with the appearance of try^+ revertants. To test this possibility, revertability of try marker in the parent strain WP-2 and in 5 spontaneous ad^+ revertants of try^- ad^- was compared on media with and without added adenine. The results are given in Table 25.

It is seen that adenine in the mutation plates had no effect on the rate of reversion of try^- either in the parent strain WP-2 or in try^- isolates extracted from try^- ad^- by selecting spontaneous adenine independent colonies.

(2) The growth rate of the di-auxotroph may be considerably slowed down so that sufficient number of divisions does not take place to permit the appearance of try^+ plate mutants. However, determinations of population densities attained after 48 hour

Table 25. Comparison of the reversion rate of try$^-$ in WP-2 and in five try$^-$ isolates extracted from try$^-$ ad$^-$ on M + T (Minimal + 1 µg. per ml. tryptophan) plates with and without added adenine.

Strain	try$^+$ revertants per plate on medium.					
	M + T			M + T + 10 µg. per ml. adenine		
	No. of plates	No. of try$^+$ revertants	try$^+$ per plate	No. of plates	No. of try$^+$ revertants	try$^+$ per plate
WP-2try$^-$	10	108	10·8	10	112	11·2
try$^-$ isolates extracted from try$^-$ad$^-$						
1	5	63	12·6	5	63	12·6
2	5	78	15·6	5	82	16·4
3	5	53	10·6	5	50	10·0
4	5	47	9·4	5	62	12·4
5	5	52	10·4	5	57	11·4

incubation at 37°C by try⁻ad⁻ on plates supplemented with 10 µg. per ml. adenine and 1 µg. per ml. tryptophan were the same as that of try⁻ grown on 1 µg. per ml. tryptophan enriched plates.

Therefore, the absence of try⁺ revertants is not due to lack of divisions on the plate.

(3) The UV irradiation employed for inducing the adenine requirement may have caused additional lesions in the genotype so that try⁻ can no longer revert. This was tested by selecting 10 spontaneous adenine independent colonies from try⁻ ad⁻ and checking their revertability. The reversion rates of these 10 isolates is given in Table 26. It was found that each one of them reverted on plates supplemented with 1 µg. per ml. tryptophan giving a range of 9·4 to 20·2 revertants per plate for different isolates. It could therefore be concluded that no additional genetical damage was responsible for the stability of try⁻ in the doubly auxotrophic strain.

The question of mutational stability of try⁻ in the doubly auxotrophic strain must be attributed, therefore, to change in genetic background with its correlated changes in metabolic properties of the system.

<u>The response of try⁻ in WP-2 and of try⁻ and ad⁻ in the doubly auxotrophic strain to UV, EMS.</u>

Mutation experiments were done to see whether the stability of try⁻ locus to spontaneous reversions, in the doubly auxotrophic try⁻ ad⁻ strain, also extends to treatment with mutagens. WP-2 try⁻ and try⁻ ad⁻ were treated, for this purpose, with ethyl methane sulphonate and UV and reversions for the two markers were

Table 26. try^+ reversions per plate, on 1 μg. per ml. tryptophan supplemented medium, for 10 try^- isolates extracted from try^- ad^-

Isolate No.	No. of plates plated	No. of try^+ revertants	try^+ revertants per plate
1	10	90	9·0
2	10	160	16·0
3	10	202	20·2
4	10	124	12·4
5	10	126	12·6
6	10	174	17·4
7	10	170	17·0
8	10	180	18·0
9	10	186	18·6
10	10	156	15·6

scored on suitably supplemented media. The results are given in Tables 27 and 28.

It is seen that try$^-$ locus in WP-2 responded positively to EMS treatment giving a high yield of mutations. try$^-$ is also known to respond well to mutagenic treatments with UV. However, in the diauxotroph try$^-$ ad$^-$, no try$^+$ revertant at all was found after treatments with EMS and UV, though ad$^+$ reversions were induced in large numbers. These results show that introduction of adenine requirement renders the try$^-$ locus in WP-2 stable not only against spontaneous but also against induced reversions. The adenine locus, however, reverts readily following mutagenic treatment.

Table 27. Frequency of try$^+$ reversions induced in WP_2 try$^-$ following EMS treatment.

Experiment No.	Duration in mins. of treatment in 0·1 M EMS	Survival	No. of bacteria screened	No. of try$^+$ revertants	try$^+$ revertants for 10^8 survivors
1	30	72·3	$1·4 \times 10^8$	490	350
2	30	80·7	$1·2 \times 10^9$	2120	176·6
	45	66·6	$1·0 \times 10^9$	3519	351·9
	60	65·3	$1·0 \times 10^9$	4809	480·9

Table 28. Relative frequencies of try^+ and ad^+ revertants following treatment of di-auxotrophic strain try^- ad^- with EMS and UV

Expt. No. (1)	Mutagenic treatment (2)	gene studied (3)	Survival % (4)	No. of bacteria screened (5)	No. of reversions (6)	Reversions per 10^8 survivors (7)
	0·1M EMS for					
1	15 min.	try^-	100	$2·1 \times 10^8$	0	0
		ad^-	100	$2·1 \times 10^8$	11	5·2
	30 min.	try^-	98·3	$2·5 \times 10^8$	0	0
		ad^-	98·3	$2·5 \times 10^8$	30	12·0
	45 min.	try^-	75·1	$2·3 \times 10^8$	0	0
		ad^-	75·1	$2·3 \times 10^8$	38	16·5
	60 min.	try^-	51·2	$1·2 \times 10^8$	0	0
		ad^-	51·2	$1·2 \times 10^8$	51	42·5
	0·1M EMS for					
2	30 min.	try^-	87·3	$3·7 \times 10^8$	0	0
		ad^-	87·3	$3·7 \times 10^8$	48	12·9
	60 min.	try^-	48·6	$3·9 \times 10^8$	0	0
		ad^-	48·6	$3·9 \times 10^8$	217	55·6

Table 28. (Contd.)

(1)	(2)	(3)	(4)	(5)	(6)	(7)
3	UV exposure for 5 seconds	try$^-$	89·0	$5·3 \times 10^8$	0	0
		ad$^-$		$5·3 \times 10^8$	11	2·07
	10 "	try$^-$	78·0	$4·6 \times 10^8$	0	0
		ad$^-$		$4·6 \times 10^8$	75	16·30
	15 "	try$^-$	71·0	$4·2 \times 10^8$	0	0
		ad$^-$		$4·2 \times 10^8$	103	24·52
	20 "	try$^-$	39·0	$2·3 \times 10^8$	0	0
		ad$^-$		$2·3 \times 10^8$	150	65·21

Table 29. Frequency of sex-linked-recessive lethals in Drosophila melanogaster raised on irradiated and unirradiated food

Experiment No.	Sex of the treated flies	Chromosome	No. of chromosomes tested	No. of lethals obtained	% Lethals
1	Males	X	1563	4	0·255
		II	1438	8	0·556
	Females	X	1453	2	0·137
		II	1378	4	0·290
2	Males	X-treated	2044	2	0·097
		X-control	1328	2	0·150

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(i) Tests for mutagenic effect of irradiated medium:* Results of two experiments done to assess the mutagenic effect of feeding irradiated food to Drosophila melanogaster are given in Table 29. In the first experiment 24 and 48 hour old larvae were transferred to irradiated food while in experiment II the males tested for sex-linked recessive lethals had developed on irradiated food from egg stage to emergence of adults.

Since no difference was found between 24 and 48 hour old larval transfers, data from these two sets of experiment I have been pooled. It will be seen that the values for sex-linked recessive lethals in experiment I are within the range usually found for the stock used. Those for autosomal lethals are twice as frequent, and this is as expected from the length relationship between the X and the second chromosome. No difference apart from that inherent in the difference of sex (Auerbach, 1941) was observed in tests on treated males and females, thus ruling out any influence of germinal selection against sex-linked lethals in the hemizygous males.

Tests for dominant lethality, replicated twice, likewise gave entirely negative results, the mean percentage of hatchability in control and treated series being 87·7 and 87·2 respectively.

In experiment II, a higher mutation rate might have been expected because of the action of transient radicals which would have been missed in the first experiment. Actually, mutation frequency was even lower here than in experiment I and not higher

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* Results of the mutagenic effect of irradiated medium and irradiated DNA have been published in Nature 208: 699-700 (1965).

Table 30. Frequency of sex-linked-recessive lethals in Drosophila melanogaster raised on normal and DNA admixed food.

Type of food and Experiment No.	Treatment	No. of chromosomes tested	No. of lethals obtained	% Lethals
Maize meal food*				
I	Control food	2361	6	0·25
	DNA food	2957	8	0·27
II	Control food	2359	8	0·33
	DNA food	1980	7	0·35
III	Control food	985	2	0·20
	DNA food	825	5	0·60
TOTAL	Control food	5705	16	0·280
	DNA food	5762	20	0·347
Bran food IV	Control food	1089	2	0·18
	DNA food	2220	5	0·22

* For composition see Materials and Methods.

than in the controls.

(ii) Tests for mutagenic effect of irradiated DNA: Experiments were done to retest the observation of Parkash (1965) that feeding of irradiated DNA to Drosophila produces a high frequency of sex-linked recessive lethals. The results are summarized in Table 30. As can be seen, no increase over corresponding controls in sex-linked lethals was observed in 3 experiments. Dr. Parkash (Personal communication) suggested that the discrepancy between his results and mine might be due to the difference in the medium used. Because of a possible competition between irradiated DNA and the nucleic acids of yeast, he had prepared his medium entirely without yeast, according to the formula already given in the section on Material and Methods. It was observed that the development of the flies on this food was considerably delayed, first emergence taking 14 days as against 9 - 10 days at 25°C on the food used in this laboratory. In 1089 chromosomes from 91 males developing on food mixed with unirradiated DNA and 2,220 chromosomes from 167 males developing on food containing irradiated DNA, the percentage of sex-linked lethals was 0·183 and 0·225 respectively. My experiments therefore give no support to the conclusion that, in general, irradiated DNA is mutagenic for Drosophila. This conclusion has subsequently been supported by several other investigators (Khan and Alderson, 1965; Kaplan, 1966; Fahmy and Fahmy, 1966; Rinehart & Ratty personal communication).
It is difficult to visualize which special conditions led to the positive results in the experiment carried out by Dr. Parkash.

(iii) Mutagenic effect of feeding unirradiated calf-thymus DNA:

The mutagenic effect in Drosophila of larval feeding of calf-thymus DNA was tested to check the observation of Gershenson (1966) that this treatment specifically induces visible mutations. Gershenson and Kiseleva (1958) have made another surprising and unlikely observation: while DNA feeding did not produce any sex-linked lethals, 5 out of the 21 genetically tested visibles were located on the X-chromosome, the rest 16 being autosomal dominant mutations.

In the present study treated P_1 males of Or.K stock that had developed on food containing 13% calf-thymus DNA were mated to $\widehat{XX}$ Cy/+ virgin females. The F_1 males were scored for sex-linked dominant and recessive and autosomal dominant visibles. A sample of the Curly F_1 males was also tested for induced 2nd chromosome lethals by the Cy/L technique as a measure of effectiveness of the DNA treatment. The results are given below:

Lethal mutations:

In 581 2nd chromosomes from 59 treated males, 12 lethals were detected. The distribution of lethals in the treated males was as follows: one male (No. 27) with 3 lethals; one male (No.42) with 2 lethals and 7 males with one lethal each. The 2·1% incidence of induced lethals is significantly higher than about 0·6% spontaneous lethals found in the stock in several periodic checks.

Visible mutations:

Amont 14,845 F_1 sons of treated males criticially examined for visible mutations, only 7 mutants were obtained. These consisted of 6 Minutes and 1 Beadex. A large number of other morphological

variants such as flies with blistered wings, stubby bristles and nipped scutellar bristles were tested. They all proved to be non-hereditary variations. The frequency of visible mutations in the DNA-fed series is within the range normally expected in the untreated population. Also, no evidence was obtained that may indicate specific induction of wing mutations.

Test of allelism between lethals from different males:

Lethals recovered from the progeny of flies that had developed on DNA mixed food were tested for allelism by intercrossing the 12 lethals. The 3 lethals from male 27 were allelic to each other; and so were the 2 lethals from male 42. The possibility of these lethals being pre-existing is excluded because the 3 lethal bearing chromosomes of male 27 were out of 19 tested chromosomes and the 2 lethals from male 42 came from 13 tested chromosomes. If a male carries a pre-existing lethal, one would expect half its progeny to carry a lethal. Since allelic lethals from the same male probably represent products of a single event, in calculating the degree of allelism between lethals of independent origin, only one lethal each from these two males was considered. Of the 36 possible crosses between 9 lethals, 11 did not yield any wild type flies. The percentage of lethals that are induced in the same narrow region of the 2nd chromosome thus works out to 30·55. This observation of a high degree of allelism among independent lethals induced by DNA feeding is in agreement with findings of Gershenson (1965) and Mathew (1965). Since several synthetic polymers produce effects analogous to DNA when injected into adult flies (Fahmy and Fahmy, 1962) it is believed that DNA produces its effect not by a specific chemical reaction with the genetic material but

rather by a macromolecular attachment which causes deletions during replication. A specificity of DNA action in producing lethals (which have been shown to be due to deficiencies) confined to narrow regions of 2nd chromosome can be expected if there were preferred regions where molecules of extranous DNA find attachment and cause deletions at subsequent replication. It is impossible to find a genetic explanation of Gershenson's observation, however, that DNA produces visibles that specifically affect the wings, particularly when it is conceded that the action of DNA is not due to any specific chemical reaction with the genetic material. It is still more difficult to imagine that a treatment produces visible mutations on the X-chromosome but no sex-linked lethals.

However, in the present study, no evidence for mutagenicity of DNA feeding in producing visible mutations was obtained. The treatment did produce a significantly higher rate of autosomal lethals showing that the absence of mutagenicity for visibles is not due to a general failure of the effectiveness of the treatment. The observations of Dr. Gershenson and coworkers probably arise from peculiarities of the Drosophila stock used in their studies. There could be unstable genes in the stock affecting wing characters that readily mutate under the unfavourable conditions of food created by DNA admixture.

DISCUSSION

DISCUSSION

As set out in the Introduction, the present study is concerned with evaluating the genetic consequences in bacteria and *Drosophila* resulting from growth on irradiated and chemically treated media. In studies on the effect of irradiated media, the organisms had received no direct irradiation and therefore any observed effect could result only from stable chemical products generated in the medium by radiation. After a brief account of basic radiochemical processes that are responsible for producing the effective chemical products, the results obtained in the two organisms will be discussed separately.

In an aqueous chemical system, such as the bacterial medium used in the present study, the indirect effects of radiation are almost exclusively mediated through water which plays a vital role as solvent and converter of radiation energy into active chemical agents. The primary step involved is radiolysis of water, the products of which can be written as follows:

(1) Decomposition into OH radicals and H atom and a solvated electron according to the reaction.

$$H_2O \longrightarrow H_2O^+ e^-$$
$$H_2O^+ \longrightarrow H^+ + OH.$$

An ejected electron can be captured by another molecule of water resulting in the production of atomic hydrogen and OH radical:

$$H_2O + e^- \longrightarrow H_2O^-$$
$$H_2O^- \longrightarrow H. + OH^-$$

(2) Formation of molecular hydrogen and hydrogen peroxide: The reaction resulting in molecular hydrogen and hydrogen peroxide

occurs only in the presence of solutes which can react with H. or OH. or both.

In the absence of solutes, however, the above mentioned reaction products are not realized because the H. and OH. change equimolar amounts of H_2 and H_2O_2 back into water according to:

$$H. + H_2O_2 \longrightarrow H_2O + OH.$$

and

$$OH. + H_2 \longrightarrow H_2O + H.$$

Any radicals from reaction (1) not used up in reaction (2) usually disappear by recombination:

$$H. + OH = H_2O$$

In addition to these basic reactions, there are several secondary chemically active entities derived from reaction of primary products with dissolved oxygen. These are:

(i) The reaction of H atoms with dissolved oxygen, leading to the formation of peroxide radical HO_2.

$$H + O_2 \longrightarrow HO_2.$$

(ii) Radical-radical interaction forming H_2; H_2O_2; and O_2 near the site of radical formation:

$$H. + H. \longrightarrow H_2$$

$$OH. + OH. \longrightarrow H_2O_2$$

$$HO_2. + HO_2. \longrightarrow H_2O_2 + O_2$$

$$OH. + HO_2. \longrightarrow H_2O + O_2$$

$$H. + HO_2. \longrightarrow H_2O_2$$

(iii) Reaction between radicals and molecular-reaction products after diffusion from electron tracks.

$$OH. + H_2O_2 \longrightarrow H_2O + HO_2.$$

$$HO_2. + H_2O_2 \longrightarrow H_2O + OH. + O_2$$

$$OH. + H_2 \longrightarrow H_2O + H.$$

$$H. + H_2O_2 \longrightarrow O_2 + OH.$$

$$H. + H_2O_2 \longrightarrow H_2 + HO_2.$$

In summary, radiolysis of water in the presence of solutes and oxygen results in H, OH and HO_2 free radicals and hydrogen peroxide. If organic solutes are present, hydrogen peroxide reacts with them to give organic peroxides. The free radicals are extremely short lived, lasting on an average, only 2×10^{-7} seconds. No biological damage to an organism can therefore result directly from free radicals produced in the suspending medium because they (the radicals) decay before they have a chance to diffuse to the biologically critical sites within the cell. However, the free radicals are chemically very reactive and initiate chains of complex chemical reactions culminating in products which may be the causative agents of the ultimate biological damage resulting from treatment with irradiated medium.

For critical evaluation of the wholesomeness of irradiated food from the genetic standpoint, a logical first step would be to identify component/s of the food material responsible for producing radiomimetic principle on irradiation. Feeding tests, with laboratory animals to elicit this information besides being laborious, time consuming and expensive, are beset with some technical difficulties. Firstly, a basic diet of known composition that is nutritionally adequate and will support normal growth and reproduction of the animal will contain such a large number of compounds that testing of each one of them separately becomes unpractical. Secondly, there is the difficulty of deciding the amount of irradiated component that

should be included in the diet. In tests on substances proposed as additives to food, it is customary to feed to animals one hundred times the amount, weight for weight, expected to be consumed by a human being, and so to show that it is harmless in this amount. This is rarely possible with feeding tests because the amount of any one item of food that would have to be consumed daily by the experimental animal would be much more than it could eat. Micro-organisms, on the other hand can grow on simple synthetic media of known chemical composition. The identification of the component of the medium responsible for generating the radiomimetic principle can, therefore, be done quickly and reliably. Micro-organisms also offer several advantages for screening potentially harmful products which can later be tested on higher forms of life.

STUDIES WITH BACTERIA

The most commonly measured criteria, of genetic damage, in bacteria are induced lethality and mutations. Both these attributes have been studied in the present study following treatment of bacteria in irradiated medium and the results obtained will now be discussed.

Lethal effect of irradiated medium.

During the course of the present study it was observed that when medium exposed to ionizing radiation (X- and gamma rays) was seeded with unirradiated bacteria, viable counts declined progressively with increasing duration of incubation; for a given dose of radiation, the lowest survival was obtained after about

8 and 4 hours of incubation in X-ray and γ-ray irradiated media respectively. The reason for this difference was investigated and it was found to result from a difference in dose rate rather than the quality of radiation. Indeed, for the same radiation type viz., gamma rays, a 4 hour incubation in irradiated medium exposed to 200 Kr produced 8 times as much killing when delivered in 11 minutes 32 seconds than when delivered in 19 hours. This indicates that a variation in dose rate at which the radiation is delivered may produce a qualitative and/or quantitative difference in the resulting bactericidal principle. From a practical point of view, for achieving the maximum elimination of spoilage organisms from radiation preserved food, it will be more efficacious if the required radiation dose is delivered at high dose rates.

A search for the effective component of the minimal medium responsible for generating cytotoxic principle on irradiation revealed that the maximum effect resulted from irradiation of glucose. Survival, of bacteria incubated in medium made up with irradiated water or salts (in solution) was hardly affected. Moreover, the bactericidal effect of medium, the glucose component of which alone had been irradiated, was more drastic than that of irradiated whole minimal medium. This suggests that unirradiated salts react with radiation product/s of glucose responsible for cytotoxicity thereby reducing its effectiveness. In agreement with these results, strong biological effect of radiation products of glucose has also been found by Molin and Ehrenberg (1964) for killing of Pseudomonas; Holsten et al. (1965) for suppression of growth of carrot cells in tissue cultures and chromosome aberrations

in Vicia faba and Tradescantia; Moutschen and Matagne (1965) for chromosome breaks in barley and Shaw and Hayes (1966) for breaks and exchanges in chromosomes of cultured human lymphocytes. Frey and Pollard (1966) have measured the peroxide content in irradiated solutions of glucose and a number of mineral salts that go into the composition of synthetic bacterial growth medium and correlated it to suppression of synthesis of DNA, messanger RNA and protein of E. coli incubated in medium containing these irradiated components. These authors have demonstrated that the concentration of peroxides is highest in irradiated glucose solution, and considerably lower in solutions of metal salts. An interesting observation, however, was that when one or more metal salts were included in the irradiation mixture along with glucose, the peroxide content was lower compared with that in pure glucose solution. If the lethal effects of irradiated medium are mediated through peroxides (and for this there is very strong evidence both from my work and the work of several other investigators already mentioned) the observations of Frey and Pollard (1966) will lend support to my explanation for greater effectiveness of irradiated glucose solution compared to salt-glucose medium.

A number of radiobiologists working on indirect effects of ionizing radiations have attributed the lethal effects of irradiated medium to hydrogen - and/or organic peroxides generated in the medium (Molin and Ehrenberg, 1964; Pollard et al., 1965; Frey and Pollard, 1966; Levison, 1966). In agreement with this, in the present study, the lethal effects of irradiated medium could be duplicated by adding reagent hydrogen peroxide to the medium at concentrations within the range of those that will be generated by

a given dose of radiation to the medium. Moreover, the bactericidal effect of irradiated or hydrogen peroxide treated medium could be completely abolished by adding catalase to the irradiated medium prior to inoculation of bacteria into it. The enzyme catalase is known to destroy peroxides (both hydroxy, and organic) and therefore the protection it confers against the biological damage of irradiated medium indicates that the lethality produced by irradiated medium is due to peroxides.

The work of Berry et al. (1965) however would seem to contradict this conclusion. Working with mammalian cell cultures, these authors demonstrated a strong cytotoxic effect when the growth medium was constituted with sugars irradiated either as dry powder or as solution in water. The cytotoxic principle was heat stable and resistent to catalase action. Berry et al. concluded that the observed cytotoxic effect in irradiated dextrose and fructose solutions was substantially due to glyoxal, a radiation decomposition product of glucose. The conclusion was based on the argument that the cytotoxicity of a given concentration of glyoxal corresponded closely to that of a radiation dose that produced this concentration. Experiments conducted during the present study to test if glyoxal could account for the bactericidal effect of irradiated medium in E. coli system showed that between 0·8 and 0·9 mg. per milliliter of glyoxal has to be incorporated into the medium to produce a response close enough to that produced by 500 Krad of X-rays to the medium. The G values (number of molecules produced for the absorption of 100 eV of radiant energy) for the production of glyoxal in glucose solution have been reported by Phillips, Moody and Mattock (1958).

Using appropriate factors it can be calculated that irradiation of glucose solution in water with 500 Krad will produce a glyoxal concentration of approximately 0·1 mg. per ml. It is thus seen that glyoxal concentrations far in excess of those as are generated in the medium are required to duplicate the effects of a given dose of radiation to the medium. Glyoxal can therefore be excluded as the major agent responsible for bactericidal effects of unstored irradiated medium in E. coli system. It can be stated in summary that the bulk of evidence at present available suggests that lethal effects resulting from treatment with irradiated medium arise from peroxides generated in the medium. The possibility cannot be ruled out, however, that other chemical species may also contribute tc the total effect, particularly in cases where the effect persists for extended periods lasting several months of storage. Glucose irradiated in the solid state and then added to the medium may be biologically active by other mechanisms than irradiated glucose solution or irradiated liquid medium. The analysis of this problem is made difficult by the fact that since reducing compounds are formed when solid carbohydrates are irradiated, a titrimetic or colorimetic peroxide determination gives very uncertain values (Molin & Ehrenberg, 1964). Furthermore several organisms are known to possess large intracellular concentrations of catalase and this factor may be important in influencing the response of an experimental system to irradiated medium effect.

In the present work the cytotoxic principle in irradiated medium was observed to persist for up to 64 days when stored at 4°C; its effectiveness relative to unstored

irradiated medium depended on the dose of radiation delivered. Furthermore, there was a positive correlation between the radiation dose and the time it took the incubated bacteria to exhaust the medium of the cytotoxic factor and resume growth. This suggests an appreciable stability in the medium of the active principle, or at least of one of them if there are several. Glyoxal, a radiation product of glucose is one such stable compound that could contribute to the smaller bactericidal effects of the stored irradiated medium. Alternatively, a fairly short-lived compound may be formed continuously, and at a decreasing rate, during the storage period. The fact that chemical reactions have been found to proceed for a long time in solutions of irradiated glucose (Ehrenberg et al., 1960) or when irradiated solid glucose is dissolved in water (Ahnström and Ehrenstein, 1959) support the latter possibility. Whatever the mechanism by which the irradiated medium retains the cytotoxic principle/s for extended periods of time, the property confers an additional advantage to food preservation by ionizing radiation; any surviving food spoiling organism that has escaped death from the radiation exposure would be eliminated through the action of irradiated medium.

Mutagenic effect of irradiated medium:

Stone et al. (1947) observed that mutation rates for resistance to antibiotics streptomycin and penicillin, increase when bacteria are grown in ultra-violet irradiated nutrient broth. However, bacterial medium irradiated with ionizing radiations has not so far been tested for its mutagenic effect. It was therefore of interest to test whether treatment of medium with ionizing radiations also

confers mutagenic properties to it. For the sake of comparison with earlier work, medium irradiated with UV was also included in the tests.

The results of the present experiments confirm the earlier observations of Stone, Wyss and coworkers reviewed in the Section Introduction that treatment with UV irradiated nutrient broth produces enhanced mutation rates for streptomycin resistance; also UV irradiated minimal medium was found to be effective in this regard. In addition, it was found that minimal medium irradiated with ionizing radiations (X- and γ rays) also stepped up mutation rates in both the forward mutation system to streptomycin resistance and reverse mutation system in which mutations from auxotrophy to prototrophy in treated bacteria are scored. The mutation rates following treatments with irradiated medium were significantly higher than controls, even at high survivals. This, together with additional evidence from reconstruction experiments which showed no selective advantage to prototrophs, clearly established that the enhanced mutation rates were due to induction and not selection of pre-existing mutants.

Wyss *et al.*(1948) suggested that mutations induced by irradiated medium resulted from peroxides generated in the medium. They failed to demonstrate an increase in mutation rate by direct treatment of bacteria in saline with hydrogen peroxide and therefore concluded that only organic peroxides are effective as mutagens. This conclusion is strengthened by the work on *Neurospora* (Dickey *et al.*, 1949; Jensen *et al.*, 1951) and *Drosophila* (Altenburg, 1954; Sobels, 1954, 1956) which showed that various organic peroxides are

indeed effective mutagens. Wagner et al. (1950) in Neurospora and Demerec et al.,(1951) in E. coli, on the other hand, obtained positive results when treatment of washed organisms with hydrogen peroxide was carried out even in the absence of any organic substance in the treatment medium. It is likely, however, that even in these cases hydrogen peroxide is converted into organic peroxides by reaction with cellular constituents before it becomes mutagenically effective.

From a study of the mutagenic effects of components of a synthetic medium, Wyss et al. (1948) concluded that the radiomimetic principle was produced from the component consisting of amino acids, vitamins and nucleic acid bases. In the present work, on the contrary, minimal medium consisting only of mineral salts in water with glucose as the energy source was found to be mutagenic on irradiation. It would thus appear that nucleic acid bases and amino acids do not have any unique property for releasing a mutagenic factor. In fact, all that is really needed is an organic source that can react with hydrogen peroxide to give organic peroxides.

STUDIES ON DROSOPHILA

Swaminathan et al. (1963) reported a slight but significant increase in the incidence of sex-linked recessive lethal and visible mutations in the progeny of Drosophila flies that had been reared on γ-ray irradiated food. A similar low but statistically significant mutagenic effect of irradiated food for sex-linked lethals has been observed by Rinehart and Ratty (1966). However,

results obtained from experiments in the present study did not provide any evidence for a mutagenic substance being produced in irradiated Drosophila food. Similar negative results have been obtained by Reddi et al. (1965) and Moutschen (Personal communication). Thus, though the evidence for mutagenicity of irradiated food is as yet contradictory, one point is clear: feeding on irradiated food produces, if any, only marginal effects in mutation rate which under certain circumstances may be pushed beyond levels of statistical significance. The factors that influence the response may include the genotype of the test stock and composition of the food irradiated. Parkash (1965) has contended that feeding of irradiated DNA is mutagenic for Drosophila and this factor (irradiated DNA) may account for the radiomimetic effect of irradiated food observed by Swaminathan et al. (1963). However, neither the present study nor the work of several other investigators (Chopra, 1965; Khan and Alderson, 1965; Fahmy and Fahmy, 1966; Kaplan, 1966; Rinehart and Ratty, Personal communication) has been able to confirm Parkash's observation. Therefore, the question of irradiated DNA being mutagenic or contributing to mutagenic effect, where observed, of irradiated food can be positively excluded.

In contrast to the marginal effects in Drosophila, striking increases in the frequency of chromosome aberrations was observed in root tip cells of plants grown in irradiated White's medium (Natarajan and Swaminathan, 1958), irradiated potato mash (Swaminathan et al. 1962), irradiated fruit juices (Chopra et al., 1963) or irradiated glucose solution (Moutschen and Matange, 1965). Dramatic

increases in chromosome breaks in cells of human lymphocytes have also been recorded when the cultures are set up in TC 199 medium irradiated with γ-rays (Kesvan and Swaminathan, 1966) or supplemented with irradiated glucose (Shaw and Hayes, 1966). The differences between the striking cytological effects in plants and cell cultures and marginal effects in Drosophila may be due to some basic differences in the two systems. Obvious among these are: (1) In plants and tissue cultures the treatment is applied directly to the tissue in which the effects are looked for, so that the cells can take up the reactive principle fairly quickly and without its undergoing metabolic changes. In feeding experiments with Drosophila, on the other hand, the irradiated food has to undergo a series of metabolic changes in the digestive system of the fly. The radiomimetic principle produced by irradiation will thus reach the gonads in a greatly modified form which may be mutagenically ineffective; (2) While chromosome breaks in plants and animal tissue cultures are scored in cell cycles immediately following the treatment, Drosophila germ cells are sampled many cell generations after treatment. Also, chromosomal aberrations in treatments with irradiated media consist mainly of chromosome breaks while translocations, which require rejoining, occur very rarely if at all. Such chromosome breaks, if induced in Drosophila, can produce dominant lethals only and these, if produced in pre-meiotic cells, are likely to be eliminated during meiosis. No increase in the frequency of dominant lethals was observed in the present work. In agreement with this Reddi et al. (1965) found no evidence for dominant lethals or large deletions of the X-chromosome in flies

raised on irradiated food. Even Rinehart and Ratty (1966) who obtained positive results for sex-linked lethals, failed to find any increase in dominant lethals.

Implications of studies at the cellular level to the problem of genetic hazards from consuming irradiated food:

Large long-term and short-term carefully designed feeding tests with different species of animals including man have been performed, especially in the U.S.A., without any indication of toxicity or histopathological changes (Report of the Working Party on Irradiation of Food, 1964). These tests have included a large enough number of diverse food materials to warrant the conclusion that radiation processing, in general, is free from harmful changes. Unfortunately, however, all feeding tests have been confined to looking for somatic effects and no serious attempt has been made to estimate the extent of genetic alterations, if any, in the progeny of the test subjects. This has mainly resulted from the fact that for drawing statistically valid conclusions, large populations have to be screened in any genetic test, particularly when the mutagenic effect is a weak one. Limitations of funds, trained personnel and laboratory space to test a large number of various types of irradiated food that are likely to be marketed, precludes such attempts. Working on the argument that, at the genetic level, there is a remarkable unity in the biological world in their response to mutagenic agents, some workers have tried to obtain information on genetic effects by studying the effects of irradiated food or food components at the cellular level. However,

there have been extreme views on the implications of the results obtained in such studies. While Swaminathan and coworkers and Steward hold that the work is relevant to radiation sterilization of food, Goldblith (1966) and Cook and Berry (1966) believe that the data already available completely clears irradiated food from any ill effects so that it "can be consumed with impunity"; the argument being that even if cytotoxic products are formed in the irradiated food, they will be rendered biologically ineffective in the animal systems which have "mechanisms for modification, alteration and digestion of the food by the alimentary tract and the detoxification and excretion mechanisms of the liver and kidney". However, Lofroth (1966) has drawn attention to the work of Baily and Duggal (1962) in which a positive correlation between gastric carcinoma and ingestion of smoked food was found and his own work in which irradiated food caused a slight, age-dependent lymphopenia in rats, suggesting that "reactive, radiation induced compounds can perhaps by-pass the detoxification process in animals". All these considerations suggest therefore that while final conclusions regarding the mutagenicity of irradiated food in animals cannot be drawn from studies at the cellular level, the latter have their use as primary screening test for selecting potentially harmful foods and food components for inclusion in large scale genetic studies on animal species.

In the present work, while clear cut cytotoxic and mutagenic effects resulted in bacteria treated with irradiated medium and irradiated glucose, no evidence for mutagenic effect was obtained when Drosophila was reared on irradiated food. As pointed out

earlier, however, the results of different workers from studies on Drosophila are not unanimous and in view of the fact that one cannot be too careful while drawing conclusions which can influence genetic hazards to man, it will be worthwhile to repeat these experiments under uniform and repeatable conditions of genetic background of test stock, food composition and other environmental factors to establish real facts. An effort to devise suitable genetic tests which will give reliable results from manageable numbers in experiments with laboratory animals and greater support for such research would be extremely prudent.

Spontaneous reversions in WP-2 try^- and a case of gene controlled mutational stability:

During the course of investigation of mutagenic effect of glyoxal, a product of glucose irradiation, some incidental observations were made on the nature of reversions to try^+ in WP-2 try^- and in a diauxotrophic derivative try^- ad^- obtained from WP-2 by UV irradiation. It is thought that the results obtained are of sufficient interest to deserve a short discussion.

It was observed that a culture of WP-2 try^- growing exponentially in minimal medium supplemented with tryptophan does not show an accumulation of try^+ revertants as would normally be expected if there were no selection against try^+. In fact, with growth of the culture, the frequency of such prototrophs showed a progressive decline. Reconstruction experiments, in which known numbers of try^+ and try^- bacteria were grown as mixed population and the relative proportion of the two types determined

at intervals, however, gave no evidence of selection against already established prototrophs.

In contrast to the behaviour in the liquid medium, when a sample of WP-2 was plated on minimal medium containing limiting amounts of tryptophan, try^+ revertants appeared readily in numbers which remained almost constant per plate irrespective of the number of bacteria initially plated.

These observations are compatible with the explanation that try^- bacteria release one or more inhibitory substances into the culture which selects against the try^+ revertants at the nascent prototroph stage. It would appear that the inhibitory compound has a threshold concentration below which it is rendered ineffective. The absence of selection against revertants on mutation plates (which contain minimal medium + limiting concentration of the growth factor) supports this idea; the inhibitory substance is diluted out and therefore fails to exert its influence. A similar inhibition of prototrophs in a histidineless mutant of E. coli, due to the production by auxotrophs of inhibitory substance/s in an unshaken his^- culture, has been demonstrated by Ryan and Schneider (1949).

The unusual characteristics of the try^- marker are, however, not shared by all mutants in the genotype of WP-2. A mutation for adenine requirement induced in WP-2, for example, showed no selection against ad^+ revertants in liquid medium. Also, the "dilution effect" (whereby disproportionately higher mutation rates per plated bacteria are obtained when the same suspension of bacteria is plated at progressively higher dilutions) observed for

try^+ reversions did not exist for ad^+ reversions.

The doubly auxotrophic strain try^- ad^- showed another striking characteristic: While the ad^- reverted readily both spontaneously and after treatment with UV and EMS, the introduction of this (ad^-) mutation into WP-2 rendered try^- completely stable to reversion. In several experiments not a single try^+ revertant was obtained either in untreated controls or in cultures that had received mutagenic treatment with EMS and UV.

This observation is similar to that of Witkin and Theil (1960) who found that this same try^- allele (try_6) acquired stability to UV mutagenesis when a requirement for streptomycin was introduced into WP-2. Glover (1956) has also quoted several cases in E. coli where reversion rates of a particular auxotroph were markedly influenced by the presence or absence of other auxotrophic or drug-resistance markers in the same strain. Still other examples where change of the genetic background has influenced mutation rates to considerable extent are those reported in Schizosaccharomyces pombe (Clarke and Loprieno, 1965) and in Aspergillus nidulans (Morpurgo and Calvori, 1966). In all these cases, however, the effect of plating medium has not been excluded. Auerbach (1962) has pointed out that the changed reversion frequency of a gene brought about by the introduction of a second auxotrophic marker, may be due to differences in the medium on which the two types of revertants are scored. One such case where the plating medium was shown to have a striking influence on the mutation rate of certain genes has been discovered in S. pombe by Clarke (1963). He showed that L. methionine markedly suppressed reversions of adenine

auxotrophs of Schizosaacharomyces pombe. In the present case, however, the possibility of plating medium conferring stability on try$^-$ has been excluded.

From this and other considerations described in detail in the Section on Results (page 81) it has been concluded that the mutational stability of try$^-$ in the doubly auxotrophic strain must be attributed to change in the genetic background with its correlated changes in the metabolic properties of the system. It seems that under these new metabolic conditions the mutation process from try$^-$ $\longrightarrow$ try$^+$ is interfered with at some stage so that try$^+$ reversion is not completed. At present, it is not possible to distinguish precisely the stage at which this inhibition becomes operative. Answers to the following questions should help to narrow down the possibilities:

(1) What is the response of the try$^-$ mutant in the doubly auxotrophic strain to different mutagens? A partial answer to this question has already been obtained when it was found that the stability of the try$^-$ is not confined only to spontaneous reversions but extends also to reversions induced by at least two potent mutagens, UV and EMS.

(2) Has the introduction of the adenine requirement suppressed mutability of other genes, besides try$^-$? The possibility of a general mutational stability of the doubly requiring strain can be excluded because adenine reverts normally.

(3) Is the suppression of try$^+$ revertants allele specific or are all try$^-$ mutants similarly affected? The specific allele used in this study, try$_6$, is the same which in the experiments of Witkin and Theil (1960) had ceased to respond to UV after the introduction

of a streptomycin requirement. This together with the fact that reversions of another try allele (try_3) were not suppressed by combination with streptomycin dependence (Glover, 1956) suggests that the try_6 allele may be particularly susceptible to this type of suppression.

(4) Is the mutability of try_6 suppressed by mutations other than those leading to adenine - or streptomycin - requirement?

On the basis of observations of Gorini and Kataja (1964) and Lederberg et al. (1964), Witkin (1965) has tentatively reinterpreted her earlier observations (Witkin and Theil, 1960) without however obtaining experimental evidence for the hypothesis. She has hypothesized that in the streptomycin dependent strain, UV-induced try^+ reversions (which, according to her, are suppressor mutations) are not obtained because the suppressor action of the mutations is overcome by streptomycin in the medium. In contrast, Glover (1956) found that in another streptomycin dependent derivative of a tryptophanless mutant of E. coli, high yields of try^+ revertants were obtained following UV treatment. Nevertheless, even if Witkin's hypothesis to explain the stability of try^- in streptomycin-dependent strains were correct, it still would not explain stability of try^- when the latter is combined with adenine requirement. For one thing, whereas streptomycin is known to affect the ribosomes and thus interfere with genetic transcription, no such mechanism can be visualized for adenine action. For another, adenine in the mutation plates had no influence on the spontaneous or induced revertability of try^- in WP-2 or a try^- extracted from $try^- ad^-$.

Still a different interpretation of my findings is suggested

by the fact that the two types of reversion show a very different pattern of appearance; tryptophan reversions arising almost exclusively on the plates, adenine reversions in the growth medium. If the introduction of adenine requirement was to impose its own pattern on the appearance of try^+ reversions, then the absence of try^+ revertants would be expected. The number of try^+ revertants present in the culture is very small and could easily escape detection. Evidence that a changed metabolic pattern can alter the kinetics of appearance of mutations, from independence of growth rate to proportionality with growth in the chemostat, is provided by the work of Kubitschek and Bendigkeit (1964). In their case, however, the change in metabolic pattern was obtained by choice of the limiting nutrilite in the growth medium.

SUMMARY

The present study is concerned with evaluating the biological effects caused by treatment of bacteria and Drosophila with irradiated and chemically treated media. Both lethal and mutagenic effects have been studied.

I. Bacteria:

(1) A sharp decline in survival of Bacillus subtilis was observed when the bacteria were treated with minimal medium that had been irradiated with 226 Krad of X-rays. The lowest surviving fraction was obtained after incubation in irradiated medium for about 8 hours.

(2) A similar lethal effect of X- and γ-ray irradiated minimal medium was found for Escherichia coli. For the same radiation dose, the maximum decrease in survival was reached after about 8 hours incubation in X- and about 4 hour incubation in gamma-ray irradiated medium.

(3) The lethal effect of gamma irradiated medium on E. coli increased when the dose to the medium was increased from 50 to 500 Krad. Medium irradiated with 1 Mrad was, however, less toxic than medium irradiated with 500 Krad.

(4) The production of the cytotoxic principle was found to be dose rate dependent. Four-hour incubation in medium exposed to 200 Krad, for example, produced 8 times as much killing when delivered in 11 minutes 32 seconds than when delivered in 19 hours.

(5) The magnitude of the cytotoxic effect was inversely related to the number of bacteria per ml. seeded into the irradiated medium;

the cytotoxic effect was greater for smaller initial inocula.

(6) After a month's storage at 4°C, the cytotoxic principle from medium irradiated with 100 Krad was exhausted by the inoculated bacteria in the first hour of incubation. After 64 days of storage the medium irradiated with 100 Krad did not produce any lethality. In medium irradiated with 500 Krad, however, the cytotoxic principle persisted, up to 64 days, though its effectiveness had been reduced.

(7) Treatment of bacteria with irradiated medium made them resistant to a subsequent treatment with irradiated medium.

(8) The lethal effect of the irradiated medium was found to arise from irradiation of the glucose component. Irradiation of only water produced a slight decrease in viable counts and irradiation of salt solution produced only a lag and no killing. When salt-glucose medium was irradiated, the bacterial killing was less than when glucose alone had been irradiated. This suggests that salts react with radiation products of glucose responsible for cytotoxicity thereby reducing its effectiveness.

(9) The lethal effect of irradiated medium could be duplicated by adding amounts of reagent hydrogen peroxide that will be obtained in the medium by irradiation. This, together with the observation that catalase completely negated the effect of irradiated medium suggests that the cytotoxic principle consists of peroxides.

(10) In a triple auxotrophic strain of E. coli, treatment with X-irradiated medium produced a significant increase over control in reversion rates to prototrophy for threonine and leucine. For the third marker, proline, no increase in mutation rate was

obtained. Proline marker is not a deletion because it does give spontaneous revertants. This shows that different genes may respond differently to treatment with irradiated medium. The mutagenic effect of irradiated medium, in this respect, is similar to that of several other chemical mutagens.

(11) Treatment with medium irradiated with UV, X-ray and gamma-rays also produced a significant increase in mutations for resistance to streptomycin.

(12) Glyoxal, a chemical product in irradiated glucose solutions, was found to be both lethal and mutagenic for bacteria. The effective concentrations of the chemical, however, were far in excess of those that will be produced in the medium by even a dose as big as 2 Mrad.

II. Drosophila.

(13) Rearing of Drosophila on food irradiated with 1Mrad of gamma-rays did not increase over control, the incidence of dominant lethals, sex-linked recessive lethals or 2nd chromosome recessive lethals.

(14) No evidence of mutagenic effect was found when Drosophila was fed on food containing irradiated DNA.

(15) Feeding Drosophila on food containing calf-thymus DNA from a sample provided by Dr. Gershenson, produced a significant increase in 2nd chromosome recessive lethals. Cross tests of lethals of independent origin showed a high degree of allelism showing that DNA acts on preferred regions of the 2nd chromosome. Contrary to the claim of Dr. Gershenson, however, no increase in the frequency

of visible mutations was obtained following development on DNA treated food.

(16) An interesting case of gene controlled mutational stability was found in WP-2, a tryptophan requiring strain of E. coli B/r; introduction of an additional requirement for adenine made the try^- locus completely stable to spontaneous reversions or reversions induced by UV and EMS. The adenine locus reverted normally in the di-auxotrophic strain.

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BIBLIOGRAPHY

BIBLIOGRAPHY

Ahnström, G. and Ehrenstein, G.V. (1959). Luminescence of aqueous solutions of substances irradiated with ionizing radiation in the solid state. Acta Chem. Scand. 13: 855-856.

Altenburg, L.S. (1954). The production of mutations in Drosophila by tertiary butyl hydroperoxide. Proc. Natl. Acad. Sci. 40: 1037-1040.

Auerbach, C. (1941). The effect of sex on the spontaneous mutation rate in Drosophila melanogaster. J. Genet. 41: 255-265.

Auerbach, C. (1962). Mutation: An Introduction to Research on Mutagenesis. Part I. Methods p.107. Oliver and Boyd. Edinburgh and London.

Baily, E.J. and Duggal, N. (1962). In. First Scand. Symp. Carcinogenesis, Stockholm.

Berry, R.J., Hills, P.R. and Trillwood, W. (1965). Demonstration of a cytotoxic agent in solutions of carbohydrates sterilized by ionizing radiation. Int. J. Rad. Biol. 9: 559-572.

Blank, I.H. and Arnold, W. (1935). The inhibition of growth of Bacillus subtilis by Ultraviolet irradiated carbohydrates. J. Bacteriol. 30: 507-511.

Bugayaki, P.L., Lafontaine, A. and Moutschen-Dahmen, J et M. (1963). Les aliments irradiés exercent ils un effect radiomimétique. 1. Atompraxis 9: 1 - 3

Chopra, V.L. (1965). Tests on Drosophila for the production of mutations by irradiated medium or irradiated DNA. Nature 208: 699-700.

Chopra, V.L., and Swaminathan, M.S. (1963). Sprout inhibition and radiomimetic properties in irradiated potatoes. Naturwissenschaften 50: 374-375.

Chopra, V.L., Natarajan, A.T. and Swaminathan, M.S. (1963). Cytological effects observed in plant material grown on irradiated fruit juices. Radiation Botany 3: 1-6.

Clarke, C.H. (1963). Suppression by methionine of reversions to adenine independence in Schizosaccharomyces pombe. J. gen. Microbiol. 31: 353-363.

Clarke, C.H. and Loprieno, N. (1965). The influence of genetic background on the action of methionine reversions by di-epoxybutane in Schizosaccharomyces pombe. Microb. Genet. Bull. 22: 11-12.

Cook, A.M. and Berry, R.J. (1966). Direct and indirect effects of radiation: their relation to growth. Nature. 210: 324-325.

Demerec, M., Bertani, G. and Flint, J. (1951). A survey of chemicals for mutagenic action in E. coli. Am.Naturalist 85: 119-136.

Dickey, F.H., Cleland, G.H. and Lotz, C. (1949). The role of organic peroxides in the induction of mutations. Proc. Natl. Acad. Sci. 35: 581-586.

Ehrenberg, A., Ehrenberg, L. and Löfroth, G. (1960). Chemical changes produced by ionizing radiations in solid carbohydrates. Risö Report, 16: 25-26.

Evans, T.C. (1947). Effects of hydrogen peroxide produced in medium by radiation of spermatozoa of Arbacia punctulata. Biol. Bull. 92: 99-109.

Fahmy, O.G. and Fahmy, M.J. (1961). Induction of mutations by deoxyribonucleic acid in Drosophila melanogaster. Nature. 191: 776-779.

Fahmy, O.G. and Fahmy, M.J. (1962). Mutagenic activity of cellular macromolecules in Drosophila melanogaster. Nature 196: 873-876.

Fahmy, O.G. and Fahmy, M.J. (1966). The nature and distribution of mutations induced by unirradiated and irradiated heterologous deoxyribonucleic acid in Drosophila melanogaster. Genetics 54: 1123-1138.

Frey, H.E. and Pollard, E.C. (1966). Ionizing radiation and bacteria: nature of the effect of irradiated medium. Radiation Res. 28: 668-676.

Gershenson, S. (1939). Induction of directed mutations in Drosophila. Dokl. Acad. Nauk SSSR., N.S., 25: 236-238.

Gershenson, S. (1965). Induction of mutations in Drosophila melanogaster by DNA. Genet. Res. 6: 156-161.

Gershenson, S. (1966). Mutagenic action of DNA and the problem of directed mutations. Genetica No.5:1-13 (In Russian).

Gershenson, S. and Kiselyeva, I.A. (1958). Induction of hereditary changes in Drosophila melanogaster. Dokl. Acad. Nauk. SSSR., N.S. 123: 554-557.

Glover, S.W. (1956). A Comparative study of induced reversions in E. coli. In "Genetic Studies with Bacteria." Publ. Carneg. Instn. 612: 121-136.

Goldblith, S.A. (1966). Radiation sterilization of food. Nature. 210: 433-434.

Gorini, L. and Kataja, E. (1964). Phenotypic repair by streptomycin of defective genotype in E. coli. Proc. Natl. Acad. Sci. 51: 487-493.

Haas, F.L. and Doudney, C.O. (1957). A relation of nucleic acid synthesis to radiation-induced mutation frequency in bacteria. Proc. Natl. Acad. Sci. 43: 871-883.

Holsten, R.D., Sugii, M. and Steward, F.C. (1965). Direct and indirect effects of radiation on plant cells: their relation to growth and growth induction. Nature. 208: 850.

Jensen, K.A., Kirk, I., Kølmark, G. and Westergaard, M. (1951). Chemically induced mutations in Neurospora. Cold Spring Harbor Symp. Quant. Biol. 16: 245-261.

Kaplan, W.D. (1966). The failure of irradiated DNA to produce mutations in Drosophila melanogaster. Drosophila Information Service 41: 101.

Kesvan, P.C. and Swaminathan, M.S. (1966). Cytotoxic and radiomimetic activity of irradiated culture medium on human leucocytes. Curr. Sci. 35: 403-404.

Khan, A.H. and Alderson,T. (1965). Mutagenic effect of irradiated and unirradiated DNA in Drosophila. Nature. 208: 700-702.

Kimball, R.F. (1955). The role of oxygen and peroxide in the production of mutation in Paramecium. Ann. New York Acad. Sci. 59: 638-647.

Kimball, R.F., Hearon, J.Z. and Gaither, N. (1955). Tests for a role of H_2O_2 in X-ray mutagenesis. II. Attempts to induce mutations by peroxide. Radiation Research 3: 435-443.

Kubitscheck, H.E. and Bendigkeit, H.E. (1964). Mutations in continuous cultures, I. Dependence of mutational response upon growth-limiting factors. Mutation Res. 1: 113-120.

Kuzin, A.M. (1963). On the role of the disturbance of metabolic processes in the radiation damage of cells. Intl. J. Rad. Biol. 6: 211-220.

Lederberg, E.M., Cavalli-Sforza, L. and Lederberg, J. (1964) Interaction of streptomycin and a suppressor for galactose fermentation. Proc. Natl. Acad. Sci. 51: 678-682.

Levison, W. (1966). Toxic effect of X-irradiated medium on chick embryo cells. Exptl. Cell. Res. 43: 398-413.

Löfroth, G. (1966). Toxic effect of irradiated food. Nature 211: 302.

Lwoff, A., Siminovitch, L. and Kjelgaard, N. (1950). Induction de la production de bactériophages chez une bactérie lysogène. Ann. Inst. Pasteur 79: 815.

McDowell, M.E. and Raica, N. (1962). Review of the United States Army's irradiated food and wholesomeness programme. U.S. Army med. Res. Nutr. Lab. Rep. 268, 39 pp.

* Mathew, C. (1965). The production of recessive lethals by calf-thymus DNA in Drosophila. Genet. Res. 6: 163-174.

Molin, N. and Ehrenberg, L. (1964). Antibacterial action of irradiated glucose. Intl. J. Rad. Biol. 8: 223-231.

Morpurgo, G. and Calvori, C. (1966). Variable frequency of back-mutation in different genotypes. Ann. Ist. Super. Sanita 2: 429-430.

Moutschen, J. and Matagne (1965). Cytological effect of irradiated glucose. Radiation Bot. 5: 23-28.

Muller, H.J. (1941). Induced mutations in Drosophila. Cold Spring Harb. Symp. Quant. Biol. 9: 151-167.

Natarajan, A.T. (1960). Stored energy and indirect biological effects of radiation. Risö Report 16: 39-40.

Natarajan, A.T. and Swaminathan, M.S. (1958). Indirect effects of radiation and chromosome breakage. Ind. J. Genet. 18: 220-223.

Parkash, O. (1965). Induction of sex-linked recessive lethals and visible mutations by feeding X-irradiated DNA to Drosophila melanogaster. Nature 205: 312-313.

Parkash, O. (1965). On the radiomimetic effect of irradiated desoxyribonucleic acid (DNA_m) on Drosophila melanogaster. Naturwissenchaften 6: 142.

Phillips, G.O., Moody, G.J. and Mattock, G.L. (1958). Radiation chemistry of carbohydrates Part I. Action of ionizing radiation on aqueous solutions of D-glucose. J. Chem. Soc. Part III: 3522-3534.

Pollard, E.C. et al. (1965). Ionizing Radiation: Effect of irradiated medium on synthetic processes. Science 147: 1045-1047.

Rappaport, J.A. (1940). Influence of thymonucleic and nucleic acids of some of their component in mutations. Dokl. Acad. Nauk SSSR., N.S., 27: 1033-1036.

Reddi, O.S., Reddy, G.M., Rao, J.J., Ebenezer, D.N. and Rao, M.S. (1965). Lack of mutagenic effect of irradiated Drosophila medium. Nature 208: 702.

Report of the Working Party on Irradiation of food, London, Her Majesty's Stationery Office (1964) pp.85.

Rinehart, R.M. and Ratty, F.J. (1965). Mutations in Drosophila melanogaster cultured on irradiated food. Genetics. 52: 1119-1126.

Ryan, F.J. and Schneider, L.K. (1949). The consequences of mutation during the growth of biochemical mutants of Escherichia coli. IV. The mechanism of inhibition of histidine-independent bacteria by histidineless bacteria. J. Bacteriol. 58: 201-213.

Sang, J.H. (1956). The quantitative nutritional requirements of Drosophila melanogaster. J. exp. Biol. 33: 45-72.

Shaw, M.W. and Hayes, E. (1966). Effects of irradiated sucrose on the chromosomes of Human lymphocytes in vitro. Nature. 211: 1254-1256.

Šmarda, J. and Čermák, V. (1964). Induction of the synthesis of phage and colicin in a culture medium irradiated by UV rays. Zeitschrift für Allg. Mikrobiologie 4: 358-366.

Sobels, F.H. (1954). Mutagenicity of dihydroxymethyl peroxide. Drosophila Information Service 28: 156-157.

Sobels, F.H. (1956). Mutagenicity of dihydroxymethyl peroxide and the mutagenic effects of formaldehyde. Nature. 177: 979-980.

Steward, F.C., Holsten, R.D. and Sugii, M. (1967). Direct and Indirect effects of radiation: the radiolysis of sugar. Nature. 213: 178.

Stone, W.S., Wyss, O. and Haas, F. (1947). The production of mutations in Staphylococcus aureus by irradiation of the substrate. Proc. Natl. Acad. Sci. 33: 59-66.

Stone, W.S., Haas, F., Clark, J.B. and Wyss, O. (1948). The role of mutation and selection in the frequency of mutants among micro-organisms grown on irradiated substrate. Proc. Natl. Acad. Sci. 34: 142-149.

Swaminathan, M.S., Chopra, V.L. and Bhaskaran, S. (1962). Cytological aberrations observed in barley embryos cultured on irradiated potato mash. Radiation Res. 16: 182-188.

Swaminathan, M.S., Nirula, S., Natarajan, A.T. and Sharma, R.P. (1963). Mutations: incidence in Drosophila melanogaster reared on irradiated medium. Science 141: 637-638.

Wagner, R.P., Haddox, C.H., Furest, E. and Stone, W.S. (1950). The effect of irradiated medium, cyanide and peroxide on the mutation rate in Neurospora. Genetics. 35: 237-248.

Witkin, E.M. (1959). Post-irradiation metabolism and the timing of ultraviolet-induced mutations in bacteria. Proc. X. Intl. Cong. Genet. Vol. I, 280-299.

Witkin, E.M. (1965). Interaction of suppressor mutations and changes at the Streptomycin locus: An old observation reinterpreted. Microbiol. Genet. Bull. 23: 5.

Witkin, E.M. and Theil, E.C. (1960). The effect of post-treatment with chloramphenicol on various ultra-violet induced mutations in Escherichia coli. Proc. Natl. Acad. Sci. 46: 226-231.

Wyss, O., Stone, W.S. and Clark, J.B. (1947). The production of mutations in Staphylococcus aureus by chemical treatment of the substrate. J. Bacteriol 54: 767-772.

Wyss, O., Clark, J.B., Haas, F. and Stone, W.S. (1948). The role of peroxides in the biological effects of irradiated broth. J. Bacteriol. 56: 51-57.

PUBLICATIONS

Chopra, V.L. (1965). Tests on *Drosophila* for the production of mutations by irradiated medium or irradiated DNA. Nature 208: 699-700.

_____ (1965). The effects of X-irradiated culture medium on bacteria. Microbial. Genet. Bull. 23: 8.

_____ (1966). The effect of X-ray irradiated culture medium in bacteria - identification of the effective component. Microbial. Genet. Bull. 25: 5.

_____ (1966). Lethal and mutagenic effect of Glyoxal. Microbial. Genet. Bull. 25: 4.

_____ (1967). Gene controlled change in mutational stability of a tryptophanless mutant of *E. coli* WP-2. Mutation Research 4: 382-384.

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Tests on *Drosophila* for the Production of Mutations by Irradiated Medium or Irradiated DNA

(1) *Irradiated medium.* Ionizing radiations are likely to find increasing use in industry for prolonging the useful storage-life of food. Before the method is declared safe and becomes widely acceptable, however, it is necessary to ensure that consumption of irradiated food is free from genetic ill-effects. Mutation rates have been shown to increase when bacteria are grown in ultra-violet-irradiated substrate[1]. Bacterial medium irradiated with ionizing radiations, however, has not been tested for its mutagenic effects. Information about the indirect genetic effects of ionizing radiations in higher organisms is scanty. Investigations of the cytological effects in root meristems of plants grown in X- and γ-ray irradiated potato mash[2] and fruit juices[3] have shown that the aberration frequencies in treated roots were strikingly higher than in corresponding controls. In order to test for similar effects in animals, these studies were extended to *Drosophila*. It was found that the rate of sex-linked lethals was increased slightly, but significantly, in the progeny of flies raised on irradiated food[4]. In view of the obvious importance of this observation it was thought necessary to re-test it, scoring both autosomal and sex-linked lethals. The former were included because it has been shown[5,6] that feeding of unirradiated calf thymus DNA has a very specific and pronounced mutagenic effect on chromosome II of *Drosophila*, while the *X*-chromosome remains largely refractory to this treatment. Induced dominant lethality was also investigated in view of the chromosome breakage observed in plant material grown on irradiated medium. The results of these investigations are reported in this communication.

Food consisting of 10 per cent dried killed yeast, 10 per cent sucrose and 3 per cent agar in water was irradiated by the Radiation Research Laboratory, Wantage. The total dose of 1 mrad of γ-rays was delivered in a single exposure lasting 70 min from a 14,000-c. annular cobalt-60 source. The Oregon-*K* (Or-*K*) stock for these experiments has been used in this laboratory for mutation experiments for a long time and has shown a spontaneous rate of about 0·3 per cent sex-linked lethals in periodic checks. In the first experiment 24- and 48-h-old larvae were transferred to the irradiated food about 36 h after the termination of radiation exposure, and the frequency of sex-linked and second chromosome (autosomal) lethals was determined simultaneously in both treated males and females. In order to keep the experiment manageable and because of the known spontaneous mutation rate of the stock, controls were not included in this experiment. In a second

experiment the usual maize meal–molasses food was used. Young Or-*K* females, mated 24 h earlier, were transferred to the irradiated food for egg laying immediately on termination of the irradiation exposure, so that the developing flies should be exposed to the full effect of the treatment including that due to any transient radicals. Males which had developed on irradiated food were tested for sex-linked lethals. Comparable controls were included in this experiment. Scoring was done by the usual techniques, namely, hatchability for dominant lethals, Muller-5 test for sex-linked lethals and *Cy*/*L* test for second chromosome lethals.

The results are summarized in Table 1. Since no difference was found between 24- and 48-h old larval transfers, data from these two sets of the first experiment have been pooled. It will be seen that the values for sex-linked lethals in experiment 1 are within the range usually found for the stock used. Those for autosomal lethals are twice as frequent, and this is as expected from the length relationship between the *X*- and the second chromosome. No difference apart from that inherent in the difference of sex[7] was observed in tests on treated males and females, thus ruling out any influence of germinal selection against sex-linked lethals in the hemizygous males. Tests for dominant lethality, replicated twice, likewise gave entirely negative results, the mean percentage of hatchability in control and treated series being 87·7 and 87·2 respectively. In the second experiment a higher mutation rate might have been expected because of the action of transient radicals which would have been missed in the first experiment. Actually, mutation frequency was even lower here than in experiment 1 and not higher than in the controls.

These experiments, therefore, do not provide any evidence for a mutagenic substance being produced in irradiated food. Similar negative results have been obtained by Moutschen (personal communication). On the other hand, Swaminathan *et al.*[4] and more recently Rinehart (personal communication) have obtained small, but statistically significant. increases of mutation rates in *Drosophila* raised on irradiated food, indicating that in certain circumstances marginal effects in mutation rate may be produced. In contrast to this, the previously mentioned experiments on plant chromosomes had shown a striking increase in the frequency of chromosome breaks

Table 1. Frequency of Lethals in *Drosophila melanogaster* raised on Irradiated or Unirradiated Food

Experiment No.	Sex of the treated flies	Chromosome	No. of chromosomes tested	Lethals (%)
1	Males	*X*	1,653	0·256
		II	1,438	0·556
	Females	*X*	1,453	0·137
		II	1,378	0·290
2	Males	*X* (treated)	2,044	0·097
		X (control)	1,328	0·150

in root meristems of wheat and barley grown on irradiated water[8], potato mash[2] and fruit juices[3]. The difference between the striking cytological effects in plants and the marginal effects in *Drosophila* may be due to some basic differences in the two systems. Obvious among these are: (1) In plants the treatment is applied directly to the tissue in which the effects are looked for, so that the cells can take up the reactive principle fairly quickly and without its undergoing metabolic changes. In feeding experiments with *Drosophila*, on the other hand, the irradiated food has to undergo a series of metabolic changes in the digestive system of the fly. The radiomimetic principle produced by irradiation will thus reach the gonads in a greatly modified form which may be mutagenically ineffective. (2) While chromosome breaks in plants are scored in cell cycles immediately following the treatment, *Drosophila* germ cells are sampled many cell generations after treatment. Also, aberrations in treatments with fruit juices consisted entirely of chromosome breaks and no evidence for translocations indicating rejoining was obtained. Such chromosome breaks, if induced in *Drosophila*, can produce dominant lethals only, and these, if produced in pre-meiotic cells, are likely to be eliminated during meiosis. In order to create experimental conditions for *Drosophila* that are more similar to those used in plants, one might determine dominant lethals in mature sperms of adult *Drosophila* males that have had irradiated glucose or fruit juices administered to them by drinking or injection. Experiments on these lines have now been started.

(2) *Irradiated DNA*. While these experiments on the effect of irradiated food were in progress, Parkash published results of an experiment in which 1·8 per cent of irradiated fish sperm DNA had been mixed into the food of *Drosophila* larva. The emerging males had 36 lethals in 635 tested *X*-chromosomes, a frequency of 5·7 per cent[9]. In a second experiment[10], 9·6 per cent autosomal lethals were obtained, but there was no control and it is not clear whether pre-existing lethals had been excluded. This seemed such a striking and important observation that I repeated the experiment, using herring sperm DNA but otherwise the same procedure as Parkash. The irradiation with 100,000 r. of γ-rays was performed by the Radiation Research Laboratory, Wantage (exposure time 31 min). The irradiated DNA was mixed with *Drosophila* food, and Oregon-*K* flies were allowed to feed and lay eggs on this food. Food mixed with unirradiated DNA served as control. Males that had developed on DNA-treated food were tested for sex-linked recessive lethals according to the Muller-5 method. The experiment was repeated thrice with negative results. In a total of 5,705 control and 5,762 treated chromosomes the percentage incidence of sex-linked lethals was 0·29 and 0·35 respectively. Dr. Parkash (personal communication to Dr. C. Auerbach)

suggested that the discrepancy between our results might be due to the difference in medium used. Because of a possible competition between the irradiated DNA and the nucleic acids of yeast, he had prepared his medium entirely without yeast, according to the following formula: sugar, 7 g; bran, 7 g; maize meal, 6 g; and agar, 0·8 g; cooked in 100 ml. water. Another experiment was carried out, using the aforementioned formula. The rest of the experimental procedure was the same as described earlier. The development of the flies on this food was considerably delayed, first emergence taking 14 days as against 9–10 days at 25° C on the food used in this laboratory. In 1,089 chromosomes from 91 males developing on food mixed with unirradiated DNA and 2,220 chromosomes from 167 males developing on food containing irradiated DNA, the percentage of sex-linked lethals was 0·18 and 0·22 respectively. My experiments therefore give no support to the conclusion that, in general, irradiated DNA is mutagenic for *Drosophila*. It is difficult to visualize which special conditions led to the positive results in the experiment carried out by Dr. Parkash.

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[1] Stone, W. S., Wyss, O., and Haas, F., *Proc. U.S. Nat. Acad. Sci.*, **33**, 59 (1947).
[2] Swaminathan, M. S., Chopra, V. L., and Bhaskaran, S., *Rad. Res.*, **16**, 182 (1962).
[3] Chopra, V. L., Natarajan, A. T., and Swaminathan, M. S., *Rad. Bot.*, **3**, 1 (1963).
[4] Swaminathan, M. S., *et al.*, *Science*, **141**, 637 (1963).
[5] Gershenson, S., *C.R. (Dokl), Acad. U.R.S.S.*, **26**, 601 (1940).
[6] Mathew, C., *Genet. Res.*, **6**, 163 (1965).
[7] Auerbach, C., *J. Genet.*, **41**, 255 (1941).
[8] Natarajan, A. T., and Swaminathan, M. S., *Indian J. Genet.*, **18**, 220 (1958).
[9] Parkash, O., *Nature*, **205**, 312 (1965).
[10] Parkash, O., *Naturwiss.*, **52**, 142 (1965).

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Gene-controlled change in mutational stability of a tryptophanless mutant of *E. coli* WP2

Mutations from try⁻ to try⁺ revertants were studied in *E. coli* B/r WP2. It was found that the rate of mutations to tryptophan independence in a liquid-batch culture grown in minimal medium supplemented with 6 μg tryptophan per ml is not proportional to growth rate. Selection against prototrophs maintains a very low level of background mutants (about 10^{-8}) that will grow on minimal agar plates. Reconstruction experiments have shown that selection operates at the stage of the nascent prototroph while completed revertants are no longer selected against.

In mutation experiments, plating of treated bacteria is routinely done on media supplemented with limiting amounts of required growth factors to allow for fixation and expression of induced mutants. When untreated WP2 is plated on medium enriched with 1 μg tryptophan per ml, which allows limited growth on the plate, the mutation rate becomes proportional to growth and new mutants arise on the plate. The average number of these plate mutants (about 15 per plate) is constant and independent of plating density. Disproportionately higher mutation rates per plated bacteria are, therefore, obtained when the same bacterial suspension is plated at progressively higher dilutions. This observation is in accord with those of DEERING[3]. This "dilution effect" has been found to be due to the fact that the final number of bacteria attained on a plate is constant over plating densities of 10^5 to about $5 \cdot 10^8$ bacteria per plate.

In order to test whether the same pattern of reversion would be obtained for other auxotrophs in this strain, an additional mutation to adenine requirement was induced by UV irradiation followed by penicillin screening. Tests of this doubly auxotrophic strain showed the pattern of ad⁺ revertants to be completely different from that found for try⁺ reversions: while almost all try⁺ revertants arise on the plates, almost all ad⁺ revertants arise in the culture. This has been concluded from the observation that the same average number of colonies per plate was obtained when a suspension of try⁻ ad⁻ bacteria was plated on tryptophan (10 μg/ml) supplemented plates with or without the addition of limiting amounts (1 μg/ml) of adenine. Also, the dilution effect observed for tryptophan revertants was absent for adenine reversions and there was a very good proportionality between the number of bacteria plated and ad⁺ revertants obtained. The reversion rate to ad⁺ varied from 3.7 per 10^8 to 19.3 per 10^8 in different experiments.

An unexpected and striking observation was the complete suppression of try⁺ reversions in the doubly requiring strain. Five experiments were carried out, each involving 7.5 to $8.5 \cdot 10^9$ plated bacteria. While the frequencies of adenine reversion were normal no try⁺ revertants at all were obtained.

This observation, though pertaining to spontaneous mutations, is similar to that of WITKIN AND THEIL[7] who found that this same try⁻ allele (try_6) acquired stability to UV mutagenesis when a requirement for streptomycin was introduced into WP2. Influence of the genetic background, but not attributable to a particular gene, on mutation rates has also been shown in *Schizosaccharomyces pombe*[2] and *Aspergillus nidulans*[5]. To explain the failure of try⁺ mutants to appear, WITKIN AND THEIL[7] suggested that in the doubly requiring strain, metabolic requirements for establishment of try⁺ were not met even when amino acids were supplied in abundance for mutation

fixation. However, in their work as also in the studies of GLOVER[4] where the genetic background was shown to strongly influence the mutability of certain genes, the effect of plating medium was not excluded. A case in which the plating medium has been shown to have a striking influence on the mutation rate of certain genes has been described by CLARKE[1]. He showed that L-methionine markedly suppressed reversions of adenine auxotrophs of *Schizosaccharomyces pombe*. The possibility of the plating medium conferring mutational stability on try$^-$ was excluded by the observation that adenine in the mutation plates did not have any effect on the appearance of try$^+$ revertants in the parent strain or in 10 singly try$^-$ strains which had been extracted from the try$^-$ad$^-$ ones by spontaneous adenine reversions. The possibility of a general mutational stability of the doubly requiring strain can be excluded because adenine revertants occurred readily.

Two other possibilities that might account for the stability of try$^-$ in the try$^-$ ad$^-$ strain were tested.

(1) The growth rate of the di-auxotroph may be considerably slowed down so that a sufficient number of divisions does not take place to permit the appearance of try$^+$ plate mutants. However, determinations of population densities attained after 48-h incubation at 37° by try$^-$ad$^-$ on plates supplemented with 10 μg adenine per ml and 1 μg tryptophan per ml were the same as that of try$^-$ grown on tryptophan-enriched plates (1 μg/ml). Therefore, the absence of try$^+$ revertants is not due to lack of divisions on the plate.

(2) The UV irradiation employed for inducing the adenine requirement may have caused additional lesions in the genotype so that try$^-$ can no longer revert. This was tested by selecting 10 spontaneous adenine-independent colonies from try$^-$ad$^-$ and checking their revertability. It was found that each one of them reverted on plates supplemented with 1 μg tryptophan per ml giving a range of 9.4–20.2 revertants per plate for different isolates. All isolates showed the "dilution effect" characteristic of the try$^+$ revertants in the parent strain WP2. It could be concluded that no additional genetical damage was responsible for the stability of try$^-$ in the doubly auxotrophic strain.

Thus the question of mutational stability of try$^-$ in the doubly auxotrophic strain must be attributed to change in the genetic background with its correlated changes in the metabolic properties of the system. It seems that under these new metabolic conditions the mutation process from try$^-$ to try$^+$ is interfered with at some stage so that try$^+$ reversion is not completed. At present, it is not possible to distinguish precisely the stage at which this inhibition becomes operative. Answers to the following questions should help to narrow down the possibilities.

(1) What is the response of the try$^-$ mutant in the doubly auxotrophic strain to different mutagens?

(2) Has the introduction of the adenine requirement suppressed mutability of other genes?

(3) Is the suppression of try$^+$ revertants allele-specific or are all try$^-$ mutants similarly affected? The specific allele used here, try$_6$, is the same which in WITKIN's experiment[7] had ceased to respond to UV after the introduction of a streptomycin requirement. This, together with the fact that reversions of try$_3$ were not suppressed by combination with streptomycin dependence[5] suggests that the try$_6$ allele may be particularly susceptible to this type of suppression.

(4) Is the mutability of try_6 suppressed by mutations other than those leading to adenine or streptomycin requirement?

Still a different interpretation of my findings is suggested by the fact that the two types of reversion show a very different pattern of appearance, tryptophan reversions arising almost exclusively on the plates, adenine reversions in the growth medium. If the introduction of adenine requirement were to impose its own pattern on the appearance of try^+ reversions, then the absence of try^+ revertants would be expected. The number of try^+ revertants present in the culture is very small and could easily escape detection. Evidence that a changed metabolic pattern can alter the kinetics of appearance of mutations, from independence of growth rate to proportionality with growth in the chemostat, is provided by the work of KUBITSCHEK AND BENDIGKEIT[6]. In their case, however, the change in metabolic pattern was obtained by choice of the limiting nutrilite in the growth medium.

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1 CLARKE, C. H., Suppression by methionine of reversions to adenine independence in *Schizosaccharomyces pombe*, *J. Gen. Microbiol.*, 31 (1963) 353–363.
2 CLARKE, C. H., AND N. LOPRIENO, The influence of genetic background on the action of methionine reversions by di-epoxybutane in *Schizosaccharomyces pombe*, *Microbial Genet. Bull.*, 22 (1965) 11–12.
3 DEERING, R. A., Mutation and killing of *Escherichia coli* WP-2 by accelerated heavy ions and other radiations, *Radiation Res.*, 19 (1963) 169–178.
4 GLOVER, S. W., A comparative study of induced reversions in *Escherichia coli*, *Publ. Carneg. Inst.*, 612 (1956) 121–136.
5 MORPURGO, G., AND C. CALVORI, Variable frequency of back-mutation in different genotypes, *Ann. Ist. Super. Sanità*, 2 (1966) 429–430.
6 KUBITSCHEK, H. E., AND H. E. BENDIGKEIT, Mutation in continuous cultures, I. Dependence of mutational response upon growth-limiting factors, *Mutation Res.*, 1 (1964) 113–120.
7 WITKIN, E. M., AND E. C. THEIL, The effect of post-treatment with chloramphenicol on various ultraviolet induced mutations in *Escherichia coli*, *Proc. Natl. Acad. Sci. (U.S.)*, 46 (1960) 226–231.

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by

Chopra, V.L.

The effects of X-irradiated culture medium on bacteria.

The effect of irradiated culture media on growth and mutation rates of Bacillus subtilis and Escherichia coli have been studied. In experiments with B. subtilis, Hayes' minimal medium, irradiated with 226 kr of X rays (140 kV, 5 MA unfiltered, dose rate 943 r/minute) was seeded with bacteria at cell densities as close to 10^6 per ml. as possible, and incubated at 37°C. In several experiments, viable counts declined with time of incubation in irradiated medium to a minimum of 2.5×10^3 after 8 hours' incubation. With smaller initial inoculum size, the minimum viability was reaced more quickly. Since B. subtilis is known to harbor a defective phage which can be induced by treatment with several mutagens, it seemed necessary to ascertain whether the killing had been due to phage induction or to a direct effect of the irradiated medium. To test this point, strains of E. coli (Gal 22), with and without λ, were incubated in irradiated M_9 medium. Killing was again observed, and the survival curves for the two strains were identical. This shows that the treatment had not induced phage λ. In agreement with this, titration for phage in supernatant of treated lysogenic bacteria gave negative results.

Preliminary experiments with auxtrophic strains of E. coli strongly suggest that reversions to prototrophy are induced by treatment with X-irradiated medium. Studies on induced mutations and identification of the component of the irradiated medium responsible for the observed effects are in progress.--M.R.C. Mutagenesis Unit, Institute of Animal Genetics, Edinburgh, Scotland.

From Microbial Genetics Bull. 25.

by

Chopra, V.L.

The effect of γ-ray irradiated culture medium in bacteria - identification of the effective component.

In a previous communication (MGB 23, p.8) I reported that X-ray irradiated medium is strongly cytotoxic for bacteria (E.coli) incubated in it. I have now tried to identify the constitutent of the medium responsible for producing the cytotoxic moiety on irradiation. Minimal (salt, glucose) medium and glucose, salts and water components of M_9 medium were irradiated separately with 200 kr of γ-rays, necessary unirradiated supplements being added subsequent to irradiation of the constituent in question.

It has been found that irradiation of water only produces a slight decrease in viable counts for the first two hours of incubation after which normal growth is resumed. Irradiation of salt solution produces only a lag and no killing. The maximum cytotoxic effect is obtained by irradiation of glucose. When M_9 salt-glucose medium is irradiated, the effect is reduced. It appears, therefore, that unirradiated salts react with radiation products of glucose responsible for cytotoxicity thereby reducing its effectiveness.

The production of the cytotoxic principle is dose rate dependent. Four-hour incubation in irradiated medium exposed to 200 kr produces 8 times as much killing when delivered in 11 minutes 32 seconds than when delivered in 19 hours. Also, the cytotoxic effect increases with increasing doses from 25 to 500 kr after which there is a drop at 1 Megarad exposure.

The bactericidal factor in irradiated medium is quite stable and is effective, in my experiments, after storage at $4^{\circ}C$ for up to 65 days.--M.R.C. Mutagenesis Research Unit, Institute of Animal Genetics, Edinburgh, 9, Scotland.

From Microbial Genetics Bull. 25.

by

Chopra, V.L.

Lethal and mutagenic effect of Glyoxal.

Glyoxal, (CHO)2, is a radiation decomposition product of glucose. Berry et al. (Int. J. Rad. Biol. 9: 558) have shown that irradiated dextrose solution is toxic to mammalian cells *in vitro* and that the level of cytotoxicity corresponds very closely to that due to the glyoxal concentration that will be produced by a given dose of radiation. I have studied the lethal and mutagenic effect of glyoxal monohydrate in *E. coli* K12 and WP2. For the study of the lethal effect a log phase culture of wild-type K12 was treated in M_9 growth medium containing glyoxal concentrations of 0.5 to 1 mg/ml. Viable counts were made at one hour intervals.

At a concentration of 0.9 mg/ml the surviving fraction dropped to 19.7% after 2 hours and to 0.23% after 4 hours incubation. This corresponds to killing produced by incubation in M_9 medium exposed to 500 kr of γ - rays. Treatment in medium containing 0.7 mg/ml glyoxal produces no killing for the first two hours of incubation and even after 4 hours treatment survival drops only to 30.3%. Concentrations of 0.5 mg/ml and lower have no effect on survival.

For mutation experiments, a log phase suspension of *E. coli* WP2 try- was treated with glyoxal at a final concentration of 1 mg/ml in M medium (Hass and Doudney, P.N.A.S. 43: 871) supplemented with 6 γ tryptophan/ml. The cells were washed twice in buffer and plated on M medium containing 0.75 γ tryptophan/ml. In all experiments where the plating density of the control and treated suspension was comparable, glyoxal treatment has given reversion rates to tryptophan independence significantly above the control level. However, lower glyoxal concentrations that do not produce lethality also do not produce mutations.

In contrast to the findings of Berry et al. for mammalian cell lines, in order to simulate the level of lethality in *E. coli* induced by a given dose of radiation to the medium, the concentration of glyoxal required is far in excess of that generated in the medium by that dose of radiation. It is also important to emphasize that even massive doses of the order of 2-3 megarads that are required to sterilize certain kinds of food will not produce glyoxal concentrations that are mutagenically effective on the *E. coli* system.--M.R.C. Mutagenesis Research Unit, Institute of Animal Genetics, Edinburgh 9. Scotland.

ABSTRACT OF THESIS

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Degree Doctor of Philosophy *Date* May, 1967.

Title of Thesis Tests for genetic effects of irradiated or chemically treated media in Drosophila and micro-organisms.

The present study is concerned with evaluating the biological effects caused by treatment of bacteria and Drosophila with irradiated and chemically treated media. Both lethal and mutagenic effects have been studied.

I. BACTERIA:

(1) A sharp decline in survival of Bacillus subtilis was observed when the bacteria were treated with minimal medium that had been irradiated with 226 Krad of X-rays. The lowest surviving fraction was obtained after incubation in irradiated medium for about 8 hours.

(2) A similar lethal effect of X- and γ-ray irradiated minimal medium was found for Escherichia coli. For the same radiation dose, the maximum decrease in survival was reached after about 8 hours incubation in X- and about 4 hour incubation in gamma-ray irradiated medium.

(3) The lethal effect of gamma irradiated medium on E. coli increased when the dose to the medium was increased from 50 to 500 Krad. Medium irradiated with 1 Mrad was, however, less toxic than medium irradiated with 500 Krad.

(4) The production of the cytotoxic principle was found to be dose rate dependent. Four-hour incubation in medium exposed to 200 Krad, for example, produced 8 times as much killing when delivered in 11 minutes 32 seconds than when delivered in 19 hours.

(5) The magnitude of the cytotoxic effect was inversely related to the number of bacteria per ml. seeded into the irradiated medium; the cytotoxic effect was greater for smaller initial inocula.

(6) After a month's storage at 4°C, the cytotoxic principle from medium irradiated with 100 Krad was exhausted by the inoculated bacteria in the first hour of incubation. After 64 days of storage the medium irradiated with 100 Krad did not produce any lethality. In medium irradiated with 500 Krad, however, the cytotoxic principle persisted, up to 64 days, though its effectiveness had been reduced.

(7) Treatment of bacteria with irradiated medium made them resistant to a subsequent treatment with irradiated medium.

(8) The lethal effect of the irradiated medium was found to arise from irradiation of the glucose component. Irradiation of only water produced a slight decrease in viable counts and irradiation of salt solution produced only a lag and no killing. When salt-glucose medium was irradiated, the bacterial killing was less than when glucose alone had been irradiated. This suggests that salts react with radiation products of glucose responsible for cytotoxicity thereby reducing its effectiveness.

(9)/

Use other side if necessary.

(9) The lethal effect of irradiated medium could be duplicated by adding amounts of reagent hydrogen peroxide that will be obtained in the medium by irradiation. This, together with the observation that catalase completely negated the effect of irradiated medium suggests that the cytotoxic principle consists of peroxides.

(10) In a triple auxotrophic strain of E. coli, treatment with X-irradiated medium produced a significant increase over control in reversion rates to prototrophy for threonine and leucine. For the third marker, proline, no increase in mutation rate was obtained. Proline marker is not a deletion because it does give spontaneous revertants. This shows that different genes may respond differently to treatment with irradiated medium. The mutagenic effect of irradiated medium, in this respect, is similar to that of several other chemical mutagens.

(11) Treatment with medium irradiated with UV, X-ray and gamma-rays also produced a significant increase in mutations for resistance to streptomycin

(12) Glyoxal, a chemical product in irradiated glucose solutions, was found to be both lethal and mutagenic for bacteria. The effective concentrations of the chemical, however, were far in excess of those that will be produced in the medium by even a dose as big as 2 Mrad.

II. DROSOPHILA:

(13) Rearing of Drosophila on food irradiated with 1 Mrad of gamma-rays did not increase over control the incidence of dominant lethals, sex-linked recessive lethals or 2nd chromosome recessive lethals.

(14) No evidence of mutagenic effect was found when Drosophila was fed on food containing irradiated DNA.

(15) Feeding Drosophila on food containing calf-thymus DNA from a sample provided by Dr. Gershenson, produced a significant increase in 2nd chromosome recessive lethals. Cross tests of lethals of independent origin showed a high degree of allelism showing that DNA acts on preferred regions of the 2nd chromosome. Contrary to the claim of Dr. Gershenson, however, no increase in the frequency of visible mutations was obtained following development on DNA treated food.

(16) An interesting case of gene controlled mutational stability was found in WP-2, a tryptophan requiring strain of E. coli B/r; introduction of an additional requirement for adenine made the try$^-$ locus completely stable to spontaneous reversions or reversions induced by UV and EMS. The adenine locus reverted normally in the di-auxotrophic strain.