non-euclidean lines on R^∞ commences and terminates at one end-point of A_1, while the other non-euclidean line on R^∞ commences and terminates at the other end-point of A_1; together these two non-euclidean lines bound a neighborhood of C_1 in R free from critical points. But this neighborhood of C_1 is smaller than the neighborhood defined in Theorem 7 and considered in the study of the critical points of $\omega(z, D, R)$. Indeed, on Γ in Theorem 8 each arc γ is a *proper subset* of an arc of Γ which (used in Theorem 7) consists wholly of images of points of C_1; the longer arc determines the larger subregion of $|w| < 1$ free from critical points. Otherwise expressed; each region of Theorem 8 involving γ and free from critical points is defined as $\omega(z, C_1, R^\infty) > 1/2$, where C_1 is counted as a bounding arc of R^∞ but once; the region free from critical points in Theorem 7 is defined as $\omega(z, C_1, R^\infty) > 1/2$, where C_1 is repeated infinitely often as a boundary arc of R^∞; even in Theorem 7 we do not here take *all* points of C_1 in every sheet of R^∞ in defining this harmonic measure.

The Principle of Gebietserweiterung applies also in the case of Theorem 8, where subregions are considered as defined on R^∞. One special case is of fairly wide applicability:

COROLLARY. *Under the conditions of Theorem 8, let the arc E of the boundary of R consist of an arc A_k, or an arc B_k of the set of arcs complementary to A, or an arc A_k plus an adjoining arc B_k; and let a circle (in the extended sense) Ω be divided into arcs Ω_1 and Ω_2 by the end-points of E. Let $E + \Omega_1$ bound a Jordan region in R, and let Ω_2 lie exterior to R. Then no critical point of $\omega(z, A, R)$ lies in the subregion of R if any bounded by E and by the circular arc orthogonal to Ω joining the end-points of E.*

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THE PRODUCTION OF MUTATIONS IN STAPHYLOCOCCUS AUREUS BY IRRADIATION OF THE SUBSTRATE

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A knowledge of the gene including its chemistry, function and mutation is of primary importance in understanding life. Muller[1] (see his bibliography also) has summarized the vast collection of information available at present on these subjects. Nevertheless, despite the interest in these problems, little information exists as to the exact chemical nature of any particular gene, much less the differences that exist between genes and

alleles. One method of attack on the nature of the gene system is through a study of mutation. Until Muller[2] showed the effectiveness of x-radiation, no method of producing gene mutations was known. Later it was demonstrated that longer wave length of radiation including ultra-violet would also produce mutations. More recently Auerbach and Robson[3] have demonstrated that mutations may result from the application of certain chemicals (including mustard gas and allied substances).

There are several difficulties in the interpretation of the effect of radiation on the mutation process. First, x-radiation is so drastic a treatment that particular kinds of effects cannot be predicted to the exclusion of others. Second, mutation from irradiation has proved to be a random process. Third, although it has been proved that the mutation rate is directly proportional to the number of individual ionizations, it has not been possible to determine how much of the effect of irradiation resulted from a direct hit on a gene as against an indirect effect from a hit on some other substance in the cell.

Another line of attack has been to compare the effects of x-rays with ultra-violet irradiation, for certain important differences exist in their effects, Stadler.[4] The reasons for these differences have not been completely determined, because here again the material changed directly by the irradiation is unknown. The work on the induction of mutation by chemicals is as yet fragmentary. It should be emphasized that in none of this work has there been evidence for selective induction of particular gene mutations. All agents so far utilized have been *general*, and, therefore, make a study of the particular change involved in mutation exceedingly difficult. As a result there exists no definite knowledge of the chemical or physical change that accompanied any particular mutation.

This paper reports experiments combining irradiation and chemical production of mutations in bacteria. This was accomplished by allowing bacteria to reproduce in a medium containing known substances which had been subjected to irradiation, and then determining if mutations had been induced. The advantage of this method in the chemical analysis of the gene lies in the fact that particular known substances can be irradiated and their effect on gene mutation studied. In addition, the chemical and physical changes that have occurred in those irradiated substances which cause an increase in mutation rate can be investigated.

Experimental.—A 24-hour broth culture of *Staphylococcus aureus* (F.D.A. strain No. 209) was divided into two portions. One half was retained as a control and the other was placed in a petri dish and exposed for 6 minutes at a distance of 50 cm. to radiation produced by a Hanovia double-U SC-2537 ultra-violet mercury vapor lamp operating at 100 milliamperes. Both portions were then plated in appropriate dilutions in nutrient agar and in nutrient agar containing various concentrations of penicillin. After

incubation for 72 hours at 37°C. the colonies were counted. The results are tabulated under Exp. 1 in table 1. All plating was done in triplicate and the figures given are averages of the 3 plates. The control organisms show a distribution of resistant forms similar to that described by Demerec.[5] Although the radiation killed about 40% of the bacteria, a greater number of resistant organisms were present among the survivors than in the unirradiated controls. The exposure to the light produced resistant individuals that had not been present originally.

TABLE 1

INCREASE IN RATE OF MUTATION TO PENICILLIN RESISTANCE BY IRRADIATION OF THE BACTERIA OR THE MEDIUM IN WHICH THEY ARE GROWN

PENICILLIN CONCENTRATION, UNITS/ML.	PLATE COUNT			
	EXP. 1. BACTERIA IRRADIATED		EXP. 2. MEDIUM IRRADIATED	
	CONTROL	IRRADIATED	CONTROL	IRRADIATED
0	221,000,000	135,000,000	114,000,000	46,000,000
0.027	483,000	531,000	27,300	182,300
0.030	86,100	269,800	7,500	40,900
0.033	4,670	56,200	4,610	33,100
0.036	1,250	12,700	2,200	16,900
0.039	1,100	5,180	415	6,500
0.042	495	4,400	317	4,600
0.045	136	1,660	12	1,360

Such individuals are regarded as mutants which differ from the normal population in an alteration of one or several genes. Ultimately, this alteration must be resolved to chemistry and physics. If chemical changes in genes can be brought about by exposure to radiation the possibility is presented that such changes might be introduced into the building materials from which the genes are produced. Therefore, experiments were devised to determine the effect of the irradiation of the substrate upon the mutation rate of organisms subsequently inoculated therein.

Nutrient broth was exposed to radiation from the ultra-violet lamp previously mentioned. After a 3-hour exposure the broth was transferred to a culture flask and an unirradiated equal portion from the same batch of nutrient broth was transferred to a similar flask. Immediately both were inoculated in an identical manner, at the rate of about one million cells per ml. from a 24-hour culture of *S. aureus*. Both flasks were incubated for 5 hours and then appropriate dilutions were plated on nutrient agar containing concentrations of penicillin varying from 0 to 0.045 Oxford units per ml. In all experiments a sufficiently large flask of nutrient agar of each penicillin concentration was prepared so that the plates of both the irradiated and unirradiated series would be poured with agar from the same flask. This eliminates the possibility of errors in dosage at any single level when comparisons are made within any experiment. The plates were counted after incubation for 72 hours at 37°C. and the averages from the triplicate platings are presented as Exp. 2 in table 1.

It is evident that neither culture had attained maximum growth in the 5 hours which had elapsed before plating. The culture growing in the irradiated broth had produced only 46 million cells or about $5^1/_2$ generations, while the culture growing in the control broth produced 114 million cells or about 7 generations. In spite of this lower total production of cells the actual number of resistant bacteria in the irradiated broth at each level of penicillin is many times greater. This fact helps to rule out selection as the cause of the phenomenon and suggests that we are dealing with induced mutation.

This experiment was repeated many times with a number of variations. The penicillin routinely employed was obtained from Commercial Solvents, but Abbott and Merck penicillins were also used. Another strain of *S. aureus* was substituted for the F.D.A. strain in a few of the experiments. For many of the tests a Hanovia analytical model quartz-mercury lamp with Type A burner was used at a distance of 20 cm. and the irradiation of the broth was continued for only 90 minutes. In most cases water lost from the broth by evaporation during exposure to the lamp was replaced, but in some cases it was not. The size of inoculum was varied and the time of plating was delayed to as long as 30 hours after inoculation. Although some quantitative differences were noted the results obtained in all cases were qualitatively similar, *viz.*, cultures grown in irradiated broth produced more mutations.

TABLE 2

INCREASE IN MUTATION RATE BY IRRADIATION OF COMPONENTS OF A SYNTHETIC MEDIUM

PENICILLIN CONCENTRATION, UNITS/ML.	PLATE COUNTS ON CULTURES GROWN IN A SYNTHETIC MEDIUM AFTER IRRADIATION OF THE INDICATED COMPONENTS			
	NONE (CONTROL)	COMPLETE MEDIUM	MINERAL SALTS	AMINO ACIDS AND VITAMINS
0	800,000,000	No growth	900,000,000	195,000,000
0.04	33,000	No growth	40,000	150,000
0.07	4	No growth	4	196
0.10	1	No growth	1	16

If the synthetic medium described by Fildes, *et al.*,[6] is subjected to the same irradiation as the nutrient broth in Exp. 2, table 1, the organisms make no growth. This medium consists of mineral salts, the amino acids found in casein, glucose, and the vitamins thiamin, niacin and biotin. In our experiments we supplemented the latter with one mg. per liter each of adenine, guanine and uracil. Each component was prepared in sterile solution at several times the final concentration so that it could be irradiated separately before combining with the other constituents of the medium. In the experiment reported in table 2, four batches of the synthetic medium were prepared: (1) an unirradiated control, (2) the complete medium irradiated, (3) a double strength solution of the mineral salts irradiated and then added to the remaining components, (4) a triple strength

solution of the amino acids and vitamins irradiated and added to the remaining components. The four media were inoculated in an identical manner and incubated for 24 hours. At that time the culture in which all the components were irradiated had made no visible growth so plate counts were made only on the other three cultures. Irradiation of the mineral salts resulted in a mutation rate no higher than the control but irradiation of the amino acids and vitamins resulted in a marked increase in the mutation rate. Further experiments showed that a good part of the toxic effect (which prevented growth when the complete synthetic medium was subjected to the amount of radiation routinely used in nutrient broth experiments) arose from the glucose. When the irradiation of the glucose was decreased to a level that permitted growth, the mutation rate of cultures grown in media prepared from it was not enhanced.

That the irradiation of the amino acids alone increased the mutation rate is indicated in table 3. Nutrient broth cultures were included in this experiment because the plating was done on agar containing streptomycin as well as on agar containing penicillin. It is evident that rate of mutation

TABLE 3

INCREASE IN THE RATE OF MUTATION TO STREPTOMYCIN AND PENICILLIN RESISTANCE

INHIBITOR, UNITS/ML.	NUTRIENT BROTH		SYNTHETIC MEDIUM	
	CONTROL	IRRADIATED	CONTROL	AMINO ACIDS IRRADIATED
0	300,000,000	260,000,000	1,250,000,000	900,000,000
Penicillin				
0.04	13,000	120,000	12,000	55,000
0.07	10	310	30	1,520
Streptomycin				
1.0	42,000	140,000	30,000	168,000
3.0	5,000	33,000	2,700	23,000

to streptomycin resistance follows the same pattern observed with penicillin resistance. The streptomycin employed in the experiment was crystalline material obtained from Merck which had a potency of 187 units per mg. Other experiments were carried out with lyophilized powder from Commercial Solvents put up in ampules containing 100,000 units.

To demonstrate that the streptomycin- and penicillin-resistant organisms were the result of different mutations, a number of colonies of the mutants were picked and transferred to nutrient agar. Similarly, a number of subcultures were isolated from the control plates. After 24-hour incubation a uniform inoculum from each isolate was streaked to sectors of plates containing streptomycin or penicillin. The plates were incubated for 48 hours and the growth of each culture on each concentration of antibiotic was noted (table 4). Due to the small inoculum used the results were generally clear-cut, although occasionally only a few colonies appeared;

these were reported as positive growth. It will be observed that mutants selected for penicillin resistance differ greatly from the controls when tested on penicillin agar but are very similar to the control organisms when tested on streptomycin agar. The streptomycin-resistant mutants differ from both the controls and the penicillin-resistant mutants. The mutant population is composed of different individuals whose gene differences are induced during growth in the irradiated substrate; the specific mutants are then separated from each other and from the normal population by plating on agar containing the inhibiting substances.

After the specificity of the resulting mutants was demonstrated, attempts were made to determine if specificity could be introduced into the induction of mutation, i.e., if the rate of mutation of one particular gene could be influenced without affecting the rate of mutation of others. Preliminary experiments indicate that this may be possible, for by suitable irradiation procedures we have been able to increase the rate of mutation to penicillin resistance without affecting the rate of mutation to streptomycin resistance.

TABLE 4

SPECIFICITY OF THE DRUG-RESISTANT MUTANTS

STRAINS	NO. TESTED	NO. OF STRAINS GROWING ON MEDIUM CONTAINING: PENICILLIN (UNITS/ML.) 0.05	0.1	0.15	1.0	STREPTOMYCIN (UNITS/ML.) 3	5	10
Penicillin-resistant	21	21	15	9	1	14	6	1
Controls	24	13	1	0	0	12	7	2
Streptomycin-resistant	29	12	2	0	0	29	29	24

Discussion.—These data are the preliminary results of the new method of investigation of gene mutation and gene chemistry. Irradiation is used to *activate* selected chemicals, which, when utilized by the cells, cause mutations. These substances might be termed *activated mutators*. The experimental methods used are very effective in detecting mutations to resistance to toxic agents, in this case penicillin and streptomycin.

Several lines of evidence indicate that these are induced mutations, not the result of selection:

1. In an immature culture there are numerically many more mutants in the irradiated broth despite a smaller total population (table 1).

2. When mutant strains were isolated and their rate of growth was followed in both irradiated and unirradiated broth it was found that they grew more slowly in the former; the rate of growth in both media did not differ significantly from that of control organisms.

3. These mutations are of independent origin and do not represent a general increase in resistance to toxic agents for, on testing, the penicillin-

resistant mutants are no more resistant to streptomycin than are the controls and vice versa (table 4).

It seems improbable that this method of treatment is limited to the production of mutations concerned with resistance. The first two things tried—mutations to resistance to penicillin and streptomycin—were induced by this treatment. With suitable tests many other types of mutation should be detected. Therefore, we believe this will prove to be a generally useful procedure, within its limitations, when used to produce other types of mutation, and mutations in other organisms.

Little need be said of the obvious advantage of this method for the study of mutation and gene chemistry. Instead of treating an exceedingly heterogeneous living organism, we are treating a selected chemical and determining if it produces mutations. If it does, we can investigate the physical or chemical changes that have occurred. These may be either or both of the following: (1) The production of different chemical compounds under the influence of irradiation. (2) Some mechanism involving a shift to a higher energy level by the absorption of a quantum of energy and subsequent effects of this energy transfer. At present we cannot decide between these alternatives, although we have determined that the mutating ability of the treated material is reduced on aging and more rapidly by heat treatment. This suggests that alternative (2) may be correct, but of course it only concerns these particular mutations. On the other hand, it is known that a definite chemical substance, nucleic acid, can induce transformation in the genetic material of microörganisms (Avery, *et al.*[7]) The possibility of producing selected mutations offers a fascinating field for further investigation. Certainly the possibility is inherent in this method. Genes must differ; therefore, we may be able to select agents which will affect only one or at most a few genes and so, indirectly, study gene chemistry. In the experiments reported here, we were able to produce two types of resistant mutants. The difference in survival in the different concentrations of the antibiotics indicates that several different alleles or different mutations are involved (tables 1, 2, 3). We have not been able to determine if the treatment produces changes in an existing gene or if it causes an error in its autocatalytic reproduction. Under ordinary circumstances genes are remarkably free from errors in copy as exemplified by the low rate of spontaneous mutation.

There are several general considerations which follow from these discoveries. For example, a study of the effect of irradiated substrate on the development of cancerous tissue may prove profitable. If any of the activated mutators prove stable this phenomenon must be considered in nutrition and in safeguarding the use of atomic energy. The most important consideration is the possibilities opened for further investigation into the gene and mutation. These experiments have shown that at least part of

the mutations produced by irradiation may be the result of an indirect effect in addition to those from a direct hit on the gene. They may explain at least part of the discrepancy between the observed normal mutation rate and that which has been calculated as attributable to direct hits on genes, Muller.[1] Natural radiation is much more important in the mutation process if it can induce mutation by an effect on the food as well as on the organism.

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