space, the dual being a discrete one. However, for infinite duration the dualism is a self-dualism in the following sense.

A Wiener differential process on the half line $0 \leq x < \infty$ *is its own cosine and sine transform, as a process.*

Finally from our approach we are able to analyze Khintchine's stationary processes very systematically, although none of the results can be strictly new, since these processes are more or less included in the theory of Hilbert space.

A full account will appear in another journal.

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*MUTATIONS INVOLVING THE REQUIREMENT OF URACIL IN CLOSTRIDIUM**

BY FRANCIS J. RYAN, LILLIAN K. SCHNEIDER AND R. BALLENTINE

DEPARTMENT OF ZOÖLOGY, COLUMBIA UNIVERSITY

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Some of the variations which occur during the growth of bacteria are due to the selection of types which arise at random by mutation. In the case of resistance to phage[1] and radiations[2] in *Escherichia coli* and penicillin resistance in *Staphylococcus aureus*[3] these changes behave like genic mutations in that there is a finite probability for each individual bacterium to undergo an hereditary change in the course of its lifetime. These changes are not induced by the unfavorable agents but occur in their absence. Phage radiation and penicillin simply select those resistant organisms which arise at random. With respect to nutritional requirements the demonstration of the genic nature of mutation is not so clear. Roepke, *et al.*,[4] found that mutants of *E. coli* with specific growth factor requirements appeared after x-radiation. However, they were unable to attribute the changes to the x-radiation because of the incidence of spontaneous mutations. This was also the experience of Gray and Tatum[5] with *E. coli* but these authors showed that growth factor deficiencies were induced in *Acetobacter melanogenum* by x-radiation. Later, when more material was available, Tatum[6] was able to show that x-radiation significantly raised the frequency of nutritional mutants in *E. coli*. By analogy with the production of biochemical mutations in *Neurospora*,[7] a sexual organism, the latter authors suggest that biosyntheses in bacteria are controlled by specific genes. We have been able to show that a strain of *Clostridium septicum* mutates to a condition where it is no longer able to synthesize the pyrimidine, uracil, but requires it in the medium for growth. Conversely, the uracil-dependent strain mutates to a state where uracil is

synthesized and need not be supplied. These mutations occur throughout the growth of the culture and even in the presence of uracil. Thus, these changes in nutritional requirement satisfy criteria for gene mutation which can be tested in an organism in which sexual reproduction has not been demonstrated.

Strain 59 Li of *Cl. septicum* will grow in a chemically defined medium which contains salts, sugar, purified Bacto-casamino acids, glutamine, tryptophane, cystine, cysteine hydrochloride and the vitamins pantothenic acid, nicotinic acid, pyridoxine, thiamin and biotin.[8] However, on this basal medium we found growth to be erratic and inconsistent among similar tubes in the same experiment.[9] The time of initiation of growth varied considerably and not infrequently growth failed to occur (table 1).

TABLE 1

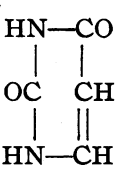
VARIATION IN THE ONSET OF GROWTH OF STRAIN 59 Li ON CHEMICALLY DEFINED MEDIUM

	APPEARANCE* OF CULTURES AT VARIOUS HOURS AFTER INOCULATION			
TUBE	8	22	34	51
1	−	−	−	−
2	−	−	+	+++
3	−	−	+	+++
4	−	−	−	−
5	−	+	+++	+++
6	−	+++	+++	+++
7	−	+	+++	+++
8	−	+++	+++	+++
9	−	+	+++	+++
10	−	−	+	+++
11	−	−	−	−
12	−	−	++	+++

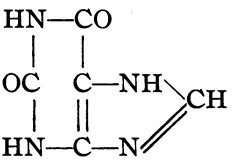
* − designates no growth; increasing densities of bacteria are represented by +, ++ and +++.

But when growth did occur, irrespective of when it started, the rate of growth during the logarithmic phase and the final crop of bacteria were the same in similar tubes. The average time for one generation to be produced during the logarithmic phase of growth was 80 ± 4(σ_M) minutes. After growth was complete the cultures contained on an average 13.0 ± 1.0 mg. of bacterial nitrogen per 100 ml.

It was found that this variation in the lag period could be eliminated by the addition to the medium of the pyrimidine, uracil,



or to a lesser extent by its related purine, xanthine,



(table 2). In the presence of an optimum concentration of 10 γ per ml. of uracil growth was completed in every case and generally in less than 18 hours. The rate of growth and the yield of bacteria were the same in the presence as in the absence of uracil (table 3). The role of uracil is in the reduction of the length of the lag phase and in the assurance that growth will occur.

TABLE 2

THE TIME IN HOURS REQUIRED BY STRAIN 59 Li TO REACH ALMOST COMPLETE GROWTH (+++) IN THE PRESENCE OF DIFFERENT COMBINATIONS OF PURINES AND PYRIMIDINES, EACH PRESENT IN THE CONCENTRATION OF 5 γ PER ML. EACH TIME IS THE AVERAGE OBTAINED FROM 3 TO 8 CULTURES

	0	ADENINE	GUANINE	XANTHINE	URACIL	XANTHINE URACIL	GUANINE URACIL	XANTHINE GUANINE
0	43	55	43	30	21	..	..	..
Adenine	..	..	37	76	37	17	33	72
Guanine	..	..	..	39	25	24	..	..
Xanthine	..	..	..	..	19	..	24	..
Uracil	..	..	..	..	..	..	..	24

TABLE 3

THE EFFECT OF URACIL ON THE GROWTH OF URACIL-DEPENDENT STRAIN, 59 Li B

URACIL, γ/ML.	MINUTES TO 2 MG. NITROGEN ± AVERAGE DEVIATION	GENERATION TIME, MINUTES	MG. NITROGEN AFTER 48 HOURS
0	1630 ± 500*	79	12.0
10	800 ± 162	78	12.5

* This figure is the average of observations on six cultures from two different experiments. Of the remaining tubes in the first experiment, one grew during the third day after inoculation, one during the fourth day and three had not grown on the fifth day when they were discarded. In the second experiment, one culture grew during the fourth day, one during the fifth and one, which had not grown, was discarded on the fifth day. None of these very long lag periods were included in the average.

One explanation of this phenomenon involves the assumption that a culture of strain 59 Li of *Cl. septicum* contains two types of organisms, one type requiring uracil in the medium and the other being independent of an external supply of uracil. The two types arise from one another by mutation. An inoculum of strain 59 Li, therefore, may contain some uracil-independent organisms. If this number is large, the inoculum on

our minimal medium will grow up rapidly; if small, growth will start slowly. If there are no uracil-independent organisms the inoculum will not grow unless one or more of its members mutates. Verification of this hypothesis demands a demonstration of the two types of bacteria and their origin by mutation.

A culture of 59 Li which, after a lag period of variable length, has grown on medium devoid of uracil will contain, on this basis, at least a reasonably large number of uracil-independent bacteria. An inoculum from such a culture proved to be independent of uracil on our basal medium and upon repeated transfer showed regular growth and a short lag period. This uracil-independent strain was plated on blood-agar and thirteen single colonies were isolated. All of these new strains behaved like their parent and grew regularly and rapidly in the absence of uracil. Indeed the addition of 10 γ per ml. of uracil to the medium on which one of these strains, 59 Li A, was grown was without effect (table 4). Therefore, the independent component of strain 59 Li has been identified.

TABLE 4

THE EFFECT OF URACIL ON THE GROWTH OF URACIL-INDEPENDENT STRAIN, 59 Li A

URACIL, γ/ML.	MINUTES TO 2 MG. NITROGEN ± AVERAGE DEVIATION	GENERATION TIME, MINUTES	MG. NITROGEN AFTER 48 HOURS
0	690 ± 40	59	14.9
10	739 ± 40	62	14.5

The dependent component has also been recovered in the form of single colony isolates obtained from blood-agar plates of strain 59 Li. Of 23 such isolates 7 were dependent. These dependent strains behaved in their growth like the parent, 59 Li. Growth was erratic in the absence of uracil but regular in its presence. The fact that these dependent strains grew at all in the absence of uracil must have been due to the presence of some uracil-independent organisms. Since each of these dependent strains was isolated from a single colony their dual composition resulted from mutation of one type to the other—probably from dependence to independence. This mutation could have occurred during growth or in the spore stage in egg-meat medium, during growth of the inoculum in complete medium or while the inoculum was suspended in the uracil-free medium.[9] The erratic nature of the lag period could have been due to a variation in the number of independent organisms in the inoculum or to a variation in the time at which mutation occurred in the uracil-free medium.

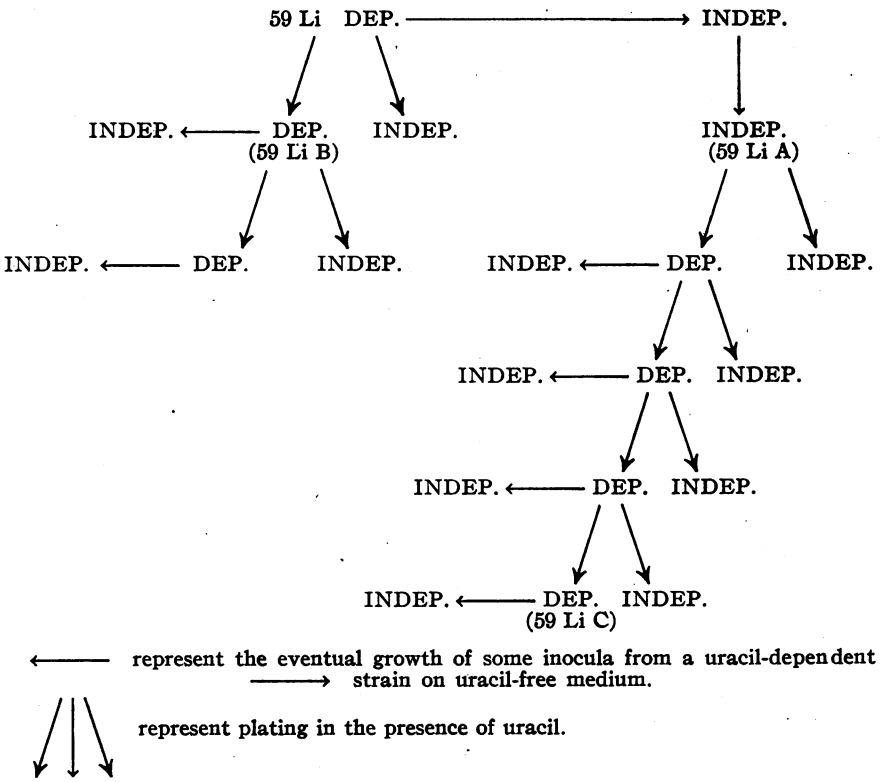
One of the new dependent strains, 59 Li B,[10] was itself plated onto blood-agar. Isolation of single colonies yielded 2 strains which were uracil-dependent and 4 which were independent of uracil. Consequently, both types of cells were present in a culture not many generations from isolation as a single colony. Since care was taken to isolate well-separated colonies in single spherical zones of hemolysis on the blood-agar plates, since even

after repeated plating *all* of the dependent strains developed independence of uracil and since microscopic observation showed that agar did not cause a clumping of bacteria, it is unlikely that any colony arose from more than one organism.

Conversely when the new independent strain, 59 Li A, was plated onto blood-agar, 1 of 64 isolated strains proved to be uracil-dependent. This strain must have arisen by mutation from a uracil-independent organism and when replated gave rise to both uracil-dependent and uracil-inde-

FIGURE 1

CHANGES IN THE URACIL REQUIREMENT OF *Clostridium septicum*



pendent strains. This repeated plating of dependent organisms was carried out two more times with the same results, making it highly improbable that the dependent colonies arose from more than one organism. A summary of these isolations is given in figure 1.

The dual compositions of the dependent and independent strains must have resulted from the mutation of one type to the other. Thus the demands of the hypothesis have been fulfilled. The uracil-independent bacteria mutate to uracil-dependence and the uracil-dependent bacteria

mutate to a condition in which they are independent of uracil. This would account for the sudden growth which sometimes occurs in uracil-free medium after a period as long as 5 days. In an organism with a logarithmic generation time of about 80 minutes and where only about 10 generations need occur between the inoculum and final growth, 5 days seems an unreasonably long lag period on any other basis. However, we have attempted and failed to demonstrate that mutations occur while the bacteria are suspended in uracil-free medium.

The chance that a mutation will occur in a culture is a function of the number of organisms present. In a very small inoculum of dependent cells suspended in uracil-free medium, mutation, and hence growth of the culture, should be a very rare event. On the other hand in the presence of uracil such small inocula should grow regularly. Consequently, the behavior of uracil-dependent inocula of various sizes was studied in the absence and presence of uracil. When the inoculum was large and contained about 10^6 organisms or more (washed free of uracil by centrifuging and resuspending in uracil-free medium three times) growth took place regularly and rapidly both in the presence and in the absence of uracil. When the inoculum contained between 10^3 and 10^5 organisms, although growth was rapid and consistent in the presence of uracil, in its absence growth was as variable as that shown in table 1. The number of organisms contained in the loop inocula which we routinely used is about 10^3. When the inoculum size is as small as 10^2 organisms growth fails to occur not only in the absence of uracil but also in its presence. Apparently in the large inoculum there was a sufficiently large number of uracil-independent organisms to permit rapid growth. As the size of the inoculum decreases this number becomes so small that the time of onset of growth from tube to tube is variable. This variability seems to be a consequence of the number of uracil-independent cells already in the inoculum and not, at least in most cases, upon mutation in organisms suspended in the uracil-free medium.

However, there is one major difficulty with the application of this interpretation by itself—the proportion of uracil-independent organisms in a uracil-dependent inoculum is too high. A summary of the proportions of two types of organisms in dependent inocula determined by isolations from the platings described in figure 1 indicate that 68% of the cells were uracil independent (70 out of 103). In other determinations the likely average proportion of uracil-independent organisms was 77%. It may be as low as 50% or as high as 100%. In the latter case growth in uracil-free medium would be regular and rapid, an event which rarely but definitely does occur with some inocula. Although these inocula are not dependent themselves they are derived from stock cultures of spores in egg-meat medium which previously and subsequently yielded uracil-dependent inocula. In view of the high average proportion of uracil-independent organisms in

uracil-dependent cultures it is difficult to understand how inocula containing about 10^3 cells would vary sufficiently to account for the observed variation in the onset of growth in uracil-free medium, especially when the lag may sometimes be as long as 5 days.

Undoubtedly some other factor is involved in the variation in the onset of growth. It is probably the result of the fact that our usual inoculum of 10^3 cells is too close to the minimum size. The fact that there is a minimum inoculum size of more than one organism indicates that our chemically defined medium is not optimum. A certain number of cells must be present to modify the medium and permit growth. In a small inoculum where the cells are in dilute suspension there may be too slow a synthesis or too rapid a loss of some essential substance. Be that as it may, we occasionally do not provide *enough* uracil-independent cells for growth to occur. At other times the marginal number of uracil-independent organisms in the inoculum may recover and grow after a variable interval in uracil-free medium. On the other hand, in rare instances, even with an inoculum of about 10^3 organisms, the dependent strain will grow up rapidly in a series of tubes containing uracil-free medium, although on further test the strain will prove to be dependent. Presumably in such cases the inoculum contained close to 100% independent cells. Although the proportion of independent organisms and, perhaps, mutation to uracil-independence are involved in the variation in the onset of growth, the major factor is probably the absolute number of uracil-independent organisms in the inoculum.

Regardless of the role that mutations in uracil requirement play in the variable growth of *Cl. septicum* their properties are in themselves interesting. The organism is able to undergo a reversible change in its ability to synthesize uracil. If we assume that there is no selection in favor of either of the two types of organisms when together in the presence of uracil, we may conclude on the basis of the frequencies of the two types that mutations to uracil-dependence are much less frequent than mutations to uracil-independence. The equilibrium proportions seem to involve a preponderance of uracil-independent organisms. About 98% (63 out of 64) of the isolates from platings of 59 Li A were uracil-independent. An independent determination of the proportion was made by comparing the number of organisms from the same culture which would form colonies in the presence of uracil, with the number which formed colonies in its absence. Ten cultures were examined and again 98% of the organisms were uracil-independent. When determined by this method cultures derived from dependent strains and grown to completion contained about 77% uracil-independent cells. Isolate counts indicated the presence of about 68% independent organisms. Judging from these proportions equilibrium must nearly be attained during the growth of the inoculum to completion. The equilibrium may sometimes be achieved in the stock culture in egg-

meat medium. Some cultures when tested within a few days after they were established from isolates may prove to be dependent upon uracil. But after a week or longer they may contain a proportion of uracil-independent cells so large as to yield inocula which grow rapidly and regularly in the absence of uracil. Other stock cultures remained uracil-dependent for periods of many months on egg-meat medium where the equilibrium proportion apparently was not achieved. Frequently as many as 5 serial transfers of some uracil-dependent strains on chemically defined medium containing uracil did not result in the assumption of equilibrium. This behavior (due to mutation rates or selection) is probably a function of variability among the organisms, but it is poorly understood.

By means of a statistical procedure devised by Luria and Delbrück[1] it is possible to discriminate between the random and the induced nature of these mutations. This procedure is based upon the clonal mode of reproduction in bacteria. A mutation occurring early in the growth of a culture will be represented at final growth by more mutant organisms than will represent a later mutation (i.e., assuming no selection). If the mutations are "spontaneous," and have a given chance of occurring per organism per unit time throughout the growth of the culture, a great deal of variation in the proportion of mutants from culture to culture would be expected at final growth. On the other hand, if the mutations are induced and each bacterium has a given chance of responding by mutation to the testing conditions, similar proportions of mutants should be found from culture to culture. To test the applicability of these two interpretations of mutation comparisons can be made between the variation in the proportions of mutants in a series of different cultures with the variation in a series of samples from the same culture.

When this method is applied to organisms mutating to a complete requirement for some growth factor, only mutations in the presence of that growth factor can be studied, for unless the factor is supplied by the investigator or excreted into the medium during the growth of the growth-factor-independent cells the mutant organisms will not duplicate. The infrequent occurrence of uracil-dependent mutants in our cultures may have been due to spontaneous mutations in the presence of uracil although the frequency with which they occurred was too small to enable demonstration of this. On the other hand mutation from uracil-dependence to uracil-independence seems to be very rapid. Dependent cultures derived from freshly isolated colonies contain a majority of uracil-independent organisms. Nothing is known of the role of selection between the two types of cells although by itself one independent strain grew faster and farther than a dependent strain (tables 3 and 4). The study of mutation to uracil-independence must also, of course, be made on cultures grown in medium-containing uracil.

The testing medium, on the other hand, since it is to contain no uracil, must have a chemically defined composition. We have found it very difficult to secure reproducible data when colony counts were made of pour plates containing our chemically defined medium in agar.[8] Therefore recourse was had to the use of 0.15% semisolid agar medium in test tubes. The colonies formed in such a loose gel were easy to count and perhaps because of the depth of medium the proper anaerobic conditions were maintained to allow for consistent results at several dilutions. A uracil-dependent strain of *Cl. septicum*, 59 Li C, was diluted with medium to which uracil had been added to contain about 10^3 cells per 2 ml. Ten 2-ml. lots of this suspension were incubated until growth was complete. Then from each of 9 tubes a 0.1-ml. sample was taken and added to 100 ml. of medium devoid of uracil. From the tenth 2-ml. tube nine 0.1-ml. samples were removed and likewise diluted to 100 ml. From each of these dilutions 1 ml. was taken and added to 9 ml. of semisolid agar medium containing 10 γ uracil per ml. in test tubes. From each of these 1 ml. of the mixed suspension was removed and added to another 9 ml. of semisolid agar plus uracil. This procedure was repeated until semisolid agar tubes were obtained containing dilutions between 10^{-3} and 10^{-7}. From the last series of tubes 1 ml. was discarded. A similar series of dilutions was made from each of the original 100-ml. dilutions in semisolid agar tubes containing medium devoid of uracil. After 24 hours' incubation the cells, which were separated by careful mixing, had formed discrete colonies which were counted. The results are shown in table 5.

There is a 0.24 probability that the distribution of proportions of uracil-independent bacteria among the 9 samples taken from the single tube was due to chance variation such as sampling error. On the other hand, there is only a 0.007 probability that the distribution of proportions among the 9 separate tubes has a similar cause. More probably the fluctuations from tube to tube reflect the distribution of mutations at different times during the growth of the culture. In this experiment it was impossible to start the growth with an inoculum of purely uracil-dependent organisms. Hence the differences from culture to culture are obscured by the uracil-independent organisms already present in the inoculum and the variation although larger than the sampling error is less than that observed in other instances.[1, 2, 3]

Hence, it appears as though mutations to uracil-independence occurred throughout the growth of uracil-dependent cultures in the presence of uracil (although not necessarily at random). These mutations were selected for and not induced by the absence of uracil in the testing medium. In our studies on the back-mutation of strains of *E. coli* which have been induced by radiation to require various amino acids for growth we have been able to demonstrate a similar spontaneity of mutation. In the case

of some strains of *E. coli* where there is no selection for or against the mutant organisms and where the frequency of mutations in a full grown culture is so small as to permit the preparation of inocula which contain no independent organisms, the variance from culture to culture is enormous and may be hundreds of times the mean. These studies will be reported elsewhere.

TABLE 5

PROPORTION OF URACIL-INDEPENDENT BACTERIA IN SAMPLES FROM DIFFERENT CULTURES, AND IN SAMPLES FROM A SINGLE CULTURE

SAMPLES FROM DIFFERENT CULTURES				SAMPLES FROM A SINGLE CULTURE			
SAMPLE NUMBER	TOTAL NUMBER OF ORGANISMS PER SAMPLE $\times 9/10 \times 10^{-5}$	NUMBER OF URACIL-INDEPENDENT ORGANISMS PER SAMPLE $\times 9/10 \times 10^{-5}$	PERCENTAGE URACIL-INDEPENDENT ORGANISMS PER SAMPLE	SAMPLE NUMBER	TOTAL NUMBER OF ORGANISMS PER SAMPLE, $\times 9/10 \times 10^{-5}$	NUMBER OF URACIL-INDEPENDENT ORGANISMS PER SAMPLE, $\times 9/10 \times 10^{-5}$	PERCENTAGE URACIL-INDEPENDENT ORGANISMS PER SAMPLE
1	0.06	0.05	83	11	28	23	82
2	10	7	70	12	28	28	100
3	15	10	67	13	46	35	76
4	16	15	94	14	48	42	88
5	3	2.3	77	15	30	21	70
6	3.1	1.5	48	16	17	15	88
7	14	13	93	17	22	20	91
8	47	34	72	18	38	27	71
9	2.2	1.6	73	19	44	41	93
Average			75				84
Variance			198				106
χ^2			21.10				10.12
P			0.007				0.24

The mutations in nutritional requirement in bacteria are similar to mutations of genes in sexual organisms. The inherited biochemical deficiencies involved are similar to those shown to be associated with single genes in *Neurospora* and they can be similarly induced by chemicals and radiation.[12] Moreover, the mutations are spontaneously reversible in the sense that the complete re-acquisition of synthetic capacity is inherited and is not induced by the absence of the required substance from the medium. The same is sometimes true of mutants of *Neurospora*.[13] These similarities allow the conception of biosyntheses of bacteria under the control of specific self-duplicating factors. These factors are capable of being brought to a state where, although they no longer enable the biosynthesis to be carried out, they continue to self-duplicate and can mutate to the original condition. An understanding of the mode of transmission of these hereditary units awaits the results of future research.

Conclusion.—The pyrimidine, uracil, is required for the growth of a strain

of *Cl. septicum*. Organisms of this strain have a given chance to back-mutate to a condition of uracil-independence in the course of their life-time. This mutation is spontaneous in the sense that it is not induced by the absence of uracil. Uracil-independent organisms can also mutate to a condition of uracil-dependence. During the growth of this strain of *Clostridium* there is a tendency to reach an equilibrium proportion of the two types of organisms which is well in favor of the uracil-independent form. This tendency is probably due to a differential in mutation rates although selection may play a role.

In 10 ml. of liquid medium an inoculum of at least ca. 1000 cells is required for the initiation of growth. The variable time until the onset of growth of the dependent strain in the absence of uracil is mainly due to the variable number of uracil-independent organisms in the inoculum. These organisms arose by mutation from uracil-dependent cells.

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[1] Luria, S. E., and Delbrück, M., *Genetics*, **28**, 491 (1943).

[2] Witkin, E. M., these PROCEEDINGS, **32**, 59 (1946).

[3] Demerec, M., *Ibid.*, **31**, 16 (1945); *Ann. Mis. Bot. Gard.*, **32**, 131 (1945).

[4] Roepke, R. R., Libby, R. L., and Small, M., *Jour. Bact.*, **48**, 401 (1944).

[5] Gray, C. H., and Tatum, E. L., these PROCEEDINGS, **30**, 404 (1944). These authors claim that x-ray treatment was responsible for the mutant strains they obtained in *E. coli*. However, the incidence of mutations in their x-ray-treated bacteria was not significantly greater from that in the control bacteria ($\chi^2 = 2.0$; $P = 0.15$). In *A. melanogenum* the difference was significant ($\chi^2 = 8.0$; $P = <0.01$).

[6] Tatum, E. L., these PROCEEDINGS, **31**, 215 (1945).

[7] Beadle, G. W., and Tatum, E. L., *Ibid.*, **27**, 499 (1941).

[8] Ryan, F. J., Ballentine R., and Schneider, L. K., in press.

[9] Unless otherwise mentioned the various strains of *Cl. septicum* were maintained as spores on egg-meat medium and inocula were prepared by transferring some egg-meat into a complete (yeast extract-tryptose) broth.[8] Loop inocula were used. Growth was measured turbidometrically by means of a densitometer calibrated in terms of mg. of *Cl. septicum* nitrogen.

[10] Proof that 59 Li A and 59 Li B were actually derived from 59 Li and are not contaminants lies in a series of similar properties. All are gram-positive anaerobic rods which grow to about the same extent on our basal medium. Hence, their vitamin requirements are not more extensive than those of 59 Li. Moreover, both can utilize pantoyl lactone instead of pantothenic acid but not β-alanine (cf. Ryan, F. J., Ballentine, R., Stolovy, E., Corson, M. E., and Schneider, L. K., *Jour. Am. Chem. Soc.*, **67**, 1857 (1945)).

[11] Redowitz, E., *Amer. Jour. Clin. Path., Technical Supplement*, **5**, 26 (1941.)

[12] Tatum, E. L., *C. S. H. Symp. Quant. Biol.*, **13**, in press (1946).

[13] Ryan, F. J., and Lederberg, J., these PROCEEDINGS, **32**, 163 (1946).