Spontaneous Mutation in Spheroplasts of *Escherichia coli*

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SUMMARY

Spheroplast suspensions containing as few as one rod in a thousand were made with *Escherichia coli* (15 *his*⁻) by the use of penicillin. These spheroplasts could be reverted to the rod state with efficiency approaching 100 %. Spontaneous mutation from histidine requirement to non-requirement was observed in these spheroplasts, in which cell division did not take place.

INTRODUCTION

The mechanism of induced mutation has been investigated profitably along several lines but less is known about spontaneous mutation (Ryan, 1963). Some studies have been made with a histidineless strain of *Escherichia coli* (15 *his*⁻) in a non-dividing condition (Ryan, 1955, 1959; Ryan & Kiritani, 1959; Ryan, Rudner, Nagata & Kitani, 1959). The method involves distributing a culture into several hundred tubes containing a defined medium without histidine. When the concentration of organisms is about 1×10^7 /ml. the cultures are so clear that overgrowth of *his*⁺ mutants which might be present is easily recognized through the development of turbidity. At 20°, *his*⁺ mutants, initially present at the time of distribution, overgrow in about 100 hr. Thereafter, for several hundred hours new overgrowths, in tubes that did not receive a pre-existing *his*⁺ mutant, occur because of *his*⁺ mutants arising during the stationary phase. Since the medium used does not contain histidine it might be expected that the *his*⁻ organisms do not grow or divide; in fact, while the total count remains constant, the number of viable bacteria usually decreases by death during a long incubation. This fact may seem to indicate that the mutation occurs in non-dividing organisms. It does not, however, exclude the possibility of a cell turnover in which a cryptic lysis and replacement might balance one another. If this were the case, mutation in non-dividing bacteria could occur as an error in replication of the chromosome (or of DNA) during the cell division required for replacement. Much effort has been expended in examining this possibility by several methods (Ryan, 1959; Ryan, Nakada & Schneider, 1961). These attempts, which included reconstruction and penicillin experiments and the determination of DNA, did not reveal any cell turnover or cell growth in the stationary phase. Each piece of evidence was, however, circumstantial and not critical; together the evidence pointed to the conclusion that the stationary-phase bacteria were not dividing; in fact they did not even seem to be engaged in a turnover of DNA. A system of *his*⁻ spheroplasts was, therefore, developed in order to investigate the role played by enzymes which digest macromolecules. This system and the evidence that mutations occur in spheroplasts are presented herewith.

METHODS

The media used were: Difco penassay broth, and penassay broth and G & T (Ryan, 1959) medium with 0.05 % glucose, both containing 20 % sucrose and 0.2 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The last two hypertonic solutions were the complete and defined media used for maintenance and enumeration of spheroplasts. To solidify them, 1.0 % agar was added. Nutrient agar or G & T agar supplemented with 0.5 % yeast extract and 0.5 % NZcase (YECA agar) were used for counting the rods. To make spheroplasts, penicillin was used at a concentration of 1000–2000 units/ml. in penassay broth.

The bacterium used was *Escherichia coli* 15 *his*⁻ *col*^{-r} (colicine-resistant and simultaneously sensitive to all T phages; Ryan, Fried & Mukai, 1955; Mukai, 1960) derived from 15 *his*⁻ *col*^{-s} which has been used for mutation experiments since 1955. This bacterium was grown in penassay broth at 37° overnight with gentle rotation. Following this, 1.0 ml. of the fully grown suspension was introduced into 4.0 ml. of penassay broth and incubated at 37° for 2–3 hr. in the same way, to bring the bacteria into the logarithmic phase. Suspensions thus obtained were used for all experiments.

The number of viable spheroplasts was determined by diluting the suspension in hypertonic penassay broth whereupon 0.5 ml. of a suitable dilution was plated in the same medium containing 1.0 % agar melted and cooled to 45°. The colonies develop from both spheroplasts and rods; by subtracting the number of rods the number of spheroplasts can be calculated.

For enumerating the rods, the suspension was diluted with 0.85 % saline solution and 0.1 ml. of the diluent was plated on nutrient agar medium. This procedure destroyed the spheroplasts but not the rods.

The procedure for making spheroplasts was almost the same as that of Lederberg (1956) and Lederberg & St Clair (1958). The efficiency of forming spheroplasts and their stability was first compared at 37° and at 20°. After introducing 1.0 ml. of cell suspension into 4.0 ml. of hypertonic penassay broth which contained 1000 units of penicillin/ml., the mixture was incubated at both temperatures. Samples were taken at 3 and at 8 hr. and the number of viable spheroplasts and rods was determined. As Fig. 1*a* shows, spheroplasts formed at 20° were more stable than those formed at 37° and the efficiency of their formation (intact spheroplasts/rods) was rather better at 20° than at 37°. Figs. 1*(b)* and *(c)* show data obtained from spheroplast preparations at 20°. The number of viable spheroplasts did not decrease appreciably during 6 hr. of incubation but the proportion of rods decreased rapidly to 10⁻³ during the first 3 or 4 hr. of treatment with penicillin, and then more gradually. In Fig. 1*(b)*, one of the two curves for rod number was obtained by diluting and plating the suspension without washing, while the other is based on the behaviour of washed cells. This result shows that there was little influence on the rod count even when penicillin was not washed out of the suspension before plating. Two sugar concentrations, 10 and 20 %, were also compared; Fig. 1*(c)* shows that there is no real difference between them. On the basis of the results described above, incubation was carried out in penicillin and 20 % sucrose for 4 hr. at 20° to create conditions suitable for making spheroplasts.

After the spheroplasts were made the suspension, which had been in penicillin

hypertonic penassay broth at 20° for 4 hr., was centrifuged in a Servall Centrifuge (type SS-1) at about 36,000 *g* for 10 min., the supernatant fluid decanted, and the organisms were resuspended in the same volume of G & T medium containing 20 % sucrose and 0.2 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ but no histidine. This procedure was repeated 3 times (two washings) in a 5° cold room and the final suspension diluted tenfold in the same medium. During this procedure there was no appreciable death of spheroplasts.

The cell suspension thus obtained, which contained from 5×10^6 to 1×10^7 spheroplasts/ml. and was clear to the naked eye, was distributed as 2 ml. portions into more than a hundred (usually about two hundred) small tubes and incubated statically at 20°. During this incubation the numbers of viable spheroplasts and rods were counted by sampling one or two clear cultures every day.

Fig. 1. (a) The effect of temperature on spheroplast formation. (b) The effect of washing twice with saline on spheroplast formation at 20°. (c) The effect of sugar concentration on spheroplast formation at 20°. Solid lines represent the viable spheroplast number and dotted lines the viable rod number.

Recovery of his⁻ spheroplasts was achieved by plating washed suspensions on penassay agar plus 20 % sucrose and 0.2 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ where the spheroplasts produced rods and visible colonies. The fraction of spheroplasts which converted into rods was calculated from the number of spheroplasts in the suspension plated (enumerated in a Petroff-Hauser counting chamber) and the number of colonies produced; it varied in five different experiments from 56 to 109 %. In the experiment reported in Fig. 1 a conversion was 109 % at 3 hr. and 80 % at 8 hr.

The method of calculating the mutation rate of his⁻ spheroplasts to his⁺ was based on the fact that if a spheroplast of *his⁺* genotype is introduced into medium of high osmotic pressure devoid of both penicillin and histidine it is able to grow and will yield rod-shaped bacteria. The culture will become turbid through the overgrowth of such *his⁺* mutants, or of whatever *his⁺* rods were present in the spheroplast population. Consequently, the mean number of mutants, *m*, can be calculated from the frequency of clear cultures, *P*₀, and the zero term of the Poisson equation:

$$P_0 = e^{-m}. \quad (1)$$

Knowing that it takes about 125 hr. for a single *his⁺* mutant spheroplast to form

rods and overgrow a culture, one can use the P_0 at this time to estimate the average number, m , of *his*⁺ mutants per culture at the time of distribution. These mutants arose, of course, during previous growth.

Thereafter, while the suspensions are incubated under conditions of histidine starvation, mutations from *his*⁻ to *his*⁺ occur among the spheroplasts, and very occasionally among the rods, which result in the continued appearance of overgrowths. These new overgrowths increase the value of m as calculated by equation (1). The rate of mutation/bacterium/hr. (μ) can be calculated (Ryan, 1955, 1959) from the equation:

$$\mu = \frac{km^1}{N_0(1 - e^{-kt})}, \quad (2)$$

where m^1 is now the average number of mutations (m at time t_2 minus m at time t_1 , which is any time after 125 hr., the time required for the overgrowth of pre-existing *his*⁺ mutants), t equals t_1 minus t_2 in hr., N_0 the number of bacteria present 125 hr. before t_1 , and k is the death rate constant calculated from the formulation:

$$N = N_0^1 e^{-kt}, \quad (3)$$

N_0^1 being the number of bacteria at time 0 and N the number at time t in hr. Equation (1) can be used to calculate m because each mutation in the stationary phase gives rise to one new mutant and where no mutations occur, the culture remains clear.

In cases in which death cannot be observed, the viable cell number remaining constant, the formulation

$$\mu = \frac{m^1}{N_0 t} \quad (4)$$

is used.

Usually the frequency of *his*⁺ mutations in an *his*⁻ culture is about 2×10^{-7} bacteria/hr. when normal rod-shaped bacteria and not spheroplasts are used. The chance of mutation/bacterium/hr. during growth is usually about 4×10^{-8} and, in a stationary phase with glucose present, about 2×10^{-9} (Ryan, 1955, 1959).

RESULTS

Representative results are shown in Fig. 2. Part (a) shows the death curves of spheroplasts and rods. In this case, the rod number is nearly constant but the spheroplast number decreases rapidly in early stages and then gradually. Fig. 2(b) shows the behaviour of m with time. The curve rises rapidly for the first 125 hr. and then gradually until 200 hr. The first rapid rise of the curve is caused by the pre-existing *his*⁺ mutants which have finished overgrowing at about 125 hr. Thereafter the gradual increase in m is due to the overgrowth of *his*⁺ mutants which arose after the cultures were suspended in medium devoid of histidine. Eventually, when the cultures contained only about 3×10^5 cells/ml., mutations among them became too rare to be detected. The average frequency of pre-existing *his*⁺ mutants in this experiment was about 2×10^{-8} and the μ for stationary phase cells was about 2×10^{-9} /bacterium/hr. Table 1 shows that these figures are not exceptional. The values calculated on the basis of spheroplast number more closely resemble those calculated for *his*⁻ bacteria which had never been converted into spheroplasts. If it were supposed that the *his*⁺ overgrowths observed were due to the small fraction of rods

inevitably present in suspensions of spheroplasts, then their mutation rates would have to be unrealistically high and mutation would unexpectedly cease when the spheroplast number became small but the rod number remained as large as ever (Fig. 2). It is, on the contrary, judged that the *his*⁺ overgrowths were due to mutations in the spheroplasts. The somewhat smaller values for the initial frequency of

Fig. 2. Stationary phase mutation. Spheroplasts of *Escherichia coli* 15 *his*⁻ made with penicillin were washed, resuspended, diluted in hypertonic G & T liquid medium without histidine and distributed as 2 ml. samples into 200 small tubes. They were incubated at 20° and 4 determinations were made at each time of the number of surviving spheroplasts and rods and also of the number of clear cultures from which *m* was calculated. (a) The number of viable spheroplasts (solid line) and of viable rods (dotted line). (b) The average number of mutations, calculated from P_0 , plotted against time. At the first break in the curve, *m* is equivalent to a frequency of *his*⁺ back mutants in the original culture of about 2×10^{-8} ; thereafter mutation occurred in the stationary phase at a rate, μ_s , of 1.7×10^{-9} until it could no longer be detected because of the small number of cells remaining.

his⁺ mutants calculated on the basis of spheroplast mutation as compared with that obtained for normal *his*⁺ cells can be attributed to a lower efficiency in the recovery of *his*⁺ spheroplasts, because of the use of synthetic medium (the *his*⁻ spheroplasts recovered on penassay agar).

Experiments were made in which spheroplasts were burst in hypotonic penassay broth and then provided with sucrose, while another group was simply suspended from the beginning in the hypertonic medium. If mutations really occur in spheroplasts, one would not expect them in the suspension of burst cells but would expect them in the control suspension. These experiments ended in failure, because in the experimental culture the rods grew on nutrients liberated from the shocked spheroplasts.

Table 1. *Mutation rates from his⁻ to his⁺ in the stationary phase and frequencies of his⁺ mutants among freshly grown his⁻ cultures estimated on the basis of the number of spheroplasts and on the basis of the number of rods present*

Expt.	Calculated on the basis of spheroplasts		Calculated on the basis of rods	
	Initial frequency of		Initial frequency of	
	$his^+ = \frac{m \text{ at 125 hr.}}{N \text{ at 0 hr.}}$	$\mu = \frac{km^1}{N_0(1-e^{-kt})}$	$his^+ = \frac{m \text{ at 125 hr.}}{N \text{ at 0 hr.}}$	$\mu = \frac{m^1}{Nt}$
1 (see Fig. 2)	1.8×10^{-8}	1.7×10^{-9}	2.9×10^{-5}	1.5×10^{-7}
2	3.5×10^{-8}	3.8×10^{-9}	4.6×10^{-5}	$5.5 \times 10^{-6*}$
3	4.6×10^{-8}	3.4×10^{-9}	2.6×10^{-5}	$5.2 \times 10^{-6*}$
values obtained with normal <i>his⁻</i> cells†	2×10^{-7}	2×10^{-9}	2×10^{-7}	2×10^{-9} $4 \times 10^{-6*}$

* In these cases the rods increased in number so that the rate was calculated as the chance per bacterium per generation ($a = (m \ln 2)/N$, Ryan, 1955) and then converted to μ on the basis of a 50 min. generation time.

† Ryan (1955, 1959).

DISCUSSION

Szybalski & Pitzurra (1959) reported mutation to streptomycin-independence in spheroplasts of *Escherichia coli* produced with LiCl. Although they did not prove that the mutants observed did not arise by mutation in the rods present, it seems likely that they took place in the spheroplasts. Their 'spheroplasts' were unlike ours, inasmuch as they did not burst in saline while in 20 % sucrose the mutations occurred infrequently, if at all, even in the presence of chemical mutagens.

The present work with spheroplasts bears out previous experiments (Ryan, 1959) in strongly supporting the conclusion that mutation from *his⁻* to *his⁺* occurs in non-dividing bacteria and that the mutated *his⁺* genotypes manifest their phenotypes in a non-dividing condition to grow in a medium without histidine.

However, the question still remains whether mutation requires DNA replication. The number of DNA-containing bodies could not be observed to increase in the spheroplasts which shrank somewhat during the stationary phase. Experiments to detect the replication of DNA by determining its quantity, and by the use of ^{14}N and ^{15}N with density gradient centrifugation, were negative. (Ryan, *et al.* 1961). Nonetheless, most investigations of induced mutation (Ryan, 1962) suggest that mutation occurs during DNA replication and support the theory that one of the mechanisms of mutation is pairing error. Errors in base pairing might be made not only during the replication of DNA but also by specific exchange when it is not replicating. This possibility is being examined by the use of isotope-marked base analogues and thymidine which may be incorporated into the DNA and induce mutations in non-dividing bacteria. It is also necessary to know whether a turnover of DNA was not observed because of isolation of the pool of DNA precursors, and whether an unusually high mutability characterizes an undetectably small turnover of DNA in non-dividing cells.

It is interesting to note that, although the *his⁻* gene reverts spontaneously and in response to ultraviolet radiation (Ryan, Fried & Schwartz, 1954) it does not respond

to 2-aminopurine, 5-bromouracil or nitrous acid under conditions where auxotrophs are induced to form (Okada & Ryan, unpublished results). This suggests that base-pair insertions or deletions of the sort discussed by Crick, Barnett, Brenner & Watts-Tobin (1961), unequal sister-strand crossing-over in a compound locus as proposed by Grigg & Sergeant (1961) or transversions as defined by Freese (1961) may be involved. Yet proflavin, which induces auxotrophs, also does not revert the *his*⁻ gene. Moreover, the possibility has not been excluded that the mutable unit under investigation is composed of RNA or protein even though it is located in a DNA-containing body (Ryan & Wainwright, 1954). Not enough evidence is as yet at hand to require rejection of the proposition that some natural mutations may result from copy-errors during the replication of the gene.

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