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APPEARANCE OF STREPTOMYCIN RESISTANCE FOLLOWING THE UPTAKE OF TRANSFORMING DEOXYRIBONUCLEIC ACID IN *PNEUMOCOCCUS*

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IN bacterial transformation, the experimenter is able to examine the physiological action of normal hereditary determinants, introduced into cells in the form of DNA, by examining the events leading to the manifestation of the newly acquired hereditary trait. Thus far, such studies are limited in number[1,2]. In one[1], the appearance of resistance to streptomycin was followed in populations of pneumococci which had been made to fix DNA from a streptomycin-resistant donor strain. The conclusion reached was that the resistant phenotype appears as a discrete change from sensitive to resistant: no stages of partial resistance could be recognized. It was found, furthermore, that the probability of a cell becoming resistant was normally distributed over a time-interval ranging from about 15 min to 90 min following penetration of the streptomycin-resistance gene, even though DNA fixation had been limited to a 5-min period. The most probable moment for a cell to become resistant was about 60 min following uptake of DNA. The interpretation of these results at the time of their publication was rendered difficult owing to the absence of information concerning the mechanism of streptomycin-resistance and the types of syntheses involved in its establishment. Later, a brief description was made of experiments showing that the discrete event, described here, is not in fact the development of typical streptomycin-resistance, but, after all, only an intermediate stage in its development[3]. Experiments documenting this contention are presented here, in conjunction with a hypothesis concerning the mode of action of the streptomycin-resistance gene. The hypothesis is a development of the recently published theory of Spotts and Stanier[4] concerning the mode of action of streptomycin and the nature of streptomycin-resistance. Since it may open some interesting new approaches to the study of gene action, and, in particular, to the question of the relationship between genes and ribosomes, the publication of these experiments, and the accompanying hypothesis on the mode of action of the streptomycin-gene, seem worth while.

(1) *The experimental demonstration of the appear[illegible] of resistance.* All investigations of the appearance [illegible] antibiotic resistance following uptake of transform[illegible] DNA use a single basic procedure. Following [illegible] period of DNA fixation which is sharply limited by [illegible] destruction of unabsorbed DNA with DNase, the [illegible] are diluted into fresh medium and incubated [illegible] 37° C. At intervals, samples are withdrawn [illegible] plated in agar containing the antibiotic. The numb[illegible] of cells able to give rise to a colony in the presence [illegible] the antibiotic are thus scored. Fig. 1, curve *a*, sho[illegible] how pneumococci transforming for streptomy[illegible] resistance develop this ability. This curve is typ[illegible] of those obtained by Fox[1] and Schaeffer[5] as well as [illegible] me. The number of cells able to give rise to colo[illegible] in streptomycin-agar rises rapidly from about [illegible] 15th min following DNA fixation. A shoulde[illegible] observed at about 80–90 min, following which [illegible] numbers increase again, but at a slower, exponen[illegible] rate characteristic of the overall population incre[illegible] of the growing culture. Various experiments[6,7] h[illegible] shown that: (1) at 90 min, virtually every cell wh[illegible] fixed a transforming molecule is able to form [illegible] colony in the presence of streptomycin; (2) that [illegible] increase observed after 90 min is due to the format[illegible] of genetically transformed daughter cells. In fa[illegible] it is established that the transmission of an acqu[illegible] gene to both daughter cells may begin as early as [illegible] second generation after DNA fixation[7], that is [illegible] about 45 min. It may, however, begin only a[illegible] third or fourth generation in some cells[7]. The e[illegible] transmission of an acquired gene is, however, [illegible] reflected immediately in the numbers of strep[illegible] mycin-resistant colony-forming units observed, ow[illegible] to the tendency of sister cells to remain attach[illegible] after cell division. Differences in the degree to whic[illegible] shoulder is observed at 80–90 min are almost certa[illegible] due to differences in the extent of chain formatio[illegible] different media.

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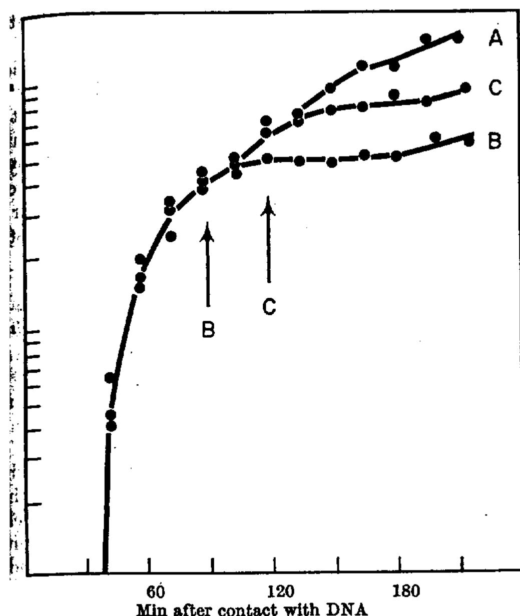


Fig. 1. Evolution of the numbers of transformed cells able to form colonies in streptomycin agar. *A*, Transformation culture growing in absence of streptomycin; *B*, streptomycin has been added 90 min after DNA uptake; *C*, streptomycin has been added 135 min after DNA uptake

If, in the agar medium used to score the resistant cells, the concentration of streptomycin is insufficient to arrest rapid growth of the recipient strain, residual metabolism in the presence of streptomycin will cause some transformants to complete the change from sensitive to resistant on the agar plate[1]. Thus, the transformed phenotype will seem to appear earlier at concentrations of streptomycin below a critical level, and the precocity of the appearance of the phenotype will appear to be a function of streptomycin concentration. Therefore, in order to avoid under-estimation of the time required for resistance to develop it is necessary to challenge the cells in agar containing streptomycin at a concentration yielding a maximally selective effect. In the experiments reported here, it was found that the rate of appearance of streptomycin-resistant cells is the same at all concentrations of streptomycin equal to or greater than 200 μg/ml. The majority of experiments were performed at 200 μg/ml., but in some the concentrations were higher.

(2) *Limitations of the procedure for demonstrating resistance.* The foregoing procedure for determining the appearance of resistance, adequate at first sight, in fact, leaves one parameter unexplored. The method reveals only when a transforming cell can form a colony at a maximally selective concentration of streptomycin. It does not tell us whether the transformant does so at once, or whether its growth and division is temporarily suspended by the challenge. The streptomycin-resistant donor strain is completely indifferent to streptomycin at the concentrations used: Is the newly resistant cell, which yields a colony, also really indifferent to streptomycin ?

(3) *Two steps in development of resistance.* To test this question, instead of challenging the transformants in streptomycin agar, a small amount of streptomycin was added to the cells in liquid medium, at a time when resistance is generally presumed to be complete. Following the addition of streptomycin, platings in agar were performed in order to determine the evolution of the numbers of resistant cells. Fig. 1 shows the results of one such experiment, in which cells which had fixed DNA for 2 min were diluted 100-fold into fresh medium and the culture divided into three portions: (*a*) no streptomycin is present in the liquid culture and platings are made directly into streptomycin agar; (*b*) 50 μg/ml. of streptomycin were added after 90 min of growth, and platings made into streptomycin agar (at 200 μg/ml.); and, (*c*) 50 μg/ml. of streptomycin were added after 135 min of growth, and platings are made in streptomycin agar. If, as is generally believed, all transformants have achieved the synthesis of the streptomycin-resistant phenotype by 90 min, there should be no difference in the numbers of streptomycin-resistant cells present in these three liquid cultures. This is, however, clearly not the case. The increase in the numbers of resistant cells present in the cultures receiving a small amount of streptomycin is almost immediately arrested by the antibiotic. Thus, the immediate replication of the newly formed resistant cells is blocked by as little as 50 μg/ml. of streptomycin. Yet these cells are able to form colonies in agar containing 200 μg/ml. or more. Other experiments showed that, in fact, streptomycin transformants become completely indifferent to streptomycin only after some 150–180 min have elapsed following DNA fixation.

Two explanations of these observations can be offered: (1) that resistance develops in two steps. First, the bacteria are altered so that streptomycin is no longer bacteriocidal, and secondly, they become completely indifferent to streptomycin. If this explanation is to be retained, it must be assumed also that cells can pass from the first state to the second in the presence of streptomycin. (2) That the cells which survive the streptomycin challenge are not genetically transformed. For example, the acquired factor can be supposed to be not yet a part of the linear array of genes of the bacterial chromosome, but transmitted via an extra-chromosomal mechanism. Streptomycin could then be supposed to block the extra-chromosomal mechanism so that the majority of the daughter cells produced in its presence would be streptomycin-sensitive and therefore die. This would be analogous to the situation found in the induction of 'petites' by acriflavine acting on yeast[8]. The eventual formation of a colony in streptomycin-agar would reflect a shift from the extra-chromosomal state to a chromosomal state, achieved through recombination at one of the numerous cell divisions which the mother cell could make.

Results of a number of types of experiments invalidate the second hypothesis. One critical argument against it is the fact that when a cell acquires a DNA particle, recombination does ensue very shortly thereafter[3,9]. Further, it is reported that when a particle of transforming DNA is genetically marked at several points, so that it is able to give rise to several types of different, recognizable recombinants, one observes that a unique recombinant type is formed from a single absorbed particle, most if not all of the time[10]. Were the acquired particle transmitted at the outset by an extra-chromosomal mechanism prior to recombination, this result could not be observed. The first hypothesis is, therefore, to be retained in considering why streptomycin arrests the multiplication of resistant cells newly formed by transformation.

Accordingly, in order to explain the results exemplified by Fig. 1, we can assume that even though from

90 min on, every cell which acquired the streptomycin-resistance gene can form a colony at high concentrations of streptomycin they do so only after a considerable period of arrested growth. In other words, at this stage of phenotypic transformation, streptomycin is a bacteriostatic substance from the effects of which the transforming cell can eventually escape. This characterizes what we shall call stage 1 in the development of resistance, while complete indifference characterizes stage 2, the definitive state.

Another type of experiment confirms this point of view, and, in addition, informs us of further characteristics of stage 1. Following a challenge of 500 μg/ml. of streptomycin for a 30-min period at 37° C, surviving transformed cells are washed on a membrane filter to eliminate unbound streptomycin, transferred to fresh medium by washing them off the membrane, and their growth followed by plating samples at intervals, in two different media: agar with and without streptomycin. In the experiment shown in Fig. 2, the resistant transformants were selected 60, 110 and 180 min after DNA fixation as described.

One may note the following features of the curves in Fig. 2. (1) There is a small lag in the onset of replication of the resistant cells selected 180 min after DNA fixation which is not observed when streptomycin is simply added at this time to a transforming culture and left there. The lag observed in Fig. 2 is almost certainly caused by the vigorous aeration of the cells during washing on the membrane filter (*Pneumococci* are microaerophilic). (2) Growth of streptomycin-resistant cells selected 60 and 110 min after DNA fixation is severely retarded, even though

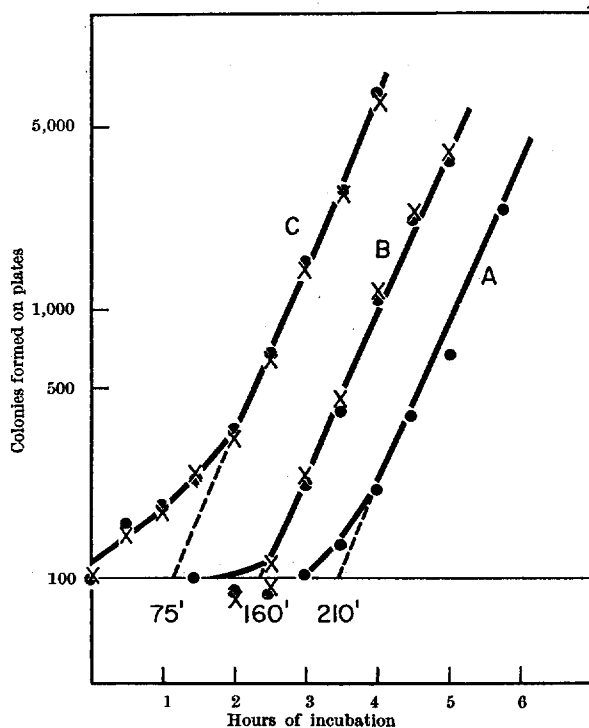


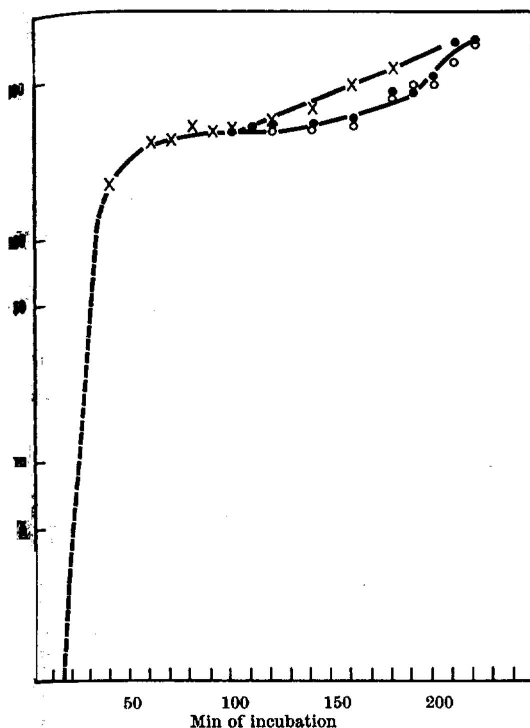
Fig 2. Onset of division of streptomycin-resistant transformants in the absence of extracellular streptomycin. The resistant transformants were selected: *A*, 60 min; *B*, 110 min; *C*, 180 min after DNA uptake by treating the transforming population with 500 μg/ml. of streptomycin for 30 min. Survivors were collected on a 'Millipore' membrane, washed, resuspended in medium, and their growth followed: ×, on plates containing no streptomycin and: ● on 200 μg/ml. of streptomycin

the antibiotic has been removed. This means the either the cells have fixed enough streptomycin that the extracellular concentration of the antibiotic is no longer critical, or that the 30-min treatment has inflicted a finite damage which is not subject to reversal by the removal of streptomycin. The latter seems more likely since the amount of streptomycin bound to bacteria is very small[11]. (3) No sensitive cells survive the selection at 500 μg/ml., and few or no sensitive progeny are formed by stage 1 resistant cells.

By extrapolating the exponential slopes of the curves of Fig. 2, one can calculate from curve *c* the delay caused by aeration, and from curves *a* and *b* the delay caused by the combined factors of aeration and streptomycin-inflicted damage. Correcting for the delay caused by aeration, one finds that the cells in *a* required 135 min of incubation to resume exponential increase, while the cells in *b* required 85 min. The difference between these two times is 50 min, which is the same as the difference in the incubation time of the two cultures prior to the streptomycin challenge. In other words, the time required for definitive resistance to develop is constant, and independent of the moment of application of the streptomycin challenge. Thus, cells which are at stage 1 in the development of resistance, and which may have arrived at this stage at very different moments, are a homogeneous population in so far as their attainment of definitive resistance is concerned. With respect to definitive resistance, primary transformants are apparently no different from their second, third or even fourth generation daughters.

In the experiment of Fig. 1, 50 μg/ml. of strepto- mycin was added to the liquid culture, while in the experiment of Fig. 2, 500 μg/ml. were added. Both concentrations arrested the multiplication of the resistant transformants. Irrespective of whether the damage to the cells was inflicted by 50 or 500 μg/ml. and of whether the streptomycin was left in contact with the survivors, the moment of onset of increase of the streptomycin-resistant cells was at about [illegible] min. This again suggests that streptomycin inflicts a finite damage on stage 1 transformants, and that the recovery is independent of the external concentration of streptomycin. To show this more clearly, an experiment was performed in which fluctuations in the numbers of streptomycin-resistant colony-forming units was followed in a control and two streptomycin-containing cultures. The latter received 50 and [illegible] μg/ml. of streptomycin, respectively, 60 min after DNA fixation. Fig. 3 shows the results of such an experiment. It can be seen that the time required for stage 1 resistant transformants to resume division after the addition of streptomycin is approximately the same, irrespective of the external streptomycin concentration. Hence, the conversion of a stage 1 resistant transformant into a definitely resistant one is essentially independent of streptomycin concentration. Further, the damage inflicted on the stage 1 cells must be finite and independent of streptomycin concentration, within the limits explored.

The most striking feature of the way in which definitive resistance develops in a transforming population is that cells destined to transform, and their immediate progeny, show this resistance at the same time, that is, about 180 min after fixation of transforming DNA. Yet some of these cells are original transformants and some are their [illegible]



...3. Evolution of the numbers of streptomycin-resistant cells ...transforming culture × in the absence of streptomycin; in the ...nce of ● 50 μg/ml.; ○, 500 μg/ml. of streptomycin added 60 ... after DNA uptake. Some of the irregularities in the curves are probably due to synchrony of division.

...d or later generation progeny. Therefore, as ...forming cells grow and divide, they must produce ...ters which are similar to themselves not only ...ypically but also with respect to the degree to ... they have developed definitive phenotypic ...nce. Since genetic integration usually occurs ...of the first two or three divisions following DNA ...on, it is difficult to imagine that this phenotypic ...rmity of transformants and their progeny is ...lished by the process of genetic integration itself. ...e other hand, DNA fixation has occurred during ...y short interval. Its penetration into the cell ... very well be the event which initiates the ... of phenotypic transformation.

...this were the case, the following mechanism of ... development of definitive resistance can be ...nced. On penetration of the DNA, the strepto-...in r gene immediately induces the formation of ...ystem which confers resistance. Since the com-...e phenotype is manifested only some 180 min after ...A fixation, we can suppose that resistance results ... the synthesis of a very large number of specific ...romolecules. As cell division proceeds, both the ...rating system initiated by the acquired gene and ... specific macromolecules which it determines are ...ributed more or less equally between sister cells. ... those cells where the resistance gene is fixed ...manently by genetic recombination, the resistance ... will also be transmitted. It is only in these cells ... the generating system will be stable enough for ... resistance to be manifested. What, then, is ... 1 resistance? As shown by Fox, it is a discrete ...ge which occurs on an average after about 60 ... have elapsed following DNA-uptake, and which ... a normal but fairly wide distribution with ...pect to the moment it occurs[1]. As shown by the foregoing experiments, it is a change which enables a cell to survive a challenge of maximally selective amounts of streptomycin, and to escape from a strong bacteriostatic effect of the antibiotic. Further, the rate at which a stage 1 resistant escapes is independent of the external streptomycin concentration in the growth medium.

A suitable explanation of stage 1 resistance was not evident so long as theories of the nature of streptomycin resistance were based on supposing the resistant cell impermeable to streptomycin. Even with the publication of a theory[12] to the effect that, in the presence of streptomycin, sensitive bacteria synthesize an abnormal membrane constituent which results in disruption of transport mechanisms, an explanation of stage 1 resistance did not seem possible. Supposing that, at the onset, the acquired resistance gene were to confer on the cell the capacity to form normal membrane substance in the presence of streptomycin, at early stages the cell membrane could be at best a mosaic, for the old membrane and membrane-forming system should still be present in the cell. It is hard to see how a mosaic membrane could confer on cells an immunity to the lethal effects of streptomycin.

The recent hypothesis of Spotts and Stanier[4] provides, on the other hand, an explanation of the nature of stage 1 resistance. According to these authors, streptomycin attacks the ribosomes of sensitive cells, causing their disruption. Resistant cells, according to the theory, contain ribosomes which do not combine with streptomycin, and are, therefore, resistant to its action. There is, indeed, some direct evidence in favour of this view[13]. In the light of this hypothesis, stage 1 resistance can be interpreted as resulting from the synthesis of adequate numbers of streptomycin-resistant ribosomes so that at least one copy of each of the different messenger RNA's of the cell which are necessary for the continuation of vital specific functions could be housed in streptomycin-resistant ribosomes. Bacteriostasis would ensue, however, at this stage owing to the destruction of residual streptomycin-sensitive ribosomes, which could still represent the majority of the ribosomes of the cell. Stage 1 resistant cells would recover their ability to divide as soon as the streptomycin-resistant ribosome population were built up to a level compatible with normal growth and division. Recovery-rate would be independent of the amount of streptomycin in the system, for recovery would result from the function of surviving streptomycin-resistant ribosomes.

The ribosome hypothesis is particularly satisfying because it explains why stage 1 resistance appears after an interval which is normally distributed over a fairly wide time-range.

There are presumably many different messenger RNA's determining vital functions which must be housed, and the probability that any one cell possesses one streptomycin-resistant ribosome-messenger RNA particle of each type would be expected to be distributed in this way, assuming random association of the RNA with ribosomes. Further, the ribosome hypothesis of streptomycin action can explain why all transforming cells and their progeny show definitive resistance at about the same time: the existing ribosomes would be shared at each division.

However, it should be mentioned that there is one fact concerning the action of streptomycin which the Spotts and Stanier theory does not explain. This is the observation that if chloramphenicol and streptomycin are added simultaneously to sensitive

cells, streptomycin has no lethal action. It appears, thus, that the lethal effects of streptomycin are the consequence of protein synthesis. It has been proposed that streptomycin does not enter cells unless a special permease is synthesized[14], following contact of cells with streptomycin, and this could account for the protective effect of chloramphenicol. Since so many of the biological actions of streptomycin can be explained by the theory of Spotts and Stanier, including the very particular way in which resistance develops following transformation, one is inclined to conclude that only a minor modification of it may be necessary in order to explain why chloramphenicol eliminates the bacteriocidal effect of streptomycin.

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