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# CIBA FOUNDATION SYMPOSIUM

ON

# DRUG RESISTANCE IN MICRO-ORGANISMS

## Mechanisms of Development

*Editors for the Ciba Foundation*

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*With 62 Illustrations*


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# INDIRECT SELECTION AND ORIGIN OF RESISTANCE

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## Adaptation of individuals and of populations

ADAPTATION to environmental changes in living organisms can take place both at an individual and at a populational level. Mechanisms of the first type are often efficient enough to cope with the altered situation, but there will be some variation in the individual responses to the changed environment. If the following two conditions are fulfilled: (i) that this variation is at least in part heritable, (ii) that there is differential reproduction of individuals showing different degrees of adaptation, then the population is also bound to change, in the sense that the frequencies with which the variously adaptable types are represented will be modified.

This mechanism is of course nothing but natural selection and its consequence is evolution. How much evolution takes place in any given population in a given time is dependent on how much heritable variation is available, and this is a property of the population; and on how much variation in the reproduction of the various types is created by the change in living conditions. The use of antibacterial drugs in concentrations at which they exert their specific effect constitutes a fairly drastic change in conditions, which is bound to affect deeply the rates of reproduction of individual cells. One should therefore expect drugs to effect major changes in the composition of a bacterial population whenever this contains—either because of original heterogeneity or because of new hereditary change—types which have different adaptability.

## Heritable changes

There would probably be full agreement so far but for the question of the heritability of individual changes in drug adaptation. In order to avoid irreconcilable disagreement at a later stage, we must agree on operational definitions of what is heritable and what is not; only the former type of change is of direct interest to the geneticists. Considering the necessity of distinguishing individual from populational adaptation, the heritability of an observed change in a population must be examined as much as possible at the level of single individuals, and not limited to that of the population as a whole. As this is often technically impossible, compromises should be attempted and their results analysed with care. For most ordinary work the following definition, which corresponds to what is routinely done in many laboratories, was found to be useful. A population is considered to contain resistant individuals, if single cells isolated (usually by plating) from it and allowed to grow into colonies in the absence of the selection medium, are found to be resistant, the test being carried out on samples from such colonies, or sub-cultures from them.

These "minimum" requirements for heritability have obvious shortcomings, but unless we accept an unequivocal definition, confusion is inevitable, as has indeed happened. Further definitions may be elaborated: this one has the advantage of corresponding to the usual procedure, and of being simple. It will fail on exceptional occasions, for highly mutable and poorly growing mutants. But if any sluggishly reversible, directly induced adaptation is taking place, it should be picked up with this procedure.

## Pre- or postadaptation

Since it has been ascertained that heritable changes in drug resistance, or any other trait, do in fact occur after treatment of the population, the question has repeatedly arisen: is the change induced by the drug itself or does this simply select

pre-existing, or independently forming, variants? These two mechanisms have also been called post- and preadaptation, respectively (Cavalli-Sforza and Lederberg, 1953).

The available data may be considered. In higher organisms the distinction between phenotype and genotype is an absolute necessity if confusion is to be avoided. The effects of environmental conditions, bringing about individual adaptation, affect the phenotype but not the genotype, i.e. the sum of the hereditary potentialities from which successive generations are moulded. Therefore, selection can be effective only when it picks up spontaneous variation, in other words, variation which occurred at a level where the selective conditions cannot act: that of the hereditary determinants. To clear the issue we have, it is true, to simplify and forget about a few systems, essentially cytoplasmic inheritance; but the cost does not seem to be too high, at least at present.

Higher organisms, on which these conclusions were developed, are probably best defined in this connexion as those in which the ratio of sizes between the adult soma and the gamete is high. In fact, some workers have developed the view that in unicellular organisms (where this ratio is low, soma and germ being of nearer orders of magnitude in size) the distinction between genotype and phenotype is an artificial one. However, experiments like those reported by Hayes (this symposium, p. 197) will be helpful in showing, if necessary, which differences exist between what is hereditarily determinant, and what is determined, even in a unicellular organism. Also, the fact that observation of simple individuals usually takes place on colonies produced from them helps to eliminate, though perhaps only partially, the effects of the "phenotype" of the individual giving rise to the colony, by the "dilution" to which it is subject when a large clone is built from it.

## Adaptation of bacterial populations

The facts on "lower" organisms are simply summarized. Early attempts were made (early at least in the short history

of bacterial genetics) to see if the model which was so fruitful for the study of evolution in higher organisms, namely the selection of spontaneous genotypic changes, could be applied to explain changes in resistance, in particular to viruses and drugs. The elegant methods of Luria and Delbrück, and of Newcombe, were used successfully and gave practically unequivocal answers in favour of the preadaptation theory.

Both the strength of these methods and the validity of the conclusions which rely on the statistical properties of clones have been questioned. While the present author would agree as to their insufficient strength, which leaves the door ajar to equivocal results or interpretations, the evidence collected later has fully confirmed the validity of the early conclusions. In view of the existence of such stronger evidence, methods of analysis such as the so-called "fluctuation test", the test of "average clones" and the "correlation between relatives" will not be considered here. They have been reviewed elsewhere (Cavalli-Sforza, 1952; Cavalli-Sforza and Lederberg, 1953). A method will be considered instead, the strength of which nobody would question, namely that of indirect selection.

## Indirect selection

Indirect selection was first introduced by Lederberg and Lederberg (1952) in streptomycin- and T1 phage-resistance. It uses sib-selection (or, in general, selection by tests on relatives) to obtain strains which are resistant to some agent, without using this agent for sorting out the resistant mutants. By substituting genetic testing for direct isolation with the drug, it can be proved, and has been proved unequivocally that the resistant cells are present in the population spontaneously, as they can be isolated from it without exposure of the population to the drug. To express the principle in simple terms, suppose we can isolate the two descendants of one cell for a great number of cells, and test for resistance to a drug one of the daughter cells, keeping the other for reproduction in the absence of the drug: if the mother cell was sensitive, apart from rare mutational events both cells should

also be sensitive. Therefore, the test on one of the two daughters will permit us to formulate with good probability the prediction that also the other daughter is sensitive. If the mother cell was resistant, both daughters should be resistant; this situation being revealed by the test carried out on one of the two daughter cells, it will be possible to verify the prediction that the untested sister should give rise to a pure colony of resistant cells.

This method has been made technically possible, for the first time, by the use of replica plating on solid media; and it has permitted the indirect isolation of streptomycin- and phage-resistant mutants. Later (Cavalli-Sforza and Lederberg, 1956), a method was developed for carrying out indirect selection in liquid cultures—which has the advantage over replica plating of leading more easily to quantitative analysis —with a view to answering the question: Have all resistant cells arisen by spontaneous mutations, or have some arisen by other mechanisms, such as mutation induced by the drug, or adaptation not controlled by nuclear determinants?

## Concentration by limiting dilution

The principle of this method (Cavalli-Sforza and Lederberg, 1956) is to concentrate spontaneous mutants to resistance by using a sample which contains few resistant cells, possibly only one, and subdividing it further. If there is in the sample before subdivision just one resistant mutant and (N−1) normal sensitives, i.e. if the relative frequency of mutant cells is $\frac{1}{N}$, after subdivision in $n$ tubes the frequency of mutants will be $\frac{n}{N}$ in the tube which happened to receive the single mutant, and zero in the other tubes. Each tube will, in fact, receive $N/n$ bacteria but only one of them contains the resistant mutant. This tube will therefore show an enrichment of mutants of $n$ times. It will be possible to identify it by incubating all tubes after addition of fresh broth, and testing samples from them for drug resistance.

When statistical fluctuations are considered, as shown by Dr. J. Pfanzagl of Vienna (personal communication), the enrichment expected is:

$$E = \frac{1 - e^{-m}}{1 - e^{-m/n}} = \frac{f_1}{f_0}$$

where E is the ratio between the relative frequency of mutant cells in tubes which have received at least one of them ($f_1$), and that one in the original suspension ($f_0$); while $m$ is the expected number of mutants in the sample which has been subdivided into $n$ tubes.

One such experiment, e.g. on 10 tubes, will give at best a tenfold enrichment; but on repeating the indirect selection experiment it is possible to isolate in a predictable number of cycles a pure culture of resistant mutants, which will never have experienced direct contact with the drug.

This experiment was successfully carried out for high degree resistance to streptomycin and for low degree resistance to chloramphenicol in *Escherichia coli*. The speed of selection observed was comparable to that predicted, as is shown in greater detail in the paper by Cavalli-Sforza and Lederberg (1956).

A short report is given here of data in which this experiment was amplified, considering that every experiment of indirect selection tests just one culture, and that the most informative stage is the first cycle (or, occasionally, the first two cycles) of indirect selection. This usually permits the counting of spontaneous mutants, and the comparison of their number with that of the resistant cells counted by direct selection, i.e. by plating in presence of the drug. In view of the greater simplicity of the system, the experiment was made on streptomycin resistance.

Samples from a number of independent saturated cultures were tested for streptomycin resistance and concentrations of resistant cells per ml. ranging from 0 to 105 resistants were found. The tests on two cultures will be considered in detail.

Table I shows the results obtained with a saturated culture of *Esch. coli* K12 no. 176 which was expected, from the assay, to contain zero streptomycin-resistant mutants. Three samples from it (of 0·5 ml., 0·25 ml. and 0·125 ml., respectively) were diluted each to 20 ml. with fresh broth. Each 20-ml. quantity was distributed into 10 tubes, in quantities of 2 ml. per tube, and the 30 cultures thus obtained were incubated to saturation. Eventually the total number of resistants per culture was counted on streptomycin agar

**Table I**

A PROTOCOL OF QUANTITATIVE INDIRECT SELECTION

From a culture which has been assayed for streptomycin resistants and found to contain zero resistants per ml., further samples have given:

| *Sample* | 0·5 *ml.* | 0·25 *ml.* | 0·125 *ml.* |
|---|---|---|---|
| *Multiplication factor* | 40× | 80× | 160× |
| Resistants | 5 | 0 | 2 |
| | 6 | 0 | 1 |
| | 0 | 12 | 10 |
| | 4 | 10 | 10 |
| | 6 | 3 | 6 |
| | 13 | 6 | 17 |
| | 5 | 5 | 1 |
| | 1 | 0 | 11 |
| | 6 | 14 | 5 |
| | 3 | 14 | 0 |
| Average per subculture | 4·9 | 6·4 | 6·3 |

(500 μg./ml.). Some resistant cells were found (Table I), but only a few, and often there were none per culture; the average number of mutants expected in such conditions from more extensive tests is $4 \cdot 8 \pm 2 \cdot 0$ per culture, and the results from the three series agree within the limits of error with this number.

Table II shows what happened instead when a culture known to contain 39·5 resistant cells (from the assay of a sample from it plated in streptomycin agar) was treated in the same way. Here the sample of 0·125 ml. was expected to contain 5 mutants and if, on subdivision into 10 tubes, each of these fell into a separate tube, 5 out of these would

be expected to contain a mutant at the beginning of growth. As the sample of 0·125 ml. was made up to 20 ml. with fresh broth, every cell inoculated was allowed to grow into a clone of 160 cells on average. There should then be about 160 resistant cells in 5 out of 10 tubes of the 0·125-ml. series. This was found to be true of 4 tubes instead of 5. In other samples, of 0·5 and 0·25 ml., there is evidence that, as would

**Table II**

A PROTOCOL OF QUANTITATIVE INDIRECT SELECTION

From a culture which has been assayed for streptomycin resistants and found to contain 39·5 resistants per ml., further samples, after addition of fresh broth to a total of 20 ml. and incubation, have given:

| *Sample* | 0·5 *ml.* | 0·25 *ml.* | 0·125 *ml.* |
|---|---|---|---|
| *Multiplication factor* | 40× | 80× | 160× |
| Resistants | 0 | 160 | 19 |
| | 44 | 59 | 7 |
| | 0 | 65 | 0 |
| | 89 | 0 | 132 |
| | 31 | 0 | 0 |
| | 59 | 0 | 184 |
| | 53 | 102 | 156 |
| | 49 | 0 | 105 |
| | 159 | 164 | 0 |
| | 3 | 82 | 0 |
| *Spontaneous mutants:* | | | |
| Expected | 19·8 | 9·9 | 5·0 |
| Found | 11 | 8 | 4 |
| Found (corr.) | 13·2 | 9·1 | 4·5 |

be expected, more than one mutant fell into some tubes (e.g. the first and ninth tube of the 0·25-ml. sample, etc.).

One can in this way count the number of spontaneous mutants in a culture and compare it with the expected one, if the hypothesis is made that all resistants observed on drug plates are the consequence of spontaneous mutation. These two values are given for each sample in the second-last and third-last lines of Table II.

Corrections have to be made, however, to account for the statistical distribution of mutants and for the possible

differences in growth rate between mutant and parent in drug-free medium. Corrected figures for the numbers of spontaneous mutants are given in the last line of Table II. When this was done for 18 cultures, no significant deviation was found from the hypothesis that all resistants are the consequence of spontaneous mutations.

## Some precautions

The following precautions should be taken in applying this test:

(1) The growth rate of mutants is usually lower than that of the normal sensitive. In 18 independent mutants, the distribution of relative growth rates $k$ given in Table III was

**Table III**

DISTRIBUTION OF THE GROWTH RATES OF STREPTOMYCIN-RESISTANT MUTANTS

$k$ = Growth rate of resistant relative to growth rate of sensitive

| $k$ | *Number of strains* |
|---|---|
| less than 0·70 | 1 |
| from 0·71 to 0·75 | 4 |
| ,, 0·76 ,, 0·80 | 2 |
| ,, 0·81 ,, 0·85 | 6 |
| ,, 0·86 ,, 0·90 | 1 |
| ,, 0·91 ,, 0·95 | 0 |
| ,, 0·96 ,, 1·00 | 4 |
| Total | 18 |

obtained, where $k$ is the ratio between the growth rate of the resistant and that of the sensitive parent in mixed culture.

If $k \neq 1$, the expected numbers of resistants per culture differ from those expected on the basis of the ratio of increase in total cell numbers (the "multiplication factors" in Tables I, II and IV). Table IV gives the expected numbers of resistants for some $k$ values. When $k$ is small the "enriched" mutants are not easily sorted out from the background of new mutants.

(2) In some circumstances, e.g. chloramphenicol resistance, first-step mutation does not determine a high level of resistance, and resistants may be incompletely recovered in tests

**Table IV**

NUMBER OF RESISTANTS EXPECTED FROM THE MULTIPLICATION OF A SINGLE CELL

$k$=Growth rate of resistant relative to growth rate of sensitive

| | *Multiplication factor* | | |
|---|---|---|---|
| $k$ | 40× | 80× | 160× |
| 0·6 | 9·2 | 13·8 | 21·0 |
| 0·7 | 13·2 | 21·4 | 34·8 |
| 0·8 | 19·1 | 33·3 | 58·0 |
| 0·9 | 27·6 | 51·6 | 96·0 |
| 0·95 | 33·2 | 64·0 | 124·0 |
| 1·0 | 40·0 | 80·0 | 160·0 |

with the drug concentrations necessary to eliminate all or most of the sensitives. There may also be interactions between sensitives and resistants in mixed populations, increasing (protection: Cavalli-Sforza and Lederberg, 1956) or decreasing (co-killing or suppression: Eagle, 1955) the count of the resistant type. Such situations can usually be revealed, and their consequences evaluated, by appropriate reconstruction experiments.

(3) Cells do not always divide regularly. For example, chains may be formed with the strain used here if static unaerated, but not aerated, cultures are employed. Where static unaerated cultures are used, situations of the type shown in Table V may be encountered. The irregularity

**Table V**

AN UNAERATED CULTURE CONTAINING 102 RESISTANTS PER ML. (STREPTOMYCIN AGAR ASSAY) TESTED BY INDIRECT SELECTION

| *Sample* | 0·25 *ml.* | 0·125 *ml.* | 0·0625 *ml.* |
|---|---|---|---|
| *Multiplication factor* | 20× | 40× | 80× |
| Resistants | 8 | 0 | 83 |
| | 47 | 10 | 24 |
| | 17 | 350 | 15 |
| | 24 | 42 | 11 |
| | 0 | 15 | 42 |
| | 132 | 24 | 0 |
| | 32 | 14 | 43 |
| | 5 | 77 | 0 |
| | 49 | 0 | 8 |
| | 27 | 0 | 610 |

in the distribution of the number of resistants per tube is immediately apparent. Thus the last tube of the 0·0625-ml. sample must have contained 8 or 9 mutants at least; the same is true of the third tube of the 0·125-ml. sample, and so on. This distribution could hardly be random. Presumably, resistant cells tend to form short chains which are not split on dilution in broth, while they are more easily broken up in agar, perhaps as a consequence of the joint action of temperature of the agar plus its chelating power. In such cases one would tend to underestimate the number of resistant mutants if the frequency of tubes showing enrichment were used for its assessment, while if enrichment ratios were used one would tend to overestimate it.

## Conclusions

Tests of adaptation in bacterial and other populations are available that permit the assessment of the relative importance of genetic and non-genetic adaptation, defining the former as the selection of spontaneous mutants. In the cases tested so far—essentially streptomycin and chloramphenicol resistance—evidence was found for the adequacy of the hypothesis of genetic adaptation, and no need arose for additional alternative explanations.

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## DISCUSSION

*Hinshelwood:* This is a beautiful method with streptomycin, and I admire the experiment very much. But what level of chloramphenicol resistance can one get by the indirect method?

*Cavalli-Sforza:* The degree of resistance that was obtained in the work with Lederberg was perhaps two or three times the original level. You

cannot get in one step a high increase in resistance to chloramphenicol, and you must therefore work on what occurs in nature. In the case of chloramphenicol, in fact, some trouble was encountered and indirect selection took a little longer. The level of resistance of the particular mutant selected indirectly was such that it did not give 100 per cent survival with the concentration of the drug that would kill all of the sensitives, it gave only 12–20 per cent survival.

*Hinshelwood:* As regards chloramphenicol resistance, by means of growing mass cultures in different biochemical media (in different sugars, broth and synthetic medium and so on) we can change what may be called the natural level of resistance in the range from 10 to 20 or 30 parts per million; and in the same way resistance to proflavine and certain other drugs changes. On the other hand, the resistance can be raised to 800 or 1000 by the direct action of the drug, so it is a very low-grade resistance indeed which is selected in your experiment. There is a great contrast, in any case, between chloramphenicol and streptomycin where the degree of resistance rises abruptly after adaptation, by whatever means, to quite a low concentration of streptomycin.

We think that there is a distinction between types of resistance. We think, moreover, that streptomycin resistance may sometimes be due to the lack of the power of cells to take up the drug.

*Cavalli-Sforza:* I think that with chloramphenicol you have to work in those conditions, because you cannot get higher resistance in just one step. It is another genetic system. You have not got any single gene capable of giving high resistance at once. You could get higher resistance only by repeating the entire process of selection on first-step mutants, and so on.

*Hinshelwood:* Still, when mutants are selected up to a certain point in your environment, the further steps should be coming in quite freely.

*Cavalli-Sforza:* The later cycles of an indirect selection experiment may be easier than the first ones; but if not, you can change the method if you want to, for instance you could go over to replica plating.

*Hinshelwood:* You would not be inclined to entertain the idea that there are really two types of adaptation?

*Cavalli-Sforza:* What was done here was to test one hypothesis. The present method can only disprove the genetical hypothesis; if it does disprove it, it leaves the field open for the alternative one, but it has not done that.

*Hinshelwood:* Have these resistant cells obtained by the indirect method been tested for streptomycin adsorption? That would be a very interesting datum to have.

*Cavalli-Sforza:* I don't think they have been tested.

*Dean:* Would you consider, if you got no selection, that the genetical hypothesis is disproved for a particular drug and a particular organism? For instance, if one did an experiment with a certain drug and a certain organism and found no selection at all, would you consider that as disproving the genetical hypothesis?

*Cavalli-Sforza:* Of course; but you have to be very careful, in using indirect selection, about those shortcomings that I mentioned, for

instance that mutants have to multiply in competition with the wild type. This is not a gratuitous assumption, it is something which you can test directly; if the mutant multiplies much more slowly than the normal, then the method is difficult to apply.

*Lederberg:* A control is needed in such a case, i.e. an artificial reconstruction of a mutant which you know was produced; you then put it in with the wild type to show that you can select it under those conditions. If you then fail to obtain similar mutants without making artificial mixtures you can conclude that no mutants *of that type* are present in untreated cultures. I emphasize *of that type*, because this consideration of differential growth rate might still come in, but you would certainly have to stretch the genetical hypothesis quite far to get selection.

*Yudkin:* Prof. Cavalli-Sforza said that mutants would be expected necessarily to be at a disadvantage, compared with the natural types. When we are dealing with drugs like some of the antibiotics which may occur in nature, it is reasonable to suggest that the resistant mutants grow more slowly than the wild type, for otherwise the sensitive strains would have disappeared at some time. But when we are dealing with drugs like proflavine, which the bacteria are most unlikely to have encountered in nature, then there is no reason to suppose that the resistant mutants have a growth disadvantage.

*Cavalli-Sforza:* I don't think that the fact that the particular strain has had experience before of one particular drug is very important.

Any mutation that arises in an otherwise homogeneous population of cells has a fitness value relative to the normal type, which of course

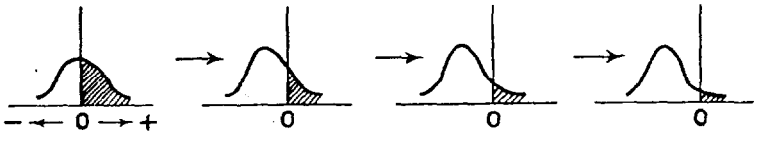


FIG. 1. (Cavalli-Sforza). An oversimplified picture of the process of genetic adaptation taking place automatically in a culture kept under fairly constant conditions, in terms of the distribution of fitness values of mutations arising in the population at various stages. It shows why most mutations are likely to be "unfavourable." The abscissa gives the fitness value of a mutation; the ordinate, the frequency of mutations having given fitness values. Arrows indicate the lapse of generations. The stippled area represents the proportion of mutations which are "favourable", i.e. have positive fitness; the white area the proportion of "unfavourable" ones.

depends on the specific environment considered. Different mutations will presumably show different fitness values; a few may have a fitness value of zero or nearly so (if we thus describe the absence of advantage or disadvantage in respect to the normal type, e.g. the mutant grows and dies at the same rate as the normal); others may have a positive fitness value (i.e. they are "favourable" mutations), and the rest a negative fitness value ("unfavourable" mutations) (Fig. 1)*. If a bacterial strain

* For greater clarity Fig. 1 was added in proof.

has had a long experience of growth in given conditions, such as normal laboratory media and transfer routine, which are fairly constant, it must have adapted genetically to it. Genetic adaptation by natural selection takes place automatically, in fact, and most of the favourable mutations must have been fixed by it, thus decreasing the proportion of favourable mutations available to the organism and increasing that of unfavourable mutations. Of course, the strain must have had time to adapt to the "usual" conditions, or in other words these must really be "usual"; therefore, the previous history of the strain may have some importance. On the other hand, the tendency of any new mutation to have a negative fitness value is not only a theoretical expectation; it is a fact, as for instance the data in the present paper have shown.

*Davis:* It seems to me that we are using the term "drug resistance" for two different concepts. When we say that one *strain* is more resistant than another we mean operationally the following: two families of cells are both grown under identical conditions and then identically tested to determine the concentration of drug that brings about a certain degree either of interference with growth or of active bactericidal action; and one family is found to require for this effect a higher concentration of drug than the other. But when we say one *cell* is more resistant than another we mean quite a different thing. If a number of cells are plated on a medium containing a borderline concentration of drug, some cells will die and others will give rise to colonies. We have a right to conclude that the ones that died were less resistant, by definition (i.e. if they would not have died in the absence of the drug). But we do not know that the more resistant survivors are more resistant in an inheritable way. They may be. They may also be simply those cells, in the inevitable range of physiological variation in a genetically homogeneous population, that happened to be able to withstand the borderline concentration of drug sufficiently to initiate colony formation. And, once initiated, the microcolony could so modify its environment as to ensure its continued growth.

No geneticist would deny that such physiological variations can affect the chance a cell has of resisting a borderline concentration of drug. Indeed, it would be safe to predict that one could shift the average level of such phenotypic resistance by varying the richness of the medium, the aeration, the stage in the history of the culture at which the organisms were harvested, etc. Furthermore, it seems inevitable that the surviving cell, in beginning to grow in the presence of the drug, would undergo further physiological changes in adaptive response to the presence of the drug; such adaptive changes not only might alter the susceptibility of the cell to the drug; they also should be passed on to the progeny as long as these progeny are grown in the presence of the drug. But such adaptive changes in resistance, in contrast to inheritable ones, would disappear after a suitable number of generations of growth in the absence of the drug.

While genetically orientated microbiologists have recognized the possibility of such adaptive influences on the resistance of a cell, they have not been much inclined to investigate the problem. I think Sir Cyril

Hinshelwood has performed a service in focussing on this interesting and neglected area of biology. However, the real rub comes in his claim that such reversible adaptive resistance, if carried through enough generations will gradually develop (by some process other than random mutation plus selection) into a stable, inheritable resistance. Most biologists would be sceptical about the existence of such gradual, non-mutational stabilization of an adaptation; and I feel that the experiments cited in support of this concept are all compatible with mutation and selection.

One further comment: as Sir Charles Harington pointed out in his introduction, the general interest in drug resistance and the *raison d'être* of this symposium have arisen from the practical importance of the problem. I would like to emphasize that the problem to which he refers is the emergence of *strains* with an inheritable increase in resistance. This is what is ordinarily meant by drug resistance. The problem of the adaptive and other non-inheritable physiological factors affecting the observed level of resistance of a cell is also interesting—but it is not the problem of drug resistance.

*Pontecorvo:* This last point which Prof. Davis has made is precisely the one I meant when I mentioned what has been done in higher organisms, particularly by Waddington. The experiment there is to expose embryos of flies to a certain concentration of drug at certain critical periods in development: a proportion of them develop into abnormal adults. Breeding is selective, i.e. only from the abnormal adults. After a few generations abnormal adults develop even without treatment or with a reduced one. In this case it is quite evident from the procedure of the experiment that what has happened is that there was initially, let us call it "physiological", variability: some individuals responded, some did not. A genetic mechanism which can be pin-pointed to particular regions of the chromosome set has taken over later on. That is precisely the transition from one mechanism to the other. It would be important to see whether this transition can or cannot be favoured by means other than selection by the stimulus; so far, I am not convinced that there has been any proof one way or the other.

*Györffy:* To raise a point concerning definition: we need to make a distinction between the terms "heritable" and "genetic"; they are not synonymous, and we must take care in using them, because "heritable" or "hereditary" means transmissible or transmitted from one generation to another; and "genetic" implies control by the genotype, by genes; and it is well known that all modifications are non-genetic changes. The "Dauermodificationen", which very often occur in micro-organisms, are "inherited" through a number of generations although they are not genetic changes. Another complication is that each bacterial cell in itself represents one generation, and if it is modified as by environmental influence it may be "inherited" through a number of generations. That again is not a real genetic change. I wonder whether we really are able, in the usual experiments, to differentiate by the criterion of the term "heritable" between a genetic change and a Dauermodification. The term "heritable" seems to me somewhat ambiguous, and it will be better when we no longer use this term in microbial genetics but use instead

the word "genetic". Then we can make the distinction that the genetic change is controlled by the genotype.

*Pontecorvo:* We have, of course, an operational test in some cases. In *Esch. coli* we can use segregation and recombination, either by a sexual process or by transduction. In other organisms, for instance ascomycetes, we have the ordinary test of sexual reproduction and segregation as well as "parasexual" segregation, etc. So we can unequivocally distinguish in the majority of cases, even in micro-organisms, a genetic change from an inheritable change which is not genetically determined.

*Davis:* It seems to me that the science of genetics must be concerned with all mechanisms of inheritance, and not simply those involving chromosomal genes. Indeed, the term "gene" was surely derived from "genetics" and not *vice versa.* I therefore wonder whether it might not be useful to use the term "genetic" to include all mechanisms of indefinitely transmitted inheritance, both chromosomal and non-chromosomal, and to use the term "genic" for chromosomal mechanisms.

*Kunicki-Goldfinger:* One should be very careful when differentiating between genic mutation and physiological, more or less stable, change, especially if recombination analysis is not possible.

In this connexion some phenomena may be pointed out which are apparently due to mutation, but which are, in fact, caused by physiological changes in bacterial cells. In an *Esch. coli* population only a very small fraction of cells can grow in the presence of lithium chloride. Not more than 1 per 100,000 cells is capable of forming a colony on media containing lithium chloride. In the majority of strains these resistant forms are not stable and their progeny are as susceptible as the parental strain. Without analysis of population during growth the change may be interpreted as being due to the selection of pre-existing mutants. In reality it is caused by a physiological adaptation in a small fraction of the heterogeneous population.

The characteristic growth curve, which Prof. Cavalli-Sforza discussed, may also be due to selection of spontaneous mutants, or to overgrowing of the culture by a new physiological variant induced by the environmental conditions. This is the case in *Brucella* grown in synthetic medium. A growth curve with many peaks is then obtained. At least some variants, whose growth resulted in the formation of additional peaks, were shown to be of non-mutational origin. Some R-variants could be obtained from homogeneous S-populations in conditions excluding cell multiplication. In this case the majority of cells were transformed into a new type. If this change is not due to semi-stable physiological adaptation, it may be caused by total mutation of almost the whole population, induced by environmental factors, which seems to me less probable.

*Hotchkiss:* Prof. Davis has pointed out quite clearly what the conceptual disagreement is. I suggest we turn more to the experimental inconsistencies. Sir Cyril has mentioned that cultures selected in low concentrations of streptomycin would be resistant to high levels of streptomycin. I know that in many organisms one may find streptomycin resistance also; so I would like to know whether a low resistance is

found in the same situation; if not, I would be concerned about possible special selective features.

The other point is rather similar, related to the case of sugar fermentation—the inconsistency between the results of Hinshelwood and Dean and those of Lederberg and Pollock. I think the cultures that Lederberg and Pollock have been examining should have a complete round trip and return to Hinshelwood's laboratory and be observed under his conditions again. I would be very pleased, for instance, to see that cultures which they had submitted to some very brief processing would also show large colonies in your media, Sir Cyril, and if they did not, then one could infer that differences in your media have prevented you from seeing this selection in favour of the more rapidly growing or rapidly utilizing mutant.