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GENETIC ANALYSIS OF DRUG-RESISTANCE

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The well-known and enormous ability of micro-organisms to adapt themselves to new conditions of life has been a frequent cause of complications in bacteriological studies.

The adaptability of micro-organisms to environment is, at a glance, greater than that of higher organisms, no doubt owing to their higher reproduction-rate and their unicellular condition. A particular case is that of adaptation to drugs, some aspects of which are discussed in this paper. This phenomenon has been known since the time when drugs active against micro-organisms were first discovered, and now certain well-established methods of chemotherapy seem to be threatened by it.

Various mechanisms have been proposed to account for drug adaptation. In order to classify these theories it is important to consider whether they assume that the change leading to an inheritable state of resistance occurs before or after contact with the drug.

DRUG-ADAPTATION THEORIES

Genetics of the higher organisms has shown that essentially two forces act in determining changes of a closed and initially homogeneous population : (*a*) mutation, namely a spontaneous change affecting the units of inheritance, followed by (*b*) selection of a fitter type produced by mutation. Can this mechanism of mutation and selection account for drug adaptation in micro-organisms ? If so, we must assume that the change leading to resistance happens *before* drug contact, by mutation (which need not always be gene mutation ; chromosome mutation, segregation of heterokaryons or heterozygotes, and other types of change may be at play). For this type of mechanism, it does not matter whether the genetic change, e.g., mutation, leads immediately to actual resistance, or only to a potential state of resistance which will become manifest later, in the presence of the drug only.

On the other hand, consideration of the unicellular condition of micro-organisms, and other reasons, have led a number of authors to consider an alternative mechanism as possible, or even likely. The mechanism of adaptation proposed by these authors is based on the hypothesis that micro-organisms surviving concentrations of the drug usually lethal to most of the others have developed a capacity for increased resistance *after*

contact with the drug and because of it. There could be a similarity between this hypothetical mechanism and that known as enzyme adaptation, whereby micro-organisms, when grown in the absence of a certain substrate, do not contain detectable amounts of enzymes to attack it, but do build up such enzymes in a short time when the substrate is present. Drug adaptation however differs from such a type of physiological adaptation (so called in contrast to the adaptation by mutation and selection, which is often named genetical adaptation) in two important ways. First, enzyme adaptation develops in all or most of the cells of a culture, while drug adaptation develops in an exceedingly small minority of them. Secondly, enzyme adaptation is quickly reversible in the absence of the enzyme, while drug resistance is often rather stable in the absence of the drug—sometimes completely so. Therefore it seems that models of enzyme adaptation cannot be applied as such to the case of drugs, but need some ancillary hypotheses to explain the peculiarities of drug adaptation. On the contrary, the rarity and stability of the resistants are in agreement with the mutation and selection theory, though a more thorough proof is wanted.

Methods of Discrimination

Fluctuation test

A rigorous method of discrimination between " before " and " after " theories is provided, whenever the practical conditions are met, by a test devised by Luria & Delbrück[11] working on bacteriophage resistance in *Escherichia coli*. This method, called the " fluctuation test ", is based on the idea that if resistant individuals are produced by mutation, this might have occurred in any of the generations previous to contact with the selective agent (phage, drugs, etc.). If the mutation has occurred in the last-but-one (or an earlier) generation, a clone of two (or more) mutant individuals will result. Thus, independent cultures, although containing the same total number of cells, may have experienced a different number of mutations, each of them having taken place at one or more generations before drug contact. Hence, if the mutation and selection theory is correct, a large variability of the number of resistant mutants among independent cultures of equal size must be observed. In such a case the fluctuation test is said to be positive; if no significant variability between independent cultures is found, it is negative.

When the fluctuation test is positive, a more refined approach could be that of testing the agreement between observed and expected distributions of the numbers of resistant mutants in independent cultures. Lea & Coulson[6] have worked out this theoretical distribution, but no data satisfactory from this point of view are available in the literature on drug-resistance. When this test is applied to phage-resistant mutants, the observed distribution does not correspond to expectation, but the deviation seems to indicate

that the observed variation of the numbers of mutants is even more extreme than that expected under the mutation and selection theory. There are several reasons for expecting such a lack of agreement, but it is not easy to analyse them quantitatively.

Demerec[2] first applied the fluctuation test to penicillin resistance in *Staphylococcus aureus*, and obtained results in favour of the mutation and selection theory. Similarly, Oakberg & Luria[15] obtained a positive fluctuation test in the same organism for sulfathiazole resistance ; Newcombe & Hawirko[13] found the same for streptomycin resistance in *Esch. coli*, while for streptomycin dependence, the fluctuation test was negative. It should, however, be considered that the latter type of mutants cannot reproduce in the absence of streptomycin and hence cannot form clones in drug-free cultures ; therefore this result cannot speak against the mutation and selection theory.

On the other hand, Welsch,[a] working on streptomycin resistance in *Staph. aureus*, obtained negative fluctuation tests. He showed, however, that streptomycin-resistant organisms are strongly selected against, in the absence of streptomycin, when mixed with sensitive cells.

Eriksen[5] confirmed Demerec's results for penicillin resistance, but found that, when the size of the cultures was increased, the fluctuation test became perfectly negative. It should be noticed that increasing the size of the cultures increases the average number of mutations per culture, and thus decreases the discriminatory power of the test. But even so, Eriksen's published data are not in good agreement with the hypothesis of mutation and selection when his observed variance is compared with that expected by making use of Lea & Coulson's transformation. If the mutation and selection theory is correct, it must therefore be assumed that resistant mutants are selected against in Eriksen's experiment.

The present author's impression is that, in the case of drug-resistance, the conditions for the validity of the fluctuation test may easily fail, and therefore both a positive and a negative test may occasionally be inconclusive. To give a few examples : For the test to be valid, there must be no selection against the mutants in the drug-free cultures before they are plated with the drug. On the contrary, it happens that the resistant mutants, with few exceptions, are selected against, especially when bred with sensitive individuals in the absence of the drug, as in the example given by Welsch.[a] An extreme case of selection against resistant mutants in drug-free cultures, and one which gives the most striking effects in the fluctuation test, turning the latter into a completely negative one, is that of streptomycin dependence.

Such selective differences between resistants and sensitives represent the likely explanation of the instability sometimes observed in resistant mutants.

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[a] See paper by Welsch on page 173 of this number of the *Bulletin*.

The same effect, that is, a positive test turned into a negative one, could result if there were total or partial inactivation of the drug, with growth towards the end of sensitive survivors or low-grade resistants. Such might be the case for penicillin, where both inactivation of drug and survival of sensitives (" persisters ") are ascertained. Again, a lag of the bacteriostatic or bactericidal effect, allowing some bacterial division to take place in the presence of the drug, will cancel an expected positive result. This is probably true for most drugs in appropriate concentration ranges ; Lederberg,[8] repeating an earlier experiment by Strandskov,[16] showed it to be the case for chloroaminobenzoic-acid resistance. Finally, " clumping " of the cells—so often observed for instance with staphylococci—could lead to similar results.

It may be noted that the modification of the fluctuation test given by Newcombe,[12] as a comparison between numbers of mutants in " spread " and " unspread " plates, is unfortunately subject to the same objections.

All the above-mentioned conditions may explain a negative fluctuation test, when a positive result was to be expected ; but the reverse can be explained as well, if one admits with Hinshelwood[b] and with Eriksen[5] that environmental conditions (e.g., of aeration) affect the potentiality to acquire resistance, and that such conditions change undetectably from culture to culture, although the cultures are apparently treated in the same way. If this is true, since the variability of both environmental conditions and the numbers of resistant mutants in parallel cultures would produce qualitatively the same effects, the variability of the numbers of resistant mutants would have no bearing on the main issue, that is, for or against the theory of mutation and selection.

From the general point of view of experimental design, the fluctuation test is, strictly speaking, inconclusive by itself, in that the variation between cultures cannot be divided into an environmental and a genetical portion; the test therefore relies on the mere trust that environmental variation between cultures is insignificant. It may therefore be useful to resort to other types of tests.

Correlation between relatives

One possible way of distinguishing between the alternative theories is that of considering differences of quality among resistant mutants, rather than their absolute numbers. It is well known that resistant mutants may well differ in degree, or in respect of accompanying morphological or physiological changes ; correlated with the above changes, one may also detect genetical differences among various mutations.

If the mutation and selection theory is correct in its present simple form, all descendants from an originally mutated organism should be

[b] See paper by Hinshelwood on page 3 of this number of the *Bulletin*.

identical, from any of these points of view. Therefore, clones of identical mutants should be found whenever the mutation starting the clone has occurred before the last generation in drug-free cultures. Two resistant colonies, obtained by plating a single culture of originally sensitive organisms, have a high chance of descending from the same mutation, and hence should have a higher chance of being identical than any two resistant colonies taken at random from independent cultures, which have certainly arisen by different, and probably differentiable, mutations. This type of test thus resembles that of the correlation between relatives used since Galton's time as a test of inheritance of traits in human and other populations.

The author performed such a test with chloramphenicol on *Esch. coli*, where resistance obtained after a single exposure to the drug was of a moderate degree ; but this degree can vary within relatively wide limits. On exposure to 25 μg/ml of the drug, for instance, resistant colonies are recovered which, on subsequent testing (by streaking on agar with increasing amounts of the drug), can resist as much as 80 μg/ml. The degree of resistance was used as the criterion for differentiating mutants.

Ten 5-ml broth cultures were inoculated with 1,000 sensitive cells of *Esch. coli* 30 and incubated to saturation of growth. They were then centrifuged, the sediment was collected in 0.2 ml of saline, and the whole amount of each culture was halved, each half being plated on chloramphenicol agar containing 25 μg/ml of the drug. The number of cells per culture was 1.8×10^{10}.

The two plates (A and B) obtained from each culture were incubated and colonies counted after 24 and 48 hours. Data given in table I show an obvious correlation between numbers of resistant mutants in plates from the same culture, which can be tested by the ratio of the variance between cultures to that within cultures, giving $F = 14.6$ for 9/10 degrees of freedom, which is significant at the 0.1% level. These data are for 24 hours only ; at 48 hours, two plates were not counted owing to counting difficulties, for which a fuller explanation is given below.

When the χ^2 test of the counting technique is applied to these data, a far too high value to be accounted for by chance is found ($\chi^2 = 51.44$ for 10 degrees of freedom).

This departure from an entirely correct technique resulted from the difficulty of counting the resistant colonies which was due essentially to their variability of size. On the first day, the size of the resistant colonies was very small, usually less than 1 mm; many sub-visible colonies could be seen at some magnification, apparently with a nearly continuous transition. On some plates, however, there were clear-cut categories of size and shape of resistant colonies arising from the same culture, suggesting, on mere inspection of the plates, the origin of these mutants in clones. On the second day, the originally small colonies had increased to normal size and many

TABLE I. NUMBER OF RESISTANT MUTANTS OF TEN INDEPENDENT CULTURES OF ESCHERICHIA COLI 30 PLATED ON 25 μg/ml OF CHLORAMPHENICOL

Culture No.	Number of resistant mutants			
	plate A		plate B	
	24 hr.	48 hr.	24 hr.	48 hr.
1	110	199	155	250
2	33	159	22	53
3	22	580	34	356
4	14	58	6	60
5	8	93	6	97
6	19	148	14	190
7	245	—*	150	352
8	3	58	9	112
9	18	95	19	180
10	4	119	18	—*

* Not counted (too high numbers involved)

more had appeared (see fig.1). Still more appeared on the third day and, probably, more would have appeared on the fourth, if the plates had not been too dry. No appreciable inactivation of the drug took place in these conditions, but, as mentioned later, it was observed that colonies developing on the second day were less resistant than those developing on the first day, and therefore probably had a longer lag, or a slower growth-rate, at the given drug concentration, as compared with the earlier resistants (see table II).[c]

There is thus a variation in size, especially of the colonies near the visibility limit—partly shown by the photograph of a plate after 48 hours of incubation (fig. 1), which makes the count difficult and adds extra variance to that due solely to the effects of chance distribution of the numbers of mutants into two halves.

In spite of this extra variance, the fluctuation test carried out as above remains a valid indication of the presence of a large variability of the numbers of resistant mutants in parallel independent cultures.

A further type of analysis is made possible by the fact that resistant colonies vary in degree of resistance. It has been shown by earlier work

[c] Miles (personal communication) has suggested an additional possible explanation for the discrepancy of the counts: a phenomenon of satellitism, which is not in disagreement with the aspect of some of the plates. A possible reason for satellitism here would be a slight local destruction of chloramphenicol by colonies with higher resistance, thus allowing the growth of less-resistant survivors in the neighbourhood.

that such differences are inherited, and that they are often due to different mutations. Hence, on the hypothesis of mutation and selection, differences of the average degree of resistance of colonies from independent cultures should be found, as such colonies are likely to have arisen by mutations determining a different degree of resistance. In other words, colonies from the same culture should resemble each other more closely than any two colonies taken at random from independent cultures.

FIG. 1. VARIABILITY IN SIZE OF RESISTANT COLONIES

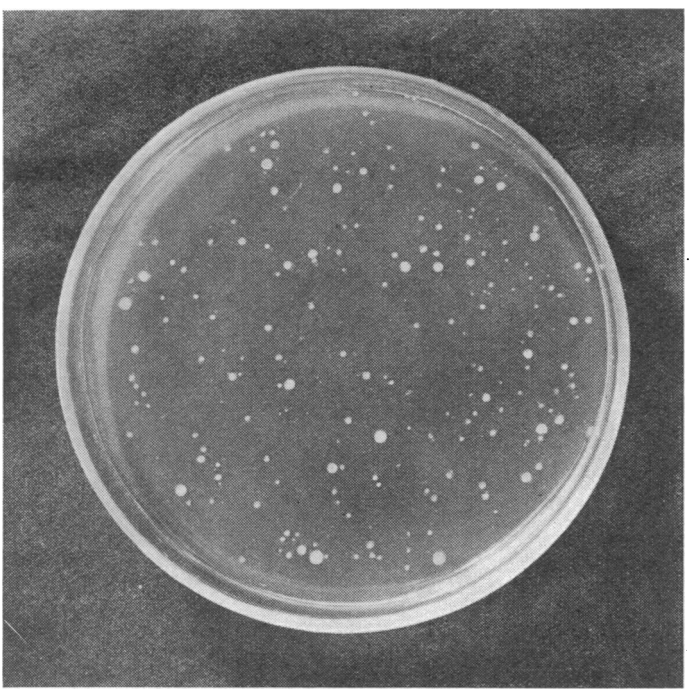


Plate containing 25 μg/ml of chloramphenicol, inoculated with 10^8 cells and incubated for 48 hours.

This test was carried out by taking two colonies of those developed after 24 hours, and two of those developed after 48 hours, from each of the 20 plates ; each colony was isolated and tested for degree of resistance by streaking on plates with increasing amounts of chloramphenicol in agar. The degree of resistance, varying from < 25 to 79, is shown in table II.

An analysis of variance (see table III) shows that the average degree of resistance varies significantly between cultures, but not between plates within cultures (the last comparison being a test of the technique), for both 24-hour and 48-hour cultures and for the total.

TABLE II. DEGREE OF RESISTANCE OF CHLORAMPHENICOL-RESISTANT MUTANTS OF ESCHERICHIA COLI 30*

Culture No.	Resistance score				Mean resistance				Total
	plate A		plate B						
	24 hr.	48 hr.	24 hr.	48 hr.	24 hr.	48 hr.	A	B	
1	4 4	4 4	4 4	4 4	4.0	4.0	4.0	4.0	4.0
2	3 5	3 0	4 4	2 0	4.0	1.2	2.8	2.5	2.6
3	2 4	4 4	4 2	2 0	3.0	2.5	3.5	2.0	2.7
4	5 6	4 2	4 4	4 4	4.7	3.5	4.2	4.0	4.1
5	4 4	4 4	4 4	4 4	4.0	4.0	4.0	4.0	4.0
6	4 4	2 2	2 3	3 2	3.2	2.2	3.0	2.5	2.7
7	3 3	3 3	3 3	3 3	3.0	3.0	3.0	3.0	3.0
8	4 4	2 2	5 3	2 2	4.0	2.0	3.0	3.0	3.0
9	5 5	2 2	3 4	4 4	4.2	3.0	3.5	3.7	3.6
10	5 4	4 3	5 5	5 3	4.7	3.7	4.0	4.5	4.2
Mean					3.9 **	2.9 **			

* Selected at random among the resistant mutants mentioned in table I
** The difference between the two means is significant at the 1% level.

Resistance score	0	1	2	3	4	5	6
Maximum tolerated dose μg/ml	<25	25	32	40	50	63	79

TABLE III. ANALYSIS OF VARIANCE OF DATA SHOWN IN TABLE II

Source of variation	24-hr. values			48-hr. values			Total		
	df	mean square	F	df	mean square	F	df	mean square	F
Between cultures .	9	1.611	3.220*	9	3.3917	5.025**	9	3.3625	2.754**
Between plates within cultures .	10	0.7000	1.400	10	1.4750	2.185	10	0.5875	<1
Within plates . . .	20	0.5000		20	0.6750		60	1.2208	

* Significant at P = 5% level
** Significant at P = 1% level

This amounts to saying that there is a positive correlation between relatives, in accordance with the galtonian test of inheritance, i.e., that there must have been, according to the simplest explanation, common ascendance, which is the same as the theory of mutation and selection.

It should not be forgotten that both the fluctuation test and the present test for the correlation between relatives rest on the same assumption,

i.e., that of the appearance of the mutants in clones. Both are therefore open to the same objection advanced by Hinshelwood and by Eriksen,[5] namely, that inappreciable differences in the environmental conditions of the parallel cultures used might well lead to the observed discrepancies in number and qualities of the resistants among cultures.

The fact that the " quality " of resistance here investigated was its intensity makes a further criterion possible, by a combination of both the fluctuation test and that of the correlation between relatives. In fact, if environmental conditions favour or disfavour future resistance in independent cultures, there would be reason, based on a hypothesis of physiological adaptation, for both the number of resistant individuals and their average resistance to be affected proportionally by those conditions. Hence, a high positive correlation between the number of resistant mutants and their average degree of resistance is expected under the hypothesis of physiological adaptation, while a correlation of zero would be the consequence of mutation and selection.

Here we have the correlation $r = -0.16$ for 24-hour values, and $r = -0.03$, approximately, for 48-hour values (calculated on logarithms of numbers of mutants). Although the sample is not large, the conclusion is in favour of the theory of mutation and selection.

Thus positive correlation between numbers of resistants from parallel cultures, positive correlation for intensity of resistance, and zero correlation between number and intensity strengthen previous conclusions that drug-resistance is due to mutation and selection.

MENDELIAN ANALYSIS

An entirely independent approach was made possible by Lederberg & Tatum's discovery of genetic recombinations in *Esch. coli*.[10] This opened the way to mendelian analysis in bacteria.

Unfortunately, there are at present difficulties imposing some restrictions on this type of experiment. First, until recently only a limited number of strains (all *Esch. coli*) was available for this type of experiment. Secondly, recombination is generally rare and, when recombinants are being selected, only one of the two or more complementary recombinants theoretically arising from each zygote is usually recovered. Thirdly, either for reasons of differential viability or of chromosome abnormalities, or because of unknown complexities of the genetical or the mating system of *Esch. coli* K-12 (the strain employed in these experiments), part at least of the map of the single chromosome of *coli* K-12 is non-linear.

Resistance Patterns

Resistance to four drugs has been investigated by this technique in some detail : streptomycin, azide, chloramphenicol, and terramycin.

Streptomycin

Streptomycin was investigated by Monod (personal communication), by Demerec,[4] and by Newcombe & Nyholm,[14] who all came to the conclusion that streptomycin resistance (or dependence) is essentially due to a single locus or group of closely-linked loci, which are linked with a gene for methionine synthesis in the standard strains. In reverse crosses (i.e., crosses in which resistance has been switched from one to the other of the two strains to be crossed) resistance behaves exactly as expected for mendelian inheritance, i.e., segregation is invariant to gene substitution. Unfortunately this most useful marker falls in the " unmappable " portion of the chromosome, as was shown by Newcombe & Nyholm's experiments.[14]

Azide

Azide resistance was introduced by Lederberg[9] as a chromosome marker, and mapped independently in several laboratories. It is an all-or-none type of resistance, although the increase of resistance is far less striking than the one observed with streptomycin (which is often complete). Even so, azide can perhaps be said to belong to the group of drugs giving the "streptomycin pattern" of resistance according to Demerec,[3] this being considered here as the capacity for developing the highest degree of resistance in a single step. Another important pattern is the " penicillin pattern ", in which resistance is not developed in a single step, but only in a number of successive steps, each one contributing a moderate degree of resistance.

Table IV shows data collected by crossing three independent azide-resistant mutants. The azide-resistance locus is linked with TL, and lies between V_1 and TL. Similar results were obtained by Lederberg and by Allen (personal communications) using mutants independent of these. Reverse crosses give results in perfect agreement with mendelian expectation, as shown in table IV.

Penicillin

Of the drugs giving the penicillin pattern, two, affecting *Esch. coli*, have been analysed so far : chloramphenicol and terramycin.

Drugs giving the penicillin pattern are of special interest in connexion with the problem of whether the change is determined before or after drug contact. When selection for resistance to these drugs is carried out in solid media, steps are clearly recognizable, as shown by Demerec.[2] But when selection is carried out in liquid media, highly-mixed bacterial populations develop, the single cells differing to a considerable extent in respect of individual, inheritable degree of resistance. In such conditions there is an overlapping of the steps and the process may seem perfectly gradual; this may, at first sight, appear to discount the theory that mutation and selection play a role.

TABLE IV. MAPPING THE LOCUS OF AZIDE RESISTANCE IN THREE INDEPENDENT MUTANTS*

Crosses: M— Lac + V_1^s Az^s TL+ (a b c d) M + Lac— V_1^r Az^r TL—

Crossing-over regions / Phenotype Lac V_1 Az	a +ss	b —ss	c —rs	d —rr	abc —rs	bcd —sr	acd +sr
Strains : 58-161 x 30 Az^r	21	46	15	15	—	1	1
CR2 x 102	24	46	20	12	3	4	—
Total	45	92	35	27	3	5	1
%	21.6	44.2	16.8	13.0		4.3	

Cross: M— Lac + V_1^s Az^r TL— (a b c d) M + Lac— V_1^r Az^s TL—

Crossing-over regions / Phenotype Lac V_1 Az	a +sr	b —sr	c —rr	d —rs	abc +rr	bcd —ss	acd +ss
Strains : 30 x 100 deriv.	44	71	31	18	2	4	—
%	25.9	41.8	18.2	10.6		3.5	

* Agreement between reversed crosses : $\chi^2_{[4]} = 1.40$

However, what is likely to happen in drug adaptation of the penicillin pattern, assuming the mutation and selection theory to be correct, can be predicted on the basis of knowledge of quantitative (polygenic) inheritance in higher organisms. This had already been postulated by Demerec on the basis of his studies of penicillin resistance in staphylococci, but it was not possible to prove the hypothesis at that time.

According to the polygenic picture, a number of loci can mutate to resistance, but each locus can contribute, by mutation, only a limited degree of resistance. Mutation occurring at more loci, in some succession, can result in a high resistance by addition of the effects of the single locus. Since there is only one chromosome in *Esch. coli*, mendelian analysis of such a polygenic system should show linkage of these polygenes between themselves and with the ordinary chromosome.

What should one expect as to the results of a cross, assuming instead the hypothesis of physiological adaptation to hold true ? An adaptation according to Hinshelwood's model would result in blending inheritance—i.e., recombinants all showing an intermediate level of resistance between those of the two parents—assuming a free exchange of cytoplasm in conjugating bacteria. At the present state of knowledge of bacterial genetics, such an assumption canot be either accepted or rejected. The demonstration of completely-blending inheritance might be fatal to the theory of mutation of nuclear genes and selection, but a non-blending inheritance would not, at present, be conclusive against either of the alternative theories. However, Hinshelwood's model of a shift in the balance of enzymes built by the resistant cells would be completely at variance with a finding of linkage between resistance and the usual chromosome markers.

Chloramphenicol

Chloramphenicol resistance was the subject of research carried out by the author in collaboration with Maccacaro.[1] In this investigation, strains obtained after long selection in liquid media, as well as those obtained with a counted number of steps by selection on solid media, were analysed by crossing to sensitives, or other resistants.

Selection curves in liquid media showed an almost perfectly continuous increase of resistance in successive sub-cultures. The speed of advance under selection varies considerably from experiment to experiment ; eventually, high levels are reached, occasionally very near the solubility limits of chloramphenicol (above 1 mg/ml ; the tolerated starting-level is about 5μg/ml).

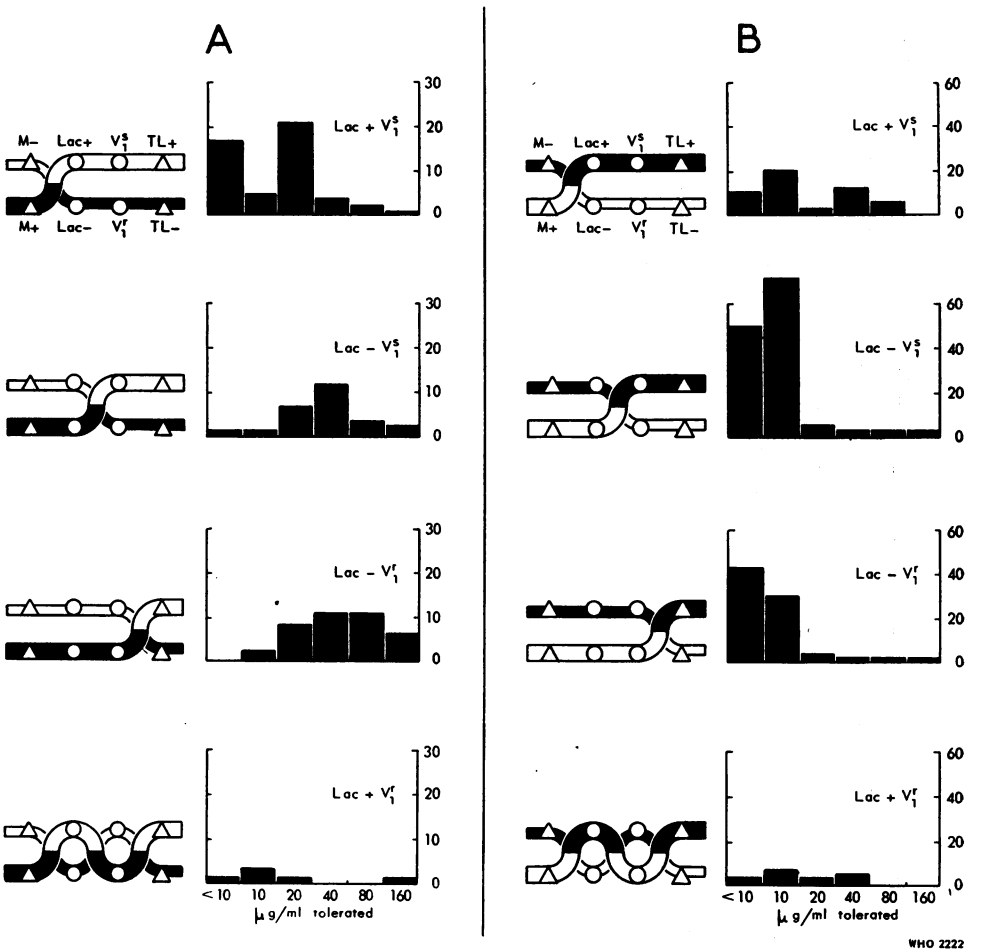
High resistants, isolated from these experiments, were crossed to sensitives, and the recombinants scored for resistance as well as for other markers, essentially lactose fermentation and virus T_1 resistance. These two markers split the region between M and TL (which are the markers used for the selection of the recombinants) into three regions.

With two markers there are four possible phenotypes ; the phenotype of a given recombinant indicates whether crossing-over has occurred in the first region (between M and Lac), in the second (between Lac and V_1), or in the third (between V_1 and TL) ; the fourth recombinant type, which is the rarest, corresponds to the triple crossover (one crossover in each region).

Fig. 2 shows the results of crosses using high resistant strains obtained by selection in a liquid medium. Two reverse crosses are indicated : in fig. 2(A), a M+Lac−V_1^rTL− strain resistant to chloramphenicol is crossed to a sensitive M−Lac+V_1^sTL+ strain. M+TL+ recombinants are selected, in the cross, and each recombinant is scored for LacV_1 and for

C-resistance. The frequency distributions of resistance levels in the various recombinants from this cross are shown on the left of fig. 2(A). Recombinants with phenotype Lac+V_1^s, which therefore have crossed over in the first segment, are those with the lowest resistance, on the average,

FIG. 2. CROSSES OF K-12 STRAINS SELECTED FOR HIGH RESISTANCE TO CHLORAMPHENICOL AND SENSITIVE (NORMAL) STRAINS



A. Cross between a sensitive M—Lac+V_1^s TL+ strain and a resistant M+Lac—V_1^r TL—strain

B. Cross between a resistant M—Lac+V_1^s TL+strain and a sensitive M+Lac—V_1^r TL—strain

Each histogram indicates the distribution of resistances of given types of recombinants; four types of recombinants are distinguished, according to scores for LacV_1 markers. The postulated type of crossing-over leading to each type of recombinant is indicated schematically left of each histogram; the strand which results in the prototrophic (M+TL+) recombinants is indicated as the thicker strand and it is the only one recovered of the two (or more) possible strands resulting from crossing over in a zygote. Resistant strands are marked black.

while those with phenotype Lac$-V_1^s$ which have crossed over in the second region (Lac$-V_1$) come next ; the most resistant ones are those with phenotype Lac$-V_1^r$ which have crossed over in the last region. Triple crossovers (phenotype Lac$+V_1^r$), which are rare, show an intermediate behaviour.

This is exactly what would be expected if there were a number of genes distributed all over the chromosome contributing to resistance. In fact, in this case, the recombinants getting most of their markers from the resistant strain (even without any assumption as to linearity) should be more resistant than the others and there should be a rough proportionality between the length of the piece of chromosome (or the number of markers) which a given recombinant obtains from the resistant parent and its degree of resistance.

This was fully confirmed by the reverse cross indicated in fig. 2(B), which represents the cross of a resistant M$-$Lac$+V_1^s$TL$+$ strain to a sensitive M$+$Lac$-V_1^r$TL$-$ strain. Here recombinants produced by crossing over in the first segment were more resistant than those of the second segment, and these, in turn, were more resistant than those in the third segment as expected. The first segment, i.e. M-Lac, contributed the greatest amount of resistance in both crosses.

When single steps, obtained by plating a large amount of sensitive cells on small concentrations of chloramphenicol and selecting resistant colonies, were crossed to sensitives, all-or-none inheritance of the trait was observed, and the gene determining resistance could be mapped satisfactorily.

To check more accurately whether inheritance of single steps was of the all-or-none type, as opposed to blending inheritance, the resistance of the sensitive parents of a first-step resistant, of a second-step resistant, and of a number of recombinants, obtained by crossing these two resistants to sensitives, was measured more accurately than in the preceding experiments, by determining the percentage of survival at various doses of chloramphenicol in parents and recombinants. The results obtained seemed to exclude blending inheritance.

Crosses between resistants gave results which were the cause of some surprise. All the first steps intercrossed so far were non-allelic, i.e., gave some sensitive recombinants in varying proportions. No clear-cut cases of increased resistance due to addition of the separately-acquired resistances were found ; but this matter has not been investigated extensively enough. [d] Thus mutation leading to first-step resistance can affect one of a number of different loci ; and such resistances are not easily additive.

Crosses between high resistants showed, in general, a decline of resistance among the recombinants ; a recombinant with resistance higher than

[d] Since this paper was read at the symposium, the author has observed a clear-cut case of addition of resistance by crossing two independent first steps in the absence of chloramphenicol.

that of either parent was never recovered. On the contrary, a few fully-sensitive recombinants were recovered. In such crosses, their recovery is reminiscent of " Dauermodifikationen ", but whatever the mechanism of the latter, here such recombinants are likely to have arisen by some kind of negative interaction, possibly a semilethal combination of characters, as these sensitive recombinants were usually poor growers even in the absence of the drug. No full explanation of this observed negative interaction has been found. Both this problem and the problem of additivity of independent resistance steps are under investigation at present.

The process of building up high resistance can perhaps be pictured approximately in the following way. A number of different loci can mutate to give a first increase of resistance. Which loci will mutate next, adding their resistance to that obtained through the first step, depends to a large extent on the locus which has mutated first, since only a few of the possible combinations of mutation at different loci seem to be able to give rise to increased resistance. Thus every selection process seems to lead to a unique combination of genes, and the resistance thus obtained can break down on crossing to a resistant individual obtained through a similar, but independent, selection process.

Terramycin

Terramycin resistance was recently investigated by similar methods. An experiment of selection for terramycin resistance on two *Esch. coli* K-12 strains gave high levels of resistance after 12 to 20 transfers.[e] The results were similar to those obtained with chloramphenicol, but the ceiling reached was lower (about 300 μg/ml ; with the lower starting-point 1 μg/ml instead of 5 μg/ml).

A considerable degree of cross-resistance is found between terramcycin and chloramphenicol, as might have been anticipated from data in the literature. Table V gives the maximum tolerated concentrations (in μg/ml) of the two drugs observed in strains selected for C- or for T-resistance ; the test was carried out by streaking on agar, and two readings (after 18 and 42 hours, respectively) were made.

Thus while T-resistance always entails high resistance to C, selection for C-resistance does not bring about any considerable increase of resistance to T.

From the point of view of the application of these drugs, the following purely speculative conclusions may be drawn : The first, and more obvious, is that the association of these two drugs will not give rise to any advantage, at least as far as prevention of resistance is concerned. The second is that, in diseases where either drug is applicable a priori, chloramphenicol should

[e] I am indebted to Dr. M. Rinaldi for carrying out the selection experiment.

TABLE V. COMPARATIVE RESISTANCE OF SELECTED ESCHERICHIA COLI STRAINS TO CHLORAMPHENICOL AND TERRAMYCIN

Esch. coli strains selected for resistance	Selection carried out for resistance to	Maximum tolerated concentration (μg/ml)			
		chloramphenicol		terramycin	
		18 hr.	42 hr.	18 hr.	42 hr.
58-161a	chloramphenicol	> 79	> 79	5.6	10
W 677		56	79	1.8	3.1
58-161b		20	56	1.8	5.6
100/2	terramycin	31	79	10	31
30		79	> 79	56	100

be tried first, since resistance obtained to it would not entirely compromise the use of terramycin, while the reverse would not be true.

Crosses between the terramycin-resistant strains and suitable sensitives were carried out. The recombinants again show a high degree of correlation between T- and C-resistance; but, while the C-resistant genes located in the M-TL region have little effect on T-resistance, the genes located left of M show a high effect on resistance to both T and C.

This is shown in fig. 3 and 4 where crosses conducted in the presence and absence of vitamin B_1 are compared. In crosses with vitamin B_1, crossovers left of M are rare (10% of the recombinants show recombination between M and B_1, the only marker of this region, giving B_1+ recombinants). In the absence of vitamin B_1, all recombinants must be B_1+ and hence this crossover, additional to that between M and TL, is compulsory.

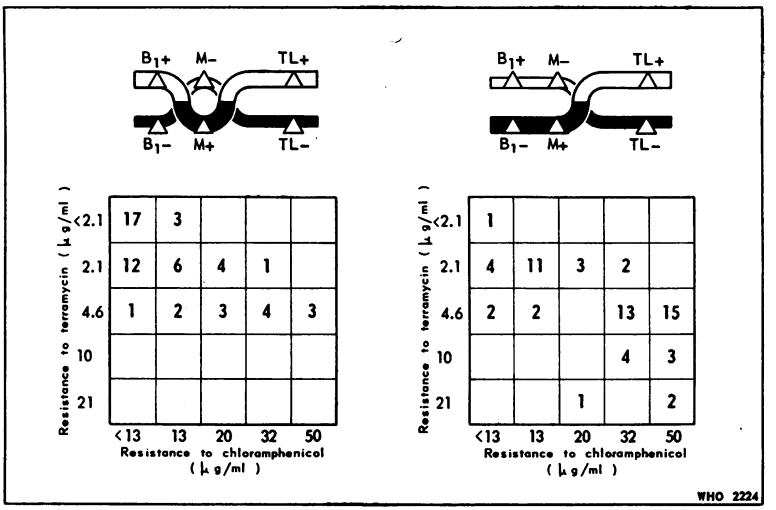
When crossover in the B_1-M region is compulsory, resistance to terramycin and chloramphenicol rises in a cross, in which the resistant strain is $B_1+M-TL+$ and the sensitive one $B_1-M+TL-$, since the B_1+ gene and surrounding regions, which include some resistant loci, come from the resistant parent. When the cross is reversed, i.e., when the $B_1+M-TL+$ parent is the sensitive one, recombinants on media without vitamin B_1 are, on the average, more sensitive than those on media with vitamin B_1. Thus here again reverse crosses give results in agreement with mendelian expectations.

When a C-resistant organism was crossed to a sensitive, and a similar comparison between recombinants from crosses with and without vitamin B_1 was conducted, C-resistance due to genes left of M could be shown to occur and to be correlated with some resistance to terramycin as well.

CONCLUSIONS

To add a few concluding remarks, it should be remembered that crossing experiments cannot throw any direct light on the problem of the origin of drug-resistance, which has to be considered rather along the

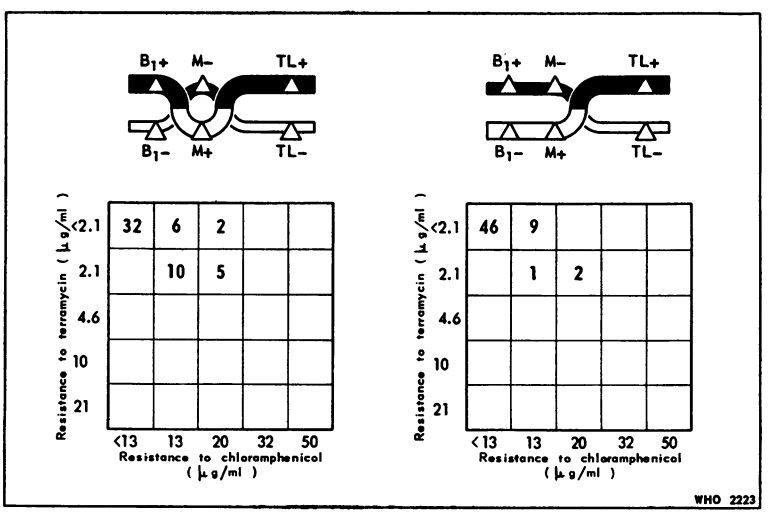
FIG. 3. CROSS BETWEEN A NORMAL (SENSITIVE) B_1+M—TL+ STRAIN AND A TERRAMYCIN-RESISTANT B_1—M+TL— STRAIN*



A
No vitamin B_1

B
Vitamin B_1 added

FIG. 4. CROSS BETWEEN A TERRAMYCIN-RESISTANT B_1+M—TL+ STRAIN AND A NORMAL (SENSITIVE) B_1—M+TL— STRAIN*



A
No vitamin B_1

B
Vitamin B_1 added

* Comparison between recombinants obtained in absence (A) and presence (B) of vitamin B_1. In the drawings the recombinant recovered is represented as the thicker strand ; the resistant parent is black, the sensitive white. The drawings indicate schematically the types of crossing-over taking place in the two conditions compared.

lines discussed in the first section of the paper. Crossing experiments, however, can give indirect information, as far as they differentiate between cytoplasmic and nuclear inheritance and, in the latter case, can show whether gene mutations have taken place. The experimental results favour the view that gene mutations play a major part in determining drug-resistance ; but whether or not these account for all of the resistance cannot be answered until (*a*) the genetical and mating systems of bacteria have been more satisfactorily explored, and (*b*) the peculiarities of interaction between these polygenes are better known.

The use of bacteria for the analysis of polygenic systems has important advantages over that of higher organisms ; first, with bacteria it is easier to utilize mutation for securing new genetic variation, which in higher organisms must usually be provided by recombination. This results in the further advantage that variability will be available in steps and will not be practically continuous, as is normally the case in higher organisms. A third factor, especially useful for the analysis of interactions, is that the work can be more easily carried out at a biochemical level. For this purpose, drugs such as the sulfonamides, for which the mechanisms of action and of microbial resistance are at least partially known, may offer particular advantages.

SUMMARY

Drug-resistance in micro-organisms and its inheritance has been explained by several theories which can be classified into two broad categories, according to whether they assume that the change leading to resistance occurs before or after contact with the drug. If it is assumed that the change happens before, the drug essentially acts as a selective agent, even if the resistant individuals were only potentially so before drug contact and need a period of adaptation in order to grow in the presence of the drug. The spontaneous change leading to potential or actual resistance is called mutation, and in such a case it is usually due, according to genetical knowledge, to a change affecting a gene, or at least the nucleus.

Many authors on the other hand maintain that the change must occur after contact, in which case the adaptation can be defined of a physiological type (in contrast to the type given above, which is usually referred to as genetical adaptation). This hypothesis does not easily account for the

RÉSUMÉ

La résistance des microbes aux médicaments et sa transmission héréditaire ont été expliquées par deux groupes de théories : selon les unes, le changement serait antérieur au contact avec la drogue; selon les autres, il lui serait postérieur. Si l'on admet que le changement est antérieur au contact, le médicament agit comme agent de sélection, même si les individus résistants ne l'étaient que virtuellement avant le contact avec la drogue et même si une période d'adaptation a préludé à leur développement. La modification spontanée conduisant à la résistance virtuelle ou actuelle est appelée mutation; elle est due le plus souvent, d'après les données de la génétique, à un changement affectant un gène ou tout au moins le noyau.

Plusieurs auteurs soutiennent d'autre part que le changement est postérieur au contact; dans ce cas, l'adaptation peut être appelée physiologique (par opposition au type précédemment décrit, connu sous le nom d'adaptation génétique). Cette hypothèse ne rend pas facilement compte du fait

fact that on exposure to the drug most cells die or do not form colonies, nor for the fact that in most cases resistance, once acquired, can be stable in absence of the drug.

The fluctuation test provides a method of discrimination between " before " and " after " theories but, both in its original design (the variation of the number of resistant colonies in parallel independent cultures) and as revised by Newcombe the conditions under which it is valid are not always met in practice in the analysis of drug-resistance. The positive and the negative results of the fluctuation test might be open to objections which are not always easily removed.

Any test designed to distinguish between the alternative theories must, of course, be based on the fact that all descendants from an originally mutated organism will form a clone of mutated individuals, so that mutants will be found in clones, in cultures where mutations have occurred before the last generation preceding drug contact. However, the available tests make use only of the absolute numbers of resistant mutants, though it may be useful to take into account the differences of morphological, physiological, or genetical type, qualitative or quantitative, which may be detected among mutants originated from different mutations. This more refined analysis can be developed into a test which can be named that of the correlation between relatives.

When more than one type of resistance can be observed, cultures experiencing few mutations only will often differ as to the type of mutations which have occurred in each of them. Each mutation, if it has occurred in the last-but-one, or an earlier generation before drug contact will have, given rise to a clone of two or more mutants all alike. Each mutant will form, on plating with the drug, a separate colony ; hence sister colonies, arising from the same culture plated in the presence of the drug must resemble each other more closely than any two colonies taken at random from parallel independent cultures. Thus if such correlation between relatives

que la plupart des cellules exposées au contact de la drogue meurent ou ne forment pas de colonies; elle n'explique pas non plus pourquoi, dans un très grand nombre de cas, la résistance acquise subsiste en l'absence de la drogue.

Le « test de fluctuation » devrait permettre de déterminer laquelle des deux hypothèses est valable; mais les conditions de sa validité ne sont pas toujours remplies dans l'analyse pratique de la résistance aux drogues, qu'il soit appliqué sous sa forme originale (variation du nombre de colonies résistantes dans des cultures parallèles indépendantes) ou sous sa forme revisée par Newcombe. Les résultats de ce test, qu'ils soient positifs ou négatifs, sont sujets à des critiques auxquelles il est souvent difficile de répondre.

Tout test destiné à établir la validité de l'une ou de l'autre théorie doit se fonder sur le fait que tous les descendants d'un mutant originel formeront un clone de mutants ; ainsi, des mutants se trouveront en clones dans les cultures où des mutations se sont produites avant la dernière génération précédant le contact avec la drogue. Or, les tests dont on dispose ne se réfèrent qu'aux nombres absolus de mutants résistants; il serait pourtant utile de tenir compte des différences morphologiques, physiologiques et génétiques, qualitatives et quantitatives qui peuvent être décelées parmi les mutants provenant de diverses mutations. Une analyse plus subtile du problème peut être faite sous forme d'un test appelé « ressemblance entre parents ».

Lorsque plus d'un type de résistance est possible, les cultures dans lesquelles ne se produisent qu'un petit nombre de mutations se différencient souvent l'une de l'autre par le type de mutation dont chacune d'elles est le siège. Chaque mutation survenue à l'avant-dernière génération précédant le contact avec la drogue — ou antérieurement — aura donné naissance à un clone de deux mutants ou plus, tous pareils. Chaque mutant, ensemencé sur des plaques contenant la drogue, formera une colonie distincte; il s'ensuit que les colonies-sœurs, provenant de la même culture ensemencée sur plaque, doivent se ressembler davantage que deux colonies

is found, we must conclude that the change has arisen before and not after drug contact. Moreover, according to the " before " hypothesis there should be no correlation between the absolute number of mutants from a culture and their average resistance, while it would be hard to explain such a lack of correlation by any " after " theory.

Such tests were performed especially with chloramphenicol on *Escherichia coli*, making use of the differences in degree of resistance which are observed among resistants which are found after the first contact with the drug. The results of these tests consistently indicated mutation and selection.

The discovery of a sexual phase in *Esch. coli* made another type of experimental approach possible, namely mendelian analysis. This was applied to drug-resistance in the case of streptomycin, azide, chloramphenicol, and terramycin. The first two drugs give a sort of all-or-none response, which can be shown to be due to well-individualized loci on the bacterial chromosome.

Chloramphenicol offers the interest of presenting the case of resistance which increases gradually and easily on repeated sub-culture with increasing amounts of the drug. Gradual resistance, also called of the " penicillin pattern " is easily explained a priori by the theory of physiological adaptation, while it can be explained on the alternative theory of genetical adaptation on the basis of a complex picture of inheritance, namely that of a number of interacting loci, each contributing by mutation a low degree of resistance, adding up when more loci have mutated to give higher and higher resistance.

This polygenic picture was borne out by the results of crosses. Crossing a highly

prises au hasard dans des cultures parallèles indépendantes. Si une telle « ressemblance entre parents » se vérifie, on peut conclure que le changement s'est produit avant et non pas après le contact avec la drogue. De plus, d'après l'hypothèse génétique (changement avant le contact), il ne doit pas exister de corrélation entre le nombre absolu de mutants d'une culture et la résistance moyenne de ces derniers; il serait difficile d'expliquer l'absence d'une telle corrélation par une théorie d'adaptation physiologique (changement après le contact).

Des tests, basés sur ce principe, ont été effectués en particulier sur *Escherichia coli* en présence de chloramphénicol; on s'est fondé sur les différences du degré de résistance constatées entre les individus résistants qui apparaissent après le premier contact avec la drogue. Les résultats de ces tests sont uniformément en faveur de la théorie mutation-sélection.

La découverte d'une phase sexuelle dans le développment de *Esch. coli* a permis d'aborder le problème par une autre voie expérimentale, celle de l'analyse mendélienne. Celle-ci a été appliquée à la résistance aux drogues telles que la streptomycine, l'azide, le chloramphénicol et la terramycine. Les deux premières de ces substances donnèrent une réponse du type « tout ou rien », que l'on peut expliquer par l'existence de loci bien distincts sur le chromosome bactérien.

Le chloramphénicol est intéressant parce qu'il illustre le cas d'une résistance qui augmente graduellement et facilement au cours de repiquages successifs, en présence de quantités croissantes de la drogue. Cette résistance graduelle, dite du « type pénicilline », est explicable a priori par la théorie de l'adaptation physiologique; elle peut être expliquée également par la théorie génétique, sur la base d'un schéma complexe de transmission héréditaire, faisant appel à l'interaction d'un certain nombre de loci dont chacun, par mutation, déterminerait un faible degré de résistance; les mutations de plusieurs loci s'additionneraient, ce qui se traduirait par une augmentation graduelle de la résistance.

Ce schéma polygénique a été suggéré par les résultats de croisements. On cons-

resistant to a suitably-marked sensitive strain, the degree of resistance of the recombinant was dependent on the length of the portion of the chromosome which the recombinant obtained from the resistant parent, thus indicating the existence of a number of loci. First steps (i.e., resistants obtained after a single exposure to the drug) show only one locus to be affected, although this locus usually differs in independently-obtained first steps. Inheritance of one-step resistance is of the all-or-none type. Although it is difficult to obtain increased resistance by recombination of first steps, one clear-cut case of addition of resistance by crossing two independent first steps in the absence of chloramphenicol has been observed.

Terramycin resistance was investigated by similar experiments. It is again of the penicillin type; but selection is slower and the results are less striking than with chloramphenicol. There is a considerable amount of cross-resistance between chloramphenicol and terramycin.

On the whole, the results of the crosses support the hypothesis of gene mutations determining resistance, full or gradual; whether, in the latter case, any room is left for non-genetic adaptation of the "after" type cannot be fully answered at present.

tata que le degré de résistance résultant du croisement d'une souche hautement résistante avec une souche sensible, convenablement marquée, dépendait de la longueur du fragment de chromosome provenant du parent résistant; de tels faits parlent en faveur de l'existence de plusieurs loci. Dans le cas d'organismes devenus résistants après n'avoir été exposés qu'une fois au contact de la drogue, on a pu montrer qu'un seul locus a été affecté; ce locus n'est pas le même chez toutes les souches considérées. La transmission de cette forme de résistance est du type « tout ou rien ». Il est difficile d'obtenir, par recombinaison, une augmentation de la résistance; ce résultat a été pourtant constaté dans un cas indubitable; à la suite du croisement de deux souches indépendantes présentant le type de résistance décrit ci-dessus, dans un milieu dépourvu de chloramphénicol, on a observé, dans la descendance, l'addition des résistances individuelles.

La résistance à la terramycine a été étudiée par des expériences analogues. Elle est aussi du type pénicilline, mais la sélection est plus lente et les résultats moins frappants qu'avec le chloramphénicol. Il existe une forte résistance croisée entre chloramphénicol et terramycine.

Dans l'ensemble, les résultats des croisements sont en faveur de l'hypothèse de mutations géniques déterminant la résistance, totale ou graduelle; il est prématuré de dire si, dans ce dernier cas, l'adaptation non génétique peut jouer un rôle.

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