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BY SIB SELECTION

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L. L. CAVALLI-SFORZA AND JOSHUA LEDERBERG

*Department of Genetics, University of Wisconsin, Madison, Wisconsin and Istituto Sieroterapico  
Milanese "Serafino Belfanti", Milan*

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## ISOLATION OF PRE-ADAPTIVE MUTANTS IN BACTERIA BY SIB SELECTION<sup>1</sup>

L. L. CAVALLI-SFORZA<sup>2</sup> AND JOSHUA LEDERBERG

*Department of Genetics, University of Wisconsin, Madison, Wisconsin and Istituto Sieroterapico  
Milanese "Serafino Belfanti", Milan*

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THE ROLE of antibiotics in provoking the development of resistant mutants of bacteria has been repeatedly discussed (CAVALLI 1952; Society of General Microbiology Symposium 1953; CAVALLI and LEDERBERG 1953; BRYSON and SZYBALSKI 1955; SEVAG, REID and REYNOLDS 1955). The two principal conceptions may be labelled the pre- and post-adaptation theories, respectively, according to whether the adaptive hereditary alteration occurs in individual cells prior to their contact with the drug, or in consequence of it. One basis of decision has been the isolation of resistant mutants by indirect (sib clone) selection (LEDERBERG and LEDERBERG 1952). The over-all result of this method is the isolation of lines of resistant mutants that have never been exposed to the drug in question, and are therefore indisputably preadaptive. This method, which depends on a technique of replica plating with a velvet sheet from one agar plate to another, gives a clear-cut qualitative result but is inherently not quantitative, so that failures are difficult to assess. In this paper, a more general procedure for indirect selection that allows of quantitative control will be described in its application to resistance to streptomycin and chloramphenicol, respectively, in *Escherichia coli*.

### INDIRECT SELECTION IN LIQUID MEDIA: LIMIT SAMPLING

The principal difficulty in assessing mechanisms of drug resistance is the rarity of resistant cells. On the preadaptation theory, a population of bacteria may already contain, say one resistant per million cells. Short of individual tests on a million isolates, on one hand, and the direct but ambiguous use of the antibiotic as a selective agent on large populations, on the other, how can these rare resistant cells (or clones from them) be extracted? The method to be described depends on the indivisibility of single cells in progressively smaller samples of these populations.

Consider, for example, a population containing  $10^9$  sensitive and  $10^3$  resistant organisms per ml. A 0.1 ml sample will contain, on the average,  $10^8$  sensitives and 100 resistants. But a  $10^{-4}$  ml sample will contain  $10^5$  sensitives and 0.1 resistant cells. What is 0.1 cell? The statement refers, of course, to the expectation that of ten samples, nine will contain no resistants, the tenth will contain one. In this tenth sample, however, the ratio of resistant to sensitive will have been raised from  $1:10^6$  to  $1:10^5$ , a tenfold enrichment. If the resistant cells can hold their own in mixed

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culture with sensitives, such a sample can be inoculated in fresh broth, and give rise to a new population with  $10^9$  sensitive and  $10^4$  resistant. The same enrichment procedure can then be reiterated until the resistant clone is obtained in pure culture, in six calculated cycles. At each stage, ten samples must be taken that have an expectation of 0.1 resistant cell each; the one which actually contains it is identified retrospectively by assays on samples of each culture. These assays further serve to guide the practice of the next cycle.

For a quantitative treatment, consider an initial culture with  $N_0$  cells of which  $R_0$  are resistant, giving a ratio  $r_0 = R_0/N_0$ . If samples of size  $q$  are taken they will contain  $m = qR_0$  resistant, and  $qN_0 = m/r_0$  total cells. If the sample is then distributed among  $n$  tubes of fresh medium, each tube will receive  $m/nr_0$  cells, and, if  $m \ll n$ , either zero or one resistant. In the latter tubes, the proportion of resistant cells in the inoculum will be  $1/\frac{m}{nr_0} = nr_0/m = r_1 = R_1/N_1$ . If the ratio of resistant cells remains unaltered during growth, the final culture will have been enriched by a factor  $E = r_1/r_0 = n/m$ .

However, if the ratio is altered owing to differential growth, the resistant ordinarily being slower, the effective enrichment  $E(t)$  will be a function of the amount by which the inoculum is permitted to grow. If we take  $t$  as the number of  $e$ -fold increases of the sensitive cells,  $k$  as the relative growth rate of the resistant, and  $R_1 = 1$ , the sensitives will grow as  $(N_1 - 1)e^t$ , and the resistant as  $e^{kt}$ . Thus  $r_1(t) = R(t)/N(t)$  will be given by

$$(1) \quad \begin{aligned} \frac{1}{r_1(t)} &= \frac{(N - 1)e^t + e^{kt}}{e^{kt}} = \left( \frac{1}{Er_0} - 1 \right) e^{t(1-k)} + 1 \\ &= \left( \frac{1}{Er_0} - 1 \right) (R_1(t))^{1-k/k} + 1, \end{aligned}$$

and approximately, if  $Er_0 \ll 1$  and  $r_1(t) \ll 1$ ,

$$(2) \quad E(t) = Ee^{t(k-1)}$$

which approaches zero asymptotically with increasing  $t$ . From (2) also,  $E(t) \leq 1$  and enrichment becomes impoverishment after  $t = \log E/(1 - k)$ . It is therefore desirable, or even essential, to limit the number of generations as much as convenient.

In the above discussion, we have taken  $R_1$  as either zero or one; actually both  $R_1$  and  $m = qR_0$  follow the Poisson series and may take any integral value. The sample with expectation  $m$  might contain no resistant at all with a probability  $e^{-m} = e^{-qr_0N_0}$ , so that none of the tubes would be "positive" (i.e., no tube will have had at least one resistant in the inoculum) and the enrichment cycle would fail entirely. For this reason, the positive tube of each cycle should be saved until the next cycle has been successfully completed.

The procedure may be formulated more generally as relying upon the calculated dispersion of  $r$  around its mean  $R_0/N_0$  and the retrospective choice of those samples in which  $r$  had been the highest. When  $N_0$  is large, its variation may be ignored,

TABLE 1

Distribution of resistant mutants in samples of 100 cultures. In addition to the numbers shown, 87 cultures had zero resistants

Tube no.	No. of $S^r$ colonies
3	9
15	1
30	4
32	2
41	<b>37</b>
44	2
47	1
57	3
67	1
78	19
83	2
84	4
100	5

while in small samples  $R$  follows the Poisson series, so that a calculable fraction of samples will have a ratio  $R_0/N_0$  which exceeds any stated value. The procedure is thus applicable, in principle, for any value of  $m$  but is most efficient for  $m < n$ .

The calculated enrichment  $E$  can also be adjusted to minimize the total number of tubes required to achieve complete enrichment,  $R = N$ , which will take  $i$  cycles where  $E^i = 1/r_0$ . The total number of tubes  $Ei$  will then be minimal for  $E \sim e$  and  $i \sim \log 1/r_0$ . As a compromise between economy of time and of material,  $E$  was chosen around 10 in the experiments reported here.

As  $m$ ,  $n$  can be established in advance, and  $k$  can be measured, it is possible to estimate with fair accuracy the enrichment expected and compare it with the enrichment observed; the agreement between the two values will supply a test that the resistant mutants are actually present in the right number in the culture untreated with the antibiotic, and thus that all of the resistant mutants recovered by direct assay with the drug are spontaneous mutants.

#### RESISTANCE TO STREPTOMYCIN

*Escherichia coli* W-1802 (a stock from the K-12 strain) was used for the experiment. A single-colony culture was used for the inoculation of 100 tubes containing 7 ml each of penassay broth, all tubes being seeded with about 100 cells. At the end of incubation half of each culture was plated on nutrient agar containing 100  $\mu\text{g}/\text{ml}$  of streptomycin, while the remaining half was stored in the refrigerator.

The plates from 87 tubes showed no growth on streptomycin, while the counts of streptomycin resistant organisms from the remaining 13 tubes were as given in table 1.

The "best" culture (i.e., with maximum  $r_0$ ) (No. 41) contained 37 resistants in the moiety which was assayed on streptomycin, and therefore about 10 resistant cells per ml. From the rest of culture 41, meanwhile kept at 4°C a sample of 0.1 ml, which was expected to contain one resistant organism, was mixed with 100 ml

of broth. From this dilution ten tubes were made, each containing 10 ml of broth, and incubated. To reduce the chance that the enrichment cycle would fail, another series of 10 tubes with 10 ml broth was prepared with an 0.2 ml sample from culture 41, thus expected to contain two resistant organisms.

After the 20 tubes were grown to saturation a sample was taken from each and plated on streptomycin agar. The estimates of resistant organisms per ml were as follows:

*First enrichment from culture 41*

Sample seeded	No. of resistant organisms per ml in each of the ten cultures									
0.1 ml	0	4	0	0	0	0	0	0	0	0
0.2 ml	0	0	33	9	11	35	0	0	6	0

It is difficult to judge whether the second culture of the first series and the fourth, fifth, ninth culture of the second series contain the same mutant as the original culture or new mutants. It is very likely instead that the third and sixth culture of the second series contain the same mutant as in the original, enriched 3.3 and 3.5 times respectively by the procedure. The expected enrichment for  $k = 1$  is  $5 \times$  in the second series, there being  $n = 10$  tubes and  $m = 2$  resistant organisms in the inoculum distributed into the ten tubes.

As a control, one of the original 100 cultures (No. 42) which showed no resistant organisms in the sample plated on streptomycin was treated in parallel to culture 41 and exactly in the same way. The results were:

*Examination of culture 42 in parallel with culture 41*

Sample seeded	No. of resistant organisms per ml in each of the ten cultures									
0.1 ml	0	0	0	0	0	0	0	0	0	2
0.2 ml	0	0	0	0	0	0	0	0	0	0

In the series from culture 42 no enrichment was expected, there being no resistant mutants to be enriched upon, and none is indeed found (except "background" mutants, e.g. in the tenth culture of the first series).

The total count in the conditions employed was  $1.7 \times 10^9$  bacteria per ml. At the beginning there were in the best culture 10 resistants per ml, or a ratio  $r_0 = 6 \times 10^{-9}$ . After the first cycle of enrichment the concentration of resistants had increased to a maximum of  $21 \times 10^{-9}$  and the best culture was chosen for a further enrichment cycle. It was thus possible to reach, by a succession of fourteen cycles conducted in a way identical or very similar to the first one just described, a concentration of resistant organisms such that plating of the culture on nutrient agar and direct scoring for resistance of the colonics thus obtained permitted a line made entirely of streptomycin-resistant cells to be indirectly selected.

The details of the successive enrichments are given in table 2. It was soon clear, from the comparison of observed and expected enrichments, that the resistant mutant was multiplying more slowly than the sensitive parent. In order to lessen this obstacle from adverse selection the following means of reducing  $t$ , the number of multiplications were tried: diluting the broth, (1/10 or 1/100) and decreasing the

TABLE 2

*Indirect selection for streptomycin-resistance. Summary of enrichment cycles*

	Total conc. of cells/ml (1)	Broth		Max. resistant assay cells/ml (3)	Relat. concentration of resistants after cycle		Observed enrichment (6)	n	m	Expected enrichment $n/m$ (9)	k (10)
		Amount (2)	Dil.		Max. (4)	Average (5)					
Original culture	$1.7 \times 10^9$	7 ml	1:1	10	$5.9 \times 10^{-9}$	—	—	—	—	—	—
Cycle 1	$1.7 \times 10^9$	10 ml	1:1	35	$2.1 \times 10^{-8}$	$2.0 \times 10^{-8}$	3.40	10	2	5.0	.94
2	$1.7 \times 10^9$	10 ml	1:1	58	$3.4 \times 10^{-8}$	$3.2 \times 10^{-8}$	1.52	10	2	5.0	.84
3	$1.7 \times 10^9$	10 ml	1:1	140	$8.2 \times 10^{-8}$	$5.9 \times 10^{-8}$	1.74	13	2	6.5	.84
4	$1.7 \times 10^9$	10 ml	1:1	456	$2.7 \times 10^{-7}$	$1.41 \times 10^{-7}$	1.72	20	1	20	.76
5	$1.7 \times 10^9$	10 ml	1:1	1400	$8.2 \times 10^{-7}$	$7 \times 10^{-7}$	2.59	20	2	10	.88
6	$3.1 \times 10^8$	10 ml	1:10	790	$2.5 \times 10^{-8}$	$2 \times 10^{-8}$	3.05	10	2	5	.95
7	$1.05 \times 10^8$	10 ml	1:50	1250	$1.2 \times 10^{-8}$	$1.2 \times 10^{-8}$	4.80	10	2	5	.99
8	$1.7 \times 10^9$	10 ml	1	$1.53 \times 10^5$	$0.9 \times 10^{-5}$	$0.53 \times 10^{-5}$	0.44	20	2	10	.78
9	$3.1 \times 10^8$	0.1 ml	1	$1.34 \times 10^5$	$4.3 \times 10^{-5}$	$43 \times 10^{-5}$	4.78	20	2	10	.93
10	$3.1 \times 10^8$	0.1 ml	1	$3.1 \times 10^5$	$1 \times 10^{-4}$	$0.98 \times 10^{-4}$	2.28	23	2	11.5	.86
11	$3.1 \times 10^8$	0.1 ml	1	$7.0 \times 10^5$	$2.3 \times 10^{-4}$	$1.19 \times 10^{-4}$	1.19	20	3	6.7	.86
12	$3.1 \times 10^8$	0.1 ml	1	$4.7 \times 10^6$	$1.5 \times 10^{-3}$	$1.13 \times 10^{-3}$	4.91	20	2	10	.95
13	$3.1 \times 10^8$	0.1 ml	1	$7.8 \times 10^6$	$2.5 \times 10^{-3}$	$1.8 \times 10^{-3}$	1.20	20	2	10	.87
14	$3.1 \times 10^8$	0.1 ml	1	$3.96 \times 10^7$	$1.28 \times 10^{-2}$	$5.5 \times 10^{-3}$	2.20	40	4	10	.90

$$k = .882 \pm .018.$$

size of the culture. The latter method was found more satisfactory. Reconstruction experiments showed in fact that while dilution of the nutrient broth with saline did not decrease proportionately to dilution the final concentration of bacteria at saturation, the disadvantage of the resistant increased at lower broth concentration. Another simple device that could reduce the number of doublings in the fresh medium, and thus improve the enrichment if  $k < 1$ , might be that of stopping growth before saturation. However, it was preferred to use saturation as the endpoint of growth because it was already known that a saturated culture, stored at 4°C, kept the ratio of the resistant cells to the total unaltered for over a week. Such a stabilization for at least 24 hours and preferably several days is essential for this kind of experiment, because the enrichment tubes must be stored until they have been assayed for resistants and the next enrichment cycle started.

The comparison of the enrichment observed (col. 4, table 2) with that expected,  $E = n/m$  for  $k = 1$  (col. 6, table 2) permits one to calculate, by the use of formula (1), the value of  $k$  (the relative growth rate of the resistants) that would explain the observed enrichment rates.  $k$  was found to be  $0.88 \pm .02$ .

Recovery of the resistant line that was selected by indirect selection permitted the value of  $k$  thus calculated to be compared with that obtained directly from reconstruction experiments. Mixtures were made in various initial proportions of  $S^*$  and  $S^r$  cells, and the ratio between the two types of cells were measured at the end of growth. An average of two experiments gave  $k = 0.856 \pm 0.013$ , in good agreement with the value obtained from the indirect selection experiment. In reconstruction experiments with mixtures of sensitive and streptomycin resistant mutants of *E. coli*, CEPPELLINI (1954) had also obtained a closely similar value of  $k$ .

It will be noticed that, for enrichment, whenever more than one "good" tube was available, the best tube (i.e. the one showing the highest assay of resistants, given as Max. in col. 2 in table 2) is to be chosen. Both average and maximum concen-

trations of resistants after each cycle are given in column 3 of table 2. Observed enrichment (col. 4) is calculated as the ratio of the average relative concentration of resistant mutants after one cycle, and the concentration of resistants in the best tube before the cycle, the best tube having of course been employed for continuing selection.

Enrichment cycles were missed three times, i.e. no resistant cell showed up in the tubes after dilution. Thus, altogether seventeen cycles were made; the third, eighth and eleventh cycles had to be repeated. As already stated the probability of "missing" a cycle is  $e^{-1} = 0.37$ , if one resistant cell is seeded;  $e^{-2} = 0.14$  if two resistant cells are seeded;  $e^{-3} = 0.05$  if both series with 1 and 2 resistant cells are employed, as in most cycles of the present experiment. Failures were 3/17, i.e. slightly more frequent than expected, but not significantly so.

At the end of the fourteenth cycle, the relative concentration of streptomycin resistant cells was estimated to be 1.28% in the best tube as shown by the assay on streptomycin agar. The culture concerned was plated on nutrient agar (without streptomycin) and 149 colonies were subcultured on nutrient agar and tested individually for streptomycin resistance by planting them on agar with and without the drug. Two colonies (1.34%) were resistant.

One resistant isolate was restreaked on plain agar, then inoculated into penassay broth from which twenty serial passages of .001 ml in 10 ml were made in the course of one month. These passages entailed a cumulative total of about 260 generations. The twentieth passage culture was then plated out on agar with and without streptomycin, giving the following assays: on plain agar,  $4.1 \pm .1 \times 10^9$ ; on agar with 200  $\mu\text{g}/\text{ml}$  streptomycin,  $4.3 \pm .1 \times 10^9$ ; on agar with 1000  $\mu\text{g}/\text{ml}$  streptomycin,  $4.2 \pm .2 \times 10^9$  per ml. In addition several hundred colonies on the plain agar plates were tested by replica plating to streptomycin agar without any sensitive reversions being detected. This indirectly selected resistant clone, which has never been exposed to streptomycin, may be concluded to be stably and completely resistant to streptomycin.

One more fact which was noticed in the present experiment is worth comment. When more than one tube of a series was "positive", a high variability of the number of resistant cells was almost always present among tubes. It must be remembered that the number of resistant cells in one of these tubes represents the total progeny of (usually) one resistant cell in a fixed time, i.e. the time necessary for the majority of the cells, the sensitive ones, to reach saturation.

This variability may reflect the variability in lag of individual cells and perhaps the variability in the time of individual divisions (KENDALL 1953). This variability is of such a size, that it may occasionally reverse enrichment into impoverishment, as has happened at the 8th cycle.

#### HERITABILITY OF VARIANCE OF STREPTOMYCIN RESISTANTS

Table 1 shows the variability in yields of resistant mutants from culture to culture, a variation that has been ascribed to preadaptive mutations by LURIA and DELBRÜCK (1943), but to physiological response to uncontrolled environmental variation by DEAN and HINSHELWOOD (1952). To test the heritability of this variation, 10

samples of one ml each were reinoculated into 7 ml fresh penassay broth, both from culture 41 (which had a calculated level of about 10 resistant per ml) and 42 (which had none). Reassays of the whole cultures gave the following results:

From culture 41: 135, 142, 181, 123, 177, 111, 138, 149, 140, 144

From culture 42: 0, 0, 8, 2, 0, 0, 0, 1, 0, 1

These results should be considered together with those of more dilute samples from the same cultures, page 370.

If the difference between the original culture 41 and 42 is to be ascribed to the physiological state of the cells, it would then be necessary to assume that this difference is heritable with the further growth of the cells in the absence of the drug.

#### INDIRECT SELECTION FOR CHLORAMPHENICOL RESISTANCE

Chloramphenicol resistance offers a particular challenge for various reasons. Resistant mutants are found which show only a moderate degree of resistance; only by the cumulation of independent mutations it is possible to reach high degrees of resistance (CAVALLI and MACCAGARO 1952). Single resistance steps do not permit complete (i.e. 100%) survival at the concentrations of drug which are necessary for a complete inhibition of the sensitive parents. The increase of resistance in "training" experiments is therefore almost perfectly gradual, a trend particularly acceptable to advocates of the post-adaptation theory.

Moreover, the survivorship varies with the medium, time of incubation, amount of background of sensitives, and other conditions. In view of incomplete survival, it has to be assumed that assays on drug agar gave a variable underestimate of the number of resistant cells. As, for a rapid application of the enrichment method here employed, it is essential that limiting dilutions are used, five series of broth tubes (each of  $n = 10$  tubes) were employed, containing respectively 2, 1,  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$  resistant cells (as estimated from the assay). Thus, even if the assay estimate were only 12.5% of the true number of resistant, the last series would contain just one resistant. Actually, later tests, which became possible when the indirectly selected resistant line was secured, showed that the estimate from the assay may even be lower than 12.5%.

An *E. coli* stock (from the K-12 line) No. 373 was employed. A single colony culture was used for the inoculation of 0.5 ml broth cultures, 50 in all, 100 cells being seeded in each tube. After saturation, 0.1 ml from each culture was plated on a penassay agar plate containing 18  $\mu\text{g}/\text{ml}$  chloramphenicol. The rest of each culture was stored in the refrigerator. Plates were incubated 48 hours, this time being necessary for the development of a good fraction of the colonies. At the time and concentration of cells and drug employed, resistant colonies were mostly of a pin-point size but there was no background growth, which would otherwise make counts difficult. Counts of resistant colonies were:

*Chloramphenicol resistance, distribution of resistant*

No. of resistant colonies	0	1	2	3-4	5-8	9-16	17-32	Tot.
No. of plates	17	14	4	5	6	3	1	50

TABLE 3

Number of chloramphenicol resistant organisms in subcultures from culture No. 40, containing originally 230 resistants/ml, from which samples were taken expected to contain 2, 1,  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$  resistant. **Boldface indicates cultures which are believed to have been seeded with at least one resistant mutant**

Expected no. of positive cultures in each series.....	Enrichment series				
	2	1	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$
Number of resistants in chloramphenicol agar plates prepared from each tube	16	15	1	0	0
	7	23	62	18	2
	7	15	<b>216</b>	0	5
	33	2	2	0	1
	9	2	1	0	25
	4	0	1	4	2
	16	49	9	7	0
	1	25	10	29	2
	27	1	1	1	0
	2	<b>131</b>	0	8	24

TABLE 4

Number chloramphenicol resistant organisms in subcultures from culture No. 39, containing originally no resistant mutant, treated exactly as culture No. 40, table 3

Expected no. of positive cultures in each series.....	Enrichment series				
	2	1	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$
Number of resistants in chloramphenicol agar plates prepared from each tube	2	39	7	14	2
	5	5	7	16	7
	2	32	1	10	6
	2	1	4	4	7
	13	18	2	19	0
	10	22	1	0	1
	1	17	5	1	0
	16	16	17	14	2
	4	47	3	18	7
	1	39	48	32	3

Culture No. 40 contained the highest number of resistants (23 in 0.1 ml, or  $r$  a little less than  $10^{-7}$ ).

From culture 40 a sample was taken and diluted so that samples of 5 ml of broth would contain each 2, 1,  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$  resistant cells. These numbers will thus represent also the approximate expectations for the numbers of positive cultures in each series. Culture No. 39 which gave no resistant colonies on the drug plate was treated in exactly the same way, as a control. The 5 ml samples were then distributed into ten small test tubes, each receiving thus 0.5 ml of broth. After incubation, 0.1 ml from each culture was spread on 18  $\mu$ g/ml chloramphenicol agar, while the remaining 0.4 ml of each culture were stored in the refrigerator. Counts of resistant colonies after 48 hours on chloramphenicol agar were as given in tables 3 and 4.

In the first cycle of indirect selection it is often difficult to distinguish whether the resistants appearing on the plates are clonal descendants of those in the original

culture, or new mutants. Assuming that the resistant cells in the original culture (No. 40) were 23 in 0.1 ml of broth, in the same amount of broth after enrichment the following numbers of resistants would be expected in the five series inoculated with culture 40.

Resistants inoculated and expected number of positive cultures	2	1	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$
Enrichment ratio	5	10	20	40	80
Expected number of resistants after enrichment in 0.1 ml of positive cultures from culture 40, table 3.	115	230	460	520	1040

If, however, 23 is an underestimate of the resistant cells due to the difficulties of assay mentioned above, the expected numbers of resistants will be overestimated.

Taking 100 to be the maximum number of "new" resistants that may be counted in 0.1 ml, one finds that while no clear enrichments are observed in the first series (with 2 organisms), in the second series (with 1) one culture, and in the third (with  $\frac{1}{2}$ ) another culture (indicated by boldface, table 3) showing enrichment are available. The limit of 100 resistants is of course arbitrary, but has at least the advantage that it is not overtaken in the control series (culture 39, table 4) where no enrichment is expected.

Unless the mutant has a smaller growth rate than the sensitive, it may be argued from the figures observed that 23 was an underestimate, and therefore that the figures of enrichment expected in the calculation above are overestimated. If this is so, the numbers of resistants expected in the first series are not sufficiently high to permit the old clones to be distinguished from the background of new mutants, as indeed seems to have occurred.

Choosing the best of the enrichment cultures, i.e. the third culture of the third series for the second cycle, and diluting it again in broth so as to have 5 ml samples containing each 2, 1,  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$  resistant cells, and distributing these in 10 small tubes as before, subculture of them and plating on chloramphenicol agar gave the counts reported in table 5.

The following facts are worth noting: underestimation of resistants in the plate

TABLE 5  
*Second enrichment cycle, from the culture indicated in table 3 as 216*

Expected number of positive cultures	Enrichment series				
	2	1	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$
Resistants in plates prepared from each of the ten cultures of the series	86	24	15	13	2
	38	26	3	2	0
	<b>178</b>	25	1	6	9
	8	<b>107</b>	0	1	5
	18	<b>814</b>	10	9	0
	<b>215</b>	7	<b>450</b>	0	2
	92	<b>208</b>	23	0	4
	<b>248</b>	<b>161</b>	4	1	0
	67	0	5	0	<b>246</b>
	42	0	3	10	<b>135</b>

TABLE 6  
*Third enrichment cycle, from the culture indicated in table 5 as 814*

Expected number of positive cultures .....	Enrichment series				
	2	1	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$
No. resistant in plates prepared from each of the ten cultures of the series	<b>292</b>	<b>210</b>	2	0	0
	3	0	0	1	3
	63	0	7	6	0
	0	<b>360</b>	0	0	4
	2	18	<b>325</b>	<b>940</b>	2
	<b>144</b>	<b>164</b>	<b>790</b>	0	0
	<b>340</b>	0	0	9	0
	0	0	0	0	0
	0	0	18	<b>544</b>	0
	<b>191</b>	0	<b>273</b>	0	0

assay is more prominent than before; the variability of counts in parallel positive cultures is remarkably high; enrichments observed are therefore somewhat erratic, but clearly higher than in the previous cycle.

The third cycle, started with the best culture of the second cycle, is also given in detail in table 6 in order to strengthen these points and show that, with increasing enrichment, the positive cultures are more clearly differentiated from those containing background mutants.

Altogether, nine cycles were necessary to reach, from the original  $10^{-7}$  concentration, a concentration of 2% resistant. At this stage, the best culture of the last enrichment cycle was plated on ordinary agar, and 317 colonies subcultured from it; they were then individually tested for resistance at the standard chloramphenicol concentration. Four colonies were found to be resistant, with a clearcut differentiation from the sensitives (see fig. 1).

Indirect selection having thus been fully achieved, it remains to be seen whether the rate of enrichment corresponds to that expected if all the resistant counted are spontaneous mutants. The incomplete survival of the resistant at the standard drug concentration interferes with this calculation. The actual survival of the indirectly selected mutant, however, could be estimated directly as soon as the mutant was isolated in pure culture: it proved to be variable from day to day, but constantly dependent on the frequency of sensitives which, if present at a high concentration, proved to provide some protection to the resistant. A typical experiment with mixtures of sensitives and resistant is given as follows:

*Counts on mixtures of resistant and sensitives*  
 2 plates at each level

No. of plated	Resistant .....	200	200	200	200	200
	Sensitive .....	$10^8$	$10^7$	$10^6$	$10^5$	
Counts on non drug-agar .....	—	—	—	—	—	228; 170
Chloramphenicol 18 $\mu$ g/ml agar ...	34; 70	6; 4	3; 0	0; 9		0; 1

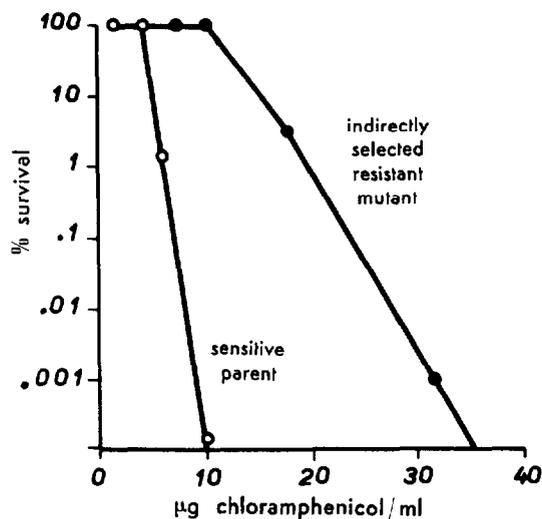


FIGURE 1.—Survival curves on chloramphenicol agar plates of sensitive parent culture and an indirectly selected resistant mutant.

TABLE 7

*Indirect selection for chloramphenicol resistance in E. coli*

1 Cycle	2 No. of positive cultures					3 Max. no. of resistants	4 Ratio dilution/ plate assay	χ <sup>2</sup>	df	P
	2	1	1/2	1/4	1/8					
I	2	1	<i>1</i>	0	0	216	1.1 ± 0.8	.8	3	non signif.
II	3	<i>4</i>	1	0	2	814	3.0 ± 1.0	10.6	4	5-2%
III	4	3	3	2	0	940	3.8 ± 1.1	3.8	4	non signif.
IV	10	9	<i>8</i>	4	0	3350	21 ± 4.6	4.0	4	non signif.
V	9	6	3	3	<i>1</i>	10,700	9.9 ± 2.2	0.9	4	non signif.
VI	3	<i>1</i>	1	1	0	52,750	1.7 ± 0.7	1.4	4	non signif.
VII	9	9	<i>6</i>	1	3	1.68 × 10 <sup>5</sup>	15.4 ± 3.4	4.9	4	non signif.
VIII	7	1	1	0	<i>1</i>	2.19 × 10 <sup>6</sup>	3.3 ± 1.0	5.5	4	non signif.
IX	6	4	1	0	0	4.2 × 10 <sup>6</sup>	3.8 ± 1.1	2.4	4	non signif.
								34.3	35	50%

Column 1: Enrichment cycle. Column 2: No. of "fertile" cultures in each of the five series of 10 tubes, each series being seeded with the number of resistant bacteria indicated at the top of each subcolumn (estimated by previous plate assay). Column 3: No. of resistant bacteria in 0.1 ml of the best "fertile" culture, i.e. the culture which was found to contain the highest number of resistant mutants by plate assay on chloramphenicol agar and was used for the next enrichment cycle. Such a culture belongs to the series indicated by italics in column 2. Column 4: Ratio between dilution assay of resistant mutants and plate assay. Column 5: Analysis of the validity of the dilution assay.

A summary of the results obtained in the nine enrichment cycles is given in table 7. The value given in the 4th column, i.e. the ratio between the resistants found by the test in liquid medium and the resistants, given by the assay on chloramphenicol agar plates may deserve some comment. It is obtained by considering that the proportions of fertile tubes at the various relative concentrations of 2, 1,  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$  constitute, in fact a "dilution assay" of the numbers of resistants in the culture to be enriched.

Estimation was carried out by maximum likelihood, and the fifth column (table 7) contains an analysis of the validity of the assay by the calculation of an (approximate) value of  $\chi^2$  for each cycle. Total  $\chi^2$  is 34.3 with 35 d.f., showing a good fit.

The ratios given in the 4th column of table 7 agree with the previously noted fact that plate counts tend to underestimate the numbers of resistants. The values estimated by dilution assay are more reliable, but unfortunately are subject to high standard errors, as is well known. This makes computation of enrichment at every step hardly worth while. However, some information can be obtained from the total enrichment observed. In nine enrichment cycles, the concentration of resistant organisms went up from  $10^{-7}$  to 0.023, i.e. there was a total enrichment, whose logarithm is 5.37 (this figure may, if any, be higher than the real one, being based on the best samples at every step).

The expected enrichment at every step can be calculated, as before, assuming equal growth rates for normals and mutants, from  $n/m$ , where  $n$  is the number of tubes into which the sample is distributed and  $m$  the number of bacteria seeded, which is given at the head of the appropriate subcolumn of column 2.

Such a value is, however, the estimate by the previous plate assay, and this, we have seen, is deficient by a factor given in column 4. The number  $m$  can therefore be obtained by multiplying the estimate from plate assay (top of the corresponding subcolumn, column 2) by the corresponding value in the fourth column. The total expected enrichment is obtained by summing the logarithms of the expected enrichments at each cycle and equals  $6.1 \pm 1.1$ .

The magnitude of this standard error does not permit a detailed comparison of the total observed and expected enrichment. The latter should be greater than the former if  $k < 1$ ; it may therefore be predicted that probably the growth rate of the mutant is slightly smaller than that of the sensitive. In view of counting difficulties of the resistant types in mixtures, no direct examination of the relative growth rates of mutant and parent in mixtures was carried out.

A comparison of the relative resistance to chloramphenicol of the normal parent and of the indirectly selected mutant by plate assays is given in figure 1. After 25 daily transfers in broth, with a multiplication factor of  $10^2$  at every transfer, for a total of 332 generations, there is no variation in resistance.

#### DISCUSSION

One of the first cogent lines of evidence on the adaptation controversy was LURIA and DELBRÜCK's (1943) finding of a high variance from culture to culture in the assay of resistant mutants. The conclusion that this variance was genetic, and arose from the stochastic event of mutation, was countered by the supposition that the

environment varies uncontrollably from one culture tube to the next. The bacteria would then differ in physiological state, and hence in their responses when later confronted with the drug. Experiments in this paper show how this question can be elaborated, by a test of the "heritability" of the variance, when samples from "high" and "low" cultures are regrown in fresh medium. These experiments were not specifically designed for the purpose, but as far as they go, they agree precisely with the expectation that "high" cultures already contain many stably resistant cells and "low" cultures do not. To give the obtained result that inocula from "high" cultures generate further "high" cultures, the resistance would have to be heritable in a new set of culture tubes, in conflict with the supposition of purely physiological variability. Whether physiological accidents in the culture tubes of plain broth ultimately cause individual mutational events is not in question here; the issue is whether such variations arise and persist independently of the drug.

The replica-plating and indirect selection procedure has provided still more direct evidence of preadaptation, as drug-resistant mutants can be isolated that have not experienced the drug (LEDERBERG and LEDERBERG 1952; BRYSON and SZYBALSKI 1955). However, certain precautions are required for the correct interpretation of negative results. For example, DEAN and HINSHELWOOD (1953) report having prepared plates of synthetic medium carrying  $2 \times 10^7$  single colonies (sic) of *Bact. lactis aerogenes*. The plates were then replicated in two passages to chloramphenicol agar, and "the congruent site on the corresponding non-drug plate (about five colonies) was picked off". Needless to say, the further experience with this isolate gave no evidence of the presence of a mutant clone: the limit of resolution of replica plating is perhaps 0.1% of the area of a plate, not  $5/2 \times 10^7$  after two passages. These authors, and LAM and SEVAG (1955) have also overlooked the occurrence of mutations during the growth of colonies which had been shown to contain resistant cells by replica plating. As with other populations of bacteria, the colony may contain resistant cells in only very small proportions, as GOLDSTEIN, MAGASANIK and UMBARGER (1955) have explicitly shown with the same material. The method of indirect selection in liquid medium described in the present paper is somewhat less liable to these troubles, as it is possible to distinguish small and large clones, which are not readily distinguished in replica platings.

Unlike replica plating, limit sampling is subject to precise quantitative formulation, though all the subtleties of population dynamics must be taken into account. The success of the procedure testifies that all of the resistant cells that are counted in the assays are already in the population being tested. The method will, of course, break down in situations, as can be devised, where the selective action of the drug is limited or delayed so that further growth and new mutations may ensue on the test plates. When either method of indirect selection should appear to fail, it should at least be shown that it is applicable to the recovery of already adapted clones artificially added to the tested populations, if the negative result of indirect selection has to testify against preadaptation.

The successful application of indirect selection to chloramphenicol resistance lends no support to the possible reservation that while single step, high level resistance is preadaptive, multistep resistance of a lower order may be directly induced by a

drug (as discussed by EAGLE 1955). However, we must exclude from our discussion phenomena of survival or persistence which entail no genetically stable variations in the survivors. For example, DEAN (1955) reports that "cultures prepared from colonies on proflavine plates are no more resistant than a normal organism". It is plausible that physiological adaptations are concerned here, and they may also be involved in the biochemical defenses which genetically resistant organisms can raise against antibiotic attack. These are, unfortunately, very obscure and we know very little about whether these defenses are, in a physiological sense, adaptive or constitutive. For the purpose of this discussion, our operational definition of resistance is the ability of a cell to survive exposure to the drug which, like the other inherent potentialities of the organism, is defined by the genotype.

## SUMMARY

Indirect selection of resistant mutants, i.e. in the absence of the toxic agent or drug can be carried out not only by replica plating but also by a procedure making use of subcultures in liquid media, and choosing the best subcultures by testing samples of them on drug media. A repetition of this process can lead to pure resistant lines which have never been in contact with the drug or toxic agent, thus proving the spontaneous origin of resistants by mutation. The procedure can be speeded up considerably by the subdivision of limiting dilutions containing one or few resistant organisms. Although more time-consuming than replica plating, this procedure is more easily amenable to quantitative analysis, to conclude whether resistant mutants obtained by direct selection (in presence of the drug) are all, or in part, the result of spontaneous mutation. Our conclusions from experiments carried out on first step streptomycin and chloramphenicol resistance in *Escherichia coli* are in agreement with the hypothesis that all resistant mutants on drug plates have a spontaneous origin. The observed rate of enrichment of resistant mutants in indirect selection has been comparable with that which was expected on the basis of the experimental conditions, and of growth rate differentials between mutant and parent.

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