ADAPTATION OF BACILLUS MEGATHERIUM TO TERRAMYCIN (OXYTETRACYCLINE)

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(Received for publication, September 10, 1956)

SUMMARY

B. megatherium cultures contain a few cells per hundred million which are able to grow in the presence of terramycin. Growth of the other cells is inhibited by the antibiotic with the result that the resistant cells overgrow the culture. The resistant cells are present in the culture, without contact with terramycin, and are able to grow in the presence of terramycin, without further modification.

The resistant cells are mutants of sensitive cells.

Adaptation of B. megatherium to Terramycin

The addition of low concentrations (0.01 to 0.1 γ/ml.) of terramycin to *B. megatherium* cultures in liquid media results in a slight decrease in the growth rate of the culture, but does not cause a lag period. On solid media the colonies grow more slowly, but the number of colonies remains the same. No adaptation results in either liquid or solid media.

Higher concentrations (0.1 to 5 γ/ml.) cause a decrease in the growth rate in liquid cultures and a great decrease in the number of colonies on solid media. The overgrowth in liquid media and the surviving colonies on solid media are resistant to much higher concentrations of terramycin than is the original culture.

The resistant colonies come from the few resistant cells present in the original culture. This resistant strain may be isolated by Lederberg's replica technique and therefore does not arise as a result of adaptation of sensitive cells to the antibiotic. The resistant strain isolated by the replica technique is indistinguishable from a strain derived from colonies surviving exposure to terramycin. The resistant cells in the culture,[1] therefore, are capable of growing in the presence of the antibiotic without further modification.

[1] Resistant cells are referred to for the sake of simplicity as though there were only one kind of resistant cell, or mutant. Actually, there is undoubtedly a whole series of

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The resistant cells arise from sensitive cells, since a few thousand cells of the sensitive culture when plated on agar containing terramycin, form no colonies, while cells from the same suspension when plated on YEP agar form colonies and every colony contains resistant cells. These resistant cells must have come from sensitive cells.

The ratio of resistant to sensitive cells rises sharply at first and then remains constant. The constant ratio varies from $1/10^8$ to $1/10^5$ depending on the strain of *B. megatherium*, and the culture medium.

Prolonged growth of the culture in the steady state apparatus in the presence of increasing concentrations of terramycin results in a strain capable of growth in the presence of 200 γ terramycin/ml. (about 1000 times the original inhibiting concentration). No further adaptation could be obtained.

All adapted strains revert sooner or later to the sensitive strain, depending on the culture medium. This reversal probably is due to overgrowth by a sensitive mutant, since it can be caused to take place by the addition of sensitive cells to the resistant strain.

The results in general are similar to those described by Fildes and Whitaker (1948), Finlay *et al.* (1950), Bryson and Demerec (1950), Hobby and Lenert (1950), Cavalli (1952), Welsch (1952), and others (*cf.* Third Symposium of Society of General Microbiologists).

Experimental Results

The effect of various terramycin concentrations on the lag period, growth rate, maximum cell concentration, and colony count is shown in Table I. As the terramycin concentration increases from 0 to 0.5 γ terramycin/ml., the growth rate decreases, but no lag occurs. The culture does not become resistant.[2] Increasing the terramycin concentration from 0.5 to 5 γ/ml. results in an increase in lag, a decrease in growth rate, and a sudden decrease in colony count to about $20/10^8$ cells. The overgrowth in this range of terramycin concentration is resistant. Higher concentrations prevent growth and do not result in resistant cultures. Concentrations over 50 γ/ml. kill the cells. The results in general confirm those of Hobby and Lenert (1950).

These results indicate that the emergence of a resistant from a sensitive culture is the result of the overgrowth of the sensitive cells by a few resistant cells. The effect of terramycin on the growth rate of sensitive and resistant cells confirms this (Fig. 1). It is evident that the resistant cells grow at about the same rate in the presence or absence of terramycin, whereas the sensitive cells grow much more slowly in the presence of the antibiotic. The result is that, in the

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mutants which differ from each other with respect to their growth rates in the presence of the antibiotic. For the present purposes, the term TR mutants refers to the group which are able to grow in the presence of >2, < 5 γ terramycin/ml.

[2] Prolonged growth in 0.2 γ terramycin/ml. does result in resistant strains.

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presence of the antibiotic, the resistant cells overgrow the culture, while in the absence of terramycin, the proportion of resistant/sensitive cells remains the same.

These results prove that some cells in the culture are able to grow in the presence of the antibiotic, but do not indicate whether the resistant cells arise as a result of the adaptation of a few sensitive cells to the antibiotic or are present in the culture before the antibiotic is added.

TABLE I

Effect of Terramycin Concentration on Growth Rate and Development of Resistance in B. megatherium

899*a* in log growth diluted to 5×10^6 B/ml. in YEP containing terramycin noted. 10 ml in test tubes. Shaken 35°. B/ml. determined by turbidity. After 30 hours, culture plated on YEP agar containing terramycin noted.

Terramycin/ml., γ.....	0	0.2	0.5	1.0	2.0	5.0	10.0	20	50
Lag, *hrs*.....	0	0	0	10	20	24	>24	>24	Indefinite
K_g-hr.$^{-1}$ (at first).....	1.8	1.6	0.9	0	0	0	0	0	0
B/ml. after 30 hrs.....	1×10^9	1×10^9	1×10^9	1×10^9	6×10^8	5×10^7	5×10^6	5×10^6	5×10^6

Overgrowth from Above Tubes Plated on Agar + Terramycin Noted Below

Terramycin/ml.	Colonies/ml.								
γ									
0.2	$>10^7$	$>10^7$	$>10^7$	$>10^7$	$>10^7$	$>10^7$	$>10^6$	$>10^6$	0
0.5	50	46	40	$>10^7$	$>10^7$	$>10^7$	40	60	
1.0	30	25	32	$>10^7$	$>10^7$	$>10^7$	34	45	
2.0	14	20	24	$>10^7$	$>10^7$	$>10^7$	30	22	
5.0	18	20	16	$>10^7$	$>10^7$	$>10^7$	22	30	
10.0	0	0	0				0	0	

The resistant strains may be isolated by the Lederberg (Lederberg and Lederberg, 1952) replica technique (Table II), however, and therefore are present in the culture and do not arise as the result of the adaptation of the sensitive cells to the antibiotic.

It is still possible to assume that the resistant cells change after contact with the terramycin before they are capable of growth in the presence of the antibiotic.

The lag period, growth rate, and colony count of a sensitive strain, a resistant strain isolated from the overgrowth in 3 γ terramycin/ml., and a resistant strain isolated by the replica technique were, therefore, determined in the pres-

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ence of various concentrations of terramycin (Table III, Fig. 2). The replica resistant strain had never been in contact with terramycin until the start of the experiment and, therefore, would be expected to show a lag period or a slightly lower growth rate, if any adaptive changes occurred before the cells

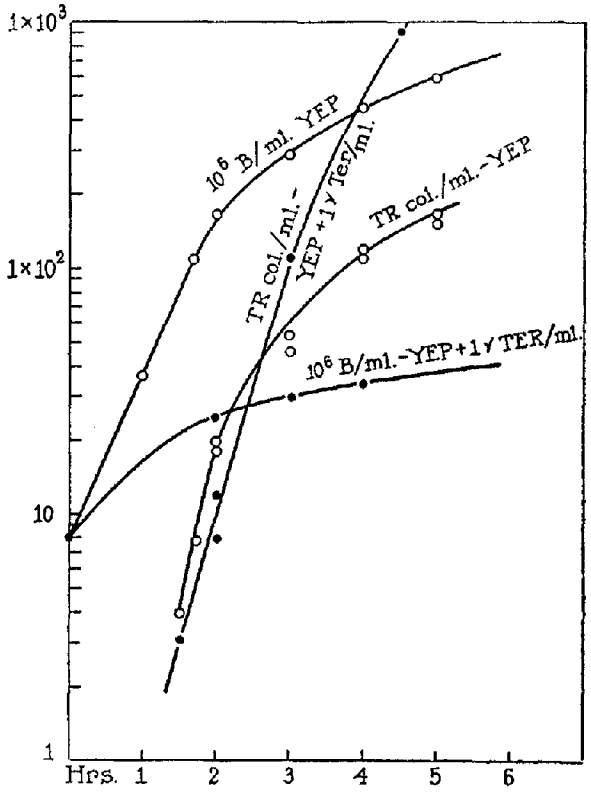

FIG. 1. Growth rate of total cells/milliliter and of TR cells/milliliter in YEP and YEP + 1 γ terramycin/ml. Cultures shaken at 35°.

were able to grow in the presence of the antibiotic. This is not the case. The replica strain, when first exposed to the antibiotic, is indistinguishable from the overgrowth strain which had grown many generations in the presence of the antibiotic. There is no indication, therefore, of any adaptive change in the cell as a result of growth in the presence of terramycin. This conclusion is confirmed by the fact that the sensitive strain may be grown in the presence of lower concentrations of terramycin (0 to 0.1 γ/ml.) indefinitely without any adaptation. In this range the growth rate of the cells is not affected. As soon as

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TABLE II

Isolation of Terramycin Resistant B. megatherium from Stock 899a Culture by Replica Technique

	Plate No.	Terramycin /ml. agar	Colonies/plate
		γ	
Stock 899*a* culture plated	1-0	0	$>10^7$
	1-3	3	45
Plate No. 1-0 printed	2-0	0	$>10^6$
	2-3	3	73
Spot picked on plate 2-0 corresponding to 1 colony on plate 2-3, stirred up in 3 ml. YEP, and plated	3-0	0	$>10^4$
	3-3	3	70
Printing, etc. repeated 7 times			
1 colony picked from 10-0. Grown up in YEP. Diluted 1×10^{-5} and plated	11-0	0	1.1×10^2
	11-3	3	1.1×10^2

It may be noted that spots corresponding to the location of colonies on the terramycin-agar were selected from the corresponding replica on YEP agar instead of from the master plate from which the replicas were made. All attempts to isolate the mutant from the master plate failed. This may be due to the fact that the master plate is necessarily 1 day older than the replica before the spot can be selected or because the sensitive colonies grow slightly larger than the resistant ones, and so overcrowd them on an old plate.

TABLE III

Comparison of a Sensitive Strain, a Resistant Strain Isolated by Replica Method, and a Resistant Strain from a Colony on 3 γ Terramycin/Ml. Agar

Terramycin/ml.	Lag, *hrs.*			Growth rate/*hr.*			Colonies/10^8 cells		
	Sensitive	Resistant		Sensitive	Resistant		Sensitive	Resistant	
		Replica	Overgrowth		Replica	Overgrowth		Replica	Overgrowth
γ									
0	0	0	0	1.9	1.7	1.7	$>10^7$	$>10^7$	$>10^7$
0.2	0	0	0	1.4	1.7	1.7	$>10^7$	$>10^7$	$>10^7$
0.5	0	0	0	1.0	1.7	1.7	50	$>10^7$	$>10^7$
1.0	10	0	0	0	1.6	1.6	30	$>10^7$	$>10^7$
2	10–20	0	0	0	1.5	1.6	14		
5	>24	0	0	0	1.1	1.1	18		
10				0	0.2	0.2	0	$>10^7$	$>10^7$
20				0				$>10^7$	$>10^7$
50				0				2	5

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sufficient terramycin is added to decrease the growth rate, the fraction of mutant cells increases. The mechanism of this result is discussed in a following paper.

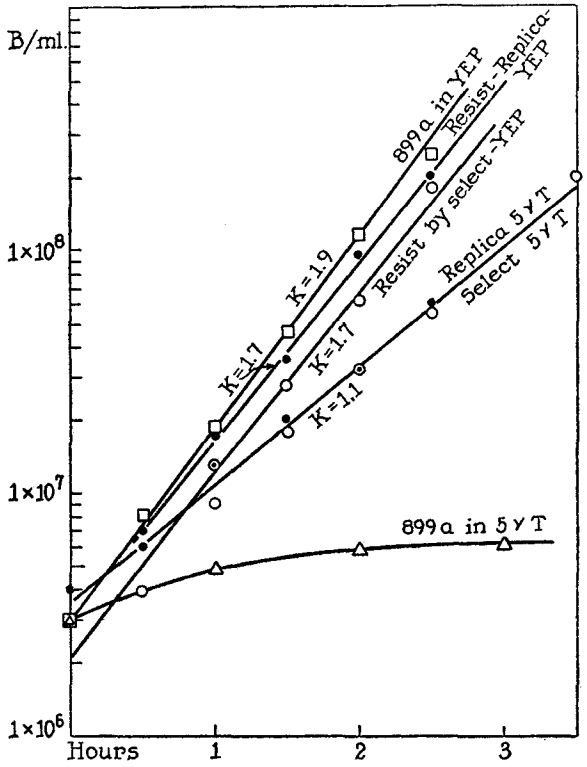

FIG. 2. Rate of growth of sensitive strain, resistant strain isolated by replica technique, and resistant strain isolated from overgrowth, in the presence of 0 and 5 γ terramycin/ml.

Origin of the Resistant Cells.—

The preceding experiments show that the resistant strain is the result of the overgrowth of the sensitive cells by resistant cells, but do not determine whether the resistant cells arise as mutants of the sensitive cells or are simply present as a mixed culture. The results of the experiments shown in Table IV, however, prove that the resistant cells arise from the sensitive cells and are not part of a mixed culture.[3] 1 ml. of the original suspension gave rise to more that 1×10^4 colonies on YEP agar, but no colonies on YEP agar containing 3 γ terramycin/ml. Ten colonies which grew from cells in the original suspension,

[3] This experiment is, of course, a modified form of Newcombe's (1949).

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picked at random from a YEP plate and grown up in YEP, all contained resistant cells. These resistant cells must have arisen from sensitive cells. It is true that the total cell concentration is much higher in the final plating than in the

TABLE IV

Tests for Origin of Resistant Cells

899*a* in log growth in YEP diluted to about 1×10^8 cells/ml. 2 ml. in test tubes and shaken at 35° for 2 hours.

	Plate No.	YEP colonies/ml.	YEP + 3γ terramycin/ml. colonies/ml.
1 ml. plated on YEP ± 3 γ terramycin/ml.	1	$>10^4$	0
Diluted to about 100 B/ml. 1 ml. plated	2	62	0
10 colonies picked from YEP plate 2			
	Colony No.		
Suspended in 5 ml. YEP, grown up to about 5×10^7 B/ml., and 1 ml. plated on agar ± 3 γ terramycin/ml.	1	$>10^6$	3
	2	$>10^6$	15
	3	$>10^6$	8
	4	$>10^6$	4
	5	$>10^6$	100
	6	$>10^6$	5
	7	$>10^6$	12
	8	$>10^6$	25
	9	$>10^6$	2
	10	$>10^6$	40

TABLE V

Effect of Sensitive Cells on Colony Count of Resistant Cells

Sensitive cells—899*a* strain in log growth.
Resistant cells—Selected by replica—log growth in YEP.
Mixed as noted and plated on YEP and (YEP + 3 γ terramycin/ml.).

Sensitive cells/ml.	0	10^7	10^7	10^8	10^8
Resistant cells/ml.	100	—	100	—	100
Colonies/ml. on YEP	38	$>10^6$	$>10^6$	$>10^7$	$>10^7$
Colonies/ml. on 3 γ terramycin	42	0	46	20	70

original, but the total cell concentration does not affect the colony count of the resistant cells (Table V).

Determination of Resistant Cells by Colony Count and by Poisson Distribution.—If the resistant colonies are descendants of resistant cells present in the suspension from which the sample is taken, then the number of resistant cells in the sample as determined by colony count should be the same as that determined by means of Poisson's ratio for replicate cultures.

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The results of a series of experiments designed to test this prediction are shown in Table VI. The number of resistant cells/sample by plate count is the same as that determined by Poisson's ratio. The experiments also show that the result depends only on the number of cells in the sample, and not on the concentration.

This result also makes it very unlikely that resistant cells can arise from sensitive cells without growth of the latter. If this process could occur, the number of colonies on the terramycin plate should increase with time, as the sensitive cells changed to resistant. This does not occur. The cells are not dead, because after 3 or 4 days many colonies do appear (owing to inactivation of the antibiotic) (*cf.* Bryson and Demerec, 1950), but these colonies are not resistant. The results of the Poisson ratio experiment confirm this. If the resistant cells

TABLE VI

Comparison of TR Colonies/Milliliter Suspension by Colony Count and by Poisson's Ratio

899*a* in log growth in YEP, about 10^8 B/ml. 1 ml. plated on 4 × 3 γ terramycin plates. Diluted to B/ml. noted in YEP + 2 γ terramycin/ml. and distributed in 10 to 20 tubes, shaken at 25° for 2 days, and number of tubes showing growth noted. Number of TR cells present in original sample calculated from Poisson's ratio.

Experiment	Sample	B/ml.	Total B	TR cells present/sample	
				From plate count of original suspension on 3γ terramycin-agar	From Poisson's ratio of total/negative tubes
	ml.				
3/20	3	1×10^6	3×10^6	2.0 ± 0.4	2.0
3/29	0.2	1×10^7	2×10^6	1.0 ± 0.2	1.2
3/26	5	1×10^5	5×10^5	0.2 ± 0.04	0.23
4/10	5	1×10^4	5×10^4	0.01	<0.2

are derived from sensitive cells without growth, then the number of tubes showing growth should increase with time. This does not happen either. Those tubes which do not show growth in 2 days (at 25°), never give resistant cell growth. After 3 or 4 days, growth does occur (owing to inactivation of the antibiotic) but the cells are sensitive. Englesberg and Stanier (1949) and Stocker (1949) also were unable to find any evidence of mutation in the absence of cell division.

The Ratio of Resistant/Sensitive Cells under Different Conditions.—Since the resistant cells are descended from sensitive cells, the ratio of resistant/sensitive must be 0 originally and increases with the growth of the clone. It soon reaches a constant value, however (owing to the difference in growth rates) (*cf.* Deskowitz and Shapiro, 1935; Novick and Szilard, 1950; and Northrop and Kunitz, 1956), and this equilibrium value remains remarkably constant either in repeated transfers or in the steady state apparatus as long as the total B concentration is fairly high (1×10^6/ml. or more).

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Fluctuations in Ratio of Resistant/Total Cells in Separate Cultures.—If a series of tubes is inoculated with 1 or 2 cells/tube and allowed to grow, the number of cells/tube and also the number of either sensitive or resistant colonies/tube fluctuate more than the error of the analytical method (Table VII). Clones derived from a single sensitive colony also differ widely in the resistant/sensitive ratio at first, but agree fairly well after several transfers (Table VIII).

This variation may be due to slight differences in the conditions in individual tubes, or to differences in the lag, growth rate, or mutation rate of the individual

TABLE VII

Fluctuations in Cells/Milliliter and TR Colonies/Milliliter from Separate Tubes and from 1 Tube

899*a* in log growth. Diluted to 0.2 B/ml. in YEP. 10 ml. in test tubes. Shaken at 25°. B/ml. by turbidity. Colonies/milliliter and TR colonies/milliliter as noted. 1 sample from each tube. Remainder mixed together and 20 samples from mixture.

Time—25°		0	4 hrs	24 hrs.
B/ml.	From 20 separate tubes.....	[0.2] calc.		$180 \pm 30 \times 10^6$
	20 measurements on mixture....			$192 \pm 1 \times 10^6$
Colonies/ml.	Average of 20 plates from separate tubes...	0	5 ± 1	
	20 platings from mixture.	0	5.3 ± 0.3	
TR colonies/ml.	Average of 20 plates from separate tubes..	0	<1	32 ± 6
	20 platings from mixture.	0	<1	28 ± 1
TR colonies/10^8B	20 separate tubes.....			14 ± 1.6
	20 platings from mixture.			14 ± 0.5

cells from which the clones grow (Dean and Hinshelwood, 1952; and Northrop, 1955).

A large part of the fluctuation is due to the difference in growth rate of the clones, since the average TR colonies/10^8 B calculated from 20 separate tubes have a smaller deviation than either colonies/milliliter or TR colonies/milliliter from separate tubes.

Proportion of Terramycin-Resistant (TR) Mutants in Various Strains of B. megatherium.—The ratio of the TR mutants to total cells for various strains of *B. megatherium* is shown in Table IX. The strain isolated from a culture of *megatherium* which had become adapted to growth in ASCM contained a much larger ratio of resistant to sensitive cells.

The ratio for 899*a* is remarkably constant as long as the culture is grown in the same medium under the same conditions, as in the steady state apparatus (Northrop, 1954).

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A culture of 899*a* growing in YEP in the steady state apparatus at a cell concentration of 5×10^7 gave resistant colony counts over a 6 weeks period

TABLE VIII

Variation in Ratio TR/Sensitive Cells from Different Resistant Colonies

899*a* streaked on 3 γ terramycin agar. 10 colonies transferred to YEP, grown up to 50×10^6 B/ml., and plated for TR colonies. Diluted 0.05/5. Let stand at 25°—18 hours. Diluted 1/10, grown up at 35° to 50×10^6 B/ml., and plated for TR colonies. Repeated.

Transfer No.	Colony No.	
	2	3
	Tr colonies/10^8 cells	
0	25	450
1	12	105
2	25	18
3	8	14
4	10	12

These were the two extreme results. The other 8 clones gave intermediate values at first. All the clones eventually gave 10 to 20 TR colonies/10^8 B. One culture in the steady state apparatus gave 300 to 500 TR colonies/10^8 cells for 12 days. The ratio then gradually decreased to the usual figure.

TABLE IX

Ratio TR Colonies/Total Cells.
Various Strains of B. megatherium in Log Growth in YEP

Strain	Terramycin/ml.			
	2.5γ	5γ	10γ	20γ
	TR colonies/10^8 B			
899*a*	25	25	20	0
AC 8*	1000	800	600	300
AC 82*	2000	1600	1500	200
SP*	1000-10,000	1000-10,000	250	100
KM	40		6	0
	(many very small colonies)			

* Strain isolated after adaptation of 899*a* to ASCM (Northrop and Murphy, 1956). These strains have lost their ability to grow rapidly on ASCM and have also lost the property of producing C phages.

which fell within the range of 10 to 20/ml., with the exception of two plates, one of 75 and one of 100 colonies.

The other strains, especially SP, show more variation in the resistant colony count in the range of 3 γ terramycin/ml. and the ratio is higher in very dilute suspensions.

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Effect of Culture Media.—The ratio of resistant/total cells in cultures of *megatherium* 899*a* depends on the culture media (*cf.* Novick and Szilard, 1951). Fewer resistant mutants are present when the culture is grown in either ammonium sulfate or asparagine media. The phage concentration and gelatinase concentration are also much lower in these media than in YEP (Northrop, 1951). This effect is probably due to an increased difference in growth rates of

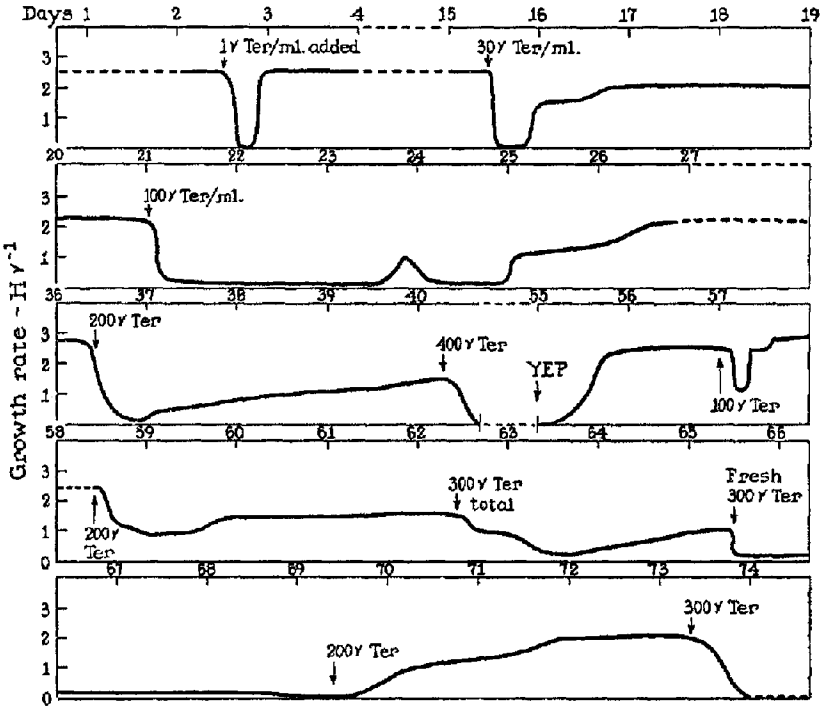

FIG. 3. Growth rate of *B. megatherium* cultures in YEP containing varying quantities of terramycin in steady state apparatus. The growth rate was calculated at 1 hour intervals from the kymograph record of the flow of culture medium as described previously (Northrop, 1954).

the sensitive and resistant cells (*cf.* Northrop and Kunitz, 1956). It is not due to a reversal of the mutation, since resistant cells may be grown in ammonium sulfate culture media for many generations.

Adaptation to Higher Concentrations of Terramycin

The result of an experiment in which 899*a* was grown in the steady state apparatus (Northrop, 1954) in the presence of varying concentrations of terramycin is shown in Fig. 3. After the addition of 1 γ terramycin/ml. to

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the sensitive strain, the growth rate drops to 0, remains there for 4 hours and, then increases rapidly to its original value. The entire cycle requires about 9 hours. The growth tube contains about 200 resistant cells and 1×10^9 sensitive cells at the beginning of the experiment. Assuming that the sensitive cells stop growing as soon as the terramycin is added (this is not quite true) and that the resistant cells continue to grow at a rate of 2.5, it would require 7.7 hours for the resistant cells to constitute 10/11 of the cell population (Northrop, 1954). Actually, it required about 9 hours for the culture to resume its original growth rate. The calculated time should be slightly shorter since the sensitive cells continue to grow for a few hours after the terramycin is added, while the calculation assumes no growth.

The calculated time for the growth rate to change from 0 to 2.5 is 3 hours $\left(M_0/W_0 = \frac{1}{10} \quad \frac{M}{W} = 10\right)$ and the observed time about 2.5.

When 30 γ terramycin/ml. were added to this culture, the growth rate again dropped to 0 very rapidly, stayed at 0 for 8 hours, and then increased to 1.5 in 4 hours. After that it gradually increased to 2.6. The entire cycle required 12 hours. The calculated time in this case is 11 hours, since the growth rate (at end of cycle) is 1.5 instead of 2.5.

The growth rate remained at about 2 for several days. 100 γ terramycin/ml. were then added. The growth rate decreased to 0 and remained there for 3 days. At this time it increased to 1.0 and then dropped rapidly to 0. This preliminary increase is due to the inactivation of the terramycin in the growth tube at 35° C. As soon as the terramycin had become partly inactive, the cells commenced to grow and fresh terramycin ran in from the storage bottle and stopped the growth in the growth tube. (The storage bottle was kept at 20° and so the terramycin remained active.) After about 24 hours, growth was resumed. It is probable that a resistant mutant appeared during the short growth period, and then overgrew the culture as before. 200 γ terramycin/ml. stopped growth for a few hours, but the growth rate returned slowly to 1.5 to 2.0. 400 γ terramycin/ml. stopped growth completely for nearly 2 weeks. At the end of this time. YEP was run in and the culture returned to its original growth rate of 2.5 in a few hours. After 30 hours in YEP, 100 γ terramycin/ml. were added as before. This time growth was resumed after 4 hours. Resistance to very high concentrations of terramycin decreases rapidly, at least as measured by the growth rate. Attempts to adapt the culture to 300 γ terramycin/ml. were repeated at intervals, but none was successful (*cf.* Wright *et al.*, 1953).

The successive steps in the adaptation are probably the result of the selection of successively resistant mutants, as in the case of penicillin resistance.

This is indicated by the fact that the lag time (*i.e.*, the time required for growth to commence again) is always about the same as that calculated on the assumption that the growth tube contains a few cells capable of growing

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in the new concentration of antibiotic. In the cases in which growth did not commence within this time, or near it, it never increased. If no resistant cells were present in the growth tube at the time of the addition of the antibiotic, none appeared, therefore.

Loss of Resistance to Terramycin.—

Strains which have developed resistance to bactericidal substances lose their resistance sooner or later, when grown on favorable media. As a rule, the longer the culture has been grown in the presence of the antibiotic, the longer it retains its resistance. This is true, in general, in the present case, although the results are somewhat irregular owing to the small number of cultures. Strains adapted to 1 γ or to 100 γ terramycin/ml. were transferred daily in YEP, ASCM, filtrate from resistant or sensitive strains, and autoclaved filtrates. The strain adapted to 100 γ terramycin/ml. showed no loss of adaptation after

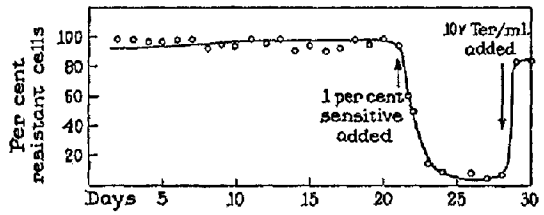

FIG. 4. Overgrowth of resistant by sensitive cells. Culture adapted to 150 γ terramycin/ml. in steady state apparatus and then grown continuously in YEP for 21 days. 1×10^8 cells/ml. 1×10^6/ml. sensitive cells added and culture plated for TR colonies as noted. 10 γ terramycin/ml. added.

125 transfers. The strains adapted to 1 γ terramycin became sensitive in YEP after 60 to 80 transfers, and in ASCM and the various filtrates somewhat more rapidly (*cf.* Foster and Pittillo, 1953).

This loss in resistance has been ascribed to back mutation, although it is extremely unlikely that such a mutation would give rise to a sensitive strain, identical with the original. The proportion of mutants in the reverted strains indicates that this is not the case, since the ratio of resistant/sensitive is much higher in some cases than in the original strain. The appearance of a few sensitive cells in a large population of resistant cells cannot be detected and direct proof of this mechanism is not possible at present. It can be shown, however, that if such a sensitive cell did arise in the mutant culture, it would overgrow the resistant cells and a sensitive culture would result (*cf.* Welsch, 1952).

The result of an experiment in which about 1 per cent of sensitive cells was added to a strain previously adapted to 150 γ terramycin/ml. and growing in YEP in the steady state apparatus is shown in Fig. 4. This resistant strain had grown for 2 months (= about 1500 generations) in the steady state apparatus,

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in YEP, with no loss in resistance as determined by colony count on agar. After the addition of the sensitive cells, the resistant cells decreased in a few days to about 10 per cent, where they remained constant. If any sensitive cells appeared in the culture, therefore, they would probably have overgrown the mutant ones. In the 2 months period, about 3×10^{12} cells had grown in the cell, without the appearance of a sensitive one; the "back mutation" rate must evidently be very low.

Attempts to Transfer the Terramycin Resistance by Phage

The recent work of Freeman, 1951; Lederberg and Lederberg, 1953; Lederberg and Edwards, 1953; Fredericq, 1953; Stocker, Zinder, and Lederberg, 1953; Woolman, 1953, and others has shown that phage particles are a special kind of transforming principle (Gratia, 1936) and can transfer genetic properties from one cell to another. Hotchkiss (1953–54) found that resistance to antibiotics is transmitted by a specific transforming principle. In order to test this possibility, lysogenic cultures of KM (the phage-sensitive *megatherium* strain) were made by adding phage from sensitive or resistant cultures to the KM. The resulting lysogenic strains, however, were indistinguishable from the original KM strain, as far as the resistance was concerned. The fraction of the total phage responsible for the production of the lysogenic strain is exceedingly small, probably not over $1/10^6$, so that, unless all the phage particles were capable of transmitting the resistance factor, a positive result would not be effected (*cf.* Bryson and Demerec, 1955).

Experimental Procedure

Culture Media.—

Yeast Extract Peptone (YEP).—50g. Difco bacto-peptone + 5 gm. Difco yeast extract + 10 ml. M/2 NaOH, dissolved in 1 liter water. Boiled 20 minutes. 24 hours at 5°. Filtered; autoclaved.

Ammonium Sulfate Culture Medium (ASCM).—Northrop and Murphy, 1956.

Terramycin.—Crystalline terramycin HCl was kindly supplied by Charles Pfizer and Company. The terramycin was dissolved in 0.01 M HCl and filtered through a Seitz filter. The activity of the substance decreases slowly on storage at 5° and there is also some loss on filtration. The inhibiting effect of solutions prepared at different times, therefore, is not exactly comparable.

Terramycin Agar.—YEP agar melted, cooled rapidly to 50°, the desired concentration of terramycin added, and 15 ml. poured into petri dishes.

Colony Counts.—

Gratia's double layer technique was used. One-tenth volume of hot 2 per cent agar[4] was added to the suspension and then 1 ml. spread on the surface of the agar (Welsch *et al.*, 1953; Northrop, 1953).

[4] The concentration of agar in the superimposed layer should be just sufficient to prevent the layer from running or slipping when the plate is turned over. 0.2 per cent of the agar used in these experiments was sufficient to do this.

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Control experiments showed that addition of the hot agar did not change the colony count. In case terramycin-agar plates were used, the colony count was the same whether or not terramycin was added to the suspension.

All colony plates were incubated at 35° for 18 hours (or at 25° for 48 hours). Longer incubation of plates containing terramycin results in the appearance of many small colonies. These colonies appear because the terramycin has been partially inactivated. They are not terramycin-resistant (Bryson and Demerec, 1950).

Cell Concentration by Turbidity.—

The turbidity was determined in a Klett-Summerson colorimeter adapted to 20 mm. tubes. The cell concentration was interpolated from the colorimeter reading by means of a standard curve prepared by plotting the cell count of a series of suspensions against the colorimeter reading. The cell concentration is nearly proportional to the colorimeter reading from 1 to 100 and then gradually falls off. During log growth in YEP the colony count is equal to the chains/milliliter and to 3 times the cells/milliliter (*cf.* Northrop, 1951).

Growth in Liquid Media.—

10 ml. of the culture was placed in 20 mm. tubes and the tubes shaken 200 to 300 times/minute in a water bath.

Steady State Apparatus.—

The steady state apparatus previously described (Northrop, 1954) was used, except for changes in the cell and wipers. The original cell foamed with some cultures (requiring a somewhat uncertain correction) (Northrop and Murphy, 1956) and mucoid organisms collected on the shoulders of the cell and on the rubber wipers themselves. The organism occasionally grew in the intake tube and in the culture medium reservoir bottle.

The cell and wipers shown in Fig. 5 obviate these difficulties.

10 per cent lactic acid and 1/5000 germ-i-tol are dropped slowly in the cell overflow through a hypodermic needle (*cf.* Northrop and Murphy, 1956) (except when a sample is taken). This prevents the accumulation of organisms outside the cell itself.

The air does not bubble through the liquid, and therefore, does not cause foaming.

The wipers were cut from a lusteroid test tube. They were cemented to the steel wire with cement made by dissolving lusteroid in acetone. The growth tube is filled with glycerine before autoclaving. This prevents the lusteroid wipers from becoming opaque and brittle. These thin wipers cause much less foaming than do the segments of rubber tubing used originally, and retain fewer organisms. The wipers are run as rapidly as possible (usually 300 to 400 strokes/minute) without causing foam; at half-hour intervals a microswitch on the kymograph axle causes the wipers to operate 600 to 700/minutes for 15 to 20 seconds. This keeps the cell walls perfectly clean even with very mucoid organisms, but some cells still adhere to the wipers in spite of the violent stirring. The number of cells which come from the wipers may be determined by running the culture media continuously through the cell at a rate of about 100 ml. per hour until the cell concentration in the overflow remains constant. This corresponds to a wash out rate of 5/hour (since the cell volume is about 20 ml.). This is 2 (or more) times the growth rate of the bacteria and so the concentration of cells in suspension

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in the tube would decrease at a rate of (5 − growth rate) and would rapidly approach 0. If cells are constantly growing and being liberated from the cell walls or wipers, however, the cell concentration in the overflow will not approach 0, but some constant value and this value represents the cells coming from the walls or wipers. With the rubber wipers, these cells amounted to about 1×10^9 per hour. With the lusteroid

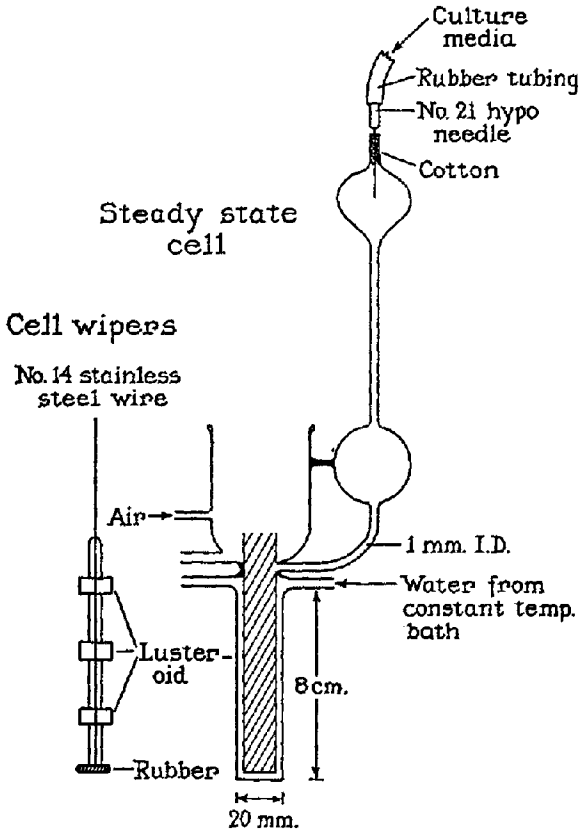

FIG. 5. Cell for use with steady state apparatus. Wipers for cell cut from lusteroid test tube. The end wiper is a segment of rubber tube.

wipers about 1×10^8 cells/hour were delivered with mucoid organisms. A culture growing in the cell at a rate of 2/hour and a concentration of 1×10^8/ml. (usual conditions) delivers $20 \times 10^8 \times 2 = 4 \times 10^9$ cells/hour. The cells derived from organisms growing on the wipers are therefore 2 to 3 per cent of the total. This is negligible if the cells do not differ *qualitatively* from those in suspension, but if they are different qualitatively, serious errors may result. In the present experiments the ratio of TR/total cells is the same in cultures derived from the wipers, as from the suspension in the cell.

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