MEASUREMENTS OF RATE OF MUTATION OF FLAGELLAR ANTIGENIC PHASE IN *SALMONELLA TYPHI-MURIUM*

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(With Plate 13 and 4 Figures in the Text)

Change of phase of flagellar antigen in *Salmonella* species appears to have the characteristics of a mutation, in that a clone, or culture grown from a single cell, consists predominantly of cells of the same phase as the parent cell, but includes after a time cells of the other phase, and that these 'mutant' cells themselves, if isolated, in turn produce clones predominantly of their own phase. Moreover, in some strains change of phase occurs in both directions with sufficient frequency for it to have been detected without the use of selective methods (Andrewes, 1922); this would appear to indicate mutation and back-mutation rates much higher than most others recorded in bacteria. The available evidence suggests that the rate of growth of a *Salmonella* organism is independent of its flagellar phase; if this is strictly true, the rate of appearance of organisms of 'mutant' phase in a culture will be determined only by the mutation rates involved. It was decided to investigate the behaviour of a *Salmonella* species to see whether it accorded with these theoretical considerations and to measure, if possible, the mutation rates concerned.

RAPID METHOD FOR DETERMINING PHASE OF NUMEROUS COLONIES

The first requirement was a method of measurement of the proportion of organisms in each phase in a culture, more rapid than the testing of individual colonies by slide agglutination. The following method, depending on the immobilization of organisms in sloppy agar containing the homologous flagellar antibody, was devised. The culture to be tested is diluted and inoculated on to agar, so as to give discrete colonies. After incubation the plate is chilled in the refrigerator and its surface is then covered with a layer of sloppy agar containing H anti-serum for one phase, the melted agar having been allowed to cool to a temperature just above its setting point so that it rapidly solidifies on the cold plate. After a further short period of incubation, each colony of non-agglutinated phase is found to be

surrounded by a wide zone of opacity with an indefinite edge, consisting of organisms swarming out into the semi-solid agar. Each colony of the agglutinated phase is surrounded by a narrow dense zone of opacity with a clear-cut outer edge. This narrow zone presumably consists of a region where motile organisms (and therefore flagellar antigen) are present in so great a concentration that all the antibody present is fixed, and further organisms swimming out from the colony are therefore free to move until they reach a region so far from the colony that free antibody is still present; here they are immobilized, and produce the sharp edge of the zone. The width of the dense zone increases if incubation is continued; it is also affected by the concentration of serum used, a high concentration producing narrow zones.

The method was tried with a stock strain of *Salm. typhi-murium* (no. C77) and was found to give satisfactory results if a number of variables were carefully controlled. The basal agar layer was of Lemco nutrient agar, cleared by filtration through paper pulp. The agar content was important; with too much the haloes were thin and slow in development, presumably because of poor formation of flagella in the colonies; with too little agar, the colonies were of such soft consistency that they disintegrated when the sloppy agar was layered on. 1.2% of a particular batch of New Zealand powdered agar was found satisfactory. The sloppy agar was of the same formula except that the agar content was reduced to 0.35%. The sera used were prepared against *Salm. cholerae-suis* var. *kunzendorf*, and against *Salm. typhi-murium* in specific phase. The use of absorbed sera was not necessary, provided that the titres for the two phases differed widely. The optimum concentration of serum in the sloppy layer was found by experiment to be rather higher than the agglutinating titre of the serum used. The inoculum was diluted so as to give about eighty colonies on a 10 cm. Petri dish, and spread with a glass rod so as to give discrete colonies. Plates were incubated until the colonies were about 1 mm. in diameter; overnight incubation

at 30° C. was usually satisfactory. To avoid smearing of the colonies by the sloppy agar it was found best to deposit a preliminary film of sloppy agar by spraying the cooled plates for 2 or 3 sec. with a paint-gun (Aerograph type M.P.); the remainder of the sloppy agar, cooled to 35–36° C., was then pipetted on. About 9 ml. was used for each 10 cm. Petri dish. Incubation of the flooded plates at 37° C. for 1–2 hr. produced optimum development of haloes. After incubation the plates were examined by transmitted light against a dark background, and the number of colonies of each phase was counted. In the early stages of the work a large number of colonies thus typed were checked by slide agglutination (after subculture if necessary) and the reliability of the method was established. In the later stages, subculture and slide agglutination were used only to check the phase of the few colonies giving equivocal appearances, e.g. dwarf colonies developing in an area where the basal agar layer was thin owing to convexity of the bottom of the Petri dish.

With strain C77, colonies of mixed phase were rare (8 in 5000 in one series of experiments). They usually showed a narrow dense sharp-edged halo surrounding half the periphery of the colony, and a wide diffuse halo round the other half. Subculture and slide agglutination confirmed that one-half of such a colony was predominantly of homologous phase and the other half of heterologous. It was at first suspected that each such colony had grown from a single bacillus which had undergone mutation at its first division on the plate, giving rise to a colony half in the parent phase and half in the mutant phase; but later such colonies were found to be very rare when almost all the colonies on a plate were of one phase, and less rare when colonies of both types were present in about equal numbers, and it was therefore concluded that such mixed colonies resulted, in most cases at least, from the confluence of two colonies of opposite phase arising very close together. In recording the proportions of colonies of each phase, such a mixed colony was therefore counted as two colonies, one of each phase.

Colonies that failed to form haloes occurred in very small numbers in some experiments. They consisted of organisms which were non-motile or almost so, and their behaviour will be discussed later. In strain C77, they never amounted to more than 2% of the colonies present.

The appearances produced by colonies of agglutinated and non-agglutinated phase, and by colonies of non-motile organisms, are illustrated in Pl. 13.

Since mixed colonies were rare, it was considered that the phase of the colony produced was a reliable indication of the phase of the organism from which it grew, and therefore that the ratio of the two types of colony on a plate was equal to the ratio of the two types of organism in the culture plated.

THEORETICAL ANALYSIS OF RATE OF INCREASE OF PROPORTION OF MUTANT-TYPE CELLS

Luria & Delbrück (1943) have made a theoretical analysis of the rate of increase of the proportion of mutant-type cells in a culture, initially wholly of 'normal' type, when it is maintained in logarithmic growth. Their analysis covers the case in which the growth rates of normal and mutant are equal and back-mutation is neglected, and they show that under these conditions the proportion of mutant-type cells increases linearly with the number of generations through which the culture goes, so long as the proportion of mutant-type cells remains small. Their argument may be illustrated as follows. The division of a bacterial cell may be regarded as the coming into existence of two new cells, and the mutation rate may be defined as the probability that a new cell so arising from a normal parent is of the mutant type. Consider a very large population of n cells, all of normal type so that the initial proportion of mutant-type cells is 0. If these cells undergo mutation at a rate m , after one generation the total number of cells will be $2n$, made up of $2nm$ mutants and $2n - 2nm$ normal cells. Thus after one generation the proportion of mutant-type cells is $\frac{2nm}{2n} = m$. If

m is a very small number, $2n - 2nm$ will be nearly equal to $2n$, and the number of normal cells may be taken as $2n$. After a second generation the total population will be $4n$ and there will be (nearly) $4nm$ mutant-type cells produced by mutation during the second generation, plus $4nm$ arising by division of the $2nm$ mutants produced during the first generation. Thus the proportion of mutant-type cells is $\frac{4nm + 4nm}{4n} = 2m$. After a third generation the total

population is $8n$, and there are $8nm$ mutant-type cells produced by fresh mutation plus $16nm$ produced by division of pre-existing mutant-type cells, so the proportion is $\frac{8nm + 16nm}{8n} = 3m$. Similarly,

after four generations the proportion is

$$\frac{16nm + 48nm}{16n} = 4m,$$

and after g generations the proportion is gm .

EXPERIMENTS ON MUTATION FROM GROUP TO SPECIFIC PHASE IN STRAIN C77

It was decided to see whether a culture of strain C77 in one phase in fact showed this linear increase in proportion of organisms in the other phase during logarithmic growth. Preliminary experiments showed

that the mutation rate for the change from group to specific was much higher than the rate from specific to group, and the rate of appearance of specific-phase cells in a group culture was therefore investigated first, since it seemed that in this case back-mutation would not cause much deviation from theory. The cultures were grown in Lemco broth at 23° C.; the lower temperature of incubation made it easier to keep the culture in logarithmic growth for long periods.

To obtain a culture initially all in one phase it was found necessary to use cultures grown from a single cell. The use of an initial population of one cell, instead of the very large number, n , postulated above, introduces some theoretical complications which will be discussed later in this paper. Presumptive single-cell cultures were made by inoculation of a series of tubes with a broth culture predominantly in the required phase so diluted that growth occurred in half or less of the tubes inoculated. A culture obtained in such a tube could then be presumed to have grown from a single cell, or less probably from two or three cells only, and was therefore almost certain to have been initially all of one phase.

The number of generations the culture had gone through was calculated from the viable count at the moment of sampling. Experiment showed that there was no detectable difference between viable and total cell counts in this organism during the logarithmic phase of growth, and the number of generations the culture had gone through in growth from a single cell could therefore be ascertained by expressing the final viable population as a power of 2. Numbers of generations recorded throughout this paper have been calculated in this way.

A series of experiments on presumptive single-cell cultures showed that after about 24 hr. growth, when the number of cells was between 10^8 and 3×10^8 (and the number of generations was therefore about 27), the proportion of specific-phase colonies was only about 1 %. To obtain statistically significant figures it was therefore necessary to maintain logarithmic growth for several days. Control experiments showed that under the conditions of the experiment this organism remained in log phase when a young culture was inoculated into fresh medium at 23° C. (i.e. there was no lag on dilution); the required continuous logarithmic growth over a period of days was, therefore, obtained by making quantitative dilutions of the culture into fresh medium twice or thrice daily. Large inocula (over 10^8 organisms) were used to ensure that the proportion of mutant-type cells in the inoculum was truly representative of that in the parent culture. The number of generations was calculated by multiplying the viable count at the moment of sampling by the product of the successive dilutions during growth and expressing as a power of 2.

The results of an experiment on these lines extending over 24 days are shown in Table 1A and in Text-fig. 1, where the proportion of specific-phase cells (and the standard error of the proportion) is plotted against the number of generations. It will be seen that up to about the 500th generation the increase is approximately linear, all the points lying within twice their standard error of a straight line passing through the origin. The mutation rate for the change group to specific, calculated from the slope of this line, is 2.9×10^{-4} . The unexpected drop in the proportion of specific-phase cells after the 500th generation will be discussed later.

Relative growth rates of the two phases

It has so far been assumed that the rate of growth of the mutant, specific-phase, cells is equal to that of the group-phase cells. To test this assumption the rates of growth were measured; two cultures of strain C77, one about 99 % specific phase and the other about 90 % group phase, were kept in continuous logarithmic growth for 48 hr. by quantitative dilution into fresh broth at intervals, and viable counts were made on both cultures from time to time. This experiment gave values of 54.4 and 54.1 min. for the generation times of the specific-phase and group-phase cultures respectively. The small difference can probably be accounted for by experimental and sampling error. It seemed therefore that the rates of growth of the two phases were at least very nearly equal. But even a very small difference in growth rate would cause a considerable error in the value of the mutation rate calculated from the proportion of mutant-type cells present after a large number of generations. A more sensitive test for differences in growth rate was therefore made. A culture of C77 containing about equal proportions of the two phases was obtained by mixing cultures in group phase and in specific phase; this culture was kept in continuous logarithmic growth for several days, and the ratio of the two phases was measured at intervals. The small change in ratio to be expected from mutation could be calculated from the previously observed rate of change in cultures initially all in one phase. Any difference in growth rates, even a difference in generation times of, say, 0.3 %, would result, in one or two hundred generations, in a marked shift towards preponderance of the more rapidly growing form. In fact, no change in proportion greater than could be expected from mutation and sampling error was found, and it was concluded that the assumption that the growth rates of the phases were equal had not caused any serious error in the measurement of the mutation rate.

Consequences of use of small initial population

In measuring the mutation rate, error arising out of differences in rate of growth of mutant and normal

Table 1A. *Strain C77. First long-period experiment on mutation from group to specific phase*

No. of generations	No. of colonies examined	Specific-phase colonies detected	Specific-phase colonies, proportion of total	Standard error of proportion	Proportion No. of generations (=mutation rate)
81	410	7	0.017	0.006	2.1×10^{-4}
134	338	8	0.024	0.008	1.8×10^{-4}
198	635	28	0.044	0.008	2.2×10^{-4}
333	222	16	0.072	0.018	2.2×10^{-4}
354	312	34	0.110	0.018	3.1×10^{-4}
476	465	67	0.144	0.017	3.0×10^{-4}
569	547	48	0.088	0.013	—*
625	518	12	0.023	0.007	—*

Table 1B. *Strain C77. Second long-period experiment on mutation from group to specific phase*

No. of generations	No. of colonies examined	Specific-phase colonies detected	Specific-phase colonies, proportion of total	Standard error of proportion	Proportion No. of generations (=mutation rate)
254	711	51	0.072	0.011	2.8×10^{-4}
410	1683	215	0.127	0.009	3.1×10^{-4}
574	279	59	0.211	0.024	3.7×10^{-4}
610	248	50	0.202	0.026	3.5×10^{-4}
799	749	129	0.173	0.014	—*
875	406	86	0.212	0.020	—*

* Mutation rate not calculated because of anomalous fall in proportion of cells of specific phase.

Text-fig. 1. Strain C77. First long-period experiment on mutation from group to specific phase. Distance from centre of dot to end of vertical stroke represents standard error of observation. Straight line corresponds to mutation rate of 2.8×10^{-4} .

will, in theory, be minimized by measuring the proportion of mutant-type cells after the smallest possible number of generations. However, experiments employing a short period of growth suffer from two disadvantages.

First, in the absence of a selective method a very large number of colonies must be examined in order to obtain a statistically significant number of mutant-

type colonies, because the proportion of mutant colonies is very small.

Secondly, the use of an initial population of one, instead of the very large number postulated in the theoretical analysis, may substantially affect the proportion of mutant-type cells present after a small number of generations. The proportion of mutant-type cells to be expected in cultures grown for a

Table 2A. *Strain C77. Short-period experiments on mutation from group to specific phase; twenty single-cell cultures tested at about the 28th generation*

Culture no.	No. of generations	No. of colonies examined	Specific-phase colonies detected	Specific-phase colonies, proportion of total
1	27.3	142	5	0.035
2	27.3	148	2	0.014
3	28.0	240	0	0
4	27.7	192	1	0.005
5	26.7	456	18	0.041
6	26.8	423	9	0.021
7	27.4	664	8	0.012
8	27.9	963	10	0.010
9	27.4	301	2	0.007
10	27.2	269	1	0.004
11	26.7	212	0	0
12	About 27	191	0	0
13	29.0	275	2	0.007
14	29.8	286	2	0.007
15	29.3	362	5	0.014
16	30.6	476	1	0.002
17	29.2	429	1	0.002
18	30.9	511	3	0.006
19	28.9	411	3	0.008
20	31.1	416	1	0.002
Totals		7367	74	

Mean number of generations 28.3

Mean proportion of specific-phase colonies (all observations combined) = $\frac{74}{7367} = 0.010$

Mutation rate = $\frac{\text{Proportion of mutant type}}{\text{Number of generations}} = \frac{0.010}{28.3} = 3.5 \times 10^{-4}$

$\chi^2 = 73.2$ on 19 degrees of freedom P is less than 0.001

Table 2B. *Strain C77. Short-period experiments on mutation from group to specific phase; five single-cell cultures tested at the 80th and 231st generations*

Culture no.	No. of generations	No. of colonies examined	Specific-phase colonies detected	Specific-phase colonies, proportion of total
21	80	550	10	0.018
22	80	1192	25	0.021
23	80	1044	10	0.009
24	80	692	9	0.013
25	80	846	25	0.030
Totals		4324	79	

Mean proportion of specific-phase colonies (all observations combined) = $\frac{79}{4324} = 0.018$

Mutation rate = $\frac{\text{Proportion of mutant type}}{\text{Number of generations}} = \frac{0.018}{80} = 2.3 \times 10^{-4}$

$\chi^2 = 11.95$ on 4 degrees of freedom P is between 0.01 and 0.02

Culture no.	No. of generations	No. of colonies examined	Specific-phase colonies detected	Specific-phase colonies, proportion of total
21	231	539	35	0.065
22	231	401	30	0.075
23	231	745	44	0.059
24	231	368	35	0.095
25	231	152	10	0.066
Totals		2205	154	

Mean proportion of specific-phase colonies (all observations combined) = $\frac{154}{2205} = 0.070$

Mutation rate = $\frac{\text{Proportion of mutant type}}{\text{Number of generations}} = \frac{0.070}{231} = 3.0 \times 10^{-4}$

$\chi^2 = 5.32$ on 4 degrees of freedom P is between 0.2 and 0.3

small number of generations from small initial populations has been analysed by Luria & Delbrück (1943); they show that in a large series of such cultures the proportion of mutant-type cells in the majority of the cultures will be smaller than the theoretical (mutation-rate) $\times$ (number of generations), while in a minority the proportion will be larger, in some cultures very much larger. This is because in most of the cultures no mutation will occur until the population is of the same order of size as the reciprocal of the mutation rate, and so the proportion of mutants will remain at zero for a number of generations. But in a few cultures a mutation will by chance occur early, while the population is still small, and so produce a proportion of

When the population in a culture becomes substantially larger than the reciprocal of the mutation rate, the expected number of mutations per generation becomes a large number, so that random variation in the number of mutations actually occurring will be negligible. So from this time onwards, the rate of increase in proportion of mutant-type cells will be virtually the same for all of a series of replicate cultures. In such a series the variation in proportion of mutants arising during the early generations will persist but it will not increase, so that after a large number of further generations the variation due to the use of a small initial population will be a small fraction only of the final proportion of mutant-type cells. Thus the error in the measurement of the

Text-fig. 2. Strain C77. Long-period experiment on mutation from specific to group phase. Straight line corresponds to mutation rate of 10^{-5} .

mutants greater than the (mutation-rate) $\times$ (number of generations) of the simplified analysis.

Several series of presumptive single-cell cultures of strain C77 in group phase were incubated for periods of about 24 hr., and tested for proportion of specific cells; the results of these experiments are shown in Table 2A. The populations range from 10^8 to 21×10^8 , and the number of generations therefore from 27 to 31, but as the expected increase in proportion of mutants in four generations is only about 0.1%, it has been considered reasonable to treat the data as though the number of generations had been the same in all the cultures. As was to be expected, there is a marked variation in the proportion of specific-phase colonies found, and a test shows that the variance is highly significant ($p < 0.001$). The mutation rate calculated from the pooled data is 3.5×10^{-4} , a value in reasonably good agreement with that obtained from the long-term experiments.

mutation rate which results from using single-cell cultures will be very small when the number of generations is large.

As the proportion of mutants increases, so also does the standard error of the measured proportion, i.e. the error due to sampling; but the variance due to the use of a small initial population remains the same. It follows that in a series of replicates the variance in the proportion of mutants due to small initial population will tend to be increasingly masked by sampling error as the number of generations increases. Table 2B and Text-fig. 3 show the proportions of mutants found in a series of five replicate single-cell cultures of strain C77, initially in group phase, tested after 80 and after 231 generations. Comparison of the results of the χ^2 tests in this experiment and in the experiments where the number of generations was about 28 shows that the variance behaves as predicted; for while in the

28 generation set the variance is so great that the probability of such a variability arising from sampling error alone is less than 1 in 1000, in the 80 generation set this probability is between 1 in 50 and 1 in 100, and in the 231 generation set between 1 in 5 and 1 in 3.

EXPERIMENTS ON MUTATION FROM SPECIFIC TO GROUP IN STRAIN C77

The rate of mutation from group to specific phase having been determined, the same methods were applied to the measurement of rate of mutation from specific to group. This was found to present more difficulty because of the slow rate of increase in proportion of group-phase cells in cultures of specific phase. As less than 0.1% were present after 24 hr. incubation, long periods of growth had to be used. In these experiments and in subsequent work on

Table 3A. *Strain C77. Short-period experiments on mutation from specific to group phase; four single-cell cultures examined at the 47th generation*

Culture no.	No. of generations	No. of colonies examined	Group-phase colonies detected
26	47	601	1
27	47	890	0
28	47	989	1
29	47	901	1
Totals		3381	3

Mean proportion of group-phase colonies (all observations combined) = $\frac{3}{3381} = 0.0009$

$$\text{Mutation rate} = \frac{\text{Proportion of mutant type}}{\text{Number of generations}} = \frac{0.0009}{47} = 1.8 \times 10^{-5}$$

For reasons of convenience subculture was made in a way which permitted the culture inoculated in the evening to come out of logarithmic phase a few hours before it was subcultured. Experience showed that even if a culture remained at 23° C. for a few hours after the end of the period of logarithmic growth, the rate at which mutations occurred per generation did not alter appreciably: and as at the time of subculturing the viable count was still about the same as the total count, no great error in the estimate of the number of generations is likely to have resulted from this modification of procedure; for even if only half the total cells at the moment of subculturing had been viable, the resulting error in the estimated number of generations would have been one generation in each 24 hr. period, that is only about 5%.

Furthermore, the subcultures were made with two particular platinum loops, previously calibrated by weighing. This method is only approximately quantitative, but even a large error in dilution entails only a small error in the calculated number of generations. Using these methods, the calculated number of generations in 24 hr. was about twenty-one, instead of the twenty-seven which would have been expected had growth been logarithmic throughout. In experiments in which periods of overcrowding intervene between periods of logarithmic growth, the theoretical relation between number of generations and proportion of mutant-type cells will hold true only if the two phases have identical lag periods, and identical growth rates under conditions of overcrowding, etc., as well as identical rates of logarithmic growth. As the results by the simplified method agree fairly well with those obtained when growth was logarithmic throughout, it is justifiable to assume that the behaviour of the two phases is identical in these respects.

Tables 3A and 3B and Text-fig. 2 show the results

Table 3B. *Strain C77. Long-period experiment on mutation from group to specific phase*

No. of generations	No. of colonies examined	Group-phase colonies detected	Group-phase colonies, proportion of total	Standard error of proportion	Proportion No. of generations (=mutation rate)
320	3228	9	0.003	0.001	9×10^{-6}
439	2746	11	0.004	0.001	9×10^{-6}
568	659	4	0.006	0.003	1.1×10^{-5}

other strains, the procedure for maintaining growth for long periods was simplified in certain respects.

It was found in control experiments that during storage of a culture for several days in a refrigerator room (temperature about 5° C.) neither the viable count nor the proportion of cells of the two phases altered significantly. It therefore seemed justifiable to interrupt the periods of continuous growth by storage in the refrigerator room for periods up to 4 days when this was convenient.

of two experiments on the mutation from specific to group in strain C77. In the long-period experiment the increase in proportion is roughly linear, and the calculated mutation rate is about 1×10^{-5} ; i.e. about one-thirtieth of the rate of mutation from group to specific. The total number of group-phase colonies detected was very small, so the possible error of this estimate due to sampling is large; it has therefore not seemed worth while to correct for the small error due to back-mutation.

Text-fig. 3. Strain C77. Second long-period experiment on mutation from group to specific phase, observations represented by large dots. Straight line corresponds to mutation rate of 3.2×10^{-4} . Short-period experiments on five single-cell cultures, observations represented by small dots.

FURTHER EXPERIMENTS ON MUTATION FROM GROUP TO SPECIFIC IN STRAIN C77

Table 1B and Text-fig. 3 show the results of a second long-period experiment on the change from group to specific using the simplified technique for continuous growth; the initial culture for this experiment was obtained by combining four of the five replicate single-cell cultures described earlier, when they had gone through 242 generations. The increase is again linear up to the 574th generation; thereafter a small fall in proportion of specific cells occurs, followed by an increase. The mutation rate (group to specific) calculated from the linear part of the graph is about 3.2×10^{-4} .

Decreases in proportion of mutant-type cells

The anomalous fall in proportion of cells of mutant phase in this experiment is too large to be accounted for by sampling error, and both here and in the similar phenomenon in the experiment shown in Text-fig. 1 it seems reasonably certain that the fall observed was not the result of technical errors, e.g. the use of too small inocula, or the mislabelling of cultures.

Perhaps the least improbable hypothesis that will account for these anomalies is that a mutation affecting rate of growth in Lemco broth has occurred

somewhat earlier than the observed anomaly. A mutation causing even a slight shortening of generation time will result in the progeny of the new mutant outgrowing the rest of the culture, so that after a number of generations this clone will make up the greater part of the whole culture. If such a mutation affecting growth rate occurs in a cell of the flagellar phase which predominates in the culture, the proportion of cells of the minority flagellar phase in the clone of more rapidly growing organisms will be lower than the proportion in the rest of the culture, since this clone was all of one phase at the time when the growth-rate mutation occurred. Thus when the clone of rapidly growing organisms begins to make up the greater part of the whole culture the proportion of organisms of the minority flagellar phase in the whole culture will diminish for a time; continued growth of the culture, now consisting mainly of the rapidly growing form, will result in a secondary increase in the proportion of cells of the minority flagellar phase, as a result of mutations of phase occurring within the rapidly growing clone. This hypothesis would account for the behaviour of the culture represented in Text-fig. 3, where the fall in proportion of specific cells is followed by an increase. The later behaviour of the culture represented in Text-fig. 1 was also studied, but certain errors in technique at about the 625th generation render these later results unreliable. No satisfactory experimental test of this hypothesis has been devised.

CONSIDERATIONS WHEN BOTH MUTATION AND BACK-MUTATION OCCUR

The analysis of Luria & Delbrück quoted above applies only so long as the proportion of mutant-type cells remains small. If logarithmic growth is maintained indefinitely in cases where mutation and back-mutation occur, the rate of increase of proportion of mutant-type will diminish for two reasons: first, because the percentage of normal-type cells present and susceptible to mutation slowly decreases, and secondly, because the cells of mutant type are themselves producing cells of normal type by back-mutation. Ultimately, the proportion of mutant type to normal type will approach an equilibrium level at which the decrease in proportion of normals by mutation is exactly balanced by the increase in proportion of normals by back-mutation. It has been shown by Bunting (1940) that for an organism which undergoes mutation and back-mutation at rates m and b , in a culture of equilibrium composition,
$$\frac{\text{number of cells of normal type}}{\text{number of cells of mutant type}} = \frac{b}{m}$$
 (if the growth rates of normal and mutant types are equal).

Results obtained with other strains of Salmonella typhi-murium

It had been hoped to demonstrate such an equilibrium in strain C77, but it became clear that this organism's mutation rates were so low that it was not a practical proposition to maintain continuous growth for a period long enough for the cultures to approximate to equilibrium. Several other strains of *Salm. typhi-murium* were therefore obtained from the National Collection of Type Cultures for investigation. They were:

- N.C.T.C. 73 *Salm. typhi-murium* var. *binns* (strain 'Binns')
- N.C.T.C. 74 *Salm. typhi-murium* (strain 'Mutton')
- N.C.T.C. 4787 *Salm. typhi-murium* var. *binns* (strain 'Timson')
- N.C.T.C. 5710 *Salm. typhi-murium* (formula IV, V: i; 1, 2)
- N.C.T.C. 5711 *Salm. typhi-murium* (formula I, IV, V: i; 1, 2)
- N.C.T.C. 5712 *Salm. typhi-murium* var. *copenhagen* (formula IV: i; 1, 2)
- N.C.T.C. 5713 *Salm. typhi-murium* var. *copenhagen* (formula I, IV: i; 1, 2)
- N.C.T.C. 5715 *Salm. typhi-murium* (formula IV, V: i; 1, 2) ('Chiefly in non-specific phase')
- N.C.T.C. 6817 *Salm. typhi-murium* (formula IV, V, (XII): i; 1, 2)

All except 74 and 5713 gave appearances on layered plates similar to those of C77. Strains 74 and 5713, which were later shown to have much higher mutation rates than strain C77, gave a rather high proportion of mixed colonies; in some of these colonies, sectors of differing phase could be detected, in others faint spreading haloes developed in addition to the small dense sharp-edged haloes, presumably because of the presence in the colonies of some mutant cells of non-agglutinated phase. However, the appearances usually suggested predominance of one phase,

and such a colony was presumed to have grown from a cell of this phase. Because of the difficulties caused by these mixed colonies, and also because its growth rate was inconveniently slower than that of the other strains, strain 74 ('Mutton') was not fully investigated. A culture of each strain in each phase was prepared by inoculating into broth the whole of an isolated colony grown on agar at 23° C. (and presumed to have grown from a single cell); these cultures were then subcultured twice daily with the calibrated loops. This method of starting the cultures introduces some error in the calculated number of generations in the first part of the experiment, since probably not all the cells, even of a young colony, are viable. The results obtained in assays of the proportion of phases at various intervals are shown in Table 4 and the results in strain 5713 are also plotted in Text-fig. 4.

For most of the strains on which sufficient data were obtained the proportion of cells of mutant phase showed an approximately linear increase; in the later stages of the experiment some anomalous results (shown bracketed in Table 4) were found, either decreases or slower rates of increase. From the findings with strain C77 it seemed that in such cases the results in the earlier parts of the experiment probably gave the best indication of the mutation rates, and the rates have therefore been calculated from those results in which the increase was linear. It has been assumed that the growth rates of the two phases are equal in the strains from the N.C.T.C., as in strain C77. The mutation rates found are summarized in Table 5. In these strains, the calculated rates of mutation from group to specific vary from about 10^{-4} to about 4×10^{-3} , and the rates of mutation from specific to group vary from about 1.5×10^{-5} to about 8×10^{-4} ; in some strains the total number of

colonies of mutant phase observed was very small, and the mutation rates shown are therefore approximations only.

Behaviour of strain 5713

Strain 5713 differs from the other strains tested in that by about the 600th generation each of the two cultures, one initially all group and the other initially all specific, contains about 14 % group cells. This is in accordance with the expectation of an

Table 4. *Strains from N.C.T.C. Long-period experiments on mutation from group to specific phase, and from specific to group phase*

Group to specific					Specific to group				
No. of generations	No. of colonies examined	Specific-phase colonies detected	Specific-phase colonies proportion	Proportion No. generations (= mutation rate)	No. of generations	No. of colonies examined	Group-phase colonies detected	Group-phase colonies proportion	Proportion No. generations (= mutation rate)
Strain 73									
73	408	3	0.007	10^{-4}	143	422	2	0.005	3.5×10^{-5}
194	135	4	0.03	1.5×10^{-4}	339	221	4	0.02	5.3×10^{-5}
727	573	12	(0.02)	—	727	491	4	(0.008)	—
Strain 4787									
73	431	5	0.01	1.4×10^{-4}	143	492	1	0.002	1.4×10^{-5}
194	94	5	0.05	2.7×10^{-4}	339	505	3	0.006	1.8×10^{-5}
511	105	9	0.09	1.7×10^{-4}	718	712	7	0.01	1.4×10^{-5}
727	487	32	(0.07)	—					
Strain 5710									
73	262	10	0.04	5×10^{-4}	143	238	2	0.009	6×10^{-5}
194	131	11	0.08	4×10^{-4}					
Strain 5711									
73	277	13	0.05	6×10^{-4}	143	537	4	0.007	5×10^{-5}
194	36	5	0.14	7×10^{-4}					
Strain 5712									
73	251	14	0.06	8×10^{-4}	143	835	8	0.01	7×10^{-5}
194	29	7	0.24	1.2×10^{-3}	339	706	17	0.024	7×10^{-5}
511	39	21	0.54	1.1×10^{-3}	675	187	5	(0.03)	—
675	42	24	0.57	8×10^{-4}					
Strain 5713									
31	249	39	0.16	5×10^{-3}	143	282	32	0.11	8×10^{-4}
73	104	24	0.23	3×10^{-3}	380	214	26	0.12	—*
194	35	26	0.74	4×10^{-3}	675	81	11	0.14	—*
511	74	60	0.81	—*	727	110	14	0.13	—*
675	72	61	0.85	—*					
727	226	190	0.84	—*					
Strain 5715									
73	445	6	0.013	1.8×10^{-4}	143	375	3	0.008	6×10^{-5}
194	123	2	0.016	8×10^{-5}	339	331	4	0.012	4×10^{-5}
511	89	5	0.056	1.1×10^{-4}					
Strain 6817									
73	450	3	0.007	10^{-4}	143	376	2	0.005	3.5×10^{-5}
194	131	3	0.023	1.2×10^{-4}	339	419	5	0.012	3.5×10^{-5}
511	117	9	0.077	1.5×10^{-4}					

* Mutation rate not calculated because culture approaching equilibrium composition.

equilibrium state. It may be shown theoretically that in the case when mutation and back-mutation occur and the rate of growth of the two forms is equal the composition of a culture will tend towards its equilibrium value exponentially (Bunting, 1940). The two curved lines in Text-fig. 4 represent the exponential approach to an equilibrium level which would be expected if the mutation rates in strain 5713 were 5.2×10^{-3} for the change from group to

specific phase and 8.8×10^{-4} for the reverse change: it will be seen that these curves give a reasonable fit with the experimental points, all but one of the observations lying within twice their standard error of the line; the exception is the value of the group-phase culture at 194 generations, which differs from the theoretical value by just over twice its standard error.

The experimental findings shown in Table 4 and

Text-fig. 4. Strain 5713. Long-period experiments on mutation from group to specific phase, observations represented by large solid circles; long-period experiment on mutation from specific to group phase, observations represented by large open circles. Lines correspond to mutation rates of 5.2×10^{-3} and 8.8×10^{-4} . Experiments on mutation from group to specific phase during growth in various media, observations represented by small solid circles.

Table 5. *Mutation rates and predicted proportion of specific-phase cells at equilibrium in N.C.T.C. strains, compared with proportion of specific-phase cells in cultures as received*

Strain	Mutation rate group to specific	Mutation rate specific to group	Specific-phase cells % at equilibrium	Specific-phase cells % in culture as received
73	1.2×10^{-4}	4.4×10^{-5}	73	7
4787	1.9×10^{-4}	1.5×10^{-5}	93	6
5710	4.5×10^{-4}	6.0×10^{-5}	88	74
5711	6.5×10^{-4}	5.0×10^{-5}	92	About 67
5712	1.1×10^{-3}	7.0×10^{-5}	94	63
5713	4.7×10^{-3}	8.6×10^{-4}	84	75
5715	1.2×10^{-4}	5.0×10^{-5}	70	9
6817	1.2×10^{-4}	3.5×10^{-5}	77	100

Text-fig. 4 are inadequate to test the expected relationship between the equilibrium composition of the culture and the two mutation rates. Some further experiments were therefore made with strain 5713 to obtain more reliable figures for the mutation rates. Five replicate single-cell group-phase cultures (obtained by the dilution method) tested at the 132nd generation contained an average of 43 % specific-phase cells; and five single-cell specific-phase cultures tested at the 90th generation contained an average of 5.8 % group phase cells. On the assumption of a linear increase in proportion of mutants, the mutation rates calculated from these figures are

3.3×10^{-3} for the change from group to specific phase, and 6.6×10^{-4} for the reverse change. But as may be seen from Text-fig. 4 the rate of increase of proportion of mutants departs significantly from the linear even by the 90th generation when the mutation rates are of this order of magnitude. When both mutation rates are known approximately it is possible to make a rough correction for this deviation from the linear; the mutation rates calculated from the short-period experiments, and thus adjusted, are 4.7×10^{-3} and 8.6×10^{-4} , values which agree fairly well with those postulated for the curves which fit the data of the long-period experiment.

The composition of a culture of strain 5713 at equilibrium predicted by these adjusted values for the mutation rates is:

$$\begin{aligned} & \frac{\text{Proportion of group cells}}{\text{Proportion of specific cells}} \\ &= \frac{\text{Mutation rate specific to group}}{\text{Mutation rate group to specific}} \\ &= \frac{8.6 \times 10^{-4}}{4.7 \times 10^{-3}} \\ &= \frac{1}{5.4} \end{aligned}$$

(The value calculated from the unadjusted values for the mutation rates is $\frac{6.6 \times 10^{-4}}{3.3 \times 10^{-3}} = \frac{1}{5}$). The mean composition of the two cultures of strain 5713 each tested after 675 and after 727 generations was 14.4 % group, 85.6 % specific, i.e.

$$\frac{\text{Proportion of group cells}}{\text{Proportion of specific cells}} = \frac{14.4}{85.6} = \frac{1}{5.9}$$

Thus the predicted value is in fair agreement with the experimental.

Measurement of the rate of mutation from specific to group in strain 5713 proved unexpectedly difficult. In some experiments inconsistent results were obtained. In this strain, the proportion of group cells in a culture initially all specific increases only to about 14 % at equilibrium; to obtain the rate of mutation the proportion of group cells must be measured while it is still increasing approximately linearly, that is, while the proportion is considerably less than 14 %. The tendency of this strain to produce colonies of mixed phase makes it difficult to measure such low proportions reliably, since the number of colonies of mixed phase in a test may be as large as the number of colonies of the minority phase, so that any error in deciding the predominant phase of a mixed colony may cause a large error in the measurement of the proportion of colonies of minority phase. This experimental difficulty may account for the inconsistent results obtained, but in some series of presumptive single-cell cultures the variance in proportion of group cells found seemed larger than could be accounted for by the combined effect of errors due to mixed colonies and variance due to small size of initial population. There is, in fact, a suggestion of heterogeneity of mutation rate in strain 5713. The technical difficulty with this strain has prevented an adequate investigation of this point.

Comparison of composition of cultures as received, and expected composition at equilibrium

From the figures for mutation rates it is possible to calculate approximately the expected composition of the strains at equilibrium. Table 5 shows the ex-

pected percentages of specific cells, together with the percentages actually found in the cultures as received from the N.C.T.C. (determined by testing 100–200 colonies of each strain); it will be seen that in all cases the specific phase may be expected to predominate at equilibrium. In five strains the specific phase predominated in the cultures as received, but in three the group phase was predominant. These three consist of the two strains described as 'var. *binns*' and one strain (5715) described as being 'chiefly non-specific' in the notes of the N.C.T.C. The data on strain 74 'Mutton', though incomplete, suggest that its mutation rates are of the same order as those of strain 5713 and that the equilibrium culture contains about 70 % specific cells.

EFFECT OF ALTERATION OF ENVIRONMENT

It has so far been assumed that mutation of phase occurs at a constant rate per bacterial generation; the absence of appreciable alteration in the proportion of phases during several days' storage of cultures at refrigerator temperature shows that at least mutation does not occur at a constant rate per unit of time regardless of rate of bacterial growth. A few experiments have been made on strain 5713 to see whether the mutation rate per bacterial generation remains constant when the rate of bacterial growth is altered by varying the medium and the temperature of incubation. In view of the reported differences in optimum pH for acid agglutination of the two phases of *Salm. typhi-murium* (Ogonuki, 1940) it seemed of interest to include media of differing hydrogen-ion concentrations. Isolated colonies of strain 5713 in group phase were inoculated into Lemco broth, peptone water at pH 6.0, peptone water at pH 10.0, and Koser's citrate medium. All were incubated at 37° C., and subcultured by calibrated loop. After sixteen subcultures, when the total number of generations was about 210 (of which the first 30 or so were on nutrient agar), the cultures were tested for proportion of cells of each phase. The results are shown in Table 6 and are also plotted in Text-fig. 4; it will be seen that they lie fairly close to the theoretical line calculated from the mutation rates in Lemco broth at 23° C., and in fact none of the four values differs from that predicted by as much as twice its standard error. So far as they go, these results suggest that in this strain mutation of phase occurs at about the same rate per bacterial generation under various environmental conditions which cause wide differences in rates of bacterial growth.

In the test on the culture grown in Koser's citrate medium about a quarter of the colonies failed to form haloes. Some of these colonies were subcultured; the growth obtained was 'rough'. Despite the

Table 6. *Strain 5713. Experiments on mutation from group to specific phase during growth in various media*

(Temperature of incubation 37° C. in all cases.)

Medium	No. of generations	No. of colonies examined	Specific-phase colonies detected	Specific-phase colonies proportion of total	Standard error of proportion
Lemco broth	210	140	86	0.62	0.04
Peptone water pH 6	210	177	92	0.52	0.04
Peptone water pH 10	210	149	77	0.52	0.04
Koser's citrate medium	210	109*	74	0.68	0.05

* 36 non-haloed colonies omitted.

difficulty of obtaining a stable suspension it was possible to detect agglutination by H sera on a slide, and all the colonies tested in Craigie tubes grew through the soft agar overnight. It seems probable that the failure of these rough colonies to form haloes resulted from the cohesive nature of their growth, which would impede swarming away of organisms from the colony. The non-haloed colonies were omitted from the calculation of proportion of phases. No similar shift towards preponderance of rough forms was met with in any of the other experiments on this or the other strains. Possibly rough variants are at a relative advantage in a synthetic medium and tend to become predominant because of more rapid growth.

NON-HALOED COLONIES

Occasional colonies that failed to develop haloes were encountered in several strains. When such a colony was plated out on agar the growth obtained resembled that of an 'O' variant in that it was not agglutinated by either specific or group H serum but was O-agglutinable (and of smooth type). These variants remained stable during several subcultures in broth or on agar. However, when such variants were inoculated into Craigie tubes (of sloppy agar without serum) it was found that they behaved in one of two ways. One sort, less frequently encountered, failed to regain motility even during prolonged and repeated incubation in sloppy agar, and thus behaved as stable 'O' variants. The other sort always grew round if incubation at 37° C. was continued for several days, though they never grew round in 18 hr. incubation as the parent motile strains invariably did. The organism recovered from the Craigie tube had regained the full motility and H agglutinability of the original parent strain. It was at first thought that these results indicated mutation to the O state and back mutation to the H form; but it was found that if several colonies of such a strain were inoculated into Craigie tubes the motile organisms recovered were all of the same flagellar

phase, indicating probably that the apparently non-motile form retained its flagellar phase characteristic; and on repetition of slide agglutination tests, using dense suspensions, it was possible to detect a trace of agglutination of the apparently non-motile form by the H serum that agglutinated the fully motile variant obtained by passage through sloppy agar. It was concluded that these forms were the result of a mutation producing very poor development of flagella but not complete loss, and that back-mutation to normal flagellar development took place with sufficient frequency for it to be detected by the use of a selective method.

When the strains other than 5710 were grown in broth, non-motile or poorly motile variants never formed more than 2% of the cultures. In strain 5710, however, by the 339th generation both the specific and the group cultures consisted predominantly of apparently non-motile forms from which normally motile forms could be recovered by passage through sloppy agar.

It seems then that, in addition to mutations affecting flagellar antigenic phase, mutations and back-mutations affecting degree of flagellar development and mutations to loss of flagella also occur in some of the strains studied; no data on the rates of these mutations have been obtained, and it is not known whether the rapid increase in proportion of poorly motile forms in strain 5710 is due to rapid mutation to this form, and slow back-mutation, or to a higher growth rate of the poorly motile variant in Lemco broth at 23° C.

DISCUSSION

The experiments here reported in general support the hypothesis that change of phase in *Salm. typhi-murium* is due to mutations and back-mutations occurring at random, at average rates per generation cycle that are constant for any particular strain. Moreover, they show that in all the strains investigated the rates are such that any culture will tend, as it grows, towards an equilibrium composition in

which the specific phase predominates, and that the number of generations required for this equilibrium to be approached will vary from several hundred in the most rapidly mutating strains, to many thousands in slowly mutating strains.

It is of interest to consider in the light of these findings the probable behaviour, as to change in proportion of the two phases, of strains of *Salm. typhi-murium* in their normal habitat. If the two phases are equally adapted to parasitic (or commensal) existence, as they are to existence under laboratory conditions, and if any antibody response by the host is without selective action, it may be expected that a rapidly mutating strain will approach its equilibrium composition during an infection lasting several days, even if the infection arose from a single cell of either phase. Thus such strains will usually be isolated in mixed phase. In the slowly mutating strains, if an infection is established by a single cell the infecting strain will consist almost entirely of one phase even after several days, and if infection in the next host arises from ingestion of only a few cells these are likely to be all of the predominant phase, so that the strain will regain its initial homogeneity of phase. Thus the slowly mutating strains will usually be isolated almost entirely in one phase; in view of the relative frequencies of the two mutations, it may be expected that a majority of such clones will be predominantly in specific phase and a minority predominantly in group phase. Unless large numbers of colonies are tested, such strains will appear to be entirely in one phase. Little has been found in the literature as to the phase of *Salm. typhi-murium* as isolated from natural infections. Tomenius (1942) reported the condition as to phase (determined by Wassén's (1930) method) of strains of *Salm. typhi-murium*, *Salm. paratyphi* B and *Salm. saint-paul* isolated in primary culture from faeces, and for each species found some strains in mixed phase and some in one phase only; in each species the number of strains in specific phase was considerably greater than the number in group phase, the figures for *Salm. typhi-murium* being specific 106, group 47, mixed 15.

Strains described as being of variety *binns* were formerly believed to exist in the non-specific phase only. Edwards (1936) reported the presence of specific cells in strains so described. The two strains investigated in the present work each contained a small proportion of specific cells when received from the National Collection of Type Cultures, and the rates of mutation of each were such that at equilibrium each strain would be predominantly of specific phase. The small proportion of specific cells in the cultures as received presumably represents the result of a slow increase of specific elements by mutation during growth while in stock culture, in strains which happened to be isolated in pure group phase.

Although strain 'Binns' (N.C.T.C. 73) was isolated in 1917 the number of generations it has undergone since isolation is probably not enormous, for it is the practice of the N.C.T.C. to subculture *Salmonella* strains only at intervals of several years. The two *binns* strains investigated do not seem to differ (with respect to phase) from strains such as 6817 in any way except that they happen to have been isolated in group phase. Edwards (1936) examined several strains of variety *binns* and found that they lacked the somatic antigen V and thus fell into the antigenic group described as variety *copenhagen*. In the present investigation, however, two strains (5715 and 6817) possessing antigen V were found to have rates of mutation from group to specific so low that they might have been described as of variety *binns* had they chanced to have been isolated in pure group phase. If the two *binns* strains investigated are typical it would seem that the label 'variety *binns*' does not describe any permanent characteristic of the strains; its use might perhaps be confined to strains such as that mentioned by Edwards & Bruner (1946) in which a specific phase could not be found even by growth in soft agar with added group serum.

In the strains investigated some correlation of the two mutation rates seems to be present, those strains which mutate most rapidly from group to specific also mutating in the reverse direction more rapidly than the other strains. Edwards & Bruner (1946) noted that in *Salmonella* strains in which one phase could be found only by the use of the serum and sloppy agar method the phase so obtained was also unusually stable; that is, the rate of mutation in each direction was low. This suggests that correlation of the rates of the two mutations of phase may be a general phenomenon in *Salmonella* species.

In all the *Salm. typhi-murium* strains examined, mutation from group to specific phase occurred with a higher frequency than the reverse mutation. Andrewes (1925), working with *Salm. typhi-murium*, *Salm. newport*, *Salm. stanley*, *Salm. paratyphi* B, *Salm. cholerae-suis* and *Salm. paratyphi* C, noted that the specific phase seemed more stable than the group. It may thus be that a more rapid rate of mutation from group to specific than in the reverse direction is a widespread characteristic of *Salmonella* species having a 'group' phase in which flagellar antigens of the 1, 2, 5 series are present.

The present work throws no light on the mechanism of mutation of phase; it may be presumed to be similar to that of other bacterial mutations. Some of the rates found are higher than most mutation rates previously recorded in bacteria, but even higher rates have been reported, e.g. that for mutation from rough to smooth in an unstable rough variant of *Salm. typhi-murium*, investigated by Deskowitz & Shapiro (1935); they found the rate of mutation to be 4.5×10^{-2} .

Nor does this work elucidate the biological function, if any, of mutability of flagellar phase. The behaviour of *Salm. typhi-murium* in regard to mutability of phase is in some ways comparable with that of *Chromobacterium prodigiosum* (*Serratia marcescens*) in regard to colonial pigmentation, as described by Bunting (1940). She found that the degree of colonial pigmentation was liable to alteration by mutation and back-mutation, that the rates of logarithmic growth of both the types were equal and that during prolonged logarithmic growth the proportion of the types tended to an equilibrium determined only by the mutation rates. But in this organism the less pigmented form, which was in a minority in the logarithmically growing culture of equilibrium composition, became predominant in cultures allowed to become overcrowded, presumably because it was better adapted to this (altered) environment. By contrast, in *Salmonella typhi-murium* the two phases seem equally well adapted to existence in any environment, with the possible exception of one containing one of the homologous flagellar antibodies. When one considers the widespread occurrence of the diphasic phenomenon in the *Salmonella* group, and its rarity in the related but non-pathogenic coliforms (Kauffmann, 1947) one is tempted to speculate on the possible value of mutation of flagellar phase to the parasite as a means of retaining its motility despite an antibody response by the host: one may compare the antigenic mutations of *Treponema duttoni* which accompany relapses. But it must be admitted that there is at present no evidence to support (or refute) this speculation.

SUMMARY

Change of flagellar antigenic phase in the *Salmonellas* is considered as resulting from mutation and back-mutation.

A rapid method of determining the phase of numerous colonies of *Salmonella typhi-murium* is described; melted sloppy agar containing antibody for one phase is layered on to plates on which colonies have grown. On brief incubation, colonies of the agglutinated phase produce narrow dense sharp-edged zones of opacity, while colonies of the other phase produce wide diffuse zones of swarming organisms.

A theoretical analysis shows that if the growth rates of the phases are equal the proportion of cells of

'mutant' phase in a culture initially all in one phase and maintained in continuous logarithmic growth will (at first) increase linearly with the number of generations at a rate proportional to the mutation rate. In one strain of *Salm. typhi-murium* experiments showed the rates of growth of the phases to be equal; the proportion of cells of mutant phase in cultures of this strain during long periods of continuous growth has been measured; linear rates of increase were found, and the mutation rates are calculated.

The variance in proportion of cells of mutant phase in replicate cultures has been measured; the findings are correlated with theoretical considerations.

Certain anomalous decreases in proportion of cells of mutant phase during prolonged growth are described, and a possible explanation is proposed.

Eight further strains have been investigated by the same methods; in the nine strains, the rates of mutation from group to specific phase range from 10^{-4} to 4.7×10^{-3} per bacterial generation cycle, and the rates of the reverse mutation from 10^{-5} to 8.6×10^{-4} . In all the strains, including two of variety *binns*, the rate of mutation from group to specific is higher than the rate of the reverse change; it is shown theoretically that such strains will tend towards an equilibrium composition in which the specific phase will predominate; in one rapidly mutating strain such an equilibrium has been demonstrated.

In a limited experiment, alteration of medium and of temperature of incubation caused no detectable alteration of rate of mutation per bacterial generation.

Stable O mutants, and poorly flagellated mutants which back-mutate to the fully motile form, are described.

The experimental findings are compared with reports in the literature on the phase of *Salm. typhi-murium* as isolated. The behaviour of strains of variety *binns* is discussed, and it is suggested that this description should not now be applied to strains in which a specific phase can be demonstrated. The biological function of mutability of phase is discussed.

I am indebted to Mr P. Armitage, of the Medical Research Council's Statistical Research Unit, for much assistance with the mathematical theory of the problem, and also for the statistical computations.

Colonies of *Salm. typhi-murium* strain C77 layered with sloppy agar containing H anti-serum for the specific phase.

Eight specific-phase colonies with sharp haloes are visible, the dark centres being the original colonies: there are two group-phase colonies with diffuse haloes, and three O colonies without haloes, one of them being partly masked by the halo of the adjacent specific-phase colony. (Diameter of Petri dish 10 cm.)

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