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Response of *Escherichia coli* to Ferrous Ions. III. Application of a Method for Estimating Cellular Division to a Study of Recovery and Mutation

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RESPONSE OF *ESCHERICHIA COLI* TO FERROUS IONS

III. Application of a Method for Estimating Cellular Division to a Study of Recovery and Mutation

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Exposure of a suspension of *Escherichia coli* to ferrous ions at 1 C has been shown to elicit a considerable reduction in the fraction of viable cells (Catlin, 1953*a*, 1953*b*). A large proportion of "nonviable" cells of the same suspension will recover viability during a period of supplementary treatment at 37 C in the presence of nitrogen. Such estimates of cellular viability or survival are based on counts of the number of colonies which grow out on an appropriate agar medium during incubation of plates inoculated with suitable dilutions of cells. Cellular division that is limited to a few generations is not detected by this method. The category of cells characterized as nonviable on the basis of failure to form macrocolonies can be subdivided, however, on the basis of differences in growth response, which are revealed by a microscopic method. Thus, three classes can be discriminated: cells having capacity for a limited number of divisions, cells having capacity for limited growth (increase in cellular mass) but not division, and cells exhibiting capacity for neither growth nor division. Such differences may reflect different kinds or magnitudes of cellular injury. A study of the growth response of cells after exposure to ferrous ions was undertaken, therefore, using a quantitative microscopic method for determining the occurrence and number of divisions undergone by cells during the first eight hours of incubation on agar (Catlin, 1954). These results are presented, together with further data on effects of ferrous ions.

EXPERIMENTAL METHODS

Bacterial strain. A streptomycin-dependent strain (Sd-4) of *Escherichia coli* was used in all experiments, and has been described (Bertani, 1951; Catlin, 1953*a*). To increase homogeneity of cellular response, single colony isolations were made periodically from the stock strain streaked on nutrient agar (Difco) containing 10 µg per ml of dihydrostreptomycin sulfate (Lilly). Test populations were grown for 20 hr with aeration at 37 C in 100 ml of nutrient broth (Difco) containing 10 µg per ml of dihydrostreptomycin.

Method of exposure to Fe^{++}. Cells were harvested aseptically by centrifugation, washed once in sterile distilled water, and resuspended in half the original volume of water. Portions of 12 or 15 ml were pipetted immediately into sterile 50-ml cylinders closed with 2-hole rubber stoppers. One hole of each stopper was fitted with glass tubing which extended nearly to the bottom of the cylinder. The second hole served as sampling port and was fitted with a removable piece of tapered glass tubing plugged with cotton. The cylinders were placed in a chipped-ice bath (1 C) and streams of humidified, high purity nitrogen (Linde, dry) were passed through the cellular suspensions for 5 to 10 minutes.

A freshly prepared solution (3.0 or 5.0 × 10^{-5} M final concentration) of ferrous sulfate, chilled to 1 C, was introduced through the sampling ports. A control, used in every experiment, consisted of a companion suspension to which was added an equal volume of sterile distilled water. The period of cellular exposure at 1 C, which was generally 30 min, was followed by a period of exposure at 37 C. The temperature change was effected by transferring the cylinders to a 37 C water bath. Nitrogen was employed continuously in some tests; in others, oxygen (Puritan, U.S.P.) was substituted for nitrogen for a period ranging from 5 to 10 min.

At intervals samples of each suspension were removed through the sampling port with the aid of sterile, long capillary, Pasteur pipettes. One ml of each sample was diluted in a 9.0-ml sterile water blank, which was adjusted to the temperature corresponding to that of the sample. Further dilutions, as required for determining

the number of viable cells, were prepared promptly in water containing inorganic salts (C medium without glucose, Catlin, 1953a). Suitable dilutions were spread in triplicate on the surfaces of plates of nutrient agar containing 50 μg per ml dihydrostreptomycin. The frequency of reversion from streptomycin-dependence to nondependence also was determined for every sample. Reversion frequency was computed from the average number of revertant colonies that appeared on 9 plates of streptomycin-free nutrient agar, each spread with 0.1 ml of the 10^{-1} dilution of cells (Catlin, 1953a).

Agar block method for estimating cellular division. Cellular suspensions, diluted 10^{-2} in sterile distilled water, were pipetted (0.1- or 0.05-ml volumes) and spread on the surfaces of prewarmed agar plates. Since cellular division may be stimulated in a crowded area by the metabolic products of adjacent cells, suspensions must be diluted and spread suitably. Nutrient-agar (either streptomycin-free or containing 10 μg per ml dihydrostreptomycin, poured in 20 ml-quantities in pressed glass dishes) was used without special clarification in order to duplicate growth conditions found on assay plates.

Inoculated plates were incubated at 37 C and at accurately timed intervals were sampled aseptically by cutting out squares of agar. This step was carried out in a walk-in incubator to avoid changes of temperature that might modify growth processes. Agar blocks, placed cell-side up on microscope slides, were exposed for two or more hours to formaldehyde (37 per cent) vapor. After fixation, the blocks were gently covered with dye-treated cover glasses and were sealed with melted paraffin. The dye mixture contained two volumes of Dienes stain (methylene blue, 2.5 g; azur II, 1.25 g; maltose, 10 g; sodium carbonate, 0.25 g; distilled water, 100 ml) and one volume of crystal violet (crystal violet, 1.0 g; maltose, 10 g; distilled water, 100 ml). This solution, filtered before use, was swabbed on clean cover glasses and allowed to dry.

In properly prepared mounts the position and number of cells in microcolonies is undisturbed. Using an oil immersion objective, the number of cells occurring singly, in pairs, and in larger aggregates can be observed and counted accurately. The numbers of generations or serial divisions were scored as follows: paired cells,

one division; aggregates containing 3-4 cells, 2 divisions; 5-8 cells, 3 divisions; 9-16 cells, 4 divisions; 17-32 cells, 5 divisions; 33-64 cells, 6 divisions. An aggregate containing more than 64 cells was scored as having undergone 7 or more serial divisions.

Generation time determinations. Using a Coleman nephelocolorimeter, model 9, and conventional nephelometric methods, the rates of growth of cells in nutrient broth containing 10 μg per ml dihydrostreptomycin were determined. Cultures in matched Coleman cuvettes were incubated with aeration at 37 C. Periodically, nephelometric readings were taken (as Coleman nephelos standard units). Growth curves plotted from these data (ordinate, $\log_{10}$ of nephelos units; abscissa, time of incubation) were used to find the minimum generation time (time required for doubling of nephelos units during the exponential phase).

Photomicrography. The following optical apparatus were used for photomicrography: Leitz periplan eyepiece (either 8 × or 10 ×), Leitz apochromatic 4-mm objective, N. A. 0.95, Spencer fluorite oil immersion 2-mm objective, 93 ×, N. A. 1.3. A yellow filter was employed. Magnification obtained with each combination of objective and eyepiece was determined using a stage micrometer. Photographs were not enlarged further.

RESULTS

Periodic sampling of a cellular suspension of *E. coli* during exposure to ferrous ions reveals rapid changes in number of viable (colony-forming) cells. Data from six experiments are presented in figure 1 to show that a consistent pattern of population response is obtained in spite of differences in survival assays. At 1 C, a rapid reduction in number of viable cells occurred. When nitrogen was used continuously during exposure to Fe^{++}, and the temperature was changed from 1 C to 37 C, a correspondingly rapid increase in number of viable cells resulted (solid line curves). Maximum recovery was usually attained within 100 minutes after the temperature change. In terms of relative recovery (difference between number of viable cells before (N_0) and after (N_t) recovery/N_t), the degree of effect was 0.48, 0.44, 0.71, 0.72, 0.80 and 0.85 for the six experiments. In general, higher relative recovery is associated with lower initial survival. The effect of a brief period of oxygenation is clearly shown by the broken-line curves.
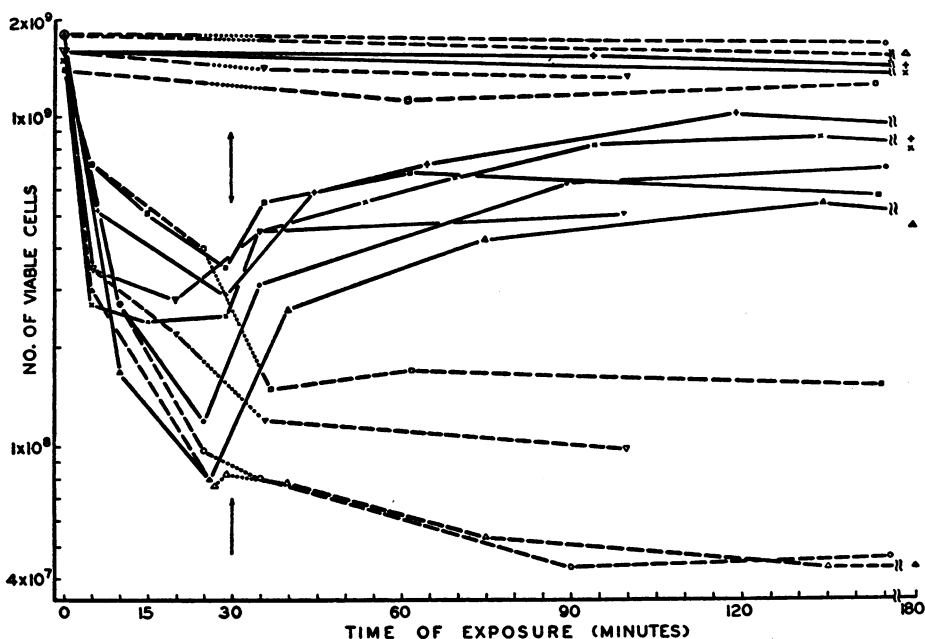
Figure 1. Effects of temperature and gas during exposure of *Escherichia coli* in 5.0 × 10⁻⁵ M FeSO₄. Temp of exposure was 1 C during the first 30 min and 37 C thereafter (arrows show time of temp change). Solid lines indicate continuous use of nitrogen. Broken lines indicate that oxygen was substituted for nitrogen during a period of 10 or 7 min (2 tests each)—beaded portions show period of oxygenation. Viability data from 6 experiments are plotted; solid and open symbols (squares, circles, and 2 sets of triangles) show results of paired tests of the same cultures; + and × symbols show results of two additional experiments using nitrogen only. The top 6 curves are controls (exposed in water) for each experiment.

When the flow of nitrogen was replaced by oxygen for a period as short as 7 min during the 1 C to 37 C change, recovery of cellular viability was presented, even though nitrogen was returned to the system during the remaining period of 37 C exposure.

The relation of time of oxygenation to time of temperature change during exposure to Fe⁺⁺ was investigated. When an 8-min period of oxygenation was used early in the 30-min period of exposure at 1 C, and nitrogen was used thereafter, recovery was about equal to that obtained in tests using nitrogen alone. When a 5- to 6-min period of oxygenation was terminated just a few minutes before the temperature change, recovery also occurred, although to a lesser degree. This is shown in figure 2, where relative recoveries obtained in three tests were 0.21, 0.44, and 0.39. Higher recoveries were not obtained, probably because an optimum degree of anaerobiosis had not been re-established during the short time before the temperature change. In simultaneous

tests of portions of the same populations, nitrogen was present until 5 or 6 min after the temperature change. Relative recoveries of 0.40, 0.56, and 0.61, respectively, were obtained during this brief period. Then, oxygen was substituted for nitrogen during 6- or 9-min intervals and a decline in viability resulted. Further exposure at 37 C in nitrogen produced little or no subsequent increase in viability.

In six experiments, observations were made of the subsequent growth response of cells removed from exposure to Fe⁺⁺ at various times before and during the recovery treatment. The agar block method for estimating cellular division and the nephelometric method of estimating minimum generation time were employed on the same samples of exposed cells. Table 1 and figures 3, 4, 5, and 6 show results of one experiment.

The survival pattern shown by the data in table 1 is similar to that of the experiments plotted in figure 1. From the results of this test alone, it could not be determined that the reduction in
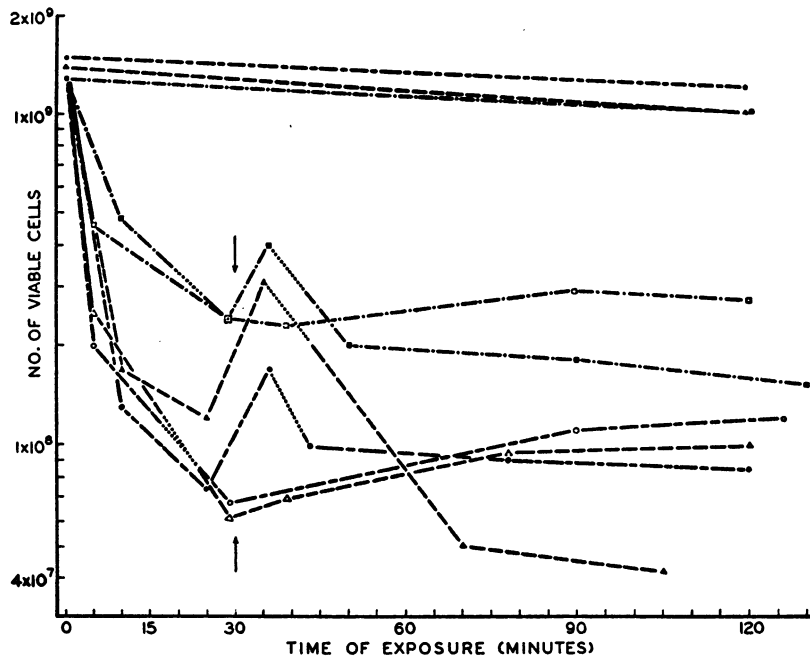
Figure 2. Effect of time of oxygenation in relation to temperature change during exposure of *Escherichia coli* in 5.0 × 10⁻⁵ M FeSO₄. Temp of exposure was 1 C during the first 30 min and 37 C thereafter (arrows show time of temp change). Nitrogen was used in each test, except during a 5- to 9-min interval (indicated by the beaded portion of curve) when oxygen was substituted. Viability data from 3 experiments are plotted. Open circles, squares, and triangles show results when oxygenation preceded the temp change; corresponding closed symbols show results using the same culture when oxygenation followed the temp change. The top 3 curves are controls for each experiment.

TABLE 1

Effects of gas and temperature on the response to Fe^{++} of Escherichia coli

Tube	Solution*	Gas Used During Various Periods			Sample Taken At:		Sd Survivors‡			Revertants	
		0–25 min	25–35 min	35 min–end	Temp†	Time	%	No. per ml	Generation time§	No. per ml	No. per 10⁸ survivors
					C	*min*			*min*		
A	Fe⁺⁺	N₂	N₂	N₂	1	24	21	3.8 × 10⁸	48	329	87
					37	62	34	6.2 × 10⁸	44	611	99
					37	120	42	7.6 × 10⁸	—	622	82
B	Fe⁺⁺	N₂	O₂	N₂	1	24	21	3.7 × 10⁸	47	338	91
					37	62	4	7.8 × 10⁷	46	400	513
C	Water	N₂	O₂	N₂	1	0	100	1.8 × 10⁹	—	167	9
					1	23	89	1.6 × 10⁹	43	122	8
					37	63	89	1.6 × 10⁹	44	111	7

* Fe⁺⁺ = 5.0 × 10⁻⁵ M FeSO₄.

† All tubes exposed at 1 C for first 30 min and at 37 C thereafter.

‡ Survivors = cells able to form macrocolonies on streptomycin agar.

§ Minimum generation time determined from growth in streptomycin broth.

generation time from 48 to 44 min was significant in the case of two samples from tube A (differences of 1 or 2 min are not significant). Results of four other experiments, however, showed a greater relative reduction in minimum generation time. In each case, the generation time determined for cells subjected to recovery treatment became equal to that of the control cells. Surviving cells of companion suspensions that were not given recovery treatment did not evidence significant reduction in generation time.

The frequency of reversion from streptomycin-dependence to nondependence was increased by exposure to Fe^{++}. As previously shown (Catlin, 1953a, 1953b) some degree of aerobiosis is required for high reversion frequency. Longer periods of oxygenation during exposure to Fe^{++} at 37 C produced higher reversion frequencies.

The photomicrographs in figure 3 show the appearance of cells on representative agar blocks of the series prepared from the experiment shown in table 1. Figure 3A shows cells (tube A, 37 C sample after exposure for 62 min in Fe^{++}) after a 3-hr period of incubation on streptomycin agar. Unenlarged cells, whose size is characteristic of resting cells of this strain, can be distinguished from the larger actively growing cells. Aggregates of two and of four cells are seen in this field. Figure 3B shows cells sampled from the same agar plate after further incubation of two hours. Three of the aggregates had undergone 4 serial divisions and the fourth group 5 serial divisions. From their arrangement, it is evident that these groups of cells represent clones derived from four different isolated cells. Cells (sampled from tube A after exposure for 24-min at 1 C to Fe^{++}) incubated on streptomycin agar for 6 and 7 hr, respectively, are shown in figures 3C and D. Aberrant cellular morphology and differences in size of microcolonies are conspicuous by this time.

Ten examples of distinctly branching or Y-shaped cells, situated among cells ranging in number from 2 to 64, were observed on slides from this one experiment. One is present in the lower left area of the largest clone in figure 3C. Branch-ing cells were produced more commonly by cells exposed to Fe^{++}, but one was seen on a control preparation. They were observed on both streptomycin agar and on streptomycin-free agar.

Dense-staining small cells are seen in figure 3E. That they do not represent merely a contaminant is suggested by the occasional finding of the same type of cell among other clones, for example figure 3F.

The interpretation of events on the basis of cytological evidences may be doubtful in many cases. In figure 3D, for example, the presence of a clone having only four cells, after 7 hr of incubation, might be due to delay in initiation of growth, to a very slow rate of division, or to the early termination of division. Because one cell shows considerable elongation, it may be inferred that growth was initiated some time earlier. Since only two serial divisions occurred, there is basis for the view that all of the cells had lost the capacity for continued division and that one, only, had retained the capacity for growth without division. Figure 3G shows a clone which has grown on streptomycin agar during an incubation period of 7 hr. The two filamentous cells evidence growth in the absence of division, whereas the other 20 cells possibly retained the capacity for division until sampled.

A considerable number of paired cells was present even after an incubation period of 6 or 7 hr. This was observed especially in the case of samples from tube B. Some cells of pairs were the size of actively growing cells, as seen in the upper left corner of figure 3C. Others, as seen in E and F, were only slightly larger than resting cells. Since bipolar distribution of metachromatic granules may simulate paired cells, and since cell walls were not specifically stained, all pairs were carefully inspected. Cells were not scored as divided unless found to be completely separate. That agglutination was responsible for the pairing seems improbable, since such cells were not observed in larger groups.

Figures 4, 5, and 6 present data obtained by the agar block method. Below each bar graph are

Figure 3. Photomicrographs of *Escherichia coli* microcolonies mounted *in situ.* Samples from Fe^{++} exposure *tubes A* or *B* of the experiment shown in table 1 were plated on streptomycin-containing (S) or streptomycin-free (P) agar and were incubated at 37 C for the designated periods. A: 37 C sample from tube A incubated 3 hr on S (800 × magnification). B: same as A after 5 hr on S (700 ×). C: 1 C sample from tube A incubated 6 hr on S (700 ×). D: same as C, after 7 hr on S (700 ×). E: 37 C sample from tube B incubated 6 hr on P (1300 ×). F: same as E, after 7 hr on P (700 ×). G: same as A, after 7 hr on S_A (700 ×).
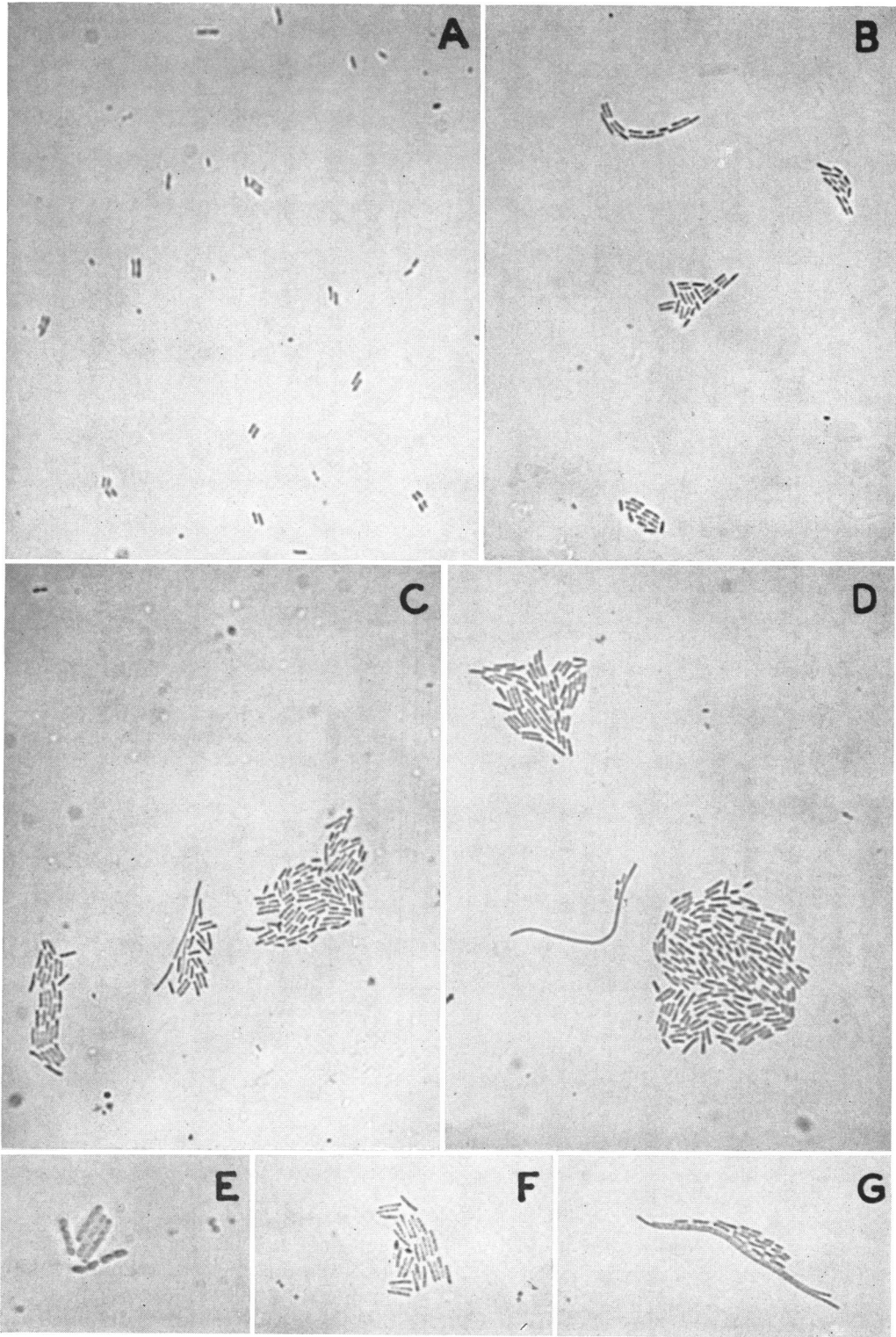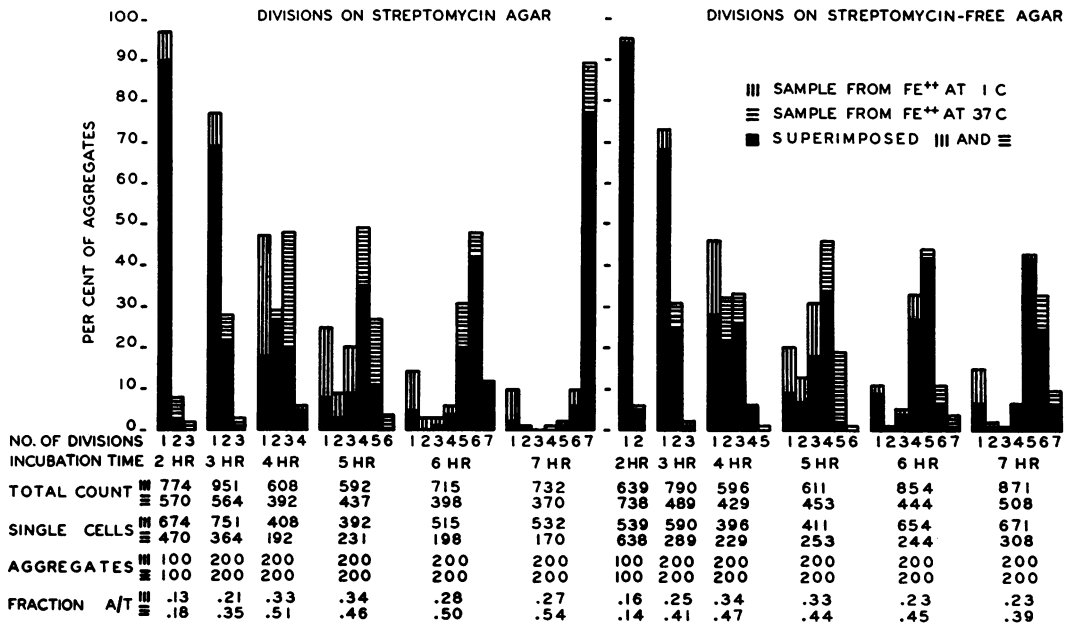
Figure 3

Figure 4. Growth response on agar of *Escherichia coli* after exposure to Fe++. Cells sampled from *tube A* of the experiment shown in table 1.

		DIVISIONS ON STREPTOMYCIN AGAR						DIVISIONS ON STREPTOMYCIN-FREE AGAR				
NO. OF DIVISIONS		1 2 3	1 2 3	1 2 3 4	1 2 3 4 5 6	1 2 3 4 5 6 7	1 2 3 4 5 6 7	1 2	1 2 3	1 2 3 4 5	1 2 3 4 5 6	1 2 3 4 5 6 7	1 2 3 4 5 6 7
INCUBATION TIME		2 HR	3 HR	4 HR	5 HR	6 HR	7 HR	2 HR	3 HR	4 HR	5 HR	6 HR	7 HR
TOTAL COUNT	III	774	951	608	592	715	732	639	790	596	611	854	871
	≡	570	564	392	437	398	370	738	489	429	453	444	508
SINGLE CELLS	III	674	751	408	392	515	532	539	590	396	411	654	671
	≡	470	364	192	231	198	170	638	289	229	253	244	308
AGGREGATES	III	100	200	200	200	200	200	100	200	200	200	200	200
	≡	100	200	200	200	200	200	100	200	200	200	200	200
FRACTION A/T	III	.13	.21	.33	.34	.28	.27	.16	.25	.34	.33	.23	.23
	≡	.18	.35	.51	.46	.50	.54	.14	.41	.47	.44	.45	.39

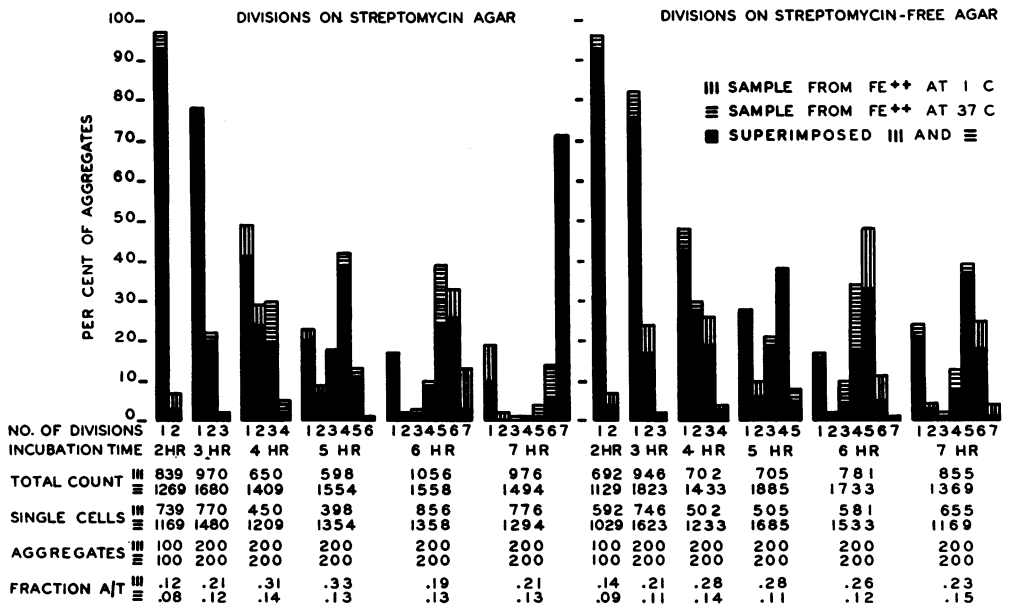Legend: III SAMPLE FROM FE++ AT 1 C ／ ≡ SAMPLE FROM FE++ AT 37 C ／ ■ SUPERIMPOSED III AND ≡

Figure 5. Growth response on agar of *Escherichia coli* after exposure to Fe++. Cells sampled from *tube B* of the experiment shown in table 1.

		DIVISIONS ON STREPTOMYCIN AGAR						DIVISIONS ON STREPTOMYCIN-FREE AGAR				
NO. OF DIVISIONS		1 2	1 2 3	1 2 3 4	1 2 3 4 5 6	1 2 3 4 5 6 7	1 2 3 4 5 6 7	1 2	1 2 3	1 2 3 4	1 2 3 4 5	1 2 3 4 5 6 7	1 2 3 4 5 6 7
INCUBATION TIME		2 HR	3 HR	4 HR	5 HR	6 HR	7 HR	2 HR	3 HR	4 HR	5 HR	6 HR	7 HR
TOTAL COUNT	III	839	970	650	598	1056	976	692	946	702	705	781	855
	≡	1269	1680	1409	1554	1558	1494	1129	1823	1433	1885	1733	1369
SINGLE CELLS	III	739	770	450	398	856	776	592	746	502	505	581	655
	≡	1169	1480	1209	1354	1358	1294	1029	1623	1233	1685	1533	1169
AGGREGATES	III	100	200	200	200	200	200	100	200	200	200	200	200
	≡	100	200	200	200	200	200	100	200	200	200	200	200
FRACTION A/T	III	.12	.21	.31	.33	.19	.21	.14	.21	.28	.28	.26	.23
	≡	.08	.12	.14	.13	.13	.13	.09	.11	.14	.11	.12	.15

Legend: III SAMPLE FROM FE++ AT 1 C ／ ≡ SAMPLE FROM FE++ AT 37 C ／ ■ SUPERIMPOSED III AND ≡

shown the number of single cells and of aggregates and the total number of cells counted for each agar block. During the early hours of incubation on agar, the fraction of aggregates/total cells partly reflects the length of the absolute lag period (time required for initiation of cellular division) and partly the fraction of survivors. At this time counts of undivided cells are most reliable. They become less reliable at later incubation times, because one tends to overlook the small, un-
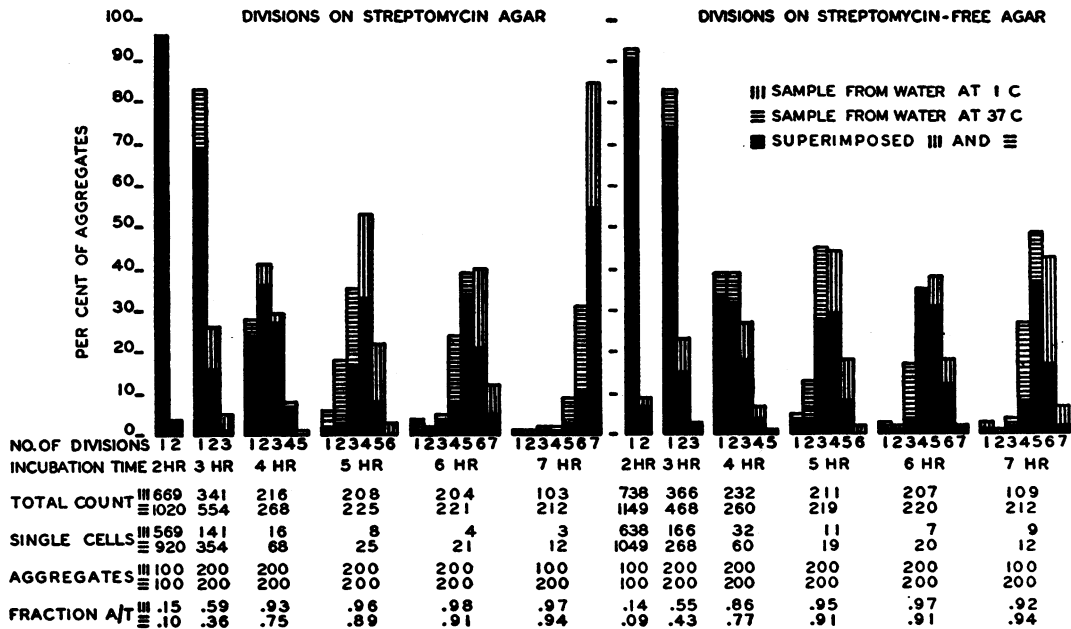
Figure 6. Growth response on agar of *Escherichia coli* after exposure to water. Cells sampled from *tube C* of the experiment shown in table 1.

stained cells when counting the cells of large clones. Nevertheless, the lower fractions of aggregates/total cells in figure 6 clearly showed a trend toward longer lag for cells of the 37 C sample, in comparison with the 1 C sample taken earlier from tube C. On the other hand, the 37 C sample of cells from Fe⁺⁺ exposure tube A (figure 4) evidenced a reduction in average lag time compared with the earlier 1 C sample.

Aggregates were grouped into seven categories, representing the number of serial divisions undergone by Fe⁺⁺ exposed and control cells. The bar graphs depict the per cent of aggregates in each category. Results for the two samples from each exposure tube are superimposed to emphasize the difference of response between the cells of tube A and tubes B and C. Cells of the 1 C samples from Fe⁺⁺ exposure tubes A and B produced similar numbers of divisions during incubation on streptomycin agar, as would be expected. Later samples from the same tubes showed divergent tendencies. Recovery treatment (tube A, 37 C sample taken at 62 min—figure 4, horizontal-lined and solid areas of bars) increased the proportion of clones numbering 65 or more cells (7 or more divisions) that were produced during 6- and 7-hour periods of incubation. In contrast, the 37 C sample from tube B (figure 5) showed a decreased proportion of large clones during com-

parable incubation periods. Numbers of divisions produced during any given incubation period were similar for the dividing cells of the 37 C sample from tube A and for the first control sample (tube C, 1 C sample taken at 23 min—figure 6, vertical-lined and solid areas of bars). However, the later (37 C) sample from tube C showed an over-all decrease in clone size in comparison with the 1 C sample from the same tube.

For approximately the first 5 hours of incubation, these streptomycin-dependent cells undergo comparable numbers of divisions on streptomycin agar and on streptomycin-free agar. Differences in clone size on the two media become apparent at the end of the fifth or sixth hour, which is about the time that exponential growth starts in tubes of aerated streptomycin broth. Conspicuous differences in morphology of cells on the two media begin to develop after about 7 hr. As incubation proceeds, the Sd cells on streptomycin-free agar gradually become filamentous and lose their viability (Bertani, 1951).

Spontaneous mutations from streptomycin-dependence to nondependence are believed to occur during the period that Sd cells are dividing on streptomycin-free agar. The occurrence of spontaneous mutations will be increased by agents that increase cellular division. Since the salts of heavy metals in high dilutions may stimu-

late bacterial growth, it was of interest to determine whether residual growth was increased by exposure of cells to Fe^{++}. The status of Fe^{++} as a mutagenic agent cannot be clarified without such data. Figures 4, 5, and 6 show that the capacity for division on streptomycin-free agar of cells from the 37 C sample of Fe^{++} exposure tube A approximated, but did not exceed that of control cells. Cells from tube B, which exhibited the highest frequency of reversion to nondependence, underwent fewer divisions than control cells. Since the procedure of plating for the agar block studies duplicates the procedure of plating for revertants, these data provide evidence that the increase in reversion frequency associated with exposure to Fe^{++} is not due to an increase in amount of residual growth produced on streptomycin-free agar.

DISCUSSION

The agar block method described represents a modification and a different application of long-existing techniques. Ørskov (1922) and later Kelly and Rahn (1932) spread bacteria from young broth cultures on agar plates. Squares of agar were cut out and placed in moist chambers for direct microscopic observation of multiplication. Kelly and Rahn examined numerous bacterial cells and reported that after transfer to agar, if multiplication started, all cells continued to multiply for the four generations observed. A comparable technique was used by Stenderup (1953) to study the effect of streptomycin on the division of individual E. coli cells and by Luria (1939) to investigate growth responses of E. coli after α- or X-radiation. Some irradiated cells divided normally, some remained undivided and unchanged, while other cells elongated, often attaining gigantic dimensions. Luria observed the formation of abortive colonies, composed of cells whose multiplication was arrested after two or three divisions. Methods of studying multiplication of individual cells on unfixed agar cultures are limited from a quantitative viewpoint. This limitation was overcome by using in situ fixation of cells on agar blocks. The Dienes method (Dienes, 1939; Leberman, et al., 1950) was adapted for staining and mounting the cells.

Using the agar block technique, a quantitative study can be made of the number of divisions undergone by cells during specified intervals of incubation. The technique is particularly useful for investigating the differential response of cells of the same population, portions of which have been subjected to different conditions of exposure to a lethal agent. Ordinary methods for estimating growth and division of bacterial cells apply at the level of the population, not at the level of the cell and, accordingly, are unable to reveal heterogeneity of response within the population. Turbidimetric methods yield average growth rate or minimum generation time determinations, but cannot distinguish between increase in cellular mass and increase in numbers of cells. Growth and division have been distinguished in recent studies of effects of chemicals and radiations (Loveless, et al., 1954; Spoerl, et al., 1954). The methods for obtaining culture dry weight (to determine the effect on growth) and microscopic count of cells (to measure the effect on division) are relatively insensitive, however, and do not detect intrapopulation differences of cellular response. Plating methods determine the number of colony-forming cells, but fail to detect cellular division that is limited to a few generations.

If counts of the number of viable cells are taken as an estimate of the capacity for division of cells after irradiation, the data will conflict with the results of short duration metabolic tests. In comparative studies of normal and X-radiated cells (E. coli strain B/r), Billen et al. (1953) found that an "essentially dead" cellular suspension (0.05 per cent survivors, based on plate counts) may respire initially at a normal rate and may produce some growth (as evidenced by turbidity increase in nutrient broth). Use of the agar block technique would show whether, with the strain and dosage employed, such a period of growth and respiratory activity after X-radiation is accompanied by cellular division, limited to a few generations.

After exposure in a solution of Fe^{++} at 1 C, cells of a population of E. coli strain Sd-4 could be divided into two categories, viable and nonviable, on the basis of capacity to form macrocolonies on agar. The nonviable fraction of the population could be subdivided into three groups, as determined by the response of the plated cells, subsequently fixed and examined in situ: (1) cells that did not divide or enlarge and showed no affinity for basic dyes, (2) cells that did not divide, but did enlarge (frequently becoming filamentous) and tend to take up basic dye—capable of "growth," (3) cells that could undergo a limited number of divisions (observed as small aggregates after an incubation time sufficient for nearby

"viable" cells to produce several hundred cells; occasionally, some cells of these aggregates also became filamentous).

The growth or division of these nonviable cells is of relatively short duration, but it provides further evidence that cellular inactivation is not complete until a certain amount of metabolic activity has occurred. This feature of duration of the events leading to inactivation provides the possibility of eliciting recovery of viability of part of a population after exposure to a physical or chemical agent by interrupting the inactivation process and changing the course of abnormal cell metabolism (for example, Kelner, 1953; Stapleton, *et al.*, 1953; Heinmets, *et al.*, 1954; Wainwright and Nevill, 1955).

Some critical step in the inactivating process initiated by Fe^{++} is oxygen sensitive at 37 C. After being subjected to Fe^{++} at 1 C, if cells are exposed to oxygen at a temperature conducive to metabolic activity either before removal from the Fe^{++} solution or as a consequence of plating, the inactivation process becomes irreversible. However, when the temperature accompanying Fe^{++} exposure was changed from 1 C to 37 C in the presence of nitrogen, a sample taken a few minutes later revealed a considerably higher fraction of viable cells than was found in an earlier sample of the same population taken at 1 C. Recovery was associated with reduction of both the proportion of undivided cells and the percentage of paired cells of the population. Thus, the capacity for recovery is not restricted to cells of only one group. In addition, populations subjected to recovery treatment exhibited a decrease in minimum generation time and a decrease in the average time required for initiation of cellular division. The recovery process, therefore, appears to be associated with a general improvement in cellular capacity for growth.

ACKNOWLEDGMENT

SUMMARY

A 90 per cent reduction in number of viable cells of a population of *Escherichia coli* strain Sd-4 (streptomycin-dependent), was found when cells were plated after a short period of exposure at 1 C in a solution of 5.0×10^{-5} M FeSO$_4$. It was

possible to interrupt the processes leading to nonviability in the case of a portion of the population by retaining the cells in Fe^{++} and raising the temperature to 37 C in the absence of oxygen. Many cells that were plated following a few minutes of this supplementary exposure were found to have "recovered viability," that is, colony-forming capacity was retained.

The nature of cellular damage produced by Fe^{++} and of this form of recovery were investigated using a quantitative method for determining the occurrence and number of divisions undergone by individual cells during specified intervals of incubation on agar plates. Observations showed that some Fe^{++} exposed cells retained the capacity for growth or even for division for a limited period after plating, but were unable to continue the division processes for the further time required for macrocolony formation, that is, they were nonviable, but not inactivated at the time of plating.

Populations subjected to recovery treatment showed a reduced proportion of two classes of nonviable cells: those that had not evidenced any growth on agar and those that had undergone division limited to a few generations. In addition, there was a general improvement in cellular capacity for growth, as evidenced by decrease in minimum generation time in broth and decrease in average time required for initiation of division on agar.

Data presented provide evidence that the increase in frequency of reversion from streptomycin-dependence to nondependence produced by exposure to Fe^{++} is not due to an increase in amount of residual growth produced by Sd-4 on streptomycin-free agar.

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