SMALL COLONY VARIANTS OF *ESCHERICHIA COLI*

MODE OF ACTION OF COPPER IN VARIANT RECOVERY AND POPULATION DYNAMICS OF CULTURES CONTAINING VARIANTS[1]

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Small colony variants of bacteria usually appear in populations exposed to stresses such as metal ions and antibiotics, or in old cultures. Much work has been done on the subject (*see* Clowes and Rowley, 1955) but the exact role of these agents remains to be elucidated. The present report deals with a small colony variant of *Escherichia coli* obtained after exposure to copper ions and originally described by Weed and Longfellow (1954). Variants recovered following exposure to copper or other metals have been reported by Yanagishima (1957) and Lindegren, Nagai, and Nagai (1958) in yeast, by Stokes and Bayne (1958) in Salmonella, and by Clowes and Rowley (1955) in *E. coli*.

Some aspects of the chemical and biological mode of action of copper in the recovery of small colony variants were investigated. Variant yield was found to be unpredictable and the causes of this variability were investigated. In doing so it became necessary to study the population dynamics of cultures containing normal and variant cells in order to elucidate the selective pressures and competitive phenomena operating in such a system.

MATERIALS AND METHODS

E. coli strain B was used. The organism was grown in the liquid medium of Weed and Longfellow (1954): Na_2HPO_4, 16.5 g; KH_2PO_4, 1.5 g; $(NH_4)_2SO_4$, 2.0 g; $CaCl_2$ (1%), 1.0 ml; $FeSO_4 \cdot 7H_2O$ (1%), 0.5 ml; $MgSO_4 \cdot 7H_2O$ (20%), 1.0 ml; water, 1,000 ml; the pH was adjusted to 7.4 with HCl prior to sterilization. All reagents used were "anal. reagent" or "Am. Chem. Soc. spec." Triple distilled water was used throughout.

In most experiments the organisms were grown in pyrex test tubes (18 by 150 mm), containing 9.0 ml medium, 0.1 ml of 30% glucose, plus water, or other additions as detailed later, to a final volume of 10.0 ml. Glucose concentration was equivalent to 3 mg per ml of final medium. Inocula were made from cultures grown overnight on nutrient agar slants, 0.1 ml of a water suspension being used. All growth experiments were carried out at 37 C, without aeration.

In agreement with Weed and Longfellow (1954), the variants isolated in the presence of copper were either unable to ferment lactose (lac^-), or fermented it slowly. For plate counts and simultaneous scoring of the number of variants, EMB agar (Difco) was used. This medium contains sucrose as well as lactose. Variants were negative on both lactose and sucrose. The normal organisms were not tested for sucrose fermentation. The final pH reached on EMB agar by the normal cells is 5, whereas it is $\sim$7 for the variants. Size and appearance of colonies on the test agar were sufficiently distinctive to permit easy scoring. Plate counts were done by the surface spreading method: 0.1 ml of the respective dilution was spread uniformly on the plates, using a bent glass rod. Plates were incubated for approximately 24 hr at 37 C, prior to counting and scoring for variants. The appearance of the small colonies, as compared to the normal, large colonies, is shown in Fig. 1. The variant colonies are perfectly round, in contrast to the serrate edges of the normal colonies. Considerable variation in colony size of variants occurred on primary isolation in different experiments as well as in different tubes from the same experiment, with colony sizes ranging from small to very minute.

When growth was measured turbidimetrically,

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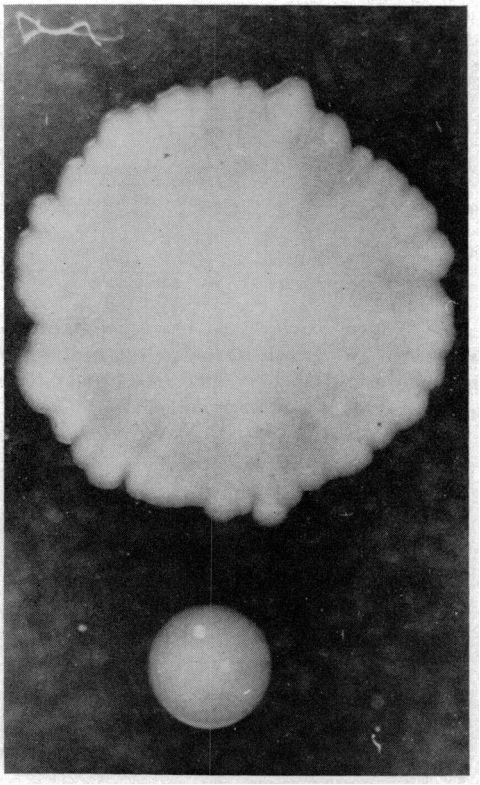

Fig. 1. Normal colony type and small colony variant on nutrient agar following incubation of 5 days at 37 C. The relative differences are just as marked after incubation for 1 or 2 days.

a Klett-Summerson photoelectric colorimeter, Klett photometer tubes, and no. 42 blue filter were used. Optical density (OD) is expressed in Klett units.

Cupric sulfate ($CuSO_4 \cdot 5H_2O$), L-cysteine, free base (Nutritional Biochemicals Corporation), and Versene (ethylenediaminetetraacetic disodium) (Eastman Chemicals Corporation) were used. Sodium styrene sulfonate (Dow) was made up in 0.05 M phosphate buffer, pH 7.2. The three latter compounds, freshly prepared for each experiment, were sterilized by filtration through an Ultrafine fritted glass filter.

Anaerobic conditions were achieved in the following manner: the experiments were run in screw-cap tubes (16 by 124 mm); the anaerobic tubes were run with caps, sealed with tape and paraffin, and covered with aluminum foil, whereas aerobic controls had cotton stoppers. Prior to inoculation, all tubes were heated in a boiling water bath for 4 min, quickly cooled, inoculated, and sealed. Maintainance of anaerobiosis in uninoculated controls was checked using the Fildes-methylene blue method (Society of American Bacteriologists, 1957); anaerobic conditions were maintained indefinitely in tubes thus sealed.

RESULTS

A) Isolation of small colony variants in the presence of copper. Optimal yield of small colony variants occurs when copper ions are present at a final concentration of 15×10^{-6} M. Lower concentrations were not as effective; this is in contrast to results of Weed and Longfellow (1954), who obtained variants in the presence of 5×10^{-6} M copper ions, using the same strain.

TABLE 1

*Growth of Escherichia coli strain B in the presence of 15×10^{-6} M copper sulfate and its relation to small colony variant recovery**

Total number of tubes used: 16

Time Needed to Obtain Full Growth	Number of Tubes	OD	Proportion of Variants in Total Population†
hr			
70	7	207	4+
		208	2+
		204	−
		215	4+
		197	2+
		212	−
		222	+
94	4	191	−
		174	2+
		188	4+
		183	−
118	3	197	+
		201	4+
		179	4+
142	1	193	+
165	1	224	4+

* Control tubes without copper reach full growth (OD 190–220) within 24 hr, corresponding to a viable count of approximately 10^9 cells/ml. Similar counts are reached by all the tubes following eventual attainment of full growth.

† 4+ = High proportion of variants (50–80% of total population); 2+ = medium proportion of variants (10–40% of total population); + = low proportion of variants (<1% of total population); − = no variants seen.

Variants were not observed in the absence of copper ions. Culture of the organism in the presence of copper ions is marked by initial inhibition, followed by growth and recovery of variants. Growth inhibition and variant yield are extremely variable, not only from experiment to experiment, but also in replicate tubes in the same experiment. This is shown by Table 1, which presents data from a typical experiment. A number of tubes were incubated until growth in each tube was completed, at which point optical density determination, plate count, and scoring for variants were done. The time needed for complete growth, eventually obtained in all tubes, varied from 70 to 165 hr; the proportion of variants varied in different tubes from 0 to 80% of the total population. The same is illustrated by another experiment where all tubes were measured after 48 hr growth; optical density at that time varied in different tubes from 5 to 216; i.e., from inhibition to full growth. It can be seen from Table 1 that in some tubes no variants were observed even though full growth had been obtained; in other experiments, variants were seen in considerable number in tubes where growth was still greatly inhibited.

It was believed this variability might constitute an explanation for conflicting results in work on small colony variants obtained by previous investigators, and an attempt was therefore made to throw light on the causes of this variability. The first experiment consisted of letting a number of cultures reach the end of the exponential growth phase; the fate of the variants in such populations was then determined. Serial sampling from each culture was done. Such sampling, by disturbing and aerating the cultures, may alter the rate and extent of population changes, as compared with undisturbed cultures. It was, however, the only approach possible because of the wide variability in proportion of variants in replicate tubes, which precludes serially independent sampling. Results from two representative cultures are given in Fig. 2. It is clear that the variants, even where present in high proportions, disappear from the population on continued incubation. After the exponential phase of growth, an equilibrium is reached, with the population census remaining fairly constant. As it can be assumed that at the equilibrium point the cells dying are balanced by those being formed it is obvious that no new

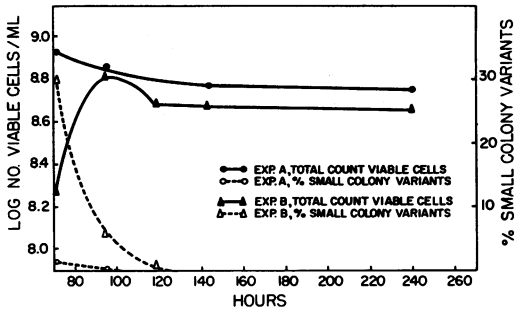

Fig. 2. Fate of variants in a population of *Escherichia coli* grown in the presence of copper (final concentration 15×10^{-6} M). All counts are means from duplicate determinations.

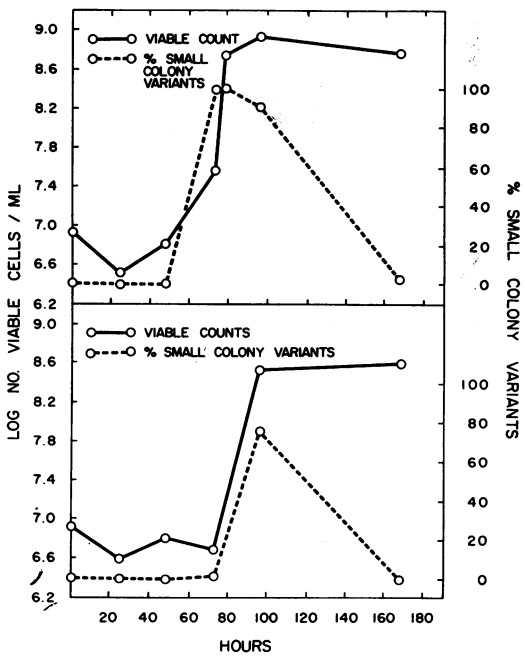

Fig. 3. Population changes in *Escherichia coli* exposed to copper ions (15×10^{-6} M final concentration). All counts are means from duplicate determinations.

variants are produced at that stage. Eventual elimination of the variants from populations occurred with regularity.

Further experiments were concerned with determining the fate of the variants during the whole growth cycle. Results are given in Fig. 3. In the presence of copper there occurs an initial drop in the viable count; a varying period of inhibition is followed by the exponential growth

TABLE 2

Behavior of two different variant isolates in the absence of copper

Time	Expt 1b			Expt 3b				
	Total count (cells/ml)	Per cent normal cells	Per cent variant cells	Total count (cells/ml)	Count, normal cells	Count, variant cells	Per cent normal cells	Per cent variant cells
hr								
0		0	100				5	95
22.5	8.57×10^8	0.3	99.7	8.94×10^8	3.09×10^8	5.85×10^8	34.6	65.4
48	3.47×10^8	0.5	99.5	3.65×10^8	2.70×10^8	9.5×10^7	74.0	26.0
144	8.5×10^7	0	100	1.59×10^8	1.58×10^8	1×10^6	99.4	0.6

phase. Roughly concomitant with, or some time following the beginning of the log phase, there is a rapid increase in the number of variants. In some, but by no means all, cases close to 100% of the population may consist of variant cells. This is followed by a dramatic and sustained decrease in the number of variants, elimination of variants from the population, and their replacement by phenotypically normal cells.

The displacement of the variants by normal cells may essentially be due to one of three possibilities: (i) replacement of variants through growth of phenotypically normal cells already present in the population, (ii) replacement of variants by normal cells by way of the variant cells giving rise to normal daughter cells, or (iii) a transformation of variants into normals via a physiological change in the absence of growth.

To decide between these possibilities, it is necessary to know more about the stability, growth, and decay of the variant cells in pure isolates. To test this, a number of variants were grown in liquid medium either in presence or absence of copper, and periodically assayed for count and colony characteristics. Table 2 records results from two experiments conducted in the absence of copper.

As far as pure culture isolates of the variants are concerned, the characters "small colony," "lac$^-$ (slow on lac)," and "resistance to copper" are preserved through numerous platings on solid media. The colonial characteristic is, however, less stable than the other two, variations in colony size occurring sometimes in early replatings from an original isolate.

Table 2 gives data from experiments with two fresh isolates. Experiment 1b represents a stable variant; experiment 3b an isolate which had partially reverted back to the normal phenotype on solid agar, a phenomenon encountered only once in the course of these investigations. In this experiment we are thus dealing with an initially mixed population.

It is apparent from the table that in a population consisting entirely of variants, no reversions to normal phenotype took place during the period of observation. The same was found true of such isolates in similar experiments conducted in the presence of copper. On the other hand, in an initially mixed population, the normal cells are able to displace completely the variant cells, thus corroborating the previous results.

These experiments show conclusively that the disappearance of the variants cannot be due to a physiological conversion of the variants to normal cells. We are, therefore, dealing with either a replacement of the variants by normals still present in the population, or with their replacement by normal daughter offspring. The data clearly favor the first hypothesis, although the second possibility may be a contributing factor at times. It can be seen from Table 2 that reduction in the census of the variants is much more rapid in mixed than in pure culture. One might thus assume that the variants are displaced because the normal cells release a substance toxic to them. This may be part of the explanation but is not the whole because the variants disappear even in mixed cultures where they initially approach close to 100% of the total population (*see* Fig. 3); an inherent instability of the variants thus must be a factor also. When normal and variant cells were plated out in close proximity, no inhibition of one form by the other was noted.

It remained to determine the growth behavior of normal and variant isolates in the presence and absence of copper. Data are given in Fig. 4.

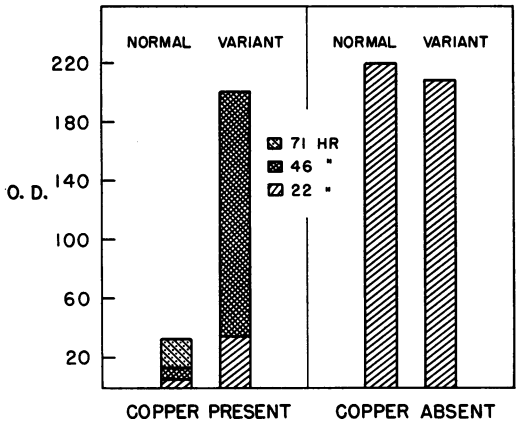

Fig. 4. Growth of normal and variant cells in the presence and absence of copper ions. Each bar represents the mean from 10 replicates (copper present) or from 5 replicates (copper absent). Final copper concentration, 15×10^{-6} M.

The variant used in this experiment was carried for 18 transfers on nutrient agar slants in the absence of copper ions; normal and variant cells were adjusted to the same optical density before inoculating the tubes.

In the absence of copper, the variants lag behind the normal cells, even on glucose. (The final population reached on a given quantity of glucose is the same with normal and variant cells, indicating no gross differences in carbohydrate metabolism such as shown, e.g., in case of the "petite" mutant by Ephrussi and his co-workers (1950–1951).) This lag was seen all during the growth cycle and is still observed after 22 hr incubation; the difference at that time is statistically significant at the 1% level. In the presence of copper, on the other hand, the variants have a decided advantage over the normal cells (*see also* Fig. 5). There is no doubt, therefore, that the initial ascendance of the variants in the population is due to their increased resistance to the toxic action of copper ions. This ascendance is quite temporary, however. Table 2 (experiment 3b), and Figs. 2 and 3 show that phenotypically normal cells eventually displace the variant cells from the population. This poses the interesting question of whether these normal-appearing (lactose-positive, large colony-forming) cells are more copper-resistant than the original wild-type cells. Data bearing on this question are given in Fig. 5. It is clear that the cells that take over after the variants disappear are considerably more resistant to the toxic action of copper than the wild-type population, although they are not nearly as copper-resistant as the variant cells. As pointed out above, there is good evidence that these phenotypically normal cells arise from cells present in the original population prior to the establishment of the variants. The present data thus give clear indication that the wild-type population is heterogeneous with respect to resistance to copper. It is very likely that these cells are able to compete with the variant cells better than the more copper-susceptible cells, and thus may be able to displace the variants before they are able to gain ascendance in the population. This explains, at least in part, the observation that the proportion of variant cells in the population frequently remains quite low, and that at times cultures wish few variants reach full growth before culturet in which many variants are present.

Two factors responsible for the great variability observed with respect to variant formation have been pinpointed: (i) competitive interplay between normal and variant cells and eventual displacement of the variants from the population

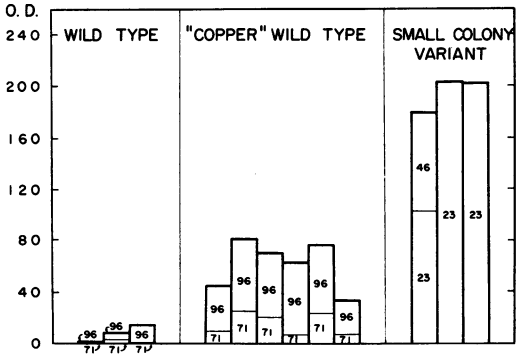

Fig. 5. Growth in the presence of copper of cells with the following characteristics: (i) Wild type. These are normal cells grown in the absence of copper. (ii) "Copper" wild type. These are phenotypically normal cells appearing in tubes containing copper after the disappearance of the variant cells (*see* Figs. 2 and 3). (iii) Small colony variants. These are variants isolated during growth in the presence of copper.

Final copper concentration: 15×10^{-6} M. All inocula were adjusted to the same optical density before seeding the tubes. Each bar represents a different isolate. Each individual bar represents the mean from triplicate determinations. The numbers inside the bars indicate the time (in hours) at which optical density was measured.

and (ii) presence in the population, prior to the establishment of the variants, of wild-type cells which differ with respect to their susceptibility to the toxic action of copper. A third important factor which also contributes to the variability, namely oxidation-reduction potential, will be discussed later.

Fig. 5 indicates that there are differences in copper resistance also between different variant isolates. Of particular interest is the fact, observed many times, that there is a direct correlation between resistance to copper on one hand and colony size and lactose fermentation on the other. The very small, lactose-negative colonies are highly copper-resistant, whereas increase in size of the colony and increase in ability to ferment lactose accompany increasing susceptibility to the toxic action of copper.

In conclusion, it can be said that the variants, due to their greatly increased resistance to copper, frequently, though not always, are able to take over temporarily, but are displaced from the population once more by phenotypically wild-type cells of a type more resistant to the toxic action of copper than the original wild-type population. Whether these latter cells were already present in small numbers in the original inoculum or whether they arose during the initial slow growth of the wild-type cells in the presence of copper is not known.

B) Considerations regarding state of copper responsible for biological effects. To understand the biological action of copper in the present system, it is necessary to determine the initial chemical state of copper before inoculation of the medium, which had a final concentration of cupric sulfate of 15×10^{-6} M. In determining the concentration of free cupric ions and other species of copper, the following complexes or precipitates must be considered (solubility products and stability constants were taken from Bjerrum (1958)): (i) precipitation of cupric phosphate, $Cu_3(PO_4)_2 \cdot 3H_2O$; (ii) precipitation of cupric hydroxide, $Cu(OH)_2$; (iii) formation of the $Cu(OH)^+$ soluble complex; and (iv) formation of the soluble $CuCl^+$ complex.

No data were found in the literature concerning the solubility product constant of cupric phosphate. Experimentally, no precipitate was formed, with the concentrations of reactants used, even on prolonged standing. No evidence has been found for the existence of a complex between cupric ions and phosphate (Genge et al., 1955). $Cu(OH)_2$ formation can be disregarded (solubility product constant is 10^{-19}). However, the $Cu(OH)^+$ and $CuCl^+$ complexes must be considered; without going into the detailed chemical equilibria, it can be stated that the concentration of free cupric ions is roughly 10×10^{-6} M; i.e., the greater part of the copper is present as the ion. It is reasonable to assume that the biological action of copper is due to the free copper ions, especially in view of the action of a number of chelating and complexing agents.

TABLE 3

Effect of cysteine on small colony variant recovery in Escherichia coli

	Experimental, + Copper, + Cysteine	Control, + Copper, No Cysteine	Control, No Copper, + Cysteine
Number of organisms per ml	1.44×10^9	1.23×10^9	1.34×10^9
Number of variants	0	1.03×10^9	0
Percentage	0	83.7%	0

Final concentrations: cysteine, 10^{-3} M; $CuSO_4 \cdot 5H_2O$, 15×10^{-6} M. Incubation period, 47 hr. Data are results from duplicate determinations.

C) Effect of cysteine on recovery of small colony variants. To study the action of copper in variant recovery, a number of chelating, radio-protective, and free radical-reacting substances, as well as other experimental conditions, were employed.

Cysteine was used in a final concentration of 10^{-3} M; such a concentration is without effect on the growth of *E. coli*, while higher concentrations were growth-inhibiting, and therefore unsuitable. Results are given in Table 3.

Cysteine abolishes both the growth inhibition and the appearance of variants occurring in the presence of copper ions. The action of cysteine in this system may be related to three possible factors: (i) cysteine-copper complex formation, resulting in removal of the active form of copper; (ii) the reducing action of cysteine might interfere with the ability of copper to pass through a continuous redox system involving $Cu^{++} \rightleftharpoons Cu^+$, which might be needed for copper to be effective in the isolation of variants; (iii) competition by cysteine with free radicals formed in copper-catalyzed autoxidations (*see* Hirsch, 1956).

The first possibility seems most likely. The

reaction taking place between $CuSO_4$ and excess cysteine is the following (Stricks and Kolthoff, 1951):

$$2Cu^{++} + 4RS^- \rightarrow 2CuRS + RSSR;$$

i.e., cysteine reduces bivalent copper immediately to the oxidation state +1, and it is the cuprous ion which forms a stable complex with excess cysteine. The concentration of free cuprous ion remaining is about 10^{-15} M (log K_S = 19.2 for the 1:1 complex; Stricks and Kolthoff (1951)).

D) Effect of Versene on recovery of small colony variants. Versene, which, in contrast to cysteine, keeps copper chelated in the divalent state, was used to determine its effect in this system. Versene chelates copper stoichiometrically in equimolar concentrations.

Concentrations of up to 15×10^{-5} M Versene were not toxic to *E. coli*, whereas concentrations of 75×10^{-5} M completely suppress growth of the organism. Concentrations of Versene of 15×10^{-6} M (equivalent to copper concentration) and 15×10^{-5} M (equivalent to 10× the copper concentration) were employed. Both concentrations abolished the toxicity of copper and prevented the isolation of variants. Thus, complexing of copper in either the cuprous or cupric form leads to a loss of its growth-inhibiting effect and prevents the recovery of variants. This indicates that the copper ions must be bound to a cell site before they can exert their effect, and that if they are already bound by the chelating agents no effect is seen. This is not something which could have been assumed, as the metal complex or chelate is frequently more active, both biologically and chemically, than the metal ion itself (Chalk and Smith, 1954; Green, Mazur, and Shorr, 1956).

E) Effect of anaerobiosis on recovery of small colony variants. The data in sections *C* and *D* show that copper must be present as the free ion in order to give its effect. This could be due to one of the following possible alternatives: (i) copper must be present as free cupric ion because of fixation to the cell in that valence state; (ii) copper must be present as the free cuprous ion because of fixation to the cell in that valence state; or (iii) copper must be able to go through a continuous oxido-reduction, $Cu^+ \rightleftharpoons Cu^{++}$.

The use of cuprous copper to test this is precluded because of the instability of the cuprous ion in the presence of oxygen. The problem was approached through the maintainance of anaerobic conditions during growth where one can expect that the copper would be reduced to, and remain reduced in, the cuprous state. Data from a typical experiment are summarized in Table 4. After 22 hr incubation, there was noticeable growth in tubes grown anaerobically, as compared to those grown aerobically, in the presence of copper, although not as much as in either the tubes grown aerobically or anaerobically in the absence of copper.

After 2 days of incubation, growth was approximately equal in the tubes grown aerobically and anaerobically in the absence of copper and in the tubes grown anaerobically in the presence of copper. However, in the presence of copper and air, growth was still inhibited in 5 out of 6 cultures; only 1 culture showed growth comparable to that of the other groups, and this culture contained variants while the others did not. Anaerobic conditions thus remove the growth inhibition by copper and prevent the

TABLE 4

Isolation of small colony variants in presence or absence of copper under aerobic and anaerobic conditions

No Copper, Aerobic	+ Copper, Aerobic	No Copper, Anaerobic	+ Copper, Anaerobic
Klett OD readings			
194	51	181	220
193	67	179	215
189	216	183	209
188	63	189	210
	53		
$\bar{x}$ = 191	47	$\bar{x}$ = 183	$\bar{x}$ = 213
Plate counts (per ml) and estimate of the number of small colony variants			
5.4×10^8 No small colony variants	7.6×10^8* 66% small colony variants	6.1×10^8 No small colony variants	1.1×10^9 No small colony variants

* Represents the count on tube no. 3 (OD 216); the other tubes in this series contained no small colony variants.

Final concentration of copper sulfate, 15×10^{-6} M. Tube contents and other experimental conditions as indicated under Materials and Methods. Plate counts are means from duplicate determinations. Period of incubation at 37 C, 50½ hr.

recovery of variants. It is clear, therefore, that oxygen is needed for copper to be active in the isolation of small colony variants. As the oxidation-reduction potential can be assumed to vary from tube to tube, the data indicate another source of the variability in variant formation found in these experiments (see above).

These results eliminate alternative (ii) as copper present in the cuprous form does not lead to recovery of variants. Data given in section *G* make alternative (iii) unlikely, although they do not rule it out completely. Alternative (i) is considered most likely to be the correct one. The main objection to this would be the assumption that copper is removed under anaerobic conditions by some substance not produced aerobically. It seems doubtful that enough cysteine (*see* section *C*) is produced anaerobically to remove the copper; preliminary qualitative tests support such a conclusion. The only likely possibility would be the formation, under anaerobic conditions, of a considerable amount of H_2S which would remove the copper as sulfide. Experiments to test this are described next.

F) Production of hydrogen sulfide by E. coli strain B under aerobic and anaerobic conditions, in the presence and absence of copper. The organism gives a negative test for H_2S on Kliger's iron agar and with test strips impregnated with ferrous sulfate (SAB *Manual of Microbiological Methods*, 1957).

Impregnation of test strips with lead sulfate gave different results; the method described by the SAB *Manual of Microbiological Methods* (1957) was modified in that test strips were encased in small, open-ended glass tubes, 25 mm long, outer diameter 9 mm, sterilized, and suspended in the test tubes by thread to prevent leaching of lead salt. Results from a number of experiments are summarized in Table 5.

H_2S production occurs toward the very end of the growth cycle; however, removal of the toxicity of copper under anaerobic conditions is seen early during growth, indicating that H_2S production, followed by removal of copper as the cupric or cuprous sulfide, cannot be responsible for the fact that under anaerobic conditions growth inhibition by copper is overcome and no variants are formed. The fact that anaerobically much less H_2S is produced than aerobically supports the same conclusion. It is possible that the reduction of H_2S production under aerobic conditions in presence of copper is due in part to precipitation of H_2S as the sulfide; if this takes place, however, it does so after the copper already has exerted its biological action.

TABLE 5

Hydrogen sulfide production under varying conditions of growth

Growth Conditions	Hydrogen Sulfide Production
No copper present:	
Aerobic	++++
Anaerobic	+
Copper present:	
Aerobic	− to + (in different tubes)
Anaerobic	Trace

Both anaerobic conditions and the presence of copper modify the sulfur metabolism of *E. coli*. This may indicate that less cysteine, the probable precursor of H_2S (Desnuelle and Fromageot, 1939) or less enzyme attacking cysteine, are formed. The data tend to confirm the opinion, expressed in section E, that not enough cysteine is produced anaerobically to remove copper by chelation.

G) Effect of a free radical "trapping agent" on growth and small colony variant recovery in the presence of copper. In the presence of copper ions and air, free radicals may be formed from intermediary metabolites in the culture. It seemed possible that the growth inhibition and the appearance of variants obtained in the presence of copper might actually be due to such substances rather than to the copper ions. This seemed a possibility because Weed and Longfellow (1952) reported that vitamin C also was active in obtaining variants in *E. coli*. The action of ascorbate might be due to hydrogen peroxide formation, involving, via a Fenton reaction, the action of free radicals.

To test this hypothesis, a free radical trapping agent was added to determine whether growth inhibition could be removed and variant recovery prevented. The trapping agent used was a vinyl monomer, sodium styrene sulfonate. A similar approach was used by Uri (1954, 1955) in his demonstration of free radical intermediates during photosynthesis by intact Chlorella, and by Parravano (1951) in experiments on initiation of free radical reactions by enzymatic redox processes.

Polarographic experiments showed that com-

plex formation between sodium styrene sulfonate and copper did not take place; the half-wave potential observed with a solution containing 2×10^{-4} M cupric ions in 0.1 KNO_3 was not changed to any significant degree by the addition of 10^{-2} or 10^{-3} M sodium styrene sulfonate. No precipitation of polymer would be expected here, as the polymer formed would be relatively small and soluble and oxygen present would act as chainbreaker.

Experiments in absence of copper showed that concentrations of sodium styrene sulfonate of from 10^{-6} to 10^{-2} M had no effect on the growth of *E. coli*.

Addition of sodium styrene sulfonate to the growth system (final concentrations, 10^{-2} M and 10^{-3} M) in the presence of copper was without consistent effect, either on growth inhibition or variant recovery.

This constitutes strong evidence that the action of copper is not due to the production, initiated in the presence of cupric ions, of highly reactive free radicals (e.g., OH·). They do not eliminate the possibility that more stable free radical species (e.g., RS·) are involved, because these probably would not react with the vinyl monomer; a more reactive scavenger would be needed to detect the presence of such free radicals. The simplest hypothesis, however, is that the action of copper is due not to a secondary production of free radicals, but to its direct interaction with the cells; this interaction must apparently occur between cell and divalent copper ion.

DISCUSSION

Cultures of *E. coli* in the presence of suitable amounts of copper ions undergo a consistent and striking series of events. After slow growth of the wild-type inoculum, small colony variants establish themselves in the cultures in varying proportions. Their establishment undoubtedly is due to their selective advantage, variant cells being much more resistant to the toxic action of copper than normal cells. On further incubation, however, the variant population is once more displaced by phenotypically normal cells which, genotypically, show a somewhat increased resistance to copper when compared to wild type. This sequence of events constitutes another example of what Braun (1958) has termed the impressive manner in which competitive cellular interactions can result in a striking regularity of population shifts, resembling in many respects processes of differentiation in higher organisms.

The data presented bear a superficial resemblance to those described by Park (1954) for interspecific competition in the flour beetle (Tribolium) where it was established that one species almost always competitively displaces the other. We are dealing here with an interesting ecosystem where first one type of cell, then another gains ascendancy, although, in contrast to the experiments of Park, we are dealing with two variants of the same, rather than with two different, species. In agreement with the findings of Park is the observation that the behavior of the different cell types in separate culture shows no relation to what occurs in mixed culture.

According to Youmans (1937) and Youmans and Delves (1942), many of the properties of small colony variants can be accounted for by an over-all lowering of the metabolic rate. Clowes and Rowley (1955) suggested that this explanation might account for the isolation of many of these variants from toxic environments, and proposed that the inhibitor (antibiotics, metal ions, etc.) permeates the variants at a lower rate, thus permitting their survival for a longer time than the corresponding, normally metabolizing, organisms. They have further suggested that the general lowering of the metabolic rate might be due to a decrease in cell wall permeability. This interpretation may well be correct; however, in the present system, behavior toward the inhibitor (copper ions) is not the only determinant affecting survival value. This is shown clearly by the fact that the variants, although highly copper-resistant, are displaced from the population by cells much more sensitive to copper.

No imputation has so far been made as to the mechanism responsible for the origin of the variant cell types. Despite some efforts to determine whether the variants are induced by copper, followed by selection, or whether one is dealing with a selective establishment of spontaneously arising mutants, the question had to be left in abeyance. The usual methods designed to help in a decision, seldom easy to make, between these two alternatives met with difficulties; the Luria-Delbrück test (1943), which depends on growth of the culture in absence of the selective or inducing substance and assay for the presence of mutants in presence of the substance, was found

inapplicable because of the strong selection against the variants in the absence of copper, and the only partial suppression of wild-type cells in the presence of copper. As pointed out in the experimental section, considerable variation with respect to sensitivity to copper exists in different wild-type as well as variant isolates; it thus appears possible that, with respect to susceptibility to copper, populations of *E. coli* exhibit the whole spectrum of reaction, ranging from susceptible to resistant; this behavior is correlated with colony size and reaction toward lactose. For this reason, and admittedly in the absence of conclusive evidence, we feel that copper acts merely as the selective agent for cells of various degrees of copper resistance. As Luria (1947) has pointed out, wholesale transformations of whole or great parts of cell populations, such as frequently observed here, are in themselves a sign that an inductive process should be seriously questioned. Weed and Longfellow (1954) suggested a direct mutagenic effect of copper, a conclusion based on experiments involving very small inocula. Variants were obtained, despite the fact that the possibility of preadaptive mutants in the inoculum had thus been ruled out. Such a conclusion would be tenable only if the copper ions were strongly selective against the normal cells. As shown here, however, slow growth by phenotypically normal cells continues in the presence of copper; during this growth copper-resistant offspring may arise on which selective forces can then act. Clowes and Rowley (1955) postulated that their K-12 variants of *E. coli* (also isolated following exposure to copper) arose by spontaneous mutation at one of several possible genetic loci.

It has been shown here that copper ions, to exert their action, must be in the divalent state, probably due to their interaction in that valence state with cell constituents. The toxicity and effect on variant recovery of copper under aerobic conditions has parallels elsewhere, for, as Lockhart (1959) points out, oxygen tension appears to mediate the inhibitory effects of a number of agents. That oxygen has an effect, under certain experimental conditions, on the mutagenicity of ferrous ions in *E. coli* has been pointed out by Catlin (1953). Haugaard, Hess, and Itskovitz (1957) have shown that the toxic effect of copper ions on the activity of certain enzymes in rat heart homogenates is augmented at high oxygen tensions. Oxygen tension also has a decisive effect on changes in a number of microbial and somatic cell populations (*see* Braun, 1958).

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SUMMARY

Populations of *Escherichia coli* strain B in the presence of copper ions undergo a series of predictable population shifts. Slow growth of the wild-type inoculum is followed by the transient establishment in the population of small colony variants in varying proportions. The variants, despite their greatly increased resistance to copper, are eventually displaced again by phenotypically normal cells of a type less resistant to copper than the variants, but more resistant to it than the original population. Copper resistance is correlated with colony size and ability to ferment lactose.

The great variability observed in the proportion of variants establishing themselves in the population can be ascribed to (i) competitive interplay between normal and variant cells; (ii) continued presence of wild-type cells with increased resistance to copper; and (iii) variations in the oxidation-reduction potential of the cultures.

Chelation of monovalent or divalent copper by cysteine or Versene, respectively, abolishes both growth inhibition and recovery of variants. Anaerobic conditions also remove the effects of copper. Free radical trapping agents had no effect. These and other experiments show that the effects of copper in the present system depend on its initial presence as the free ion and on its binding to cellular sites in the divalent state.

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