

MORPHOLOGICAL AND BIOCHEMICAL CHANGES INDUCED BY COPPER IN A POPULATION OF *ESCHERICHIA COLI*

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It has been found that if traces of copper ion are present in growing liquid cultures of *Escherichia coli*, subsequent platings of this suspension contain a variant form whose colonial morphology and chemical composition differ from the untreated parent strain. Specifically, the morphological change consists of the appearance of a small colony variant which does not revert when carried by serial transfer on noncopper containing solid media. It is the purpose of this report to describe this phenomenon with particular reference to the biochemical changes found in the *E. coli* variant.

MATERIALS AND METHODS

Strain. The "B" strain of *E. coli* used in the current work was obtained from Dr. Seymour Cohen as a culture on nutrient agar. It was lysed by T2r⁺, T4r, and T6r⁺ bacteriophages and behaved biochemically as a typical member of the *Escherichia*.

Media. The liquid medium employed had the following composition: Na₂HPO₄, 16.5 g; KH₂PO₄, 1.5 g; (NH₄)₂SO₄, 2.0 g; MgSO₄·7H₂O, 1 ml of 20 per cent solution; CaCl₂, 1 ml of 1 per cent solution; FeSO₄·7H₂O, 0.5 ml of 1 per cent solution.

The salts were dissolved in 1,000 ml of triple glass distilled water and adjusted to a pH of 7.4. Glucose to the concentration of 3 mg per ml was added at the time of each experiment. When required, CuSO₄ was added to a molarity of 5×10^{-6} . All bacterial growth experiments were carried out in a water bath of 37 C without aeration, using 18 by 150 mm Coleman spectrophotometric cuvettes as culture vessels. Nutrient agar (BBL) was obtained from commercial sources.

Estimation procedures. A. Dilution plate counts were made by inoculating the surfaces of nutrient agar plates with 0.1 ml of a serial tenfold dilution of a culture and evenly distributing the inoculum by means of a broad platinum loop. It has been found that bacterial counts made in this manner

agree with those obtained by the conventional pour plate method.

B. The bacterial population was determined also by densitometric methods. To estimate the total mass, the optical density of the culture was determined at 475 m μ . Additional density measurements were made at 260 m μ in order to determine the degree of absorption in the ultraviolet region of the spectrum.

Biochemical procedures. Oxygen uptake was measured by conventional means in the Warburg respirometer at 37 C using washed whole cells. Ribonucleic acid was determined by the orcinol reaction (Kerr and Seraidarian, 1945), and the Dische test (Dische, 1930) was used for the estimation of the deoxyribonucleic acid. It was found that whole organisms could be used without previous extraction for these latter two determinations. The values were in excellent agreement with those obtained using hot trichloroacetic acid extraction of the whole organism. In preparation of the cells for the ribonucleic acid and deoxyribonucleic acid determinations, care was taken to wash the cells in the cold, and all centrifugations were done at 4 C. Delay was avoided since ribonucleic acid was lost readily from the cell. The washed organisms then were placed in 3 ml of acetone at 64 C for 18 hours, after which the remainder of the acetone was evaporated in a drying oven at 100 C. The dried material was powdered finely before adding the reagents.

Ultraviolet irradiation of cultures. The source of ultraviolet light was a Model SL "Mineralight". This is a low intensity, cold ultraviolet source emitting approximately 90 per cent of its light at 2537 A. The open surfaces of the plates, inoculated as for counts (see above), were exposed at a distance of 8 inches from the lamp. In experiments to determine survival rates the exposure times ranged from 2 to 12 seconds, whereas a constant exposure time of 8 seconds was used in studies designed to establish changing resistance

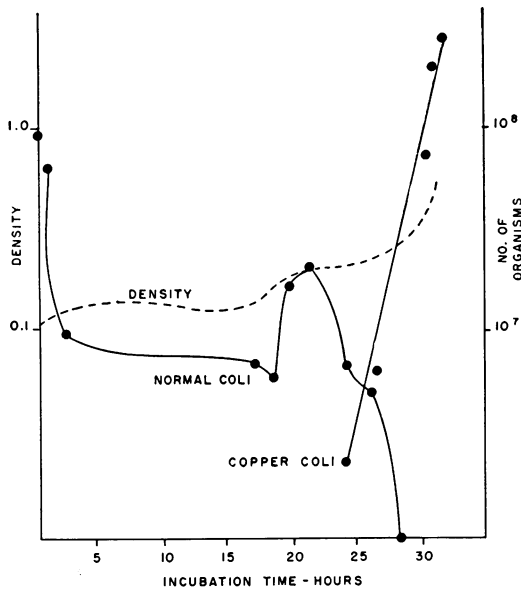


Figure 1. Appearance of small colony forms of *Escherichia coli* ("copper" organisms) in the presence of 5×10^{-6} M copper sulfate.

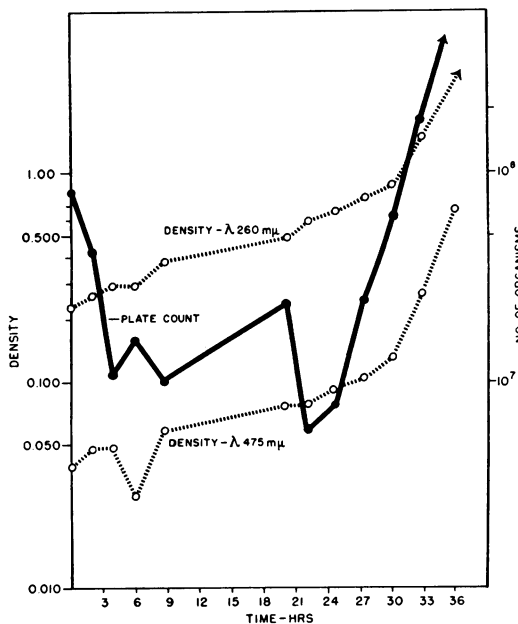


Figure 2. Relationships between densities and plate counts in liquid cultures of *Escherichia coli* containing copper sulfate.

among members of a growing culture. To avoid light reactivation (Dulbecco, 1949; Kelner, 1949), all preparations were irradiated and incubated in the absence of visible light.

RESULTS

Production of the small colony variant. The small colony variant of *E. coli* (hereafter referred to as the "copper" organism) developed as shown in figure 1.

In the first two hours after inoculation of the copper containing culture media, the count of viable bacteria fell to 10 per cent of its original value. Beginning at about the end of the next 18 hour period, during which the plate count did not change appreciably, there was a rapid decline in the number of *E. coli* giving normal sized colonies. Concomitant with this fall, small colony forms appeared.

It seemed advisable to follow closely the density changes at both $475 \text{ m}\mu$ and $260 \text{ m}\mu$ during the period in which the precipitous fall in plate count occurs in order to determine any changes in mass and nucleic acid content which might be taking place. In figure 2 are plotted the density readings at $475 \text{ m}\mu$ and $260 \text{ m}\mu$ as well as the plate counts of a liquid culture of normal cells in the presence of copper sulfate. This figure shows that during the initial phases there was the usual loss of approximately 90 per cent of viable cells. During this same period, however, the absorption at $475 \text{ m}\mu$ doubled and that at $260 \text{ m}\mu$ increased almost threefold, while the number of viable organisms remained low. Therefore, there was appreciable synthetic activity in the culture, even though this was not obvious at the time, from the counts of viable normal or "copper" organisms. Examination of the culture by measurements at $475 \text{ m}\mu$ and $260 \text{ m}\mu$ indicated that there was continuing activity from the beginning of the experiment.

The length of the period which precedes the appearance of small colonies is related to the concentration of cupric ions in the culture. Figure 3 illustrates an experiment in which *E. coli* were grown in the presence of concentrations of cupric ion from 10×10^{-6} to 25×10^{-6} M, and in which the optical densities were determined at frequent intervals. It will be seen that as the concentration of copper increased the static period was prolonged, but eventually the density of the cultures rose. It should be noted that at 25×10^{-6} M copper ion full growth had not been attained even after 5 days of incubation. However, small colony forms appeared in each culture after growth had finally been achieved.

Experiments were performed then to determine whether the "copper" organism, after cultivation

on copper-free solid media, would lose its capacity to grow in the presence of appreciable amounts of copper. It was found that following five passages in the absence of copper, the organism grew rapidly when introduced into media containing copper in concentrations ranging from 5 to 50×10^{-6} M. Indeed, growth proceeded in these cultures at almost the same rate as that of the normal organisms in copper-free media. Thus, the resistance of the small colony form to copper does not depend on the continued presence of the metal ion.

The colonies of the "copper" organisms were one-sixth to one-tenth the size of those of the normal organism, and after 36 hours growth there was a well defined margin which was conspicuously different from the serrated edges of the normal *E. coli* colony of the same age. This is illustrated in figure 4.

No clear-cut differences in form and size of the individual normal and "copper" organisms have been observed in wet or stained preparations.

In interpreting the mechanism of copper action on *E. coli*, the process of selection has to be considered. Some evidence that this phenomenon is probably not a matter of simple selection can be derived from an experiment which consisted of following the fate of a very limited number of cells when introduced into the synthetic medium containing copper. Each of a series of 15 cultures was inoculated with 150 cells of normal *E. coli*. Beginning at about the thirty-second hour, growth, as measured by density, began to appear in all tubes. Furthermore, when these cultures were plated out after full growth had been attained, all colonies were of the small variety. This experiment has been repeated with identical results. Since it seems unlikely that there were sufficient naturally occurring variants in the initial inocula to have accounted for these results, it would appear that an effect of copper on at least some of the normal *E. coli* had taken place.

A limited number of experiments were performed to learn whether other strains of *E. coli* were susceptible to this effect of copper. Under the conditions stated above small colonies have been produced and maintained in *E. coli*, strain K₁₂. On the other hand, *E. coli*, strain 15, did not yield small colonies under these conditions; moreover, much larger concentrations of copper were required to inhibit growth to a comparable degree.

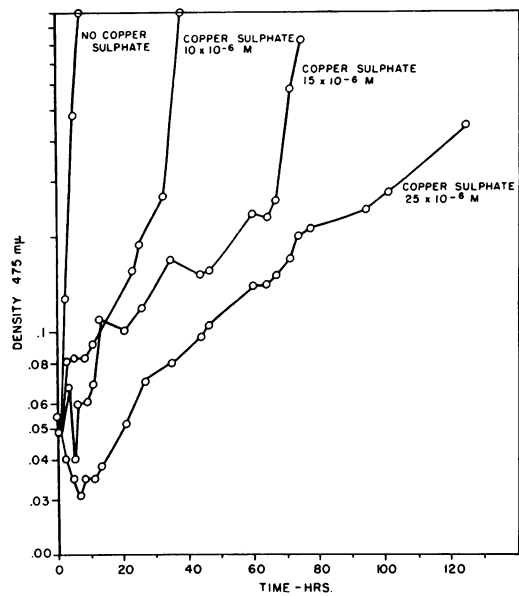


Figure 3. Growth rates of normal *Escherichia coli* in the presence of increasing concentrations of copper sulfate.

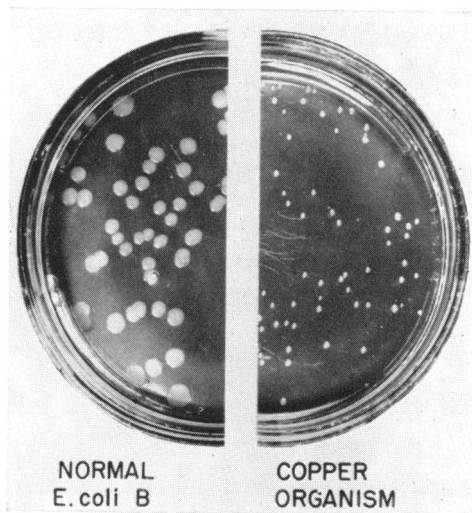


Figure 4. Thirty-six hour growth on nutrient agar of the 30th serial transfer in the absence of copper.

Maintenance of the "copper" organisms in a stable form. The small colony form has been maintained without reversion to normal type for seventy transfers on copper-free solid media. To insure a control for colony size, both normal and "copper" organisms were inoculated on opposite halves of the same agar plate, permitting com-

parison under identical conditions. It should be stated that in liquid media the "copper" organism slowly reverted to the large colony type during the course of a few transfers. These reverted organisms were not examined in detail; therefore, it cannot be stated that they resembled the parent large colony form in all respects.

At frequent intervals throughout the study, the small colony organisms were examined to reassure ourselves that they were *E. coli*. In each instance they were found (1) to give typical biochemical reactions of *Escherichia* (except lactose utilization), (2) to be susceptible to infection by the T6r⁺ and T4r bacteriophages, and (3) to give the usual reactions with specific antisera.

Comparative biochemical studies of normal and "copper" coli. Both the normal and "copper" organisms used in the experiments described below were saline suspensions of cells derived from serial transfers on copper-free nutrient agar.

A. *Utilization of lactose.* The "copper" organism, unlike the normal *E. coli*, cannot multiply in the presence of lactose as the sole carbon source. Separate tubes of media containing 3 mg of lactose per ml were inoculated with 5×10^7 normal and 5×10^7 "copper" coli, respectively. In the culture of normal organisms, growth began in 2½ hours and was complete in 6½ hours, whereas

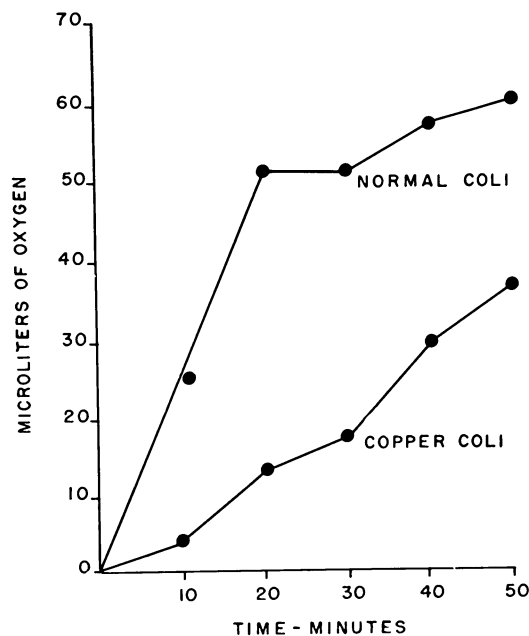


Figure 5. Oxygen uptake of normal and "copper" *Escherichia coli*.

in the culture of the "copper" cells there was no visible growth in 8 hours, but it did become evident after 18 hours. When this growth was more complete, it was found that only 3 per cent of the organisms were of the small colony variety. It may be noted that the "copper" organisms grown in a liquid medium containing glucose instead of lactose retained this small colonial form for at least one passage.

B. *Utilization of oxygen.* Evidence has been obtained that the rate of oxygen uptake of the "copper" organisms is slower than that of the normal *E. coli*. Figure 5 illustrates the findings of a typical experiment in which equal masses of the two types of organism were compared over a 50 minute period for oxygen uptake in the utilization of glucose. These findings suggested that associated changes might be found in nucleic acid metabolism of the "copper" organisms, and the studies mentioned below were initiated.

C. *Nucleic acid content of normal and "copper" *E. coli*.* The desoxyribonucleic acid/ribonucleic acid ratios for the normal and "copper" organisms in a series of 4 experiments are shown in table 1. The ratios in the "copper" coli are nearly twice those in the normal; the higher ratios were dependent on increased amounts of desoxyribonucleic acid and decreased amounts of ribonucleic acid, and therefore there was relatively more desoxyribonucleic acid for a given amount of ribonucleic acid in the changed organisms than in the normal. These determinations were made on organisms harvested at the time full growth was attained. In order to determine whether these ratios remained the same at other points during growth, aliquots of the culture were taken at intervals and studied. It was found that the ratios changed as growth proceeded and that the maximum difference between the two types of organisms occurred at the end of the logarithmic phase of growth.

TABLE 1

Desoxyribonucleic acid (DA)/ribonucleic acid (RNA) ratios in organisms derived from comparable regions of the growth curve

	EXPERIMENTS			
	1	2	3	4
	<i>DNA/RNA ratios</i>			
Normal coli	0.25	0.26	0.20	0.22
"Copper" coli	0.46	0.44	0.35	0.38

TABLE 2

Density and desoxyribonucleic acid (DNA) relationships during growth of normal and "copper" *Escherichia coli*

TIME min	NORMAL COLI		"COPPER" COLI	
	Density	DNA/ml	Density	DNA/ml
0	0.410	9.4	0.410	10
80	0.780	13.4	0.770	16.1
140	1.430	19.9	1.150	19.8
225	1.520	19.8	1.320	23.6
285	1.480	19.7	1.288	24.6
420	1.480	19.8	1.30	24.3

One may determine the relationship between desoxyribonucleic acid synthesis and the increase in total bacterial mass by estimating at intervals the desoxyribonucleic acid concentration and the density of the culture. The results of such an experiment are shown in table 2. The data show that the normal organism reached a higher density than the "copper" organism, but that in the latter, desoxyribonucleic acid synthesis proceeded beyond that of the normal. In fact, if desoxyribonucleic acid is calculated on the basis of equal densities, one finds that after 285 minutes there is 37 per cent more desoxyribonucleic acid in the "copper" organism.

*Effects of ultraviolet irradiation on the normal and "copper" E. coli.*¹ The resistance of the "copper" organism to ultraviolet irradiation was considerably greater than that of normal *E. coli*. This was established by exposing open plates, inoculated as in the usual fashion for counting, to an ultraviolet light source before incubation. As short an exposure time as 4 seconds was sufficient to prevent colony development in 80 per cent of the normal bacteria, whereas even 12 seconds exposure failed to diminish the survival rate of the "copper" coli (figure 6).

It was of interest to correlate the development of resistance to ultraviolet light with the appearance of small colony forms in a liquid culture of *E. coli* containing copper. Quadruplicate plates were made at the intervals shown in figure 7, and one pair was allowed to incubate in the usual fashion, whereas the other received an 8 second

¹ We are indebted to Dr. John Athens of the Department of Biophysics, Army Medical Service Graduate School, for his collaboration in this phase of the studies.

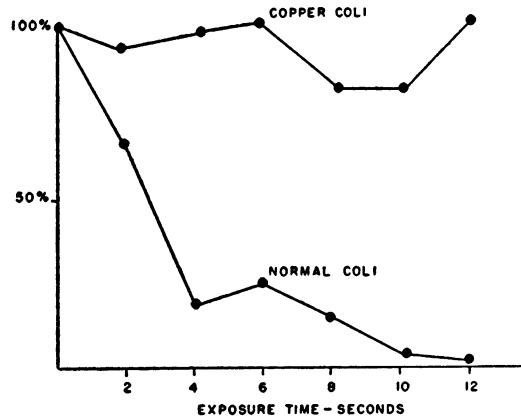


Figure 6. Relative numbers of survivors of normal and "copper" *Escherichia coli* after exposure to ultraviolet irradiation.

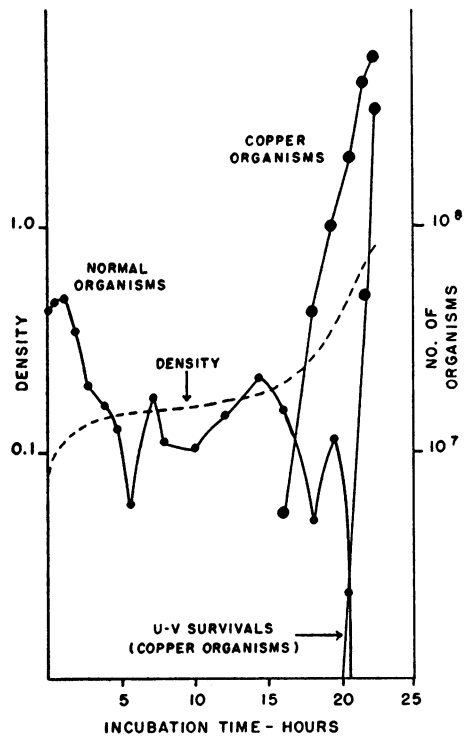


Figure 7. Relationship of ultraviolet resistance to the appearance of small colony forms.

exposure to ultraviolet light. It was found that no organisms survived the irradiation until a population of small colony producing forms had become well established. This experiment is consistent with findings already shown in figure 6.

Neither of the above types of experiments

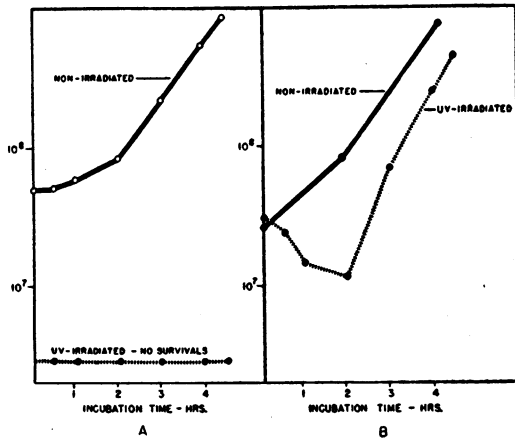


Figure 8. Resistance to ultraviolet irradiation as related to growth of (A) normal *Escherichia coli* and (B) "copper" *Escherichia coli*.

provided information concerning possible variation in resistance along advancing stages of the growth curve. It seemed worthwhile to determine whether such changes might exist since it had already been found that the relative amount of ribonucleic acid fell significantly as growth proceeded. Two cultures were set up in which noncopper containing liquid synthetic medium was inoculated with normal and "copper" coli, respectively. Samples were taken from each at intervals, plated out, and irradiated for 8 seconds. Figure 8A shows that with an identical amount of ultraviolet light applied to all plates there were no survivors among the normals at any point on the growth curve. However, in the case of the "copper" organism (figure 8B), there were always survivors, and the proportion of these in the total number of viable organisms increased significantly in the terminal part of the growth curve.

DISCUSSION

Superficially there appears to be a parallel between this phenomenon described for copper and the production of small colony forms in yeast by the action of acriflavine as described by Ephrussi *et al.* (1949). A valid comparison awaits a detailed analysis of the morphological and genetic aspects of the effects of copper on bacteria.

The particular aim of this study has been to investigate the chemical changes produced in *E. coli* by exposure to copper ions. From the data it appears that the changes observed are quantitative, not qualitative. A shift in balance of alter-

nate pathways of metabolism could result in significant chemical changes without any qualitative additions or subtractions to the system. It would be very difficult to attribute a primary role to any of the several quantitative differences that have thus far appeared.

One might suspect, on the basis of the oxygen consumption data, that a shift had occurred in the normal balance between the phosphogluconate oxidative pathway and the anaerobic fermentative scheme in the utilization of glucose. This in turn might have resulted in an alteration in the relative production of ribose and deoxyribose since the former is a product of the gluconate pathway and the latter of the fermentative scheme (Cohen, 1951; Lanning and Cohen, 1952). Any shift therefore in favor of the fermentative pathway might have resulted conceivably in smaller ribonucleic acid production relative to the amount of deoxyribonucleic acid synthesized. One might reverse this order of reasoning by invoking a concept of decreased demand for ribonucleic acid with a subsequent change in the balance of the alternate pathways of glucose utilization. Although no experimental evidence has been accumulated to prove any single course of events, it is this line of thought that led to the deoxyribonucleic acid/ribonucleic acid analyses described above. It can only be said at this point that the results are consistent with either series of related events.

The inability of the "copper" organism to use lactose for growth is not understood. Whether it is an example of altered protein metabolism with secondary alterations in adaptive enzyme formation, or whether it represents a more specific change is not clear.

SUMMARY

The presence of small amounts of copper ions in a liquid synthetic medium inoculated with *Escherichia coli* resulted in the appearance of a small colony variant. The changes noted persisted in the absence of copper.

The small colony variant possessed significant differences in lactose, glucose, and nucleic acid metabolism as well as increased resistance to ultraviolet irradiation when compared to the normal *E. coli*.

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