STUDIES ON BACTERIAL REDUCING ACTIVITY IN RELATION TO AGE OF CULTURE

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Received for publication October 18, 1951

Bacterial cells in the logarithmic phase of growth are characterized by an ability to reproduce in fresh media without the initial lag period required by organisms in later phases of development. It would seem that the latter lack certain chemical or physiological properties prerequisite to cell division. The type of changes taking place during what may be called the rejuvenation of the cell has only been incompletely investigated. Penfold (1914) set forth the hypothesis that during the lag phase bacteria formed a substance necessary for cell multiplication, which would diffuse from the cells at the end of the logarithmic phase of growth. Lodge and Hinshelwood (1943) observed that a substance appearing during the lag period could be transferred with inocula or sterile filtrates to fresh media, in which it would reduce the lag of newly introduced organisms.

One characteristic which differentiates the rapidly multiplying cell from older cells has long been recognized to be a higher rate of oxygen uptake, generally referred to as endogenous respiration. This has been confirmed recently by Bielig, Kausche, and Haardick (1949) with triphenyltetrazolium chloride and in this laboratory with sodium nitrate (Kopper, 1951*a*). It was felt that further investigation of this property of cells might yield valuable knowledge regarding the nature of factors associated with anabolic cell activities. The present paper deals with the effect of certain environmental changes on what is here termed the reducing activity of bacteria of different growth phases.

MATERIALS AND METHODS

The organisms were grown on meat extract agar at 37 C for 4 hours (young cells) or at room temperature for 18 hours (old cells). They were then washed with distilled water and centrifuged. Following one more washing and centrifugation, the bacteria were suspended in distilled water. Suspensions were adjusted turbidimetrically to give a reading of 20 at a wavelength of 550 mμ in the Leitz photoelectric colorimeter. By means of nutrient agar plate surface counts, this optical density was found to correspond to about 2×10^9 and 3×10^9 viable cells per ml for young and old bacterial cultures, respectively. Unless indicated otherwise, 10^{10} young cells or 3×10^{10} old cells in a volume of 0.1 ml were used in the experiments.

Reducing activity was generally measured with 2,3,5-triphenyltetrazolium chloride in accordance with the method of Kun and Abood (1949). Color intensities were estimated in a Leitz photoelectric colorimeter at a wavelength

of 415 mμ and compared with standards prepared with known amounts of reduced 2,3,5-triphenyltetrazolium chloride.

In general experimental procedure the bacteria were added first to 15 ml centrifuge tubes. The volume was then made up to 0.5 ml with distilled water. This was followed by the addition of 1 ml of M/15 phosphate buffer of pH 7, unless a different hydrogen ion concentration was desired. After incubation for 5 minutes in a 37 C water bath, 0.5 ml of a 0.1 per cent solution of 2,3,5-triphenyltetrazolium chloride was added. The tubes were returned to the water bath for 15 minutes. Five ml of acetone were then added, the tubes stoppered, vigorously shaken to extract all the dye from the bacterial cells, and centrifuged. The clear supernatants were decanted and readings taken as described. Under these conditions 10^{10} young cells reduced 170 to 200 μg, 3×10^{10} old cells 80 to 110 μg of 2,3,5-triphenyltetrazolium chloride.

In one series of experiments the time required to reduce methylene blue was determined in evacuated Thunberg tubes. The organisms were allowed to act on 0.2 ml of a 1:2,000 dilution of methylene blue in 4 ml of distilled water.

RESULTS

A linear relationship exists between the number of bacteria, irrespective of age, and the amount of 2,3,5-triphenyltetrazolium chloride reduced. Figure 1 shows the values obtained with young *Escherichia coli* cells. A long lag period was encountered with low bacterial concentrations (2×10^9 or less). This has also been observed in studies on the reduction of formaldehyde (Kopper, 1951*b*) and sodium nitrate and methylene blue (Kopper, 1951*c*).

The effect of changes in pH on the reducing activity of young and old cells of *E. coli* is illustrated in figure 2. The optimal pH of about 8.5 for old cells is in agreement with the value found by Bielig, Kausche, and Haardick (1949) with their strain of *E. coli*. It is noteworthy that young cells follow a pH activity curve that has shifted toward the region of lower pH levels, reaching its highest point at about 7.5.

The figures presented in table 1 indicate considerable differences in the effect of various concentrations of sodium chloride on the reducing activity of young and old cells.

One per cent NaCl was shown to be the optimal salt concentration for the reduction of 2,3,5-triphenyltetrazolium chloride and methylene blue by old cells. At 0.5 and 2 per cent NaCl reducing activity remained fairly high; at 5 per cent NaCl or in the complete absence of the salt it was hardly measurable. Young cells, on the other hand, were little affected by the absence of, or changes in, the amount of NaCl in the suspending fluid up to a concentration of 1 per cent, but above 1 per cent a rapid decrease was noted. The differences in the reducing activity of young and old bacteria in the presence of higher concentrations of NaCl may be explained by their differential susceptibility as described by Sherman and Albus (1923). This explanation cannot be applied, however, to the results with low concentrations or in the absence of the salt. The latter effect could be duplicated with other salts of monovalent cations, such as sodium

sulfate, potassium nitrate, ammonium chloride, sulfate and oxalate, and Sorensen's phosphate buffers, but not with barium chloride, calcium chloride, and magnesium sulfate.

Bacteria in M/15 phosphate buffer of pH 7 were preheated at various temperatures for 10 minutes, rapidly cooled, then incubated at 37 C prior to the addi-

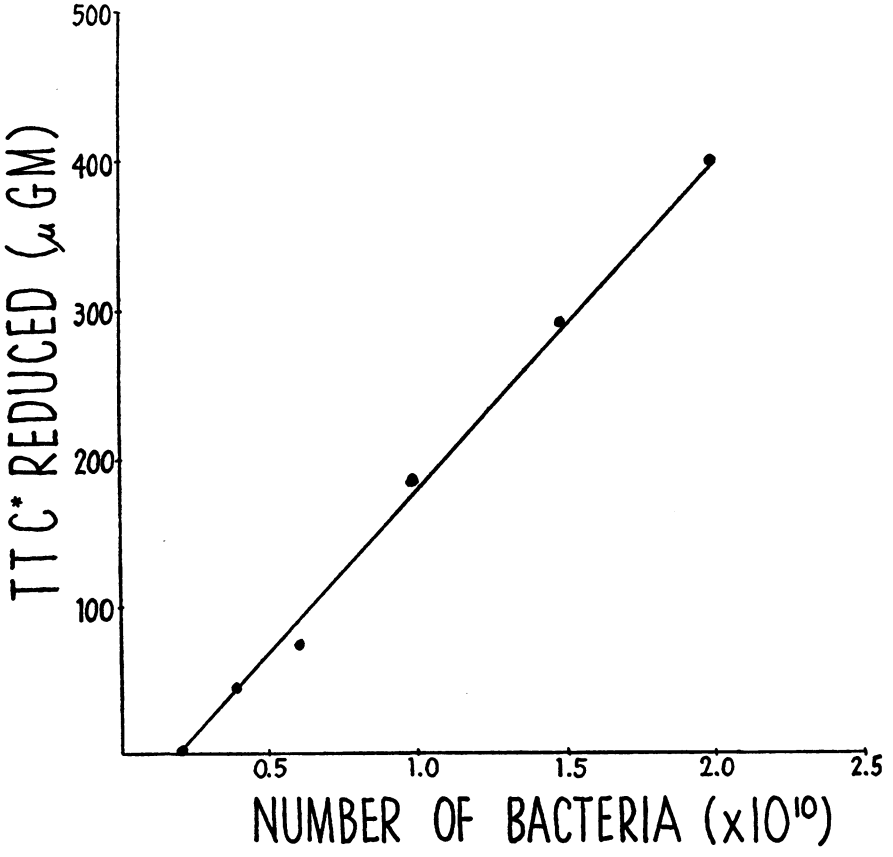

Figure 1. Relation of bacterial concentration to the reduction of 2,3,5-triphenyltetrazolium chloride by young cells of *Escherichia coli*.

* TTC = 2,3,5-triphenyltetrazolium chloride.

tion of 2,3,5-triphenyltetrazolium chloride. The effect of this treatment on cellular reducing power and viability is demonstrated by the data of table 2.

Relative decreases in the number of viable cells were about the same with increasing temperatures regardless of age of culture. The rate of loss of reducing activity at the lower temperatures was roughly proportionate to this decrease for old bacteria, but was much higher for young bacteria.

Data on the effect of a number of chemical compounds on cellular reducing activity are presented in table 3. The organisms were exposed to solutions of

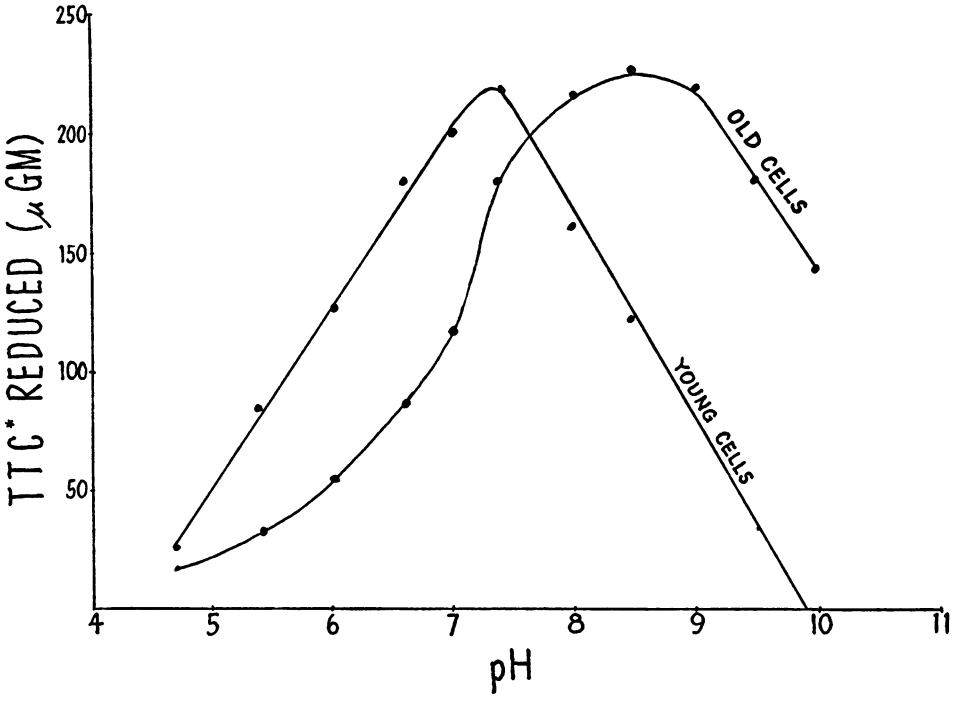

Figure 2. Effect of pH on the reduction of 2,3,5-triphenyltetrazolium chloride by young and old cells of *Escherichia coli*.

* TTC = 2,3,5-triphenyltetrazolium chloride.

TABLE 1

Effect of NaCl on the reducing activity of young and old* cells of Escherichia coli*

CONCENTRATION OF NaCl	AMOUNT OF 2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE REDUCED†		TIME OF DECOLORIZATION OF METHYLENE BLUE	
	Young cells	Old cells	Young cells	Old cells
per cent	*μg*	*μg*	*min*	*min*
0	46	0	10	70
0.2	40	16	5	12
0.5	41	35	5	6
1.0	27	56	5	5
2.0	trace	27	15	9
5.0	0	trace	50	38

* 5×10^9 cells of a 4-hour culture, 1.5×10^{10} cells of an 18-hour culture.

† The cells were suspended in saline prepared with distilled water of pH 5.8 and incubated with 2,3,5-triphenyltetrazolium chloride for 30 minutes.

the various chemicals in distilled water for 15 minutes at 37 C. They were then centrifuged, washed in distilled water, and following centrifugation resuspended in phosphate buffer. Their reducing activity was determined in the usual way.

With the exception of aureomycin none of the substances listed in table 3 exerted any deleterious effect on the old cells. Young cells, on the other hand, were affected by aureomycin, sodium iodoacetate, quinone, and 2,4-dinitro-

TABLE 2

Effect of preheating on the reducing activity of young and old cells of Escherichia coli

TEMPERATURE OF PREHEATING	DECREASE IN AMOUNT OF 2, 3, 5-TRIPHENYLTETRAZOLIUM CHLORIDE REDUCED		DECREASE IN NUMBER OF VIABLE BACTERIA	
	Young cells	Old cells	Young cells	Old cells
C	*per cent*	*per cent*	*per cent*	*per cent*
46	33	14	10	10
48	71	35	21	19
50	75	40	44	37
52	81	49	70	68
54	90	78	99	99
56	100	85	99.98	99.98

TABLE 3

Effect of treatment with chemicals on the reducing activity of young and old cells of Escherichia coli

NAME OF CHEMICAL COMPOUND	AMOUNT ADDED	DECREASE IN AMOUNT OF 2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE REDUCED		DECREASE IN NUMBER OF VIABLE BACTERIA	
		Young cells	Old cells	Young cells	Old cells
		per cent	*per cent*	*per cent*	*per cent*
Penicillin	30,000 units	0	0	—	—
Aureomycin	1.0 mg	100	questionable*	70	30
Aureomycin	0.1 mg	0	0	—	—
Chloromycetin	1.0 mg	0	0	0	0
Chloromycetin	0.1 mg	0	0	—	—
Sulfanilamide	0.01 millimole	0	0	0	0
Sulfanilamide	0.001 millimole	0	0	—	—
Sodium fluoride	0.01 millimole	0	0	0	0
Sodium fluoride	0.001 millimole	0	0	—	—
Sodium iodoacetate	0.01 millimole	50	0	43	0
Sodium iodoacetate	0.001 millimole	0	0	—	—
Quinone	0.001 millimole	100	0	99	0
Quinone	0.0001 millimole	0	0	—	—
2,4-Dinitrophenol	0.001 millimole	73	0	25	0
2,4-Dinitrophenol	0.0001 millimole	0	0	—	—

* Some 2,3,5-triphenyltetrazolium chloride was reduced, but the yellow color of aureomycin, which was adsorbed on the bacteria and later extracted with acetone, made an accurate colorimetric reading impossible.

phenol. In the case of the last compound, a known inhibitor of synthesizing processes, the decrease in reducing activity exceeded appreciably the loss in cell viability. It is noteworthy that of the two antibiotics active against gram-

negative bacteria in table 3, chloromycetin proved to be 4 times as effective as aureomycin in preventing the reproduction of *E. coli* in nutrient broth. This is in contrast to their effect on the reducing activity of the organism and, thus, might shed some light on the differential mode of action of these antibacterial agents.

DISCUSSION

The present work demonstrates the effects of environmental factors on the reducing activity of young and old bacterial cells. Some of these, such as the results of treatment with high concentrations of NaCl or with sodium iodoacetate or quinone, may be ascribed to the greater susceptibility of young cells to the toxic properties of these agents. Others, such as the effects of low temperatures and of 2,4-dinitrophenol, indicate that the reducing activity of young cells may be considerably lowered without affecting their viability, which might lead one to speculate that the damage inflicted on the cell could be repaired in a nutritive medium such as nutrient agar, in which plate counts were made.

The response of the cells to changes in pH and in salt concentration appears of particular interest. This perhaps may be explained by assuming that a relationship exists between the aging process and increases in cell permeability. According to Heilbrunn (1947), there appear to be only two investigations recorded in the literature which deal with this very same problem. Weber (1931) found threads of *Spirogyra* and guard cells of leaves of *Ranunculus ficaria* more permeable to urea in the "adult", i.e., fully functioning stage, than in the "embryonic" stage. No other compounds were tested. In the course of the present study it was observed that young cells of *E. coli*, while far more vigorous than old cells in reducing sodium nitrate, methylene blue, and tetrazolium salt, all ionizable compounds, were unable to take up formaldehyde. Since the reduction of HCHO by old cells of *E. coli* had been established previously (Kopper, 1951*b*), it would seem logical to conclude that HCHO was somehow prevented from diffusing into that region of the young cell where it could be reduced. The same mechanism may be operative in causing the rapid decline in the reduction of 2,3,5-triphenyltetrazolium chloride at alkaline pH when the salt in increasingly larger amounts is converted into undissociated base.

It may be speculated that a relative impermeability of the young cell would serve the purpose of retaining selectively chemical compounds fulfilling vital functions in reproduction. In this respect it is noteworthy that old cells require a definite concentration of NaCl for optimal reducing activity whereas young cells displaying no such need are thus much more independent of this environmental factor.

Further progress in understanding the basic differences between young and old microbial cells will depend on the isolation and chemical identification of the factor or factors that stimulate the reducing activity and shorten the lag period of microorganisms.

SUMMARY

The reducing activity of 4-hour and 18-hour cells of a strain of *Escherichia coli* toward 2,3,5-triphenyltetrazolium chloride was studied under various environmental conditions.

In general, a linear relationship was found to exist between the number of bacteria and 2,3,5-triphenyltetrazolium chloride reduction. An appreciable lag, however, was encountered with low bacterial concentrations.

The optimal pH of the reaction was about 7.5 for young cells and 8.5 for old cells.

For old cells a 1 per cent concentration of NaCl was optimal in 2,3,5-triphenyltetrazolium chloride reduction. In the complete absence of the salt no 2,3,5-triphenyltetrazolium chloride was reduced during the course of the experiments. Young cells, on the other hand, reached their optimal activity in the absence or at low concentrations of NaCl. Concentrations of the salt higher than 1 per cent exerted a strongly inhibitory effect on them.

Exposure of bacterial suspensions to temperatures of 46 to 56 C for 10 minutes caused a far more rapid decline in the reducing activity of young cells than of old cells without affecting cell viability in the same way.

A number of chemical substances were allowed to act upon the bacteria. Young cells proved to be more susceptible than old cells to the action of aureomycin, sodium iodoacetate, quinone, and 2,4-dinitrophenol. The last compound effected a marked decrease in reducing activity without causing a proportionate loss in cell viability.

REFERENCES

BIELIG, H. J., KAUSCHE, G. A., AND HAARDICK, H. 1949 Über den Nachweis von Reduktionsorten in Bakterien. Z. Naturforsch., **4b**, 80–91.

HEILBRUNN, L. V. 1947 An Outline of General Physiology. W. B. Saunders Co., Philadelphia and London. Second revised edition, p. 144.

KOPPER, P. H. 1951*a* Relationship of growth phase to reducing activity of bacterial cells. Nature, **167**, 951–952.

KOPPER, P. H. 1951*b* The reduction of formaldehyde by bacterial cells. J. Gen. Physiol., **34**, 573–582.

KOPPER, P. H. 1951*c* The reducing power of bacterial cells. Bact. Proc., **1951**, 71.

KUN, E., AND ABOOD, L. G. 1949 Colorimetric estimation of succinic dehydrogenase by triphenyltetrazolium chloride. Science, **109**, 144–146.

LODGE, R. M., AND HINSHELWOOD, C. N. 1943 Physicochemical aspects of bacterial growth. Part IX. The lag phase of *Bacterium lactis aerogenes*. J. Chem. Soc., **1943**, 213–219.

PENFOLD, W. J. 1914 On the nature of bacterial lag. J. Hyg., **14**, 215–241.

SHERMAN, J. M., AND ALBUS, W. R. 1923 Physiological youth in bacteria. J. Bact., **8**, 127–139.

WEBER, F. 1931 Harnstoff-Permeabilität ungleich alter Stomata-Zellen. Protoplasma, **14**, 75–82.