GROWTH, REPRODUCTION, AND DEATH RATES OF *ESCHERICHIA COLI* AT INCREASED HYDROSTATIC PRESSURES[1]

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ABSTRACT

ZoBELL, CLAUDE E. (University of California, La Jolla) AND ANDRE B. COBET. Growth, reproduction, and death rates of *Escherichia coli* at increased hydrostatic pressures. J. Bacteriol. **84**:1228–1236. 1962.—Pressures ranging from 100 to 500 atm were found to retard the growth and reproduction of *Escherichia coli* in nutrient medium. Reproduction (as manifested by cell division or an increase in the number of viable cells) was retarded more than was growth (as manifested by increase in cell size or the formation of biomass). When incubated near the threshold of pressure tolerance (about 475 atm at 30 C), some cells of *E. coli* grew into long slender filaments showing little evidence of fission or cell division. Compression prolonged the lag phase of *E. coli* in nutrient medium, particularly at pressures higher than 400 atm. The inhibiting effects of pressure on reproduction and growth were found to be less at 30 than at 20 or 40 C. Pressures of 400 to 1,000 atm accelerated the death rate of *E. coli* cultures in nutrient medium. The lethal effects of pressure were greater at 40 than at 30 C and greater at 30 than at 20 C.

Moderate hydrostatic pressures (100 to 1,000 atm) have been shown to affect the viability and physiological activities of microorganisms (Johnson, Eyring, and Polissar, 1954; Morita and ZoBell, 1956; Oppenheimer and ZoBell, 1952). Pressures of 200 to 600 atm inhibited the growth of virtually all freshwater and soil bacteria examined by ZoBell and Johnson (1949). Similarly, ZoBell and Oppenheimer (1950) found that, with the exception of a few species from the deep sea, most marine bacteria failed to grow at pressures ranging from 200 to 600 atm, the limiting pressure depending upon species, size of inoculum, chemical composition of the medium, temperature, and other factors. From water depths of 7,000 to 10,400 m, ZoBell and Morita (1957, 1959) recovered bacteria that were capable of growing when compressed to 700 to 1,000 atm, but great and unexplained differences were found in the pressure response of deep-sea microflora. Also lacking obvious explanation are the observations of Kriss (1961) that most deep-sea bacteria which he examined grew better at 1 atm than when compressed to the pressure of the environment from which they were taken. He reported the prolonged survival and growth of many garden soil bacteria at pressures exceeding 1,500 atm.

The following studies were undertaken in an effort to reconcile some of the apparently anomalous observations and to learn more about the mechanism by which pressure affects microorganisms. Because of the wealth of published information concerning the factors that influence the reproduction and survival of *Escherichia coli* under various experimental conditions, this organism was selected for study.

MATERIALS AND METHODS

The stock culture of *E. coli* (ATCC 11303) was maintained by monthly transplantation on nutrient agar slants. The inoculated slants were incubated at 30 C for 24 hr, then stored at 4 C until subcultures were needed for experiments. At 1 atm, this culture grew at temperatures ranging from 15 to 45 C, most rapidly at 43 C as determined in a polythermostat (Oppenheimer and Drost-Hansen, 1960), which provided for 18 different temperatures simultaneously. Biomass formation was measured with a Bausch & Lomb Spectronic 20 nephelometer at 420 mμ.

All reproduction, growth, and death-rate tests were made in a medium consisting of 0.5% Phytone (BBL), 0.5% peptone (Difco), 0.2% yeast extract (Difco), 0.5% NaCl, 0.25% $K_2HPO_4 \cdot 3H_2O$, and 0.1% KNO_3, dissolved in

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distilled water. After autoclave sterilization, the medium had an unadjusted pH of 7.3 to 7.5. Nitrate served as the primary hydrogen acceptor, since little free oxygen was available in the piston-stoppered tubes in which the cultures were tested for pressure tolerance.

Test cultures were prepared by incubating *E. coli* in this medium for 18 to 20 hr at 30 C. Enough of the test culture, in the negative-acceleration phase of growth, was introduced into a large flask of nutrient medium to give 2 to 5×10^4 viable cells per ml, for testing the effects of pressure on reproduction and growth.

Such inoculated nutrient medium was dispensed in several dozen 10-mm Pyrex test tubes. The latter were sealed with no. 000 neoprene stoppers, so inserted as to exclude air bubbles. When pressed into steel pressure vessels, the stoppers function as pistons, subjecting the contents of the tubes to virtually the same pressure as that of the external hydraulic fluid. Tests with sterile media of various kinds have established that such piston stoppers effectively seal the tubes against either chemical or microbial contamination by the external hydraulic fluid. Pressure was applied to the cultures by the procedures and apparatus described by ZoBell and Oppenheimer (1950) and ZoBell (1959).

Each pressure vessel, filled with hydraulic fluid, was equilibrated to the temperature at which the test was to be made in a constant-temperature water bath. Then, after introducing 12 to 24 stoppered tubes per vessel, pressure was applied, the needle valve was closed, and the loaded vessel was returned to the water bath for incubation. From 1 to 2 min were required to load, close, and pressurize a vessel, and about the same time to decompress, open, and recover the culture tubes for observation.

As reported by ZoBell (1959), compressing such a system to 1,000 atm at ambient temperatures between 5 and 50 C results in temperature increases of 2.2 to 3.9 C, the heat of compression being less at lower pressures and temperatures. The temperature increases due to heat of compression are of short duration when the pressure vessel is immersed in a constant-temperature water bath, the thermal time constant T-63 being only 2 or 3 min in our experiments. The temperature within the pressure vessel was determined with a thermistor connected with a strip recorder.

After incubating or holding for the desired length of time, the pressure vessels were decompressed to permit examination of the treated cultures. The examinations consisted of measuring turbidity nephelometrically, plating appropriate dilutions on EMB Agar (Difco) for colony counts, and observing preparations by both phase contrast and electron microscopy.

EMB Agar was selected for plating cultures of *E. coli* after it was found to give colony counts comparable with results on Phytone-yeast extract-agar and other solid media tried for this purpose. There was no evidence that pressure selectively influenced the ability of *E. coli* to develop on EMB Agar. EMB has the advantage of revealing the presence of contaminating organisms, a matter of considerable importance when working at increased pressures, since there is always a possibility of introducing contaminants while manipulating the piston stoppers in and out of the 10-mm test tubes employed in the experiments. Any experimental culture showing contaminants on the EMB Agar was discarded.

RESULTS

Filament formation. At increased pressures, *E. coli* had a tendency to grow into long filaments, with less and less incidence of fission or cell division as the pressure was increased to the threshold of tolerance. At 400 atm and 30 C, 50 to 75% of the cells exceeded 10 μ in length, some being longer than 100 μ, as compared with 1 to 2 μ for cells grown at 1 atm. The filaments were of normal width, i.e., about 0.6 μ. They appear to be single unsegmented cells, not chains (Fig. 1). Only occasionally could we find a slight indentation in filaments fixed with osmic acid immediately after decompression of the cultures. Fission seemed to take place primarily at the end of the long filaments.

Plating pressurized cultures on EMB Agar demonstrated that reproduction or cell division (as indicated by colony counts) was retarded more by pressure than was growth or increase in biomass [as indicated by optical density (OD)]. Table 1 summarizes results obtained in nutrient medium at 30 C. There was only negligible growth at 500 and none at 525 atm. Note from the data (Table 1) that the ratio of colonies to OD decreased with increasing pressure. Direct microscopic examination of pressurized cultures

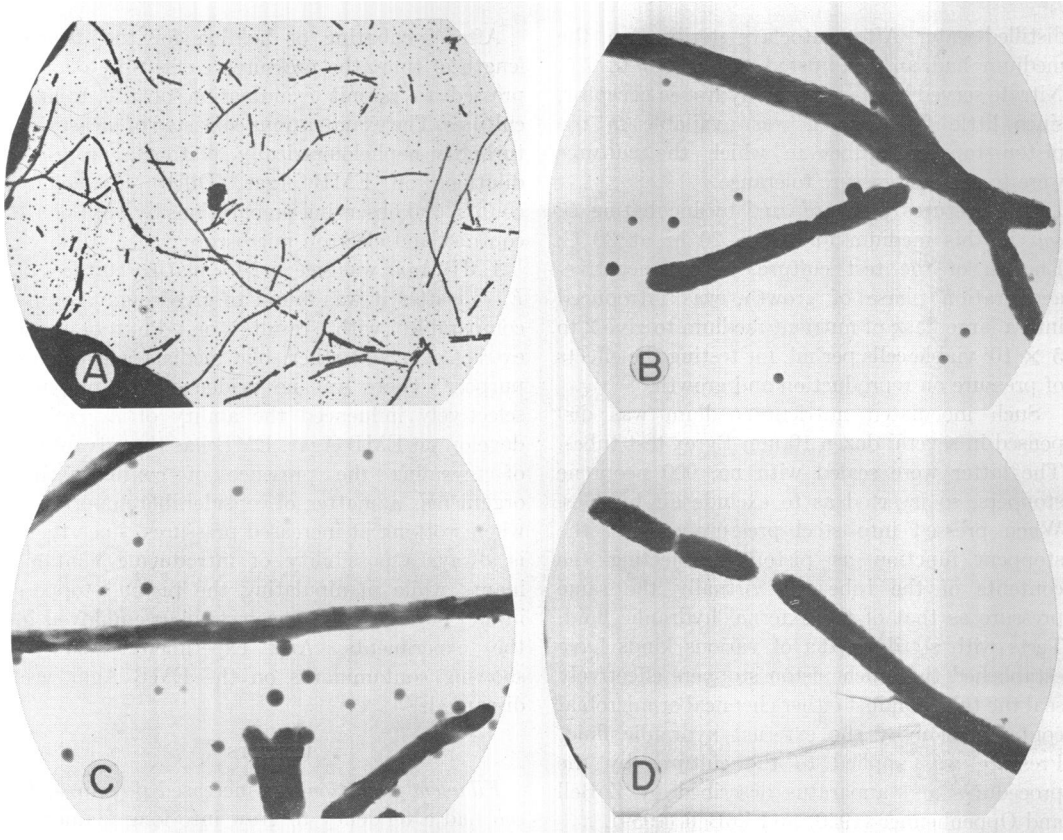

FIG. 1. *Electronmicrographs of Escherichia coli that developed at 450 atm and 30 C. Magnification (970 × for A and 6,100 × for B, C, and D) is indicated by cell width, viz., 0.6 μ.*

TABLE 1. *Colony count, optical density (OD), and ratio of the number of colonies to the optical density of Escherichia coli cultures in nutrient medium after different periods of incubation at different pressures at 30 C*

Period of incubation	Determination	Hydrostatic pressure (atm)				
		1	100	200	300	400
hr						
4	Colonies × 10^3.....	2,300	1,600	1,000	370	90
	OD × 10^{-3}.....	n*	n	n	n	n
	Ratio.....	—	—	—	—	—
6	Colonies × 10^3.....	29,000	16,000	5,000	1,300	470
	OD × 10^{-3}.....	41	29	13	n	n
	Ratio.....	707	551	385	—	—
10	Colonies × 10^3.....	800,000	770,000	490,000	120,000	4,700
	OD × 10^{-3}.....	384	357	300	174	n
	Ratio.....	2,083	2,156	1,633	689	—
12	Colonies × 10^3.....	760,000	760,000	460,000	190,000	7,000
	OD × 10^{-3}.....	409	409	375	292	30
	Ratio.....	1,858	1,858	1,226	650	233

* Denotes an optical density of less than 0.013.

revealed the presence of proportionately more and longer filaments with increasing pressure.

Lag phase. Moderate pressures prolonged the lag phase of *E. coli* in nutrient medium, particularly at pressures higher than 400 atm (Table 2). Only after 7 days of incubation at 500 atm was there any evidence of either growth or reproduction at 30 C. Holding *E. coli* (2×10^4 viable cells per ml) in nutrient medium for 7 days at 525 atm resulted in complete loss of viability. Viable cells could be detected in the decompressed medium neither by transplanting on EMB Agar nor by incubating the decompressed medium at 1 atm for 1 week at 30 C. That decompression was not responsible for the loss of viability in these experiments was demonstrated by compressing *E. coli* cultures to 1,000 atm for 1 min and quickly decompressing them to 1 atm ten successive times within a period of less than 1 hr, with little lethal effect.

TABLE 2. *Effect of hydrostatic pressure on lag period of Escherichia coli, starting with 2×10^4 cells per ml of medium at 30 C*

Pressure	Lag period	Pressure	Lag period
atm	*hr*	*atm*	*hr*
1	ca. 1	450	20–28
300	1–2	475	72–84
400	2–3	500	168–180
425	7–9	525	(Lethal)*

* No viable cells in system after 180 hr.

Effect of pressure on reproduction rates. Pressures of 100 to 400 atm retarded the rate of reproduction or cell division of *E. coli*, as indicated by colony counts (Fig. 2). Such pressures also limited the total number of cells that could be detected, regardless of the period of incubation, albeit part of the cells were somewhat longer than those which developed at 1 atm. Only after several days of incubation was there any evidence of reproduction at 500 atm, and no reproduction occurred at 525 atm at 30 C.

At pressures up to 200 atm, as at 1 atm, cells of *E. coli* reproduced more rapidly at 40 than at 30 C. The maximal stationary phase was reached in 5 to 10 hr at 40 C (Fig. 3) as compared with 10 to 15 hr at 30 C. But at 400 atm, a short temporary increase in the number of viable cells was followed by a definite decrease during the first 10 hr, after which lag phase the cells divided until almost half as many were formed at 400 atm as at 1 atm. Direct microscopic observations showed the presence of a good many long filaments in cultures incubated at 200 to 400 atm, but the biomass was less than in cultures incubated at 1 atm.

When incubated at 20 C (Fig. 4), *E. coli* reproduced more slowly at all pressures tested than it did at 30 or 40 C. Pressures up to 200

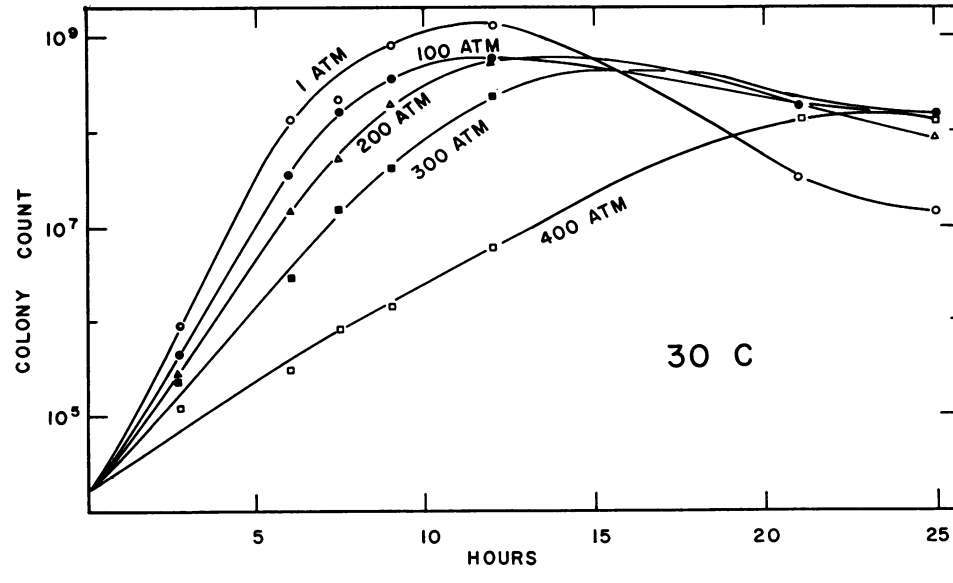

FIG. 2. *Effect of pressure on the reproduction of Escherichia coli in nutrient medium at 30 C, as indicated by colony counts on EMB Agar.*

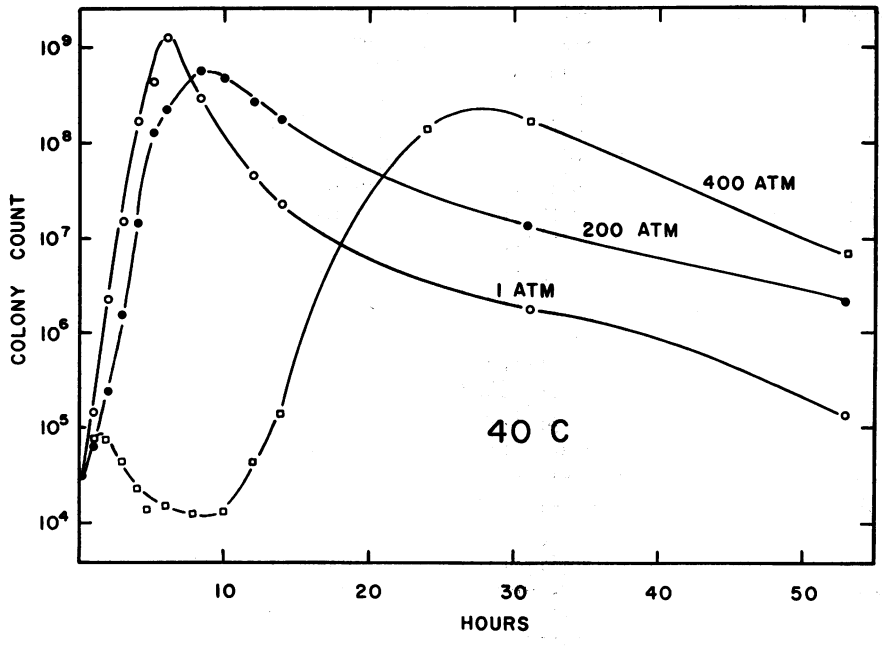

FIG. 3. *Effect of pressure on the reproduction of Escherichia coli in nutrient medium at 40 C, as indicated by colony counts on EMB Agar.*

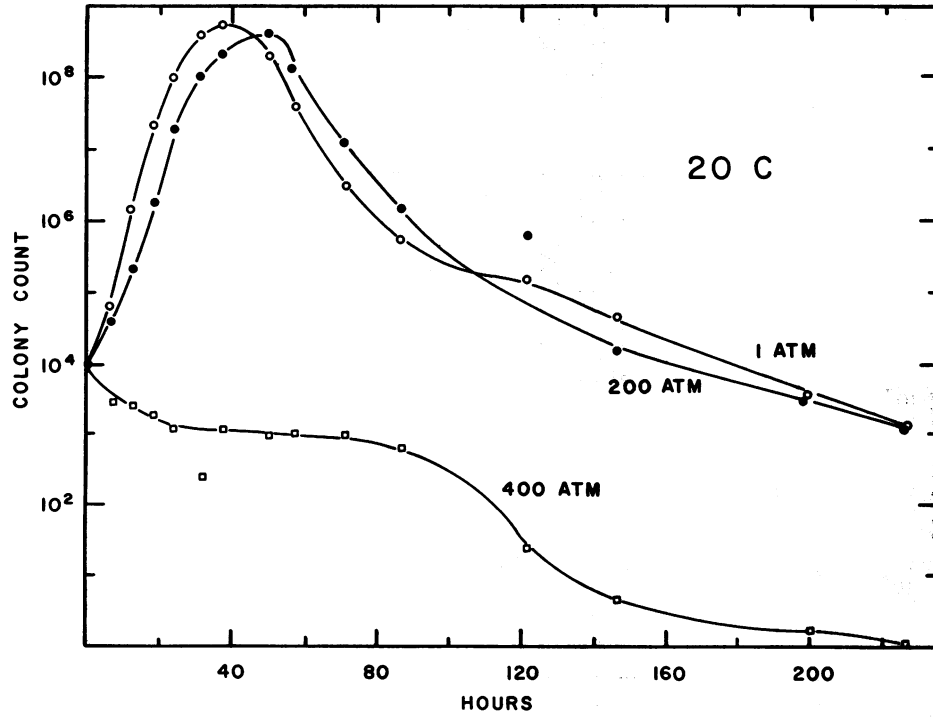

FIG. 4. *Effect of pressure on the reproduction of Escherichia coli in nutrient medium at 20 C, as indicated by colony counts on EMB Agar.*

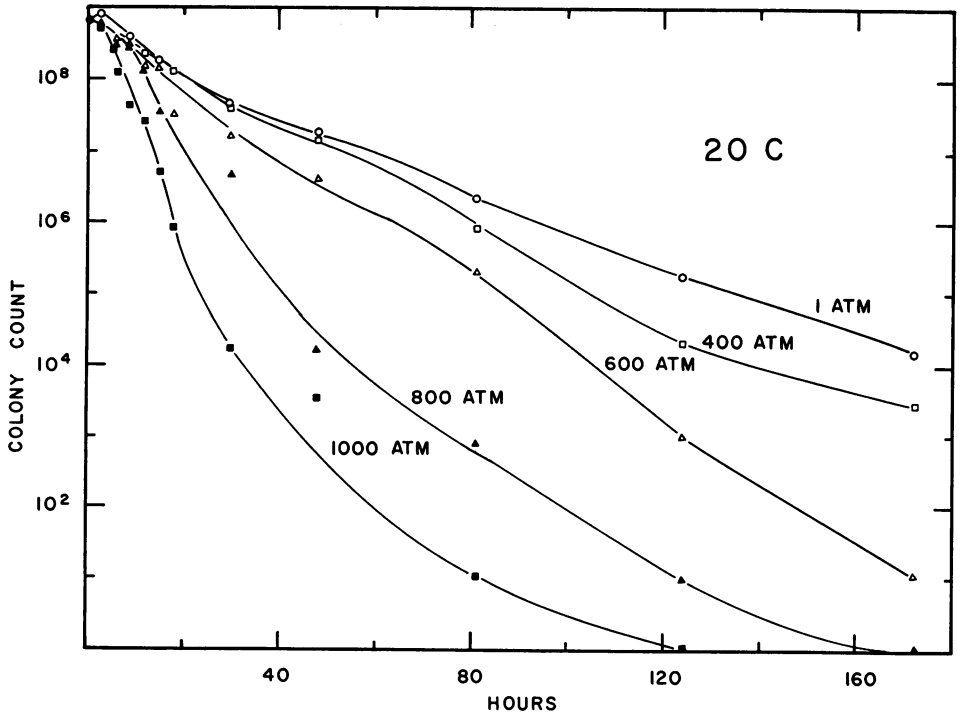

FIG. 5. *Death curves of Escherichia coli in nutrient medium at different pressures at 20 C.*

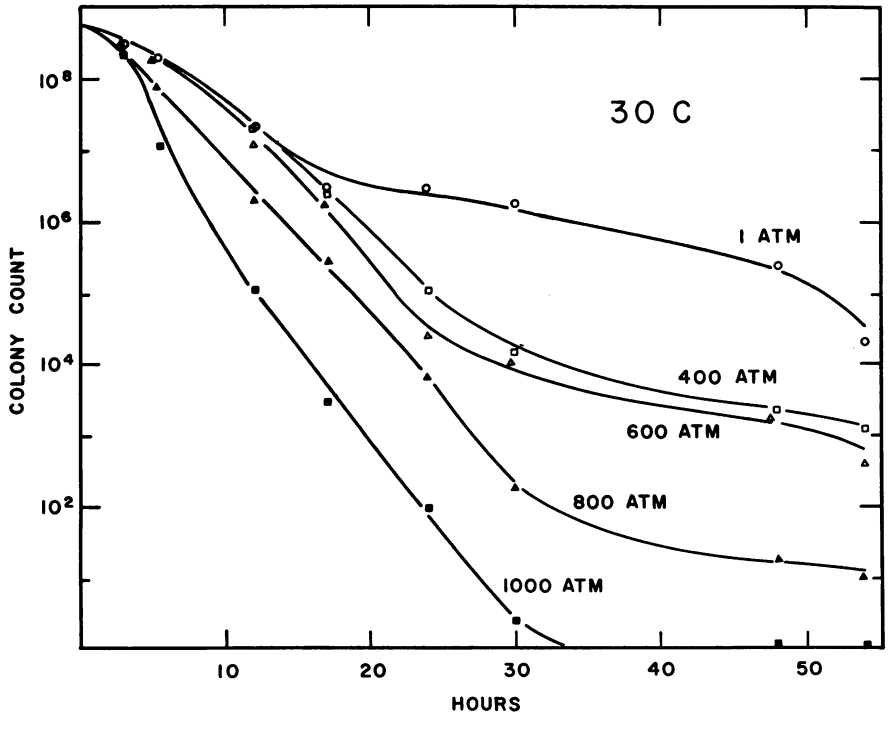

FIG. 6. *Death curves of Escherichia coli in nutrient medium at different pressures at 30 C.*

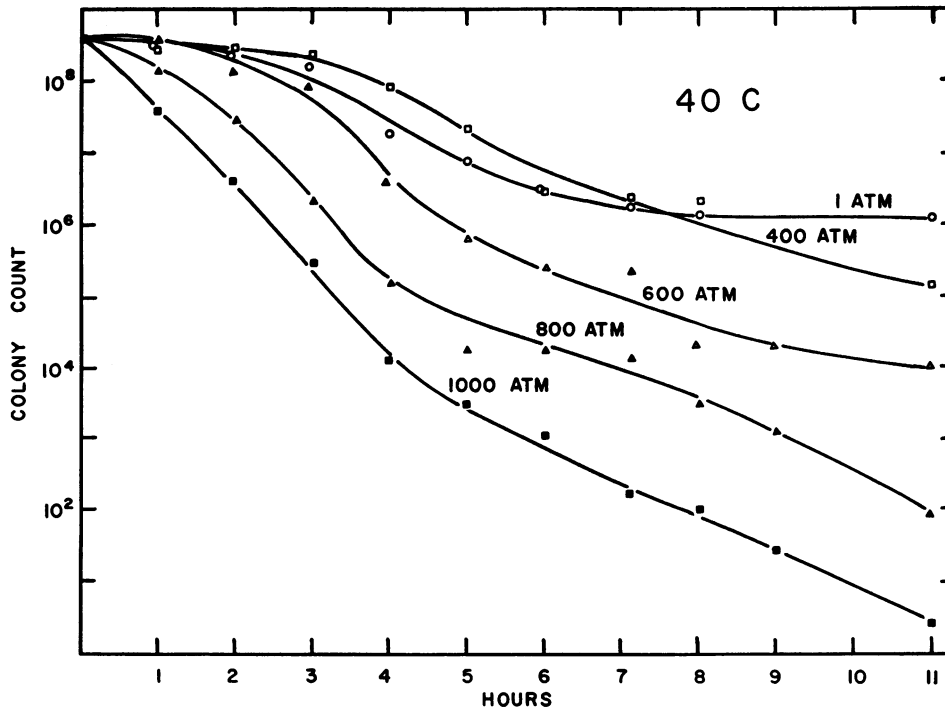

FIG. 7. *Death curves of Escherichia coli in nutrient medium at different pressures at 40 C.*

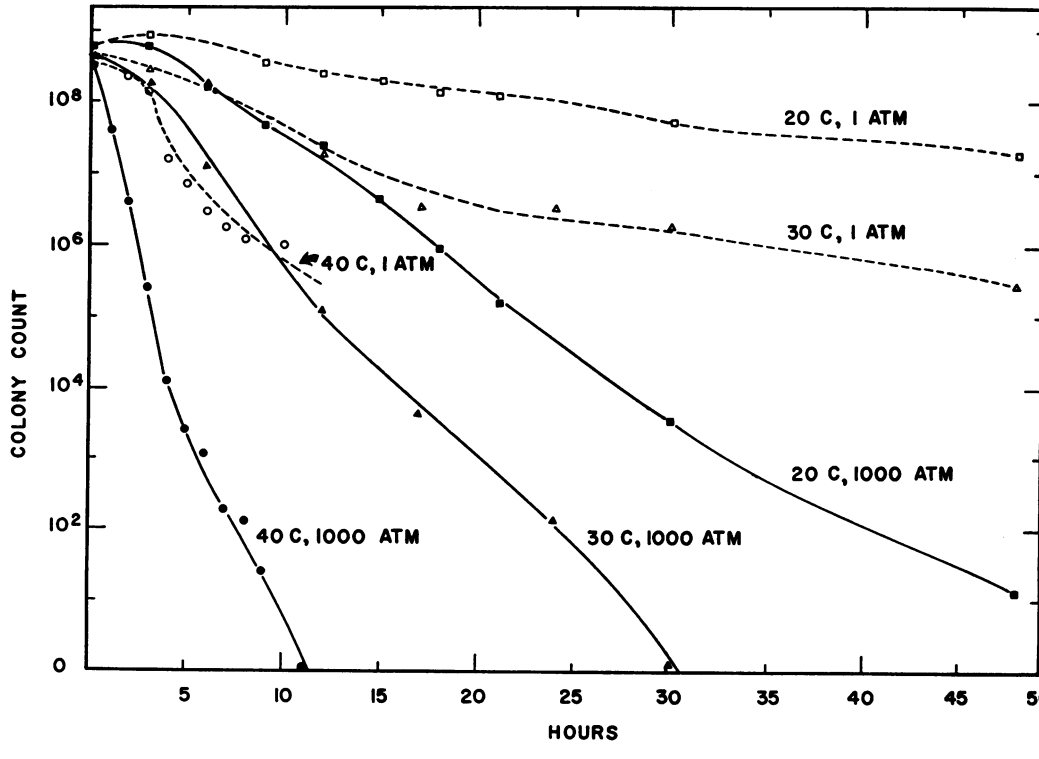

FIG. 8. *Death curves of Escherichia coli in nutrient medium at different temperatures at 1 and at 1,000 atm.*

atm retarded the rate of reproduction somewhat, but at pressures higher than 400 atm there was no demonstrable cell division. Nor was there any growth (increase in cell size or length) at 400 atm. At 400 atm, *E. coli* cells slowly died off at 20 C.

Effect of pressure on death rates. Pressures of 400 atm and higher promote the death rate of *E. coli* in nutrient medium. Test cultures containing 5 to 9 $\times$ 10^8 viable cells per ml, in the maximal stationary phase of growth, were used in the death-rate studies. Such cultures, grown at 1 atm and 30 C, were dispensed in 10-mm tubes for pressure treatment. After different periods of time, pressurized cultures were analyzed for the number of surviving cells, by plating on EMB Agar.

From Figs. 5, 6, and 7, it will be noted that, at all temperatures tested, *E. coli* died off more rapidly when pressurized than when at 1 atm. Pressurized cells as well as those held at 1 atm died off more rapidly at 40 than at 30 C and more rapidly at 30 than at 20 C. Figure 8 compares the effect of temperature on the death rates of *E. coli* at the three different temperatures at 1 and at 1,000 atm.

DISCUSSION

Our results on the effects of temperature on the disinfection of *E. coli* cultures by pressure are somewhat at variance with those of Johnson and Lewin (1946), as well as with those of Johnson and ZoBell (1949), both of whom found that higher temperatures had a protective action against the adverse effects of high pressure. The variance may be due to differences in the cultures or in the chemical composition of the media used in the different experiments. Since various chemical compounds are known to affect the pressure sensitivity of microorganisms (Johnson et al., 1954), further experiments are indicated, to identify those responsible and the mechanisms by which they interact with cell components at different pressures and temperatures. Haight and Morita (1962) found that pressures of 100 to 1,000 atm decreased certain enzymatic activities of *E. coli* at temperatures between 37 and 45 C, but adverse effects of pressure were less at higher pressures.

Interference with cell division, filament formation, and the death of cells appear to be manifestations of the effects of pressure on certain macromolecules, probably enzymes responsible for deoxyribonucleic acid (DNA) synthesis, cell division, or vital metabolic activities. Microscopic examination of specially stained filaments formed at increased pressures shows that they contain less chromatin or nuclear material per vol of cell substance than normal cells grown at 1 atm. Preliminary tests indicate that *E. coli* filaments formed at 450 atm have less DNA per unit of cell protein than have cells grown at 1 atm. This will be the subject of a subsequent paper, since effects on DNA and ribonucleic acid (RNA) synthesis may provide clues to the mechanisms by which increased pressure influences the morphology, reproduction, and disinfection of bacteria.

ZoBell and Oppenheimer (1950) observed abnormal morphology and filament formation in cells of *Serratia marinorubra* and other marine bacteria grown at 400 to 600 atm. Filament or long-thread formation by *Proteus vulgaris* growing in the presence of small amounts of penicillin was reported by Fleming et al. (1950). Filament formation by *E. coli* resembling that found in our pressurized cultures was reported to result from sublethal doses of ultraviolet radiation (Curry and Greenberg, 1962), unfavorable pH (Wahlin and Almaden, 1939), various antibiotics (Pulvertaft, 1952), magnesium excess (Webb, 1949), folic acid analogues (Nickerson and Webb, 1956), and a number of other chemicals (Loveless, Spoerl, and Weisman 1954). According to Webb and Nickerson (1956), abnormal morphology and filamentous growth resulting from various combinations of chemicals bear a relationship to DNA synthesis. Katchmann, Spoerl, and Smith (1955), however, questioned whether interference with synthesis of DNA or RNA is responsible for filament formation.

Possibly, pressure inactivates certain enzymes affecting DNA synthesis or cell division. As will be detailed in a separate communication, pressure interferes with the activity of lactase and certain other carbohydrases of *E. coli*. Morita and ZoBell (1956) showed that the succinic dehydrogenase system of *E. coli* is inactivated by pressure of 200 to 600 atm, and Morita (1957) showed that its formic and malic dehydrogenases are also inactivated by moderate pressure.

His observation of precipitates in ampules of bacterial cultures held at 500 atm led Kriss (1961) to conjecture that apparent lack of growth (as indicated by lack of turbidity and low colony counts) was due to the precipitating or agglomerating action of pressure on suspended

bacterial cells. We found, however, that pressure had no such action on *E. coli* cells in any phase of growth, nor did pressure alter the normal ratio of cells appearing singly, in pairs, or multiplets: the average ratio at 1 atm being 79:15:6, respectively, as compared with 82:13:5 at 400 atm.

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