INFLUENCE OF TIME AND TEMPERATURE OF INCUBATION ON HEAT RESISTANCE OF ESCHERICHIA COLI[1]

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Field and laboratory observations (Frazier *et al.* 1935) long have indicated that the temperature of incubation of Swiss cheese starter cultures significantly influences their ability to develop following the rather severe heat exposure to which they are subjected during manufacture. If it were true that the temperature of growth had an effect on heat resistance of bacteria, this fact would be of significance in various fermentations, both commercial and natural, and would prove of general interest from the standpoint of the physiology of bacteria. Therefore, an investigation was initiated to determine the influence of incubation temperature and time on the thermal resistance of certain Swiss cheese starter cultures. For the purpose of comparison, similar studies were undertaken with a typical strain (H-52) of *Escherichia coli.* The results of the investigations on *E. coli* are presented in this paper.

Because it produced less acid, was able to develop under a wider variety of environmental conditions, and could be counted fairly accurately by the plate method, *E. coli* gave results which were more conclusive than those obtained with the lactic starter cultures and indicated that *E. coli,* a favorite subject for experimentation, is an ideal organism for studies on the heat resistance of vegetative cells.

Despite the apparent importance of the relationship between the growth temperature and thermal resistance of bacterial cells,

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few attempts have been made to correlate them. That the growth temperature may directly influence the thermal relationships of protozoa has been demonstrated by Dallinger (1887), who by gradually increasing the incubation temperature of three flagellates over a seven-year period raised their maximum temperature from 23° to 70°C. At the same time the optimum and minimum temperatures were raised to such a degree that when cells growing at 70°C. were placed at 15°C. they perished.

Sherman and Cameron (1933) found that when young cultures of *E. coli*, grown at 45°C., were placed in sterile media at 10°C., there was a far greater destruction of cells than when young cultures grown at 10°C. were placed in sterile media at 45°C. The same investigators reported (1934) that young cells of *E. coli* from cultures growing slowly exhibited greater resistance to various deleterious factors than did the cells from cultures growing rapidly. The growth rate was reduced by using low incubation temperatures, dilute media or media of increased osmotic pressure.

Frazier and coworkers (1935) demonstrated that a slight elevation in incubation temperature from 35–37° to 38–39°C. markedly increased the ability of *Lactobacillus helveticus* to develop at high temperatures.

According to Anderson and Meanwell (1936) a thermoduric streptococcus in the lag and early logarithmic phases of growth showed increased resistance to heat when the incubation temperature was reduced below the optimum.

Dorner and Thöni (1936) found that after cells of *Bacterium acidi-propionici* had reached the mature, heat resistant stage, there was little difference between the thermal resistance of cells grown at 22° and at 30°C.

Claydon (1937) reported that at 10°C. *Streptococcus lactis* cultures grew more slowly than at higher temperatures, but attained greater thermal resistance.

Theophilus (1935) demonstrated that spores formed at the optimum temperature for growth were definitely more heat resistant than those formed at temperatures either below or above the optimum.

The influence of age of bacterial cells on their thermal resistance has been more widely studied.

Numerous investigators including Reichenbach (1911), Schultz and Ritz (1910), Sherman and Albus (1923, 1924), Behrens (1923), Hückel (1926), Ørskov (1925), Daránÿi (1927), Gates (1929), Jensen (1928), Robertson (1927, 1928), Stark and Stark (1929, a, b), Sherman and Stark (1929), Fabian and Coulter (1930), Hammer and Hussong (1931), Frazier and Wing (1931), Heiberg (1932), Dorner and Thöni (1936), and Claydon (1937) have demonstrated by various methods that young cells are far more susceptible to adverse environmental influences than are older, more mature bacterial cells.

EXPERIMENTAL

In order to maintain the most uniform conditions possible during these investigations, all cultures were incubated in thermostatically controlled water baths, and the time and temperature of incubation were carefully controlled. Moreover, all cultures were carried in freshly prepared, sterile reconstituted skim milk always prepared from the same lot of skim milk powder, and, unless otherwise indicated, one per cent of inoculum was used. Stock cultures, transferred weekly, were incubated at 37°C. for 24 hours after which they were kept at 10°C. until the time for the next transfer.

When it was necessary to inoculate cultures for studies of heat resistance, an inoculating culture was prepared by transfer of one per cent of inoculum from the most recent stock or mother culture to a six-ounce bottle containing 100 cc. of milk. Unless otherwise indicated, this bottle was then incubated under the same conditions as the stock or mother culture and, following incubation, was placed at 10°C. for 36 hours. Then, at varying intervals, inoculations were made from the inoculating culture into triplicate tubes, each containing 10 cc. of milk. The tubes were incubated for the periods and at the temperatures desired. When the triplicate tube cultures were of the required age, they were removed from the water bath. One cubic centimeter of culture from each of the triplicates was placed in a test tube

containing 3 cc. of sterile 2 per cent sodium citrate. The contents were thoroughly mixed; 0.1 cc. was transferred to a tube containing 10 cc. of milk at a temperature of 10°C.; and a sample was removed for the plate count. The tubes were then placed in a mechanically stirred water bath at a temperature of 54°C.; one minute was allowed for the temperature rise, and then heat treatment was carried out at 53°C. for 30 minutes, after which treatment the tubes were immediately cooled and samples removed for plate counts. Plain nutrient agar was used, and plates were incubated at 37°C. for 48 hours.

Curran and Evans (1937) have shown that the indicated percentage survival of cells during heat treatment may be greater if some medium is employed which is superior to plain nutrient agar. Addition of a fermentable carbohydrate to the agar was undesirable because of the resulting gas formation. The results obtained with plain nutrient agar were very uniform and it appeared to be most suitable for these particular studies.

When larger samples were desired, one-fourth per cent of the culture to be heat shocked was transferred to Erlenmeyer flasks containing 450 cc. of milk. The temperature of the milk in the flasks was raised to 53°C. in a period of about five minutes, maintained at 53°C. for 30 minutes and then lowered. Plate counts were made before and after heat treatment.

Percentage survival of mature cells grown at various temperatures

In a preliminary experiment, inoculating cultures were prepared from stock cultures, incubated, respectively, at 28°, 30°, 30.5°, 38.5° and 40°C. for varying periods, and then transfers were made from the inoculating cultures to flasks of milk. The inoculated flasks were heated at 53°C. for 30 minutes, and the heat resistance of the respective cultures was determined. Table 1 shows the percentage survival during heat treatment of cultures which had been incubated for varying periods at the six different temperatures.

These results demonstrate that during the maximum stationary phase of growth the maximum percentage survival of cells at 38.5° and 40°C. is distinctly greater than that obtained at lower

temperatures such as 28°, 30° and 30.5°C. Heat resistance appears to be lowest at 28°C., the lowest incubation temperature employed.

TABLE 1

Heat resistance of Escherichia coli grown at different temperatures for varying periods and then heat shocked at 53°C. for thirty minutes

TEMPERATURE OF INCUBATION	TIME OF INCUBATION	PLATE COUNT		PERCENTAGE SURVIVAL
		Before heating	After heating	
°C.	*hours*	*nos. per cc.*	*nos. per cc.*	
28	38	1,467,000	120,000	8.2
	42	1,445,000	120,000	8.3
	48	1,540,000	93,000	6.0
	54	1,447,000	113,000	7.8
	60	1,333,000	107,000	8.0
30	37	1,674,000	577,000	34
	42	1,393,000	383,000	28
	46	1,233,000	377,000	31
	51	1,357,000	320,000	24
30.5	42	1,510,000	427,000	28
	48	1,390,000	300,000	22
	54	1,257,000	367,000	29
38.5	21	1,341,000	875,000	65
	24	1,188,000	948,000	79
	27	1,145,000	952,000	83
40	10	1,413,000	267,000	19
	12	1,480,000	533,000	36
	16	1,623,000	753,000	46
	20	1,143,000	827,000	72
	24	1,352,000	770,000	57

Heat resistance of cultures of Escherichia coli carried continuously for two weeks at 28° and 38.5°C., respectively

The results shown in table 2 indicate the heat resistance of cultures of *E. coli* after only one incubation period at various temperatures. However, it was considered possible that the heat resistance of a culture might be altered by numerous successive transfers at a definite temperature. Therefore, mother

cultures were inoculated from the stock cultures and carried in tubes containing 10 cc. of milk. According to the results of the previous experiment, shown in table 1, the maximum heat resistance at 28° was maintained from at least the 38th to the 60th hour, and at 38.5° it occurred around the 24th to the 30th hour. In order to make the transfers at the two temperatures at about the time of maximum heat resistance, the 28° culture was transferred every 48 hours and the 38.5° culture every 24 hours. Heat resistance determinations were made after the first transfer and after two weeks of successive transfers at the two temperatures.

TABLE 2

Heat resistance of cultures of Escherichia coli carried at 28° and 38.5°C., respectively, and heat shocked at 53°C. for thirty minutes

INCUBATION OF MOTHER CULTURE		NUMBER OF TRANSFERS	PLATE COUNT		PERCENTAGE SURVIVAL
Temperature	Time		Before heating	After heating	
degrees	*hours*		*nos. per cc.*	*nos. per. cc.*	
28	48	1	1,133,000	253,000	22.3
		1	1,327,000	130,000	9.9
38.5	24	1	1,540,000	960,000	62.3
		1	1,417,000	1,083,000	76.4
28	48	7	1,400,000	250,000	17.9
		7	1,570,000	160,000	10.2
38.5	24	14	1,250,000	1,040,000	83.2
		14	1,490,000	1,210,000	81.2

The cultures to be heated were transferred to flasks of sterile milk and then heat shocked at 53°C. for 30 minutes. Plate counts were made before and after heating. According to the results shown in table 2, the maximum resistance at 28° is again far lower than at 38.5° after both one and numerous transfers at the two temperatures. There seems to be little doubt that when *E. coli* is grown under the conditions of this experiment, thermal resistance during the maximum stationary phase, the time when a culture is considered by most workers to be at the peak of its heat resistance, is far greater when a higher incubation tempera-

ture is used. The results further suggested that a slight increase in heat resistance occurred as a result of numerous successive transfers at the higher temperature.

Influence of age of cells and incubation temperature on heat resistance of E. coli

Next a study was made of the progressive changes taking place in the heat resistance of cultures grown at 28° and 38.5°C. during periods varying from the time of inoculation to the end of the

TABLE 3

Heat resistance of cultures of Escherichia coli grown at 28°C. for varying periods and then heat shocked at 53°C. for thirty minutes

AGE OF CULTURE	PLATE COUNT		PERCENTAGE SURVIVAL
	Before heating	After heating	
hours	*nos. per cc.*	*nos. per cc.*	
0	25,200	1,600	6.4
1.5	33,800	11,500	34.0
3	40,350	550	1.4
6	297,000	320	0.11
9	730,000	140	0.02
12	1,350,000	540	0.04
15	2,730,000	5,120	0.19
18	3,410,000	58,000	1.7
21	3,000,000	55,000	1.8
24	3,170,000	71,800	2.3
27	3,240,000	83,000	2.6
30	2,710,000	129,000	4.8
33	2,560,000	184,000	7.2
36	3,200,000	184,000	5.8

maximum stationary phase. Because of the large number of samples to be heat shocked and plated at one time, triplicate tubes were inoculated from the respective inoculating cultures at varying intervals. The inoculating cultures were grown at the temperature to be used in the experiment. The results contained in tables 3 and 4 and figures 1 and 2 reveal certain changes in heat resistance undergone by *E. coli* as it passes through the various growth phases. The heat resistance at the 0 hour is naturally that of the inoculating culture. When active reproduc-

tion commences, the heat resistance decreases and according to the actual numbers of survivors shown in the table, it would seem that the survivors are cells which had not reproduced in the culture. As the rate of reproduction diminishes, there is a corre-

TABLE 4

Heat resistance of cultures of Escherichia coli grown at 38.5°C. for varying periods and then heat shocked at 53°C. for thirty minutes

AGE OF CULTURE	PLATE COUNT		PERCENTAGE SURVIVAL
	Before heating	After heating	
hours	*nos. per cc.*	*nos. per cc.*	
0	25,170	18,500	74
1	32,300	5,450	17
2	78,700	1,000	1.3
3	233,000	6,700	2.9
4	617,000	3,300	0.53
5	990,000	3,300	0.33
6	1,365,000	3,300	0.24
7	2,113,000	3,300	0.15
8	2,540,000	6,700	0.27
9	3,150,000	60,000	1.9
10	2,910,000	53,000	1.8
11	2,820,000	140,000	5.0
12	2,466,000	290,000	12
13	2,680,000	460,000	17
14	2,780,000	540,000	19
15	2,740,000	1,260,000	46
16	2,514,000	1,160,000	46
17	2,114,000	1,380,000	65
18	2,900,000	1,520,000	52
19	2,660,000	2,120,000	80
20	2,320,000	1,340,000	58
21	2,780,000	1,780,000	64
22	2,840,000	1,720,000	61
23	2,880,000	1,993,000	69
24	3,360,000	1,880,000	56

sponding rise in heat resistance until a peak is attained well along in the maximum stationary phase of growth. There follows a period which varies with the incubation temperature, during which period both the numbers of organisms and the heat resistance remain fairly constant, and after which there is a slow

and gradual decline in numbers and in resistance of the cells. Again the heat resistance of the 38.5° culture is far greater than that of the 28° culture. The one and one-half hour sample at 28° indicates an abrupt and brief increase in heat resistance comparable to that reported by Anderson and Meanwell (1936) for a thermoduric streptococcus.

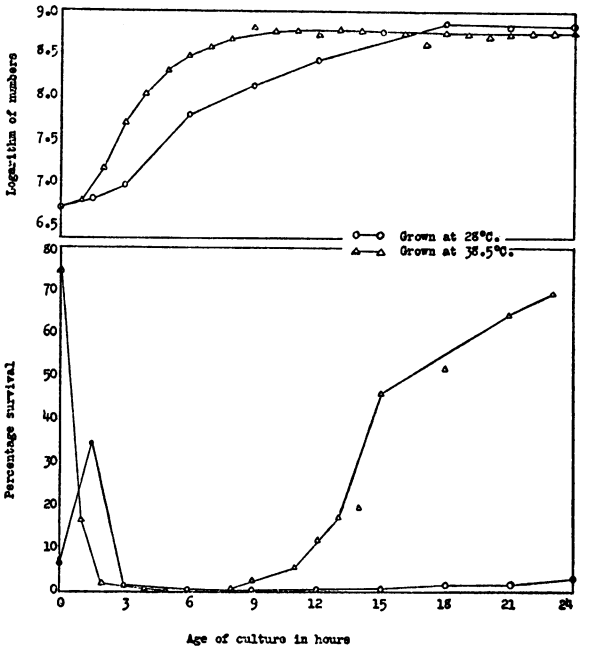

FIG. 1. Upper part shows growth curves of cultures of *Escherichia coli*; lower part shows influence of time and temperature of incubation on percentage survival of cells during heat treatment at 53°C. for thirty minutes. Age of cultures, 0 to 24 hours.

Heat resistance of cultures of E. coli during the initial stationary growth phase

The observation that there was an increase in resistance during the early hours of incubation at 28°C. led to a more thorough investigation of the heat resistance of *E. coli* during the first few hours of growth at 28° and at 38.5°C. Inoculating cultures, prepared from stock cultures, were incubated, respectively, at

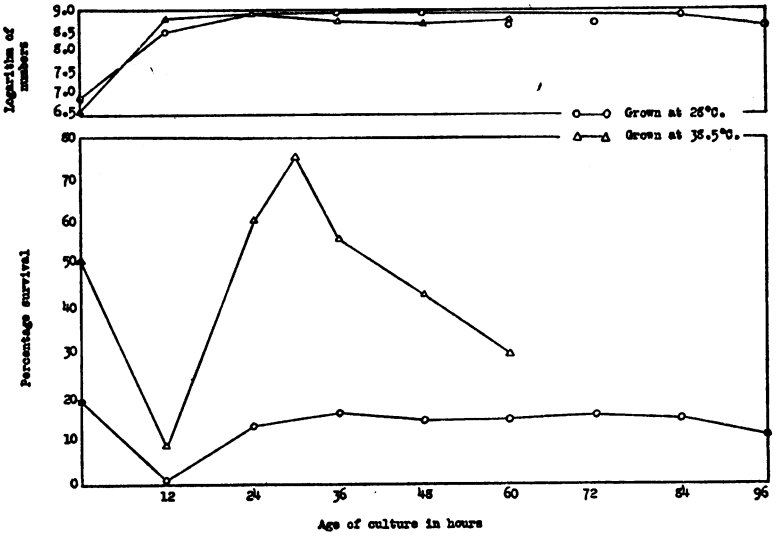

FIG. 2. Upper part shows growth curves of cultures of *Escherichia coli*; lower part shows influence of time and temperature of incubation on percentage survival during heat treatment at 53°C. for thirty minutes. Age of cultures, 0 to 96 hours.

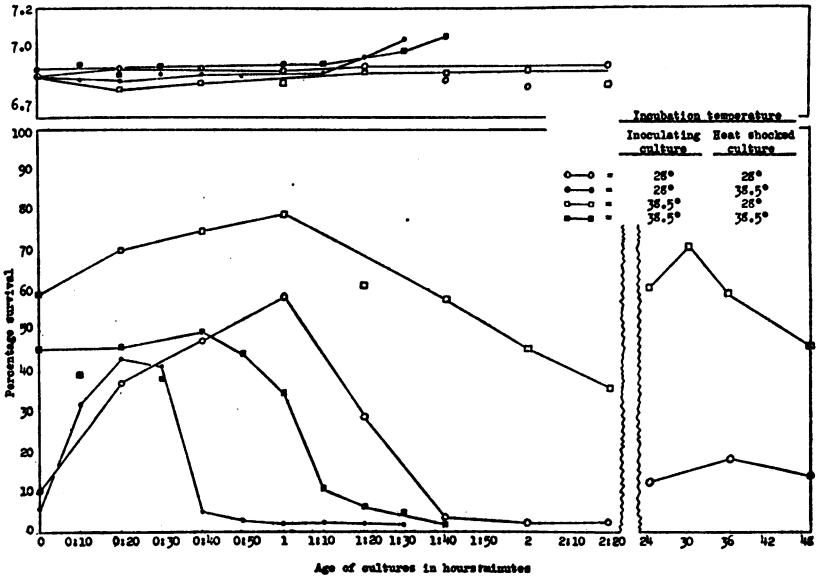

FIG. 3. Upper part shows growth curves of cultures of *Escherichia coli*; lower part shows influence of time and temperature of incubation on percentage survival of cells during heat treatment at 53°C. for thirty minutes.

28° for 48 hours and at 38.5°C. for 24 hours. Triplicate tubes were inoculated from these at varying intervals and incubated at 28° and 38.5°C., respectively. To determine the heat resistance of the tube cultures during the first three hours, the percentage survival was calculated. The results are contained in figure 3. The highest percentage survival obtained during the maximum stationary phase of growth at 28° and 38.5°C., respectively, is also shown for purposes of comparison.

Contrary to expectations, it was found that just before or during the "period of physiological youth" there was a distinct, abrupt rise in heat resistance of the culture, and then, a short time before reproduction commenced, a rapid decline in resistance to the low level characteristic of the phase of logarithmic growth.

However, the maximum percentage survival during the initial stationary phase of a culture inoculated from one grown at 38.5°C., generally exceeded that obtained with the subculture from the 28°C. inoculating culture. The greatest thermal resistance was demonstrated by 28° cultures inoculated from a 38.5°C. inoculating culture. When a 38.5°C. inoculating culture was transferred to fresh media with incubation at 38.5°C., no increase in heat resistance occurred during the lag phase of this subculture. These results, therefore, suggest that apparently some factor inherent in the inoculum plays an important rôle in determining the degree of heat resistance during the lag phase of growth.

DISCUSSION

It has been a common belief that a culture of bacteria, in any but the logarithmic growth phase, when placed in a medium advantageous for growth, passed through a stationary or lag phase and underwent a process of "biological rejuvenescence" which was followed by active reproduction. It has been believed that the heat resistance of the culture was notably low during the period of physiological youth and remained low as long as reproduction took place at a rapid rate. It is now apparent that still another change is manifest during the early life of a culture, and that this transitory change is characterized by a very decided increase in heat resistance. This increase is apparently more

marked when the culture is placed at a temperature below the optimum for growth.

In these experiments, it was found that during the period of most active reproduction at both 28° and 38.5°C. the heat resistance declined to its lowest point. Then as the cultures entered the maximum stationary phase, their heat resistance again rose. At 28°, however, the maximum percentage survival rarely reached more than 20 per cent and was usually less than 10 per cent, while at 38.5°C., 50 to 80 per cent was usually reached. At 30° the percentage survival was generally about 25 to 30 per cent. The maximum resistance at 40° and 42° was similar to that at 38.5°C. At the higher temperatures, therefore, the maximum percentage survival during the maximum stationary phase in every case exceeded that obtained at the temperatures below the optimum for growth. There are two possible explanations for such an effect of incubation temperature on heat resistance. The temperature at which the organisms are grown may in some manner influence the physico-chemical properties of the cells and thus render them more or less resistant to heat. The fact that the difference between heat resistance of 28° and 38.5° cultures was evident on the first transfer at the two temperatures would tend to discount this theory. Another explanation might be the following: At 38.5°C. reproduction and accompanying changes take place at about twice the rate at 28°C. Therefore, a greater accumulation of mature cells may be possible at 38.5° than could take place at any one time at 28°C. As a result, the maximum percentage survival during the maximum stationary phase would be decidedly greater at the higher incubation temperature.

In addition to their interest from the standpoint of bacterial physiology, the results reported above are of significance for various other reasons. *E. coli* has long been used to determine the efficiency of different methods of destroying vegetative cells. It is apparent that the resistance of *E. coli* to heat and possibly to other adverse factors varies markedly with the time and temperature of incubation and that these, together with the medium in which the organisms are grown before or following heat or

other exposure, will play an important part in determining the number of surviving cells.

As indicated by Anderson and Meanwell, organisms other than *E. coli* may increase decidedly in heat resistance during the early hours of growth. Therefore, the temperature at which milk is held previous to pasteurization may profoundly influence the efficiency of the process. The survival of organisms during other processing treatments, intended primarily to decrease the number of vegetative cells in a liquid, may be affected in the same manner.

It has been shown by Elliker (1937) that the culture medium and time and temperature of incubation might have a significant effect on the activity of Swiss cheese starter cultures. Lactobacilli growing more rapidly in a favorable medium generally demonstrated greater heat resistance than did those growing more slowly in a poor medium. Furthermore, when the same organisms were grown in a poor medium, a slight elevation of the incubation temperature and, therefore, a consequent increase in rate of growth usually resulted in a more heat resistant culture. In the present studies with *E. coli*, greatest heat resistance during the maximum stationary phase was exhibited at the higher temperatures where rate of growth had been comparatively rapid.

The results of Anderson and Meanwell and the observations reported in this paper indicate that starter cultures may also exhibit an increase in resistance during the initial stationary phase of growth and that this increased thermal resistance may significantly influence the behavior of the starter organisms during the early stages of the cheese making process, particularly if the cheese be one like Swiss where comparatively high temperatures are employed in the making process.

Future investigations may determine whether or not other species of bacteria demonstrate the same changes in thermal resistance as do the thermoduric streptococcus used by Anderson and Meanwell and *Escherichia coli*.

SUMMARY

Cultures of *Escherichia coli* exhibited a decided increase in heat resistance, as evidenced by percentage survival of cells during

heat treatment, while in the initial stationary phase of growth. The increase in heat resistance was more marked in cultures incubated at 28° than in those incubated at 38.5°C. The time and temperature of incubation of the culture used for inoculum decidedly influenced the degree of increase in heat resistance during the initial stationary phase of growth of the subculture.

The heat resistance of all of the cultures decreased as reproduction commenced and their resistance fell to a minimum during the period of most active reproduction. The resistance then increased again to a second peak as the rate of reproduction decreased and the culture entered the maximum stationary phase of growth.

Growth at and above the optimum temperature resulted in cultures whose heat resistance during the maximum stationary phase was distinctly greater than was true of cultures incubated at temperatures below the optimum for growth.

Possible reasons for the variations between heat resistance of cultures grown at high and low temperatures and the practical significance of the results are discussed.

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