FACTORS WHICH INFLUENCE THE GROWTH OF HEAT-TREATED BACTERIA

II. Further Studies on Media[1]

F. E. NELSON[2]

Kansas Agricultural Experiment Station, Manhattan, Kansas

Received for publication May 22, 1944

In the preceding publication in this series (Nelson, 1943), the medium used for enumeration of bacteria by the plating procedure was shown to be a factor in the apparent survival of heat-treated bacteria. The suggestion was made that factors other than the gross chemical composition of the medium might influence the initiation of growth of bacteria following heat treatment. The present studies were undertaken to determine the possible effect of certain variations in the preparation of media upon the viability of pure cultures of bacteria which have been subjected to heat treatments approaching those lethal for the various test organisms.

METHODS

The cultures and the plating methods were the same as certain of those employed in the previous studies (Nelson, 1943). The synthetic basal medium contained 1.0 g of $NH_4H_2PO_4$, 1.0 g of K_2HPO_4, 5.0 g of glucose, and 15.0 g of agar per liter and was adjusted to pH 6.8 by colorimetric means. Peptone, in the amounts indicated, was added to this basal medium either before autoclaving at 15 pounds' pressure for 20 minutes or immediately before the cooled agar was poured into the plates. In the latter case, the peptone was added as a sterile 10 per cent solution in distilled water. The plates were incubated ± 3 hours at 36.5 C in a carefully controlled, water-jacketed incubator and counted with the aid of a Quebec colony counter.

RESULTS

Preliminary results, using 0.5 per cent quantities of neopeptone, Bacto peptone, and tryptone added to the basal medium prior to autoclaving, indicated that tryptone was the most satisfactory of these peptones for permitting growth of the greatest numbers of heat-treated bacteria; hence this peptone was chosen for use in further studies.

The influence of the presence of varying concentrations of tryptone in the synthetic basal medium and also of added thioglycollic acid upon the development of heat-treated bacteria is shown by the data in table 1. Counts on tryptone glucose extract milk agar (Committee, 1941) are included for comparison. The basal medium supported growth of the heated lactic streptococci only in the 1:10 dilution, and that only because of the milk added with the

[1] Contribution No. 223, Department of Bacteriology.

[2] Present Address: Iowa State College, Ames, Iowa.

inoculum. Data on counts of unheated control cultures are not presented. All of the supplemented media provided adequate conditions for the development of these control organisms. The unheated lactic streptococci failed to grow on the unsupplemented medium because the necessary 1:100,000 dilution of the inoculum did not furnish sufficient milk. The counts on the unheated controls were relatively constant for each series of platings on media of varying composition and ranged from 15 to 25 millions per ml, the exact range varying somewhat from series to series.

The results show that a decrease in concentration of tryptone from 0.5 to 0.2 per cent results in a marked reduction in count of heat-treated bacteria; further decrease in tryptone content of the medium results in a further decline in count in nearly all series. The magnitude of these reductions seemed in no way

TABLE 1

Effect of varying the tryptone content of a synthetic basal medium on the apparent survival of heat-treated bacteria

ADDITIONS TO THE SYNTHETIC MEDIUM	PLATE COUNTS PER ML OF HEAT-TREATED CULTURES (000'S OMITTED)							
	Escherichia coli (55 C for 8 min)		*Streptococcus liquefaciens*		*Streptococcus zymogenes* (62 C for 15 min)		*Streptococcus durans*	
	No. 1	No. 2	No. 1 (62 C for 8 min)	No. 2 (62 C for 15 min)	No. 1	No. 2	No. 1 (65 C for 12 min)	No. 2 (65 C for 10 min)
Control	0.46	0.32	<10	2.8	0.46	<0.1	<0.01	<1.0
0.5 per cent tryptone	6.5	16.0	810	21.0	15.0	7.5	5.3	310
0.2 per cent tryptone	3.0	4.6	340	12.0	6.3	4.3	2.9	160
0.04 per cent tryptone	1.0	0.89	72	2.7	2.2	4.4	0.80	140
0.01 per cent tryptone	0.74	0.39	44	2.1	0.40	4.1	0.40	69
0.01 per cent tryptone plus 0.01 per cent thioglycollic acid	14.0	25.0	360	9.5	2.7	4.4	0.10	130
Tryptone + glucose + extract + milk agar	3.6	10.0	280	12.0			2.7	220

associated with the number of colonies developing on the individual plates. This fact, coupled with the absence of decrease in counts of the unheated control cultures, seems to furnish evidence that the observed effect is due to something other than the gross change in chemical composition of the medium.

The addition of 0.01 per cent thioglycollic acid to the medium containing 0.1 per cent tryptone resulted, in all instances except two, in large increases in count as compared with results with the same medium without the added thioglycollic acid. In several instances, the medium supplemented with thioglycollic acid yielded larger counts of heat-treated bacteria than were obtained using tryptone glucose extract milk agar, despite the greater complexity and higher concentration of nutrients in the latter medium. Again, this would indicate that the mere presence of certain nutrients in the medium is probably

not always the controlling factor in determining the ability of heat-treated bacteria to initiate proliferation.

Comparisons of counts on basal media supplemented with varying amounts of tryptone, added both before sterilization of the medium and after sterilization just before plating, are shown in table 2. Again, no significant differences in counts on the various media were obtained when the unheated control cultures were plated, the counts varying from 15 to 25 millions per ml in different series; therefore, counts on these control cultures are not presented. In the case of the heat-treated cultures, the addition prior to final sterilization of 0.5 per cent tryptone, and sometimes of 0.2 per cent tryptone, resulted in considerably higher counts than were obtained with the addition of the same amounts of peptone at the time the plates were poured. When only 0.01 per cent of tryptone was used, however, the difference usually was in favor of the medium in

TABLE 2

Effect of time of adding tryptone to basal synthetic medium on the apparent survival of heat-treated bacteria

TIME OF ADDITION OF TRYPTONE	AMOUNT OF TRYPTONE ADDED	PLATE COUNT PER ML OF HEAT-TREATED CULTURE (000'S OMITTED)				
		Streptococcus liquefaciens (62 C for 15 min)	*Streptococcus zymogenes* (62 C for 15 min)		*Streptococcus durans*	
			No. 1	No. 2	No. 1 (65 C for 12 min)	No. 2 (65 C for 10 min)
	%					
Before sterilizing agar	0.5	60.0	15.0	7.5	5.3	310
	0.2	14.0	6.3	4.3	2.9	160
	0.04	2.8	2.2	4.4	0.8	140
	0.01	2.0	0.4	4.1	0.4	69
Immediately before plating	0.5	3.2	7.7	4.9	2.3	190
	0.2	3.0	3.9	4.3	1.3	170
	0.04	2.6	1.7	3.6	1.2	140
	0.01	2.7	1.9	3.9	0.8	98

which the addition was made at the time the plates were poured. No studies were made using amounts of tryptone in excess of 0.5 per cent. These data also indicate that factors other than the gross chemical composition of the medium are operative in permitting the initiation of growth of bacteria which have been exposed to sublethal heat.

DISCUSSION

The results of these investigations furnish additional evidence that heat-treated bacteria are more exacting in their requirements for initiation of growth than are unheated control bacteria. Not only the presence of adequate kinds of nutrients but also the quantities of these nutrients and the order in which the peptone supplement is added to the basal medium seem to be of significance in determining the viable population in a heat-treated culture.

A possible explanation for the increased counts obtained with the media supplemented with larger amounts of tryptone added before autoclaving may be the greater capacity of larger quantities of this ingredient to bring about and maintain a reducing potential in the medium, and thus facilitate the development of heat-treated bacteria which otherwise would not form colonies. The high counts obtained when 0.01 per cent thioglycollic acid was added to the medium supplemented with 0.01 per cent tryptone may be considered additional evidence that the reducing character of the medium is a factor in permitting the initiation of growth of heat-treated bacteria. The importance of the reducing character of the medium in the initiation of growth of certain types of bacteria has been recognized for some time (Dubos, 1929; Allen and Baldwin, 1930 and 1932). Quastel and Stephenson (1926) suggested the use of thioglycollic acid, as well as cysteine and glutathione, for the establishment of anaerobic conditions in culture media; and Brewer (1940) made use of this suggestion in formulating his clear liquid medium which has been used rather extensively for the cultivation of anaerobic bacteria and the testing of surgical supplies and similar materials for sterility.

Dubos (1929) showed that heating of the complete medium resulted in the establishment of reducing conditions which affected the test organism, *Clostridium sporogenes*, in the same way as did addition of reducing substances such as cysteine or thioglycollic acid. Hartelius and Nielsen (1941) ascribed the favorable effects of heating the media which they studied to the reaction of glucose with ammonium hydroxide, sodium hydroxide not being effective. Smiley, Niven, and Sherman (1943) obtained similar results by heating glucose or arabinose with either sodium hydroxide or ammonium hydroxide, with *Streptococcus salivarius* as the test organism. Pyruvic acid or acetaldehyde added to a medium containing glucose which had been sterilized separately had the same effect as the addition of the alkali-treated sugars. Neither of these observations would explain the results of the studies reported in this paper, as here the peptone was the portion sterilized separately, and glucose and ammonium phosphate were present in the basal medium and were sterilized together. Snell (1942) observed that pyridoxine was changed in such a way as to become available to *Streptococcus lactis* R when this compound was autoclaved with the medium or with certain amino acids. These results were obtained using uninjured cultures, but some similar action might be involved in the present studies in which more sensitive heat-treated bacteria were employed. The greater counts obtained when the larger amounts of tryptone are added before autoclaving, rather than just before the plates are poured, may be due to the influence of the relatively high temperature during autoclaving in altering the physico-chemical character of the complete medium in a manner different from that resulting from autoclaving separate portions of the medium. The favorable effect of the addition of thioglycollic acid to the medium seems to substantiate the hypothesis that the establishment of a more reduced potential is at least an important factor in permitting the development of heat-treated bacteria of the types studied.

The data indicate that more consideration probably should be given to the physico-chemical characteristics of the media used for the enumeration of heat-treated bacteria of the types studied. This factor may be of considerable significance in counts of bacteria in pasteurized milk and other heat-treated products. Conditions such as partial drying of the medium and exposure to sunlight, where photochemical oxidation may result, may owe some of their inhibitory effect to the creation of a more oxidizing condition in the medium.

CONCLUSIONS

Variation of the tryptone content of the medium, addition of thioglycollic acid, and differences in the time of addition of peptone in the preparation of the medium may influence the numbers of certain nonsporulating bacteria which develop into countable colonies following sublethal heat treatment.

The observed variations in colony counts of heat-treated bacteria may result from alterations in the physico-chemical character of the medium; a more reduced potential is considered favorable for the initiation of growth of heat-injured cells and thus for the formation of greater numbers of countable colonies.

REFERENCES

ALLEN, W. P., AND BALDWIN, I. L. 1930 The effect of the oxidation-reduction character of the medium on the growth of an aerobic form of bacteria. J. Bact., **20**, 417–439.

ALLEN, W. P., AND BALDWIN, I. L. 1932 Oxidation-reduction potentials in relation to the growth of an aerobic form of bacteria. J. Bact., **23**, 369–398.

BREWER, J. H. 1940 A clear liquid medium for the "aerobic" cultivation of anaerobes. J. Bact., **39**, 10.

Committee of the Laboratory Section, American Public Health Association. 1941. Standard methods for the examination of dairy products. 8th ed. Am. Pub. Health Assoc., New York City.

DUBOS, R. 1929 The initiation of growth of certain facultative anaerobes as related to oxidation-reduction processes in the medium. J. Exptl. Med., **49**, 559–573.

HARTELIUS, VAGN, AND NIELSEN, NIELS. 1941 Bildung von Hefewuchsstoff durch Erwärmung von Zucker mit Ammoniumhydroxyd. Biochem. Z., **307**, 333–340.

NELSON, F. E. 1943 Factors which influence the growth of heat-treated bacteria. I. A comparison of four agar media. J. Bact., **45**, 395–403.

QUASTEL, J. H., AND STEPHENSON, M. 1926 Experiments on "strict" anaerobes. I. The relationship of *B. sporogenes* to oxygen. Biochem. J., **20**, 1125–1137.

SMILEY, K. L., NIVEN, C. F., JR., AND SHERMAN, J. M. 1943 The nutrition of *Streptococcus salivarius*. J. Bact., **45**, 445–454.

SNELL, E. E. 1942 Effect of heat sterilization on growth-promoting activity of pyridoxine for *Streptococcus lactis* R. Proc. Soc. Exptl. Biol. Med., **51**, 356–358.