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OBSERVATIONS ON THE PHYSIOLOGY OF AEROBIC THERMOPHILIC SPOROGENOUS BACILLI IN SUPPLEMENTED CASEIN HYDROLYZATE MEDIA

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OBSERVATIONS ON THE PHYSIOLOGY OF AEROBIC THERMOPHILIC SPOROGENOUS BACILLI IN SUPPLEMENTED CASEIN HYDROLYZATE MEDIA

By

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HISTORICAL INTRODUCTION

The influence of environmental temperature on growth and multiplication of all forms of life cannot be overestimated, and a great bulk of work has disclosed much pertinent knowledge of the temperature requirements and tolerances of a wide variety of bacteria. The study of bacterial growth at temperatures higher than 40 C has received an enormous amount of attention of investigators. Excellent and exhaustive reviews, including the early and descriptive work, and the later more quantitative work, are available (Robertson, 1927, Casman, 1933, and Gaughran, 1947).

Definition of optimum growth temperature for bacteria is based upon rate of increase in number of cells, but it is customarily described from simple observations considered by some as arbitrary and inadequate (Dorn and Rahn, 1939, and Hansen, 1933). Determination of temperature requirements for growth of bacteria is usually made by incubation of suitably inoculated media at a variety of temperatures, and after lapse of time, examining the media for best apparent growth, or estimating the crop of cells, total or viable, or determining the concentration of a metabolic product. Dorn and Rahn (1939) have shown that the temperature optima for rate of fermentation and rate of growth are different for a thermophilic streptococcus. Hansen (1933) found that the rate of growth and maximum cell crop possessed different temperature optima, for a thermophilic member of genus Bacillus.

Observations of differences in growth temperature requirement, or tolerance, have compelled the bacteriologist to adopt vocabulary descriptive of these relationships: thermophilic bacteria which grow readily at 55 C and 37 C are commonly called facultative (Morrison and Tanner, 1924); those which grow at 55 C but not at 37 C are termed obligate (Cameron and Esty, 1926); those which withstand elevated temperatures, but which do not presumably grow at such temperatures are called thermoduric (Robertson, 1927).

Imsenecki and Solnzeva (1945) divided thermophilic bacteria into two groups: the "stenothermal thermophiles", which grow well at 60 C, but not at 30 C; and the "eurithermal thermophiles", which grow well at 60 C as well as at 30 C. Gordon and Smith (1949) showed that many organisms of genus Bacillus were capable of growth over a wide range of temperatures. Their method of study was examination of agar slants for growth, a method previously criticized by Dorn and Rahn (1939) and Hansen (1933).

Thermophilic members of genus Bacillus have been much studied because of their ubiquity, relative ease of handling, importance in industry, and possible taxonomic significance. Practically every material which has ever been in contact with soil has been shown to yield thermophiles, even fresh snow (Golikowa, 1926) and deep ocean bottom mud (Bartholomew and Rittenberg, 1949). Hansen (1933) explains the ubiquity of the thermophiles by their slow but continuous and substantial growth at temperatures as low as 20 C. Imsenecki and Solnzeva (1945) and Gaughran (1946) failed to find that their organisms grew at temperatures as low as described by Hansen.

The present classification of the thermophilic members of genus

Bacillus is chaotic. It is based on cell measurements, condition of sporangia, and a few biochemical characters, which are acknowledged to be quite variable. Smith, in Bergey's manual (Breed, et al, 1948), stated:

> The data on the species of this group are so meager that it is not possible to offer a rational system of classification. Many of the characters used for separating the various species are probably as variable in this group as they have been found to be in the mesophilic group. Lacking a knowledge of the limits of variability and lacking other pertinent data, the present arrangement is regarded as temporary only.

In an attempt to elaborate a reasonable scheme of classification, Gordon and Smith (1949) examined about 200 strains of thermophilic sporogenous bacilli, and proposed the recognition of two general types, as regards temperature relationships: (1) those which grow at the usual temperatures (30 C to 40 C), but not at 65 C; and (2) those which fail to grow at the usual temperatures, but grow well at 65 C. They adopted the name Bacillus coagulans Hammer (Hammer, 1915, Hussong and Hammer, 1929, and Searles and Hammer, 1932) as representative of the former type, and Bacillus stearothermophilus Donk (Donk, 1920) as representative of the latter group. Some of the cultures they studied, which had been identified as Bacillus thermoacidurans Berry (Berry, 1933) were shown to grow at 37 C and 55 C, but failed to grow at 65 C, and were therefore called B. coagulans; others so labeled grew well at 65 C, and were therefore called B. stearothermophilus. Numerous other named strains studied were considered to be misnamed.

It has been shown that as thermophilic bacteria grow rapidly at elevated temperatures, they also die rapidly (Hansen, 1933, Imsenecki and Solnzeva, 1945). Stained smears reveal enormous numbers of "ghost

cells", and the visible count was found to vary from the total count by 50 to 100 per cent. As the cells grow rapidly, and autolyze, enzymic material accumulates rapidly in the medium. The production of amylase from thermophilic members of genus Bacillus is said to be commercially feasible (Imsenecki and Solnzeva, 1944). Fierer (1927) indeed, contended that the diastase and catalase of some thermophilic soil bacteria were active at temperatures where those enzymes of mesophilic bacteria were denatured. Gaughran (1949), using resting cells and manometric methods, showed by detailed quantitative analysis of the respiratory enzymes of thermophiles, that the energies of activation are of the same magnitude as for "mesophilic" enzymes.

Studies of growth processes, using resting cells, were criticized by Rahn and Schroeder (1941), because the cells are static and thus incapable of producing new enzymes for those which are either consumed or thermally inactivated. Investigations of growth processes of thermophiles might be facilitated by the use of a simple satisfactory medium.

The literature abounds with reports of simple media which are satisfactory for growth of common soil saprophytes of the genus Bacillus, as well as the supposedly more fastidious organisms. Glinka-Tschernorutsky (1933), for example, found that many strains of Bacillus mycoides grew well in a medium composed of $MgSO_4$, K_2HPO_4, NaCl, $FeSO_4$, $CaCl_2$, and glucose, when supplemented with any of several ammonium salts. Growth was improved, however, by the addition of 15 amino acids. Indeed, growth was better in this inorganic salt - amino acid medium than in peptone broth, as measured by total nitrogen (Kjeldahl). Werner (1933), Stührk (1935) and Heigener (1935) were able to grow many of their strains of

Bacillus subtilis in a medium similar to the inorganic salt medium of Glinka-Tschernorutsky. Gladstone (1939) found that Bacillus anthracis was exacting in its amino acid requirements, expecially as regards omitting one amino acid at a time from a defined medium containing 17 amino acids, inorganic salts, and glutamine. He did not mention that the organism required vitamins, although Landy (1939) and Brewer, et al (1946) found growth factors essential. Stokes and Woodward (1943) showed that B. brevis produced especially large amounts of tyrothricin in a medium composed of inorganic salts, glucose and asparagine. Brewer, et al (1946) found the growth of their strains of B. anthracis was vastly improved over that in Gladstone's medium, when supplied 18 amino acids in the proportion in hydrolyzed casein, supplemented with nucleic acid or components of nucleic acid.

No reports of simple media suitable for cultivation of thermophiles was found. Gaughran (1947) stated in his review of the thermophilic microorganisms, "The field of essential nutrilites for these organisms has been untouched."

The objects of the present work were: (1) to determine the vitamin requirements of representative thermophiles of genus Bacillus; (2) to ascertain if the vitamin requirements varied with temperature of growth; (3) to attempt to cultivate these thermophiles in completely synthetic media; (4) to study the growth curves of a stenothermophile, in the optimal simple medium, and in a natural medium; (5) to observe changes in morphology engendered by changes in composition of medium and in temperature of cultivation; and (6) to assay for the production of an amylase active at elevated temperatures.

EXPERIMENTAL

Cultures

Source and maintenance of cultures. A collection of "flat-sour organisms" was obtained from the National Canners Association, Washington, D. C., through the kindness of Dr. John Yeasir. These are unidentified numbered cultures isolated from a variety of spoiled foods. Two cultures were obtained from the American Type Culture Collection, Washington, D. C., through the kindness of Dr. R. E. Gorden. One culture was obtained from the U. S. Food and Drug Administration, through the kindness of Dr. Arthur P. Dunnigan. One strain was a University of Maryland stock culture. Six cultures were isolated from soil and mouse feces, by enrichment in nutrient broth (24 hours at 55 C) and subsequent streaking on dry nutrient agar plates, in such a manner as to obtain isolated colonies. Repeated streaking of single colonies was considered to assure purity. This collection of cultures was considered representative.

Taxonomic study of the cultures employed was limited to demonstration that all were facultative, sporogenous, gram-positive to gram-variable rods, and to determination of growth at 35 C, 55 C and 65 C. This last was done by the simple method of smearing a drop of 20 to 24 hour broth culture (55C) over the entire surface of a tube of slanted agar, and incubating at the three temperatures. Growth at 55 C and 65 C was always quite prompt, while at 35 C sometimes did not appear until 3 days. All cultures at 35 C were examined up to 3 weeks. Table 1 shows the sources,

Table 1

Number, source, and growth temperature range of cultures.

Number	Source	Isolated from	Growth at 35 C	Growth at 55 C	Growth at 65 C
10	Univ. Md.	?		+	+
26	NCA*	corn		+	+
1356	NCA	hominy		+	+
1492	NCA	pumpkin		+	+
1503	NCA	peas		+	+
1518	NCA	corn		+	+
1792	NCA	corn		+	+
1805	NCA	corn		+	+
2156	NCA	corn		+	+
4103	NCA	string beans		+	+
4298	NCA	corn		+	+
NRS 91	ATTC**	?		+	-
1215	NCA	hominy		+	
1264	NCA	pumpkin		+	
1460	NCA	peas		+	
1508	NCA	corn		+	
1734	NCA	beets		+	
1863	NCA	milk		+	
4160	NCA	milk		+	
3401-1	FDA***	tomato juice		+	
NRS 27	ATTC****	?		+	
E-1	isolated	fertile soil		+	
E-2	isolated	fertile soil		+	
E-3	isolated	fertile soil		+	
C-1	isolated	clay soil		+	
C-2	isolated	clay soil		+	
MF-1	isolated	mouse feces	+	+	

*National Canners Association.
**American Type Culture Collection, identified as B. stearothermophilus.
***U. S. Food and Drug Administration, tentatively identified as B. thermoacidurans.
****American Type Culture Collection, identified as B. coagulans.

and temperature requirements of cultures studied.

Maintenance of cultures. Stock cultures were kept in the refrigerator on agar slants of the following composition: tryptiocse, 1 per cent; yeast extract, 0.2 per cent; NaCl, 0.5 per cent; agar, 2.0 per cent; final pH 7.2 It was found necessary to transfer stock cultures frequently in order to obtain growth when subcultured in broth for preparation of test cell suspensions. In order to maintain some strains, it was found necessary also to alternate broth and agar subcultures. This alternation was accomplished by the use of the medium above without [illegible] duplicate set of cultures was covered with sterile light mineral oil, and kept in the refrigerator. These oil-preserved cultures were viable after 18 months.

Materials and general methods

Detection of growth responses. In determining suitability of media, and testing constituents of the media, for growth of the organisms, advantage was taken of the rapidity of growth, and the rapid rate of fermentation. A Fisher electrophotometer, AC model, using the 425 B filter supplied with the instrument, was used to detect turbidity as a measure of growth. Orientation experiments showed that most of the organisms rapidly dissimilated glucose with the consequent accumulation of abundant acid in the medium. Accordingly, the final pH was estimated colorimetrically.

Incubation. For incubation at 35 C and 55 C, double-walled air incubators, without forced draft, were used. Thermometers, immersed in water, in several places in the incubators, showed that the differences were in the range of plus or minus 1 C, which was not considered critical.

For incubation at 20 C, a refrigerated incubator was employed, equipped with forced draft circulation and an adequate heater. For incubation at 45 and 65 C, a closed water bath, approximately 11 x 14 x 21 inches was used, heated with a looped lo-lag immersion heater, 40 inches long, controlled by a bimetallic thermoregulator, using a mercury switch. The bimetallic portion of the regulator was completely immersed, 4 inches from any portion of the heating element. Temperature fluctuations, within the bath, and at different times, were at a minimum, ±0.25 C, probably because of the small size.

Glassware. Pyrex glassware (except a few serological pipettes), glass-distilled water, aluminum caps, and 16 x 125 mm test tubes were used throughout. Glassware was chemically cleaned, using Orvis and Na_3PO_4, followed by soaking in aqua regia. After thorough rinsing, the glassware was baked at 120 C to 180 C for a few hours, and then sterilized by autoclaving or baking.

Selection of medium for screening of vitamins. In order to select a medium, it was necessary at the outset to obtain an estimate of the amount of nutrient material required for growth of the organisms. They were studied at 55 C only for this purpose. Nutrient broth and trypticase soy broth (BBL) were prepared in dilutions of 1:1, 1:2, 1:3 and 1:4. It was found that the cultures tested grew fairly well in nutrient broth diluted 1:1 with distilled water, but only sparsely when diluted 1:4; whereas they grew promptly and well in trypticase soy broth diluted 1:4. This corresponds to about 0.5 per cent nitrogenous materials, with reasonable concentration of accessory substances supplied by the soy bean extract. The organisms were found to grow readily in 0.5 per cent casein hydrolyzate medium, when supplied 0.1 per cent yeast extract.

Because of original low cystine content, and losses of cystine and tryptophane upon acid hydrolysis of casein, it was necessary to fortify the medium for continued use with these amino acids. Following the usual practice, glucose was added. Although Gordon (1947) and Gordon and Smith (1949) expressed the opinion that glucose is specifically inhibitory to the "obligate" thermophiles, especially at 65 C, these organisms were found not to grow in the absence of glucose in the casein hydrolygate medium. The acid sensitivity of the thermophiles is generally accepted, and proved in one instance by Hansen (1933): therefore, it was decided to buffer the medium strongly. The addition of 0.5 per cent K_2HPO_4 was found satisfactory. The addition of trace elements is also the common practice in the use of casein hydrolyzate media, and traces of the following compounds were incorporated in the early work, until the inadvertent omission revealed that better growth was obtained without them: H_3BO_3, KI, $MgSO_4$, $FeSO_4$, $MnSO_4$, $CuSO_4$, $ZnSO_4$. (For quantities used, see note with table 2.) No attempt was made to determine the need or optimal concentration of inorganic salts. Table shows the effect of glucose, phosphate, and the trace elements on the growth of 10 stenothermophiles in the casein hydrolyzate medium above, supplemented with the required vitamins.

Sterilization of ingredients of basal medium. A 10 per cent solution of K_2HPO_4 was sterilized by autoclaving. Solutions of nitrogenous materials and 20 per cent glucose were sterilized by filtration through porcelain funnels (Selas 02); concentrated vitamin solutions and other chemicals added as substitutions for vitamins, were filtered through sintered glass funnels (Corning UF). All filters were cleaned with aqua regia.

Table 2

Effect of addition of glucose, phosphate, and trace salts* on growth of 10 stenothermophiles**, at 55 C and 65 C, in casein hydrolyzate and in "natural" medium.

	Growth response at 55 C	65 C
Natural medium		
no glucose 0.1 % K_2HPO_4	30***	28***
0.5 % glucose 0.1 % K_2HPO_4		
0.5 % glucose 0.5 % K_2HPO_4		
Casein hydrolyzate medium		
no glucose 0.1 % K_2HPO_4 with trace elements*		
0.5 % glucose 0.5 % K_2HPO_4 with trace elements		
0.5 % glucose 0.5 % K_2HPO_4 without trace elements		

*A salt mixture was made by dissolving the following compounds in 500 ml water, without the aid of heat: H_3BO_3, 25 mg; KI, 2.5 mg, $MgSO_4.7H_2O$, 10 g; $FeSO_4.7H_2O$, 0.5 g; $MnSO_4.4H_2O$, 0.5 g; $CuSO_4.5H_2O$, 2.5 mg; $ZnSO_4$, 2.5 mg. The mixture was filtered (porcelain) and refrigerated to prevent decomposition. One-half ml was added to each 100 ml of completed casein hydrolyzate medium.

**Cultures: 10, 26, 1356, 1492, 1503, 1518, 1792, 2156, 4298, and NRS 91.

***Figures represent average turbidities of 3 serial 24-hour transfers, of all 10 organisms. Turbidity measured with the Fisher electrophotometer, AC model, using the 425 B filter. Figures obtained by subtracting the light transmittance from 100.

Note: The natural medium was trypticase soy broth diluted 1:4; the casein hydrolyzate medium contained biotin, niacin and thiamine.

Preparation of basal medium. Small amounts of the casein hydrolyzate medium were prepared by dissolving (with boiling) the following substances in 95 ml water: l-cystine, 1 mg; dl-tryptophane, 10 mg; NaCl, 100 mg; vitamin-free casein hydrolyzate[1], 0.5 g. After filtration, the medium was dispensed into 100 ml pyrex bottles or suitable flasks, and 5 ml of a sterile 10 per cent solution of K_2HPO_4, and 2.5 ml of a sterile 20 per cent solution of glucose were added. After addition of vitamins, or other chemicals under test, the medium was pipetted into sterile test tubes. The final pH of the medium was 7.2 to 7.4. Large amounts of the medium were more conveniently prepared by making a tenfold concentration of the casein hydrolyzate, NaCl, cystine and tryptophane, which after filtration was added aseptically in the proper amount to previously measured and autoclaved water.

Unless otherwise specified, the basal medium used throughout the work was prepared as described above, and had the following composition: casein hydrolyzate, 0.5 per cent; NaCl, 0.1 per cent; cystine, 0.001 per cent; glucose, 0.5 per cent; K_2HPO_4, 0.5 per cent; tryptophane, 0.01 per cent.

Different batches of the medium, prepared from the same lot of casein hydrolyzate, and prepared from different lots of casein hydrolyzate, using different inocula, produced non-identical growth responses. A similar observation was made by Woolley and Hutchings (1940).

Preparation of inoculum. Cultures were grown 20 to 24 hours at 55 C in centrifuge tubes containing 5 ml of medium of the following composition: trypticase, 1 per cent; yeast extract, 012 per cent; NaCl,

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[1] Three lots from National Dairy Research Labs., Inc., Oakdale, Long Island, and lot 388461 of Difco Casamino Acids, were tried and found satisfactory.

0.5 per cent; glucose 0.5 per cent; K_2HPO_4, 0.5 per cent, final pH about 7.4 (Sterile glucose and phosphate solutions were added after autoclave sterilization of the rest of the medium.) The centrifuge tubes were covered with aluminum caps during incubation, and before harvesting the cells by centrifugation, the tubes were closed with sterile rubber diaphragm stoppers, of the type used in serum bottles. This obviated the occurence of fragments of gauze or cotton in the medium, materially lessened the possibilities for contamination, and enormously speeded up the subsequent operations. The sedimented cells were washed twice with 10 ml of 0.9 per cent NaCl, and finally resuspended in 5 to 9 ml of saline for use. An attempt was made to standardize the cell suspensions only roughly, and no measurements were made on this account. Such cell suspensions contained relatively old cells, and practically no spores. One drop from the tip of 1 ml serological pipet contained from 350 to 10,000 viable cells, as determined by plate count at 55 C in medium of the same composition as that used to yield the crop, with the addition of 2 per cent agar. Orientation experiments showed that the speedier dropwise initial inoculation was in no way inferior to the more laborious loopwise method and that repeated subculture was unnecessary before harvest of cells for test. Toennies and Gallant (1948) reported the same findings in their quantitative studies on bacterimetry.

Conduct of tests. The tubes of completed media, containing different amounts of vitamins, or other tests substances, were preheated to the temperatures of incubation, and inoculated rapidly with a drop of cell suspension. Four serial 24-hour transfers were made with a 4 mm

loop. Growth response was observed by turbidity measurement, and by colorimetric estimation of the final pH attained. All tests were made by cultivating the organisms under test at two temperatures (e. g., 35 C and 55 C; 45 C and 55 C; 55 C and 65 C), because of the possible taxonomic significance of the ability to grow well at stated temperatures, and in the anticipation of finding differences in requirements at different temperatures.

Unless otherwise specified, the natural medium (used as a comparison with the casein hydrolyzate medium) had the following composition: Trypticase, 1.0 per cent; yeast extract, 012 per cent; NaCl, 0.5 per cent; glucose, 0.5 per cent; K_2HPO_4, 0.5 per cent; final pH 7.4.

Screening of vitamins. The following vitamins, in the ranges of concentration indicated, were added to the basal medium, singly, and in many combinations: choline, calcium-D-pantothenate, riboflavin, pyridoxin, niacin and thiamine, 0.1 to 10.0 µg per ml; pteroylglutamic acid[1] and biotin (free acid), 0.1 to 1.0 µg per ml; i-inositol and p-aminobenzoic acid, 1.0 to 10 µg per ml. Only biotin, niacin and thiamine were found to be essential and stimulatory.

Substitutions for vitamins. It was found that many of the thermophiles required biotin and niacin. Biotin can be replaced in the nutrition of some bacteria by desthiobiotin, oxybiotin, oleic acid, pimelic acid, aspartic acid, and tween-80. Niacin can be replaced for some bacteria by the amide, various derivatives of pyridine, and coenzymes I and II. In this work, oleic acid, pimelic acid, tween-80 and desthiobiotin were tested for biotin activity; nicotinamide and diphosphopyridine nucleotide[2] were tested for niacin activity.

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[1] Folvite, obtained from Dr. Benjamin Carey, Lederle Labs., Inc., Pearl River, N. Y.

[2] Lot Co 4902, Schwarz Labs., New York.

Morphological observations. Stained smears of all cultures, in all media, were frequently made to observe changes in morphology and spore yield engendered by changes in composition of the medium and in temperature of incubation. Smears were often stained by both the Gram method and with Giemsa solution, and occasionally by spore stains. Spores, when present, were easily seen without special staining methods.

Tests for high temperature amylase. All cultures were tested for the production of extracellular or soluble amylase, especially which might be active at elevated temperatures. A representative stenothermophile, B. stearothermophilus NRS 91, was grown at 65 C in a natural medium, a potato decoction, and in the optimal casein hydrolyzate medium and the cell-free medium tested for evidence of a high temperature amylase.

Growth curves of a stenothermophile. A representative stenothermophile, organism 10, was planted in the optimal casein hydrolyzate medium and in trypticase yeast extract glucose phosphate medium, and incubated at 20 C, 35 C, 45 C, 55 C and 65 C. Growth curves, as determined by plate counts, were plotted, to observe if a stenothermophile grew well but slowly at low temperatures, as did a eurithermophile in the studies of Hansen (1933).

III

RESULTS

A. Vitamin requirements

. Essential vitamins for stenothermophiles.

All of the stenothermophilic cultures studied required vitamins for good continued growth in the casein hydrolysate medium employed. Table 3 shows that the organisms may be arranged into 3 groups, depending upon their need for niacin, biotin, and thiamine. One group of 5 cultures required all three vitamins, another group of 5 required biotin and niacin, and a third group of 2 required only biotin.

In order to discover the optimal amounts of these three vitamins, the organisms were cultured serially, at the two temperatures, in the basal medium containing different concentrations of the vitamins. The responses of the organisms, according to groups, are presented in tables 4, 5 and 6. Because of the remarkable similarity found in response of individual cultures, a tabulation of averages for each group is included. Comparison of turbidities at 55 C and 65 C, indicates that at 65 C, the stenothermophiles require a higher concentration of vitamins than at 55 C.

Organisms 4103 was the only organism which exhibited a difference in vitamin requirement, depending upon temperature of cultivation. This organisms did not at all times require biotin at 55 C, although it did at 65 C. This property was not stable. Occasionally the organism grew better (higher turbidity measurement), but this was not correlated with

Table 3

Vitamin requirements of 12 stenothermophiles in casein hydrolyzate medium, 55 C and 65 C.

Group	Culture numbers	Vitamins supplied*						
		biotin niacin thiamine	biotin niacin	biotin thiamine	thiamine niacin	biotin	niacin	thiamine
	10	+	-			-		-
	1356	+						
	1518	+						
	NRS 91	+						
	26	+	+					
	1492	+	+					
	1792	+	+					
	2156	+	+					
	4298	+	+					
	1805	+	+					
	4103	+	+	+	-	+	-	-

* Concentrations: thiamine, niacin, 1 ug per ml; biotin 0.04 ug per ml.

Note: Organism 4103, of group 3, occasionally did not require biotin at 55 C, but uniformly did at 65 C. See text.

the demand for biotin, although this change in amount of growth was an equally variable property. The culture was repeatedly "purified" by streaking on very dry agar plates, which were picked as early as colonies appeared, usually in 12-18 hours; a new culture was taken from the original slant received; the phase of the culture did not apparently vary. Yet, consistency could not be obtained in biotin requirement. Especially cleaned glassware, filters, caps, and bottles were used, but these precautions likewise failed to produce duplicatable results.

Examination of the tables indicates that for best continued growth in the casein hydrolyzate medium, the concentrations of vitamins were: biotin, 0.04 ug per ml; niacin and thiamine, each 1 ug per ml. It is seen also that groups 2 and 3 organisms, which do not require all three vitamins, are stimulated by their presence.

Responses of the organisms to increments of vitamins were not linear. When supplied suboptimum amounts, however, the organisms did not attain the usual low pH, although growth was substantial, as measured by turbidity. All organisms except 4103 and 1805, in the optimal medium described, produced a final pH of about 4.5, that is, they were methyl red positive.

None of the casein hydrolyzate media employed supported more than about 60 per cent as much growth as a more concentrated natural medium, although about 100 per cent of the growth in a diluted natural medium (see table 2). The incorporation of 10 times the optimal concentrations of vitamins did not improve growth under the conditions described.

The addition of 0.05 per cent Biopar E[1] to the casein hydrolyzate medium produced practically identical turbidity as the natural medium.

[1] A liver extract, supplied for use by Dr. L. L. Lachet, Armour and Co., Chicago

Table 4.

Growth response of Group 1 stenothermophiles to varying amounts of biotin, niacin and thiamine, in casein hydrolysate medium, at 55 C and 65 C.

°C	Organism		Vitamins, concentration, mg per ml											Natural Medium
		biotin	0	0.0000004	0.00004	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
		niacin	1.0	1.0	1.0	0	0.001	0.01	0.1	1.0	1.0	1.0	1.0	1.0
		thiamine	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0	0.00001	0.001	0.01	1.0
55	10	turbidity*	0	2	24	0	9	24	47	0	26	43	50	54	76
		final pH**		7.4	5.0		7.2	6.8	4.8		6.6	5.0	4.8	4.4	4.4
	1356	turbidity	0	0	23		10	21	46	0	22	42	48	50	75
		final pH			5.0		7.2	6.8	5.0		6.6	5.0	4.8	4.4	4.4
	1503	turbidity	0	2	20	0	11	20	53	0	20	40	51	54	76
		final pH		7.4	6.0		7.0	6.8	4.6		6.6	5.0	4.8	4.4	4.2
	1518	turbidity	0	3	15	0	11	23	48	0	26	32	48	53	76
		final pH		7.4	7.0		7.2	7.0	4.8		6.8	6.0	4.8	4.4	4.4
	NRS 91	turbidity	0	2	15	0	10	20	53	0	27	43	50	54	75
		final pH		7.4	7.0		7.2	7.0	4.8		6.4	5.0	5.0	4.4	4.4
65	10	turbidity	0	0	20	0	5	20	48	0	22	29	46	49	70
		final pH			6.0		7.4	6.8	5.0		6.8	5.0	5.0	4.4	4.4
	1356	turbidity	0	0	18	0	2	12	40	0	21	38	39	48	70
		final pH			6.2		7.4	7.2	5.0		6.6	5.2	5.0	4.4	4.4
	1503	turbidity	0	0	20	0	2	19	44	0	20	33	42	48	70
		final pH			6.0		7.4	7.0	4.8		6.6	5.0	5.0	4.4	4.4
	1518	turbidity	0	0	14	0	0	17	39	0	20	30	34	46	74
		final pH			6.2			7.0	5.2		6.6	5.0	5.0	4.4	4.4
	NRS 91	turbidity	0	0	15	0	2	17	43	0	20	36	43	46	72
		final pH						7.0	4.6		6.6	5.0	5.0	4.4	4.4
55	AVERAGES	turbidity	0	2	20	0	10	21	45	0	20	43	49	52	75
		final pH		7.4	6.0		7.2	7.0	5.0		6.6	5.0	5.0	4.4	4.4
65	AVERAGES	turbidity	0	0	19	0	2	17	42	0	20	34	42	47	70
		final pH			6.0		7.4	7.0	5.0		6.6	5.0	5.0	4.4	4.4

*Average of replicate turbidities of serial 24-hour transfers. Figures obtained by subtracting light transmittance from 100.

**Approximate final pH estimated colorimetrically.

Natural medium was trypticase yeast extract glucose phosphate broth.

Table 5

Growth response of Group 2 stenothermophiles to varying amounts of biotin, niacin and thiamine, in casein hydrolysate medium, at 55 C and 65 C.

°C	Organisms	Vitamins, concentration, µg per ml											Optimal	Natural Medium
		biotin	0	0.0000004	0.00004	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	
		niacin	1.0	1.0	1.0	0	0.001	0.01	0.1	1.0	1.0	1.0	1.0	1.0	
		thiamine	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0	0.00001	0.001	0.01	1.0	
55		turbidity*	0	2	24	0	17	24	45	30	36	43	50	52	75
	26	final pH**		7.4	5.2		7.0	7.0	4.8	5.0	5.0	5.0	4.8	4.5	4.4
		turbidity	0	2	41	0	15	45	49	50	50	47	51	53	75
	1492	final pH		7.4	5.0		7.0	6.0	5.0	5.0	5.0	5.0	5.0	4.5	4.4
		turbidity	0	3	20	0	18	20	24	20	20	20	20	33	44
	1792	final pH		7.4	5.4		7.0	7.0	6.0	6.0	6.0	6.0	6.0	4.5	4.4
		turbidity	0	0	42	0	11	12	40	36	42	44	47	50	75
	2156	final pH			5.0		7.0	7.0	5.0	5.0	5.0	5.0	5.0	4.4	4.4
		turbidity	0	0	35	0	15	20	44	38	40	45	47	48	75
	4298	final pH			5.0		7.0	7.0	5.0	5.0	5.0	5.0	5.0	4.4	4.4
65		turbidity	0	0	23	0	5	20	40	30	36	42	46	48	70
	26	final pH			5.0		7.4	7.0	5.0	5.0	5.0	5.0	5.0	4.4	4.4
		turbidity	0	0	39	0	6	22	46	43	44	42	44	51	70
	1492	final pH			5.0		7.4	7.0	5.0	5.0	5.0	5.0	5.0	4.4	4.4
		turbidity	0	0	12	0	11	18	23	20	20	20	21	23	44
	1792	final pH			6.0		7.4	7.0	5.0	5.0	5.0	5.0	5.0	4.8	4.4
		turbidity	0	0	40	0	0	12	32	36	42	42	42	43	70
	2156	final pH			5.0			5.0	5.0	5.0	5.0	5.0	5.0	4.4	4.4
		turbidity	0	0	20	0	4	20	37	40	40	44	45	48	70
	4298	final pH			6.0		7.4	7.0	5.0	5.0	5.0	5.0	5.0	4.4	4.4
55		turbidity	0	2	34	0	13	25	44	40	40	44	50	52	75
	AVERAGES	final pH		7.4	5.0		7.0	7.0	7.0	5.0	5.0	5.0	5.0	4.4	4.4
65		turbidity	0	0	34	0	3	19	41	40	40	42	44	49	70
	AVERAGES	final pH			5.0		7.4	7.0	5.0	5.0	5.0	5.0	4.4	4.4	4.4

*Average of replicate turbidities of serial 24-hour transfers. Figures obtained by subtracting light transmittance from 100.

**Approximate final pH estimated colorimetrically.

Natural medium was trypticase yeast extract glucose phosphate broth.

Table 6

Growth responses of Group 3 stenothermophiles, to varying amounts of biotin, niacin and thiamine, in casein hydrolysate medium, at 55 C and 65 C.

°C	Organism	Vitamins, concentration, µg per ml												Optimal	Natural medium
		biotin	0	0.000000[illegible]4	0.00004	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	
		niacin	1.0	0.0	1.0	0	0.001	0.01	0.1	1.0	1.0	1.0	1.0	1.0	
		thiamine	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0	0.0[illegible]0001	0.001	0.01	1.0	
55	1805	turbidity*	0	0	15	23	38	38	40	25	30	30	35	38	85
	4103**	turbidity	0	0	33	41	43	41	43	37	37	40	40	45	75
65	1805	turbidity	0	0	9	22	33	35	37	28	30	37	37	43	80
	4103	turbidity	0	0	20	35	36	38	35	37	37	34	38	38	70
55	AVERAGES	turbidity	0	0	12	32	41	41	41	32	32	35	35	44	82
65	AVERAGES	turbidity	0	0	17	32	36	36	36	32	32	34	34	40	72

*Average of replicate turbidities of serial 24-hour transfers. Figures obtained by subtracting light transmittance from 100.

**Organism is variable in its need for biotin. See text.

In neither the casein hydrolysate medium, nor in the trypticase yeast extract glucose phosphate medium did these organisms lower the pH more than 0.2.

Essential vitamins for eurithermophiles.

Of the 15 cultures of eurithermophiles studied, 8 were found to grow on continued subculture, in the casein hydrolyzate medium containing no vitamins, at 35 C, 45 C, and 55 C. No further study was made of these cultures. The other 7 cultures were all found to require biotin, niacin and thiamine for good continued growth at the three temperatures above. A few experiments indicated that growth was less prompt in the casein hydrolyzate media than in the natural medium at all temperatures, and accordingly, the transfers and readings of turbidity were made at intervals of 48 hours. Some of the cultures appeared to grow slightly more abundantly, but apparently no more rapidly, in 1 per cent casein hydrolyzate. This concentration was used occasionally. In other respects, all of the media used in studies of the eurithermophiles were the same as for the stenothermophiles.

Orientation studies showed that in the media employed, these organisms were as active as the stenothermophiles, in the dissimilation of glucose, and that in 48 hours, grew somewhat more abundantly. Therefore, examination of final pH was limited to testing with methyl red.

Table 7 shows the growth response of the 7 eurithermophiles, in casein hydrolyzate medium, upon the addition of varying amounts of biotin, niacin and thiamine. Examination of this table shows that the organisms produced somewhat more turbidity, and were considerably more fastidious as to concentration of vitamins, than the stenothermophiles. This is especially marked when grown at 55 C. It is seen also, that the optimum concentrations of the vitamins concerned, are

Table 7

Growth response of 7 eurithermophiles, to varying amounts of biotin, in casein hydrolyzate medium, at 45 C and 55 C.

°C	Organisms	Vitamins, concentration, µg per ml: biotin	0	0.0000004	0.000004	0.04	0.04	0.04	0.04	0.04	0.04	0.04	Optimal	Natural medium
		niacin	1.0	1.0	1.0	0	0.01	0.1	1.0	1.0	1.0	1.0		
		thiamine	1.0	1.0	1.0	1.0	1.0	1.0	0	0.01	0.0	1.0		
			T* MR**	T MR	T MR	T MR	T MR	T MR	T MR	T MR	T MR	T MR		T MR
45	1215		0	14 -	60 +	0	55 +	60 +	0	35 +	50 +	65 +		80 +
	1264		0	17 -	62 +	0	60 +	67 +	0	45 +	60 +	70 +		80 +
	1460		0	15 -	65 +	0	60 +	65 +	0	35 +	55 +	70 +		80 +
	1508		0	10 -	40 +	0	10 -	26 +	0	20 +	30 +	45 +		60 +
	1734		0	50 +	58 +	0	40 +	58 +	0	40 +	50 +	64 +		70 +
	4160		0	18 -	60 +	0	54 +	6 +	0	54 +	65 +	70 +		80 +
	NRS 27		0	10 -	50 +	0	60 +	60 +	0	0	50 +	70 +		80 +
55	1215		0	24 +	52 +	0	0	0	0	37 +	45 +	60 +		80 +
	1264		0	30 +	50 +	0	30 +	40 +	0	4 +	48 +	65 +		80 +
	1460		0	15 -	60 +	0	0	0	0	0	0	68 +		80 +
	1508		0	0	30 -	0	0 -	28 +	0	0	2	33 +		55 +
	1734		0	0	0	0	0	0	0	0	0	64 +		70 +
	4160		0	0	30 -	0	30 -	30 -	0	10 -	10 -	60 +		75 +
	NRS 27		0	10 -	50 +	0	55 +	55 +	0	0	58	65 +		80 +
45	AVERAGES OF ALL		0	15	58	0	53	58	0	25	51	63		76
55	AVERAGES OF ALL		0	10	37	0	16	20	0	13	15	60		74

* T is the average of replicate turbidities of 5 serial 48-hour transfers.

** MR: methyl red test. + means pH of about 4.5 or below.

Natural medium was trypticase yeast extract glucose phosphate broth.

the same as for the stenothermophiles. Again, 10 times these concentrations did not improve growth, although 0.05 per cent Biopar E made the casein hydrolysate medium entirely comparable to the natural medium, as determined by production of the same turbidity.

Bacillus coagulans NRS 27 was tested by the same means, in media containing varying amounts of biotin, niacin and thiamine, at 35 and 55 C. The results indicate that this organism, which may be typical of the group had about the same requirements at 35 C as at 45 and 55 C. Table 8 presents the data of this experiment.

B. Vitamin replacements

. Niacin replacement by niacinamide and diphosphopyridine nucleotide.

Bacteria for which niacin is a growth factor can usually utilize niacinamide as well. Johnson (1945), however, found that Leuconostoc mesenteroides 9135 did not use the amidein conventional concentrations, but demanded the free acid. Conversely, Koser, et al (1941) found that for some members of genus Pasteurella, niacin would not satisfy the requirement for the amide. The concept that niacin is converted to the amide, and is then synthesized into diphosphopyridine nucleotide (DPN) suggests that organisms which require niacin should probably grow well or better when supplied coenzyme I (DPN). Koser and Kasai (1948) found the contrary case with 2 strains of Leuconostoc mesenteroides. They found that very large amounts of the coenzyme supported slow and slight growth at 24 C and 30 C, but failed to support any growth at 37 C.

The 10 cultures of stenothermophiles which had been found to

Table 8

Growth of B. coagulans NRS 27, at 35 C and 55 C, in casein hydrolyzate medium** with combinations of niacin, thiamine and biotin.

Vitamin concentration, µg/ml			Growth response	
Niacin	Thiamine	Biotin	37 C	55 C
1	1	0.04	66 *	53 *
1	1	0.004	62	46
1	1	0.00004	41	25
1	1	0.0000004	10	0
1	0.01	0.04	0	0
1	0	0.04	0	0
0.1	1	0.04	66	53
0.01	1	0.04	62	51
0	1	0.04	0	0

*Average of replicate serial 48-hour transfers. Figures represent turbidity as measured with Fisher Electrophotometer, 100 minus reading of light transmittance, using 425 B filter.

**Concentration of casein hydrolyzate, 1 per cent.

require niacin were tested in the casein hydrolysate medium, at 55 C and 65 C, for their ability to use niacinamide and DPN as replacements for niacin. All cultures respond as readily to the amide as to the free acid. None of the organisms responded well to the presence of DPN in usual concentrations. Tenfold increase in concentration of DPN supported from 20 to 100 per cent as much growth as did niacin, at 55 C, for all cultures, but at 65 C, even this amount of DPN failed completely to satisfy the niacin demand for 3 cultures. For the other 7 cultures, elevated concentration of DPN supported from 80 to 100 per cent of growth as did niacin. Table 9 shows the response of the 10 cultures to varying amounts of niacinamide and DPN. Negative tubes were reinoculated, and a fresh tube of the same medium was inoculated from a tube showing good growth, in order to eliminate the possibility of technical errors.

Because of the low order of activity of the DPN preparation, it seemed advisable to test it for activity for another niacin-demanding organism. Micrococcus pyogenes var. aureus (carried as Staphylococcus aureus 209, in the University of Maryland stock culture collection) was chosen because this organism has been found to require niacin or DPN (Knight, 1937a, 1937b, and Landy, 1938), and was found to grow well on serial subculture in the optimal casein hydrolysate medium employed. Table 10 shows the ability of the DPN preparation to satisfy the niacin demand of the micrococcus.

At the time of preparation and use of the DPN solution, no knowledge of its purity was available, but these preparations usually are a little more than 50 per cent pure, and it was with this in mind

Table 9

Response of 10 stenothermophiles which require niacin, when supplied niacinamide and DPN, in casein hydrolysate medium, at 55 C and 65 C.

Organism	°C	Niacin	Niacinamide		Diphosphopyridine nucleotide**		
		1 µg	1 µg	0.1µg	20 µg	2 µg	0.2 µg
10	55	50*	51	45	50	42	20
	65	42	42	40	40	27	13
1356	55	44	45	40	44	24	20
	65	42	42	38	42	23	10
1503	55	50	50	45	50	30	20
	65	43	42	40	25	25	10
1518	55	51	51	44	40	31	16
	65	43	42	40	30	25	5
NRS 91	55	50	50	45	50	33	22
	65	36	36	35	34	30	5
26	55	50	51	46	48	40	20
	65	40	41	36	0	0	0
1492	55	48	47	40	42	38	25
	65	33	35	33	30	30	20
1792	55	30	29	25	20	20	20
	65	20	25	20	0	0	0
2156	55	51	50	45	5	2	0
	65	40	40	38	0	0	0
4298	55	46	45	42	46	32	22
	65	37	36	35	35	30	5

*Average of turbidities of 3 serial 24-hour transfers.

**Prepared by dissolving the coenzyme in distilled water, and filtering through sintered glass (Corning UF). Amounts indicated are uncorrected for activity, which was found to be about 50 per cent.

Table 1(

Response of Micrococcus pyogenes var. aureus to niacin DPN preparation, at 35 C, in casein hydrolysate medium containing biotin, 0.04 µg per ml, and thiamine, 1.0 µg per ml.

Vitamin or coenzyme, contents per m

niacin	DPN	Turbidity
1 µg	0	70*
0	0	0
0	20 µg	79
0	2 µg	65
0	0.2 µg	10

*Turbidity of third serial 24-hour transfer. Figures represent light transmittance subtracted from 100.

that the concentrations were prepared. The stated purity was later found to be 64 per cent. Examination of table 10 reveals that 2 µg per ml of DPN preparation (uncorrected for DPN content) gives about the same turbidity with the micrococcus as did 1 µg per ml of niacin. This indicates that the DPN preparation had approximately 50 per cent activity. Possible losses in filtration, errors in weighing and dilution may account for the disparity.

The eurithermophiles were found completely able to use niacinamide in place of niacin, as were the stenothermophiles. The eurithermophiles were found generally more able to use the DPN as supplied than the stenothermophiles. Table 11 indicates the responses of the niacin-demanding eurithermophiles to varying amounts of niacinamide and the DPN preparation. Tubes of medium prepared at the same time as the medium used for testing the stenothermophiles and the micrococcus were used. It is seen that 2 µg per ml of the DPN preparation (equivalent to approximately 1 µg DPN, as estimated in the test with the micrococcus) produced practically the same turbidity as did 1 µg per ml of niacin.

2. Biotin replacement by desthiobiotin, oleic acid, pimelic acid, and Tween-80.

The identification of biotin as a growth factor for bacteria has introduced the important problem of mechanism of its utilization. One approach to this has been to present to the organisms requiring biotin, certain homologs, derivatives, or compounds which were found by elimination or chance to have biotin activity in biotin-free media, or were found to stimulate synthesis of the vitamin by microorganisms. It was

Table 11

Response of eurithermophiles to niacinamide and DPN as replacements for niacin, in casein hydrolyzate medium, at 45 C and 55 C.

Organism	Temp	Concentration of compound, µg per ml						
		Niacin	Niacinamide			DPN		
		1.0	0.1	0.01	0.001	20	2	0.2
1215	45	58*	50	35	21	60	55	40
	55	52	45	30	18	56	50	30
1264	45	58	45	40	30	62	60	40
	55	55	40	30	18	58	58	30
1460	45	55	50	40	20	65	50	33
	55	50	45	30	20	63	44	24
1508	45	47	48	32	10	60	40	35
	55	42	40	20	10	50	40	35
1734	45	70	60	35	20	70	40	35
	55	60	38	20	15	60	40	30
4160	45	65	55	38	30	68	60	50
	55	60	45	35	20	60	50	50
NRS 27	45	70	50	30	20	70	47	30
	55	60	40	20	10	50	37	20**

* Figures for niacin and niacinamide represent the turbidity of the cultures on the third serial transfer; those for the DPN, represent turbidities on the second serial transfer.

**All cultures except this one were methyl-red positive.

found, for instance, that desthiobiotin could replace biotin in cultivation of yeasts, but failed for some members of genus Lactobacillus (Dittmer, et al, 1944 and Lilly and Leonian, 1944). It has been shown that desthiobiotin is probably an intermediate in the synthesis of biotin by a strain of Penicillium chrysogenum (Tatum, 1945), although desthiobiotin acts as an antibiotin compound in the growth of Lactobacillus casei (Dittmer, et al, 1944, and Tatum, 1945).

Among the other compounds that have been shown to be able to replace biotin in the growth of microorganisms, pimelic acid and oleic acid were available for use. duVigneaud, et al (1942) showed that the pimelic acid requirement of a strain of Corynebacterium diphtheriae could be satisfied by biotin; Hutchings and Boggiano (1947) and William, et al (1947) showed that oleic acid could replace biotin in the nutrition of certain lactic acid bacilli. Tween-80, presumably because of its oleic acid content, was reported to possess biotin activity (Williams, et al, 1947).

As a preliminary test, three stenothermophiles were grown in the optimal casein hydrolysate medium, changed by the substitution of varying amounts of oleic acid, pimelic acid, Tween-80, and desthiobiotin. Also, the medium containing biotin as well as the challenge substances was used in order to see if antibiotin activity was present, or if the compounds tested were toxic in the concentrations used.

Table 12 shows that at 55 C and 65 C, 1 µg per ml of oleic acid or pimelic acid and 2 µg per ml of Tween-80 exhibited neither antibiotin effect, or biotin activity; that desthiobiotin exerts no antibiotin effect, but is instead a suitable substitute for biotin, at a

Table 12

Oleic acid, pimelic acid, Tween-80 and desthiobiotin as replacements for biotin, in casein hydrolyzate medium at 55 C and 65 C.

Contents of medium, µg/ml		Organisms					
		1536		1503		NRS 91	
Biotin	other	55 C	65 C	55 C	65 C	55 C	65 C
0.04	none	45*	44	50	49	50	44
0.04	oleic acid, 1.0	46	44	50	45	50	44
0	oleic acid, 1.0	2	2	5	2	5	2
0	oleic acid, 10.0	0	0	0	0	0	0
0.04	pimelic acid, 1.0	44	43	51	48	50	44
0	pimelic acid, 1.0	2	0	2	0	2	0
0	pimelic acid, 10.0	0	0	0	0	0	0
0.04	Tween-80, 2.0	44	43	50	42	48	40
0	Tween-80, 2.0	0	0	0	0	0	0
0.04	desthiobiotin, 2.0	47	46	50	47	50	46
0	desthiobiotin, 2.0	48	46	51	47	50	46

*Figures represent the average of the turbidities of 4 serial transfers, and are obtained by subtracting the percent light transmittance from 100.

concentration of 2 μg per ml. Since the concentration of desthiobiotin used was so much higher than the usual concentration of biotin employed, all of the 12 stenothermophiles were tested in lower amounts, 1 to 0.04 μg per ml. The results of these tests appear in table 13. Reference to this table reveals that desthiobiotin at a concentration of 0.04 μg per ml, is suitable as a biotin replacement at 55 C and 65 C for 9 cultures; that it is suitable at 55 C but not at 65 C for 2 cultures; and that it is unsuitable at either temperature for 2 cultures.

Since the 3 stenothermophiles initially studied showed no response to the presence of oleic acid, pimelic acid, or Tween-80, these substances were not tried with the eurithermophiles. Table 14 shows the ability of desthiobiotin, at a concentration of 0.04 μg per ml, to replace biotin for all 7 eurithermophiles, at 45 C and 55 C, as evidenced by turbidity measurement and attainment of low pH. Maximum response was with 1.0 μg per ml desthiobiotin, although 0.04 μg per ml satisfied the requirements sufficiently to produce the usual turbidity and low pH.

Attempts to grow stenothermophiles in chemically defined media

A principal aim of this work was to devise a completely synthetic medium which would support good continued growth, which might facilitate studies of such features as spore formation and germination, proteolysis, amylolysis, and need for inorganic ions. No such medium was found.

Since the thermophiles here studied were doubtless soil organisms,

Table 13

Response of 12 stenothermophiles to varying amounts of desthiobiotin in casein hydrolyzate medium, at 55 C and 65 C.

		Vitamins, concentration in µg per ml							
		(Control) Biotin		Desthiobiotin					
		0.04		1		0.4		0.04	
	Organism	55 C	65 C	55 C	65 C	55 C	65 C	55 C	65 C
Group 1	10	50*	42	48	38	48	38	46	36
		+**	+	+	+	+	+	+	+
	1356	44	42	42	41	42	41	41	40
		+	+	+	+	+	+	+	+
	1503	50	43	50	34	48	34	48	32
		+	+	+	+	+	+	+	+
	1518	51	43	51	42	48	40	48	40
		+	+	+	+	+	+	+	+
	NRS 91	50	36	50	36	50	36	50	36
		+	+	+	+	+	-	+	-
Group 2	26	50	40	50	0	47	0	47	0
		+	+	+	-	+	-	+	-
	1492	48	33	[illegible]		[illegible]		[illegible]	0
		+	+	-		-	-	-	-
	1792	30	30	30	25	20	20	15	10
		+	+	+	+	-	-	-	-
	2156	51	40	10	7	6	[illegible]	0	0
		+	+	-	-	-	-	-	-
	4298	46	37	46	36	46	35	46	35
		+	+	+	+	+	+	+	+
Group 3***	1805	33	32	30	30	25	20	20	20
	4103	41	40	38	36	38	36	36	32

*Average turbidity of 3 serial 24-hour transfers.
**Results of "methyl red test". + means pH about 4.5 or below. - means pH above 4.5
***These organisms are never methyl-red-positive in the casein hydrolyzate media.

Table 14

Response of 7 eurithermophiles to desthiobiotin as a replacement for biotin, in casein hydrolyzate medium, at 45 C and 55 C.

Organism	Temp	Concentration of compound, µg per ml biotin	desthiobiotin		
		0.04	1.0	0.4	0.04
1215	45	58*	60	55	40
	55	52	56	50	30
1264	45	68	62	60	40
	55	55	58	58	30
1460	45	55	65	50	33
	55	50	63	44	24
1508	45	47	48	48	48
	55	42	46	44	42
1734	45	70	67	60	60
	55	60	65	50	52
4160	45	65	65	60	50
	55	60	55	50	45
NRS 27	45	70	65	50	50
	55	60	62	48	45

*Figures represent the turbidity of the third serial transfer. All cultures methyl-red positive.

organisms, and members of genus Bacillus, it was anticipated that they would be readily cultivatable in some of the simple media used for other members of the genus. Duplication of these media, and inoculation with washed cells, showed that the usual inorganic salts with ammonium compounds and glucose, with biotin, niacin, and thiamine, failed entirely to support growth. In some instances, enormously heavy inoculation from a young agar slant culture resulted in some growth which did not continue, however, on second subculture.

The suitability of casein hydrolyzate suggested the use of the constituent amino acids, in the approximate proportions found in casein. Even when such a mixture was prepared, in two-fold and four-fold concentration, the media failed to support continued, or even very good, growth. The addition of 0.01 per cent yeast extract did not render the defined media satisfactory. This observation suggested that a marked deficiency existed. Supplementation of such complete amino acid mixtures as described by Brewer, et al (1946) with glutamine, nucleic acid, asparagine, urea, ammonium salts, purines and pyrimidines, did not improve the growth of the organisms. In addition to the use of the media above, attempts were made with a large variety of mixtures of amino acids, inorganic nitrogen compounds, and organic compounds. The amino acids in the mixtures were selected because of essentiality for the rat (Mitchell, 1946), or because of structural formula, or merely arbitrarily. All such mixtures failed to support growth even upon massive inoculation. The addition of 0.01 per cent casein hydrolyzate did not greatly improve the medium.

In contrast to the unproductiveness of many materials added to the amino acid media, the addition of 0.1 per cent Biopar E supported fair growth. But since this material is a liver derivative, and contains doubtless more undefinable substances than casein hydrolysate, this finding was not further pursued.

The amino acid mixtures employed contained no alpha-hydroxyglutamic acid, which, according to Mitchell (1946) is the second most abundant amino acid residue in casein. Inclusion of this amino acid in amino acid or in inorganic salt mixtures with the required growth factors, might result in growth of the organisms.

Growth curves of organism 10, a stenothermophile.

Morrison and Tanner (1922, 1924) and Hansen (1933) suggested that generally thermophiles grow at temperatures far below the supposed minimum. These observations are at variance with those of Gaughran (1946) and Insenecki and Solnzeva (1945), who noted that even upon prolonged incubation, proliferation was not detectable. Apparently the disparity in findings resolves itself into differences in strains or species.

The growth curves of a eurithermophile, organism 1460, were reported by Hansen (1933) to show that the highest total viable crop was obtained at 20 C, although the growth rate was enormously higher at 55 C. Since the present study included organisms which failed after 3 weeks at 35 C to show visible growth, a representative strain was chosen for study of growth curves. This also presented an opportunity to ascertain if the optimal casein hydrolyzate medium

was as satisfactory for growth (as determined by rate of growth in the medium) as an excellent natural medium (the trypticase yeast extract glucose phosphate broth previously described.) The media were prepared in 100 ml pyrex bottles with the usual precautions of adding glucose and phosphate after appropriate sterilization of the rest of the media, and were incubated 2 days for sterility, at 30 C. The inoculum was 0.2 ml of a 20-hour culture at 55 C of organism 10 in casein hydrolysate medium. The bottles were stationary, and without aeration beyond that given when they were periodically shaken to obtain an aliquot for plate count. Plate counts were made in trypticase yeast extract glucose phosphate medium, solidified with 2.0 per cent agar. Plates were counted after 24 to 36 hours incubation. Counts were made periodically from cultures, in both media, incubated at 20 C, 35 C, 45 C, 55 C and 65 C. Table 15 shows the plate counts at different times, in each medium at each temperature. The logarithms of the numbers were plotted against time, as is shown in figures 1, 2, 3, and 4, so that points on a straight line, in the rapidly multiplying phase, may be used to calculate the generation times in the different media at the different temperatures.

The curves are not smooth, recalling the statement of Mudge (1930) that thermophilic bacteria multiply in a cyclic manner, the cycles composed of individuals of different heat resistance. It is seen from the graphs (figures 1 to 4) and table 15 that the organisms failed to multiply within 220 hours, at either 35 C or 20 C.

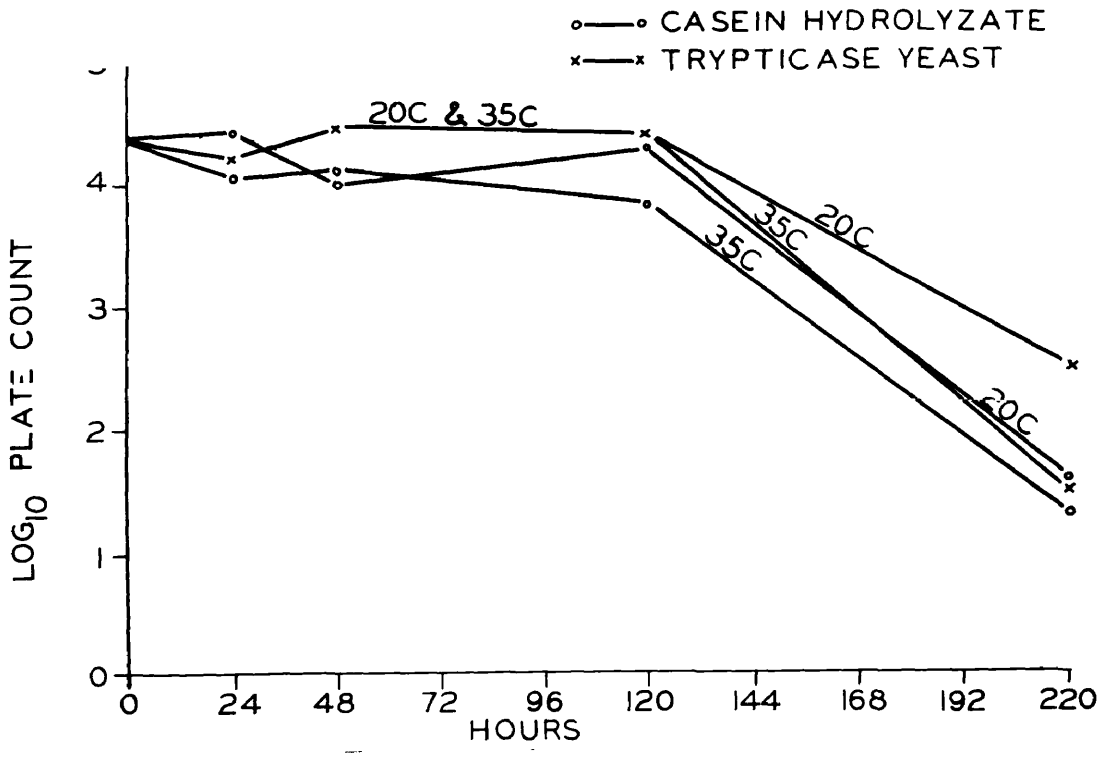

Figure 1. Growth curves of organism 10 at 20 C and 35 C, in casein hydrolyzate and in trypticase yeast extract media.

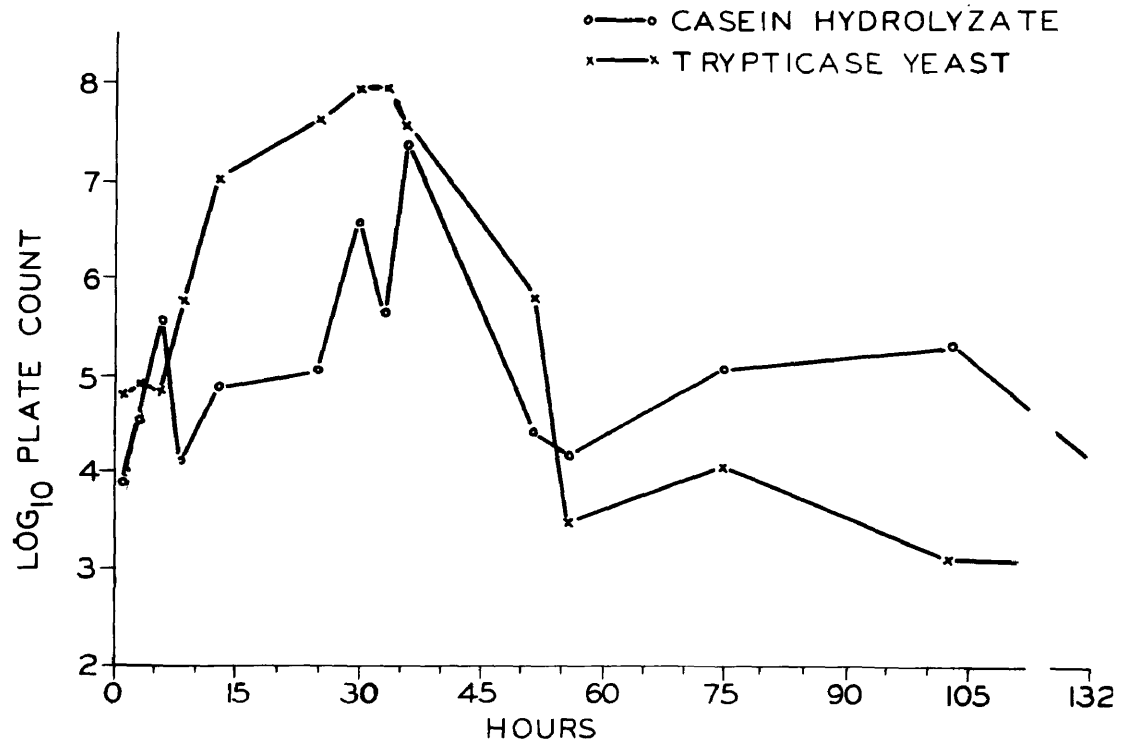

Figure 2. Growth curves of organism 10 at 45 C, in casein hydrolyzate and in trypticase yeast extract media.

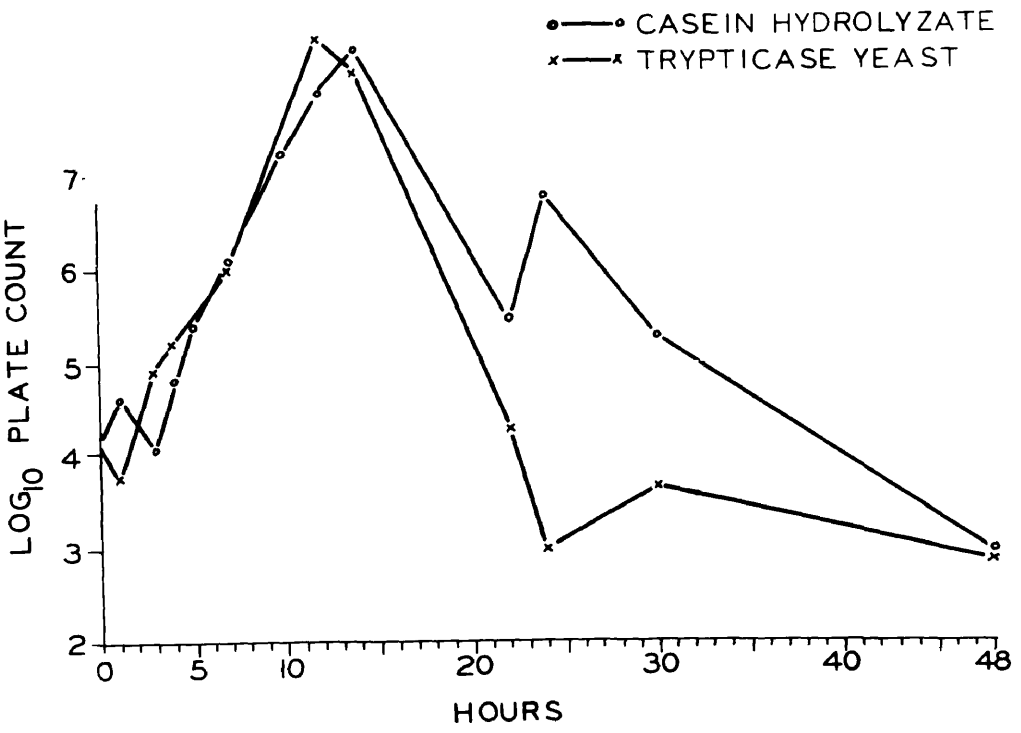

Figure 3. Growth curves of organism 10 at 55 C, in casein hydrolyzate and in trypticase yeast extract media.

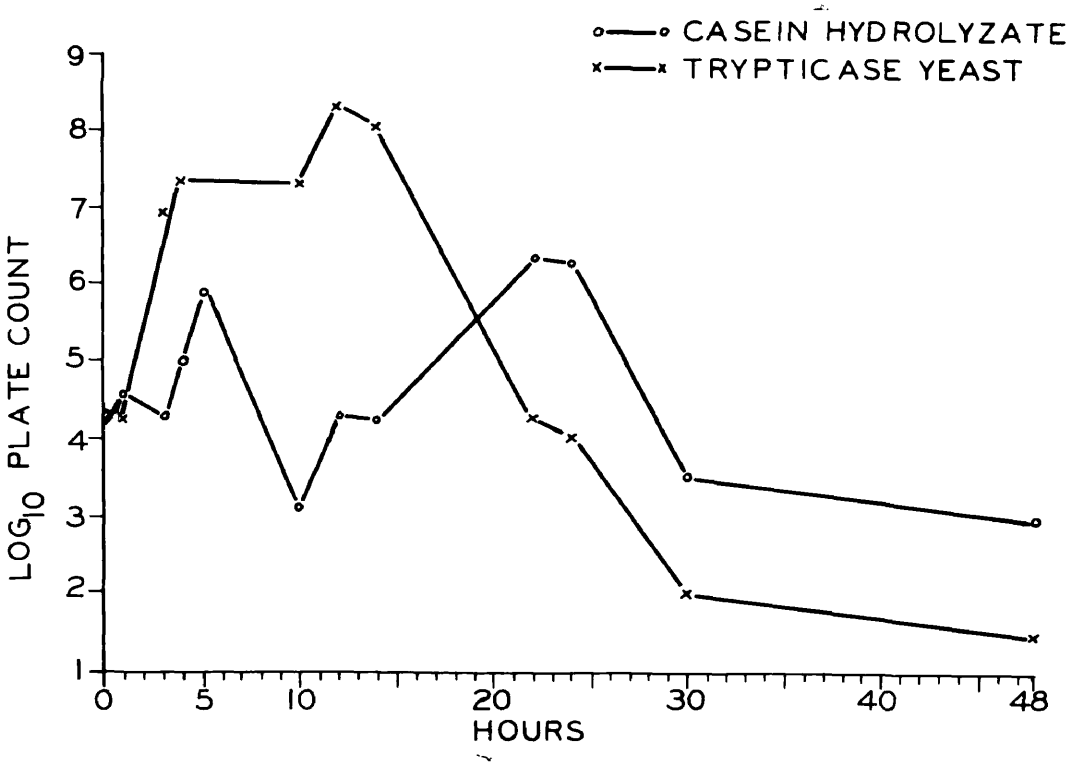

Figure 4. Growth curves of organism 10 at 65 C, in casein hydrolyzate and in trypticase yeast extract media.

Table 15

Plate counts of organism 10, a stenothermophile, in optimal casein hydrolyzate medium and in trypticase yeast extract medium, at 20 C, 35 C and 45 C.

	Time (Hours)	Plate counts: Casein hydrolyzate medium	Plate counts: Trypticase yeast extract medium
20 C	0	22,500	22,000
	24	11,000	16,500
	120	20,000	26,000
	220	50	30
35 C	0	21,500	20,500
	24	11,000	16,500
	48	13,500	29,000
	120	7,000	26,000
	220	20	30
45 C	0	1,500	6,000
	1	8,300*	62,000
	4	34,000	76,500
	6	320,400*	61,000*
	8	12,900	500,000
	13	75,500	11,100,000*
	25	122,600	43,000,000
	30	3,750,000	92,500,000
	33	410,000	93,100,000
	36	26,250,000	41,000,000
	52	25,500	685,000
	56	15,000	3,000
	75	125,000	11,500
	103	210,000	1,240
	132	70,000	1,100

*Plate counts from which generation times were calculated.

Table 15, continued

Plate counts of organism 10, a stenothermophile, in optimal casein hydrolyzate medium and in trypticase yeast extract medium, at 55 C and 65 C.

	Time (Hours)	Plate counts: Casein hydrolyzate medium	Plate counts: Trypticase yeast extract medium
55 C	0	17,000	12,000
	1	45,500	6,000*
	3	14,200	94,000
	4	70,000*	195,000*
	5	270,000	—
	7	1,370,000*	1,100,000
	10	19,000,000	—
	12	64,000,000	300,000,000
	14	210,000,000	124,000,000
	22	325,000	22,500
	24	6,500,000	2,000
	30	230,000	5,100
	48	1,200	850
65 C	0	17,000	20,000
	1	48,500	18,000*
	3	19,500*	8,450,000
	4	100,000	22,000,000*
	5	805,000*	—
	10	1,400	20,800,000
	12	20,000	200,000,000
	14	19,000	124,000,000
	22	2,500,000	20,000
	24	2,000,000	11,000
	30	3,800	100
	48	1,200	29

*Plate counts from which generation times were calculated.

Table 16 gives the generation times calculated from the formula

$$g = \frac{t \log 2}{\log b - \log B}$$

in which t is the time interval, b is the plate count at end of interval, and B is plate count at start of interval.

Comparison of generation times and viable crop yields of the natural and the casein hydrolyzate media reveals that the natural medium was productive of both more generations in a time interval, and more total generations (to produce a higher viable count), although in this feature, the casein hydrolyzate was not markedly deficient.

The shortest generation time for this organism, 17.5 minutes at 65 C, in the natural medium, was found to be somewhat longer than that which Hansen found for his eurithermophile (16 minutes at 55 C in natural medium), and very much longer than reported by Mudge (5 to 8 minutes at 62.5 C in milk).

Morphological Observations

While the present classification of the thermophiles is primarily based on the criterion of cell dimensions, particularly width (Breed, et al, 1948), it is generally agreed that these organisms exhibit unusually wide variations in this property. Gordon and Smith (1949) found morphology to be entirely unreliable as a taxonomic character.

Throughout the course of the present study, stained films were prepared in order to see if temperature of incubation affected the shape of the cells, and to see if changes in composition of media

Table 16

Generation times of organism 10, at 45 C, 55 C and 65 C, in casein hydrolysate and in trypticase yeast extract media.

Medium	Temperature °C	Interval (Hours)	log b	log B	Generation time (minutes)
Casein hydrolysate	45	1 to 6	5.5051	3.9191	56.6
	55	4 to 7	6.1367	4.8451	38.5
	65	3 to 5	5.9031	4.2788	22.2
Trypticase yeast extract	45	6 to 13	7.0414	4.7853	51.5
	55	1 to 4	5.2900	3.7782	36.2
	65	1 to 4	7.3424	4.2553	17.5

brought about any alterations. The general results were that cells grown in the casein hydrolysate media were indistinguishable from those grown in the natural medium, and that at the lower temperatures, a tendency to uniformity was found. Spore formation was somewhat better at the lower temperatures, also. In the media containing suboptimum amounts of vitamins, which supported only a fraction of the growth, and fermentation of which the organisms are capable, the cells appeared in all respects the same as in the optimal casein hydrolysate and the natural media.

Slides were more frequently stained by one of the polychrome blood stains, Giemsa or Wright, because more cells were revealed. Possibly those which appeared very poorly stained by the method of Gram were slightly more stainable with the polychrome stain. Frequent appearance of both vacuoles and granules in both young and old cells, grown at all temperatures possible, makes the Gram stain a less satisfactory one for examining the smears of the thermophiles.

Figure 5 is a photomicrograph of Bacillus coagulans NRS 27, grown 24 hours in optimal casein hydrolysate medium, at 35 C. Figure 6 is a reproduction of the appearance of the same organism in the same medium, but cultivated 24 hours at 55 C. Both slides stained by polychrome blood stain. The relative uniformity in dimension of cell is evident in the former, and the great variation in size and shape is evident in the latter.

Figure 7 shows the relative uniformity in cell dimension of B. stearothermophilus NRS 91, when grown 24 hours in optimal casein hydrolyzate medium, at 55 C. There are unusually numerous swollen sporangia in this photomicrograph. Figure 8 shows the appearance of the same organism in the same medium for the same time, but cultivated at 65 C, where the marked variation in cell dimension is again apparent. Both slides stained by polychrome blood stain.

Figure 9 is a photomicrograph of a slide stained by the malachite green-safranin method, to show spores. The organism is culture 10, grown at 55 C for 72 hours in optimal casein hydrolyzate medium. Unusually numerous spores are seen. The sporangia are sometimes swollen, and the spores are present in almost any portion of the cell.

Bacillus coagulans NRS 27, when measured with an ocular micrometer, using stained cells grown at 35 C usually was 0.5 to 0.7 μ by 4.0 to 5.0 μ, while the cells grown at 55 C were mostly less than 0.5 μ x 5.5 to 7.4 μ. Staining by Gram was most irregular at 55 C, but at 35 C, quite uniform, especially in early culture. B. stearothermophilus NRS 91, when grown at 55 C was usually 0.8 by 5.0 to 6.0 μ, but when grown at 65 C varied from 0.5 to 1.0 μ by 4.0 to 8.0 μ. Greater variations were often seen with this and all other cultures.

The percentage of spores in the cultures was so small as to make quantitative estimation unreasonable, but the qualitative observations revealed that the casein hydrolyzate medium and natural medium were approximately equally satisfactory for spore formation,

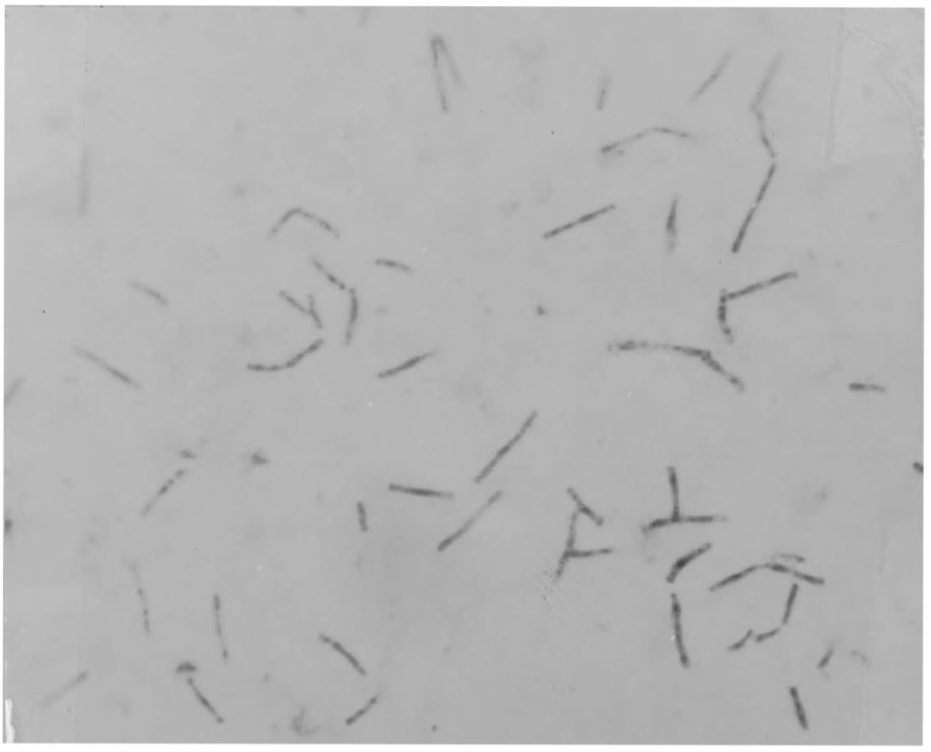

Figure 5. B. coagulans, NRS 27, grown at 35 C, 24 hours, in casein hydrolyzate medium. Cells stained by Giemsa. 1800 X.

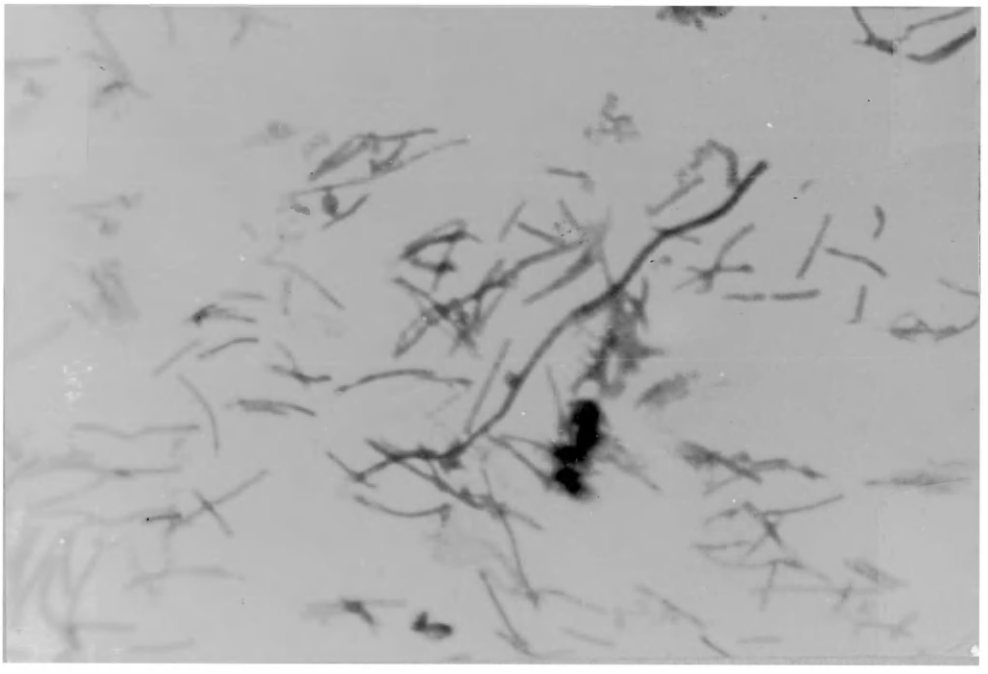

Figure 6. B. coagulans NRS 27, grown at 55 C, 24 hours, in casein hydrolyzate medium. Cells stained by Giemsa. 1800 X.

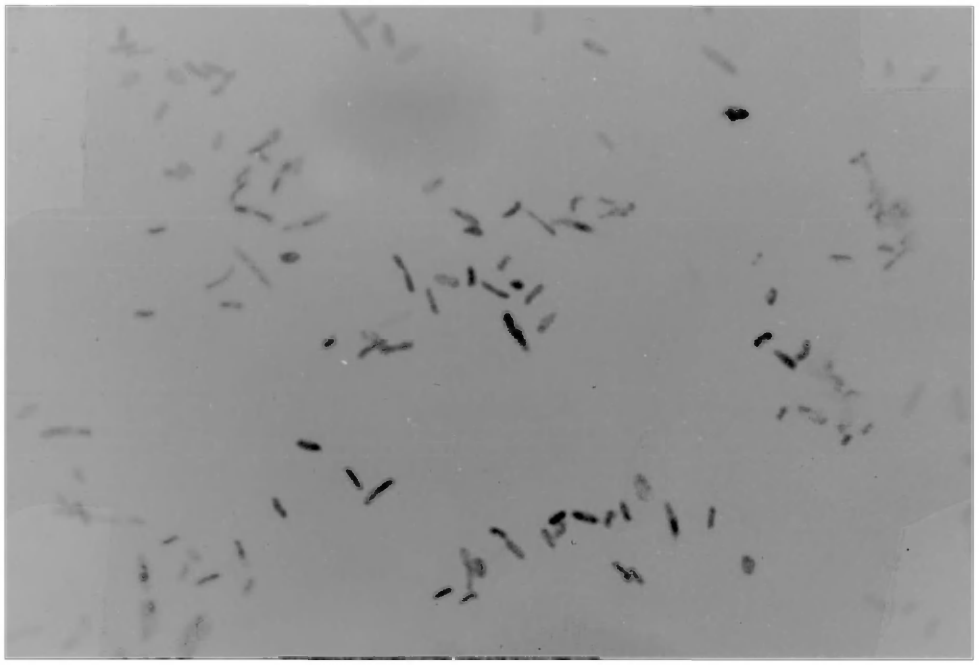

Figure 7. B. stearothermophilus NRS 91, grown at 55 C, 24 hours, in casein hydrolysate medium. Cells stained by Giemsa. 1800 X.

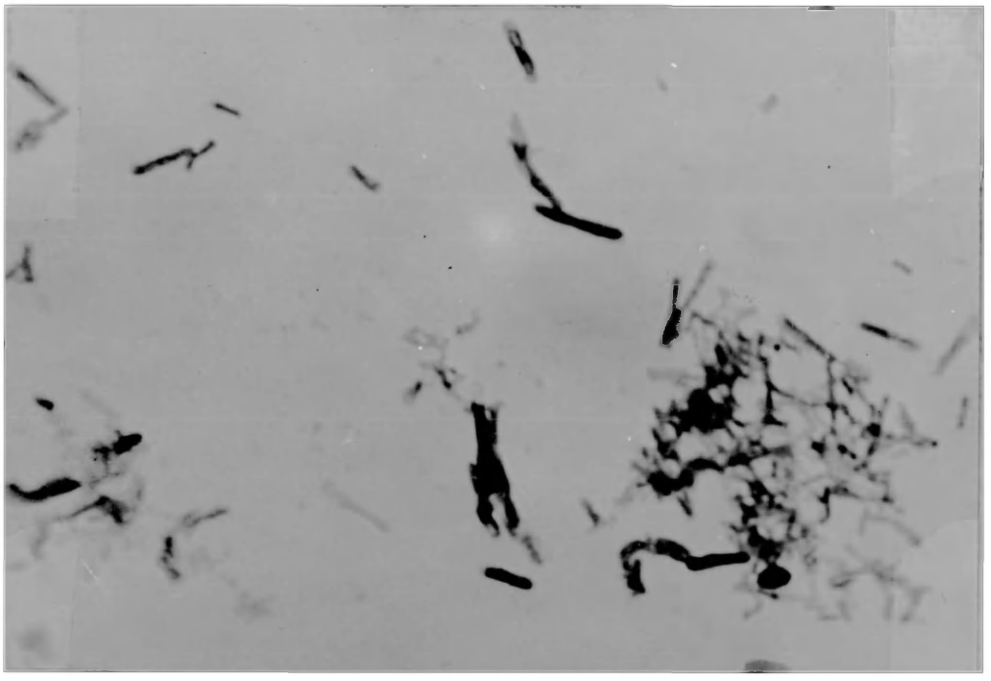

Figure 8. B. stearothermophilus NRS 91, grown at 65 C, 24 hours, in casein hydrolysate medium. Cells stained by Giemsa. 1800 X.

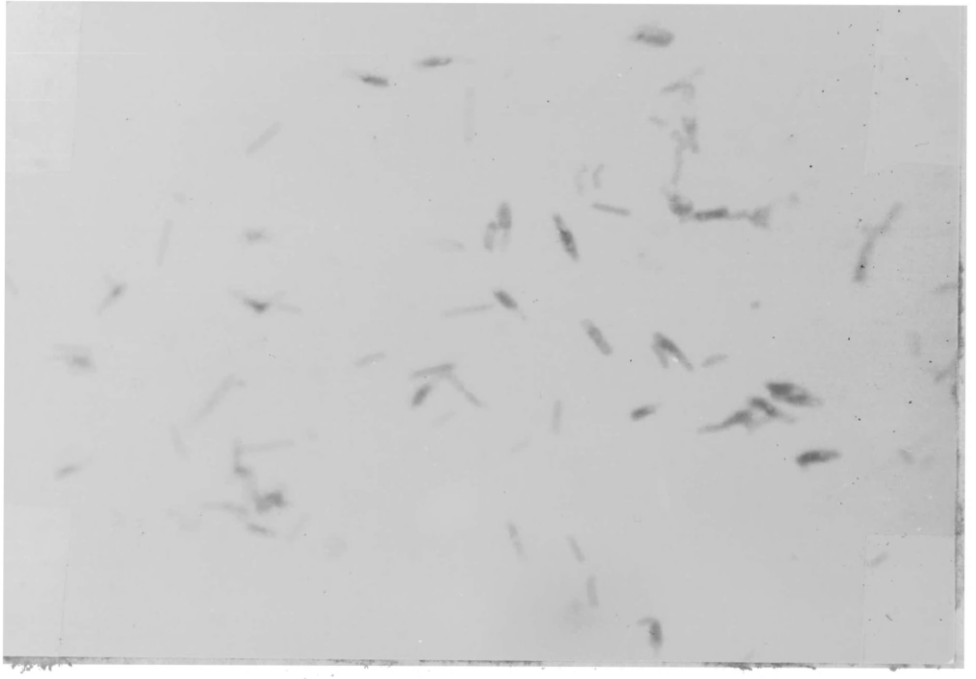

Figure 9. Organism 10, grown at 55 C, 72 hours, in casein hydrolysate medium. Stained by malachite green-safranin method. 1800 X.

and that at the higher temperatures, fewer spores were produced. In rare instances did the spore count rise above 5 per cent.

Assay of a cell-free preparation from B. stearothermophilus NRS 91 for amylolytic activity

Imsenecki, et al (1942) and Imsenecki and Solnzeva (1944) reported that the production of amylase from thermophilic bacteria is commercially practicable. The enzyme which Imsenecki, et al (1942) described possessed a temperature optimum of 70 C to 100 C. The production of such an enzyme would be of inestimable commercial value in the United States. No such enzyme is available, and frequently none is found upon repeated trials (Larson, 1947).

Since the thermophiles are mostly quite active in starch hydrolysis, one was chosen in an attempt to demonstrate amylolytic activity above the usual temperatures. As is pointed out by Tauber (1949) and Imsenecki, et al (1942), amylase is produced in the absence of starch, if abundant proteinaceous material is available, although the Russian workers stated that a more powerful enzyme is produced when the organisms are grown in the presence of starch.

Bacillus stearothermophilus NRS 91 was chosen because it grew so readily and was active in starch hydrolysis, and was an identified culture. It was subcultured 20 times at 65 C in a medium composed of 2 per cent tryptiease, 0.5 per cent yeast extract, 0.5 per cent K_2HPO_4, and 0.01 per cent soluble starch (Baker's), before being inoculated into the three test media below. (1) Tryptiease

soy broth containing 0.01 per cent starch; (2) 1 per cent casein hydrolyzate medium containing only 0.1 per cent glucose, and 0.2 per cent starch; and (3) the potato decoction described by the Russian workers. This was prepared by autoclaving 100 g of very finely diced clean whole potatoes and 10 g of $CaCO_3$ in 1 liter of water for 2 hours. The liquid was decanted into a sterile container, and inoculated with a fresh culture when cooled to 65 C.

All media were dispensed into half-gallon mason jars, which withstood autoclaving well, covered with deep petri dishes. Control jars remained sterile. The casein hydrolyzate medium on the second day received new vitamins, in order to replenish those which might have been either used up or inactivated. At the end of 18 hours, no starch was detectable (iodine) in any of the three media; at the end of 36 hours, growth was abundant in all media. At the end of 48 hours, cells were removed by decantation and centrifugation. The supernatant fluids received a little toluene for preservation, and were immediately tested for amylase activity. Then, the supernatant fluids were sterilized by filtration through porcelain (Selas 02), and stored in sterile flasks in the refrigerator. Amylolytic activity was again tested, at 37 C, 40 C, 55 C and 65 C. A control was made by using a dilution of saliva (1:100). The very crude test described by Hawk et al (1947) was employed in order to obtain an indication of the magnitude of activity present. This crude method defines a "unit" of amylase as the amount required to bring 5 ml of 1 per cent soluble starch to the achromic point in

10 minutes, under conditions of test.

Table 15 presents findings in testing amylolytic activity of the toluene-preserved and the cell-free (filtered) preparations, at the 4 temperatures described, at pH 6.6 and pH 6.8. These data show that the demonstrable enzyme never exceeded 4 units per ml; that it was least abundant in the casein hydrolyzate medium; that a considerable amount was lost either by storage in the refrigerator or by filtration or both; that it was active only at the conventional temperatures.

Since the amylolytic property of the preparations was so slight, it seemed useless to attempt to purify or to concentrate it, or to use a more elaborate and accurate titrimetric method, such as that of Sandstedt, et al (1939). None of the other stenothermophilic cultures showed any more accumulation of enzyme than NRS 91, or promise for continued work.

Table 17

Amylolytic activity* of 3 crude preparations from B. stearothermophilus, at 37 C, 40 C, 55 C and 65 C at pH 6.6 and pH 6.8

Medium	temperature / ml of broth / treatment	pH	37 C 1.0	37 C 0.5	37 C 0.25	37 C 0.1	40 C 1.0	40 C 0.5	40 C 0.25	40 C 0.1	55 C 1.0	55 C 0.5	65 C 1.0	65 C 0.5	Crude Units
Casein hydrol.	centrifuged only	6.6	+	+			+	+						-	2
		6.8	+												2
	filtered (sterile)	6.6	+												1
		6.8	+												1
Trypticase soy broth	centrifuged only	6.6	+	+											1
		6.8	+	+											1
	filtered (sterile)	6.6	+	+											2
		6.8	+	+											2
Potato decoction	centrifuged only	6.6	+	+											4
		6.8	+	+											4
	filtered (sterile)	6.6	+	+											2
		6.8	+	+											2
saliva	diluted 1-100	6.6	+												100
		6.8	+				+				-		-		100

*As detected by iodine test (achromic point), at end of 10 minutes, in NaCl and phosphate buffers.

IV

DISCUSSION AND SUMMARY

Vitamin requirements. It was anticipated that growth of the thermophiles at 65 C would take place readily only if they were supplied unusually large amounts of vitamins, and that they would exhibit different demands at different temperatures. It was found that these organisms required vitamins in amounts exceeding those of mesophilic bacteria, and somewhat less than many yeasts, but no temperature-correlated differences in vitamin requirements were found. The optimum amounts found were: biotin, 0.04 µg per ml; thiamine and niacin, 1.0 µg each per ml, in the supplemented casein hydrolyzate medium employed. Suboptimum amounts of the required vitamins (as little as one-hundredth of the optimum) supported from 25 to 80 per cent of total growth, as detected by turbidity measurement, but did not permit the organisms to attain the low final pH attained in the optimal medium. This is especially noted at the higher temperatures of cultivation, in both the stenothermophiles and the eurithermophiles. Possible thermal destruction of the accessory factors is more important in relatively low concentrations.

Vitamin replacements. The finding that all of the thermophiles studied were able to use niacinamide as readily as the free acid is the usual finding with niacin-demanding bacteria. The

finding that for the stenothermophiles DPN even in massive amounts is apparently not satisfactory as a displacement for niacin is unusual, although not new. A fresh or more potent, or even differently prepared, DPN preparation might in the future prove to be entirely suitable for these organisms. The indicated inability of some organisms to use the coenzyme, requires, as suggested also by Koser and Kasai (1948), that consideration be given to the possibility that there are other routes by which niacin is useful in the metabolism of the cell. There exists the possibility that the DPN molecule, as supplied, is of such a size and in such an undissociated state that it is unable to enter the cell; there exists also the possibility that niacin is used for some process other than synthesis into coenzymes. Koser and Kasai, however, in their cultures of Leuconostoc, found evidence for DPN itself, or some product with DPN activity. In view of the fact that the eurithermophiles were able to utilize DPN as supplied (at 55 C), it is possible that at 65 C a considerable amount of thermal alteration occurs to the DPN itself, although coenzymes are generally regarded as being at least fairly thermostable.

The optimal concentration of biotin was found to be relatively high, 0.04 µg per ml, and for all of the eurithermophiles at 45 C and 55 C, the same amount of desthiobiotin was found entirely suitable as a displacement for biotin. Desthiobiotin, in the same concentration as was found optimal for biotin, was suitable for the replacement of biotin at 55 C and 65 C for 9 of the 12 stenothermophiles, suitable at 55 C but not at 65 C for

2 cultures, and unsuitable at either temperature for 2.

Chemically defined media. It was anticipated that these soil saprophytes would be readily cultivable in simple definable media. It is regrettable that none was found or devised that would support good continued growth, for in such a medium, the fate of niacin and biotin, progress of spore formation and germination, demand for inorganic ions, and kindred problems would have been studied with more success than with the casein hydrolyzate medium. Doubtless a suitable completely defined medium will be found.

Growth curves. Accepting the growth curves of organism 1460, reported by Hansen (1933) as typical of the eurithermophiles, and the growth curves here reported of organism 10 as typical of the stenothermophiles, it may be concluded that some thermophilic bacteria are incapable of multiplication at usual, or lower temperatures. No stenothermophiles were isolated from any of the soil or feces samples tested, and it may be that the stenothermophiles are relatively infrequently found. Ordinarily, no differentiation is made beyond observation of growth at 55 C, and the present classification gives no aid in identifying organisms isolated. The study of growth curves in the optimal casein hydrolyzate medium, as challenged by the natural medium used, afforded an opportunity to compare the productivity of the simpler medium. It was found that the natural medium was superior in that it supported more generations in a time interval, and more total generations (gave a higher viable count). The differences were not so great as to

indicate that the casein hydrolyzate medium was markedly deficient. The shortest generation time found for the stenothermophile was 17.5 minutes at 65 C, in the natural medium. This is somewhat longer than that found by Hansen (16 minutes at 55 C in a natural medium), and a great deal longer than that found by Mudge (5 to 8 minutes at 62.5 C in milk).

Morphological variations. The studies of the effect of temperature of cultivation and composition of medium on size and shape of the organisms indicate that the casein hydrolyzate and the natural media used produce cells which are indistinguishable, that spore formation is about the same in both media, and that spore formation is depressed at the higher temperatures. It was also observed that at the higher temperatures of incubation (i.e., 55 C for the eurithermophiles, and 65 C for the stenothermophiles), the cells vary greatly in size and shape, whereas at the lower temperatures, the cells are relatively uniform. These findings support the opinion of Gordon and Smith (1949) that measurement is an unreliable criterion for identification of the thermophiles. They propose instead, differentiation on basis of temperatures of growth.

Amylase. Employing the medium advocated by the Russian workers for the production of an amylase which is active at elevated temperatures, as well as an excellent natural medium (trypticase soy broth, BPL), and the casein hydrolyzate medium, it was found that none of the organisms in any medium produced detectable

amount of enzyme, secreted into the medium or accumulated upon autolysis, which was active at a temperature above 40 C. Undoubtedly, the workers who were able to extract so potent and useful an enzyme used a kind of thermophile of which there was no representative in this study.

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