THE EFFECT OF TEMPERATURE ON THE NUTRITIONAL REQUIREMENTS OF FACULTATIVE AND OBLIGATE THERMOPHILIC BACTERIA

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The first report dealing with the effect of temperature on the nutritional requirements of microorganisms seems to be that of Mitchell and Houlahan (1946*a*). In the course of their genetic studies on *Neurospora* a mutant strain was isolated which required riboflavin for growth. They noted that this requirement was a function of the incubation temperature. When the mutant was grown at temperatures below 25 C, it synthesized riboflavin at a rate approaching that of the wild type strain while at temperatures above 28 C an external source of the vitamin was necessary. Since this report papers have appeared describing temperature sensitive mutants of *Neurospora* requiring adenine (Mitchell and Houlahan, 1946*b*; McElroy and Mitchell, 1946), uridine (Houlahan and Mitchell, 1947), and sulfanilamide (Emerson, 1947). Maas (1950) has reported on a temperature sensitive mutant of *Escherichia coli* requiring pantothenic acid.

Similar studies of the effect of the temperature of incubation on nutritional requirements have been made on nonmutant strains of *Lactobacillus arabinosus* by Borek and Waelsch (1951), on *Escherichia coli* by Ware (1951), and on *Pasteurella pestis* by Hills and Spurr (1952). In general the findings of these workers have shown that at the higher incubation temperatures the organisms under study required additional metabolites for growth.

A search of the literature revealed a paucity of information concerning the nutritional requirements of thermophilic bacteria. Cleverdon *et al.* (1949*a*) studied the vitamin requirements of a strain of *Bacillus coagulans* at 37 C and 55 C. Employing a vitamin-free casein hydrolyzate medium they found that niacin, thiamin, and biotin were required for growth at both temperatures, growth and sporulation being most abundant at the lower temperature of incubation. In a study of the vitamin requirements of twelve obligate thermophilic strains of *Bacillus stearothermophilus*, these same workers (Cleverdon *et al.*, 1949*b*) noted that biotin, niacin, and thiamin were the only vitamins found to be essential for continued growth of the cultures at 55 C and 65 C in the casein digest medium employed. One group of five cultures required only biotin and niacin, another group of two cultures required only biotin, and a third group of five cultures required all three vitamins. With the exception of one strain no temperature correlated difference in vitamin requirements was noted. This strain grew well at 55 C in the absence of biotin although at 65 C it uniformly required biotin. Here also as in the case of *B. coagulans*, growth and spore formation were most abundant at the lower temperature of incubation.

In order that there may be no confusion regarding the terms applied to thermophilic bacteria in the present study the classification proposed by Cameron and Esty (1926) has been followed. It is as follows: *facultative thermophiles:* growth at 37 C and 55 C; *obligate thermophiles:* growth at 55 C but not at 37 C.

The work reported herein was undertaken to determine the effect of the temperature of incubation upon the nutritional requirements of several strains of facultative and obligate thermophilic bacteria with the hope that the information obtained might give some clues to the nature of thermophily. Before the study could proceed the development of a completely synthetic medium supporting good growth had to be realized.

METHODS AND MATERIALS

Cultures. Eight strains of *B. stearothermophilus* and four strains of *B. coagulans* were selected at

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random for study. One strain of a thermophilic mutant of *B. globigii* described by Mefferd and Campbell (1952) was also included. All cultures, with the exception of the thermophilic mutant of *B. globigii*, were obtained from the National Canners Association through the courtesy of Mr. C. W. Bohrer. The species characteristics of all *B. coagulans* and *B. stearothermophilus* cultures were confirmed according to the classification scheme of Gordon and Smith (1949). Stock cultures were maintained on Difco nutrient agar slants.

Media. In earlier work on the growth of these strains from small inocula it was found that a glucose-mineral base medium containing 0.3 per cent basamin (a yeast autolysate sold by Anheuser-Busch Company) gave good results. Employing the reported value of the amino acid and vitamin content of basamin, a synthetic medium equivalent to 0.3 per cent basamin was devised. The formula of the medium finally adopted is given in table 1. This medium supported good growth of all cultures through six serial transfers. The amino acids and vitamins used were products

TABLE 1

Synthetic medium employed for the determination of the nutritional requirements of facultative and obligate thermophilic bacteria

CONSTITUENT	AMOUNT	CONSTITUENT	AMOUNT
L-Arginine	10.5 mg	Pantothenic acid	100.0 μg
L-Cystine	4.8 mg	Pyridoxal	7.5 μg
L-Glutamic acid	10.0 mg	Biotin	0.9 μg
L-Histidine	4.5 mg	Folic acid	6.0 μg
DL-Isoleucine	14.0 mg	Glucose	200.0 mg
L-Leucine	19.2 mg	Sodium acetate	50.0 mg
L-Lysine	19.5 mg	Na_2HPO_4	250.0 mg
DL-Methionine	6.0 mg	KH_2PO_4	100.0 mg
L-Tryptophan	6.0 mg	NH_4Cl	100.0 mg
DL-Valine	14.4 mg	NaCl	100.0 mg
Thiamin·HCl	15.0 μg	Mineral supplement*	0.1 ml
Riboflavin	15.0 μg	Distilled water	100.0 ml
Nicotinic acid	150.0 μg		

* Mineral supplement: $MgCl_2$, 0.5 g; $FeCl_3$, 0.5 g; $CaCl_2$, 0.5 g; distilled water, 100 ml.

Materials. Pyrex glassware, cotton plugs, and twice distilled water were used throughout. Flasks and pipettes were cleaned by soaking overnight in potassium dichromate cleaning solution, rinsed thoroughly in tap water, then distilled water, and allowed to air dry. Owing to the large number required for each determination the tubes were washed thoroughly with Dural H detergent, rinsed thoroughly in tap water, then in distilled water, and placed in a hot air oven at 200 C for twelve hours. The hot air treatment of tubes was employed for two reasons, namely, to ensure that any thermophilic spores which might be present from previous runs were destroyed, and to oxidize any traces of organic material remaining in the tubes. Preliminary experiments with tubes treated in this manner compared with tubes cleaned with acid cleaning solution showed no differences in requirements of three test cultures.

from commercial sources. Prior to use all amino acids were recrystallized twice according to the methods of Dunn and Rockland (1947).

For the determination of the nutritional requirements of the cultures the procedure followed was that of omission of individual amino acids and vitamins from the complete medium. The respective media were pipetted into 7 inch tubes, in 10 ml amounts, and autoclaved at 15 pounds pressure for 15 minutes. The final pH of all media was 7.0 to 7.2. Preliminary experiments comparing heat sterilized media and sintered glass filtered media showed no significant differences in the requirements of three test cultures.

Preparation of inocula. The cultures were grown in a medium containing 2 per cent tryptose, 0.5 per cent basamin, 0.25 per cent Na_2HPO_4, 0.1 per cent KH_2PO_4, and 0.1 per cent NaCl. After 24 hours' incubation at 55 C the cells were har-

vested by centrifugation, washed three times with phosphate buffer of pH 7.0 (62 ml M/15 Na_2HPO_4 + 38 ml M/15 KH_2PO_4), and resuspended in buffer. Cells thus prepared were kept in the ice box until needed. In no case were cells kept over 5 days. In order to have the inocula as standard as possible the suspensions were adjusted prior to use to an optical density of 0.061 on a Klett-Summerson photoelectric colorimeter employing the no. 42 blue filter.

The tubes of media were inoculated with 0.1 ml of the cell suspensions prepared as described above. One series of tubes was incubated at 36 C, one series at 45 C, and one series at 55 C. After 48 hours' incubation growth response was determined by turbidimetric measurement with a Klett-Summerson photoelectric colorimeter, employing the no. 42 filter. The complete medium was used as a control in all cases.

RESULTS AND DISCUSSION

Table 2 summarizes the nutritional requirements of each strain at each incubation temperature. The data presented are representative of results obtained in at least five, and in most instances, ten separate experiments.

It is of interest to note that all previous reports in the literature dealing with the effect of temperature on the growth requirements of microorganisms have shown that as the incubation temperature is increased there is an increase in the growth requirements of the particular organism under study. The explanation for these findings presented by nearly all authors is that at the higher temperature the enzyme(s) responsible for the synthesis of a particular metabolite(s) required, say compound X, undergoes thermal inactivation and thus the organism requires an exogenous source of compound X before growth can take place.

An alternate explanation of temperature sensitive mutants of *Neurospora* has been advanced by McElroy and Mitchell (1946). They postulate that *Neurospora* may possess normally two pathways for the synthesis of essential metabolites, each pathway having a different environmental optimum.

An examination of table 2 reveals that the strains of thermophilic bacteria employed in this study fall into three groups in so far as the incubation temperature affects the growth requirements. One group, designated as group A (*B. coagulans*, strain 2; *B. stearothermophilus*, strains 1356, 1503, 3656), showed no differences in growth requirements regardless of the incubation temperature. Group B which is comprised of six cultures (*B. coagulans*, strains 32, 1039; *B. stearothermophilus*, strains 1373b, 3084, 4259; *B. globigii*, strain 1) showed additional requirements as the temperature of incubation was increased. Group C containing three cultures (*B. coagulans*, strain 12; *B. stearothermophilus*, strains 3690, 5149-5) showed additional requirements as the incubation temperature was lowered.

In attempting to explain these data several possibilities must be considered. It has been well established that genes are responsible for the *potential* capacity of an organism to produce enzymes, which in turn synthesize the metabolites necessary for growth. Thus, the complete inactivation of a gene responsible for the synthesis of an essential metabolite imposes upon the organism a requirement of an external source of the substance which it can no longer produce. Consider *B. coagulans*, strain 2, as an example of the cultures which fall into group A. This strain requires, among other things, histidine for growth at all three temperatures. This finding may be explained by assuming that the gene necessary for the production of the enzyme responsible for the synthesis of histidine, if present, has been inactivated; thus this strain can no longer make histidine and now requires an external source of this metabolite before growth can occur. If the gene is absent, or has been inactivated, one would not, therefore, expect temperature to exert any influence on the histidine requirement of the organism.

As an example of group B cultures consider *B. coagulans*, strain 1039. This strain requires histidine, methionine, and nicotinic acid for growth at 55 C but not at 36 C or 45 C. In this case it may be that the gene responsible for the synthesis of histidine, for example, rather than being inactivated has become modified in some manner such that it now produces an enzyme with the same specificity but with altered physical properties which lead to a rapid inactivation of the enzyme at the higher temperature. Theoretically such enzymes might be obtained by the breaking of certain hydrogen bonds, thus giving altered physical properties without affecting the spatial configurations which confer specificity. At the lower temperatures the normal specificity and

TABLE 2

*The effect of incubation temperature on the nutritional requirements of thermophilic bacteria**

ORGANISM	NUTRITIONAL REQUIREMENTS		
	Incubation temperature		
	36 C	45 C	55 C
Bacillus coagulans 2 (F)	Histidine, thiamin, biotin, folic acid	Histidine, thiamin, biotin, folic acid	Histidine, thiamin, biotin, folic acid
12 (F)	Histidine, leucine, valine, thiamin, biotin, folic acid	Histidine, leucine, thiamin, biotin, folic acid	Histidine, leucine, thiamin, biotin, folic acid
32 (F)	Methionine, tryptophan, thiamin, biotin	Histidine, methionine, tryptophan, thiamin, biotin, folic acid	Histidine, methionine, tryptophan, thiamin, biotin, folic acid
1039 (F)	Thiamin, biotin, folic acid	Thiamin, biotin, folic acid	Histidine, methionine, thiamin, nicotinic acid, biotin, folic acid
Bacillus stearothermophilus 1356 (O)	No growth	Leucine, thiamin, nicotinic acid, biotin	Leucine, thiamin, nicotinic acid, biotin
1373b (O)	No growth	Glutamic acid, histidine, methionine, leucine, biotin	Glutamic acid, histidine, methionine, leucine, biotin, pyridoxal
1503 (O)	No growth	Valine, nicotinic acid, biotin	Valine, nicotinic acid, biotin
3084 (F)	Biotin, folic acid	Biotin, folic acid	Thiamin, biotin, folic acid
3656 (O)	No growth	Histidine, nicotinic acid, biotin	Histidine, nicotinic acid, biotin
3690 (F)	Methionine, leucine, thiamin, nicotinic acid, biotin, folic acid	Methionine, thiamin, biotin, folic acid	Methionine, thiamin, biotin, folic acid
4259 (F)	Biotin, folic acid	Methionine, histidine, nicotinic acid, biotin, folic acid	Methionine, histidine, nicotinic acid, biotin, folic acid
5149-5 (F)	Methionine, thiamin, biotin, folic acid	Biotin, folic acid	Biotin, folic acid
Bacillus globigii 1 (F)	Grows in a glucose-glutamic acid-mineral medium	Thiamin, nicotinic acid	Thiamin, nicotinic acid

(F)—Facultative thermophile.
(O)—Obligate thermophile.
* For data on numerous other strains see Campbell (1952).

efficiency would be maintained, but at the higher temperature the enzyme molecules would unfold and collapse, rendering the enzyme inactive. It is possible of course that at the higher temperature one of the precursors necessary for the synthesis of histidine may be shunted off into another series of reactions and thus not be available for histidine synthesis.

B. stearothermophilus, strain 3690, as an example of group C cultures, requires leucine and nicotinic acid at 36 C but not at 45 C or 55 C. These data may be explained on the assumption that the genes responsible for the synthesis of these metabolites are not inactivated but are modified in some way such that they produce enzymes which require a higher temperature before they can be activated and thus operational. Therefore, at the lower temperature of 36 C the enzymes are not active, and before growth can take place these metabolites must be supplied preformed in the medium. The removal of precursors for these substances by some form of shunt metabolism, active at 36 C but not at 45 C or 55 C, also possibly may explain the experimental findings.

SUMMARY

The effect of the temperature of incubation on the nutritional requirements of eight strains of *Bacillus stearothermophilus*, four strains of *Bacillus coagulans*, and one strain of a thermophilic mutant of *Bacillus globigii* has been determined. All strains gave good growth in a synthetic medium containing amino acids, vitamins, glucose, and salts. The cultures fell into three groups in so far as the incubation temperature affected the growth requirements. One group showed no differences in growth requirements regardless of the incubation temperature. A second group had additional requirements as the temperature of incubation was increased. A third group required additional metabolites as the incubation temperature was lowered. These findings are discussed in terms of the gene-enzyme relationship and shunt metabolism.

REFERENCES

BOREK, E., AND WAELSCH, H. 1951 The effect of temperature on the nutritional requirements of microorganisms. J. Biol. Chem., **190**, 191–196.

CAMERON, E. J., AND ESTY, J. R. 1926 The examination of spoiled canned foods. 2. Classification of flat sour, spoilage organisms from non acid foods. J. Infectious Diseases, **39**, 89–105.

CAMPBELL, L. L., JR. 1952 Physiological studies on thermophilic bacteria. Ph.D. Dissertation, University of Texas, Austin.

CLEVERDON, R. C., PELCZAR, M. J., AND DOETSCH, R. N. 1949*a* Vitamin requirements of *Bacillus coagulans*. J. Bact., **58**, 113.

CLEVERDON, R. C., PELCZAR, M. J., AND DOETSCH, R. N. 1949*b* The vitamin requirements of stenothermophilic aerobic sporogenous bacilli. J. Bact., **58**, 523–526.

DUNN, M. S., AND ROCKLAND, L. B. 1947 The preparation and criteria of purity of the amino acids. Advances in Protein Chem., **3**, 296–382.

EMERSON, S. 1947 Growth response of a sulfonamide-requiring mutant strain of *Neurospora*. J. Bact., **54**, 195–207.

GORDON, R. E., AND SMITH, N. R. 1949 Aerobic sporeforming bacteria capable of growth at high temperatures. J. Bact., **58**, 327–341.

HILLS, G. M., AND SPURR, E. D. 1952 The effect of temperature on the nutritional requirements of *Pasteurella pestis*. J. Gen. Microbiol., **6**, 64–73.

HOULAHAN, M. B., AND MITCHELL, H. K. 1947 A suppressor in *Neurospora* and its use as evidence for allelism. Proc. Natl. Acad. Sci., U. S., **33**, 223–229.

MCELROY, W. D., AND MITCHELL, H. K. 1946 Enzyme studies on a temperature sensitive mutant of *Neurospora*. Federation Proc., **5**, 376–379.

MAAS, W. K. 1950 A temperature-sensitive pantothenicless mutant of *Escherichia coli*. Bact. Proc., 128–129.

MEFFERD, R. B., AND CAMPBELL, L. L., JR. 1952 Studies on a thermophilic variant of *Bacillus globigii*. Texas Repts. Biol. Med., **10**, 419–420.

MITCHELL, H. K., AND HOULAHAN, M. B. 1946*a* *Neurospora* IV. A temperature-sensitive riboflavinless mutant. Am. J. Botany, **33**, 31–35.

MITCHELL, H. K., AND HOULAHAN, M. B. 1946*b* Adenine-requiring mutants of *Neurospora crassa*. Federation Proc., **3**, 370–375.

WARE, G. C. 1951 Nutritional requirements of *Bacterium coli* at 44°. J. Gen. Microbiol., **5**, 880–884.