TABLE III. Antibacterial Spectrum of Pool 2 from Abalone Juice.

Test organism	Conc., mg %	Abalone juice pool 2	Colonial growth of test organisms on plate* 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Control†
Staph. aureus, Pen. sensitive	5	+	0	0	0	0	0	0	0
		—	4	4	4	4	4	0	0
Staph. aureus, Pen. resistant	5	+	1	0	0	0	0	0	0
		—	4	4	4	4	4	0	0
Staph. aureus, Pen. resistant	10	+	0	0	0	0	0	0	
		—	4	4	4	4	4	0	0
Strep. pyogenes, beta hemolytic	5	+	2	0	0	0	0	0	0
		—	4	4	4	4	4	0	0
Salmonella paratyphi A	10	+	0	0	0	0	0	0	0
		—	4	4	4	4	4	4	0
Salmonella paratyphi B	10	+	1	0	0	0	0	0	0
		—	4	4	4	4	4	0	0
Salmonella typhi	10	+	1	1	0	0	0	0	0
		—	4	4	4	4	4	4	0

1-4, etc. indicate degree of bacterial growth. 0 = no bacterial growth.
* See Table I. † Not inoculated.

structure of the active agent and *in vivo* studies are in progress.

Summary. Ten fractions have been isolated from abalone juice by ion exchange chromatography (Cellex-D) and tested for *in vitro* antibacterial activity against *Staphylococcus aureus*. Four fractions of effluent showed high activity. One active fraction was tested and found effective against *Staphylococcus aureus*, (including a penicillin-resistant strain), *Streptococcus pyogenes*, beta-hemolytic strain, and *Salmonella typhi*, and *paratyphi A and B*. The action for *Staphylococcus aureus* seemingly was bactericidal.

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Increased Nutritional Requirements of *Saccharomyces cerevisiae* as a Result of Incubation at 38°C.* (26156)

HERMAN C. LICHSTEIN AND WILLIAM J. BEGUE
Department of Bacteriology, University of Minnesota, Minneapolis

During nutritional studies on yeasts, it was noted that certain strains grew poorly or not at all at 38°C in a chemically defined medium which supported excellent growth at 30°C. In contrast, some of the strains grew equally well at 30°C or 38°C when inoculated into a complex medium. In most instances where such a phenomenon had been demonstrated previously, an increase in temperature was accompanied by an increase in nutritional requirement(1-7). The present paper is concerned with identification of the substance(s) in the complex medium which

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TABLE I. Effect of Temperature of Incubation on Growth of Yeasts in a Complex Medium.*

Yeast strain	Turbidity (24 hr) 30°C	38°C
S. cerevisiae Java	86	67
" (Fleischmann strain 139)	110	78
" (ATCC† 2338)	84	72
S. globosus (" 10600)	99	3
S. carlsbergensis (" 9080)	91	47
S. fragilis (" 10022)	87	90
S. intermedius (" 2360)	77	2

* ACG medium consisting of 1.0% yeast extract, 1.0% casitone, 0.5% K_2HPO_4, and 0.5% glucose (pH 7.0).

† ATCC = American Type Culture Collection, Washington, D. C.

permitted growth of yeasts at the higher temperature.

Materials and methods. Several species and strains of *Saccharomyces* were used, but most of the studies were performed with *S. cerevisiae* (Fleischmann Strain 139). The yeasts were maintained in a complex medium (see Table I for composition); the experimental medium was a modification of the one introduced by Snell, *et al.*(8) and contained 10^2 μg/liter of biotin.

All yeasts were cultivated in the complex medium at 30°C and were transferred at approximately 24-hour intervals 3 or 4 times prior to use in an experiment. The cells were harvested by centrifugation and washed 3 times with 5.0 ml of sterile distilled water.

The experimental culture tubes were 22 x 150 mm screw-cap tubes with caps loosely fitted. Materials to be investigated were added aseptically along with 4.0 ml of medium and distilled water to bring the final volume to 5.0 ml. Inoculum was added in the amount of 0.05 ml. The criterion for growth was turbidity, as measured in a Klett-Summerson photoelectric colorimeter (660 mμ) using the medium as a blank. Incubation for about 24 hours was carried out in water baths where temperatures were maintained to within $\pm$ 0.5°C.

Results. Cultivating the organisms listed in Table I in Snell's medium at 38°C gave rise to somewhat surprising results, since only *S. fragilis* proliferated at this temperature. Since it was known that some of these strains were capable of growth at 38°C in a complex medium, it was necessary to compare their growth at 30°C and 38°C in a complex medium. The results (Table I) revealed that all but 2 of these strains were capable of growth at 38°C. These data suggested pH and/or nutrition as major factors related to growth of yeasts at 38°C. Further experiments in which Snell's medium and ACG medium at pH's of both 4.0 and 7.0 were tested for their capacity to support growth at the elevated temperature made clear that pH is not a factor in the growth response of these organisms at 38°C.

Before the effect of nutrition could be inspected, it was necessary to investigate the influence of inoculum size and to standardize the number of yeast cells to be used in subsequent experiments because 1) preliminary results employing bulk inoculations were somewhat erratic; 2) a rough titration of inoculum size suggested that initial number of cells present affected the expression of the temperature phenomenon; and, 3) past experience in this laboratory(9) as well as others(10) has shown that inoculum size can be of critical importance in nutritional work.

A suspension of yeast cells was prepared as previously described and a series of dilutions inoculated into several media and incubated at the 2 temperatures. Fig. 1 shows that inoculum size was an important factor in the phenomenon under investigation. For ex-

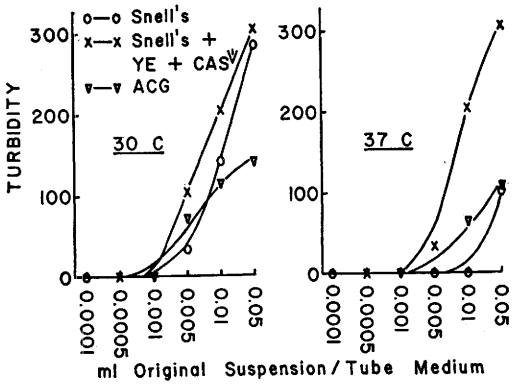


FIG. 1. Influence of inoculum size on growth of *Saccharomyces cerevisiae* (139) at 30°C and 38°C in several types of media.

[1] YE = yeast extract, 1.0%; CAS = casitone, 1.0%. All media adjusted to pH 4.0. Incubation time, 24 hr. Inoculum was 0.05 ml of dilution desired.

ample, at 30°C with an inoculum size of 0.01 ml, growth was obtained in the 3 media used, whereas at 38°C only Snell's medium plus yeast extract and casitone supported good growth. Standardization of inoculum size for all further studies resulted in using 0.05 ml of a washed suspension of yeast cells having a turbidity of 30.

As to the nutritional aspect, it was necessary first to determine whether the growth promoting substance(s) was organic or inorganic in nature. To this end an ashed mixture of yeast extract and casitone was tested and found to have no effect on growth of the organism at 38°C.

Inasmuch as a mixture of yeast extract and casitone was effective in allowing proliferation of *S. cerevisiae*(139) at 38°C, it was important to determine which of the 2 complex materials possessed the active substance (s). Moreover, since yeast extract is often regarded as a source of vitamins and other growth factors, a mixture of known B-vitamins was included.

Good growth recovery at 38°C was obtained with addition to Snell's medium of a vitamin solution, a mixture of yeast extract and casitone, yeast extract alone, or high concentrations of casitone (Table II). The growth recovery obtained upon addition of yeast extract suggested that a vitamin might be the stimulatory substance. This was substantiated by the results using the mixture of known B vitamins. Examination of the spectrum of B vitamins present in the vitamin mixture revealed that pantothenate was the most active single organic material concerned with the ability of yeasts to proliferate at 38°C (Table III). Repeated experiments demonstrated that addition of this vitamin supported growth at 38°C to the extent of 40 to 82% of that obtained at 30°C.

TABLE II. Growth of *Saccharomyces cerevisiae* (139) at 30°C and 38°C in Media of Different Composition.

Additions to Snell's medium	Turbidity (24 hr) 30°C	38°C
None	252	75
Vitamin solution*	246	188
" " , diluted 1:10	262	206
Yeast extract + casitone, 50 mg each	271	256
Idem 5.0 " "	299	256
" 0.5 " "	266	105
Yeast extract, 50 mg	275	276
" " , 5.0 "	296	237
" " , 0.5 "	266	121
Casitone, 50 mg	315	242
" , 5.0 "	273	60
" , 0.5 "	254	44

* 1.0 ml of vitamin solution contains: 20 μg *p*-aminobenzoic acid, 20 μg pantothenate, 40 μg pyridoxine, 20 μg nicotinic acid, 20 μg riboflavin, 20 μg thiamine, 10 μg folic acid.

Since growth was determined as a function of the total mass of synthesized protoplasm (*i.e.*, turbidity), it was important to evaluate growth by measurement of number of viable yeast cells. For this purpose the conventional plating method was employed. Results showed an excellent correlation between turbidity and viable count measurements at 38°C. Furthermore, death of the yeast cells occurred at a more rapid rate at 38°C than at 30°C in media lacking pantothenate. Since such death did not commence until at least 36 hours of incubation, the use of turbidity as a measure of growth coupled with reading the tubes at about 24 hours appears both adequate and accurate.

The results presented thus far suggest that the synthesis of pantothenate normally carried out by *S. cerevisiae*(139) at 30°C is in some manner impaired at 38°C. As a consequence of this, and the results obtained by Maas and Davis(6), it was desirable to inspect the precursors in the accepted synthesis of pantothenate by microorganisms, namely β-alanine and pantoic acid.

Since β-alanine is an obligatory requirement for growth of *S. cerevisiae*(139) in absence of pantothenate, it was inspected only briefly. Its presence did not alter the temperature effect in the usual concentration and the possibility of a competitive inhibitor of β-alanine being synthesized at 38°C was ruled out, since increasing the concentration of this amino acid almost 200-fold had no effect on growth.

Experiments designed to explore the efficacy of pantoate in reversing the temperature phenomenon revealed that while pantoyl lactone had very little activity, sodium pantoate was as effective as pantothenic acid itself al-

TABLE III. Comparative Effectiveness of Pantothenate, Pantoyl Lactone, and Sodium Pantoate in Permitting Growth of *Saccharomyces cerevisiae* (139) at 38°C.

Additions to Snell's medium	Concentration of additions, μM/ml	Turbidity (24 hr) 30°C	38°C
None	—	222	32
Pantothenate	10^{-5}	223	35
"	10^{-4}	220	40
"	10^{-3}	224	105
"	10^{-2}	224	120
"	10^{-1}	222	140
"	10^{0}	223	145
"	10^{1}	230	139
"	10^{2}	234	143
Pantoyl lactone	10^{-1}	228	33
" "	10^{0}	222	31
" "	10^{1}	226	41
" "	10^{2}	222	67
Sodium pantoate*†	10^{-2}	224	33
" "	10^{-1}	226	54
" "	10^{0}	226	118
" "	10^{1}	220	123
" "	10^{2}	230	143
Yeast extract	0.1 g/ml	305	274

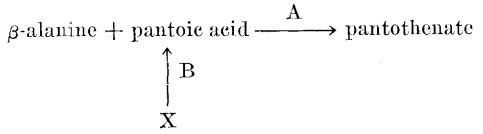
* NaCl controls were negative.
† Prepared from commercially available pantoyl lactone after the method of Maas(5).

beit at a much higher concentration (Table III). Yeast extract was considerably more active than either pantoate or pantothenate. The poor activity of pantoyl lactone when compared to pantoic acid is consistent with the observations of Maas(11), that the latter has about 3 times the effectiveness of the former both in reversing salicylate inhibition and supporting growth of a pantoate auxotroph of *Escherichia coli*.

Studies of the Java and ATCC 2338 strains of *S. cerevisiae* revealed that the former responded in a manner similar to the 139 strain, whereas only yeast extract was effective in promoting growth of the 2338 strain at 38°C.

Discussion. The results of experiments designed to elucidate the nature of the observed temperature sensitivity revealed that the effect of complex materials such as yeast extract was largely replaceable by pantothenic acid. These findings led to an inspection of the synthesis of pantothenate in the hope that the biochemical lesion might be identified.

The accepted pathway of pantothenate biosynthesis is the coupling of β-alanine with pantoic acid:



β-Alanine was studied at concentrations up to 200 times greater than that normally used in the chemically defined medium with entirely negative results. Pantoic acid, on the other hand, was as effective as pantothenate in preventing the temperature effect, but the concentration required was 100 to 10,000 times as great. These findings, in light of the pathway cited above, suggest that since pantoic acid effectively overcomes the temperature effect, either enzyme A is not temperature sensitive or possibly another mechanism of pantothenic acid biosynthesis is operative in this organism. Lansford and Shive (12) considered the possibility that pantonine and β-alanine would couple to yield pantonyl-β-alanine with subsequent deamination of the pantonyl moiety to yield pantothenic acid. Possibly the enzymatic synthesis of pantonine from pantoic acid is temperature sensitive.

The large amount of pantoic acid required as compared to the quantity of pantothenic acid needed, suggests that a competitive type of enzyme inhibition might be involved in the temperature phenomenon. The site of such competition might be in one of the series of reactions leading to formation of pantoate (site B), or conceivably in an entirely unrelated biochemical reaction which is temperature sensitive, thus resulting in accumulation of a substance that exercises its effect either at the site of β-alanine-pantoate coupling (site A) or, again, in a reaction leading to synthesis of pantoate. The identity of such a presumed inhibitor has not been established.

Summary. Data were presented revealing that the paucity of growth of certain strains of yeast at 38°C is somehow related to a reduced ability to carry out the biosynthesis of pantothenic acid. Results were discussed in the light of the accepted pathway of panto-

thenate biosynthesis. Since the organisms are genetically endowed with the enzymic make up to synthesize pantothenate, this provides another example of the fact that environment plays an important role in genetic expression.

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A Method of Crystallization of Human Myoglobin.* (26157)

WILLIAM H. LUGINBUHL (Introduced by H. B. Pierce)
Department of Pathology, College of Medicine, University of Vermont, Burlington

Myoglobin was first crystallized by Theorell(1) using material of equine origin. Human myoglobin was first crystallized by Drabkin(2). Additional methods of crystallizing human myoglobin have been described by Theorell and de Duve(3) and by Rossi-Fanelli(4). In these methods hemoglobin and other proteins are salted out of aqueous extracts of muscle by lead acetate, phosphate, or ammonium sulfate. Concentration of phosphate or sulfate is then gradually increased by dialysis(3) against concentrated solutions of these salts or by evaporation(4) and crystallization is thereby induced. These steps are performed at a constant hydrogen ion concentration near pH 6.6, the isoelectric point of human myoglobin(5).

We have had difficulty in regularly obtaining crystalline human myoglobin by these methods. We have developed a simple method of isolation and crystallization of human myoglobin that depends on precipitation of contaminating proteins from aqueous extracts of muscle at pH 8 by saturation with ammonium sulfate. Myoglobin remains in solution under these conditions. After precipitation of these contaminating proteins and adjustment of the pH to 7, myoglobin crystallizes out of solution.

Procedure. Skeletal muscle from surgical or autopsy sources was frozen within one hour of operative removal or post mortem examination, and stored at −60°C. Cases with a post mortem interval before autopsy of more than 4 hours or with debilitation were avoided. While still frozen, samples weighing approximately 100 g were chopped into small pieces and mixed in an electric blender with 2 ml of distilled water per gram of tissue. These and all subsequent steps were performed at 4°C. After blending, the mixture was adjusted to pH 8 with 2N sodium hydroxide and extracted for one hour with constant stirring. Insoluble materials were then removed by centrifugation at 16,000 g for 30 minutes and the supernate filtered through coarse filter paper.

The filtrate was then made 80% saturated by addition of solid ammonium sulfate, C. P. grade. During this and subsequent additions of ammonium sulfate, the solution was maintained at pH 8 ± 0.05 by additions of 2N sodium hydroxide or 2% acetic acid. The material was then centrifuged at 16,000 g for 30 minutes.

The supernatant fluid was brought to

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