INVESTIGATIONS OF THE THERMOPHOBIC CHARACTER IN NEUROSPORA CRASSA, ESPECIALLY OF THE RELATIONSHIPS BETWEEN TEMPERATURE AND CARBOHYDRATE UTILIZATION

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ABSTRACT

The thermophobic character is a temperature mutant which is unable to grow at 35°C on certain sugars in tube culture. This character is controlled by a single gene located far away from the centromere on the chromosome of Paba linkage group.

A limited growth of thermophobic strain on lactose minimal medium can be forced at 35° after activation at 25° for as little as 2 hours. The experiments show that the inhibition of growth at higher temperature is not due to nutritional deficiency, change of permeability of cell membrane to certain sugars or inhibition of hydrolytic enzymes of oligosaccharides. At 40° the thermophobic strain can not grow on any sugar, while the normal strain can grow on certain sugars but not others, just as the thermophobic strain did at 35°. The difference of effective temperature between the strains is only 5°C. This led to the studies of the common step of carbohydrate degradation, e.g., glycolysis and Krebs' cycle. All Krebs' acids were found to have a strong thermophobic effect at 35° on the thermophobic strain on sucrose. But the manometric studies of certain of the enzyme systems in Krebs' cycle reveal no significant differences between the strains.

In liquid culture on lactose at 35° there appears a morphological difference between the strains, i.e., semicolonial for the thermophobic strain and usual filamentous for the normal. This led to the studies of cell wall material in Neurospora, and it was found that there is a difference in response to chlorozinc iodine staining. The staining reaction is quantitatively related to the length of unacetylated end group of chitin. Estimates show that the chitin of the thermophobic strain has a length of half molecule only about 1/5 that of normal. A length of chitin half molecule less than 100 acetylglucosamine units will induce a growth of semicolonial form in liquid culture, and will have very little measurable growth in tube culture. The length of chitin molecule is influenced by glucose-acetate ratio. Temperature and type of sugar will influence the ratio greatly, and will thus change the habit of growth of both strains, but different quantitatively as the thermophobic gene causes a shortness of chitin molecule from the start.

CONTENT

INTRODUCTION

During the study of adaptive enzyme in Neurospora on different sugars Dr. Emerson found that at 35°C some of the wild strains, 1A for instance, are unable to grow in growth tube culture on some sugars, such as lactose and inulin, but are able to grow normally when glucose, sucrose, or maltose is used in nutrient medium. The inhibitive effect of these sugars appears only at 35°C or higher temperature, and vanishes totally when the temperature is as little as three degrees lower. The inability to grow on certain carbohydrates at 35°C is inherited as an unit character (Emerson and Cushing, 1946). This differential temperature effect is known as thermophobic character.

This thermophobic character in Neurospora is interesting not only because it may help us to understand the mechanism of gene action in relation to changes in environment, but also, it may throw some light on the biochemistry of carbohydrate utilization in Neurospora. Two conditions are necessary for the expression of the character, namely, a certain kind of sugar, and a critical culture temperature. Fortunately, the mode of inheritance of this seemingly complicated character is quite simple; it is due to a single gene. The main work would therefore be concentrated on the mechanism of the action of this temperature sensitive gene.

MATERIAL AND METHODS

This thermophobic character is widely distributed in the Neurospora crassa strains. Eleven strains were selected at random from the California Institute of Technology stock collections and tested in small 100mm length growth tube on 0.5% lactose minimal medium. Six of them showed the thermophobic character. Their growth rates, which are little lower than otherwise in such short tubes in which one end is sealed, are shown in ~~the~~ Table 1. The difference of growth rate at 35°C between the thermophobic and non-thermophobic strains is obvious. At 25°C, on the other hand, there is practically no difference in growth rate between them.

The sugars other than lactose, with which the thermophobic strains are able to show their differential temperature effect are fructose, galactose(monosaccharides), melibiose (disaccharide), raffinose (trisaccharide) and inulin (polysaccharide). There are differences in the degree of the differential temperature effect among these sugars. With melibiose and inulin, the effects are as clear as with lactose. While with galactose, the growth is also poor at 25°C, it is still much better than at 35°C. On the other hand, with fructose and raffinose, the thermophobic strains grow much better at 35°C than on lactose medium, but in comparison with the growth at 25°C, they show the thermophobic character clearly. All the strains grow almost equally well at both 25° and 35°C on glucose, sucrose, maltose, cellobiose, trehalose and soluble starch. The results are summarised in Table 2.

All the strains listed in Table 2 are p-aminobenzoic acidless mutants except 25a, Peach and Em 5256A which are normal strains as far as the chemical reqirement is concerned. The 25a and Peach are thermophobic, and Em 5256A is not. For the sake of simplicity only 25a, as a representative of thermophobic strain, and Em 5256A, as a normal one for comparison,

were chosen for further experimentations.

Synthetic Fries containing biotin was used exclusively as basal medium for the culture. Two methods of cultures were generally used for the measurement of Neurospora growth, namely, the flask method for mycelium dry weight and the tube method of Ryan, Beadle and Tatum (1943) for linear growth of mycelium. In the present case, the result of flask culture in lactose liquid medium at high temperature shows wide fluctuation. By the conventional method, 72 hours growth in 25cc medium, only a few mg of dry mycelium could be obtained. With longer times of incubation the dry weight of thermophobic strain would even occasionally exceed that of normal strain. When thermophobic strains are grown on lactose in liquid culture at 35°C there is an initial lag phase which may vary in length from culture to culture, suggesting that partial adaptation must occur before appreciable growth appears (Emerson, unpublished). This partial adaption might be due to the accumulation of lactase in mycelium as tested by the experiment later. For this reason, the tube method was used exclusively for both the measurement of mycelial growth and for differentiation of the thermophobic from the normal. However, liquid culture was used when a quantity of mycelium was wanted.

In lactose liquid culture at 35°C, although there is no consistent difference on a dry weight basis between the thermophobic and normal strain, there is a pronounced morphological difference. The thermophobic strain invariably grows in a semicolonial form with patches of mycelium suspended throughout the medium. Such mycelial pads are not easily collected without the help of filtration. The normal strain, on the other hand, forms a thin mycelial pad on the surface of medium as usual. This morphological difference in liquid culture led to an investigation of the probable difference in the chitin structure of the contrasting

Table 1

Growth rate on 0.5% lactose medium in mm per hour.

Strain	Culture temperature 35°C	25°C	Remark	Origin of strain
1633A	0.16	0.89	Thermophobic	LA X La
1633-2367a	0.16	1.00	"	"
38113A	0.15	1.41	"	1A X 25a
71301a	0.11	1.16	"	Abb4 X 25a
25a	0.21	1.12	"	LA X La
Peach A	0.13	1.07	"	Lindegren
830A	1.79	1.13	Normal	LA X La
$Z35_1$ 8	1.42	1.39	"	Zaloka
La	2.02	1.13	"	Lindegren
Abb4	1.74	1.56	"	Abbott
Em 5256A	2.33	1.57	"	Emerson

Table 2

Growth rate in mm per hour on different sugar (0.5%) medium.

Strain	Glucose 35°C	Glucose 25°C	Fructose 35°C	Fructose 25°C	Galactose 35°C	Galactose 25°C	Melibiose 35°C	Melibiose 25°C	Sucrose 35°C	Sucrose 25°C	Maltose 35°C	Maltose 25°C
1633A	2.72	1.21	0.64	2.00	0.12	0.41	0.15	1.54	2.71	1.87	1.84	1.75
38113A	2.29	1.84	0.38	1.97	0.12	0.52	0.12	1.10	2.52	2.42	--	--
71301a	2.41	1.69	0.95	2.12	0.01	0.17	0.14	1.19	2.29	2.35	1.59	1.81
25a	2.79	2.15	0.50	2.09	0.00	0.43	0.09	1.41	2.54	2.02	1.69	1.89
830A	3.20	1.43	2.54	1.97	0.14	0.09	1.54	1.36	3.32	1.52	--	--
1633-2367a	1.88	1.50	0.86	1.82	0.05	0.02	0.13	1.05	1.79	1.50	--	--
Em 5256A	3.06	2.04	2.58	2.00	0.50	0.42	3.68	1.36	3.20	1.92	2.83	1.88
Peach A	2.10	1.79	0.25	1.64	0.02	0.11	0.06	1.10	1.59	1.72	--	--

Strain	Cellobiose 35°C	Cellobiose 25°C	Trehalose 35°C	Trehalose 25°C	Raffinose 35°C	Raffinose 25°C	Inulin 35°C	Inulin 25°C	Soluble starch 35°C	Soluble starch 25°C	Remark
1633A	2.06	1.86	2.04	1.75	0.44	1.97	0.12	1.76	1.69	1.92	Thermophobic
38113A	--	--	--	--	0.29	1.39	0.13	1.45	1.28	0.98	"
71301a	1.40	1.25	1.88	1.56	0.36	1.91	0.11	1.82	2.52	1.83	"
25a	1.38	1.64	1.30	1.88	0.35	1.97	0.13	1.82	1.36	1.92	"
830A	--	--	--	--	2.08	1.76	2.02	1.54	2.71	1.83	Normal
1633-2367a	--	--	--	--	0.31	1.52	0.12	1.43	2.00	1.56	Thermophobic
Em 5256A	3.30	1.62	3.38	1.92	2.12	1.52	2.02	1.47	2.92	1.93	Normal
Peach A	--	--	--	--	0.16	1.39	0.06	0.94	--	--	Thermophobic

strains which will be described later.

The incubation temperatures generally used for the differentiation of strains are 25 and 35°C. Sucrose, lactose and glucose are three of the sugars most commonly used in experiments.

GENETICS OF THE THERMOPHOBIC CHARACTER

1. Position of the thermophobic locus

Three crosses have been made between thermophobic strains and the normal one in order to test its mode of inheritance. Spores were dissected in order from a number of asci in each hybrid. Asci in which more than half spores did not germinate are not included in the summarized data. However, the number of the total asci dissected will be mentioned for reference. In some instances growth occurred before heat activation of ascospores, and in some cases an excess of wild-type cultures then resulted. Since this result may be due to contamination, data from asci including such possible contaminants are also omitted from the summaries.

a. Em 5256A (t^+ p^+) X 1633-2367a (t^- p^-)

Em 5256A is a normal strain, and 1633-2367a is both thermophobic and p-aminobenzoic acid (Paba) requiring. Fifty one asci were dissected but only 37 asci have been counted. The results of classification of these 37 asci are shown in Table 3.

Each number on top of the Table represents a pair of spores, and the

Table 3

Classification of the asci from the cross, Em 5256A (t^+p^+) X 1633-2367a (t^-p^-).

Type of asci show second division segregation	Representative type of ascus 1	2	3	4	Frequency
tp	t^-p^-	t^+p^+	t^-p^-	t^+p^+	22
p	t^+p^-	t^+p^+	t^-p^-	t^-p^+	3
t	t^+p^-	t^-p^-	t^-p^+	t^+p^+	4
none	t^-p^-	t^-p^-	t^+p^+	t^+p^+	8
Total					37

t^- Thermophobic
t^+ Normal

p^- Paba requiring
p^+ Normal

Table 4

Crossover values of the gene loci involved in the cross,

Em 5256A (t^+p^+) X 1633-2367a (t^-p^-).

Asci show crossover between:	Frequency	Percent	Percent of cross-over chromatid	Loci distance
p and centromere	22	59.50	31.78	33.81
t and centromere	26	70.27	39.20	43.26
p and t	4	10.81	7.44	9.47
Double(2 and 4 strand)	3	8.11	2.03	

Coincidence = 0.63

series number 1, 2, 3 and 4 represent the order of spore pairs of an ascus. Permutations between spore pairs number 1 and 2, and 3 and 4, and between adjacent pairs (1,2) and (3,4) are all neglected for the reason of simplicity of presentation, since these represent only different orientations of first or second division spindles. Table 4 shows the calculation of the crossover values of the gene loci involved. Since there is no significant difference between the second division segregation classes p and t in Table 3, it is impossible to determine for sure which one is the double crossover class. In other words, the order of p and t in the linkage map can not be asigned without the presence of another nearby locus. However, in order to carry out further calculations, an arbitrary sequence of loci will be assigned for the present purpose. Thus, the p class of second division segregation with frequency of 3 asci in Table 3 is asigned as the double crossover class. The sequence of the loci involved would then be centromere, p and t.

The double crossover asci (Table 3 and 4) could rèpresent either two strand double or four strand doubles with equal probability. If we suppose that the occurrence of the second crossover is independent from the first, then three strand doubles with spores distributed as p^+t^+, p^-t^-, p^+t^-, p^-t^+ should occur as frequently as the combined total of two strand and four strand doubles. The absence of this class may be due to chance. Thus the 8.11 percent of asci showing double crossover represent 2.03% of double crossover chromatid if 2 and 4 strand doubles occur equally often.

b. 830A (t^+p^-) X 25a (t^-p^+)

From the hybrid perithecia of this cross, 50 asci have been dissected.

in order and 36 asci were counted. The results and calculations are shown in Table 5 and 6 respectively.

c. Em 5256A (t^+) X 25a (t^-)

Fifty two asci have been dissected from the hybrid perithecia of this cross and only 39 were counted. Their scores and the calculations are presented in Table 7 and 8 respectively.

From the calculations, sex and t show a recombination value of 51.28% which indicates that they are not linked.

d. Conclusion

From the genetic data shown above, it is quite clear that the thermophobic gene t in both 25a and 1633-2367a is independent from sex but is linked with the Paba gene. They also show a great distance from the centromere in all the three crosses, although the numerical values vary from 34.62% to 45.84% which might be due to the chance variation of the small sample taken. The genetic analysis of the Paba strains by Zimmer (1946) showed that there are three Paba genes located on the same chromosome. These three genes may be designated as p_1, p_2, and p_3. From the data, taken from Zimmer's and mine, it is possible to construct a tentative chromosome map of these four gene loci and the centromere (data in Table 9), as:

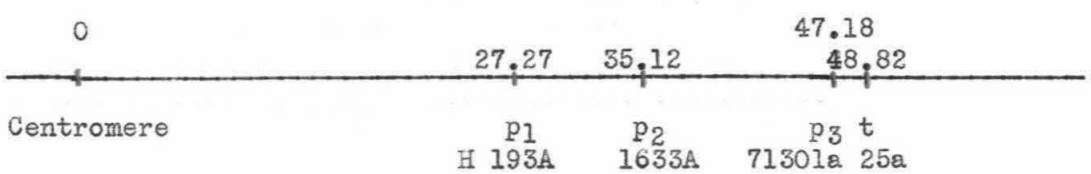


Table 5

Classification of the asci from the cross, 830A (t^+p^-) X 25a (t^-p^+).

Type of asci show second division segregation	Representative type of ascus 1	2	3	4	Frequency
tp	t^-p^+	t^+p^-	t^-p^+	t^+p^-	20
p	t^+p^-	t^+p^+	t^-p^-	t^-p^+	6
t	t^+p^-	t^-p^-	t^+p^+	t^-p^+	6
p and t	t^-p^+	t^+p^-	t^+p^+	t^-p^-	1
none	t^-p^+	t^-p^+	t^+p^-	t^+p^-	3
Total					36

Table 6

Crossover values of the gene loci involved in the cross,

830A (t^+p^-) X 25a (t^-p^+).

Asci show crossover between:	Frequency	Percent	Percent of cross-over chromatid	Loci distance
p and centromere	20	55.56	32.64	37.50
t and centromere	26	72.22	45.84	55.56
t and p	6	16.67	13.20	18.06
Double(2 and 4 strand)	6	16.67		
Double(3 strand)	1	2.78	4.86	

Coincidence = 0.72

Table 7

Classification of the asci from the cross, Em 5256A (t^+) X 25a (t^-).

Type of asci show second division segregation	Representative type of ascus 1	2	3	4	Frequency
ta	t^-a	t^+A	t^-a	t^+A	0
t	t^-a	t^+a	t^-A	t^+A	26
a	t^-a	t^-A	t^+a	t^+A	3
t, a	t^-a	t^+A	t^-A	t^+a	1
a and t	t^+a	t^+a	t^-A	t^-A	5
none	t^-a	t^-a	t^+A	t^+A	4
Total					39

Table 8

Crossover values of the gene loci involved in the cross,

Em 5256A (t^+) X 25a (t^-).

Asci show crossover between:	Frequency	Percent	Percent of cross-over chromatid	Loci dist.
t and centromere	27	69.23	34.62	34.62
Sex and centromere	4	10.26	5.16	5.16
Sex and t	30	76.92	38.46	51.28
Double	5	12.82		

Table 9

Summarised genetic data of the Paba linkage group.

Region between loci:	% crossover	No. asci counted	Strains involved	Author
Centromere and p_1	27.27	11	H 193	Zimmer
Centromere and p_2	28.57	21	1633A	Zimmer
	23.08	26	1633A, La	Pao
	33.79	37	1633-2367a, Em5256A	Pao
	37.50	36	830A, 25a	Pao
Weighted average	31.67	120		
Centromere and p_3	37.50	24	71301a	Zimmer
	26.93	26	71301a, LA	Pao
Weighted average	32.00	50		
Centromere and t	39.20	37	Em5256A, 1633-2367a	Pao
	34.62	39	Em5256A, 25a	Pao
	45.84	36	830A, 25a	Pao
Weighted average	39.74	112		
p_1 and p_2	7.50	74	H 193, 1633A	Zimmer
	8.51	47	292-3, 1633A	Pao
Weighted average	7.85	121		
p_2 and p_3	8.68	23	252-8a p_{23}, La	Pao
	11.77	17	248-2A p_{23}, La	Pao
	14.29	42	1633A, 71301a	Pao
	12.00	146	1633A, 71301a	Zimmer
Weighted average	12.06	228		
p_2 and t	9.47	37	Em5256A, 1633-2367a	Pao
	18.06	36	830A, 25a	Pao
Weighted average	13.70	73		

It should be emphasized here that this chromosome map is subject to change especially the order of the loci, p_2, p_3 and t, when data involving more loci are available. The data presented here are not conclusive for the order of these three loci.

2. Heterocaryon tests

As shown in Table 1, the thermophobic character is widely present in Neurospora strains, but whether they are due to the effect of the same locus or are due to different genes was not evident. However, none of the heterocaryon tests involving the following thermophobic strains: 1633A, 38113A, Peach A, 25a, 71301a, 1633-2367a, 10075a, 1186a, 27947a, S-1-4, S-1-8, S-2-7, S-2-8, S-3-1 and S-3-5, resulted in enhanced growth as tested in growth tube by mix inoculation on lactose medium at 35°C. The S strains used here have a very complicated pedigree history. S-1 group involves strains 27663a, 34508A, Em 5256A, 27947a and 18637A; S-2 group, 38706A, 27947a, 34508A and 18637A; and S-3 group, 37301A 27947a, 34508A and 18637A. From this result the conclusion could be either that all the thermophobic strains so far tested possess the same gene, or that the t gene has a dominant effect upon its normal allele.

When all the thermophobic strains mentioned here were traced back to their origin (Beadle and Tatum, 1945), it was found that they were all from the cross LA X La of which La is normal and LA is thermophobic. It is therefore most likely that the thermophobic character in the strains mentioned here is derived from the same stock LA.

THE MECHANISM OF THE THERMOPHOBIC GENE ACTION

During the investigation of this problem, many assumptions have been put to experimental test and a large amount of data were thus accumulated. Much of the data give only negative answers to the assumptions. These are included since they help to rule out certain possibilities.

1. Interaction of alternating high and low temperatures

In order to get some information concerning the nature of high temperature effect, experiments have been carried out on the interaction of successive high (35°C) and low (25°C) temperatures.

Growth tubes containing 0.5% lactose were inoculated with 25a and incubated at 25°C for one or two days until the growth rate was constant. They were then transferred to 35°C where upon the growth was observed to continue at an even higher rate than at 25°C during the first day, then to slow down gradually for two or more days until a minimal level of growth, characteristic of the higher temperature, was reached. If the culture was first put at 35°C for two or more days during which there was very little growth and then transferred to 25°C, growth started almost immediately without any kind of apparent after effect of the previous high temperature inhibition, as shown in Fig. 1.

Growth rates were also determined in cultures incubated at alternate high (35°C) and low (25°C) temperatures. The following sequences were used: 4 hours at 25°C and 20 hours at 35°C, 8 hours at 25° and 16 hours at 35°, 12 hours at each temperature, 16 hours at 25° and 8 hours at 35°, and

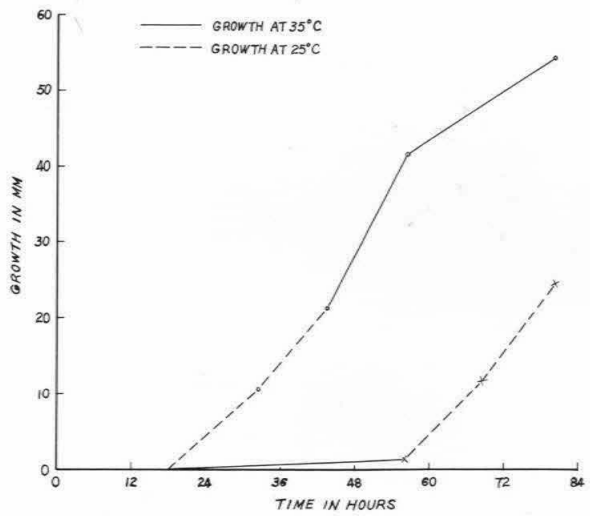


Fig. 1. Growth curves show the effect of alternating high and low temperatures on strain 25a on 0.5% lactose minimal medium.

20 hours at 25° and 4 hour at 35°C. Each sequence was repeated until growth reached the end of the tube. From the results (Figure 2) it is evident that the longer the time at 25°C during a day the better will be the growth at 35°C. However, the relation is apparently not linear. From 4 to 8 hours a day at 25°C, there is a steady improvement in growth at 35°C on succeeding days. And the improvement becomes less and less as the time at 25° is increased beyond 8 hours a day. This point could even be more clearly represented by the three day averages of the growth rates at 35°C on 0.5% lactose, melibiose, inulin and raffinose minimal media after activation for different periods of time at 25°C, as shown in Table 10 in which the growth rate of the normal strain Em 5256A at 35°C is included for comparison. The growth rate of the thermophobic strain 25a at 35°C reaches a maximum, which is equivalent to the rate obtained with the normal strain, when the growth-tube was activated more than 8 hours a day at 25°C. This fact seems to indicate that something accumulates during the activation period at 25°C and is used up at 35°C. After 8 hours incubation at 25°C, the accumulation seems to reach a level that will support the maximum growth at 35°C, or in other words, it is no longer a limiting factor for growth.

The proportionality between the length of activation at 25°C and the 2.5 days growth at 35°C after activation was also measured. The growth was started at 25°C for two days to establish the maximum rate at that temperature, and then the growth tubes were transferred to 35°C for two days by the end of which the rate of growth reaches the minimal characteristic of that temperature. Then the growth tubes were transferred back to 25°C for activation period of from 2 to 24 hours. After activation, the tubes were transferred back to 35°C, and the total growth resulting in 2.5 days

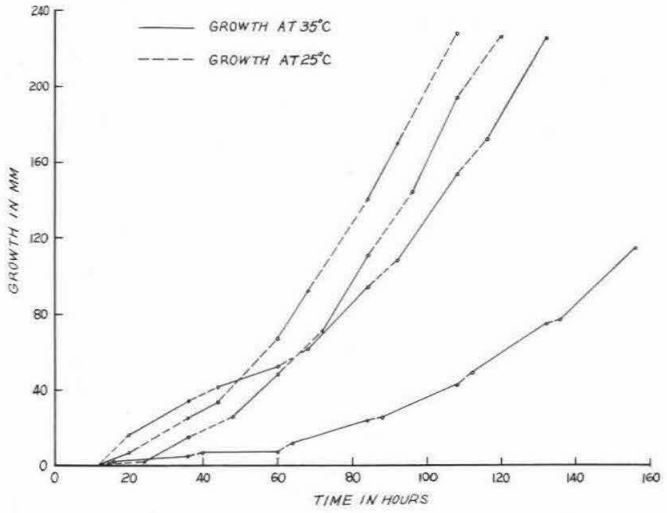


Fig. 2. Growth curves of 25a on 0.5% lactose minimal medium with different lengths of activation at 25°C in every 24 hour period.

Table 10

Three day average growth rate of 25a at 35°C after activation at 25°C for from 4 to 20 hours a day (growth rate in mm/hr).

Sugar	25a Length of activation at 25°C each day					Em 5256A 35°C
	4 hrs	8 hrs	12 hrs	16 hrs	20 hrs	
Lactose	1.67	2.96	3.42	3.67	--	3.54
Melibiose	--	3.13	3.38	3.38	3.08	3.71
Inulin	1.75	3.50	3.46	3.83	3.58	3.88
Raffinose	2.21	4.21	4.46	4.17	4.04	4.50

Table 11

Two and half days total growth (in mm) of 25a at 35°C after activation at 25°C.

Length of activation at 25°C in hours	Two and half days total growth at 35°C after activation									
	First treatment					Second treatment				
	1	2	3	4	Average	1	2	3	4	Average
2	16	25	19	21	20.2	10	12	19	33	18.5
4	39	44	27	32	35.5	27	22	29	25	25.8
6	53	48	49	58	52.0	43	49	38	36	41.5
8	66	69	60	62	64.3	38	50	50	40	44.5
10	74	77	72	78	75.3	39	39	62	55	48.8
12	85	73	83	85	81.5	67	37	53	50	51.8
24	--	--	93	87	90.0	--	--	76	84	80.0

at that temperature was measured. The same tubes were used again for a second activation, but in the reverse order, namely, the tube which had 2 hours activation at 25°C the first time would have 24 hours activation the second time and so on. Each treatment was duplicated four times. The results are presented in Table 11 and the averages were plotted in Fig. 3.

It is quite evident from the curves in Fig. 3 that each curve consists of two straight lines on which the observed points fall closely. This result could mean that the total growth at 35°C after activation is directly proportional to the substance accumulated at 25°C. When the accumulation has reached a certain level then the rate of further accumulation is changed abruptly. The different rates of accumulation could be expressed by the slopes of the respective straight lines, or the growth per unit time (mm/hr) of activation. The curve of the first activation growth consists of two straight lines with slopes, 7.27 and 0.73 mm per hour respectively. While for the curve of the second activation growth, the slopes of the two lines are 6.25 and 2.17 mm per hour respectively. The significant difference between the results of first and second activations, seems to indicate that the capacity to produce (at 25°C) the substance (required at 35°C) is somewhat related to the previous history of the mycelium treated.

Moreover, it should be pointed out here that the intersecting point of the two lines from the first activation lies near the 10 hours point, a time which agrees well with the result shown in Fig. 2 previously. In contrast, the two slopes from second activation intersect near the 6 hours point, a difference of 4 hours from the previous one. An explanation for such a difference will be suggested later (p. 101).

With certain assumptions, it is possible to derive some expressions which will theoretically describe the growth curve of the thermophobic

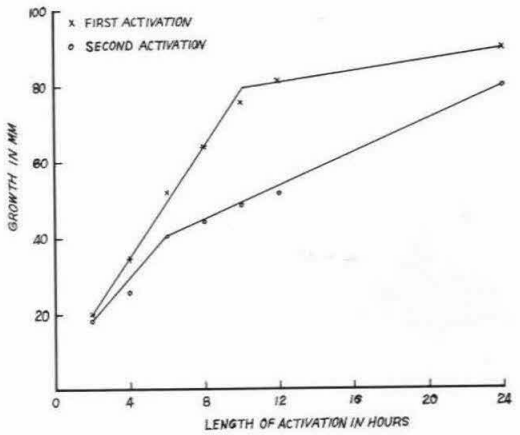


Fig. 3. Shows the linear relationships between the length of activation at 25°C and total growth at 35°C afterward of the thermophobic strain 25a on 0.5% lactose medium.

strain at 35°C after activation at 25°C. The correctness of the assumptions made could thus be checked by a comparison of the theoretical curves derived with the observed ones.

Let the substance produced during the activation period at 25°C be called E without regard to its possible chemical nature. The growth at 35°C is then supposed to be dependent on the quantity of this substance E. This is usually true when a substance becomes the limiting factor of the growth. Then it is reasonable to assume that the growth rate is directly proportional to the quantity E present. This could be expressed as:

$$\frac{dG}{dt} = AE$$

where G is the growth, t the time, and A a constant. At 35°C after a certain period of time, the growth will eventually be slowed down to its minimum. So we would suppose that the E is gradually used up, and finally exhausted or nearly so. Then the quantity E will be changed as a function of time.

If the E is consumed at a constant rate a at 35°C then the quantity E at time t will be,

$$E(1-a)^t.$$

The growth rate will thus be,

$$\frac{dG}{dt} = AE(1-a)^t.$$

After integration,

$$G = AE\left(\frac{(1-a)^t}{\log(1-a)} + C\right)$$

where G is the total growth, and C is an integration constant which could be determined by supposing that when t = 0, then G = 0. Therefore C will be:

$$-AE/\log(1-a).$$

Then,

$$G = \frac{-AE(1-(1-a)^t)}{\log(1-a)}$$

$$= K(1-(1-a)^t) \qquad \text{where } K = -AE/\log(1-a).$$

When t is infinite, G will equal to K. So that the constant K is the maximum total growth.

With a simple rearrangement, the equation could be expressed as,

$$(1-G/K) = (1-a)^t.$$

Taking the logarithm of each side, we get,

$$\log(1-G/K) = t \log(1-a).$$

When the value of $\log(1-G/K)$ is plotted against the time, a straight line passing through the origin with a slope of $\log(1-a)$ results.

From the growth data $\log(1-G/K)$ was calculated with the values shown in Table 12. The average values were plotted against the time in Fig. 4, and the points fall very nicely on the theoretical straight line with a slope of -0.02125. From the slope, the value was calculated to be 4.78% per hour. It means that in each hour 4.78% of the E material was consumed for growth.

For the sake of completeness of evaluation, we also may assume that at 35°C substance E is also produced in a rate of b. Then the total quantity of E after time t will be,

$$E(1-a)^t + b(1 + (1-a) + (1-a)^2 + (1-a)^3 + \ldots\ldots\ldots + (1-a)^t) =$$

$$E(1-a)^t + b/(1-(1-a)) = \qquad \text{since } (1-a) < 1$$

$$E(1-a)^t + b/a$$

And the growth rate will be,

$$\frac{dG}{dt} = A(E(1-a)^t + b/a)$$

On integration, we get the general formula,

Table 12

t (in hours)	- Log(1 - G/K) 1	2	3	4	5	Average
12	0.26440	0.29499	0.18046	0.13312	0.23210	0.22101
24	0.60206	0.58503	0.45593	0.35458	0.48945	0.49741
36	0.83268	0.78516	0.83565	0.64397	0.61439	0.74237
48	0.98716	0.91009	1.06048	0.98297	0.84164	0.95647
60	1.53760	1.16749	1.23657	1.17393	1.04576	1.23227
72	---	1.38722	1.85387	1.50864	1.34679	1.52413
Total growth, K in mm	68	73	103	163	111	

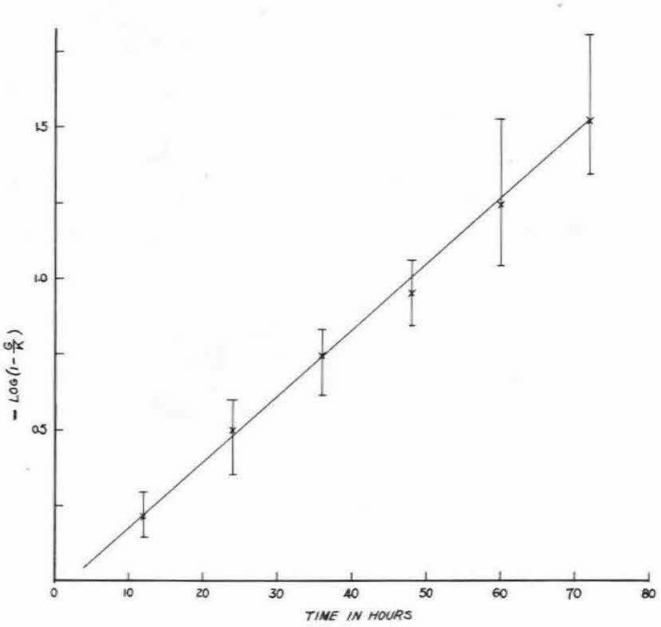


Figure 4

$G = K(1-(1-a)^t + Abt/a)$ Where as before $K = -AE/\log(1-a)$.

When b is 0, the equation goes back to,

$G = K(1-(1-a)^t)$.

When a = b = 1, that means, the rate of production is equal to the rate of consumption, then

$G = At$

Growth will here be directly proportional to the time, in other words a constant rate of growth is obtained. In fact, the growth rate of thermophobic strain is extremely low at 35°C. Therefore, the b value here must be very small and could thus be neglected without significant change of the shape of the curve.

From the foregoing calculations, it might be concluded that the consumption of substance E is in a constant rate, ~~of the total quantity E~~. If we assume that the E substance is the enzyme, then there is reason to believe that the amount of enzyme destroyed at 35°C might be directly proportional to the amount of the enzyme present. However in relation to the mechanism of such an enzyme inactivation at 35°C, the formula could tell nothing. Nevertheless, from the fact that the growth recovers immediately without any kind of noticeable lag period when the growth tube is transferred back from 35°C to 25°C, it seems less possible, therefore, to have an explanation that the inactivation is due to the production at 35°C some enzyme inhibitor which would be expected to have some residual effect at 25°C. Besides, the constant rate of destruction namely about 4.78% per hour from calculation seems to indicate that the inactivation process might be a kind of autointoxication of the enzyme. The decreasing of enzyme quantity E will thus follow the negative compound interest law. Furthermore, the autointoxication of the enzyme must not be reversible,

otherwise the inactivation at 35°C will give an acceleration of activation afterward. This obviously is not the case so far as the data have shown.

Since we have no idea about the nature of the substance E, in addition to enzyme it also could be nutritional factor of some sort, or just simply carbohydrate. These possibilities will be tested in the following experiments.

2. Nutritional deficiency at high temperature

The utilization of carbohydrate by the organism is a complex process in which a large number of enzymes and coenzymes are involved. It is not without reason, thus, to suspect that something which is indispensible in the synthesis of this special substance is retarded by increasing temperature or that this special substance is used by a side reaction which happened to have a very high temperature coefficient. If this is true, it would be expected that the thermophobic effect could be removed by supplying the deficient substance from without in excess quantity. A vitamin mixture, yeast nucleic acid, yeast extract, hydrolysed caseine and indole acetic acid have been tested, and the results, as presented in Table 13 show that there is no improvement at all in growth of 25a on 0.5% lactose medium at 35°C as a result of such additions.

The H^+ and OH^- ions might be regarded as special chemical agents. Deficiency or excess of either ions would shift the pH value toward alkaline

or acid to such an extent that the Neurospora would become unable to grow. Media adjusted to different pH's were tested on both normal and thermophobic strains.

The minimal 0.5% lactose medium was adjusted to different acidities from pH 4.8 to 8.98 by McIlvaine's citrate-phosphate buffer, or by 1N NaOH when a pH higher than 7.5 was wanted. Then agar was added and the media autoclaved under 15 lb pressure for 15 minutes. The growth rates of both 25a and Em 5256A at 25° and 35°C are summarised in Table 14.

From the Table 14, it is evident that the optimum pH range is from pH 5 to pH 7. At 25°C the normal strain Em 5256A can endure pH as high as 8.98 which will totally inhibit its growth at 35°C. In other words, normal strain becomes thermophobic on very alkaline medium. While for thermophobic strain 25a, the growth at 25°C becomes decidedly worse when pH rises above 8. At 35°C the thermophobic effect becomes more apparent when pH goes either lower than 5 or higher than 8.

Although high pH does cause the normal strain to show thermophobic effect on lactose medium, it is not necessary to conclude that the deficiency of H^+ ion or excess of OH^- ion is the cause of the thermophobic effect, since the pH on acid side can not remove the thermophobic effect of the thermophobic strain.

Table 13

Growth rate on 0.5% lactose minimal with different additional supplements.

In 15cc medium add:	Growth rate(mm/hr) of 25a at: 25°C	35°C
Vitamin mixture 1 drop	3.21	0.27
50 γ IAA	3.17	0.11
800 γ IAA	3.04	0.14
30mg hydrolysed casein	3.00	0.33
75mg yeast nucleic acid	1.53	0.14
75mg yeast extract	1.61	0.11
Control	1.71	0.30

Table 14

Growth rate(mm/hr) on 0.5% lactose minimal with different pH's.

	pH 25°C						pH 35°C					
Strain	4.3	5.0	5.9	7.0	8.08	8.98	4.3	5.0	5.9	7.0	8.08	8.98
25a	1.04	1.46	0.88	1.04	0.33	0.63	0.00	0.00	0.32	0.35	0.10	0.00
Em 5256A	1.46	1.46	1.75	1.06	1.15	0.98	1.69	2.02	2.19	1.90	1.73	0.00

3. Permeability of cell membrane to the different sugars under high and low temperatures

High temperature might cause a change of the cell membrane of the thermophobic strain in such a way that the permeability to sugar would be decreased as the temperature rises. If this is true, then the thermophobic effect can be adequately explained as due to partial starvation.

Considerable quantity of mycelium of both normal and thermophobic strains were obtained from three days culture in 2% sucrose minimal at 25°C. The mycelium having been washed on the Buchner funnel with distilled water, was put back in freshly prepared minimal medium without sugar and was thus fasted at 25°C for 40 hours in order to get rid of the accumulated sugars in the mycelium. After fasting, the mycelium was filtered through the Buchner funnel and washed with distilled water. The mycelial cake was cut into small blocks ready for experiment.

Minimal medium containing 2% of different sugars was prepared. One of the small blocks of mycelial pad was placed in each 125cc flask containing 50cc of the medium, and was dispersed by vigorous shaking. Three temperatures, 10, 25 and 35°C were used for incubation. Starting from zero time, samples were taken out at scheduled time intervals up to 36 hours, and were washed with distilled water three times and dried in an 80°C oven for at least 12 hours. The dry weight was then taken. For quantitative determination of sugar content, the dried mycelium was first soaked in 10cc of water and then ground with about 0.6g of sea sand in a motar. The ground material was washed quantitatively into a centrifuge tube, with another 20cc of distilled water. Extraction was continued by heating the ground mycelium suspension in a boiling water bath for 15 minutes, and then the insoluble material was centrifuged down. Five cc

samples of the clear supernatants were taken out and analysed for reducing substances by Hassid's ceric sulfate titrating method (Hassid,1937). The figure thus obtained was converted into mg of glucose-equivalent per 100mg of dry mycelium, and the results are plotted in Fig. 5.

It is interesting to point out that the general shape of the curves in Fig. 5 are surprisingly similar to the curves of the absorption of strong electrolytes, like Na^+, Rb^+ and K^+, by Nitella (Brooks and Brooks, 1941). They all show three phases in the absorption processes an immdeiate and sharp increase (Phase I, as called by Brooks and Brooks), a sharp decrease (Phase II), and a gradual increase leading to equilibrium with a final slow decrease (Phase III). It is obvious that the interpretation of such a curve for absorption process of strong electrolytes in Nitella could hardly be borrowed here without some sort of amendement. However, no intention is made to formulate a hypothesis to explain the mechanism of sugar absorption in Neurospora. The important thing to be considered here is the temperature effect on sugar absorption and the relative difference in amount of sugar absorbed by the two strains concerned.

Apparently, there is no regular temperature effect to be seen in the data in Fig. 5. This is especially true in relation to the lactose absorption. It follows that the thermophobic character of 25a could not be due to the hindrance of sugar absorption at high temperature.

When the peaks of the curves are compared, it is easy to see that sucrose is usually absorbed to a greater extent than lactose, and that the normal strain Em 5256A always has a higher sugar content in the mycelium than that in thermophobic 25a, no matter whether sucrose or lactose is used for absorption. The strain difference towards lactose is, however, more marked than that towards sucrose.

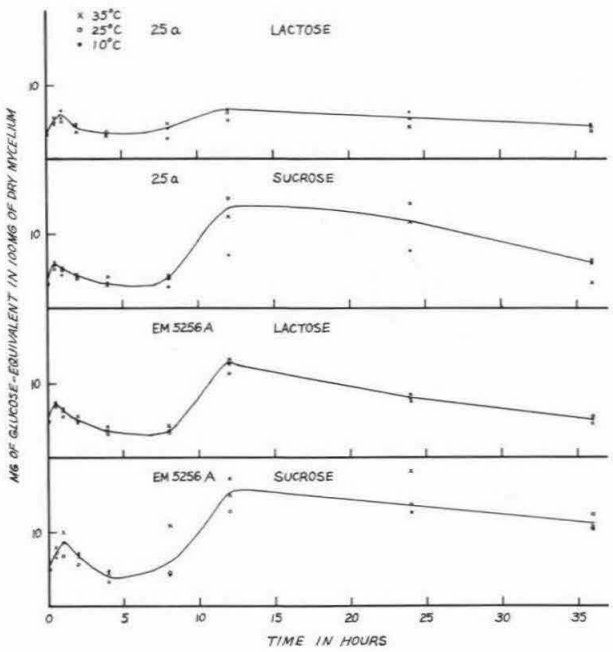


Fig. 5. Curves showing the absorption of sugars at 10, 25 and 35°C.

Since sucrose in the medium is rapidly hydrolysed to monosaccharides by the secreted invertase, the absorption of sucrose would not be simply as such, but rather an absorption of a mixture of sucrose, glucose and fructose. For this reason experiments were also made on glucose, fructose, galactose, mixture of equal amounts of glucose and fructose, and of glucose and galactose. These were done at two temperatures, 25 and 35°C. The same type of curve was obtained without a single exception for all these monosaccharides or mixtures of them used. For the purpose of comparison, only the figures which represent the reducing sugar content in mycelium after incubation for 12 hours in the medium are presented in Table 15.

In the normal strain Em 5256A the sugar content is consistently higher at 35°C than that at 25°C. While in the thermophobic strain 25a, there is practically no difference between high and low temperatures in monosaccharide absorption, but there is a consistently lower sugar content in the mycelium at 25°C than that at 35°C when disaccharides, sucrose or lactose, or even mixture of monosaccharides were used for incubation. Since the magnitude of the difference due to temperature is not very large, it is doubtful whether this could be counted as the main cause of thermophobism. Furthermore, the absorption of fructose by 25a is as low as the absorption of lactose, but the growth at 35°C is much better on fructose than on lactose medium.

Table 15

Reducing sugar content in mycelium after 12 hours incubation in 2% solution of different sugars.

Sugar	Strain	Temperature °C	Mg of glucose equivalent in 100mg of dry mycelium after 12 hrs of incubation
2% Glucose	25a	35	13.37
"	"	25	13.91
"	Em 5256A	35	17.60
"	"	25	16.57
2% Fructose	25a	35	6.10
"	"	25	5.59
"	Em 5256A	35	13.22
"	"	25	10.68
2% Galactose	25a	35	5.41
"	"	25	5.99
"	Em 5256A	35	9.40
"	"	25	8.38
1% Glucose, 1% Fructose	25a	35	7.77
" "	"	25	11.23
" "	Em 5256A	35	19.10
" "	"	25	15.86
1% Glucose, 1% Galactose	25a	35	9.95
" "	"	25	12.89
" "	Em 5256A	35	18.80
" "	"	25	11.12
2% Sucrose	25a	35	12.36
"	"	25	14.80
"	Em 5256A	35	15.08
"	"	25	12.90
2% Lactose	25a	35	5.26
"	"	25	6.21
"	Em 5256A	35	13.03
"	"	25	11.34
Average	25a	35	8.60
	"	25	10.09
	Em 5256A	35	16.60
	"	25	12.41

4. Hydrolytic enzymes

It has been assumed by Willstaetter and Oppenheimer (1922) and others that lactose could be fermented without previous hydrolysis to glucose and galactose in some species of Saccharomyces (Willstaetter and Oppenheimer, 1922; Myrbaeck and Vasseur, 1943: and Leinbowitz and Hestrin, 1945) and Lactobacillus (Hoff-Jorgensen, Williams and Snell, 1947). They based their assumption on the faster rate of fermentation of the disaccharide as compared with its monosaccharide components. But it seems to be not so in Neurospora crassa, since the preliminary tests on both liquid and solid media show that growth is much better in the mixture of glucose and galactose than that in lactose. In Neurospora lactose is likely hydrolysed to its components first and then used.

The experiments just mentioned have shown that the substance E is neither a reparable nutrient factor nor the disaccharide as such within the cell. Then there is another possibility that it could be hydrolytic enzyme of the disaccharide. Since sucrose has been frequently used for comparison in growth, both invertase and lactase were thus compared between thermophobic and normal strain extensively.

a. Comparison of invertases between thermophobic and normal strains.

The invertase is present in both culture medium and mycelium in large quantity, and is, thus, quite easy to be prepared. It can be successfully extracted (according to Holmbergh's method, 1933) first by adsorption on starch in water-alcohol mixture in deep freezer for one hour with occasional shaking and then eluted from starch by water at room temperature with vigorous shaking for 10 minutes. The strength of the invertase was measured by incubating the enzyme preparation in a sucrose buffer solution

(0.2 M acetate buffer, pH usually 4.5) at a certain temperature, and samples were taken at definite time intervals for the determination of monosaccharides by the colorimetric method of Tauber and Kleiner (1932). Tauber and Kleiner's method is characterized by its applicability in the presence of disaccharides. Relative enzyme strength in preparations could be calculated by comparing the colorimetric readings of samples with same interval of incubation.

It was at once observed that the alcohol concentration is important in adsorption of invertase by starch. There was very small adsorption when the water and 95% alcohol ratios were either below 5 : 95 or above 50 : 50. Between these two ratios, there are two maximum peaks of adsorption, one at 44 : 56, and another at 16 : 84. It was first supposed that the invertase might consist of two components, each with its own optimum alcohol concentration for adsorption on starch. Large quantities of invertase were extracted from ground mycelium with its own medium of a 10 days old culture in 2% sucrose minimal at 25°C of both 25a and Em 5256A by starch adsorption in 44 : 56 water-95% alcohol mixture, then the water elution was divided into equal parts (5cc each). In each part, except the one which was saved as control, water and 95% alcohol were added to make the final volume to 100cc with different water-95% alcohol ratios. Adsorption was made on one gram potato starch for one hour in deep freezer with occasional shaking. After then starch was centrifuged down, and supernatant was discarded. The starch was washed down in a 125cc Erlenmeyer flask with 10cc water and shaken on a shaking machine for 10 minutes. After the elution was completed, the starch was centrifuged down and discarded, and the supernatant was saved at this time as the enzyme preparation.

To each preparation was then added 10cc of 0.4 M acetate buffer at pH 4.28 and 10cc sucrose solution whose concentration will make the final sucrose concentration one percent. The whole preparation was incubated at 35°C with the unadsorbed enzyme control. One cc samples were taken at one hour intervals for the determination of monosaccharides. The relative enzyme strength was calculated by dividing the colorimetric reading of readsorbed preparation by that of the control. The absolute activities of controls are 1mg sucrose hydrolysed per hour for Em 5256A invertase, and 2mg per hour for 25a. The results as shown in Fig. 6 show clearly again the two peak curves with peaks at same locations as before for both strains 25a and Em 5256A.

It is clear then, that the two adsorption peaks can not be due to two components of invertase, rather there must be two minima of invertase solubility in water alcohol mixture. So far as the adsorption experiment goes, there is therefore evidently no difference between the invertases from the thermophobic and normal strains.

Furthermore, the pH effect and temperature effect on invertase activity were also compared between the two strains. No apparent difference was found, as judged by the results presented in Figs. 7 and 8. The optimum pH lies between 5 and 6 for invertases from both strains. The activity of both invertases increases as the incubation temperature rises. The relation between invertase activity and temperature seems linear below 35°C, and curves upward when temperature goes higher than 35°C. The same is true for invertases from both strains.

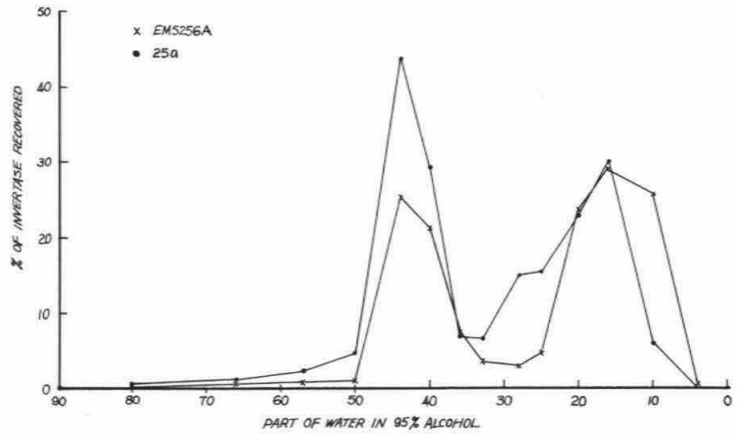


Fig. 6. Shows the adsorption efficiency of invertase on potato starch in solvent containing different proportion of alcohol.

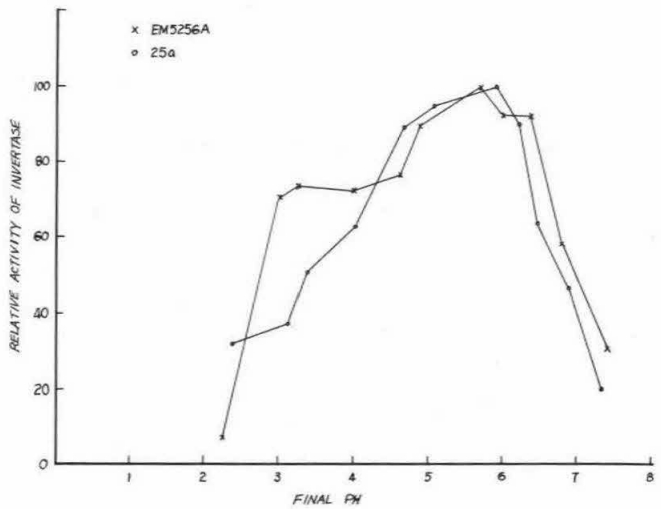


Fig. 7. pH effect on invertase activity.

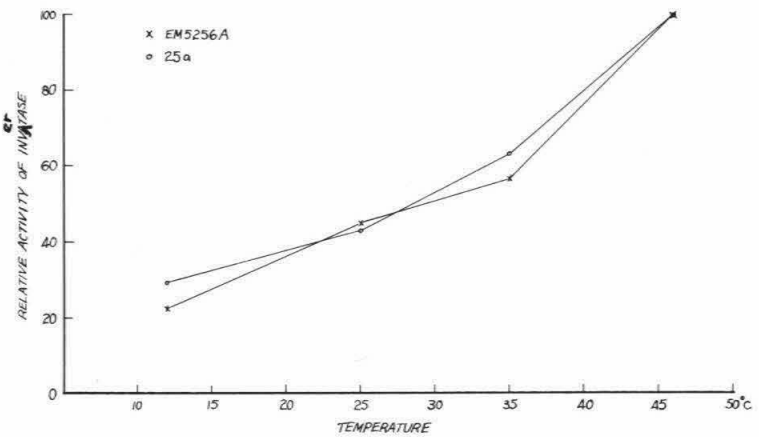


Fig. 8. Temperature effect on invertase activity.

b. Comparison of lactases between thermophobic and normal strains

If there is any difference of enzymes between normal and thermophobic strains, the difference, as expected from the analyses of transferring experiments mentioned previously, should be in lactases. The experiments on invertases reveal no difference at all between the two strains, No difference has been found either in lactases between the two strains. This is shown by the results of experiments with lactases similar to these with invertases, Figs. 9, 10 and 11.

However, the differences between invertase and lactase are quite obvious. The first adsorption peak for lactase is at same point as for invertase, namely at 44 : 56 water-95% alcohol mixture, while the second peak does not coincide with that for invertase. For invertase, it lies at the 16 : 84 point, in contrast the adsorption curve for lactase rises steeply as the water-alcohol ratio goes down below 10 : 90 (Fig. 9). This difference indicates that the invertase is more alcohol soluble than lactase.

Since the absolute activity of lactase is very low, the adsorption experiment was made directly from the aqueous filtrate of the ground mycelium. About 18g of 25a mycelium and 43g of Em 5256A in wet weight were used. Eighty cc filtrate of each was obtained. For each water-alcohol combination, only 5cc of the filtrate was used for lactase adsorption. The relative efficiency of adsorption was calculated from colorimetric reading of each sample divided by the corresponding reading of 5 : 95 water-alcohol class which has the highest efficiency of lactase adsorption. The absolute activity of this most efficient class is about 1mg of lactose hydrolysed per 25 hours for both 25a and Em 5256A lactases.

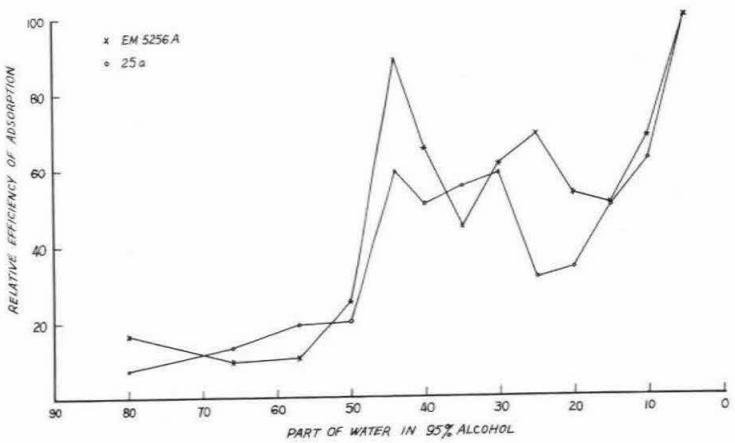


Fig. 9. Adsorption efficiency of lactase on potato starch in solvent containing different proportions of alcohol.

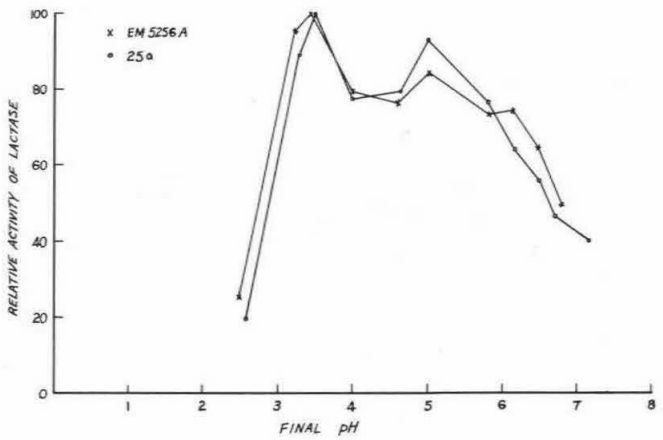


Fig. 10. pH effect on lactase activity.

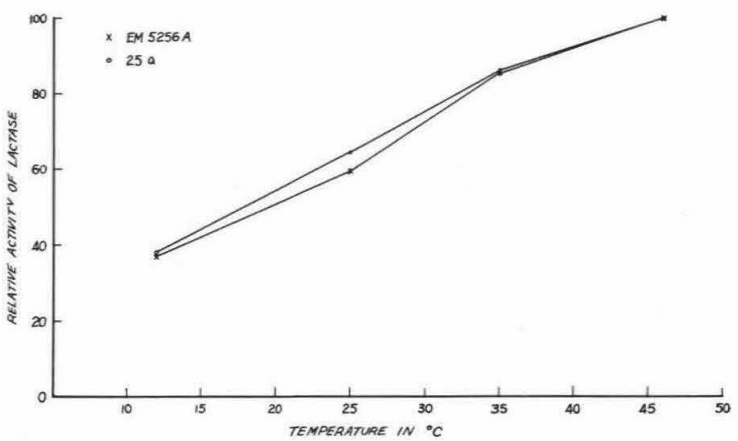


Fig. 11. Temperature effect on lactase activity.

The optimum pH for lactase is also different from that for invertase. The former lies between 3 and 4, and the latter, between 5 and 6 (Fig. 10).

The temperature effect on lactase shows a higher temperature coefficient below 35°C and lower above, just opposite from the effect on invertase.

Furthermore, lactase occurs only in the mycelium. In the medium it is hardly detectible, except when the culture becomes very old (more than two weeks) when autolysis, as it is believed, might set in. The strength of lactase in the mycelium is also very low as compared with that of invertase. When their hydrolysis rates on respective sugars are compared, the lactase invertase ratio varies from 1 : 25 to 1 : 135 as shown in Table 16. The medium used for this experiment was 2% lactose minimal. Each flask contained 50cc of medium. Each set consisted of three flasks, one for measuring mycelium dry weight, one for determination of invertase and one for lactase activity. It should be pointed out here that even in the lactose medium a large, even larger than that in sucrose medium as shown in Table 16, amount of invertase was produced in the mycelium, before the beginning of autolysis. The autolysis shows its effect by a loss in dry weight and in invertase activity as clearly indicated in 18 days old culture of strain Em 5256A in lactose minimal.

The reason for using cultures older than two weeks is that both invertase and lactase accumulate as culture becomes aged.

Table 16

Relative quantity of lactase and invertase in mycelium.

Strain	Temp. °C	Age of culture (day)	Dry wt.(mg) from 50cc medium of 2% Lactose	2% Sucrose	Lactase-Invertase ratio from lactose medium L : I		Invertase from medium of Lactose	Sucrose
Em5256A	25	14	311.3	271.7	1	57	3.79	1
"	35	"	121.7	115.6	1	111	2.66	1
25a	25	"	271.2	--	1	51	--	--
"	35	"	193.7	133.2	1	134	1.17	1
Em5256A	25	18	273.0	218.0	1	25	0.86	1
"	35	"	100.5	100.0	1	32	0.41	1
25a	25	"	300.0	--	1	86	--	--
"	35	"	178.2	--	1	135	--	--

5. The effect of temperature higher than 35°C

A water bath was used for the experiments at temperatures higher than 35°C. The growth stops immediately for both strains when 46°C was used no matter what kind of sugars was used in the medium. At 40°C however, some interesting facts appeared. At that temperature, the normal strain Em 5256A shows the characteristic thermophobic effect, namely it fails to grow on lactose but grows quite well on glucose, sucrose and maltose. The results of experiments involving alternating temperatures of 25 and 40°C, as presented in Fig. 12, are quite characteristic of the thermophobic strain between 25 and 35°C, mentioned previously.

At 40°C the thermophobic strain 25a fails to grow not only on lactose but also on maltose, sucrose and glucose as well. The results of transferring experiments between 25 and 40°C on glucose, maltose and sucrose media show exactly what had happened in transferring experiment of the strain on lactose medium between 25 and 35°C, namely each activation at 25°C forced a limited growth at 40°C, as shown in Fig. 13.

The activation process for the normal strain Em 5256A on lactose medium can be carried out not only at 25°C, but also at 35 and 37°C as well. However, the efficiency of activation is decreasing as the activation temperature goes higher, as clearly shown by the results in Table 17. The cultures were started at 25°C for one day, and then were transferred to 40°C water bath for four days. The growth rate at the end of that time was reduced to only one or two millimeters per 12 hours, as shown in the first column of the Table. The growth tubes were then transferred to 25, 35 and 37°C for activation for 12 to 24 hours. After that, the tubes were transferred back to 40°C water bath and growth was measured in 12 hour periods for 4 days more. The growth decreased to only few millimeters

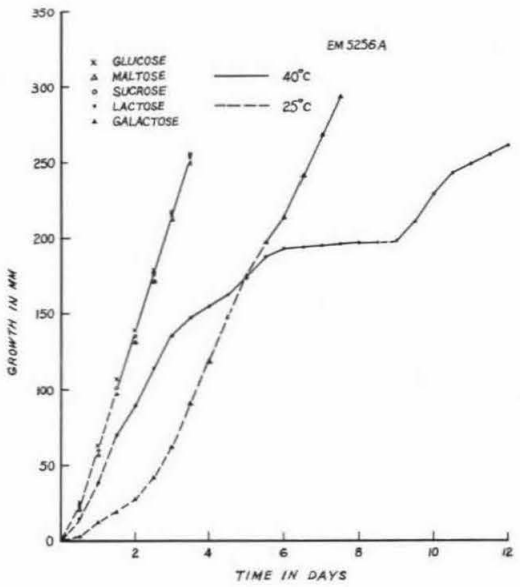


Fig. 12. Shows the thermophobic effect of lactose on the normal strain Em 5256A at 40°C.

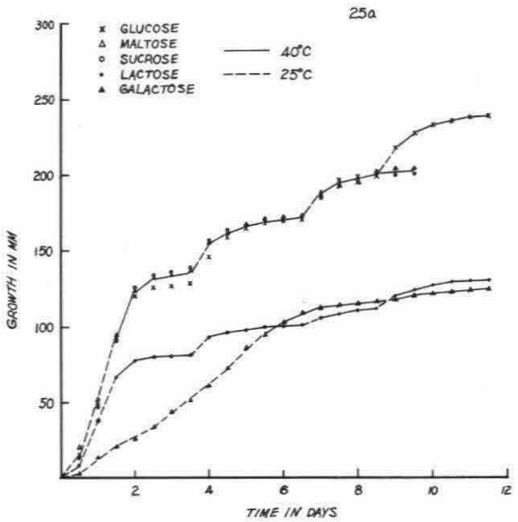


Fig. 13. 25a becomes thermophobic on all kind of sugars at 40°C.

Table 17

Efficiency of activation temperatures on Em 5256A on 0.5% lactose minimal.

Growth rate at 40°C just before activation (mm/12 hrs)	Growth in mm during activation at: 37°C 12hrs	24	35°C 12	24	25°C 12	24	2.5 days growth at 40°C after activation (in mm)
1	0						25
1	0	8					31
1			0				27
1.5			0.5	7			30.5
2					1		66
1.5					2	18	91

per day after 2.5 days incubation at 40°C. Therefore the 2.5 days total growth after activation is a good measure of the growth due to activation. It is evident from the figures presented that the efficiency of activation increases with decreasing temperature of activation.

Now the difference between the normal strain Em 5256A and the thermophobic strain 25a can be differentiated not only on lactose medium at 35°C but also on glucose, maltose and sucrose media at 40°C as well. This is not only an additional method to differentiate the mutant strain from the normal, but also seems to have some significance in relation to mechanism responsible for thermophobic character. Thus, the difference between the normal and the thermophobic may be fundamental. All strains grow normally on all sugars at 25°C. At 35°C, the thermophobic strains show their differential ability in using some sugars, such as lactose, melibiose, raffinose and inulin. When temperature rises 5 degrees higher, i.e., at 40°C, the thermophobic strains no longer grow on any sugar, while the normal strains show in their turn a temperature differential effect on some sugars just as the thermophobic strains did at 35°C. Naturally, it would cause one to suppose that the enzymes or rather the proteins in general in thermophobic strain have a higher sensitivity on thermodenaturation than those in the normal strains. The difference of sensitivity is only about 5 degrees centigrade.

However, as mentioned previously no noticeable differences in invertases and lactases between the thermophobic and normal strains have been found, the attention was then directed to the lower common steps, namely, glycolysis and Krebs' cycle, in the process of break-down of carbohydrate.

6. Thermophobic effect of Krebs' acids

The process of break-down of disaccharide following the hydrolytic degradation to its constitutional monosaccharides consists of glycolysis and the Krebs' cycle. The important intermediates in glycolysis are the three carbon compounds. Glycerol and β-sodiumglycerolphosphate have been tested in tube culture and they show no thermophobic effect on sucrose medium. In contrast all Krebs' acids tested show clearly thermophobic effect with 25a on sucrose medium without a single exception.

a. Effects in growth tube culture

The Krebs' acids used for the test are lactate, pyruvate, acetate, citrate, succinate, fumarate, malate and malonate. To each growth tube, containing 10cc of 0.5% sucrose minimal agar media, various amounts of acids or their sodium salts were added. The normal strain Em 5256A grows quite normally in the presence of these acids up to 100mg per growth tube at both 25 and 35°C, even on a medium without sugar.

While with the thermophobic strain 25a, growth was also normal at 25°C with these acids, but at 35°C the growth slows down rapidly after incubation for 2 to 3 days. This slowing down of growth can be effectively reversed by activation at 25°C for 12 hours with the sole exception of the acetate effect. This reactivation by low temperature in the presence of these acids is exactly the same as that on the lactose medium. The results are summarised in Fig. 14. Citrate, succinate and malonate are not active in salt form up to 100mg per growth tube (containing 10Øcc of medium) but in acid form, 10mg per growth tube gives a very significant thermophobic effect. It should be pointed out here that malonic acid which shows its thermophobic effect clearly, does not participate in the Krebs' cycle itself,

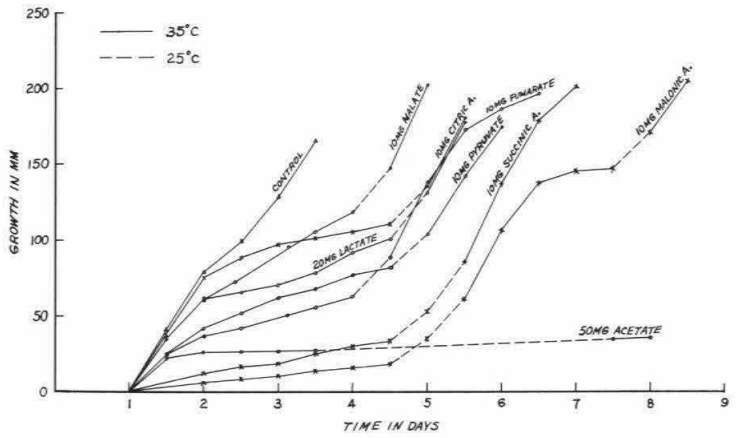


Fig. 14. Thermophobic effect of Krebs' acids on 25a on 0.5% sucrose medium at 35°C. The data illustrated are from experiments employing the minimal quantity of acid (or salt) that give a clear thermophobic effect on test. Higher concentrations give similar results.

on the other hand it is a specific inhibitor of succinic acid dehydrogenase.

The behavior of acetate which is usually supposed to be one of the common intermediates in the metabolism of carbohydrate and fats, deserves much attention. Although, at low concentration, say 10mg per growth tube, it shows no apparent thermophobic effect, once the concentration reaches a point which will inhibit the growth at 35°C, the inhibition, as a rule, is never reversed by transferring to 25°C. The growth inhibition of acetate is thus always absolute. Inoculation has been made in a petri dish culture containing an inhibitive amount of acetate. At 35°C growth started for a while and then stoped. Then the petri dish was transferred to room temperature (23-25°C) for several days. Growth was not resumed. However, new growth does start in room temperature in several points and spreads in the form of a ring with a diameter from one to two centimeters and then stops again. Inoculum taken from the ring growth to a new medium containing no acetate initiates a new growth promptly and normally. But the inoculum taken from other part starts no growth at all. Therefore the acetate inhibition does kill the mycelium but apparently not all cells. Since chitin has an acetyl group on it, the acetate effect will be treated in more detail later in relation to the cell wall material.

Monochloroacetic acid is also active, but its amino derivative, that is glycine, is inactive up to 100mg per growth tube as shown in Fig. 15. Other amino acids which are related to the Krebs' acids were also tested. Among them alanine whose keto acid is very active, has no thermophobic activity, but the two dibasic amino acids, glutamic and aspartic, are active (Fig. 15).

On the basis that the calcium salts of these Krebs' acids are relatively insoluble it is possible that calcium might antagonize the thermophobic

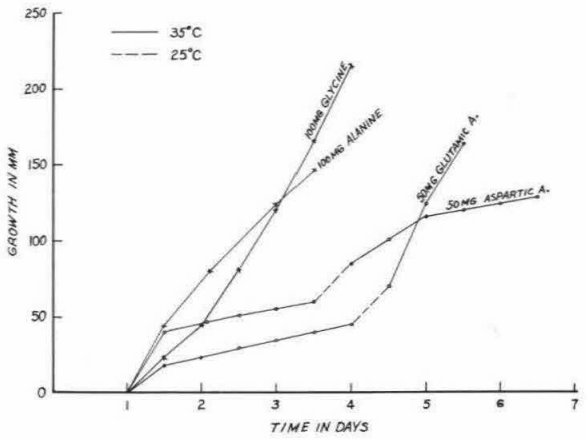


Fig. 15. Thermophobic effect of some amino acids related to the Krebs' acids on 25a on 0.5% sucrose medium at 35°C.

effect of the Krebs' acids accumulated during the biological degradation of carbohydrates. Calcium chloride was tested on both sucrose and lactose media with strain 25a in order to see whether it counteracts the thermophobic effect of lactose or not. Surprising enough, the calcium chloride is not only without any indication of amending effect on lactose, but itself also shows the characteristic thermophobic effect on sucrose medium when the concentration of $CaCl_2$ reaches 50mg per growth tube (Fig. 16). These tests have been repeated twice more, once with negative result but once with a result exactly the same as in the first test. It may be, therefore, concluded that the Ca ion is also thermophobic.

The general occurrence of the thermophobic effect of the Krebs' acids seems to indicate that something is wrong in the Krebs' cycle of the thermophobic strain. However, some alteration in the terminal step of oxidation, namely the hydrogen transfer process, might also show the same effect. If this is true, then a substance which can help the hydrogen transfer process should have counteracting effect on thermophobism. Thus methylene blue which has been widely used in biological oxidation experiments was tried on thermophobic effect of lactose and Krebs' acids. The effect is only relative. After activation at 25°C, there is more growth at 35°C in the presence of methylene blue than without, as shown in Fig. 16. Dyes other than methylene blue but with reduction-oxidation potential close to it, such as neutral red and indigo tetrasulfonate have also been tested without improvement of growth of 25a on 0.5% lactose minimal at 35°C after activation. Riboflavin, cytochrome C and pyridine with adenine were also tested in the same way. They all gave negative results. It was therefore concluded that the terminal oxidation process could not be the cause of thermophobism.

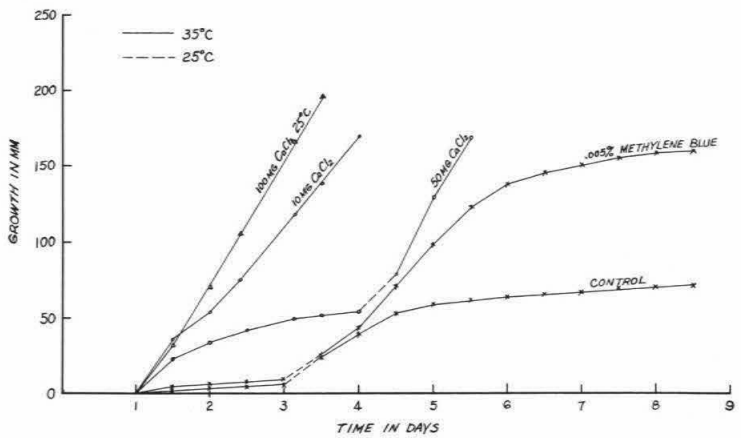


Fig. 16. Thermophobic effect of calcium chloride on 25a on 0.5% sucrose medium at 35°C, and the effect of methylene blue on growth on lactose at 35°C after activation at 25°C.

b. Studies on certain of the enzyme systems in Krebs' cycle

From the preceding experiments, the cause of thermophobism seemed to have been narrowed down to the Krebs' cycle proper. Hence an attempt was made to identify the critical enzyme involved. Warburg's manometric method was used for the respiratory enzymes in the cycle (Umbreit et al, 1945). Mycelium was lyophilized and then ground to powder and stored under refrigeration. Mycelium homogenate was thus very easily prepared by simply rubbing the dried mycelium powder in a buffer solution. The insoluble material is easily removed, if desired, by centrifugation.

(1) Basal respiration

When a mycelium homogenate was used as enzyme preparation, only feeble basal respiration was observed for both strain 25a and Em 5256A and was thus neglected in the first part of this study. A large part of the work on lactic and succinic acid dehydrogenases and keto acid decarboxylases was done before the supernatant was tried as enzyme preparation. As soon as the insoluble part of mycelium homogenate was removed by centrifugation and the supernatant was used, there is a surprisingly high basal respiration in 25a preparation which lasts for more than 4 hours without an appreciable decrease in rate of O_2 uptake and obviously would interfere with the observation of any other oxidative enzyme system. Supernatant of Em 5256A preparations on the other hand, have a basal respiration as low as in homogenate. Figure 17 shows the basal respiration of 25a and Em 5256A supernatants and their mixture in the presence of methylene blue. An enormous increase in basal respiration was observed by adding 25a supernatant to the normal Em 5256A supernatant even when the supernatant of 25a made up as little as 5% of the resulting mixture. In these mixtures, there is always a noticeable

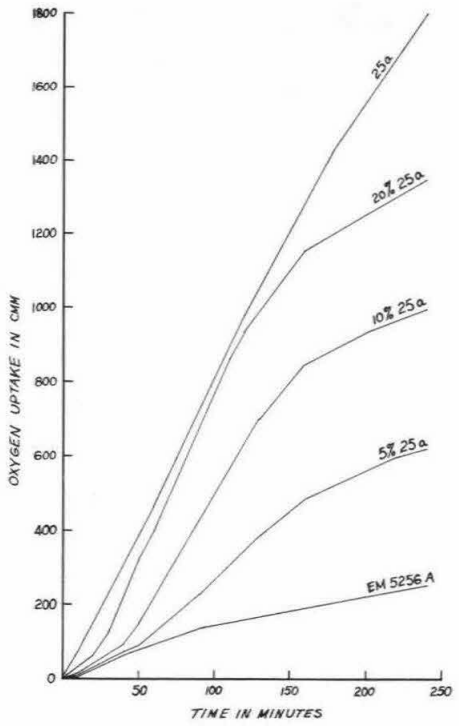


Fig. 17. Basal respiration of 25a and Em 5256A supernatants and their mixtures in the presence of methylene blue.

lag period in O_2 uptake whose length is inversely proportional to the quantity of 25a supernatant added. This could mean that the basal respiration involves several steps from the removal of hydrogen from the substrate to the terminal oxidation by atmospheric oxygen. If we suppose that the first step, removing hydrogen from the substrate, works at a greater rate than the process in terminal step which is thus the limiting factor of the rate of O_2 uptake, then a small amount of the enzyme required to catalyse the first step would need a longer time to saturate the last step, and result in a lag period. The lag phase should consequently increase with decreasing amounts of the enzyme needed for the first step. This interpretation is supported by the fact that in the presence of methylene blue, which is widely used for substitution of terminal oxidation, the basal respiration is greatly enhanced by as much or more than 300 percent as shown by the data in Table 18. The change in slope of all curves at 106 minutes seems to indicate that degradation of the enzyme is beginning.

The difference in basal respiration between homogenate and supernatant suggests and inhibition of basal respiration by insoluble material. The data summarised in Table 18 show the effect of the insoluble matter in the homogenate, of added boiled supernatant, and of dialysis. The insoluble matter has a very strong inhibitive effect on the basal respiration of 25a. The effect is the same no matter whether the insoluble matter is from 25a or Em 5256A. More interesting is the fact that the inhibitive effect disappears completely after boiling in a water bath for 10 minutes. This thermolabile nature of the inhibitive effect of the insoluble substance suggests that it might be a protein attached to some sort of insoluble material. The boiled supernatant from both strains increases the respiration. Most probably, this is due to the increase of simple thermostable substrate

Table 18

Basal respiration of ground mycelium supernatant.

Strain	Enzyme system: Preparation (Lyophilized mycelium : buffer=1:11)	Cofactor (Boiled supernatant)	Inhibitor (ppt.)	Methylene blue (0.1%)	Duration of run (hrs)	Temp. °C	Total O_2 uptake (cmm)
25a	2cc Homogenate	--	--	--	2	35	153.9
"	" "	--	--	0.2cc	2	"	174.3
"	2cc Supernatant	--	--	--	2	"	674.0
"	" "	--	--	0.2cc	2	"	936.0
"	2cc Rehomogenate	--	--	--	2	"	115.3
"	" "	--	--	0.2cc	2	"	100.1
25a	1cc Supernatant	--	--	--	1.5	35	143.9
"	" "	--	--	0.2cc	1.5	"	490.0
"	" "	1cc 25a	--	--	1.5	"	209.8
"	" "	" "	--	0.2cc	1.5	"	598.5
"	" "	1cc Em 5256A	--	--	1.5	"	187.9
"	" "	" "	--	0.2cc	1.5	"	476.0
25a	2cc Supernatant	--	--	--	1	27.8	172.7
"	" "	--	--	0.2cc	1	"	518.0
"	" "	--	0.8cc 25a	--	1	"	73.3
"	" "	--	" "	0.2cc	1	"	109.9
"	" "	--	0.8cc Em5256A	--	1	"	79.5
"	" "	--	" "	0.2cc	1	"	73.2
25a	2cc Supernatant	--	--	--	4	35	528.0
"	" "	--	--	0.2cc	4	"	1468.0
"	" "	--	0.8cc 25a Boil	--	4	"	824.0
"	" "	--	" " "	0.2cc	4	"	1311.0
25a	1cc Dialysed super.	--	--	--	2	35	10.1
"	" " "	--	--	0.2cc	2	"	10.7
"	" " "	1cc 25a	--	--	2	"	34.1
"	" " "	" "	--	0.2cc	2	"	56.1
Em 5256A	1cc Supernatant	--	--	--	0.5	35	8.6
"	" "	--	--	0.2cc	0.5	"	21.3
"	" "	1cc 25a	--	--	0.5	"	19.0
"	" "	" "	--	0.2cc	0.5	"	21.7
"	" "	1cc Em 5256A	--	--	0.5	"	15.1
"	" "	" "	--	0.2cc	0.5	"	15.8

in the supernatant. After dialysis of 25a supernatant for 24 hours against a large quantity of water in the refrigerator, most of the high basal respiration is lost, but it can be restored to some extent by adding boiled supernatant. It is clear, therefore, that the process of dialysis causes loss of substrate and some degradation of the enzyme.

One more point should also be mentioned here in connection with the degradation of the enzyme concerned with basal respiration. Only very feeble basal respiration was observed from the supernatant prepared from the 25a mycelium which was ground with dry ice and stored in deep freezer. The enzyme must be lost quantitatively in passing through this procedure of preparation.

Furthermore, it is curious that this basal respiration is present only in the mycelium harvested from sucrose medium at 25°C. Mycelium from cultures on lactose at 25°C or sucrose at 35°C shows very feeble basal respiration (less than 50cmm O_2 uptake in 1.5 hours run with 2cc supernatant in the presence of methylene blue).

Although the basal respiration itself is interesting enough to deserve a full investigation, the more important thing to be found out here is whether it has anything to do with thermophobic character. Eight odd number isolates, half of them are thermophobic, from two asci of the cross between 25a and Em 5256A were cultured in 2% sucrose at 25°C, and their basal respirations were tested. Only isolate No. 947-1 has a basal respiration as high as 25a, and the other three have only very feeble O_2 uptake. The four isolates from another ascus No. 948 all have very low basal respiration. And the isolate 947-1 with high basal respiration happened not to be thermophobic. Therefore, it is safe to conclude here that the

high basal respiration seems to be inherited in a somewhat complex manner and is independent of the thermophobic character concerned.

(2) Succinic acid dehydrogenase

The mycelium homogenates of both strains have high activity on succinate. On the same dry weight basis, the Em 5256A homogenate has higher activity than that of the 25a homogenate, as shown by the data in Table 19. The mycelium used here was from the culture in 2% sucrose at 25°C. The temperature effect on succinate dehydrogenase is somewhat peculiar. In comparison with the activity at 27.8°C, the activity is lower at 35°C but higher at 38°C. This is true for both strains. It is true that test at different temperatures were carried on different days, but otherwise all conditions were comparable; the enzymes were prepared fresh for each experiment from the same lot of lyophilized mycelium.

Methylene blue has an obvious positive effect on activity, while for cytochrome C, its effect, if any, is quite small. From the data presented here there appears no qualitative difference between the succinic acid dehydrogenases of the two strains. The quantitative difference in succinic dehydrogenase activity of the two strains seems real, but it was not tested in enough different preparations to be established. It is doubtful if one should put too much weight on this quantitative difference as a cause of thermophobism.

(3) Lactic acid dehydrogenases

Similar experiments have been done on lactic acid dehydrogenases of the two strains, and their data are summarised in Table 20. Their activities on dry weight basis of mycelium used are nearly the same. Further, no

Table 19

Activity of succinic acid dehydrogenase.

Strain	Enzyme system: Methylene blue (0.1%)	Enzyme system: Cytochrome C (1.3mg per cc)	Total O_2 uptake(cmm) in 2 hrs run: 27.8°C	35°C	38°C
25a	--	--	110.8	103.7	166.9
"	0.2cc	--	153.1	143.9	255.5
"	--	1cc	135.2	117.5	189.5
Em 5256A	--	--	210.5	148.0	262.6
"	0.2cc	--	241.4	178.2	344.5
"	--	1cc	203.8	165.4	233.0

2cc homogenate contains 0.22g lyophilized mycelium
pH 7.6, 0.1 M phosphate buffer
Substrate: 0.5cc 0.5M sodium succinate.

Table 20

Activity of lactic acid dehydrogenase.

Strain	Enzyme system: Methylene blue (0.1%)	Enzyme system: Cytochrome C (1.3mg per cc)	Total O_2 uptake(cmm) in 2 hrs run: 27.8°C	35°C	38°C
25a	--	--	80.6	89.2	122.3
"	0.2cc	--	199.7	268.9	338.1
"	--	1cc	190.9	213.5	251.5
Em 5256A	--	--	98.2	98.2	150.5
"	0.2cc	--	196.1	274.5	387.0
"	--	1cc	211.5	193.6	234.1

2cc homogenate contains 0.22g lyophilized mycelium
pH 7.6, 0.1M phosphate buffer
Substrate: 0.5cc 0.5M potassium lactate.

other obvious differences could be found between the two strains as to the temperature effect, responses to methylene blue and cytochrome C. For both strains, there is almost no temperature effect between 27.8 and 35°C, but there is some increase of activity at 38°C. The methylene blue effect here is stronger than that for the succinic acid dehydrogenases. Moreover cytochrome C here shows a positive effect on activity nearly as great as that of methylene blue.

The cytochrome C effect here is somewhat puzzling. Efforts have been made to detect the cytochrome oxidase activity in mycelium homogenate of both strains in the presence of cytochrome C and hydroquinone or cytochrome C and dimethyl-p-phenylenediamine, but all without success. However, the increase of O_2 uptake of the lactic acid dehydrogenase activity in the presence of cytochrome C, does indicate that there is cytochrome oxidase present in the preparation. In yeast, Dixon and Zerfas (1939) found that the crude preparation reduces both methylene blue and cytochrome C readily in the presence of lactate. When the preparation is further purified, it retains the ability to reduce methylene blue but not cytochrome C. The authors interpreted this as due to the presence of an additional catalyst which present in the crude preparation is necessary for the reduction of cytochrome, possibly a hydrogen carrier acting between the dehydrogenase and cytochrome C. It is therefore possible that the failure of detecting the cytochrome oxidase activity in Neurospora preparation may be due to the absence of such a hydrogen carrier between the substrate (hydroquinone or dimethyl-p-phenylenediamine) and cytochrome C. Moreover, the ineffectiveness of cytochrome C in the succinic dehydrogenase system may be, as suggested by Pappenheimer and Hendee (1949), due to the fact that the succinic dehydrogenase itself is one of the cytochromes, called b.

(4) Keto acids decarboxylases

When the mycelium supernatant prepared from dry ice ground material was tested on pyruvate, a high rate of CO_2 evolution was at once observed for both strains. Other keto acids also have been tried on supernatant preparation of Em 5256A. It has very high activity too on α-ketobutyrate, and appreciable activity on α-ketoglutarate, ~~and α-ketosuccinate~~. As no significant relationship to the thermophobic character is evident, these keto acids have not been tested on 25a preparations.

c. Conclusion

The results of transferring experiments between high and low temperature seem to indicate that some sort of inactivation of certain enzymes might be involved in the thermophobic strain. The effect of temperature higher than 35°C gives a hint that the temperature inactivation is not confined to a single enzyme. Therefore, it was supposed that the protein in general of the thermophobic strain might have a comparatively higher sensitivity to thermodenaturation. On this basis, invertases and lactases from both strains were compared extensively and, unfortunately, no significant difference has been found.

Surprising enough, all of the Krebs' acids without a single exception were found to produce the thermophobic response in 25a at 35°C on sucrose medium. This seems strongly to indicate that something is wrong in the

Krebs' cycle of the thermophobic strain. These acids were tested on mycelium homogenates manometrically in the Warburg apparatus, and lactic and succinic dehydrogenases, and pyruvic decarboxylase activities were found. No enzyme activity on fumaric or citric acid was detected. The activity of lactic and succinic acids dehydrogenases from both strains was compared with or without the addition of methylene blue or cytochrome C at three different temperatures and no appreciable difference between strains has been found. The only very significant difference found in this manometric study was the basal respiration which was proved to have nothing to do with the thermophobic character itself.

It is therefore quite clear that the problem should be approached from a different angle.

7. Cell wall material and thermophobism in Neurospora

As mentioned previously, in lactose medium the difference in growth of thermophobic and normal strains is not as consistent in liquid flask culture as in tube culture on solid medium. But in the flask culture, instead of obtaining a difference on dry weight basis of mycelium, an apparent morphological difference was immediately observed, i.e., the semicolonial growth of thermophobic strain. It is obvious that one can not compare the growth rate by the tube method between colonial and non-colonial forms. The tremendous difference in tube culture experiments between the strains may result solely from morphological differences. Since there is not much difference on dry weight basis between the thermophobic and normal strain in liquid lactose medium, it is therefore most likely that the semicolonial form is due to the partial inhibition of cell elongation. Cell elongation would obviously involve the cell wall material. For this reason the nature of the cell wall material of Neurospora was investigated.

a. Staining reaction of the cell wall material

The main constituent of the cell wall of most fungi is chitin, a nitrogenous polysaccharide. Only in certain intermediate groups, namely Didymium squamulosum, a myxomycete, and in the Peronosporae(Pythaceae), and Saprolegniae, belonging to the oomycete group of Phycomycetes, is chitin absent (Schmidt, 1936; Von Wettstein, 1921, cf Foster, 1949). The naphthol reaction of chitin after Schulze and Kunike (1924, cf Klein, 1932) gives violet color, which is characteristic for the presence of chitin, for both normal strain Em 5256A and thermophobic strain 25a. The test consists of dissolving dried mycelium in 60% H_2SO_4 and adding a few drops of 0.5%

α-naphthol in 50% alcohol. The violet color develops immediately. This shows the presence of chitin in both strains.

There is another method of testing for chitin called the chlorozinc iodine reaction (Von Benecke, cf Klein, 1932). The color reagent is made by dissolving 1.6g of iodine, and 10g of KI in 14cc of water and 60g of $ZnCl_2$ in another 14cc of water and then mixing the two solutions together. When this color reagent is tested on fresh mycelium from sucrose medium at 25°C, the difference between the two strains shows up as a color difference: the normal strain is stained greenish blue, while the thermophobic strain 25a is stained brownish blue. The difference becomes much more prominent when the mycelium is cultured at 35°C or in lactose medium. The staining reaction for the normal strain is always the same no matter what conditions of culture are used. While for the thermophobic strain, the brown color increases in the following order of the condition of culture:

Sucrose 25°C < Sucrose 35°C ≦ Lactose 25°C < Lactose 35°C.

The mycelium from lactose culture at 35°C is stained almost pure brown in color.

This color reaction was also tested on 40 isolates from the cross, Em 5256A X 25a. All of the 24 thermophobic ones give the same staining reaction as the thermophobic parent 25a, while the remaining 16 normal isolates behave as the normal parent Em 5256A. The complete correlation between thermophobic character and differential staining reaction immediately suggests the significance of the chemical nature of the cell wall on thermophobism. Therefore, a close study of the chitin is necessary for the understanding of the differential staining reaction.

b. Chemical nature of chitin

The cell wall material of Neurospora crassa is free from cellulose. This was proved by the following experiment. Lyophilized mycelium powder was extracted with a well known cellulose solvent called cuprammonium hydroxide solution which, as recommended by the American Chemical Society (cf Otto, 1943), contains approximately 31g of copper and 165g of ammonia per liter. The preparation of the solvent involves: dissolve 6.25g of $CuSO_4.5H_2O$ and 2.67g of NH_4Cl in 50cc of water, then add 10cc 20% NaOH. The bluish powderous precipitate formed was filtered on a Buchner funnel by applying mild suction. Dissolve the bluish precipitate in a mixture of 33.5cc of ammonium hydroxide (28-29% NH_3) and 16.5cc of water, and the resulting cellulose solvent is deep blue in color and has a very strong pungent odor of ammonia. This solvent dissolves filter paper or cotton rapidly. The dissolved cellulose can be reprecipitated instantly by pouring into an acid solution. The recovered cellulose stained dark blue with the chlorozinc iodine color reagent just as the original filter paper or cotton. The cuprammonium extract of Neurospora mycelium was precipitated in 5N sulfuric acid solution, and the precipitate was centrifuged down and washed with water. The white precipitate thus obtained was stained brownish orange with chlorozinc iodine reagent. This indicates that there is no cellulose in the cell wall material of Neurospora.

The chitin was purified by the method used by Scholl (1908, cf Foster, 1949) with some minor modifications. The dried (lyophilized or dried in an 80°C oven for at least 12 hours) ground mycelium was first suspended in a mixture of one part ether and one part 1% NaOH alcoholic (95%) solution for at least 8 hours and then the solvent was filtered off and discarded. The residue was then exhaustively extracted with 9% NaOH

in boiling water bath for 30 minutes. The resistant fraction was filtered with suction, and washed twice with water. Since the material was sticky, it was impossible to separate it from the filter paper. They were dried together in an 80°C oven. The filter paper was easily dissolved away by cuprammonium solution and the cell wall material (believed to be chitin in quite pure form) was left free. It was then first washed in water, decolorized in 1% hydrochloric acid for few minutes, soaked in water for few hours and finally dried in oven.

The dried cell wall material, at this stage, can also be stained with chlorozinc iodine reagent. The material from normal strain Em 5256A stains dark blue or almost black. The material from the thermophobic 25a was stained differently according to the culture conditions, namely dark blue or nearly black from sucrose medium at 25°C and grey or greyish brown from lactose culture at 35°C. The same staining reaction can be obtained with aqueous iodine-potassium iodide solution without zinc chloride. In the later tests, iodine-KI solution will be used exclusively instead of chlorozinc iodine reagent.

This alkali resistant fraction was found by Scholl (1908, cf Foster) to be not the chemically pure chitin but chitin accompanied by other nitrogen-free substances. These associating substances are of the hemicellulose type and can be selectively destroyed by oxidation, leaving the chitin intact (Scholl). This is accomplished by treating the material with 1% $KMnO_4$, allowing it to stand until the $KMnO_4$ is completely converted to MnO_2. The liquid is filtered off and the brownish mass treated with dilute HCl (1:40) to dissolve the MnO_2. When the Neurospora material which would be stained black by iodine solution has been treated through this last selective oxidation procedure, the material is stained grey or brown by the iodine solution instead of usual black. This strongly

indicates that the iodine staining reaction is attributable to these non-nitrogenous substances.

The same thing can be shown in other way. Since the chitin is very resistant to acid, it is possible to remove the nitrogen-free part by mild hydrolysis with very dilute acid. It was found that the staining reaction is completely lost after digesting in a boiling water bath with 1N HCl for 5 minutes. The hydrolysed product was found most likely to be glucose. In order to make a colorimetric test for monosaccharides (Tauber and Kleiner, 1932) as used before, of the hydrolysate, the effective dilution of acid for hydrolysis should be found since the presence of 3mg of NaCl in 2cc sample will interfere with the test. A series of dilutions, from 1N to 1/100 N of hydrochloric acid was tested for the ability to remove the iodine stainable part by heating in a boiling water bath for 10 minutes, and then testing the residue by the iodine reaction. It was found that the material hydrolysed with acid of a concentration higher than 1/5 N can no longer be stained by iodine. With acid concentration below 1/20 N, it was stained dark blue, and with 1/10 N, light blue. The 1/10 N HCl would thus be the most convienent concentration for hydrolysis. When this concentration of acid is neutralized with NaOH, it contains 5.845mg of NaCl per cc. Since the NaCl content should be kept within the safety range, it was decided to use only 0.4cc of the sample with a NaCl content (about 2.3mg) which is within the safety range for colorimetric determination of monosaccharides. Standard glucose solution was used for comparison. It is thus possible to convert the colorimetric reading of an unknown sample to glucose equivalent. Twenty eight mg of chitin from Em 5256A mycelium which had been cultured in 2% sucrose for 4 days at 35°C was hydrolysed in 5cc of 1/10 N HCl in boiling water bath, and 0.4cc of

aliquot was taken in 5 minutes interval for the analysis of monosaccharides split off. For the iodine staining test a parallel set with only a few mg of chitin was sampled at 5 minutes intervals. From the data presented in Table 21, it is evident that the staining reaction coincides directly with the degree of hydrolysis. The quantity of monosaccharides in the hydrolysate reaches its maximum value at 15 minutes boiling, at which time the residual chitin no longer stains with iodine. The colorimetric readings were converted into total glucose equivalent in the hydrolysate as shown in column 3 of the Table 21.

By this analysis the chitin of Em 5256A contains about 11.18% of monosaccharides. Since the monosaccharide residues must have been split from polysaccharide chains, this figure should be reduced by one molecule of water for each monose residue. However, for the purpose of a rough idea, this figure is good enough without such a correction.

The hydrolysate obtained in this way gives a very strong Molisch test for carbohydrate, but gives negative Seliwanoff test for ketose sugar and Tollens' phloroglucinol test for pentoses. From this evidence, the monosaccharide produced by hydrolysis is mostly likely glucose.

The iodine staining reaction was not changed by soaking the chitin in 1N HCl at room temperature for days. Once the iodine stainable part is removed by hydrolysis, it could not be recovered by recooking in 9% NaOH for another half hour. Therefore, this dilute acid hydrolysable part is quite resistant to the treatments used in the preliminary preparation of the material.

Having reached this point, we are in a position to postulate the relation of the easily hydrolysed part to the chitin molecule proper.

Table 21

Staining reaction and degree of hydrolysis of the non-nitrogenous part of the chitin. Twenty eight mg of Em 5256A chitin boiling with 5cc 1/10 N hydrochloric acid.

Time in boiling water bath (minutes)	Colorimetric reading of monose analysis (dilute to 10cc)	Total glucose equivalent in hydrolysate (mg)	Iodine staining reaction
0	7	--	Dark blue
5	25	1.300	Blue
10	206	2.339	Light blue
15	425	3.080	Colorless
20	450	3.131	Colorless

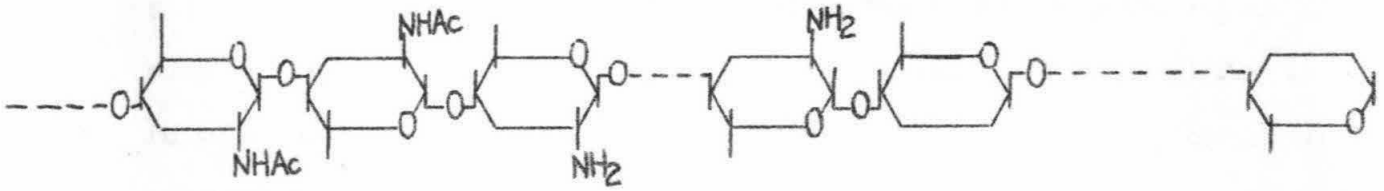
Since the hydrolysable fraction is resistant to alkali and to the cellulose solvent (cuprammonium solution), it must be joined to chitin chemically rather than in some sort of physical association. The fact that it can be digested to glucose by very mild hydrolysis without destroying the chitin itself strongly suggests that a number of glucose residues (probably in relatively short chains with cellulose-like linkages) make up the ends of the long chitin chains. Since this terminal part is not yet aminated and acetylated, it could take up iodine to give the dark blue color characteristic of cellulose. This postulate suggests a mechanism whereby the chitin molecule increases in size. Glucose molecules would first be linked successively to the end of the chain, and amination and acetylation would lag some way behind. Since the resistance of chitin proper to acid hydrolysis has been explained by the difficulty in approach of the catalyst hydrion to the glycosidic group when a positively charged nitrogen atom is situated on an adjacent carbon atom (Stacey, 1946), the reverse may also be true that the positively charged nitrogen atom may also hinder the building up of a glucosidic linkage. Hence amination would be expected to take place after the glucosidic linkage had been completed.

The chitin from Em 5256A (grown in 2% sucrose at 35°C for four days with aeration) has been hydrolysed in 1/10 N HCl for 15 minutes in a boiling water bath and the hydrolysate was tested colorimetrically for reducing sugar (Tauber and Kleiner, 1932), glucosamine (Morgan and Elson, 1934) and N-acetylglucosamine (Elson and Morgan, 1933) simultaneously. The N-acetylglucosamine used for standard solution was synthesized from glucosamine and Ag-acetate according to the method of Stacey for synthesis of N-acetylchondrosamine (Stacey, 1944). The tests were positive for

reducing sugar and glucosamine but negative for N-acetylglucosamine. The method for testing glucosamine will give positive reaction for N-acetyl-glucosamine but not vice versa. The reducing sugar test will give positive reaction with glucosamine and N-acetylglucosamine too. When the colorimetric reading for reducing sugar was converted to glucose equivalent, and the reading for glucosamine to mg of glucosamine by comparison to their respective standards, there is as calculated, 12.61% of glucosamine in this hydrolysable end group of the chitin molecule.

The chitin was dissolved in 60% sulfuric acid and the solution was then neutralized with NaOH. The resulting solution gives positive test for acetylglucosamine.

From the results just reported it is probable that the end of a chitin molecule is rather heterogeneous as diagramatically represented below:



The importance of the heterogenety of the end group on the further lengthening of the chitin molecule can immediately be seen. If chitin has anything to do with the thermophobic character, it would most probably be the end group of the chitin that is most directly concerned.

c. Chitin formation in relation to environment

In order to get some information about chitin formation in relation to environment and the length of end group of chitin molecule, an extensive experiment was carried out. The environmental factors made use of were carbon sources, sucrose vs. lactose in two percent solution; temperature, 25 vs. 35°C; acetate concentration 0, 0.5, 1, 2 and 4 percent potassium acetate. Each flask contained 50cc of medium with various constitutions. The normal strain Em 5256A and the thermophobic 25a were used for comparison. Mycelium was harvested at 4 days intervals, up to 12 days, and dried in an 80°C oven for at least 12 hours. Having taken the dried weight of the mycelium each dried pad was soaked in a large quantity of water, and the softened pad was then ground into paste. The paste was passed through the same procedures as described in previous section for analysis of chitin and the glucose content of chitin. The glucosamine content was not analysed in this experiment, and all the reducing sugar was considered as glucose. When the dried weight of mycelium was below 100mg the chitin content was not analysed due to insufficient quantity of material for a reliable analysis. For the same reason, the glucose content of chitin was not analysed, when the chitin yield was below 20mg. The data are summarised in Table 22. Only those which seem to have some important bearing on the main problem will be discussed in detail.

It is interesting to point out, first of all, that acetate itself has a very strong temperature effect on the dry weight of mycelium of both strains in sucrose medium. In four days old cultures, there is a slight increase in dry weight at 25°C with increasing acetate concentration up to 1%, above which there is a rapid decrease. When acetate concentration reaches 4% 25a makes little or no growth, whereas, the normal strain

Table 22

Chitin formation in relation to sugars, temperatures, concentrations of acetate and ages of culture. Each culture flask contains 50cc of medium.

Strain	Sugar (2%)	KAc (%)	Temp. °C	4days old culture Dry wt. (mg)	Chitin (%)	Glucose (%)	8days old culture Dry wt. (mg)	Chitin (%)	Glucose (%)	12days old culture Dry wt. (mg)	Chitin (%)	Glucose (%)	Iodine staining reaction
25a	Lact.	0	25	13.0	--	--	82.0	7.32	1.25	167.5	7.47	3.52	Black
"	"	.5	"	39.0	--	--	192.0	16.41	0.80	381.5	15.08	3.81	Black
"	"	1	"	34.0	--	--	53.5	--	--	41.5	--	--	--
"	"	2	"	5.5	--	--	41.0	--	--	26.5	--	--	--
"	"	4	"	0	--	--	0	--	--	14.0	--	--	--
25a	Sucr.	0	25	105.5	10.43	--	155.5	13.51	3.12	190.0	15.52	4.44	Black
"	"	.5	"	118.0	10.17	--	188.5	15.39	3.59	191.0	16.74	3.78	Black
"	"	1	"	132.0	9.09	--	199.5	15.52	4.00	194.0	17.78	3.62	Black
"	"	2	"	20.0	--	--	230.0	13.27	4.88	315.0	18.10	4.08	Black
"	"	4	"	1.0	--	--	4.5	--	--	108.0	11.80	0.59	Black
5256A	Lact.	0	25	22.5	--	--	61.0	--	--	248.5	10.87	5.52	Black
"	"	.5	"	33.0	--	--	35.0	--	--	27.0	--	--	--
"	"	1	"	31.0	--	--	24.0	--	--	30.0	--	--	--
"	"	2	"	29.0	--	--	18.0	--	--	21.0	--	--	--
"	"	4	"	2.5	--	--	22.0	--	--	16.0	--	--	--
5256A	Sucr.	0	25	103.0	7.77	--	216.5	10.85	8.84	240.0	13.97	8.36	Black
"	"	.5	"	169.0	9.47	--	268.5	13.40	7.20	282.0	14.01	6.65	Black
"	"	1	"	163.5	7.95	--	304.5	14.78	5.98	285.0	15.70	5.56	Black
"	"	2	"	88.0	--	--	232.5	13.32	7.47	295.5	17.76	5.06	Black
"	"	4	"	60.0	--	--	222.5	12.37	7.02	361.5	14.11	5.09	Black
25a	Lact.	0	35	6.0	(8.92)	(1.86)*	26.0	--	--	86.5	--	--	--
"	"	.5	"	18.0	--	--	16.0	--	--	19.0	--	--	--
"	"	1	"	4.0	--	--	19.5	--	--	14.0	--	--	--
"	"	2	"	1.0	--	--	19.5	--	--	9.0	--	--	--
"	"	4	"	0	--	--	0	--	--	0	--	--	--
25a	Sucr.	0	35	73.0	6.85	--	180.5	11.91	10.01	196.5	18.56	8.56	Grey
"	"	.5	"	367.0	11.69	5.63	375.5	--	--	262.0	26.75	4.05	Brown
"	"	1	"	385.0	11.03	2.02	370.5	20.64	2.56	209.5	36.79	2.60	Brown
"	"	2	"	3.0	--	--	57.0	--	--	14.0	--	--	--
"	"	4	"	0	--	--	0	--	--	0	--	--	--
5256A	Lact.	0	35	46.0	(9.26)	(3.64)*	210.0	12.61	6.32	247.5	14.75	5.71	Black
"	"	.5	"	28.0	--	--	17.0	--	--	19.0	--	--	--
"	"	1	"	20.0	--	--	14.5	--	--	17.5	--	--	--
"	"	2	"	18.0	--	--	13.5	--	--	14.0	--	--	--
"	"	4	"	11.0	--	--	14.5	--	--	13.0	--	--	--
5256A	Sucr.	0	35	132.0	7.96	2.75	242.5	15.46	7.00	189.0	21.68	7.63	Black
"	"	.5	"	337.0	10.69	2.89	226.0	15.70	4.50	206.5	20.58	3.65	Black
"	"	1	"	402.0	8.71	1.26	230.5	14.96	2.86	170.5	23.17	2.66	Black
"	"	2	"	354.0	8.20	1.00	279.5	15.39	2.84	211.0	22.74	2.67	Black
"	"	4	"	215.0	5.58	2.42	320.5	10.13	1.83	206.0	20.12	2.64	Black

* Borrowed from Table 23.

Em 5256A, which seems to be more tolerant to high concentration of acetate, has still quite good growth at that concentration. At 35°C, the acetate effect is phenomenal. For 25a, growth increases from 73mg without acetate to 367mg with 0.5% acetate and 385 mg, 1% acetate. The fresh mycelium harvested from the medium containing acetate is very brittle. When the acetate concentration reaches 2%, 25a makes a very poor semicolonial growth somewhat like the growth in lactose medium, with a dry weight of only 3mg. There is no growth at all with 4% of acetate. On the other hand, in the normal strain, Em 5256A, without acetate the dry weight is 132mg; with 0.5% acetate, 337mg; even at 4%, it still is 215mg. For cultures older than four days, the dry weight of slow growing ones increases to 12 days, while for the rapidly growing ones, it begins to decrease at the 8th days' harvest.

In lactose medium, acetate in low concentration helps the growth of both strains at 25°C, but inhibits the growth of both even at the lowest concentration (0.5%) tested at 35°C.

It should be emphasized here that acetate (within certain concentration limits) has a positive temperature effect (resulting in increased growth) in the presence of sucrose and a negative temperature effect in the presence of lactose no matter which strain is concerned.

The chitin content of mycelium increases, as a rule, with the age of culture, and with temperature. The aging process causes a decrease in mycelium dry weight but an increase in chitin content. Thus the synthesis of cell wall material still continues when the cell stops to grow. The acetate effect on chitin production is of great interest. At 25°C acetate has practically no influence on either strain. While at 35°C, there is still no influence of acetate concentration on the chitin content of mycelium

of the normal strain Em 5256A, but in the thermophobic strain 25a, the chitin content increases rapidly with increasing concentration of acetate, for example from 18.56% chitin without acetate to 36.79% with 1% acetate in the 12 days old cultures in sucrose at 35°C. Without acetate, the chitin content of 25a at 35°C is pretty close to that of Em 5256A, but there is decidedly higher chitin content in 25a mycelium from the cultures with acetate. The acetate therefore has a differentially stimulating effect on chitin synthesis depending upon temperatures and strains concerned.

The glucose content of chitin is usually slightly lower in lactose culture than that in sucrose culture. There are very small differences for Em 5256A between 25 and 35°C. For 25a, the glucose content is usually higher at 35°C than that at 25°C. The most interesting point here is the acetate effect which shows too a prominent differential temperature response. At 25°C, there is practically no acetate effect with 25a in sucrose medium while with Em 5256A there is a slight decrease in ~~chitin~~ glucose content as the concentration of acetate increases. At 35°C, a colossal effect appears. The glucose content drops rapidly as the acetate concentration increases. For Em 5256A in sucrose medium, the decrease of glucose reaches a minimum value of about 2.7% at 1% acetate above which there is no further change. For 25a, a slightly lower value of 2.6% is reached at 1% acetate, but whether this is the minimum value or not is not known since analysis was not carried out for higher concentrations of acetate because of lack of material. It is evident then that the presence of acetate in the medium will greatly help the acetylation process of the glucose residues on the end group of the chitin molecule.

From these data, it is possible to figure out the approximate average half length (suppose it is a linear molecule) or average length of the

branch (suppose it is a branched molecule) of chitin molecule with some supplementary assumptions based upon the known facts. For the sake of simplicity, let us call it a chitin half molecule, regardless whether it is a half molecule of a linear chain molecule or it is a branch of a much branched molecule. Let x be the number of acetylglucosamine residues and y be the number of glucose residues of the end group, in the half molecule of chitin, then:

$$\text{Half molecule of chitin} = x(C_6H_9O_4NHCOCH_3)y(C_6H_{10}O_5),$$

and its molecular weight will be

$$203.192x + 162.140y.$$

Then evidently the percent of glucose in this half molecule will be

$$\% \text{ glucose} = 180.156y \,/\, (203.192x + 162.140y) = a.$$

By simple rearrangement, thus

$$x = \left(\frac{0.885}{a} - 0.799\right)y$$

where a is known from experimental data. Then, if y can be estimated, x can be calculated immediately from the formula. Actually it is possible to establish certain limits to the length of the terminal glucose chains (y) from the intensity of the staining reaction of the chitin with iodine. Three arbitrary grades of color were used: nearly black, greyish and brownish. Degradation by mild hydrolysis shows that these colors represent decreasing numbers of glucose residues. When material presumably having the longest glucose chains (Em 5256A grown in sucrose medium in the absence of acetate, see Table 21) has been partially hydrolyzed, so that approximately three quarter of the hydrolysable glucose has been removed, the staining reaction of the residual chitin has been altered from black to grey. From this we may assume that the longest glucose chains have something like four

times the number of glucose residues as those which stain grey. Further, when chitin which stains brown is submitted to mild hydrolysis to remove terminal glucose residues, the staining reaction is completely lost. It is therefore reasonable to suppose that there are at least two terminal glucose residues in chitin which stain brown. If we can further assume that the brittleness of the fresh mycelium is an inverse function of the length of the chitin chain, it is possible to carry the analysis further. It has been assumed that non-brittle mycelium will have at least twice as long chitin chains as the most brittle. Table 23 gives rough estimates of the chain length both for terminal glucose residues (y) and for the acetylglucosamine residues (x) in half molecules of chitin of the two strains under varying cultural conditions. Data upon which these estimates are based are also included in the Table.

Even though the estimates of lengths of chitin half molecules or branches, are admittedly inexact, it follows inescapably that the chitin molecule of the thermophobic strain is either very much smaller, or very much more branched than that of the normal chain.

It should be noted that the chitin half molecule of Em 5256A has approximately the same number of glucose residues at both 25 and 35°C on sucrose in the absence of acetate. With acetate, the number of glucose residues is progressively reduced to a limiting value of 32 at 25°C as compared with 16 at 35°C. Why there should be these limiting values in the normal strain and not in the thermophobic strain is not yet known, but it may will represent a fundamental difference between the two strains.

With a hope that the difference in length of the chitin half molecule between thermophobic and normal strain at critical conditions, namely on

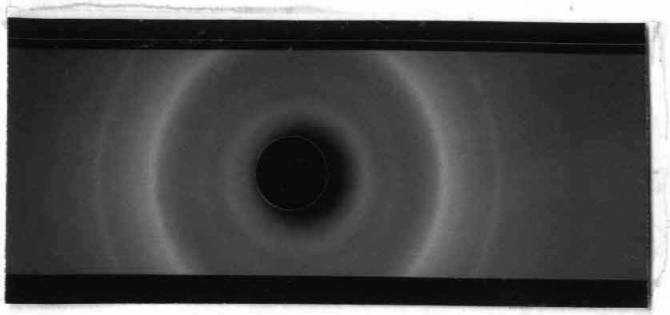
Table 23

Estimation of the number of acetylglucosamine and glucose residues in a chitin half molecule. (All cultures are from sucrose medium. "a" values are taken from Table 22).

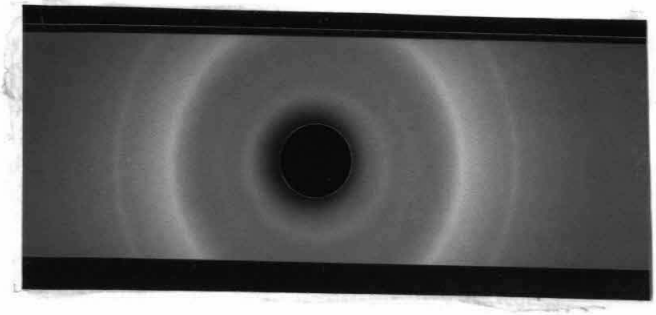
Strain	Age (day)	Temp. °C	KAc %	% of glucose in chitin a	$\frac{0.885}{a} - 0.799$ x'	No. glucose residues y	No. acetyl-glucosamine x (= x'y)
25a	8	35	0	10.01	9.181	14	128
"	"	"	1	2.58	33.621	2	67
25a	12	35	0	8.56	9.531	14	133
"	"	"	.5	4.05	21.061	3	63
"	"	"	1	2.60	33.221	2	66
5256A	12	25	0	8.36	9.781	52	528
"	"	"	.5	6.65	12.511	42	525
"	"	"	1	5.56	15.111	34	514
"	"	"	2	5.06	16.691	32	534
"	"	"	4	5.09	16.591	32	531
5256A	8	35	0	7.00	11.841	48	568
"	"	"	.5	4.50	18.861	28	528
"	"	"	1	2.86	30.151	18	543
"	"	"	2	2.84	30.371	18	547
"	"	"	4	1.83	47.501	12	570
5256A	12	35	0	7.63	10.801	48	518
"	"	"	.5	3.65	23.431	22	516
"	"	"	1	2.66	32.451	16	520
"	"	"	2	2.67	32.341	16	518
"	"	"	4	2.64	32.721	16	524

lactose medium at 35°C might be revealed by X-ray diffraction patterns, the chitins of both strains were purified by the usual method (including the mild acid hydrolysis), and a powder X-ray picture of the groud chitin was taken with a wave length of 1.54 Å from a copper taget and a distance of 57mm. The diffraction patterns from both strains are identical as shown in Fig. 18. They are also identical with the X-ray diffraction pattern of chitin obtained by Heyn (1936) with the flattened sporangiophore wall of Phycomyces and by Clark and Smith (1936) with ground chitin. The calculation of unit dimensions confirms that of Meyer (1935) with a = 4.55 Å and b = 9.64 Å by the formula $d = N\lambda/2\sin\theta$ where N is the first order. Since the X-ray diffraction picture only reveals the dimensions of a repeating unit of the macromolecule, it is incapable of revealing difference in the macromolecular structure. (Thanks are due to Dr. Y. C. Tang who took and interpreted the X-ray pictures).

The shortness of end glucose chain of chitin half molecule of the thermophobic strain 25a was tested by another experiment. When cultured on 2% lactose at 35°C in liquid culture there should be a big difference between the chitins produced by strain Em 5256A and 25a. Under these conditions the chitin of 25a should have very few glucose residues per terminal group whereas chitin from Em 5256A should have at least 16 as judged by the degree of the staining with iodine. Another difference under the conditions is that the mycelium of 25a is semicolonial while that of Em 5256A is not, suggesting that the chitin of 25a is composed of ~~sam~~ smaller, or more branched molecules. In these circumstances one should expect a more rapid increase in the glucose content of the chitin of the thermophobic strain than that of the normal strain when a large amount of

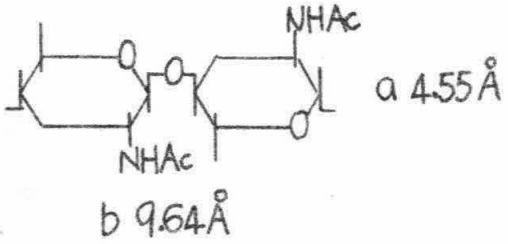


A



B

Fig. 18. X-ray diffraction patterns of purified chitin powder taken with a wave length of 1.54 Å from a copper taget and a distance of 57mm. A. Chitin of the thermophobic 25a cultured on 2% lactose medium at 35°C and B. Chitin of the normal strain Em 5256A cultured under the same conditions.



glucose is made available to the cultures.

The two strains were cultured at 35°C in 500cc or one liter flasks containing respectively 400cc and 700cc of lactose medium. When the cultures were of 5 and 10 days old, a quantity of glucose was dumped into each culture to make a final glucose concentration of 2%. Then the mycelium was harvested at 2 or 3 hour intervals up to 12 hours starting from zero time. The mycelium was washed and dried and passed through the same procedures of analysis for chitin and glucose content of chitin as previously reported. The data shown in Table 24 clearly indicate a rapid increase of glucose content of 25a chitin after glucose has been dumped in. There is practically no change at all of glucose content of chitin from cultures of Em 5256A of different ages. But the difference is quite apparent between the 5 and 10 days cultures of 25a. There are two possibilities to account for the dependency upon age of the increase in glucose content in 25a. Either the number of acetylglucosamine units of the chitin may be reduced with age, or there may be an increase in the number of glucose residues on the ends of the molecules. Since the rate of increase of glucose content of chitin of the aged culture is much slower than that of the younger culture, the latter alternative seems therefore more likely to be the case. Moreover, from the experiments on invertases and lactases mentioned previously, these enzymes are accumulated quantitatively on aging of the culture. An increasing in lactase content together with a slowing down of general metabolism of the cell during aging, would possibly create a condition in which there is a relatively greater abundance of glucose than is present in younger cultures and in consequence, would increase the number of glucose residues on the end of chitin molecule.

Table 24

Rate of glucose coupling on the end of chitin molecule in relation to the length of the end piece of the chitin molecule. Cultures raised on 2% lactose minimal medium at 35°C. The experiment was also carried out at 35°C.

Strain	Sampling interval after adding glucose (hours)	5 days old culture %chitin	5 days old culture % Glucose	10 days old culture % Chitin	10 days old culture % Glucose
25a	0	8.92	1.86	10.45	5.38
	2	--	--	9.91	5.60
	3	9.62	2.56	--	--
	4	--	--	8.97	5.74
	6	10.17	3.68	11.40	5.99
	8	--	--	8.65	7.34
	12	10.33	3.17	8.65	6.14
Em 5256A	0	9.26	3.64	9.95	3.36
	2	--	--	10.05	3.16
	3	9.05	3.21	--	--
	4	--	--	9.52	3.36
	6	9.81	3.55	9.60	3.56
	8	--	--	7.98	3.56
	12	10.63	2.84	--	3.23

It is quite natural that the formation of chitin should be influenced by the relative concentrations of its main components, namely glucose and acetate. Reasons have already been given for believing that chitin molecules increase in size by coupling free glucose molecule to terminal glucose residues, followed by amination and acetylation. When the supply of glucose is in excess, then the chitin would have a large number of acetyl-glucosamine residues with a long non-acetylated end piece of hemicellulose nature. When the supply of glucose is limited or the supply of acetate is in excess, then chitin with relative short half molecule or branch will be formed with an end piece containing only few glucose residues. The acetate effect here is temperature sensitive, i.e., high temperature accelerates the acetylation process and makes both end piece and chitin half molecule short. The probable reason for this will be discussed later.

d. Acetylation process

The fact that the chitin half molecule from medium containing acetate at 35°C has, as a rule, a shorter end piece of few glucose residues indicates that the glucose residues have been acetylated with greater rapidity at 35°C than at 25°C. In the case of an excessive supply of acetate from without, the rapidity of acetylation is obviously attributable to the high concentration of acetate present. When the thermophobic strain is grown without an external supply of acetate, the rapid acetylation could be due to either a higher accumulation of acetate through metabolism or an increased activity of the enzyme concerned with acetylation. The acetylation process was studied in vitro by two methods, but neither gave positive results. Both homogenate and supernatant of lyophilized mycelium were used as enzyme preparation with glucosamine and potassium acetate as

substrates. The colorimetric method of Morgan and Elson (1934) was used for the detection of N-acetylglucosamine. The test gave a positive result when the enzyme preparation has not been boiled. Unfortunately, the test was also positive without adding substrates and no increase of N-acetylglucosamine could be detected with the additional substrates. It is, therefore, quite clear that a hydrolytic enzyme of chitin has been dealt with instead of the acetylation process of glucosamine.

In liver the acetylation process can be detected by a detoxication of sulfanilamide (Lipmann, 1945). The unacetylated sulfanilamide was determined by the colorimetric method of Bratton and Marshall (1939). This method has been tried for the study of enzymatic acetylation process in Neurospora homogenate and supernatant in the presence of potassium acetate and sulfanilamide as substrates hoping to detect the quantitative difference of acetylation process between the thermophobic and normal strains. The method is capable of detecting as little as 1γ consumption of sulfanilamide per cc with good reliability. No acetylation of sulfanilamide was detected with Neurospora preparations. Acetylphosphate which was synthesized according to the method of Lipmann and Tuttle (1944) has been used instead of potassium acetate in the study of enzymatic acetylation of sulfanilamide by Neurospora homogenate or supernatant. No acetylation of sulfanilamide could be detected by this method either.

Moreover, the acetylation process has been tested on growth tube cultures. Kaplan and Lipmann (1948) have shown that there is a widely distributed coenzyme called coenzyme A for the acetylation process in animals, plants and microorganisms. One of the important chemical constituents of coenzyme A is pantothenic acid (Novelli, Kaplan and Lipmann, 1949). It was thought that the pantothenic acid might thus also be thermophobic.

Calcium-pantothenate was tested by growth tube culture with practically no effect at all for either thermophobic or normal strains on sucrose medium up to 6mg per 10cc of medium. The ineffectiveness might be due to the limiting factor of the substrate acetate. Therefore Ca-pantothenate was tested in the presence of amounts of acetate too small to give a thermophobic effect to see whether it can enhance the thermophobic effect of acetate or not. The results show that it does not.

If the thermophobic character is due to the accumulation of acetate in cell through metabolism, then it would be expected that the excess of acetate might be removed by detoxication of sulfanilamide through acetylation. No improvement of growth was observed from the thermophobic strain on lactose medium at 35°C in the presence of either sulfanilamide alone or sulfanilamide and Ca-pantothenate together.

The main source of acetate in vivo is probably from pyruvate. In Neurospora, there is strong activity of pyruvate decarboxylase. The product of this reaction is acetaldehyde. If this is the very end product of the decarboxylation, as found in yeast, the process would be called straight decarboxylation (cf Baldwin, 1948). In animal tissue, the end product is acetate, and the process is called oxidative decarboxylation. It was thus interesting to see if the assumed excess of acetate in thermophobic strain would be due to the oxidative decarboxylation instead of the usual straight one. Pyruvate and acetaldehyde were tested manometrically on mycelium homogenate to see if there is the difference of oxygen uptake between the strains. Both strains, as has been mentioned, have very strong decarboxylation activity on pyruvate, but the oxygen uptake if there is any, is negligibly small for both strains with either pyruvate or acetaldehyde. It seems therefore that the pyruvate decarboxylation in

Neurospora is a straight one.

There is another enzyme found in liver and yeast called aldehyde mutase which brings about the Cannizzaro reaction (cf Sumner and Somers, 1943). Here, two molecules of an aldehyde undergo an oxido-reduction. One molecule is oxidized to an acid and the other is reduced to an alcohol. This was tested manometrically on homogenate of both strains using acetaldehyde as substrate in carbonate buffer. Since the oxidative activity on acetaldehyde is negligibly small, the test was thus run under air instead of nitrogen. No quantitative difference between the strains was observed.

The experiments mentioned in this section neither show that there is excess of enzyme concerning with acetylation nor indicate that more acetate is accumulated in the thermophobic strain.

e. Glucose-acetate ratio

Acetate has the thermophobic effect on both strains on sucrose medium although the effective concentration of acetate for the normal strain is about five times higher than that for the thermophobic strain. In order to see whether or not there is any definite critical ratio of molar concentrations between glucose and acetate that will manifest the thermophobic effect, media with different combinations of glucose and acetate concentrations were tested on both strains for thermophobic effects. The critical glucose-acetate molar concentrations that will cause thermophobic effect have been selected and plotted in Fig. 19. Each strain can tolerate a certain maximum concentration of acetate, namely, about 0.1 M for the thermophobic strain and 0.43 M for the normal one. Acetate concentrations higher than these will stop the growth of the respective strains not only at 35°C but also at

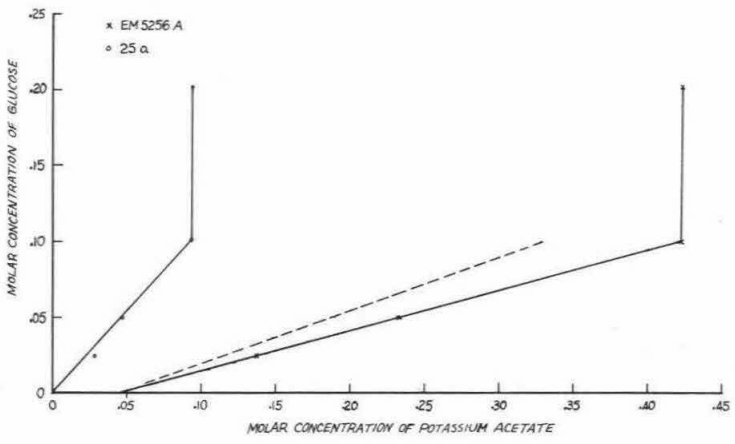


Fig. 19. Curves showing the glucose-acetate critical ratios of 25a and Em 5256A.

25°C as well, and the effect can no longer be cancelled by increasing the concentration of glucose. Below that fatal concentration of acetate, the thermophobic effect of acetate can be removed by increasing glucose concentration, and the straight line relationship shows that the critical ratio of glucose-acetate is always the same no matter what absolute concentration of acetate was used below the fatal concentrations. The critical glucose-acetate ratios are 1.02 for the thermophobic strain 25a and 0.21 for the normal strain Em 5256A. The ratio for 25a is thus about 5 times higher than that for Em 5256A. Surprisingly enough, the calculation shown in Table 23 indicates that at 35°C on sucrose medium, the thermophobic strain has the chitin with a length of half molecule only about one-fifth of the normal strain. This seems to be more than a mere coincidence due to chance.

If the difference between the critical ratio of glucose to acetate necessary to produce the thermophobic effect in the two strains were due solely to an increased rate of acetate production by 25a, then the amount of acetate produced by 25a must be directly proportional to the amount of glucose added to the medium. The excess acetate produced by 25a at each concentration of glucose must then be equivalent to an added external acetate concentration equal to the difference between the concentration necessary to produce the thermophobic effect in Em 5256A and that in 25a. The broken line in Fig. 19 represents the external concentration of acetate equivalent to the excess acetate produced by 25a at different sugar concentrations. From the curve it appears that for every mole of glucose added to the medium, 25a must produce an amount of acetate inside the cell which is equivalent to 2.7 moles added to the medium. Not only does this

seem like an excessive production of acetate, but it would mean that these must be a linear relation between acetate formed and glucose added over the entire range of concentrations tested. In this connection it may be noted that in liquid culture at 35°C with sucrose as carbon source, acetate stimulates growth (dry weight) of 25a, suggesting that acetate is not already present in excess. However, it should be noted that different phases of growth are measured by the liquid culture and the growth tube methods and it is not known how results of one type of experiment should be related to those from the other. It should also be remembered that a search for enzymes in 25a which might lead to enhance acetate production, especially at higher temperatures has not uncovered any differences between the two strains. This negative evidence is not conclusive but does support the supposition that the thermophobic characteristics of 25a are not primarily due to increased acetate production.

f. Conclusion

Studies on the cell wall materials of the thermophobic strain 25a and normal strain Em 5256A have shown striking differences between the chitinaceous material in the two strains. The molecular size (or structure) of the difficultly hydrolysable portion (probably true chitin) is different: 25a having an estimated maximum number of acetylglucosamine residues per half molecule or per branch of about 100 to 150, whereas Em 5256A has an estimated minimum number of over 500. The readily hydrolysable portion presumably constitutes terminal chains of glucose residues which are on the average about four times as long in Em 5256A as in 25a.

It has also been shown that when acetate concentration is high in

relation to glucose concentration, the end pieces in both strains become shorter, and in 25a the chitin half molecule itself is also reduced in size. When the glucose-acetate ratio reaches a critical value (1.02 for 25a and 0.21 for Em 5256A) growth in length of hyphae is almost completely inhibited (at 35°C on solid medium). It is tempting to postulate that the failure of growth at the critical ratio is a direct result of the shortness of the terminal end chains, presumably because of the inability of the organism to add additional glucose residues to chain unless there is a definite minimal number of unsubstituted glucose residues already present at the end of the chitin chain. It is possible that the thermophobic effect of such sugars as lactose and galactose occurs because these sugars give rise to relatively less glucose and consequently to a low glucose-acetate ratio when they are broken down.

DISCUSSION AND CONCLUSIONS

1. Origin and genetics of the thermophobic gene

The pedigree study of the thermophobic character among the Neurospora strains of California Institute of Technology collections indicates that the gene in each instance is traceble to strain Lindegren A (Beadle and Tatum, 1945). LA is not a vigorous strain, and is conidialess. Its sister strain La on the other hand is a vigorous strain with abundant conidia and is non-thermophobic. These two strains were extensively used by Beadle and Tatum in inducing chemical mutants by various means (1945). This is the reason why the thermophobic character is so widely distributed among the Neurospora strains of California Institute of Technology collections. The origin of the thermophobic character of LA is not known.

That LA was thermophobic was first inferred from pedigree studies of the known thermophobic strains and was proved to be by tube culture on lactose minimal medium. This was found after all the experiments have been done on the thermophobic strain 25a, so that this original thermophobic strain LA has never been used in investigation here. The thermophobic character is controlled by a single gene, t, linked with the three para-aminobenzoic acidless loci, p_1, p_2 and p_3. All of these four loci are quite far away from the centromere with the nearest one p_1 a distance of about 27.27% crossover units from the centromere. The order of the other three loci can not be determined with certainty, until more loci are found in this linkage group. However, a tentative chromosome map was constructed with a probable loci sequence of: centromere, p_1, p_2, p_3 and t.

2. Mechanism of the thermophobic gene action

Experiments and results described individually in the previous sections have been left without a unified explanation to link them together. It will now be attempted to show how all the experimental evidences can be related to a single system.

In earlier studies, three conditions were necessary for the detection of the thermophobic character, namely, a certain kind of sugar as carbon source, an agar medium, and a critical culture temperature. The question why this character should require these conditions for its expression, can now be answered from new experimental evidences. The experiment on the antagonistic interaction between acetate and glucose shows that there is a definite critical molar ratio of glucose-acetate which just causes inhibition of growth. This critical ratio changes with temperature. For instance, a ratio which inhibits the growth at 35°C, will lead a normal growth at 25°C. In other words, the critical ratio has a positive temperature effect, namely, increase of temperature would have a tendency to increase the critical ratio. More simply, the thermophobic effect is thus attributable to the effect of acetate.

The critical ratio is different for the thermophobic and normal strains. The thermophobic strain has a critical ratio about five times higher than that of normal strain at 35°C. Thus the difference between thermophobic and normal strains is rather quantitative. Probably, this should be expected from the nature of so-called temperature mutants in Neurospora, as Emerson (1950) has suggested that a gene which causes the change in the relative temperature coefficient of a certain reaction of biological importance will be expressed as a temperature mutant.

Acetate is universally recognized as a common intermediate of carbohydrate metabolism (Bennet-Clark, 1933). Chughtai et al (1947) have shown that appreciable quantities of acetate are present inside the cells of Aspergillus niger though none could be detected in the external glucose medium. The most probable route for the generation of acetate from hexose is through the three carbon compounds namely lactic and pyruvic acids (glycolysis cycle). However, the possibility of a C_4-C_2 split of hexose is not ruled out completely (Allsopp, 1937) and from C_4 and C_2 fractions acetate also could be generated. Although attempts have been made to detect the route of acetate formation in Neurospora without success, it seems to be without doubt that acetate must be formed through the metabolism of carbohydrate anyway. Thus, in vivo, the acetate produced would establish a glucose-acetate ratio with the available glucose either from without or from the convertion of other carbohydrate. The relative growth of Neurospora on medium containing monosaccharides other than glucose indicates that this fungus can not use them efficiently. This low efficiency of utilization might be due to the fact that they should be converted first to glucose and then metabolized. If this is so, then the relative efficiency of utilization of these monosaccharide would mean the relative efficiency of their convertion to glucose. It becomes obvious then that hexose of low convertion efficiency would establish a low glucose-acetate ratio in vivo. This ratio would become critical when the culture is grown at a relatively high temperature, and as a consequence normal growth would be impaired. Moreover, the high temperature would facilitate the consumption of carbohydrate, and thus increases the out put of acetate and decreases the available glucose and consequently an even lower glucose-acetate ratio than critical

could be established. Since the critical ratio for the normal strain is much lower than that for the thermophobic strain, it is easy to visualize that within a certain range of temperature a particular sugar could be thermophobic to the thermophobic strain but not to the normal.

For the oligosaccharides the same argument could be applied with an additional complication, namely, the hydrolytic enzymes involved here in splitting the polysaccharides to its monosaccharide components. There is strong evidence to show that Neurospora is unable to use certain oligosaccharides as such without first hydrolyzing them. If glucose is one of the components of the oligosaccharide, then the decisive factor to whether this oligosaccharide would be thermophobic or not will be the ability of the hydrolytic enzyme to split it. Therefore, sucrose is not thermophobic because there is very strong invertase activity in both medium and cell, and lactose is thermophobic, because there is very low lactase activity in the order of one-hundredth of that of the invertase in the mycelium.

When there is no glucose among the components of the polysaccharide, as in inulin, then the decisive factor for it to be thermophobic will be the combined effect of hydrolytic activity and the convertion ability of the hexose. Fructose is thermophobic in less extent than inulin. Thus it would be expected that in Neurospora the inulase activity must be low as was observed by Emerson (personal communication).

Since the Krebs' acids can either be transformed to acetate, probably through the common intermediate oxalacetate, or can function as inhibitive agents to prevent the incorporation of acetate into Krebs' cycle, they should all increase the total free acetate, it would thus not be surprising that they should all have thermophobic effect on 25a on sucrose medium.

Calcium ions have been widely used in the industry of biological synthesis of many acids as oxalic, citric, fumaric, malic and others as a trapping agent to the end that very much larger proportion of glucose are transferred into the acids (cf Foster, 1949). On this basis, the thermophobic effect of $CaCl_2$ observed here might thus be interpreted as to help the accumulation of acetate or its precusor acids.

It is not necessary to assume that all of the acetate produced is accumulated or used as such. Neurospora can grow though to a quite limited extent in a medium with acetate as sole carbon source. The central role of acetate in the di- and tri-carboxylic acid metabolism of fungi was first proposed by Chrzaszcz and Tiukow (1930) and has since then gained general credence. Methylene blue has a positive effect on oxidation process and may thus help the further oxidation of acetate in some extent. This will lower the acetate accumulation and would therefore help to permit more growth at high temperature after activation at 25°C.

Probably it is wrong to regard the thermophobic character as purely a growth character, since with flask liquid culture no consistent difference in the dry weight of mycelium can be observed in lactose medium at 35°C. Instead, an apparent morphological difference can be noticed between the strains in liquid cultures. Therefore, the thermophobic character is actually a morphological temperature mutant. However, it would be quite difficult to score the morphological difference, semicolonial growth in the thermophobic strain from the usual filamentous growth in the normal, in a quantitative way. It is obvious then that the colonial form of growth would hardly give any linear measure of growth in growth tube culture. Hence the growth measurement of thermophobic effect of a strain in growth

tube culture is actually an inverse measure of the degree of development of the colonial habit. A difference in length in growth tube is much more easy to be observed and measured than a difference of growth form in liquid culture. That is the reason why this character was first discovered in tube culture and studied so extensively as a quantitative growth difference but without positive result until the morphological difference in liquid culture was noticed, and lead to studies of the Neurospora cell wall material.

The study of Neurospora chitin reveals the molecular structure of chitin as a molecule bearing a heterogeneous end group of glucosamine and glucose residues. This end group is stained by chlorozinc iodine to a deep blue color. When this end group is removed by mild acid hydrolysis or by $KMnO_4$ selective oxidation, the chitin thus treated would be no longer stained by iodine. Therefore, the iodine stainability of chitin becomes a good measure of the length of the unacetylated end group in a qualitative manner. This was confirmed by quantitative measurement of glucose hydrolysed off by diluted acid. This finding immediately suggests the way of chitin molecule formation through the sequence of glucose coupling, amination and acetylation.

The more important finding is that the length of end group is closely related to the expression of thermophobic effect or in other words to the degree of colonization of the mycelium. The mycelium of 25a in a semicolonial form has an end group of only few glucose residues, while in the filamentous form it has more than 16. On the other hand the normal strain in normal form of growth has an end group of 40 to 50 glucose residues. Now the acetate effect or the effect of glucose-acetate ratio on the expression of thermophobic character of a strain becomes evident. Low glucose-acetate ratios will obviously lead to the acetylation of most of the glucose residues

on the end group.

The relation between the length of unacetylated end group of chitin and the glucose-acetate ratio seems to be not very simple. As shown by the result presented in Table 24, the rate of glucose coupling on the end of chitin molecule is a function of the length of end group. The shorter the end group is, the greater rate of glucose coupling will be. And a longer end group will evidently facilitate the acetylation process and thus can endure lower glucose-acetate ratio. Therefore, this is the reason why there is big difference in critical glucose-acetate ratios between thermophobic and normal strains. The normal strain has an end group nearly 4-5 times longer than that of the thermophobic strain, and thus can endure a critical glucose-acetate ratio about 5 times lower than that for the thermophobic strain.

Now, most likely, the substance E previously postulated to accumulate during activation at 25°C (p. 21) is to be identified with the coupled glucose residues on the end group. At 25°C more glucose units are coupled than are acetylated, and consequently the end group increases in length. Since the rate of glucose coupling is inversely proportional to the length of end group, it would be naturally to have a change in the rate of accumulation of E (or coupled glucose) when a certain level of E is reached, as shown by the abrupt change of slope of the straight line relation between the total growth (or E) at 35°C and length of activation period at 25°C (Fig. 3). It is obvious that the rate of accumulation of E should be delicately balanced by the rate of glucose coupling and the rate of acetylation process. It is not without reason thus to believe that these rates of glucose coupling and acetylation should also be influenced by other things than the length

of the end group. This might be the reason why the accumulation rate of E varies with the previous history of culture as shown in Fig. 3. Without the support of further experimental evidences no detailed explanation can be offered to explain how the rate of accumulation is influenced by various internal and external conditions.

Since E is really a number of glucose residues coupled to the chitin proper, and thus can not be extracted by hot water, then its accumulation would naturally not be detected in test for the accumulation of reducing sugar in the cell by the method used in permeability experiments (p. 29).

In the activation experiments, the length of the end group has been expressed as a substance E. The quantity of E then is inversely related to the extent of colonial growth. Since the tube growth measurement here is actually a measure of colonization, then indeed, E should be quantitatively correlated with the growth measurement as expressed in Fig. 4. It is remarkable that the growth curve relationship should so adequately express a morphological character of this sort.

It is probable that the relation between the length of end group and the degree of colonial growth is by no means direct. The calculation presented in Table 23 indicates that the immediate cause of the colonial habit is the molecular structure of chitin as a whole. Estimates show that the normal strain has a chain length of chitin half molecule of 500 to 600 N-acetylglucosamine residues while the thermophobic strain only has 100 to 200. When this chain length is below 100, the growth will begin to show semicolonial form. This shortness of chain length of half chitin molecule of thermophobic strain could mean that there really is a short straight chain molecule, or that there are many short branches on each

molecule. The shortness of chitin molecule of thermophobic strain must be a fairly direct expression of the thermophobic gene. As a result of shortness of chain, there must be more end groups per unit weight of chitin. When a limited quantity of glucose molecules are distributed to many end groups, each group would naturally acquire fewer glucose residues. This shortness of end group would in turn tend to retard the lengthening of the chitin chain. Therefore any environment which would result in a shortening of the end groups would thereby prevent the effective lengthening of the chitin chain and in extreme cases the chitin chain would be sufficiently short to result in the expression of the semicolonial or colonial form of growth. It is then easy to see that the length of end group should have a significant influence upon the total length of chitin molecule, and then in turn to influence the growth morphology.

The temperature effect of thermophobic is attributable to the acetate effect. But the reason for that is not quite clear. In flask culture, the dry weight increases enormously for both thermophobic and normal strains in the presence of acetate at 35°C but much less at 25°C when the glucose supply is in excess. This seems to indicate that the acetylation process of the chitin end group has a relatively high temperature coefficient. No comparable evidence has been found in the literature so far as the author is aware. Since attempts to find the enzymes responsible for acetylation in Neurospora were unsuccessful, no further evidence for the dramatical acetate effect is available. However, it should be pointed out here that the enzymatic process of acetylation of the end group of chitin in vivo should be somewhat different from the usual one for the reason that one of the substrate here, i.e., the end group of chitin, is quite fixed on the cell wall and one dimension of its free motion is thus

fixed.

As to the functional mechanism of the thermophobic gene, the gene most likely first causes the shortness of the chitin half molecule. Perhaps the simplest way to account for this is on the assumption that the thermophobic gene promotes the production of an enzyme which catalyzes the formation of 1-6 glycosidic linkage in addition to the usual 1-4 linkage of the chitin molecule. This would result in chitin molecules of very branched form, with each branch much shorter than half molecule of a straight chain.

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