RESPIRATORY BLOCK IN THE DORMANT SPORES OF *NEUROSPORA TETRASPERMA*

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(WITH FIVE FIGURES)

Introduction

SHEAR AND DODGE (**26**) have shown that the ascospores of *Neurospora tetrasperma* are dormant, but that they may be induced to germinate by a short period of heating. This process of heat activation was studied by GODDARD (**7**), who found that heating the dormant spores to temperatures of over 50° C. for a few minutes induced germination two to three hours after returning them to room temperature. Further, he found that the activation is reversible. If the respiration of the activated spores was prevented for several hours by anaerobic conditions or the addition of cyanide, upon returning to conditions favorable for respiration, the spores failed to germinate; that is, they had been de-activated. If the de-activated spores were re-activated by a second heat treatment, they germinated normally. The activation of the spores induced a large increase in the respiratory rate (8 to 40 times), and this high rate of respiration had to continue for two to three hours if germination was to occur. Upon germination, a second increase in the respiratory rate was found, nearly doubling that of the activated spores. Thus, he recognized three phases in the rate of respiration of the ascospores: (1) that of dormant spores, (2) that of activated spores (one-half hour to two hours after the heat treatment), and (3) that of germinating spores (the spores in which germ tubes may be seen under the microscope).

In this paper we have tried to determine what constitutes the respiratory block of dormant ascospores of *Neurospora tetrasperma,* or, conversely, what part of the respiratory mechanism undergoes heat activation. It is to be realized that activation and germination occur readily in distilled water, so that an external substrate is not essential for activation or germination. We may assume concerning the respiratory systems of dormant and activated spores, either (a) that the dormant and activated respiratory mechanisms are qualitatively dissimilar or even independent, and that heat treatment causes a *de novo* appearance of a system inactive in dormant spores, or (b) that the same mechanism is functional in dormant and activated spores, but that in dormant spores the rate is limited by the slowest process in the total reaction chain, and that this slow reaction is greatly accelerated by the heat treatment, allowing the total respiratory chain to proceed at the rate found in activated spores. If the first assumption is correct, the respiratory block is the inactivity of the reaction which prevents the second

respiratory mechanism from functioning, while if the second assumption is correct, the limited activity of a certain reaction constitutes the respiratory block.

We have accepted the WARBURG-KEILIN scheme of cellular respiration as a working theory, and we have summarized this scheme in abbreviated form in diagram I. For reviews of this theory, see KEILIN (**10, 11**) and

DIAGRAM I

PHAEOHEMIN—CYTOCHROME—DEHYDROGENASE THEORY

(1)	Reduced phaeohemin	+ O_2		Poisoned by CO		Oxidized phaeohemin		
(2)	Oxidized phaeohemin	+ Reduced cytochromes		Poisoned by HCN		Reduced phaeohemin	+ Oxidized cytochromes	
(3)	Oxidized cytochromes	+ Reduced dehydrogenases				Reduced cytochromes	+ Oxidized dehydrogenases	+ H_2O
(4)	Reduced substrate	+ Oxidized dehydrogenase				Oxidized substrate	+ Reduced dehydrogenase	

Oxidized substrate may undergo reaction with a second dehydrogenase, or it may undergo decarboxylation and then oxidation. Flavine enzyme, coenzymes, etc., omitted. Either (3) or (4) inhibited by urethanes.

MELDRUM (**17**). Certain workers have long believed that the first reactions in respiration are identical with the first steps of alcoholic fermentation. The older literature supporting this opinion is reviewed by KOSTYCHEV

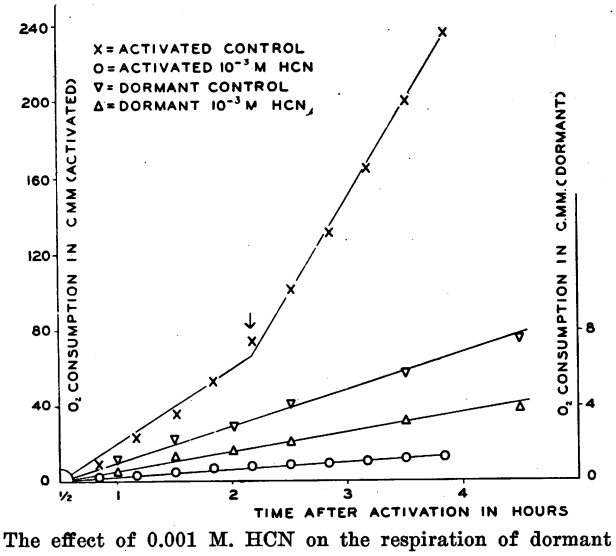

FIG. 1. The effect of 0.001 M. HCN on the respiration of dormant and activated ascospores, 6.48 mg. dry weight of spores per vessel. The arrow indicates the beginning of germination.

(**13**) and, more recently, by TURNER (**32**). Since in the work which follows we have had to discuss certain reactions which are usually considered as part of the mechanism of alcoholic fermentation, we have summarized the MEYERHOF-KIESSLING theory of alcoholic fermentation in diagram II. For a review of this theory, see MEYERHOF (**19**) and MEYERHOF AND KIESSLING (**22**).

DIAGRAM II

MEYERHOF-KIESSLING SCHEME OF ALCOHOLIC FERMENTATION

Initial phase

(1) Glucose + Hexosediphosphate + $2H_3PO_4$ $\rightleftharpoons$ 4 triosephosphate

(2) 4 triosephosphate $\overset{\text{poisoned by iodoacetate}}{\rightleftharpoons}$ 2 phosphoglyceric acid and 2 glycerophosphoric acid

Stationary phase

(3) 2 phosphoglyceric acid $\overset{\text{poisoned by NaF}}{\rightleftharpoons}$ 2 phosphopyruvic acid

(4) 2 phosphopyruvic acid + glucose $\longrightarrow$ hexosediphosphate and 2 pyruvic acid

(5) 2 pyruvic acid $\overset{\text{carboxylase, co-carboxylase}}{\longrightarrow}$ 2 acetaldehyde + 2 CO_2

(6) Glucose + $2H_3PO_4$ + 2 acetaldehyde $\overset{\text{catalyzed by hexosediphosphate}}{\longrightarrow}$ 2 triosephosphate and 2 acetaldehyde

(7) 2 triosephosphate and 2 acetaldehyde $\overset{\text{poisoned by iodoacetate}}{\longrightarrow}$ 2 phosphoglyceric acid and 2 alcohol

Methods

The *Neurospora tetrasperma* culture was the same strain used in the earlier work (**7**), and the same method of collecting and separating the ascospores was followed. The cultures were grown at 25° C. for three to four weeks in 500-ml. Blake bottles on 35 to 50 ml. of agar 0.5 per cent. for glucose and Difco malt extract. The spores were stored at constant relative humidity over saturated NH_4Cl/solid NH_4Cl. In the earlier experiments the spores were stored at room temperature, but in the later experiments at about 4° C. Spores stored at room temperature gave fairly uniform results for several weeks; those stored at 4° C. have given very uniform results over several months. In the experiments reported here, the spores from more than four thousand cultures were used, and we wish to thank Miss EUGENIA SHERIDAN who has carried out much of the laborious culture work.

All measurements were made with the FENN (**6**) volumetric micro-respirometer, at 25° C., unless stated to be at 15° C. This instrument is a

closed system, and is very satisfactory for the low rates of dormant spores. The rate of shaking was 180 complete cycles per minute, through an arc of 1.5 cm. A small spherical glass bead was included in each vessel to enhance stirring. The high density (about 1.18) of the spores caused some shaking difficulties, but in none of these experiments was the rate of shaking limiting. Carbon dioxide was absorbed in KOH insets, unless respiration was measured in the presence of carbon dioxide; in this case, WARBURG'S indirect method (5) was used. In the HCN experiments (KREBS, 14), KOH/KCN mixtures were used in the insets. The spores were suspended in M/60 phosphate buffers at a pH of 5.4 or in distilled water. The substrates and poisons were neutralized and made up in the same buffer. The poisons or substrates were added to the vessels at the beginning of an experiment, with other vessels serving as controls. All experiments were run in duplicate, and the experiments reported are only a few of many which gave similar results. The volume of cells used in an experiment was determined by centrifuging to constant volume in hematocrit tubes. Very uniform results may be obtained with *Neurospora spores* by this means. In a few cases, both dry weight and hematocrit determinations were made on the same suspension, and it was found that the volume multiplied by 0.36 equals the dry weight. In most experiments, the $-Q_{O_2}$ was calculated from volume determinations by the use of this factor.

The spores as collected were dormant. Activation was accomplished by heating the spore suspension in a test tube for twenty minutes at 54–56° C. in a water bath, and then cooling to room temperature. Zero was taken as the time at which the spores were removed from the 54° C. bath.

Pure nitrogen for anaerobic experiments was obtained by passing tank nitrogen over freshly reduced copper in an electric furnace. A small piece of yellow phosphorus was used in the inset to absorb any oxygen not swept out of the vessel with nitrogen. Carbon monoxide was prepared by the dehydration of concentrated formic acid with hot sulphuric acid, and was washed with KOH. Gas mixtures were made by volume, and were not checked by analysis.

The hexosediphosphate was a gift from the Winthrop Chemical Company. It was converted to the sodium salt, decolorized with charcoal, and re-precipitated as the calcium salt with alcohol. The calcium salt of the hexosediphosphate was converted to the sodium salt by adding the theoretical amount of sodium oxalate and filtering. The solution of sodium hexosediphosphate was neutralized, when necessary, with HCl. The methyl glyoxal was prepared by M. P. SCHUBERT of the Rockefeller Institute. The pyruvic acid was made by the method of HOWARD and FRASER (9), and was distilled at low pressure.

Results

The respiratory increase on activation and germination

A few typical results are listed in table I. The respiratory rate of activated spores varies from about 8 to 40 times that of the dormant spores,

TABLE I

Respiration of dormant, activated, and germinating spores
Oxygen consumption as $-Q_{O_2}$*

Spore lot	Spore age	Dormant	Activated 1–1.5 hr. after activation	Germination 3–5 hr. after activation
	wk.			
A	3	0.25, 0.27	10.86	19.45
A	9	0.53, 0.59	4.48	9.62
B	3	0.25, 0.24, 0.27	10.36	19.60

* $-Q_{O_2} = \frac{\text{cu.mm. oxygen consumed per hr.}^1}{\text{dry wt. in mg.}}$

1 The symbols Q_{O_2}, Q_{CO_2}, etc. are by convention negative when the gas is consumed and positive when it is produced.

depending upon the age of the spores. The $-Q_{O_2}$ of dormant spores increases from about 0.25 when first collected to 0.50–0.70 when several weeks older, while the $-Q_{O_2}$ of activated spores decreases from about 10 when first collected to about 5–6 several weeks later. If the spores are stored at 4° C. the values remain practically constant at 0.5 and 5 to 6 respectively for several months. When the spores have been stored for 3 to 4 months at room temperature the $-Q_{O_2}$ values of dormant and activated spores have decreased to about 0.08 and 1 to 2 respectively, and only a few per cent. of the spores germinate after a heat treatment.

Approximately two hours after activation, if respiration has been allowed to proceed normally at 25° C., and four hours, if at 15° C., a second change in respiratory rate occurs, and this corresponds with the first appearance of the germ tubes. The respiratory rate of germinating spores also varies with the age of the spores, but in such a manner that the absolute value is about twice that of the activated rate. The average value from ten experiments with spores of various ages was 1.98 times the activated rate, and the range was from 1.79 to 2.15. The number of spores germinating seemed to be quite independent of the rate of oxygen consumption as long as the $-Q_{O_2}$ for activated spores was 4 to 5 or higher.

Effect of oxygen and carbon dioxide pressure on respiration

It is possible that the permeability of the dormant spores to carbon dioxide or oxygen limits the respiratory rate. If permeability to oxygen

TABLE II

EFFECT OF O_2 AND CO_2 PRESSURE ON DORMANT SPORES
OXYGEN CONSUMPTION AS $-Q_{O_2}$

GAS PRESSURE	OXYGEN*			CO_2†	
	A	B	C	D	E
mm. Hg.					
0	—	—	—	0.33	0.71
37	0.15	—	—	0.30	0.65
75	0.15	—	0.66	0.37	0.66
150	0.17	0.27	0.65	0.30	—
190	—	—	—	—	0.58
300	0.16	0.26	—	—	—
750	—	—	0.77	—	—

* CO_2 pressure = zero.
† O_2 pressure = 150 mm.

were limiting, we would expect an increase in the respiratory rate by raising the partial pressure of oxygen. The data in table II show that raising the oxygen pressure from 37 to 300 mm. has practically no effect on the rate. If the retention of CO_2 by the spores were limiting, it would be expected that increased pressures of CO_2 would decrease the rate. The data in table II show that this is not the case. The results reported in table III

TABLE III

EFFECT OF OXYGEN TENSION ON ACTIVATED AND GERMINATING SPORES
OXYGEN CONSUMPTION AS $-Q_{O_2}$

OXYGEN PRESSURE	A 25° C.		B 25° C.		C 15° C.	
	ACTIVATED	GERMINATING	ACTIVATED	GERMINATING	ACTIVATED	GERMINATING
mm. Hg.						
75	—	—	6.04	6.90	3.07	7.55
150	6.94	10.17	7.91	15.65	3.15	7.03
300	—	—	8.00	16.60	3.23	—
450	8.90	11.40*	—	—	—	—
750	8.92	10.25*	—	—	—	—

* High partial pressures prevent normal germination.

show the effect of oxygen tension on germinating and activated spores. At oxygen tensions below 20 per cent., the oxygen diffusion rate may be limiting the rate of respiration in activated and germinating spores at 25° C. but not at 15° C., where the absolute rate is lower. It seems very improbable that the permeability of the dormant spores to CO_2 or oxygen governs their rate of oxygen consumption.

THE PHAEOHEMIN-CYTOCHROME SYSTEM

The work of WARBURG and KEILIN, which is briefly summarized in diagram I indicates that the cyanide-carbon monoxide sensitive respiration of most cells is catalyzed by the phaeohemin-cytochrome system. From diagram I it can be seen that both cyanide and carbon monoxide poison the phaeohemin enzyme. In an attempt to determine whether the rate of respiration of dormant spores was limited by the activity of the phaeohemin enzyme, we have studied the effect of cyanide and carbon monoxide on the respiration of dormant and activated spores. The results are shown in figures 1 and 2 and in tables IV and V. It is apparent from these results

TABLE IV

PERCENTAGE INHIBITION OF RESPIRATION OF SPORES BY HCN

HCN CONCENTRATION	DORMANT	ACTIVATED	GERMINATING
1.0×10^{-4} Molar	7.2; 5.4; −7.5	41.6; 23.2	55.6; 53.4
1.0×10^{-3} Molar	49.2; 45.3	86.8; 86.8	94.4; 94.1†
1.0×10^{-2} Molar	35.5*	87.5	95.6†

* Result not very reproducible; HCN poisoning is not reversible at 1.0×10^{-2} molar. Lower concentration always reversible. 1.0×10^{-2} molar sometimes stimulates dormant spores slightly, while 1.0×10^{-3} molar always inhibits.

† Controls germinating; HCN prevents poisoned spores from germination.

TABLE V

PERCENTAGE INHIBITION OF RESPIRATION OF SPORES BY CARBON MONOXIDE*

GAS MIXTURE	DORMANT	ACTIVATED	GERMINATING
90% CO†/10% O_2	5.6; −5.0; 7.7; 15.4	53.3; 43.4; 50.2; 31.6; 38.3; 42.3	54.7; 17.1; 26.0; 8.8
95% CO‡/5% O_2	21.4; −6.0	73.3	———
Av.	5.7	43.15	23.64

* In the dark.

† Controls 90% N_2; 10% O_2.

‡ Controls 95% N_2; 5% O_2; these results at 15° C.

that the respiration of the activated spores is much more sensitive to these poisons than is the respiration of the dormant spores. Not only are the dormant spores less sensitive to these poisons, but about 50 per cent. of the respiration is entirely resistant to cyanide. A comparison of the absolute rates of respiration of cyanide poisoned dormant and activated spores is of interest. The results from one experiment gave the following: the $-Q_{O_2}$ values for dormant spores and dormant spores plus 10^{-3} and 10^{-2} molar HCN were 0.27, 0.14, and 0.17 respectively. The corresponding values for

activated spores were 10.86, 0.65, and 0.30. These $-Q_{O_2}$ values show that while most of the activated respiration is poisoned by HCN, cyanide does not bring the respiration quite as low as that of cyanide poisoned dormant spores. Cyanide at 10^{-3} and 10^{-2} molar inhibits 93.5 and 97.1 per cent. of the increased respiration due to activation (the increased respiration equals

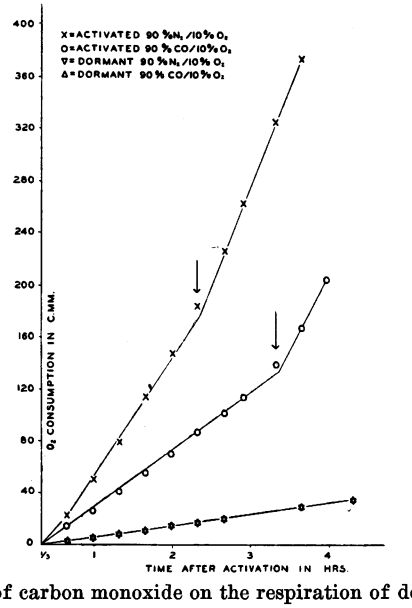

FIG. 2. The effect of carbon monoxide on the respiration of dormant and germinating ascospores, 7.95 mg. of spores per vessel in the activated experiment and 14.90 mg. of spores per vessel in the dormant experiment. The arrows indicate the beginning of germination.

the activated respiration minus the dormant respiration). The cyanide resistant respiration may be catalyzed by WARBURG'S flavine enzyme since it is known (**30**) that the enzyme is not cyanide sensitive. However, it is clear that 93 to 97 per cent. of the increased respiration due to activation is cyanide sensitive, so that the activity of the flavine enzyme (if it is present) cannot account for the increased respiration on activation. The carbon monoxide results show that the dormant respiration is almost completely carbon monoxide resistant, while the activated respiration is definitely carbon monoxide sensitive.

If the rate of respiration of the dormant spores is limited by the concentration of active phaeohemin, the dormant respiration should be as sensitive to cyanide and carbon monoxide as is the respiration of the activated cells. However, if the concentration of the phaeohemin is as high in the dormant spores as in the activated spores, only 1/8 to 1/40 of the enzyme is essential to maintain the observed rate, and poisoning a large percentage

of the enzyme with carbon monoxide or cyanide will have little or no effect on the dormant rate, while a large inhibition of the activated rate may be expected. This is what has been found experimentally. Cyanide at 10^{-4} molar should combine with about 50 per cent. of the phaeohemin, but it has no effect on the dormant respiratory rate. Higher concentrations, as 10^{-3} molar, will combine with 95 per cent. or more of the enzyme. The affinity of carbon monoxide for phaeohemin is much less than is that of cyanide, and it may be impossible, at pressures of atmospheric or less, to tie up sufficient phaeohemin in dormant cells to produce an appreciable inhibition. Following this line of argument, we have interpreted our cyanide and carbon monoxide results to mean that the respiratory rate of the dormant spores is not determined by phaeohemin activity.

In the WARBURG-KEILIN scheme, cytochrome is believed to act as a carrier between phaeohemin and the dehydrogenases. No specific cytochrome poison is known. Cytochrome activity may be followed spectroscopically in some cases, but the black spore pigment has made that impossible in this work. One method of attack on phaeohemin-cytochrome systems is the one introduced by BARRON (**2**), and used by many workers, of "short circuiting" both the phaeohemin oxidase and cytochrome by the addition of autoxidizable dyes (such as methylene blue, pyocyanine, and thionine) to the cell suspensions. These dyes are reduced by dehydrogenase-substrate systems and re-oxidized directly by atmospheric oxygen, and therefore these dyes may replace both oxidase and cytochrome. Another dye which RUNNSTROM (**25**) has used in paraphenylenediamine. This dye is not autoxidizable. It is reduced by the dehydrogenase-substrate systems, and its oxidation is catalyzed by phaeohemin. This dye will supplement cytochrome, but not phaeohemin. We have added such dyes to dormant spores, and no significant change in the respiratory rate has been detected, as is seen in table VI. These results may be interpreted to mean that the respiratory block is not

TABLE VI

EFFECT OF DYES ON DORMANT SPORES
OXYGEN CONSUMPTION AS Q_{O_2}

DYE	CONTROL	EXPERIMENTAL
Methylene blue 0.01%	0.23	0.31
Pyocyanine M/5000	0.31	0.24
Pyocyanine M/10,000	0.31	0.31
Paraphenylenediamine M/1000	0.21	0.28*
Paraphenylenediamine 10 mg. per vessel	0.37	0.46†

* Not corrected for autoxidation of the dye.
† Corrected for autoxidation of the dye.

in the phaeohemin-cytochrome system, or that the spores are impermeable to the dye, and that no conclusions can be drawn from these experiments.

To prove definitely that the oxidase is phaeohemin, light reversal of carbon monoxide poisoning should be demonstrated. Attempts to obtain light reversal of carbon monoxide poisoning were largely unsuccessful, probably because of the black pigment in the spore walls. The results all show slightly less inhibition by carbon monoxide in the light (7 cm. from 100-watt Mazda lamps) than in the dark, but the differences were hardly larger than the experimental error and are not reported here.

Paraphenylenediamine may be used to measure the activity of phaeohemin, by the method of KEILIN (**10**). In this method, a large amount of dye (10 to 23 mg.) is added to the cell suspension, and the oxygen used in oxidation of the dye is measured. Proper controls are used to correct for the autoxidation of the dye and for the cellular respiration (or this may be poisoned with urethane, etc.). The rate of the catalyzed oxidation of the dye is a measure of phaeohemin activity. Such an experiment was tried with dormant spores with negative results. This may mean that there is no phaeohemin activity (which would conflict with our CO and HCN results), or, more likely, that the cells are impermeable to the dye.

DEHYDROGENASE-SUBSTRATE SYSTEMS

It seems to be clearly established from the work of KEILIN (**10**) and others that the respiratory substrates do not react directly with oxidized cytochrome, but that the substrates first react with intermediary enzymes, the dehydrogenases of THUNBERG (**31**). The usual method of determining dehydrogenase activity is the methylene blue technic of THUNBERG. An attempt to use this method with *Neurospora* spores failed because the black spores removed all of the dye from solution, and the spore color made it impossible to determine whether the dye was reduced or not. (An attempt to determine this spectroscopically also failed.) The dye may not be extracted by centrifuging with water, though it can be extracted with acetone.

We know no truly specific dehydrogenase poison. Though SVENSSON (**29**) has shown that urethane will poison dehydrogenases, other enzymes are also poisoned by urethane. We have studied the effect of ethyl urethane on the respiration of dormant and activated spores, and a typical experiment is shown in figure 3. A higher concentration of urethane (5 per cent.) caused at first a marked decrease in respiration of dormant spores, followed by stimulation. The stimulation is probably associated with irreversible injury. If the percentage inhibition of respiration by 2.5 per cent. urethane is calculated from the data in figure 3 for dormant, activated, and germinating spores, the following results are obtained: 48, 19, and 62 per cent.

respectively. The greater sensitivity of the dormant than of the activated spores to urethane might be interpreted to mean that the dehydrogenase systems are limiting the dormant rate. However, we have not been able to obtain more direct evidence on this point. The results reported later on carboxylase activity seem to be sufficient to explain the respiratory increase following activation, without the necessity of a change in dehydrogenase

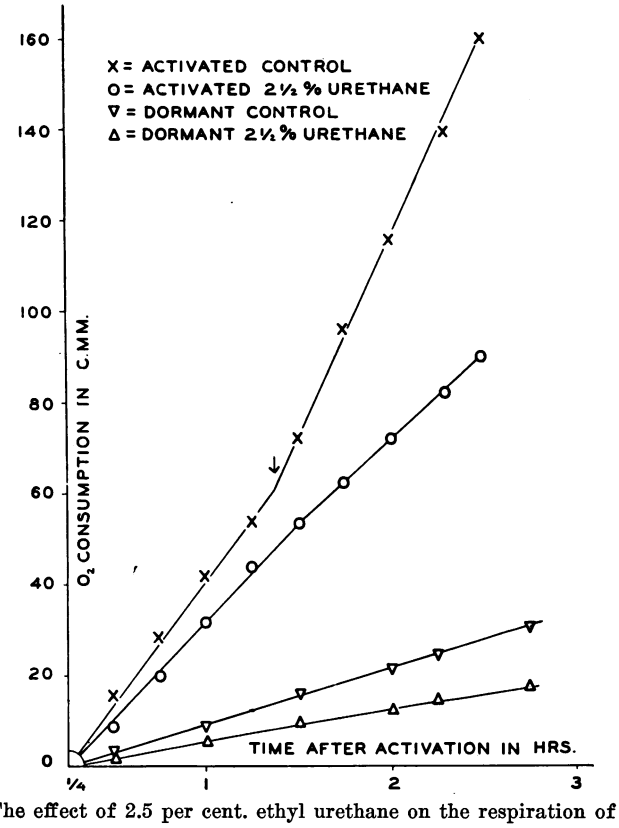

FIG. 3. The effect of 2.5 per cent. ethyl urethane on the respiration of dormant and activated spores. The arrow indicates germination; the poisoned activated spores did not germinate. 10.1 mg. of spores per vessel in the activated experiment, and 40.4 mg. of spores per vessel in the dormant experiment.

systems. The low sensitivity of activated spores to urethane remains without explanation.

Since spores may undergo activation and germination in distilled water, it is clear that the amount of stored food is not limiting the respiration of dormant spores. However, it is possible that though sufficient stored substrate is present, the rate at which it undergoes hydrolysis, phosphorylation, or other anaerobic reactions may be limiting the rate of respiration. If this

were the case, the addition of the proper intermediate substrate should cause a marked increase in the respiratory rate of dormant spores. It is seen from the results in table VII that the only substrates which have any

TABLE VII

EFFECTS OF SUBSTRATES ON OXYGEN CONSUMPTION OF DORMANT SPORES
VALUES GIVEN AS $-Q_{O_2}$

SUBSTRATE	CONCENTRATION	SUBSTRATE RESPIRATION	CONTROL	PERCENTAGE STIMULATION
Glucose	M/50	0.345	0.355	-2.8
Sodium hexosediphosphate	M/50	0.344	0.355	-3.2
Acetaldehyde	M/50	0.707	0.355	99.2
Above three together	M/50	0.737	0.355	107.6
Ethyl alcohol	M/20	1.150	0.529	117.5
Acetaldehyde	M/50	1.152	0.529	117.8*
Alcohol plus acetaldehyde	M/20 + M/50	1.178	0.529	122.8
Methyl glyoxal	M/50	0.420	0.357	15.0
Sodium succinate	M/20	0.550	0.528	4.16
Pyruvic acid	M/20	0.296	0.273	8.40
Sodium glycerophosphate	M/20	0.286	0.273	4.78
Sodium acetate	M/40	0.301	0.303	-0.6

* Stimulations as high as 228% have been obtained at M/20, and 297% at M/50 acetaldehyde.

appreciable effect are acetaldehyde and ethyl alcohol. The increase caused by these compounds is not additive. Though the stimulation in respiration caused by these compounds is marked, it is far short of the stimulation induced by heat treatment. The possible significance of acetaldehyde and alcohol stimulation will be discussed later. It is seen from diagram II that alcoholic fermentation depends upon the presence of glucose, hexosediphosphate, and acetaldehyde. In an experiment these substances were added to one vessel containing dormant spores, and each substance independently to other spores, and the oxygen consumption followed. The increased oxygen consumption was no larger than was obtained from acetaldehyde alone, as may be seen from table VII. The failure of the added substrates to bring about respiratory stimulation may be due to impermeability of the spores or because the proper substrate or combination of substrates has not been found. The results obtained above with added substrates have not ruled out the possibility that the respiratory block is located in the anaerobic reactions which act on glycogen and which form the actual respiratory substrate.

Action of sodium fluoride, iodoacetate, and iodoacetamide on oxygen consumption

Blackman's theory of plant respiration postulates that the first step in aerobic respiration is the conversion of carbohydrates to trioses or triose-phosphates in reactions essentially similar to those of alcoholic fermentation. This theory has recently been reviewed by Turner (**32**). One approach to the possible location of the respiratory block between glycogen and the actual respiratory substrate is to study the effect of certain poisons, known to inhibit alcoholic fermentation and muscle glycolysis, on oxygen consumption. In this section we report some results on the action of sodium fluoride, iodoacetate, and iodoacetamide on oxygen consumption. We cannot say definitely what steps of the respiratory mechanism are inhibited by these poisons, but it is possible that the same reactions which are poisoned in anaerobic metabolism are also inhibited in respiration, even though the concentrations required to inhibit respiration are higher than those which inhibit fermentation. The question has been discussed at some length with special attention to iodoacetate by Turner (**32**).

Sodium fluoride poisons alcoholic fermentation and has been shown by Lohmann and Meyerhof (**15**) to poison the breakdown of phosphoglyceric acid to pyruvic acid and H_3PO_4 (see diagram II). It is not claimed that this is the only reaction poisoned by fluoride. Lundsgaard (**16**) has shown that iodoacetate poisons muscle glycolysis and alcoholic fermentation, and it has been shown by Meyerhof and Kiessling (**20, 21**) that it does not inhibit the breakdown of phosphoglyceric acid to acetaldehyde and CO_2, but that it does inhibit reactions 2 and 7 of diagram II. It is possible that other reactions may also be poisoned by iodoacetate. It is well known that iodoacetate combines with –SH groups of glutathione (**4, 23**) and proteins (**8, 24**), but the work of Smythe (**27**) indicates that its inhibition of fermentation is not due to destruction of –SH groups. Goddard (**7**) has shown that iodoacetamide inhibits respiration of *Neurospora* spores, Smythe (**27**) that it inhibits alcoholic fermentation, and Stannard (**28**) that it inhibits muscle glycolysis. The mechanism of the action of this compound is unknown, though it is established that it reacts rapidly with –SH groups (**8, 27**). The results obtained with these three poisons on the spores are shown in table VIII. The results of using iodoacetate and iodoacetamide on activated and germinating spores are recalculated from an earlier paper (**7**).

The great sensitivity of all phases of the respiration to sodium fluoride is the most interesting result from these experiments. If the respiration is over pyruvic acid, this result is to be expected, but this does not explain the inhibition of the respiration of ethyl alcohol and acetaldehyde by fluoride. It is possible that neither of these compounds is directly undergoing oxidation, but they may be entering into dismutation reactions similar to

TABLE VIII

EFFECTS OF POISONS AND SUBSTRATES ON OXYGEN CONSUMPTION OF SPORES

VALUES AS $-Q_{O_2}$

POISON	SUBSTRATE	DORMANT			ACTIVATED			GERMINATING		
		CONTROL	EXPERIMENTAL	PERCENTAGE INHIBITION	CONTROL	EXPERIMENTAL	PERCENTAGE INHIBITION	CONTROL	EXPERIMENTAL	PERCENTAGE* INHIBITION
NaF M/10	None	0.32	0.067	81.5	5.68	1.61	64.7	15.75	0.76	95.2
NaF M/10	Acetal. M/50	0.34	0.19	73.8†	—	—	—	—	—	—
None	" "	0.34	0.70	—	7.31	7.71		14.4	16.7	—
NaF M/10	Alcohol M/20	0.54	0.15	78.6‡	—	—	—	—	—	—
None	" "	0.54	0.89	—	—	—	—	—	—	—
Iodoacetate M/200	———	0.47	0.46	2.0	4.07	4.11	−1.0	7.36	3.46	53
Iodoacetate M/100	———	0.47	0.43	8.5	—	—	—	—	—	—
Iodoacetamide M/200	———	0.50	0.30	40.0	4.07	3.70	8.2	7.36	2.43	67

* In no case did the poisoned spores germinate. These figures are obtained by comparing germinating controls with poisoned cells, at the same time periods after activation.

† Inhibition calculated against the rate with acetaldehyde from the experiment below.

‡ Inhibition calculated against the rate with alcohol from the experiment below.

reactions 6 and 7 of diagram II. This would seem probable enough for acetaldehyde but much less probable for alcohol, unless it is first converted to acetaldehyde.

The respiration of the germinating spores is sensitive to iodoacetate (pH 5.4) and the spores are rapidly killed by the poison. The oxygen consumption of dormant and activated spores is unaffected by iodoacetate and it is probable that it does not penetrate the cells. If dormant or activated spores stand for an hour in iodoacetate (0.005 M) and are then washed on the centrifuge, they germinate normally. The respiration of dormant and germinating spores is poisoned by iodoacetamide, while the activated spores are unaffected (see table IX). This cannot be due to failure to penetrate the

TABLE IX

ANAEROBIC CO_2 PRODUCTION OF ACTIVATED SPORES IN THE PRESENCE OF SODIUM FLUORIDE, IODOACETAMIDE, AND ETHLY URETHANE

MEDIUM	$Q_{CO_2}^{N_2*}$	PERCENTAGE INHIBITION
Control	5.30	
NaF M/10	1.44	73.0
Control	7.18	
Iodoacetamide M/200	5.82	19.0
Iodoacetamide M/1000	7.07	1.5
Control	8.13	
Ethyl urethane 2½ per cent.	7.35	10.0

* $Q_{CO_2}^{N_2} = \frac{\text{cu. mm. } CO_2 \text{ per hour}}{\text{dry wt. mg.}}$ (under anaerobic conditions).

$Q_{CO_2}^{N_2}$ and percentage inhibition calculated on the linear part of the curve.

activated spores, for if activated spores are exposed to iodoacetamide and then washed in the centrifuge, the respiration proceeds normally until the time when the controls germinate; then the respiration of the poisoned washed cells falls off and no germ tubes are formed. There seems to be a real difference in the respiratory mechanisms of dormant and germinating spores on the one hand and of activated spores on the other, with reference to iodoacetamide poisoning. No explanation of this effect seems possible at this time.

ANAEROBIC CO_2 AND CARBOXYLASE ACTIVATION

The marked inhibition of the oxygen consumption by sodium fluoride suggested that reactions similar to those occurring in alcoholic fermentation might be part of the respiratory scheme. The best approach to such reactions seemed to be through a study of the anaerobic CO_2 production. Re-

sults typical of many experiments are shown in fig. 4. It may be seen from the figure that no measurable anaerobic CO_2 is liberated by the dormant spores. Even when 40 mg. of dry weight of dormant spores were used, no CO_2 evolution was detected which was greater than the experimental error ($\pm$ 1.0 cu.mm. per hour). The activated spores evolved CO_2 anaerobically quite rapidly as may be seen in fig. 4 and table IX. The rate of anaerobic CO_2 production by the activated spores is linear for a short period after activation and gradually falls to zero at about three hours after the heat

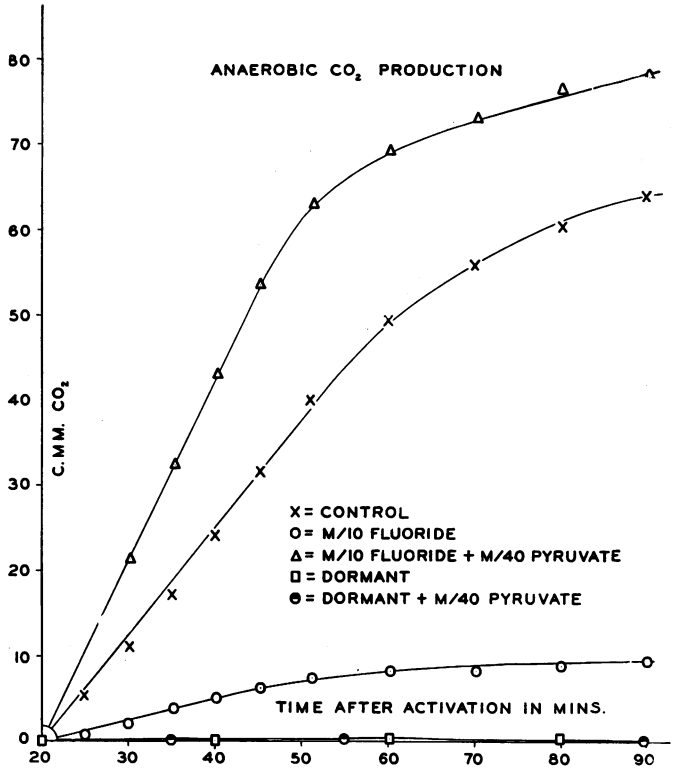

FIG. 4. Anaerobic CO_2 production of dormant and activated spores. The effect of 0.1 M NaF on inhibition of CO_2 production of activated spores. The effect of pyruvate and NaF together on activated spores and the failure of pyruvate to affect dormant spores. 19.5 mg. of spores per vessel.

treatment. The rate of anaerobic CO_2 formation by activated spores during the linear phase is about the same as the rate of oxygen consumption. Compare tables I and IX. Though the dormant spores liberate no CO_2 under anaerobic conditions, it was expected that they would produce CO_2 aerobically. Aerobic CO_2 production was measured by WARBURG's indirect method (see DIXON, 5) with the suspension in equilibrium with an atmos-

phere of 5 per cent. CO_2 and 95 per cent. oxygen. Only a few experiments were made, and a typical one gave a $Q_{CO_2}^{O_2}$ of 0.59 and a respiratory quotient of 0.85 $\left(Q_{CO_2}^{O_2} = \frac{\text{cu.mm. } CO_2 \text{ evolved per hr., in the presence of } O_2}{\text{mg. dry weight}} \right)$.

Formation of anaerobic CO_2 is often accompanied by formation of alcohol or organic acids. No attempt has been made to determine whether the activated spores produce alcohol under anaerobic conditions. Acid formation may be determined manometrically by the displacement of CO_2 from a bicarbonate buffer according to the following equation:

$$HA + NaHCO_3 \rightarrow NaA + CO_2 + H_2O$$

Under anaerobic conditions the difference in CO_2 liberated from bicarbonate buffer and from phosphate buffer is a measure of the acid formed. We carried out such an experiment with activated spores in M/60 phosphate buffer with pH 5.4 and in 0.0025 M $NaHCO_3$ in equilibrium with 5 per cent. CO_2, with pH 7.4. The average from two vessels was 52.8 c.mm. in one hour in phosphate buffer, and 52.5 cu.mm. in bicarbonate buffer. We may be sure that under anaerobic conditions the activated spores do not form any acid.

If the block in anaerobic CO_2 production of the dormant spores was anterior to pyruvic acid formation (see diagram II), it was to be expected that the addition of pyruvic acid to dormant spores would induce anaerobic CO_2 production. The pyruvic acid solution used was half neutralized, so that the $pH = P_{ka} = 2.5$, and one half of the acid was present in undissociated form. Under these conditions (and in unneutralized pyruvic acid or in sodium pyruvate solutions), no anaerobic CO_2 could be detected from dormant spores (see fig. 4). This failure of dormant spores to produce CO_2 from pyruvate suggested that there was no active carboxylase in dormant spores.

Experiments on the effect of three poisons on anaerobic CO_2 production are shown in table IX. In each case the $Q_{CO_2}^{N_2}$ and the percentage inhibition were calculated on the linear part of the curve (fig. 4). The results with NaF are striking, and are in agreement with the MEYERHOF-KIESSLING theory of alcoholic fermentation. This experiment and the one which follows make it clear that the anaerobic CO_2 is not merely the physical release of bound CO_2 due to heating, nor to the escape of previously formed CO_2 due to changes in cell permeability. From the previous discussion, it seems quite clear that iodoacetamide penetrates the spores, and it was surprising that it had such a small effect on anaerobic CO_2 production. The small effect of ethyl urethane is not surprising. This experiment was included

for comparison of its effect on oxygen consumption and anaerobic CO_2 production.

The fact that dormant spores liberate no anaerobic CO_2 even when pyruvic acid is added, and the ready production of CO_2 by activated cells suggests that carboxylase is absent or inactive in dormant spores, and that this enzyme is activated on heat treatment. If this is true, we should be able to demonstrate carboxylase activity by restoring the ability of NaF-poisoned activated spores to produce anaerobic CO_2 by the addition of pyruvic acid. NaF is known to poison anaerobic CO_2 formation from glucose (see diagram II), but not from pyruvic acid, since it does not poison carboxylase. The increased CO_2 production on the addition of pyruvic acid over that of NaF-poisoned cells should be an approximate measure of carboxylase activity. The results from one such experiment are shown in figure 4. It is clear that pyruvic acid raises the amount of CO_2 production above that of the unpoisoned controls; therefore, in the controls the rate of CO_2 production is not limited by carboxylase concentration, but by the rate of pyruvic acid formation. We interpret the failure of pyruvic acid to induce anaerobic CO_2 production in dormant spores and its pronounced effect on NaF-poisoned activated spores as definite evidence that the enzyme carboxylase is activated (formed?) on heat treatment of the spores.

It will be recalled that heat activation as it affects germination and increased respiration is reversible; that is, under anaerobic conditions activated spores return to secondary dormancy. These de-activated spores respond to a second heat treatment in the usual manner. The falling off in the rate of anaerobic CO_2 production, with or without added pyruvic acid, is surprisingly similar to the de-activation of the germination mechanism under the same conditions. An experiment was undertaken to determine whether carboxylase activation was likewise reversible. After the activation of the spores, a sample was set aside in a hanging drop (in air) for a germination test. The spores were placed in four vessels, and anaerobic CO_2 production measured. After the rate had fallen off (170 min.), two vessels were removed from the bath and spore samples were taken for germination tests. (The samples were too small to affect the subsequent readings.) The two vessels were placed for twenty minutes in a thermostat bath at 55° C. for re-activation. After the re-activation, samples were again removed for germination tests, the vessels replaced in the bath, anaerobic conditions established, and the CO_2 production again measured. During this period, readings were continued on the other two vessels. The experiment was continued for six hours after the first activation, and by this time the rate of CO_2 production had fallen approximately to zero in all vessels. The experimental results are shown graphically in figure 5.

Examination of figure 5 shows that the activation, de-activation, and

re-activation of the spores as concerns anaerobic CO_2 production (and presumably carboxylase activity) is closely parallel to the reversible activation-de-activation of the germination mechanism.

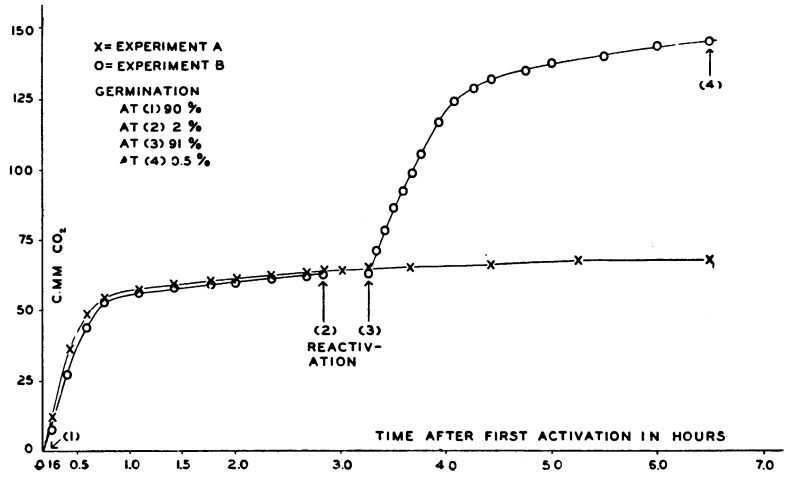

FIG. 5. The effect of a second heat treatment on the anaerobic CO_2 production. In experiment A, the readings were uninterrupted. In experiment B, the vessels were removed at arrow no. 2, re-heated for twenty minutes at 55° C., allowed ten minutes for temperature equilibrium, and readings commenced at arrow no. 3. Germination tests were made by removing the spores to air and counting several hours later, at each point indicated by an arrow.

Discussion

It is possible that the respiratory rate of dormant spores is limited by cell permeability and that activation brings about a reversible change in the cell surface. The authors do not believe that this explanation is the correct one for the following reasons:

(1) The respiratory rate of the dormant spores is independent of the partial pressure of oxygen over the range of 37 to 300 mm. of mercury. This result indicates that the rate of oxygen entrance is not limiting the respiratory rate.

(2) The rate of oxygen consumption of dormant spores is independent of the partial pressure of carbon dioxide over the range of 0 to 190 mm. of mercury. This indicates that outward diffusion of carbon dioxide is not limiting the rate of oxygen consumption.

(3) The spores are not completely impermeable to carbon dioxide, for it is formed under aerobic conditions. The complete failure of dormant spores to liberate carbon dioxide anaerobically cannot be explained on a basis of permeability to carbon dioxide, for the anaerobic CO_2 production would be as high as the aerobic CO_2 production.

(4) The production of anaerobic CO_2 by activated spores might be interpreted as the release of preformed CO_2 due to a change in cell surface, but this seems improbable, for the anaerobic CO_2 production is poisoned by NaF and the NaF poisoning is overcome by pyruvic acid.

(5) Ethyl alcohol and acetaldehyde cause a marked increase in the respiratory rate of dormant spores. This does not seem to be consistent with the view that the respiratory rate is limited by the cell surface.

The much greater sensitivity of the activated spores to carbon monoxide and to cyanide suggests that the activity of phaeohemin is not limiting the rate of respiration. The evidence in this paper does not definitely establish the fact that the oxidase is phaeohemin (indophenol oxidase). To prove this point, light reversal of CO poisoning would be necessary, and this is difficult to obtain with spores with black walls. Proof of phaeohemin might be obtained by the catalysis of paraphenylenediamine, but the spores appear to be impermeable to this dye. This dye could be tried on ground cells, but the authors decided to limit this paper to results obtained with intact living cells. The limited permeability of the spores has made it difficult to obtain any definite information concerning the dehydrogenase systems. It is possible that information may be gained on this point from ground cells, but how useful such information would be in attempting to interpret the changes which occur on activation is open to question. No information has been obtained on cytochrome, for the black spore wall has prevented spectroscopic determinations, and the impermeability of the spores has prevented the use of artificial carriers, such as paraphenylenediamine. It is possible that a portion of the dormant respiration is catalyzed by WARBURG'S flavine enzyme. However, the activated respiration is probably not catalyzed by this enzyme, since the respiration catalyzed by the flavine enzyme (**30**) is cyanide- and carbon monoxide-stable.

Since increased respiration and normal germination are obtained when the ascospores are activated in distilled water, the respiratory block of the dormant spores cannot be caused by absence of stored food. However, it is possible that the reactions converting the stored food into the substrate actually respired may be limiting the respiratory rate. The only added substrates, out of a long list tried, which produced an appreciable increase in the rate of oxygen consumption of dormant spores were acetaldehyde and ethyl alcohol. These substrates stimulated the rate from 100 to 300 per cent., while the difference between dormant and activated respiratory rates is of the order of 1000 to 4000 per cent. The significance of the increase brought about by acetaldehyde or alcohol is not clear. The acetaldehyde and ethyl alcohol respiration is NaF sensitive. This is rather surprising, because NaF does not poison most dehydrogenase systems, nor the phaeohemin-cytochrome system. However, an old result of MEYERHOF'S (**18**) indi-

cates that succinic dehydrogenase is poisoned by NaF. Acetaldehyde may be oxidized by the xanthine oxidase. Ethyl alcohol will undergo a coupled oxidation by xanthine oxidase when hydrogen peroxide and catalase are present (**12**).

The failure of dormant spores to produce CO_2 anaerobically in the presence of pyruvic acid, and the fact that pyruvic acid restores the anaerobic CO_2 production to NaF-poisoned activated spores, seems clearly to establish the fact that carboxylase is inactive in dormant spores and active in heat-treated spores. The reversible activation of the system producing anaerobic CO_2 on heat treatment parallels almost completely the effect of this heat treatment on germination and respiration. The absolute rate of anaerobic CO_2 production during the linear portion of the curve is of the same order as the rate of oxygen consumption. It is difficult to determine whether or not the respiratory block is the absence of active carboxylase, but the agreement between carboxylase activity and the respiration and germination of the spores seems too close to be fortuitous, particularly so when it is recalled that activation and de-activation for one component corresponds with activation and de-activation for the other two. Taken all together, it seems probable that carboxylase activation by heat treatment of the spores leads to a marked increase in the respiratory rate and to germination. It is, of course, possible that other enzymes are similarly activated on heat treatment, and that the inactivity of one or several of these enzymes constitutes the respiratory block.

Since there seems to be no carboxylase activity in dormant spores, but a measurable respiratory rate, it seems clear that the respiratory system of dormant spores is not a system involving carboxylase. From the discussion presented it appears that the respiratory mechanism of the activated spores includes carboxylase as one essential step. If this interpretation is correct, we are forced to the conclusion that there are two qualitatively different respiratory mechanisms, namely, the dormant mechanism of which carboxylase is not a part, and the mechanism which comes into play after the heat treatment, which so closely parallels carboxylase activity that we may assume that carboxylase is one step in the system. The phaeohemin-cytochrome system and other enzymes may be common to the two systems.

If we accept the interpretation that carboxylase is an essential part of the respiratory mechanism of activated spores, then it seems clear that in this system anaerobic processes similar to those in alcoholic fermentation are a part of the respiratory mechanism. It is difficult to understand how carboxylase functions in the respiratory mechanism. It cannot be merely the formation of acetaldehyde, for though the addition of this substance causes a respiratory stimulation, it is far short of the maximum rate. Nor can it be merely the accumulation of pyruvic acid that limits the rate, for the addi-

tion of pyruvic acid does not inhibit the respiration of activated spores. AUHAGEN (1) has shown that a co-carboxylase is essential for carboxylase activity; activation might be the formation of co-carboxylase. If co-carboxylase were identical with a co-enzyme of respiration, the parallelism between carboxylase activity and respiration would be explained, without the necessity of assuming that carboxylase was part of the respiratory mechanism. But AUHAGEN has shown that co-carboxylase is not identical with co-zymase, nor is it found in muscle, which lacks carboxylase but which contains a respiratory co-enzyme. To the authors' knowledge, WARBURG'S respiratory co-enzyme from horse blood has not been tried with carboxylase.

The zymase complex including carboxylase is widely distributed in the tissues of higher plants, which are never known to produce alcohol in nature. KOSTYCHEV (13) and BLACKMAN (3) assume that the function of the zymase system is to convert sugars into the respiratory substrates. The BLACKMAN theory assumes that these substrates are trioses. It is possible that carboxylase functions in the respiratory scheme in higher plants, converting three carbon atom keto acids into two carbon atom compounds which are then respired.

Summary

1. An attempt has been made to locate the respiratory block in dormant *Neurospora tetrasperma* ascospores.

2. The respiratory rates at various partial pressures of oxygen and carbon dioxide indicate that permeability of the spores to gases is not limiting.

3. Studies on the cyanide and carbon monoxide sensitivity of dormant and activated spores indicate that phaeohemin (indophenol oxidase) activity is not limiting.

4. The dormant spores produce no CO_2 anaerobically, while the activated spores have a $Q_{CO_2}^{N_2}$ of 5 to 8, though the rate falls to zero after 3 to 4 hours.

5. It is suggested that no active carboxylase is present in dormant spores, but that carboxylase is reversibly activated on heat treatment. The activation-de-activation of carboxylase parallels the effect of activation and de-activation on respiration and germination.

6. The results in this paper are interpreted to mean that two qualitatively different respiratory systems are present, namely, the dormant system, which functions in the absence of carboxylase, and a second system, active in heat treated spores, which passes over the enzyme carboxylase. The respiratory block is then the inactivity of the enzyme carboxylase.

7. These experiments indicate that reactions similar to those of alcoholic fermentation are a part of the respiratory mechanism in *Neurospora.*

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