CONTROL MECHANISMS IN RESPIRATION AND FERMENTATION

Edited by

BARBARA WRIGHT

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Preface

This Symposium was organized with the purpose of defining and clarifying basic mechanisms which control and interrelate aerobic and anaerobic metabolism. It appeared both useful and appropriate, particularly in view of the general aims of this Society, to approach the problem as broadly as possible. Hence papers are included dealing with presumably related metabolic problems in micro-organisms, marine eggs, insects, parasites, plants, and tumor cells. The reader of this volume will discover, however, that the phenomenon, for example, of a sudden increase in respiratory activity, apparently has as many causes as the number of creatures in which this interesting situation exists. In the germinating bacterial spore, the sudden availability of an electron acceptor, dipicolinic acid, is considered to be a critical stimulant; in the diapausing insect, the release of a hormone and the level of cytochrome *c* are implicated as important controlling factors; in the newly fertilized marine egg, an increase in the reduced pyridine nucleotide level is correlated with a burst in respiratory and synthetic activity. In a variety of biological materials, glycolytic activity will be shown to be influenced by such agents as virus infection in animal cells, hormonal regulation of the activity of an enzyme, or by the level of intracellular inorganic phosphate in tumor cells.

Hopefully, such comparisons will result in the recognition of variations on a theme, or in the eventual discovery of certain common denominators. At present they certainly accentuate the difficulties in distinguishing between primary and secondary causes, and even between cause and effect. The reader of this volume can decide how applicable these generalizations are in his own area of interest.

It is a pleasure to thank the participants, the chairmen, and particularly Dr. John Buck for encouragement and help in the successful organization of this symposium. The Society of General Physiologists

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Control Mechanisms in Respiration and Fermentation

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Electron Transport in Spore Germination

HARLYN O. HALVORSON [1]

It is appropriate, in a symposium on the control of respiration and fermentation, that attention be directed toward those biological systems in which absolute control is exerted over metabolic activity. A wide spectrum of metabolic states between actively metabolizing vegetative organisms and those that show no perceptible metabolism are known in biology. The best known example of the latter case is that of the bacterial endospore. This represents a phenotypic development of vegetative growth leading to the dormant state and to a highly specialized and complex structure which is resistant to adverse environmental conditions. At the same time, the spore is endowed with a built-in trigger mechanism, enabling it to germinate rapidly and resume vegetative growth. The present paper attempts to summarize our present knowledge on the metabolic activity of the dormant state and the control mechanisms governing the conversion of the dormant state to a vegetative cell.

Metabolic Activity of Bacterial Spores

Reports on the metabolic activity of spores have varied considerably, largely depending on the extent to which metabolically active, germinative forms have been removed. Vegetative cells of *Bacillus* have Q_{O_2} values of about 60 to 100. For example, a Q_{O_2} value of 2.4 in the presence of glucose has been reported in a preparation of *Bacillus megaterium* spores containing approximately 10 per cent germinating forms (Levinson and Hyatt, 1955). Crook (1952),

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using a microrespirometer and well-washed suspension of spores of *Bacillus subtilis*, found a Q_{O_2} value of less than 0.3. Unfortunately, he did not report the percentage of germinating forms.

When precautions are taken to prepare fresh well-washed suspensions of spores, no oxygen uptake can be detected in the presence of glucose (Spencer and Powell, 1952; Church and Halvorson, 1957). These findings, as well as some of the factors affecting the respiratory activity of spores of *Bacillus cereus* strain T, are summarized in Table 1–1. Spores were washed 12 to 15 times until they were freed

TABLE 1–1

Respiratory Activity of Spores and Vegetative Cells of *Bacillus cereus* Strain T on Glucose

Organism	Q_{O_2}*
Vegetative cells	60–100
Spores	
Dormant	<0.05
Heat-shocked	20
Aged, heat-shocked	50
Germinated	50–70

* 30° C.

of germinating forms. When dormant spores were tested at very high densities (30 mg per Warburg cup), there was no detectable oxygen uptake after 60 minutes in the presence of glucose. Based on the limits of the manometric technique employed, the Q_{O_2} value of dormant spores was estimated to be less than 0.05. The vegetative cells had a Q_{O_2} value of 60 to 100. When spores were heat-shocked for one hour at 65° C, their Q_{O_2} value on glucose was 20. Similar elevation in respiratory activity occurred when spores had been stored for four months in the deep freeze. Activation of the dormant glucose oxidizing system was also observed when spores were germinated either by the addition of L-alanine or by high concentration of glucose (Church and Halvorson, 1957; Murrell, 1955; Murty and Halvorson, 1957). Levinson and Hyatt (1955) have shown that germination leads to full metabolic activity under nutritional conditions where outgrowth of the germinative cells and presumably protein synthesis is insignificant.

Although spores were originally thought to be devoid of enzymes, it is now clearly recognized that a wide variety of enzymes is present in the bacterial spore. The increasing number of these in recent years suggests that the spore may contain as many enzymes as its

parent vegetative cell. The enzymes of the spore fall into three groups: (1) enzymes which are active in the intact spore, (2) enzymes whose activity *in vivo* requires activation, and (3) enzymes which can be demonstrated only in disrupted spores (Halvorson, 1957b).

The findings on the metabolic activity of spores can be summarized as follows. The overall respiratory activity of a freshly prepared spore is negligible, if not completely inactive. These spores contain metabolic systems for glucose metabolism which can be activated either by heat, chemicals, aging or by germination.

Comparison of Vegetative Cells and Spores

A clearer description of the control mechanism governing the dormant state of spores and of the nature of differentiation accompanying both sporulation and germination is possible only in so far as the difference between vegetative cells and spores is understood. In the last decade our knowledge of these differences has increased considerably.

The chemical differences between spores and vegetative cells have been recently reviewed elsewhere (Halvorson and Church, 1957a; Murrell, 1961) and will not be enumerated here. Two constituents, calcium and dipicolinic acid (DPA), are of particular interest to the problem of dormancy. The level of calcium in spores is approximately 10 times that of vegetative cells (Curran *et al.*, 1943). DPA, which is formed just prior to or coincidental with heat resistance, is present to the level of 5 to 15 per cent of the dry weight of the spore. Decreases in either calcium or DPA lead to reduction in the heat resistance of bacterial spores (Curran *et al.*, 1943; Slepecky and Foster, 1958; Church and Halvorson, 1959; Black *et al.*, 1960).

Comparisons of the enzyme contents of spores and vegetative cells are somewhat complicated by the fact that a number of enzyme activities are masked and require prior activation of the spore before they can be adequately measured. The recognition of dormant enzymes and of improved methods of rupturing spores has permitted a more extensive analysis of their distribution. Table 1–2 summarizes those enzymes which have been identified and assayed under optimal conditions in both spores and their homologous vegetative cells. The enzyme changes are both qualitative and quantitative. Ribosidase and X-transferase are present in the spore but not in the vegetative cell, whereas the reverse is true of glutamic transaminase and succinic cytochrome *c* reductase. Five enzymes are present in higher concentrations in the spores than in vegetative cells, whereas

seven enzymes decrease in the spore. The most impressive differences between spores and vegetative cells involve enzymes active in electron transport. These will be discussed below.

Not all the changes in enzyme patterns can be attributed to induction or repression of the enzyme-forming systems present in vegetative cells. Several examples exist in which enzymes of the spore

TABLE 1–2

Comparison of the Enzyme Constitution of Vegetative Cells and of Spores

Enzyme	Organism	Units of Enzyme * Veg. Cell	Spore
Enriched in spore			
Adenosine deaminase †	*B. cereus*	0.021	0.093
Ribosidase †	*B. cereus*	0	0.146
Alanine racemase ‡	9 strains *Bacillus*	1–4	10–84
Pyrophosphatase §	*B. megaterium*	0.070	1.88
Soluble DPNH oxidase ||	*B. cereus*	0.018	0.078
X-transferase #	*B. cereus*	0	0.059
Glutamo-transferase #	*B. cereus*	0.16	0.22
Decreased in spore			
Glutamic transaminase **	*B. mycoides*	0.049	0
Catalase ††	*B. cereus*	321	16.5
DAPA decarboxylase ‡‡	*B. sphaericus*	1.25–1.87	0.25–0.50
Particulate DPNH oxidase ||	*B. cereus*	6.58	0.024
DPNH cyt. *c* reductase ||	*B. cereus*	0.98	0.06
Diaphorase ||	*B. cereus*	1.87	1.47
Succinic cyt. *c* reductase ||	*B. cereus*	0.35	0
Glutamine synthetase #	*B. cereus*	0.63	0.23
Acetokinase #	*B. cereus*	0.19	0.06

* Micromoles per hour per milligram dry weight cells.
† Powell and Strange (1956).
‡ Stewart and Halvorson (1953).
§ Levinson *et al.* (1958).
|| Doi and Halvorson (1961a).
Krask (1961).
** Hardwick and Foster (1953).
†† Lawrence and Halvorson (1954).
‡‡ Powell and Strange (1957).

differ qualitatively from the homologous enzymes of the vegetative cell. For example, it has been observed that a heat-resistant glucose dehydrogenase (Bach and Sadoff, 1960) and a heat-resistant catalase (Sadoff, 1960) are formed during sporulation 3 to 4 hours prior to the development of the mature spore. These enzymes were immunologically and electrophoretically identical with the analogous heat labile enzymes from germinating spores or vegetative cells.

Electron Transport Systems in Spores and Vegetative Cells

That spores and vegetative cells differed in their electron transport systems was first suggested by Keilin and Hartree (1947), who found that spores had less than 6 per cent of the cytochromes present in vegetative cells. More recently Hachisuka and co-workers (1956) observed similar differences and found that overall germination of the spore was characterized by a development in the respiratory system. Spencer and Powell (1952) found that the flavin content, on the other hand, does not vary during germination. Nakada and co-workers (1957) observed that spores are less sensitive to cyanide than are vegetative cells and that germination is accompanied by cytochrome synthesis. The low content of cytochromes and the

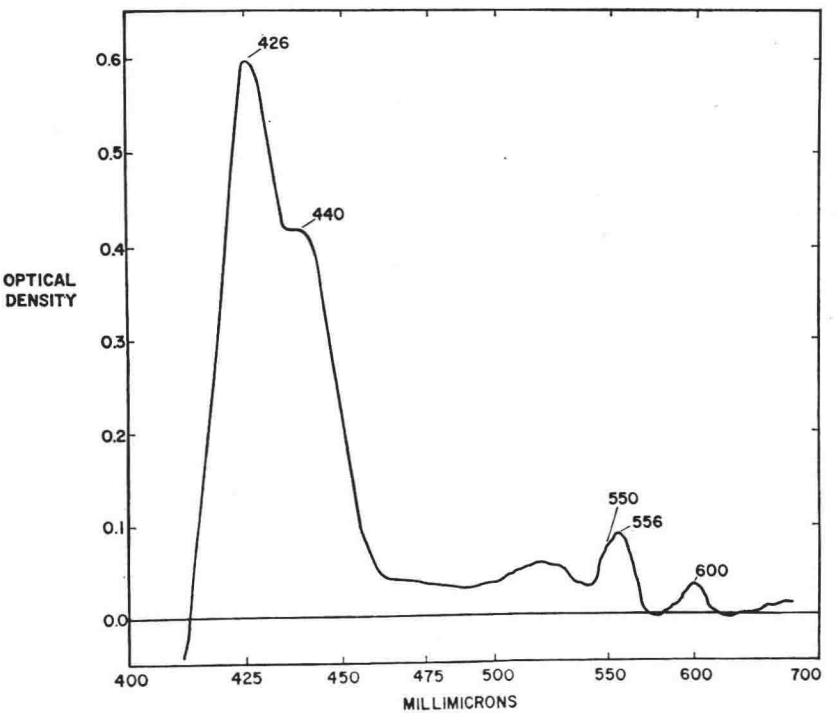

Fig. 1–1. Difference spectrum of the particulate DPNH oxidase of *Bacillus cereus* vegetative cells. The sample cuvette held 2.6 mg of enzyme protein, 7.5 mg of deoxycholate, and 15 μM of phosphate (pH 7.3) in a total volume of 0.225 ml. The reference cuvette contained the identical materials. The sample cuvette was reduced by the addition of dithionite. (Data from Doi and Halvorson, 1961a.)

presence of a cyanide-resistant respiration suggested that spores utilize primarily a flavin system for respiration. This conclusion is supported by the data in Table 1–2.

The DPNH oxidase activity of vegetative cells is primarily associated with a particulate fraction. The electron transport particles of vegetative cells of *Bacillus cereus* (Fig. 1–1) have a normal complement of cytochromes characteristic of other systems and similar to that observed in other strains of bacillus (Smith, 1954). Absorption peaks at 600, 556, 550, 510 to 530, 440, and 426 mμ are observed in these particles which correspond to the α band of cytochrome *a*, cytochrome *b* type, cytochrome *c*, and the β bands, and the Soret bands. Schaeffer (1952), using whole cells of *Bacillus cereus*, observed absorption bands at 600, 554 to 556, 548, and 528 mμ.

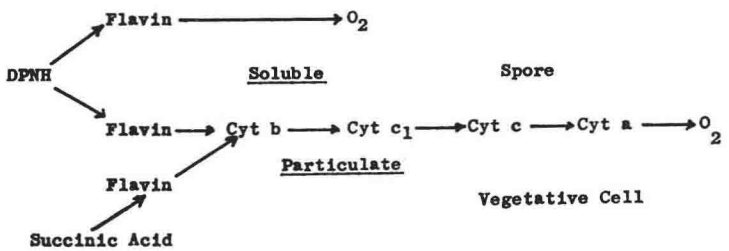

Fig. 1–2. The soluble and particulate electron transport system of spores and vegetative cells.

When spore particles (Doi and Halvorson, 1961a) were examined spectroscopically, no cytochrome bands could be detected. Only a low level of flavin absorption was observed in the difference spectrum of spore particles. When whole spores were examined microspectroscopically, a faint absorption was observed in the cytochrome *b* region. The enzymic activities of spores also differ from those of vegetative cells. Succinic cytochrome *c* reductase, for example, is completely absent in the particulate fraction from spores. In addition, the spore particles contain a diaphorase which in contrast to that from vegetative cells cannot use ferricyanide as the electron acceptor. It thus appears that spores contain either altered or immature electron transport particles.

Spores, on the other hand, contain four times higher concentration of a soluble DPNH oxidase than is present in vegetative cells. The soluble DPNH oxidase was purified from spores of *Bacillus cereus* (Doi and Halvorson, 1961b) and found to be identical to the DPNH oxidase previously isolated from vegetative cells of *Bacillus*

subtilis (Lightbown and Kogat, 1959). Both enzymes are stimulated by FMN.

The collective data suggest the following electron transport systems for spores and vegetative cells (Fig. 1–2).

Breaking of Dormancy

The significance of dormant enzyme systems capable of furnishing a supply of both energy and carbon compounds for biosynthesis can be understood from the findings that the overall germination process is an energy-requiring reaction (Halvorson and Church, 1957b). An understanding of the metabolic requirement for the germination process has in recent years been the subject of our own investigations. There are two stages in this process: (1) an activation reaction and (2) a triggered germination of the activated spores.

Activation

One of the frequently encountered phenomena in the germination of bacterial spores is the need for pretreatment with heat. Curran and Evans (1945) observed that spores which did not germinate or whose germination was delayed in the absence of heat overcame this dormancy when heat-activated. For example, in *Bacillus coagulans* (Murrell, 1961) heat activation increases the viable count by 100-fold. An example of the effect of heat activation on spores of *Bacillus cereus* is shown in Fig. 1–3. The rate of germination increases almost linearly with the time of heat treatment up to 4 hours at 65°C. Heat activation also reduces appreciably the lag prior to germination. Although both lag and germination rate are greatly influenced by heat shock, prior heat activation apparently has little effect on the total number of spores germinating. The effect of heat activation on the rate of alanine-induced germination resembles the effect of heat activation on alanine deamination by intact spores (O'Connor and Halvorson, 1959).

The duration of heat activation necessary for optimum germination decreases with the age of the spore preparation (Powell, 1950). Both prolonged storage of spores and heat activation lead to simpler requirements for germination stimulants, decrease the concentration of germinating agents required for optimum germination, and activate dormant enzyme systems of the spore.

The biochemical effects of heat activation are not well understood, although several consequences of the activation have been recog-

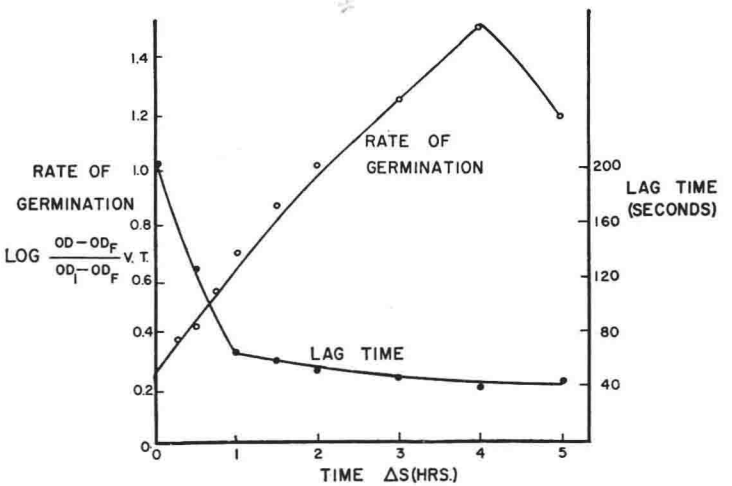

Fig. 1–3. Effect of heat activation on lag time and rate of L-alanine-induced germination. Spores of *B. cereus* strain T were heat-shocked at 65° C for various periods of time, washed, and germinated at 30° C in carbonate-bicarbonate buffer pH 9, containing 100 μM of L-alanine per milliliter. (Data from O'Connor and Halvorson, 1961b.)

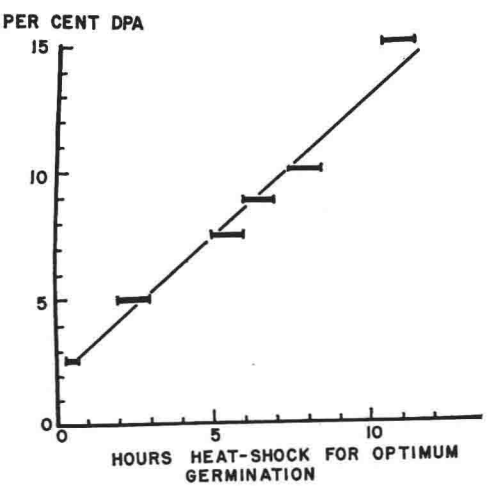

Fig. 1–4. Dependence of duration of heat shock on the intrasporal DPA content. Spores with various contents of DPA were heat-shocked for various periods at 65° C, washed, and resuspended in *M*/50 Tris buffer, pH 8.0. Germination was initiated by addition of 15 mg/ml of L-alanine at 30° C. The bars indicate the approximate time of heat shock required to obtain the maximum germination rate. (Data from Keynan *et al.*, 1961.)