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Comparision of Soluble Reduced Nicotinamide Adenine Dinucleotide Oxidases from Cells and Spores of *Clostridium botulinum*[1]

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ABSTRACT

GREEN, J. H. (Michigan State University, East Lansing), AND H. L. SADOFF. Comparison of soluble reduced nicotinamide adenine dinucleotide oxidases from cells and spores of *Clostridium botulinum*. J. Bacteriol. **89**:1499–1505. 1965.—The properties of purified reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidases from cells and spores of *Clostridium botulinum* 62-A have been studied to determine whether they are the same or different proteins. The spore $NADH_2$ oxidase was very heat-stable, whereas the vegetative enzyme was readily denatured at 70 C. The spore oxidase exhibited less affinity for the substrate than did the vegetative protein, but possessed a tightly bound cofactor. Atabrine was a noncompetitive inhibitor for both enzymes, but was less inhibitory to the spore $NADH_2$ oxidase. The enzymes could be separated from each other by gel filtration or chromatography on a diethylaminoethyl-cellulose column. The molecular weight of the spore oxidase was estimated to be 200,000 or greater, whereas that of the vegetative enzyme was 100,000 or less. Neither $NADH_2$ oxidase would cross-react with its heterologous antibody in a precipitation reaction. The conclusion drawn from this investigation is that the two $NADH_2$ oxidases are distinctly different proteins.

Simmons and Costilow (1962) have described a heat-stable reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase in spore extracts of *Clostridium botulinum* 62-A. They noted that the corresponding enzyme in extracts of cells and germinated spores was heat-labile, and suggested that the spore and cell enzymes might be the same protein, differing only in heat resistance. This study was undertaken to investigate the potentialities of the spore-cell enzyme system as a model for the study of heat resistance, and to compare some physical, catalytic, and immunochemical properties of the two oxidases.

MATERIALS AND METHODS

Vegetative cells and spores of *C. botulinum* 62-A (ATCC 7948) were used for these experiments. Cells and spores were produced in 18-liter quantities by use of cultural techniques and media described by Day and Costilow (1964). Vegetative cultures were incubated from 7 to 10 hr at 35 C and then harvested immediately. Spore cultures were incubated for 48 to 60 hr at 35 C and then kept at 4 C until phase microscopic examination revealed that the spores were free from their sporangia. Vegetative cell and spore cultures were harvested in a cooled, closed Sharples Super Centrifuge (model T-1P) with a standard clarifier rotor. Cells and spores were washed three to five times in cold 0.85% NaCl solutions and stored as a paste at −20 C.

Preparation of extracts and initial purification. Homogenates were prepared at maximal speed in a Servall Omnimixer with a medium-sized cup (50-ml capacity) which was cooled by immersion in an ice bath. Cells or spores, 10 to 12 g, were mixed with 45 g of Superbrite 110 beads (Minnesota Mining and Manufacturing Co., St. Paul, Minn.) and sufficient buffer to fill the cup. The buffer used was 0.067 M tris(hydroxymethyl)-aminomethane (Tris), *p*H 7.7. The duration of the run was usually 10 min. Cell debris and glass beads were removed by centrifugation, washed, and often rehomogenized for recovery of more enzyme. The supernatant fluids were then pooled and referred to as initial extracts. These extracts were centrifuged for 6 to 8 hr at 32,000 × *g*, and the supernatant fluid was saved. The nucleic acids were removed from extracts by treatment with ribonuclease and deoxyribonuclease (Calbiochem), 1 mg each per 100 ml of extract, and were incubated at 37 C for 2 hr in the presence of 0.005 M Mg^{++} (Hatch et al., 1961). The degradation products formed were separated from the enzyme

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during subsequent ammonium sulfate and gel filtration procedures.

Both vegetative and spore $NADH_2$ oxidases exhibited some degree of thermal stability, and this property was used in their purification and in the destruction of toxin. The extracts were placed in a boiling-water bath and, when the treatment temperature was approached, they were transferred to a water bath of the proper temperature. Vegetative extracts were treated for 3 min at 50 C. Spore extracts were treated for 30 min at 75 C. Denatured material was removed by centrifugation at 32,000 × *g*.

Assay procedures. $NADH_2$ oxidase activity was assayed by the reduction in absorbance at 340 mμ in a Beckman DU spectrophotometer. To a diluted portion of enzyme in a 3-ml cuvette, sufficient reagents were added to achieve the following final concentrations in a volume of 3.0 ml: 10 μM flavin adenine dinucleotide (FAD), 0.067 M phosphate buffer (*p*H 7.7), and 125 μM $NADH_2$. It was found necessary to mix the enzyme and FAD and incubate them at room temperature for several minutes to observe maximal activity. A unit of enzyme activity, defined as 0.01 absorbance change per min, corresponds to 4.8 mμmoles of $NADH_2$ oxidized per min. To distinguish between vegetative and spore $NADH_2$ oxidase, the enzymatic activity was tested before and after heating a portion of the sample at 70 C for 10 min. Spore enzyme was not affected by this heat treatment, whereas vegetative enzyme was completely inactivated.

The assay procedure for diaphorase was identical to $NADH_2$ oxidase assay except 10 to 100 μg/ml of methylene blue were added to the mixture. The diaphorase was estimated as the activity above the measured $NADH_2$ oxidase activity for the same sample.

Protein was determined by turbidimetric procedures (Stadtman, Novelli, and Lipmann, 1951), the Folin-phenol technique (Lowry et al., 1951), or a spectrophotometric estimation (Warburg and Christian, 1942).

Purification of $NADH_2$ oxidases. Purification of the vegetative and spore $NADH_2$ oxidases was achieved by a sequence of ammonium sulfate fractionation, gel filtration on Sephadex G-100 or G-200 (Pharmacia, Uppsala, Sweden), and ion-exchange chromotography on diethylaminoethyl (DEAE)-cellulose columns. Tris buffer, 0.01 M and *p*H 7.7, was used in all gel filtration and chromatographic procedures. Flodin's (1962) methods and calculations were used with Sephadex columns of 3.5 by 100 cm or 2.5 by 60 cm dimensions. The DEAE columns, 1 by 20 cm, were prepared according to Peterson and Sober (1962), and a linear NaCl gradient of 0.07 to 0.2 M was used to elute the proteins from the column.

Immunological methods. Vegetative-cell or spore $NADH_2$ oxidases eluted from Sephadex columns were suspended in Freund's complete adjuvant (Difco), and 0.5- to 1-ml suspensions (1 to 2 mg/ml of protein) were injected into rabbits by subcutaneous and intramuscular routes. FAD, 30 μM, was added to the vegetative enzyme in another immunization series in which intradermal injection was used. In subsequent injections, the suspensions were prepared with Freund's incomplete adjuvant and administered once a week for 4 to 6 weeks. Over a period of a year, a total of 12 rabbits were injected with vegetative protein and four with spore protein.

Blood was collected by cardiac puncture, placed in clean tubes (25 by 150 mm), slanted, incubated at room temperature for 2 to 4 hr, and then stored overnight at 4 C. Serum was decanted from these tubes and clarified by centrifugation. Further treatment of the serum was necessary to remove serum enzymes that oxidized $NADH_2$. A fraction which precipitated on the addition of ammonium sulfate at 40% saturation was prepared from the serum proteins. This fraction was suspended in 0.85% NaCl solution and used in the serological reactions described below. Control serums were prepared from blood drawn from nonimmunized rabbits and treated in a similar manner.

The method of Cohn and Torriani (1952) was used to determine cross-reactions between spore and vegetative-cell $NADH_2$ oxidases. Vegetative and spore enzymes eluted from Sephadex G-100 columns were serially diluted in 0.85% NaCl solution. The dilutions were then distributed into several sets of clean test tubes (13 by 100 mm). To each identical set of dilutions, an equal volume of antiserum, control serum, or 0.85% NaCl solution was added. All tubes were incubated at room temperature for 1 to 2 hr, stored at 4 C overnight, and any precipitate formed was removed by centrifugation. The supernatant fluid from each tube was collected and assayed for $NADH_2$ oxidase activity.

RESULTS

Purification of vegetative and spore $NADH_2$ oxidase. Several procedures in sequence were utilized to purify the spore and vegetative $NADH_2$ oxidases, but the highest purification ratios obtained were 40-fold for vegetative enzyme and 80-fold for spore enzyme. Tables 1 and 2 present the purification of the vegetative and spore oxidases.

The prolonged centrifugation of either initial extract (6 to 8 hr at 32,000 × *g*) removed the particulate $NADH_2$ oxidase which made up 10 to 15% of the total activity. Methylene blue stimulated the oxidation of $NADH_2$ by the suspended particles but had no effect on the activity of the supernatant fluid. The diaphorase activity in vegetative-cell extracts was equal to the $NADH_2$ oxidase activity, but in spore extracts it was equal to 65% of the $NADH_2$ oxidase activity.

Properties of vegetative and spore $NADH_2$ oxidase. The difference in thermal stability between the vegetative and spore oxidases is illustrated in Fig. 1. The spore enzyme had a

TABLE 1. *Purification procedures and typical results for vegetative cell NADH$_2$ oxidase from Clostridium botulinum*

Procedure	Vol	Activity	Protein	Specific activity	Purification	Yield
	ml	*units/ml*	*mg/ml*	*units/ml*		%
Initial extract	246	396	30.7	12.9	1.0	100
Centrifugation for 7.5 hr at 32,000 × *g*	238	360	18.5	19.5	1.53	87.5
Ribonuclease-deoxyribonuclease at 37 and 50 C; 3-min treatments	222	267	7.8	34.0	2.65	61.5
$(NH_4)_2SO_4$, 0–90%	100	550	17.0	32.3	2.52	56.5
$(NH_4)_2SO_4$, 55–70%	10	4,500	49.5	91.0	7.1	46.3
Sephadex G-100	3	510	1.33	380.0	29.5	1.57

TABLE 2. *Purification procedures and typical results for spore NADH$_2$ oxidase from Clostridium botulinum*

Procedure	Vol	Activity	Protein	Specific activity	Purification	Yield
	ml	*units/ml*	*mg/ml*	*units/mg*		%
Initial extract	310	96	20.5	4.7	1.0	100
Centrifugation for 6 hr at 32,000 × *g*	300	87	5.0	17.3	3.6	88
Ribonuclease-deoxyribonuclease at 37 and 75 C; 30-min treatments	280	60	0.9	66.5	14.0	56
$(NH_4)_2SO_4$, 0–90%	100	138	2.0	69.0	14.7	46.5
$(NH_4)_2SO_4$, 45–55%	7	1,740	11.2	155.0	33	44
Sephadex G-100	3	330	0.95	345	73	3.3

half-life of 35 min at 85 C, which is of the magnitude of the stability of proteins in intact spores. At 70 C, the half-life of the vegetative cell enzyme was less than 1 min.

A detailed study was performed to determine the range of ammonium sulfate concentration necessary to precipitate the two oxidases. Vegetative or spore proteins which had been initially precipitated by 90% saturated ammonium sulfate were dialyzed, and were suspended in 0.01 M Tris buffer (*p*H 7.7); calculated amounts of ammonium sulfate were added to these suspensions to give 5 or 10% increases in saturation in the range of 33 to 90% saturation. Assays were performed to determine NADH$_2$ oxidase activity and protein concentration of precipitates which formed within these increments (Fig. 2). The spore enzyme precipitated within the range 45 to 60% saturation with a peak at an ammonium sulfate concentration of 55% of saturation. The vegetative preparations precipitated over a wide, asymmetric band of ammonium sulfate concentrations with a peak at 70% of saturation. NADH$_2$ oxidase of vegetative-cell origin was partially inactive after ammonium sulfate fractionation and required FAD for maximal activity.

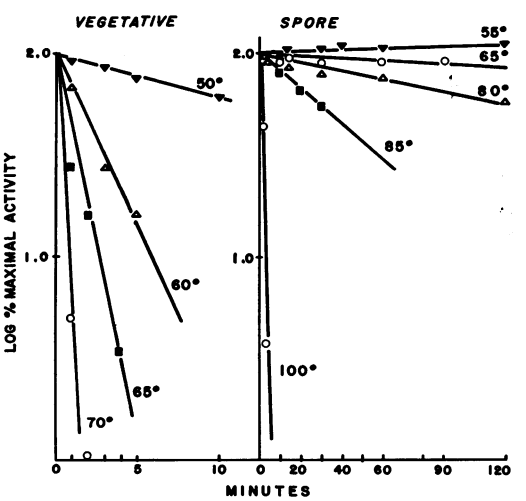

FIG. 1. *Heat inactivation of NADH$_2$ oxidases derived from vegetative cells and spores of Clostridium botulinum 62-A.*

The vegetative enzyme which precipitated over the 45 to 60% saturation range was subsequently shown to be similar to spore enzyme by its heat

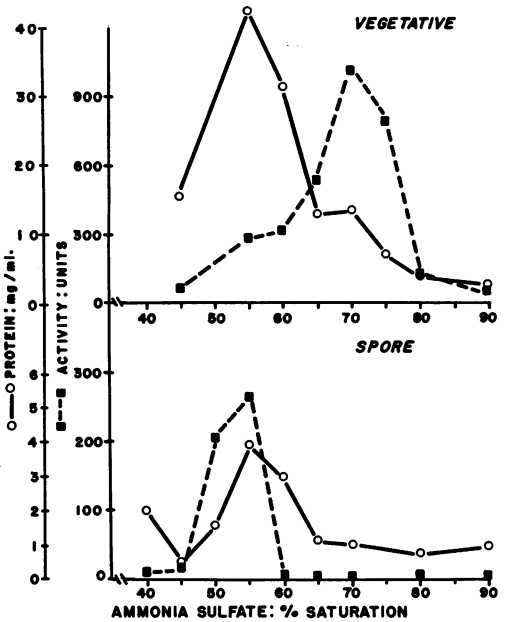

FIG. 2. *Solubility profiles of $NADH_2$ oxidase and protein from extracts of vegetative cells or spores of Clostridium botulinum 62-A. The proteins were precipitated with increasing concentrations of ammonium sulfate at 0 C.*

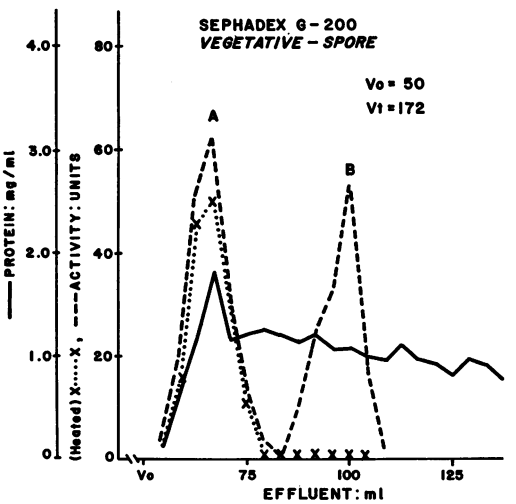

FIG. 3. *Elution profiles for $NADH_2$ oxidase activity (dashed line) and protein (solid line) from a Sephadex G-200 column loaded with a mixture of vegetative and spore extracts. The fractions from the column were heated to 70 C for 10 min, and the remaining activity is shown by the dotted line. Peak A, therefore, represents spore enzyme and peak B, the vegetative enzyme.*

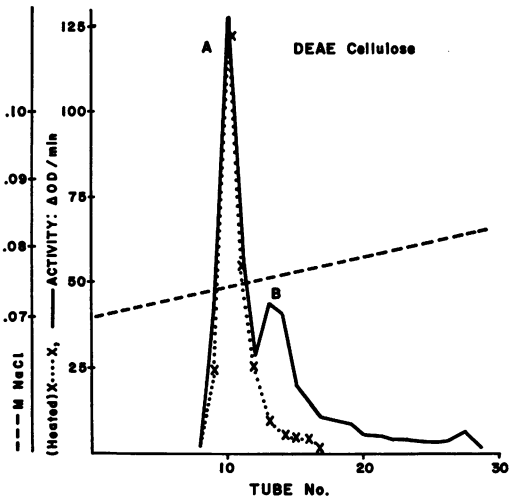

FIG. 4. *Profiles for $NADH_2$ oxidase activity (solid line) eluted with a linear NaCl gradient from a DEAE-cellulose column loaded with a mixture of vegetative and spore extracts. The fractions were heated at 70 C for 10 min, and the remaining activity is shown by the dotted line. Peak A, therefore, represents spore enzyme and peak B, vegetative enzyme.*

resistance and immunochemical properties, and amounted to approximately 10% of the activity in the extract.

Gel filtration elution profiles were established for vegetative and spore oxidases by assaying the protein and enzymatic activity of fractions from Sephadex G-100 and G-200 columns. A complete resolution of the enzymes was achieved by gel filtration. The following tests were made. Equal activities of vegetative cell and spore $NADH_2$ oxidase were mixed and placed on Sephadex G-100, G-200, and also DEAE-cellulose columns. The effluent fractions from these columns were assayed for proteins, total $NADH_2$ oxidase activity, and for heat-resistant $NADH_2$ oxidase activity. These elution profiles are shown in Fig. 3 and 4.

The spore $NADH_2$ oxidase was excluded, or nearly excluded, from Sephadex G-100 and G-200. The vegetative $NADH_2$ oxidase was retained by either Sephadex G-100 or G-200. This implies that the spore enzyme is equal to or greater than the greatest exclusion size of the G-200 gel, which corresponds to a molecular weight of 200,000. The vegetative $NADH_2$ oxidase was retained by both Sephadex G-100 and G-200, and thus has a molecular weight of approximately 100,000.

The elution profile for spore enzyme on DEAE cellulose is quite narrow compared with the

vegetative enzyme, which has a tendency to trail. These results indicate differences in ionic charge between the spore and vegetative $NADH_2$ oxidases.

Determination of enzyme characteristics. Studies of enzyme kinetics, *p*H optima, and cofactor requirements were performed on the vegetative-cell and spore $NADH_2$ oxidases. Figure 5 presents Lineweaver and Burk (1934) plots of the relationship between substrate concentration and the reaction rates from which the kinetic values were determined. Table 3 summarizes the Michaelis constants, K_m and V_{max}, for spore and vegetative enzymes and the K_i values for various concentrations of Atabrine, a noncompetitive inhibitor. The K_m of the spore enzyme is approximately eight times that of the vegetative enzyme, implying a greater substrate binding by the vegetative enzyme. The spore enzyme was less inhibited by Atabrine than the vegetative $NADH_2$ oxidase.

The *p*H optimum for either of the two enzymes lies between 7.3 and 7.6, although both enzymes were active over the range *p*H 5.5 to 9 (Fig. 6). The spore enzyme showed greater activity than the vegetative enzyme in the range below optimal *p*H; the converse was true above the optimal *p*H.

Differences in affinity for FAD were noted between vegetative and spore $NADH_2$ oxidases.

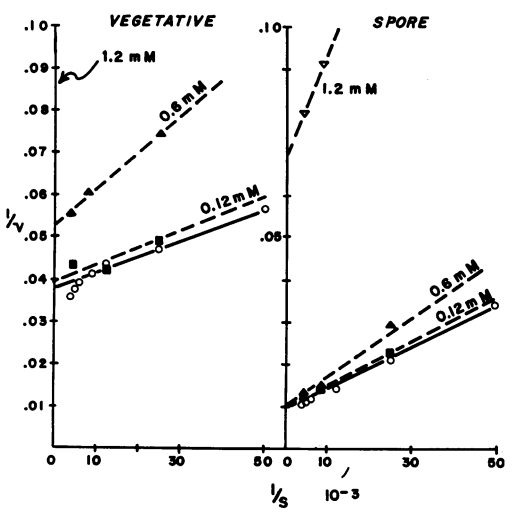

FIG. 5. *Effect of various concentrations of substrate and inhibitor on the reaction velocities of both vegetative-cell and spore $NADH_2$ oxidases. The effect of substrate is shown by the solid lines and substrate in various Atabrine concentrations by the dashed lines.*

TABLE 3. *Enzyme kinetic constants determined for vegetative-cell and spore $NADH_2$ oxidases from Clostridium botulinum*

Determination	Inhibitor concn (Atabrine)	Vegetative $NADH_2$ oxidase	Spore $NADH_2$ oxidase
	mM	*M*	*M*
K_m	—	7.9×10^{-6}	5.9×10^{-5}
V_{max}	—	130	280
K_i	0.12	1.2×10^{-5}	5×10^{-5}
	0.60	2.3×10^{-5}	9×10^{-5}
	1.2	—*	4.5×10^{-4}

* Completely inhibited.

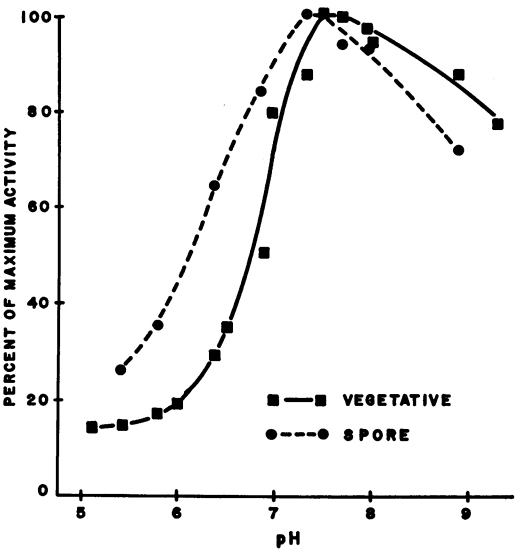

FIG. 6. *Relative activity versus pH for $NADH_2$ oxidases derived from vegetative cells and spores of Clostridium botulinum 62-A.*

The vegetative enzyme lost half or more of its activity after ammonium sulfate fractionation, and the activity could be restored by the addition of FAD but not flavin mononucleotide (FMN). After elution from Sephadex columns, the vegetative $NADH_2$ oxidase was nearly free from its cofactor. Attempts to separate the partially purified spore $NADH_2$ oxidase from its cofactor by treatment with acid-ammonium sulfate (Warburg and Christian, 1938) were unsuccessful. The cofactor remained strongly bound to the spore $NADH_2$ oxidase, and only 5% stimulation could be achieved by the addition of FAD. Acidification below *p*H 3 inactivated the enzyme irreversibly.

Serological studies. Serological studies were performed to ascertain any structural relation-

ship between vegetative-cell and spore $NADH_2$ oxidases. If these two enzymes had one or more common antigenic sites, a cross-reaction would be expected between either protein and the heterologous antibodies. These investigations show that there was no cross-reaction between the purified $NADH_2$ oxidases from vegetative cells or spores and heterologous antisera. Rabbits receiving spore enzyme produced high levels of antibody, necessitating a 10- to 100-fold dilution of the precipitated immune globulin prior to use in the Cohn and Torriani (1952) procedure. The undiluted antibody did not react with vegetative-cell $NADH_2$ oxidase. Rabbits receiving antigen derived from vegetative cells elaborated only low titers of antibodies against the vegetative $NADH_2$ oxidase. Neither the inclusion of FAD nor the route of injection had any significant effect on the antibody titer. These results indicate major differences in the antigenic properties of these enzymes.

DISCUSSION

Vegetative-cell and spore $NADH_2$ oxidases of *C. botulinum* are different proteins, and they can be differentiated by their heat resistance, solubility, molecular size, charge, enzyme kinetics, cofactor binding, and immunochemical properties. Their only nearly common property observed in this study was the hydrogen ion concentration of optimal enzyme activity. The above results are not unique, for the differences between the vegetative-cell and spore catalases of *Bacillus cereus* are also well documented (Sadoff, 1961; Norris and Baillie, 1964). In both cases, the thermal stability of the spore enzyme in extracts is of the same order of magnitude as its stability in the intact spore. Furthermore, since the thermal resistance of either $NADH_2$ oxidase or spore catalase does not diminish on purification, these proteins can be considered intrinsically stable. The stable $NADH_2$ oxidase derived from spores of *C. botulinum* is at least twice the molecular size of its labile vegetative counterpart. In contrast, the glucose dehydrogenase from spores of *B. cereus* undergoes reversible dissociation and molecular size reduction as its thermal stability increases (Sadoff, Bach, and Kools, 1965). Thus, it would appear that there is no single mechanism of aggregation or disaggregation which renders spore proteins stable.

Vegetative oxidase was not detected in extracts of well-washed spores. However, sporelike $NADH_2$ oxidase was detected in exponentially growing vegetative cells and was identified by its heat resistance, gel-filtration characteristics, and by its precipitation with serum against the spore protein. The presence of sporelike enzyme in the extracts was not due to the presence of spores in the vegetative cultures. While the significance of this relatively high level of sporelike protein in vegetative cells is unknown, its presence accounts for the spread which has been observed in the solubility, gel filtration, and chromatographic properties of the enzyme in crude extracts of vegetative cells.

Cells and spores of *C. botulinum* possess approximately equal levels of $NADH_2$ oxidase, of which 10 to 15% is particulate. The diaphorase activity in either cells or spores is approximately equal to the $NADH_2$ oxidase activity.

Vegetative cells of *B. cereus*, on the other hand, have more than 60 times the $NADH_2$ oxidase of spores, and it is essentially all (99%) particulate (Doi and Halvorson, 1961). The $NADH_2$ oxidase activity of *B. cereus* spores, albeit low, is primarily due to soluble enzyme, and it is the diaphorase which constitutes 95% of the $NADH_2$-oxidizing ability of spores.

The spore and vegetative $NADH_2$ oxidases of *C. botulinum* differ dramatically in their ability to bind cofactor. The relative insensitivity of the spore enzyme to inhibition by Atabrine, and the failure to achieve extensive dissociation of the enzyme and cofactor suggest that FAD may be covalently bound to the spore protein. The heat resistance of the spore $NADH_2$ oxidase could then be due to the bound flavin's maintaining the structural integrity of the active center, for it has been shown by Burton (1951) that even readily dissociable FAD enhances the heat resistance of other flavin-linked enzymes.

ACKNOWLEDGMENT

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LITERATURE CITED

BURTON, K. 1951. The stabilization of D-amino acid oxidase by flavin adenine dinucleotide, substrates and competitive inhibitors. Biochem. J. **48**:458–467.

COHN, M., AND A. M. TORRIANI. 1952. Immunological studies with β-galactosidase and structurally related proteins of *Escherichia coli*. J. Immunol. **69**:471–491.

DAY, L. E., AND R. N. COSTILOW. 1964. Physiology of the sporulation process in *C. botulinum*. J. Bacteriol. **88**:690–694.

DOI, R. H., AND H. HALVORSON. 1961. Comparison of electron transport systems in vegetative cells and spores of *Bacillus cereus*. J. Bacteriol. **81**: 51–58.

FLODIN, P. 1962. Dextran gels and their applications in gel filtration. Dissertation, Uppsala, AB Pharmacia, Uppsala, Sweden.

HATCH, F. T., A. R. LARRABEE, R. E. CATHOU, AND J. M. BUCHANAN. 1961. Enzymatic synthesis of the methyl group of methionine. I. Identification of the enzymes involved in the system isolated from *Escherichia coli*. J. Biol. Chem. **236**: 1095–1101.

LINEWEAVER, H., AND D. BURK. 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. **56**:658–666.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.

NORRIS, J. R., AND A. BAILLIE. 1964. Immunological specificities of spore and vegetative cell catalases of *Bacillus cereus*. J. Bacteriol. **88**:264–265.

PETERSON, E. A., AND H. A. SOBER. 1956. Chromatography of proteins. I. Cellulose ion exchange adsorbants. J. Am. Chem. Soc. **78**:751–755.

SADOFF, H. 1961. Some properties of the spore catalase and some heat resistant enzymes in bacterial glucose dehydrogenase, p. 180–194. *In* H. O. Halvorson [ed], Spores II, Burgess Publishing Co., Minneapolis.

SADOFF, H. L., J. A. BACH, AND J. W. KOOLS. 1965. Significance of multiple forms of glucose dehydrogenase in relation to its heat resistance, p. 97–110. *In* L. L. Campbell and H. O. Halvorson [ed.], Spores III. American Society for Microbiology, Ann Arbor.

SIMMONS, R. J., AND R. N. COSTILOW. 1962. Enzymes of glucose and pyruvate catabolism in cells, spores, and germinated spores of *Clostridium botulinum*. J. Bacteriol. **84**:1274–1281.

STADTMAN, E. R., G. D. NOVELLI, AND F. LIPMANN. 1951. Coenzyme A function in an acetyl transfer by the phosphotransacetylase system. J. Biol. Chem. **191**:365–376.

WARBURG, O., AND W. CHRISTIAN. 1938. Bemerkum über gelbe Fermente. Biochem. Z. **298**:368–377.

WARBURG, O., AND W. CHRISTIAN. 1942. Isolierung und Kristallisation des Garungsferments Enolase. Biochem. Z. **310**:384–421.