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# CYANIDE-RESISTANT ELECTRON TRANSPORT IN SPORULATING *BACILLUS MEGATERIUM* KM

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## SUMMARY

The NADH oxidase activity of stage V mother-cell membranes, isolated from sporulating *Bacillus megaterium* KM, shows a greater inhibition by cyanide and displays this response at lower concentrations of cyanide than the stage V forespore inner membrane. Comparison of the effects of various respiratory inhibitors reveals that the difference in cyanide sensitivity between these membranes is located on the oxidase side of the 2-heptyl-4-hydroxyquinoline *N*-oxide-sensitive step. Both membranes contain cytochromes $a+a_3$, *b*-562, *b*-555, *c* and *d*, with three potential oxidases: cytochromes $a+a_3$, *o* and *d*. Cyanide difference spectra suggest that cytochromes *b*-562 and *d* may be the components involved in the cyanide-resistant electron transport pathway. Membrane ascorbate-*N*,*N*,*N'*,*N'*-tetramethylphenylenediamine and ascorbate 2,6-dichlorophenolindophenol oxidase activities are highly sensitive to cyanide. Evidence is presented for terminal branching of the respiratory chain with branches differing in cyanide sensitivity. The cyanide sensitivity of the NADH oxidase of membranes prepared from various stages of sporulation is compared. Morphogenesis of the mother-cell plasma membrane to a cyanide-sensitive form during stages II and III of sporulation is postulated.

## INTRODUCTION

Bacterial sporulation may be regarded as an example of prokaryotic cellular differentiation [1] and many of the biochemical and morphological changes have been reviewed [2–4]. Electron microscopical observations [5–8], have shown that early in sporulation a membrane septum is laid down asymmetrically disposed to one cell pole. The smaller compartment (forespore), destined to become the mature

Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; HOQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; TMPD, *N*,*N*,*N'*,*N'*-tetramethylphenylenediamine.

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spore, is engulfed within the larger compartment (mother-cell) by a continued proliferation of the initial membrane septum towards the cell pole. After engulfment is complete, the forespore exists within the mother-cell cytoplasm as a discrete cell containing at least one complete genome and bounded by a double membrane. Thus, at least three separate membranes can be recognized in the sporulating *Bacillus*; viz. the mother-cell plasma membrane, the forespore inner membrane and the forespore outer membrane. The development of methods for isolating forespores during sporulation [9, 10] has facilitated investigations of membrane modification during sporulation [10–12].

The endogenous respiration of stage V forespores isolated from *Bacillus megaterium* KM, is markedly more resistant to KCN than the endogenous respiration of the stage V sporangial protoplast [11]. Andreoli et al. [13] have reported the respiration of isolated stage III *Bacillus cereus* forespores to be unaffected by 0.33 mM KCN. Cyanide-resistant respiration has been observed in Bacilli [14] and in several other bacterial genera [15–20]. This paper describes further investigation into the cyanide sensitivity of stage V protoplasts and isolated stage V forespores by comparing the susceptibility of the oxidase activities of the mother-cell and forespore membranes to respiratory inhibitors. The results indicate terminal branching of the respiratory chain and reveal the existence of a cyanide-resistant pathway of electron transport. Low temperature reduced minus oxidised difference spectroscopy has led to the discovery of cytochrome *d* in this organism.

## MATERIALS AND METHODS

### *Methods*

The organism used was a sporogenic strain of *B. megaterium* KM which retains its lysozyme sensitivity throughout sporulation. Synchronous sporulation, in which the population of cells in any given sporulation stage rises from 0 to 90 % within 90 min, was achieved by transferring exponentially growing cells from a rich medium to a minimal salts medium as described by Ellar and Posgate [10]. Vegetative organisms were harvested during the exponential growth phase and all subsequent sporulation stages were harvested when phase contrast microscopy of the culture indicated that 90 % of the cells had attained that particular stage. These experiments were performed in conjunction with calcium transport studies [11, 21] and the buffer used, unless otherwise stated, was 25 mM Tris · HCl, pH 7.3, containing 0.6 M sucrose, 0.09 mM $CaCl_2$, 15 mM $MgCl_2$, 1 mM $K_2SO_4$ and 0.01 mM $MnSO_4$ (sucrose salts buffer).

*Preparation of protoplasts.* Cells were washed once in sucrose salts buffer and protoplasts prepared by lysozyme treatment (250 μg/ml) at 37 °C, essentially as described by Ellar and Posgate [10].

*Isolation of forespores.* Forespores, released from protoplasts by selective rupture of the mother-cell membrane by mild sonication, were isolated by differential centrifugation as described by Ellar and Posgate [10] and Eaton and Ellar [22]. Forespores were separated from the mother-cell components by centrifugation at 11 600 × *g* for 3 min at 4 °C, and were washed four times in sucrose salts buffer.

*Isolation of spores.* Mature spores released at the end of the sporulation sequence were harvested by centrifugation (6 000 × *g* for 3 min) and washed six times in deionised water (4 °C).

*Isolation of membrane fractions*

*Vegetative and stage II membranes.* Membranes isolated from vegetative or stage II organisms were prepared either by mild sonication of protoplasts or by homogenisation of organisms with glass beads in a Braun cell homogeniser. Protoplasts were sonicated for up to 10 × 1 s pulses with a 1.2 cm (0.5 inch) sonic probe (Dawe Instruments Ltd., London, W.3, U.K.) operating at maximum output at 4 °C. Membranes, recovered by centrifugation at 37 600 × *g* for 45 min at 4 °C, were washed twice in sucrose salts buffer.

Vegetative or stage II organisms were resuspended to a final volume of 7.5 ml in sucrose salts buffer and homogenised with 40 g glass beads (0.10–0.11 mm diameter) in a Braun cell homogeniser (model MSK) for 2 × 30 s, with a liquid $CO_2$ cooling system operating. After removal of the glass beads by filtration through a coarse sintered-glass filter, the homogenate was centrifuged at 10 000 × *g* for 15 min at 4 °C to deposit cell walls. To recover membranes, the supernatant was centrifuged and the pellet washed once in sucrose salts buffer at 144 000 × *g* for 75 min at 4 °C.

*Stage III and stage V mother-cell membranes.* Protoplasts from stage III or stage V cells were prepared and disrupted by sonication as described above. After centrifugation to remove forespores, the supernatant containing the mother-cell components, was centrifuged at 37 600 × *g* for 45 min at 4 °C to deposit mother-cell membranes. These were then washed twice in sucrose salts buffer. After removal of mother-cell membranes, the supernatant containing mother-cell cytoplasm was centrifuged at 144 000 × *g* for 75 min at 4 °C, and the supernatant from this step designated the mother-cell soluble fraction.

*Forespore and spore inner membranes.* Stage V forespores and mature spores were disrupted in the Braun cell homogeniser as described above for vegetative organisms. After filtration, the homogenate was repeatedly centrifuged at 25 000 × *g* for 2-min intervals at 4 °C to deposit the spore integuments. This process was carried out until the supernatant was observed by phase-contrast microscopy to be free of contaminating spore integuments. From other experiments (ref. 11, Koncewicz and Ellar, unpublished work) it is known that both spore coats and spore outer membranes are components of the integument fraction. Spore and forespore inner membranes can conveniently be separated and purified by centrifuging the 25 000 × *g* supernatant at 144 000 × *g* for 75 min at 4 °C. The supernatant from this step was designated the spore- or forespore-soluble fraction. The inner membrane pellet was washed once in sucrose salts buffer.

*Measurement of oxidase activities.* Oxidase activity was measured in air-saturated sucrose salts buffer at 30 °C using an oxygen electrode (Rank Bros., Bottisham, Cambridge, England) in a final assay volume of 3 ml. Under the conditions used, the oxygen concentration was not limiting in any of the assays. Substrates were used at a final concentration of 0.5 mM NADH, 10 mM ascorbate plus 0.1 mM TMPD and 10 mM ascorbate plus 0.3 mM DCPIP. Correction was made for the low non-enzymatic oxidation of ascorbate-DCPIP and ascorbate-TMPD. Generally NADH oxidase measurements were performed at 0.2–0.65 mg membrane protein per assay.

Inhibitors were added as small volumes (30 μl or less) of aqueous or ethanolic solutions directly to membranes respiring NADH, or were preincubated with mem-

branes for 3 min prior to the addition of substrate. When necessary, ethanol controls were carried out and inclusion in the assay of up to 1 % (v/v) ethanol had no effect on respiration. KCN was freshly prepared for each experiment, and was adjusted to pH 7.5 with HCl.

*Measurement of difference spectra.* A split-beam recording spectrophotometer built in the Johnson Research Foundation (Philadelphia, Pa., U.S.A.) with a liquid nitrogen attachment, as described by Bonner [23] was used to measure difference spectra at liquid nitrogen temperatures. Reduced minus oxidised spectra were taken using either NADH (2 mM) or $Na_2S_2O_4$ as reductant. NADH reduced plus 250 $\mu$M KCN aerobic vs. aerobic spectra were obtained by placing aerated membranes in the reference cell and a cooled aerated membrane suspension preincubated with 250 $\mu$M KCN in the sample cell. NADH (2 mM) was added to the sample cell and the cuvettes immediately frozen in liquid nitrogen. NADH reduced plus 250 $\mu$M KCN anaerobic vs. NADH reduced plus 250 $\mu$M KCN aerobic spectra were obtained by placing membranes preincubated with 250 $\mu$M KCN and NADH (2 mM) in the sample cell and a cooled aerated membrane suspension preincubated with 250 $\mu$M KCN in the reference cell. NADH (2 mM) was added to the reference cell and the cuvettes immediately frozen in liquid nitrogen.

*Determination of protein.* Protein was estimated by the method of Lowry et al. [24] with 0.1 ml of 10 % (w/v) sodium dodecyl sulphate included in the assay to aid solubilisation of preparations.

*Chemicals.* NADH and HOQNO were obtained from Sigma (London) Chemical Co., Ltd. All other reagents were of analytical grade.

## RESULTS

### *The effects of KCN on the NADH oxidase activities of stage V mother-cell and forespore inner membranes*

We have shown previously [11] that the endogenous respiration of stage V protoplasts is inhibited by KCN to a much greater extent than the endogenous respiration of stage V isolated forespores. Mother-cell membranes possessed an NADH oxidase activity of 204 nmol $O_2$/min per mg protein, which was inhibited 72 and 81 % by 1 and 10 mM KCN, respectively. Forespore inner membranes possessed similar NADH oxidase activity (140 nmol $O_2$/min per mg protein), but in contrast this was inhibited only 32 and 57 % by 1 and 10 mM KCN, respectively. Mother-cell and forespore soluble fraction oxidases constituted approx. 9 and 10 %, respectively, of the total NADH oxidase activity of the isolated components of the stage V mother-cell and forespore. These soluble NADH oxidases, although cyanide resistant, do not represent quantitatively a major electron transport pathway in this organism. Thus, the cyanide sensitivities of the mother-cell and forespore inner membranes closely reflected the cyanide sensitivities of their respective cell types [11].

Fig. 1 shows the inhibition of NADH oxidase when KCN was added to membranes respiring NADH, or of membranes preincubated with KCN prior to the addition of NADH, when the respiratory carriers would be in the oxidised form. For both mother-cell and forespore inner membranes approx. 5–10 % greater inhibition was achieved by preincubating the membranes with cyanide. The mother-cell

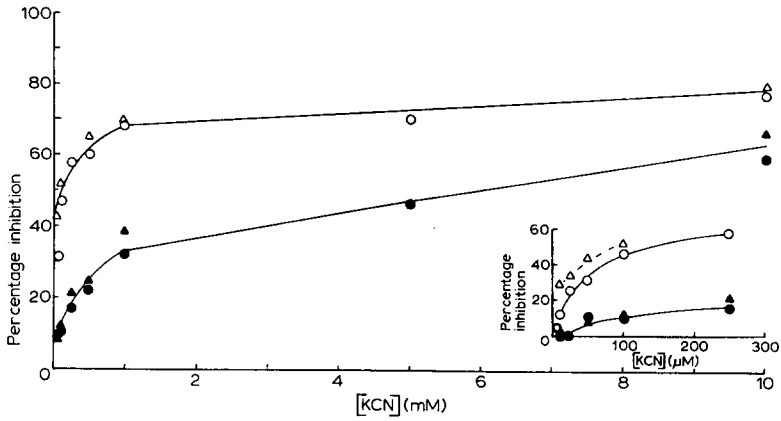



Fig. 1. The effect of KCN on the NADH oxidase activities of stage V mother-cell and forespore inner membranes. Membrane fractions were prepared and NADH oxidase activity was measured as described in Materials and Methods. Membranes were either preincubated with cyanide for 3 min prior to the addition of NADH: △, mother-cell membrane; ▲, forespore inner membrane; or cyanide was added to membranes respiring NADH; ○, mother-cell membrane; ●, forespore inner membrane.

membrane showed a greater degree of inhibition by cyanide than the forespore inner membrane and displayed this response at lower cyanide concentrations. At 25 $\mu$M KCN the mother-cell membrane was inhibited 25–35 %, while the forespore inner membrane was uninhibited. The difference in response of the two membranes was greatest between 0.25 and 1 mM KCN.

Since the nature of the forespore isolation system yielded mother-cell and forespore membranes by different methods, a control experiment was performed in which mother-cell membranes were isolated as described above, but were then homogenised with glass beads and recovered as for inner membrane preparations. The response of these membranes to cyanide was identical to that of mother-cell membranes prepared using mild sonication only.

*Inhibitor studies*

In order to localise the section of the electron transport chain resulting in differing cyanide sensitivity, the effects of various respiratory inhibitors on the NADH oxidases of the two membranes were compared. HOQNO (40 $\mu$M), which acts between menaquinone and the cytochromes in *B. megaterium* [25], inhibited the forespore inner membrane NADH oxidase to a somewhat greater extent (76 %) than the mother-cell membrane (58 %). Both membranes were inhibited approx. 28 % by 100 $\mu$M rotenone. These observations apparently localise the cyanide-resistant electron transport pathway on the oxidase side of the HOQNO-sensitive step. Similar situations have been described in other bacterial species (*Azotobacter vinelandii* [16]; *Beneckea natriegens* [17] and *Chromobacterium violaceum* [20]. It is of interest that the relatively cyanide-resistant forespore inner membrane was also resistant to inhibition by azide, while the more cyanide-sensitive mother-cell membrane showed 20 % inhibition by 10 mM $NaN_3$.

*Oxidase activities using non-physiological electron donors*

In some cases [17, 20, 26] non-physiological electron donors such as ascorbate-TMPD and ascorbate-DCPIP have been used in attempts to identify different bacterial cytochrome oxidases. Ascorbate-TMPD oxidase has been found to represent a highly cyanide-sensitive oxidase in several organisms (see above references). Ascorbate-TMPD and ascorbate-DCPIP oxidase activities of the mother-cell membrane varied somewhat between preparations, with typical values of 46 and 56 nmol $O_2$/min per mg protein, respectively, and were low compared with NADH oxidase activity and with activities observed in other bacteria [17, 26]. In two experiments neither ascorbate-TMPD nor ascorbate-DCPIP oxidase activities were detectable in forespore inner membrane preparations assayed at protein concentrations of up to 2 mg per assay. In the third experiment ascorbate-DCPIP oxidase activity was found in inner membrane preparations with a specific activity of approximately half the corresponding mother-cell membrane activity and with similar high sensitivity to cyanide. Room temperature reduced minus oxidised difference spectra of stage V mother-cell membranes using either ascorbate-TMPD or ascorbate-DCPIP as reductant have shown that both substrates preferentially reduce cytochromes *c* and $a+a_3$ in this organism (Hogarth, C., unpublished work).

Ascorbate-DCPIP and ascorbate-TMPD oxidases of stage V mother-cell membranes were highly sensitive to cyanide with maximum inhibition, approaching 80–90 %, achieved at cyanide concentrations of 10 $\mu$M (Fig. 2) and $I_{0.5}$ values for both oxidases of 2.3 $\mu$M. Double reciprocal plots of the fractional inhibition against cyanide concentration for ascorbate-DCPIP and ascorbate-TMPD oxidases yielded straight lines. HOQNO (40 $\mu$M) had no effect on mother-cell membrane ascorbate-TMPD and ascorbate-DCPIP oxidases.

*Cytochrome components of stage V mother-cell and forespore inner membranes*

The cytochrome complement of *B. megaterium* has previously been shown to include cytochromes $a+a_3$, *b*, *c* and *o* [12, 25, 27, 28]. The cytochrome components

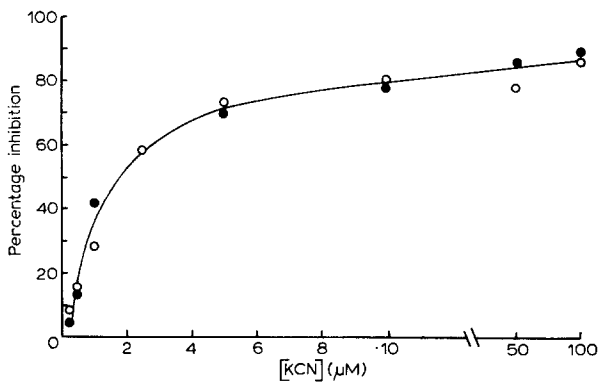



Fig. 2. Effect of cyanide on ascorbate-DCPIP and ascorbate-TMPD oxidases. Ascorbate-DCPIP and ascorbate-TMPD oxidases were measured as described in Materials and Methods. Cyanide was added to respiring membranes and at these concentrations had no effect on the non-enzymic rate of oxidation. Ascorbate-DCPIP oxidase (○) was assayed at 1.07 mg protein per assay. Ascorbate-TMPD oxidase (●) was assayed at 0.80 mg protein per assay.

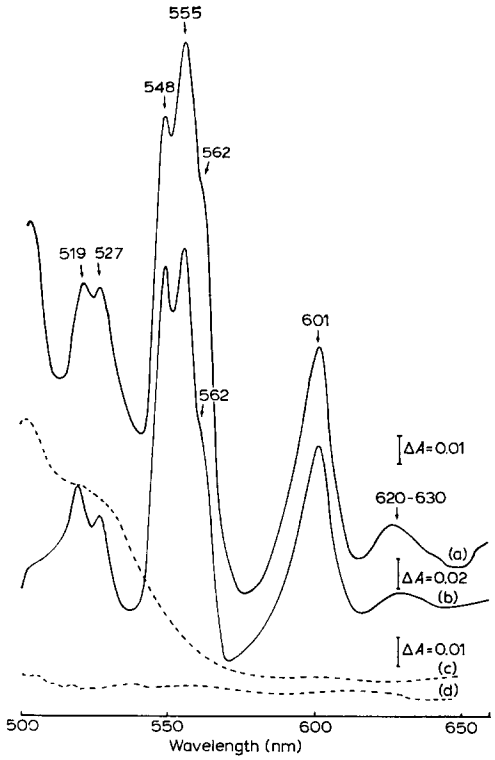



Fig. 3. Reduced minus oxidised difference spectra of stage V mother-cell and forespore inner membranes. Dithionite reduced minus oxidised difference spectra were carried out at liquid nitrogen temperatures as described in Materials and Methods. a, forespore inner membrane, 8.8 mg protein/ml; b, mother-cell membrane, 7.1 mg protein/ml; c, forespore inner membrane base line; d, mother-cell membrane base line.

of stage V mother-cell and forespore inner membranes were compared by dithionite reduced minus oxidised difference spectra, performed at liquid nitrogen temperatures. Both membranes were found to contain cytochromes $a+a_3$, *b* and *c* (Fig. 3), with maxima at 601, 555 and 527 nm, and 548 nm and 519 nm, respectively. The increased resolution achieved at liquid nitrogen temperatures revealed a distinct shoulder on the *b*-555 peak at 562 nm and therefore a second *b* type cytochrome, and a broad absorption peak with a maximum at 620–630 nm, typical of cytochrome *d* (Fig. 3). In both membranes the height of the cytochrome *d* adsorption peak was approx. 10–15 % of the $a+a_3$ peak (601 nm). All the cytochromes of both membranes were fully reducible by NADH (Fig. 4). Although direct quantitative measurements of cytochrome contents are not possible in liquid nitrogen difference spectra, the inner membrane contained proportionately more of the *b*-562 component and less of the cytochrome *c* component than the mother-cell membrane. The forespore inner membrane is an intensely red membrane with a low cytochrome and high protein and carotenoid content, and produces a sloping base line in liquid nitrogen difference spectra (Fig. 3), making it less than ideal for spectral investigation.

Dithionite reduced plus CO minus dithionite reduced liquid nitrogen spectra

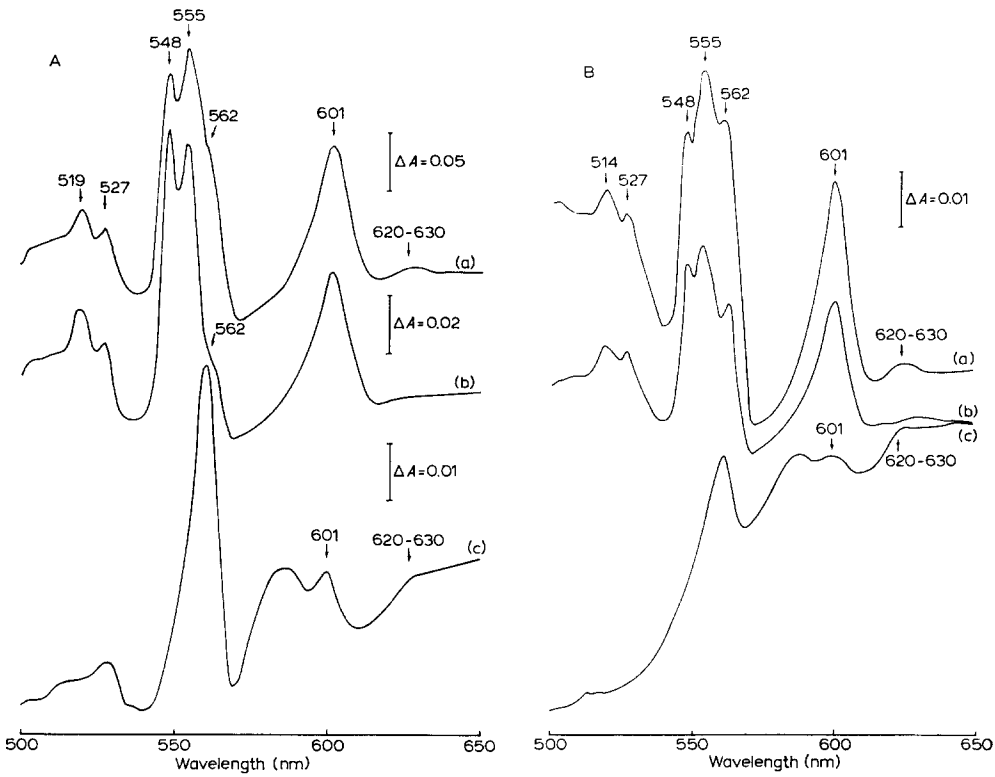



Fig. 4. Cyanide difference spectra of stage V mother-cell (A) and forespore membranes (B). Difference spectra at liquid nitrogen temperatures were performed as described in Materials and Methods. a, NADH reduced minus oxidised difference spectra; b, NADH reduced plus 250 $\mu$M KCN aerobic vs. aerobic difference spectra; c, NADH reduced plus 250 $\mu$M KCN anaerobic vs. NADH reduced plus 250 $\mu$M aerobic difference spectra. Spectra of forespore inner membranes were carried out at a protein concentration of 6.7 mg/ml and for mother-cell membranes, spectrum a at 8.8 mg/ml and spectra b and c at 4.4 mg/ml.

revealed cytochrome *d* (identified by its peak at 630–640 nm) as an additional CO binding component as well as $a+a_3$ and *o*, previously shown to be CO binding components in this organism by Broberg and Smith [27] and Wilkinson and Ellar [12]. This suggests the presence of three potential oxidases in this organism.

*Cyanide difference spectra of mother-cell and forespore inner membranes*

Dixon plots [29] for the cyanide inhibition of mother-cell and forespore inner membranes revealed biphasic curves for both membranes and indicated two modes of inhibition with widely differing values for $K_i$.

In plant mitochondria [30] and *Pseudomonas* AMI [31] the presence of 250 $\mu$M KCN and 300 $\mu$M KCN, respectively, have been shown to result in complete inhibition of oxidation of cytochromes $a+a_3$. Thus, in our organism the presence of 250 $\mu$M KCN might be expected to prevent oxidation of cytochrome $a+a_3$, while some other component of the electron transport system transfers electrons to oxygen to account for the residual cyanide-resistant respiration.

In liquid nitrogen difference spectra (trace b, Fig. 4) the presence of cyanide in aerobic conditions for both membranes resulted in a decrease in the peak heights of components *b*-562 and *d*, and to a much lesser extent $a+a_3$, relative to the NADH reduced minus oxidised spectra (trace a, Fig. 4), indicating that these components remained to some extent oxidised in the presence of 250 $\mu$M KCN.

In NADH reduced plus 250 $\mu$M KCN anaerobic vs. NADH reduced plus 250 $\mu$M KCN aerobic spectra (trace c, Fig. 4) only those cytochromes which can remain oxidised in the presence of 250 $\mu$M KCN will result in absorption peaks. For both membranes, peaks corresponding to cytochromes *b*-562 and *d* resulted. For the mother-cell membrane these peaks, in comparison with NADH reduced minus oxidised spectra (trace a, Fig. 4) represented almost complete oxidation of cytochromes *b*-562 and *d*, while for the forespore inner membrane they represented approx. 40–50 % oxidation. For both membranes a very small absorption peak due to cytochrome $a+a_3$, representing approx. 5–10 % of the total $a+a_3$, and an additional peak with maximum 585–588 nm due to the cytochrome $a_3$-cyanide complex [30] were present. For forespore inner membranes, cyanide difference spectra (trace c, Fig. 4) showed a change in the slope of the base line which was probably a freezing artifact.

These results indicated that cytochromes *b*-562 and *d* may be the components involved in the cyanide-resistant electron transport pathway and that this resistant pathway exists in both membranes. However, this experimental approach gives no information on the relative quantitative contribution of the resistant pathway to the total NADH oxidase of the two membranes.

*Cyanide sensitivity of NADH oxidase in membranes prepared from various stages of sporulation*

While homogeneous preparations of plasma membranes are readily obtained from vegetative and stage III organisms [10], membranes prepared by these methods in stage II before the forespore is engulfed, will contain potential mother-cell plasma membranes and potential forespore inner membranes. However, at this time the smaller compartment represents only 10 % of the sporangial volume. The NADH oxidase activities of vegetative and stage II membranes, (prepared either by homogenisation of cells or sonication of protoplasts) and mature spore inner membranes exhibited similar cyanide inhibition to the stage V forespore inner membrane NADH oxidase (Fig. 5). The stage III mother-cell membrane was inhibited by cyanide to the same extent as the stage V mother-cell membrane (Fig. 5). Thus, it would appear that the mature spore inner membrane retains the cyanide resistance properties of the vegetative cell plasma membrane throughout sporulation. This is in agreement with Tochikubo [32] who found that spore membranes had similar inhibitor sensitivities to vegetative membranes in *Bacillus subtilis*. Modification of the mother-cell plasma membrane, to account for the increased sensitivity to cyanide, must take place after stage II and during stage III of sporulation, and probably after completion of engulfment of the forespore.

The cytochrome complements of these membranes were examined by liquid nitrogen difference spectroscopy (data not given). Although no absolute difference was observed, the relative proportions of the various cytochromes change throughout sporulation. Cytochromes $a+a_3$, *d* and *b*-555 were present to approximately the same degree throughout sporulation; however, the shoulder representing cytochrome *b*-562

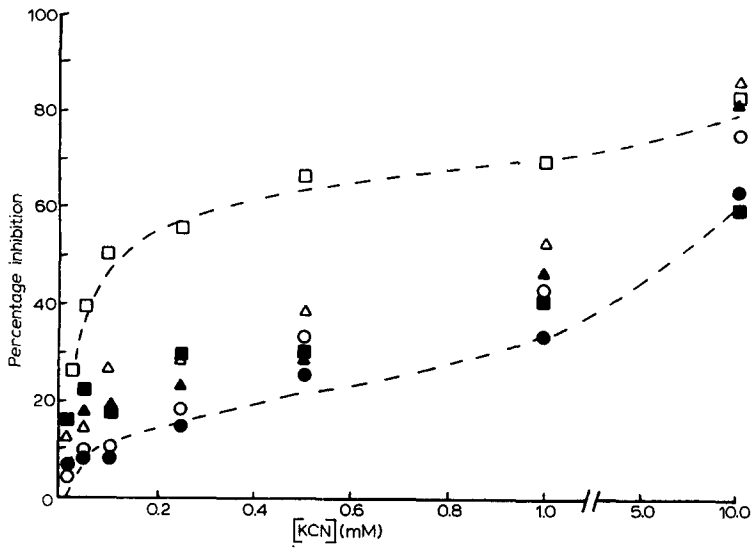



Fig. 5. The cyanide sensitivity of membranes from various stages of sporulation. Membrane fractions were prepared either by disruption of organisms with glass beads or by sonication of protoplasts as described in Materials and Methods. Mature spore inner membranes were prepared in 0.05 M Tris · HCl, pH 7.5 [12] and were resuspended and assayed in 0.05 M $KH_2PO_4$ (pH 7.5). NADH oxidase measurements were carried out as described in Materials and Methods. Cyanide was added to membranes respiring NADH, with the exception of mature spore inner membrane assays, where membranes were preincubated with cyanide prior to the addition of NADH. Open symbols, membranes prepared by sonication of protoplasts. Closed symbols, membranes prepared by disruption of organisms by homogenisation with glass beads. ○ and ●, vegetative membranes, 0.2 and 3.0 mg protein per assay, respectively; △ and ▲, stage II membranes, 1.25 and 1.88 mg protein per assay, respectively; □, stage III mother-cell membranes, 0.84 mg protein per assay; ■, mature spore inner membranes, 0.98 mg protein per assay. Dotted lines corresponding to stage V mother-cell and forespore inner membranes are included for reference.

which was present only to a small extent in vegetative membranes, increased by stage II, was found in the stage V forespore inner membrane to a greater degree than in the stage V mother-cell membrane and had become the major *b*-type cytochrome component of mature spore inner membranes. Cytochrome *c*, which is absent in vegetative membranes and appears by stage II of sporulation [12], was present to a somewhat lesser extent in the stage V forespore inner membrane than the stage V mother-cell membrane and contributed only a shoulder to the mature spore inner membrane spectrum.

## DISCUSSION

Although cyanide-resistant respiration occurs at all stages of growth of *B. megaterium* KM, the contribution of the cyanide-resistant pathway to membrane NADH oxidase varies during sporulation and between the compartments of the sporulating cell after engulfment. For exponential phase and stage II membranes and stage V forespore and mature spore inner membranes, 60–80 % of the respiration was possible in the presence of 500 $\mu$M KCN, while stage III and stage V mother-

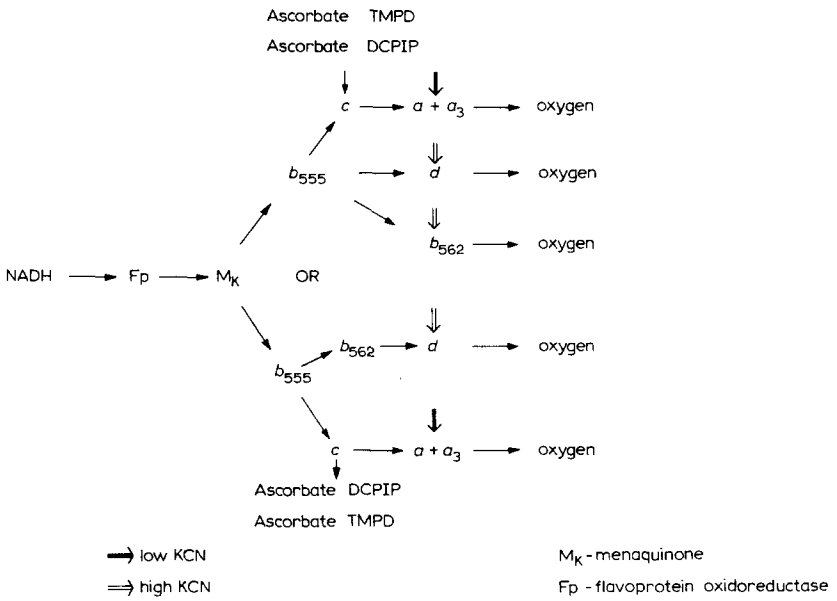
cell membranes were comparatively cyanide sensitive and only 35–40 % NADH oxidase activity occurred in the presence of 500 $\mu$M cyanide. The response of stage V mother-cell and forespore inner membranes to cyanide reflected the cyanide sensitivity of their corresponding cell types. We have thus shown that membrane fractions isolated from the two compartments of the same cell possess functional differences and that this difference is not a result of different methods of preparation.

Cyanide-resistant respiration occurs in a wide variety of genera from both prokaryotes and eukaryotes, and a number of mechanisms have been proposed as responsible (for a review see Henry and Nyns [33].) Hydroxamic acids specifically inhibit the cyanide-resistant alternate oxidase of branched, mitochondrial respiratory systems [34]. Such an alternate oxidase does not appear to be operative in *B. megaterium* KM, as salicylhydroxamic acid did not enhance inhibition of membrane NADH oxidase by cyanide.

In some bacterial systems cytochrome *d* appears to be involved in cyanide-resistant respiration. Arima and Oka [15] found a close relationship between the degree of resistance to cyanide and the amount of cytochrome *d* ($a_2$) in *Achromobacter*, while Jones and Redfearn [16] and Ackrell and Jones [35] proposed that cytochrome *d* may act as a cyanide-resistant oxidase in *A. vinelandii*. Recent publications [18, 19] have implicated cytochrome *d*, which increases during stationary phase, in the cyanide resistance of *Escherichia coli*. We have found cytochrome *d* to be present in our organism in approximately the same proportions throughout sporulation. The presence of this cytochrome alone, is therefore an insufficient explanation of the cyanide resistance displayed by this organism. The occurrence of cytochrome *d* has not previously been reported in strains of *B. megaterium* and has been found in only a few species of *Bacillus* [36, 37]. Cytochrome *d* is usually found in combination with cytochromes *a* and *o* rather than $a+a_3$ and *o*.

Although cytochrome *o* is claimed to be cyanide resistant in *Acetobacter suboxydans* [38, 39], it is not usually associated with cyanide resistance and has indeed been shown to be more cyanide sensitive than cytochrome *d* in some organisms [16, 18]. The cyanide sensitivity of cytochrome *d* is reported to vary greatly between organisms [18].

In NADH reduced plus 250 $\mu$M KCN anaerobic vs. NADH reduced plus 250 $\mu$M KCN aerobic difference spectra (trace c, Fig. 4) cytochromes *b*-562 and *d* can remain oxidised by air, while cytochromes *c* and $a+a_3$ show almost complete reduction. Thus 250 $\mu$M KCN results in almost complete inhibition of oxidation of cytochrome $a+a_3$. This makes it unlikely that biphasic Dixon plots may be due to one oxidase whose inhibited form is still capable of significant turnover. We therefore propose that cytochromes *b*-562 and *d* are components involved in a cyanide-resistant electron transport pathway in *B. megaterium*. The peak due to cytochrome *b*-562 in Fig. 4 (trace c) is asymmetrical and this may suggest incomplete reduction of cytochrome *b*-555. Cytochrome *c*-548 showed complete reduction under these conditions (trace c, Fig. 4). Possible arrangements of the electron transport system during stage V of sporulation of *B. megaterium* are shown in Scheme 1. The section of the electron transport chain prior to the cytochromes is based on Kröger and Dadáck [25]. It is not known whether both cytochromes *b*-562 and *d* can act as terminal oxidases or whether *b*-562 transfers electrons to cytochrome *d*. Ascorbate-TMPD and ascorbate-DCPIP oxidases are highly sensitive to cyanide, showing maximal inhibition at 10




Scheme 1. Proposed electron transport system of *Bacillus megaterium* KM during sporulation. The section of the electron transport chain prior to the cytochromes is based on Kröger and Dadáck [25]. This scheme does not include cytochrome *o* but there is some evidence to suggest that cytochrome *o* may be cytochrome *b*-562 in *B. megaterium* [25, 27, 32].

$\mu$M and $I_{0.5}$ values of 2.3 $\mu$M.

For the mother-cell membrane respiring NADH, the dominant oxidase is the cytochrome $a+a_3$ oxidase, with the alternative oxidase representing only a small proportion. In contrast, for forespore inner membranes the dominant oxidase is the alternative oxidase with only a small contribution from the $a+a_3$ pathway. The more cyanide-sensitive stage V mother-cell membrane contains somewhat more cytochrome *c* and considerably less cytochrome *b*-562 than the stage V forespore inner membrane. Cytochrome *b*-562 increases throughout sporulation and becomes the dominant *b*-type cytochrome in the dormant spore inner membrane, and cytochrome *c* is absent from the cyanide-resistant exponential phase membrane.

A functional difference between vegetative membranes and stages III and V mother-cell plasma membranes and between stage V mother-cell membrane and the forespore inner membrane has been shown. Membrane modification is undoubtedly involved in spore morphogenesis and here we have demonstrated that the mother-cell plasma membrane undergoes a functional change during stage II-III while the forespore inner membrane remains closely similar, in this respect at least, to the vegetative cell plasma membrane. The greater sensitivity of the plasma membrane of sporulating cells to cyanide as compared to vegetative cells may account for the observation by Lang et al. [40] that vegetative cells will grow in 0.3 mM KCN but will not sporulate.

In comparing the features of *B. megaterium* respiration reported here with the composition of the respiratory chain in other bacteria, it is important to consider those characteristics which distinguish the sporulating cell from its vegetatively

growing counterpart. Sporulation in Bacilli is initiated when growth is reduced by depletion of the carbon source or limitation of other essential metabolites. At the time of this transition from exponential growth, the citric acid cycle is derepressed and an increase in oxygen consumption occurs. These observations suggest a shift from glycolytic to oxidative energy metabolism [41]. From stage III of sporulation, the former vegetative cell contains a second cell (forespore) destined to become the mature spore, which is enclosed by two membranes with reversed polarity relative to each other [42]. These forespore membranes have both been shown to possess NADH oxidase activity. Since the mother-cell membrane also possesses NADH oxidase activity, it is difficult to determine the extent to which oxygen is available to the forespore inner membrane even in a well aerated culture. If, however, this membrane exists in a relatively anaerobic environment by comparison to the mother-cell membrane, the fact that the cyanide-resistant oxidative pathway is dominant in the forespore could be an important advantage. Cytochrome *d* is often associated with conditions of reduced oxygen availability [43–45]. From their results with *Kurthia zopfii* including the very low P/O ratios under oxygen-limited conditions, Meyer and Jones [45] suggested that the cyanide-resistant oxidase in this organism which is probably cytochrome $a_2(d)$ (Meyer, D. J. and Jones, C. W., personal communication) might have a high affinity for oxygen, enabling respiratory chain phosphorylation to continue at low oxygen concentrations.

Jones et al. [46] have noted that the physiology of different microorganisms may to some extent be reflected in differences in respiratory chain composition and consequently in their efficiency of energy conservation. They observed that three species of *Bacillus* (*B. licheniformis*, *B. megaterium* and *B. subtilis*) growing vegetatively, belong to a group of microorganisms which lack both pyridine nucleotide transhydrogenase and cytochrome *c* and which therefore possess only two proton translocating loops. In terms of respiration-linked proton translocation, these organisms are potentially less efficient energy conservers than other organisms in which the presence of cytochrome *c* provides a third proton translocating loop.

These observations by Jones et al. [46] provide a valuable framework in which to view our results. Since as pointed out above, sporulation occurs in a relatively poor environment, the *Bacillus* may no longer be able to afford the luxury of relatively poor energy conservation which characterized the vegetative cell. Accordingly, the incorporation of cytochrome *c* into the membranes during sporulation of *B. megaterium* KM may reflect this change in the environment and result in a necessary increase in the efficiency of energy conservation through the provision of a third proton translocating loop.

In the organism used in this study, the vegetative cell respiration displays a similar cyanide resistance to that of the forespore inner membrane. It will be important to determine whether this pattern is a general feature of sporulating Bacilli. It is possible that the cyanide sensitivity of the vegetative cell membrane may vary in different *Bacillus* species and with the growth conditions used. If an example can be found where the vegetative cell membrane is relatively cyanide sensitive, it will be of interest to determine whether there is a distinct change to a more cyanide-resistant membrane in the spore form of this organism.

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