THE EFFECT OF RATE OF RESPIRATION ON SENSITIVITY TO CYANIDE AND CARBON MONOXIDE IN *BENECKEA NATRIEGENS* GROWN IN BATCH AND CONTINUOUS CULTURE

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1. Introduction

Respiration of cell-free extracts of the marine bacterium *Beneckea natriegens* was reported to be inhibited by 10 μM cyanide when ascorbate-TMPD* was the substrate but, with succinate as the electron donor, 1 mM KCN caused only 50% inhibition [1]. These results were explained in terms of a terminal branching of the electron transport system. *Beneckea natriegens* had been shown to possess 4 different potential terminal oxidases: cytochrome a_1, cytochrome *d*, cytochrome *o* and a CO-binding cytochrome *c* [2], and it was suggested by Weston and Knowles [2] that this may be further indication of a branching of the respiratory chain at the terminal oxidases, although they did not ascribe any particular cytochrome to the cyanide-insensitive pathway. Studies of cyanide sensitivity of whole cells of *B. natriegens* grown at different oxygen concentrations in chemostat culture failed to reveal changes in the sensitivity of the maximum potential respiration rate (i.e. respiration in the presence of excess substrate) to cyanide [3], although the relative content of cytochromes *o* and CO-binding *c* varied [4]. Further experiments showed that growth of glucose-limited cultures was resistant to inhibition by cyanide while harvested cells, supplied with excess substrate, were sensitive (Linton, Harrison and Bull, in preparation). This could be explained if it were the actual electron flux through the respiratory system on which the sensitivity to cyanide depended. This latter possibility has been investigated in the present work using harvested cell suspensions.

* TMPD = *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride.

2. Materials and methods

2.1. *Organism and media*

Beneckea natriegens strain III was provided by C. J. Knowles, University of Kent at Canterbury. The organism was grown in continuous culture under glucose or glycerol limitation in a minimal medium containing (per litre) (A) Na_2HPO_4, 1.5 g; KH_2PO_4, 1.5 g; $(NH_4)_2SO_4$, 3.0 g; NaCl, 23 g; KCl, 0.745 g; 1 ml trace element mixture from a stock solution containing $CaCl_2 2H_2O$, 0.66 g; $ZnSO_4 7H_2O$, 0.18 g; $CoSO_4 5H_2O$, 0.16 g; $MnSO_4 4H_2O$, 0.15 g; $CaCl_2 6H_2O$, 0.18 g; H_3BO_3; 0.10 g; $Na_2MoO_4 2H_2O$, 0.30 g pH 7.3 (925 ml). (B) Glucose or glycerol 2.0 g; $MgSO_4 7H_2O$, 0.2 g; trisodium citrate 0.146 g; 0.020 g $FeSO_4 7H_2O$ pH 7.3 (75 ml). Components A and B were autoclaved separately and mixed on cooling. Details of the growth conditions in continuous culture have been described [4]. Erlenmeyer flasks fitted with side arms to facilitate direct absorbance measurements were used to grow the organism in batch culture and the minimal medium described above was used with glucose as the sole source of carbon and energy.

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2.2. *Measurement of respiration rate of harvested bacteria*

Respiration rates were measured by diluting fresh samples from the chemostat in complete basal medium minus the carbon and nitrogen source (pH 7.2), adding known quantities of glucose and following the oxygen uptake in an oxygen electrode respirometer cell (Rank Bros., Bottisham, Cambridgeshire [5]). Measurements were made at 31°C unless stated otherwise.

2.3. *Inhibitor studies*

Solutions of known concentrations of KCN were injected into the oxygen electrode cell containing bacteria suspended in a solution buffered to pH 7.2. After a 2 min incubation period substrate was injected into the cell and the oxygen uptake recorded. Preliminary experiments had shown that between 0.5 and 1.0 mins were required after exposure to cyanide, to attain a constant rate of respiration.

Carbon monoxide gas was bubbled briskly through the buffer (pH 7.2) for 5 min which was sufficient to saturate the solution. The saturated CO solution was diluted to give final concentrations of CO from 5 to 50% saturation. The bacteria suspended in aerated saturated solution at pH 7.2 were diluted with the CO solution in the oxygen electrode cell, darkened by covering with aluminium foil and left in contact with the CO for 2 min before the substrate was injected into the cell.

For studying the combined effect of KCN and CO, CO saturated buffer was introduced into the oxygen electrode cell containing diluted culture and the container closed immediately. KCN was then injected into the suspension. A 2 min incubation period was allowed before substrate was injected into the electrode cell.

3. Results

3.1. *Respiration of glucose*

The effect of cyanide concentration on the respiration rate of *Beneckea natriegens* freshly harvested from a glucose-limited chemostat culture and supplied with excess (133 μM) glucose is shown in fig.1a. Respiration was sensitive to cyanide, over the whole range of cyanide concentrations tested down to 5 μM. The experiment was repeated using a limiting glucose concentration of 44 μM which gave an initial respiration rate of only 85% of the maxi-

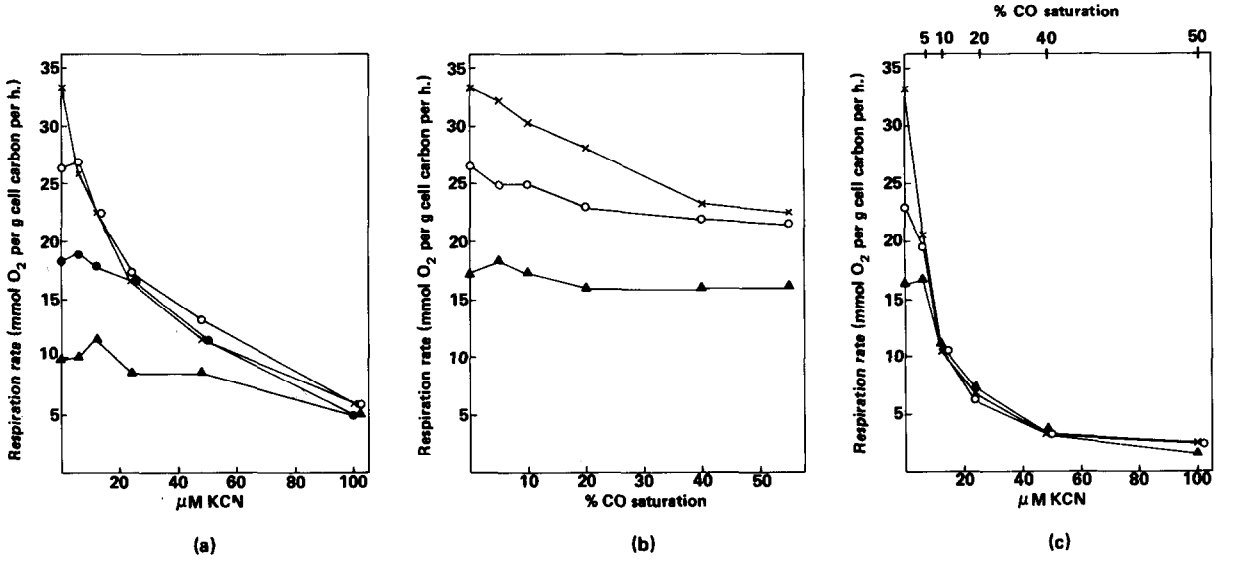

Fig.1. The effect of respiration rate on the sensitivity of respiration of cells harvested from a glucose grown culture of *B. natriegens* to cyanide and carbon monoxide. (a) The effect of KCN. (b) The effect of CO. (c) The effect of a combination of KCN and CO. The respiration rate was varied by injecting glucose at the following concentrations (X——X) 133 μM; (o——o) 44 μM; (●——●) 22 μM; and (▲——▲) 11 μM into the oxygen electrode cell.

mum. In this case 6 μM cyanide had no effect on respiration rate but higher concentrations caused inhibition. With 22 μM glucose, giving a respiration rate of 70% of the maximum, cyanide concentrations of up to 12 μM had no effect and with 11 μM glucose, giving a respiration rate of 45% of the maximum, cyanide had little effect at concentrations below 24 μM. Above these 'critical' cyanide concentrations the curves were almost completely superimposable (except the 11 μM glucose case).

The effect of carbon monoxide at various glucose concentrations is shown in fig.1b and it can be seen that it had little effect on respiration rate. However, carbon monoxide in combination with cyanide caused much greater level of inhibition than cyanide alone (fig.1c). Once again, at sub-maximal respiration rates there was a range of cyanide concentrations below which cyanide had no effect and above which the response curve was superimposable on that of the maximum respiration rate.

3.2. *Respiration on glycerol*

In order to investigate whether the phenomena observed above were unique to glucose, and possibly associated with its active transport, the experiment was repeated on bacteria grown on glycerol (reported to be taken up by facilitated diffusion) [6]. The pattern of results was very similar to that obtained with glucose: The maximum respiration rate (obtained with 352 μM glycerol) was the same and concentrations of glycerol of 85, 44 and 22 μM gave a similar family of curves as that shown in fig.1a for glucose. The combination of cyanide and carbon monoxide also gave a remarkably similar pattern of curves to that obtained with glucose.

3.3. *Effect of temperature*

A series of experiments made in order to test whether the change in sensitivity was a function of respiration rate itself or of substrate concentration. The respiration rate was altered, in the presence of excess (133 μM) glucose by changing the temperature of the oxygen electrode cell. Fig.2 shows that lowering the temperature, and thus the respiration rate, rendered the bacteria more insensitive to cyanide. Again at sub-maximal respiration values there was a range of low cyanide concentrations to which the bacteria were completely insensitive.

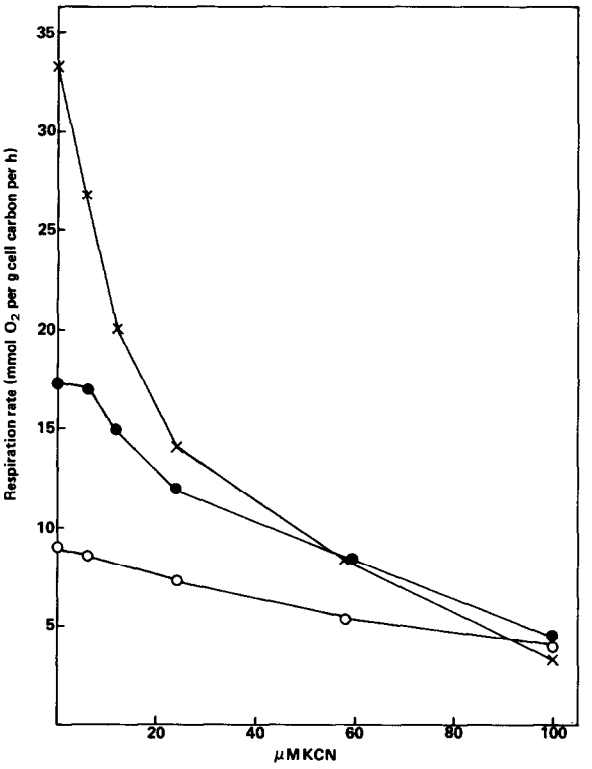

Fig.2. The effect of reduced incubation temperature on the sensitivity of the respiration of glucose grown cells to KCN. (×——×) 31°C; (•——•) 25°C; (○——○) 5°C.

3.4. *Effect of cyanide on bacteria from batch cultures*

Earlier reports of insensitivity to cyanide by *B. natriegens* were based on bacteria grown in batch culture [1]. Therefore, *B. natriegens* was grown on glucose in batch culture and the effect of cyanide tested on bacteria harvested from the logarithmic phase and the early and late stationary phases. Fig.3 shows that the bacteria harvested in the stationary phase had a much lower maximum respiration rate and were also less sensitive to cyanide.

3.5. *Cyanide sensitivity of bacteria grown in the presence of cyanide*

The effect of exposure to cyanide during growth was investigated by adding 50 μM KCN to the medium supplied to a glucose-limited chemostat culture. We have shown previously that growth and in situ

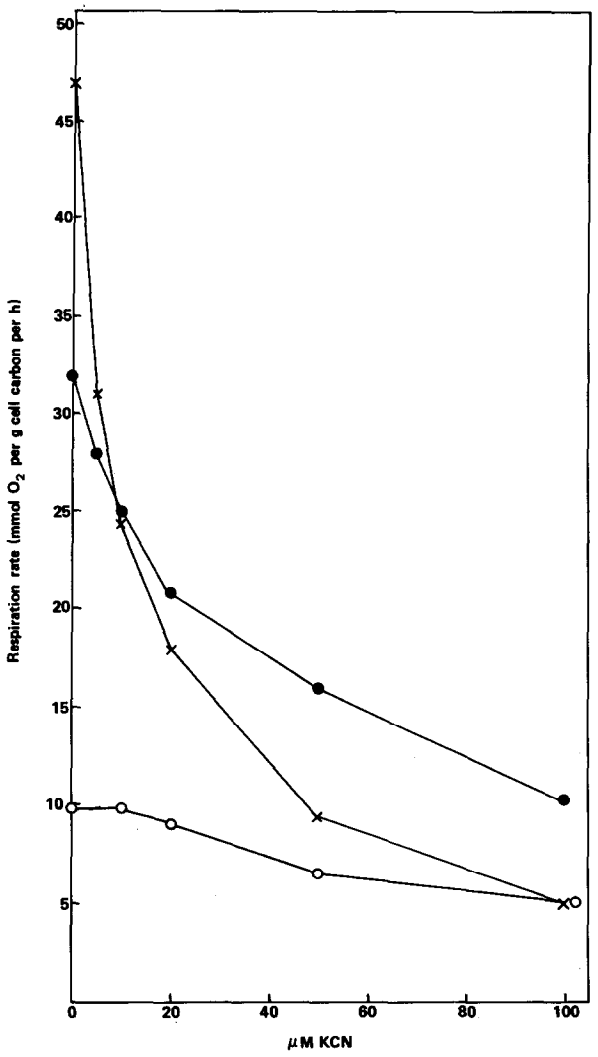

Fig.3. Cyanide sensitivity of the respiration of *B. natriegens* taken from log phase early and late stationary phase batch culture. Log phase (X——X); early stationary phase (16 h) (●——●); late stationary phase (24 h) (○——○).

respiration in chemostat culture in the presence of excess oxygen is unaffected by 50 μM KCN but the level of CO-binding cytochrome *c* in the bacteria is increased significantly [4]. The maximum potential respiration rate of harvested bacteria grown in the presence of KCN was very similar to that obtained in the absence of KCN and a similar pattern of sensitivity to cyanide and to combined cyanide and carbon monoxide was also obtained, this being dependent on the respiration rate of the bacteria.

The effects of KCN and CO singly and in combination at maximal respiration rates indicated that the combined effect was additive (table 1).

4. Discussion

The results reported here indicate a far greater dependence of respiratory sensitivity to cyanide on the actual respiration rate than has been reported previously for whole cells of any species. At maximum respiration rates the effect of KCN and carbon monoxide appeared to be at least additive, that is the combined effect of KCN and CO was equal to a summation of their separate effects. This type of response is usually found when the two inhibitors act on the same enzyme [7].

The relationship between cyanide concentration and respiration rate at different initial respiration rates was unexpected. There have been few systematic studies reported on the effect of respiration rate on cyanide sensitivity but Jones [4] reported pure uncompetitive inhibition for physiological substrates, but non-competitive inhibition for ascorbate dichlorophenolindophenol oxidation via cytochrome *o* in *Azotobacter vinelandii.* The relationship found in *B. natriegens* in this work was neither that of simple competitive nor of non-competitive inhibition. At sub-maximal respiration rates there is a range of cyanide concentrations to which respiration is completely insensitive but at the point where these curves meet that obtained in the presence of excess glucose, respiration becomes sensitive and followed the same curve as that obtained with excess glucose.

A similar family of curves was obtained with glycerol as the substrate and it is suggested, therefore, that the effect was not due to inhibition of glucose transport by cyanide. Moreover, decreasing the respiration by lowering the incubation temperature also gave a similar series of curves from which it may be concluded that respiration rate rather than substrate concentration is the important factor.

These results indicate an apparent titration of cyanide against a limiting component of the respiratory chain. At maximal respiration rates this component is limiting for respiration rate therefore low cyanide

Table 1
The effect of KCN and CO on the maximum respiration rate of *B. natriegens* harvested from continuous cultures grown on glucose in the presence and absence of cyanide and on glycerol

KCN concentration (μM)	Inhibition (%) (A)	CO concentration (% saturation)	Inhibition (%) (B)	KCN + CO (μM %)	Inhibition observed (%)	Inhibition A + B (%)
			Glucose-grown bacteria (in presence of 50 μM KCN)			
6	21	5	3	6 + 5	24	24
12	35	10	15	12 + 10	54	50
24	56	20	18	24 + 20	76	74
48	75	40	20	48 + 40	89	95
100	91	50	24	100 + 50	93	115
			Glucose-grown bacteria			
6	24	5	4.5	8 + 5	39	28
12	34	10	11.2	12 + 10	69	45
24	52.5	20	18	24 + 20	79	70.5
48	66	40	30.25	48 + 40	91	96
100	82	50	34	100 + 50	92	116
			Glycerol-grown bacteria			
6	21.4	5	2.0	6 + 5	24	23.4
12	36	10	15.4	12 + 10	53.4	51.4
24	57	20	18.4	24 + 20	76.7	75.4
48	75.4	40	19.4	48 + 40	88	94.8
100	90.5	50	24.7	100 + 50	90	115.2

concentrations cause inhibition. At sub-maximal rates there is an excess of this component which can be titrated with and probably binds to cyanide before any restriction of respiration occurs. This would suggest that the potential respiration is limited by a cyanide binding terminal oxidase in this organism. *Beneckea natriegens* grown in the presence of 50 μM KCN contained more CO-binding *c*-type cytochrome than when grown in its absence, but the maximum respiration rate was not increased. If CO-binding cytochrome *c* were the rate-limiting component then either a higher respiration rate or an increased resistance to cyanide would be expected when it is induced. Therefore, this CO-binding *c*-type cytochrome would not appear to be related to the cyanide-sensitivity or the rate-limiting component in the respiratory system of this organism.

Cytochrome *d*, has been shown by Weston and Knowles to be a major terminal oxidase in the *B. natriegens* even though it is barely detectable in cytochrome difference spectra [2]. In fact, the turnover number of terminal oxidases is generally so high that respiration may be routed entirely through a cytochrome which is barely detectable in most instruments presently available [9]. Thus, it is possible that cytochrome *d* was the main terminal oxidase in the cells studied here but that it was present at such low concentrations that the maximum respiration rate was limited by this component. This cytochrome then, would bind with cyanide and respiration rate would depend on the number of unbound sites.

The results reported here are not consistent with an alternative cyanide-insensitive pathway functioning as a *major* respiratory pathway, as all the cells tested were cyanide-sensitive at high respiration rates. However, there was in each case, a residual cyanide-insensitive respiration amounting to some 10% of the

maximum. This may represent the cyanide-insensitive pathway that Weston and Knowles [2] detected in cell free extracts of *B. natriegens.*

So far this phenomenon has not been investigated fully for any other organisms but we have found that a similar relationship between cyanide sensitivity and respiration rate may exist for *Pseudomonas extorquens* grown on methanol (J. Linton, unpublished data). There is no reason to assume that *Beneckea natriegens* would be unique in this feature and thus cyanide sensitivity of micro-organisms should always be investigated in relation to their respiration rate.

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