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THE RESPIRATORY CHAIN OF *AZOTOBACTER VINELANDII*

II. THE EFFECT OF CYANIDE ON CYTOCHROME *d*

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SUMMARY

1. Cyanide causes a slow disappearance of the oxidized band (648 nm) of cytochrome *d* in particles of *Azotobacter vinelandii* and inhibits the appearance of the reduced band (631 nm). No effect of cyanide is found on the reduced band of cytochrome *d*.

2. The kinetics of the disappearance of the 648-nm band of cytochrome *d* with excess cyanide deviates from first-order kinetics at lower temperatures (22 °C) indicating that at least two conformations of the enzyme are involved. At higher temperatures (32 °C) the observed kinetics of the cyanide reaction are first order with a $k_{on}=0.7\ M^{-1}\cdot s^{-1}$ and with an estimated k_{off} of approximately $5\cdot10^{-5}\ s^{-1}$.

3. The value for the k_{off} ($7\cdot10^{-4}$–$14\cdot10^{-4}\ s^{-1}$ at 32 °C) determined from the rate of reduction of cyanocytochrome *d* by $Na_2S_2O_4$ or NADH is one order of magnitude larger than the k_{off} value found when the enzyme is in its oxidized state.

4. No effect of cyanide is found on the spectrum of cytochrome a_1.

INTRODUCTION

In a previous paper[1] on the respiratory chain of *Azotobacter vinelandii*, it has been concluded from spectra of particles reduced by internal substrates that the oxidized (648 nm) and the reduced (631 nm) absorption bands of cytochrome *d* are only indirectly related. Therefore we proposed a second oxidized conformation of cytochrome *d*, that scarcely absorbs in the red region. In order to study the properties of both conformations we investigated the effect of ligand binding to the oxidase part of the chain.

Microspectroscopic studies[2–6] of aerobically grown microorganisms revealed the disappearance of the oxidized band of cytochrome *d* upon addition of cyanide without the formation of the reduced band, and a shift by carbon monoxide of the reduced band 7 nm to the red. In contrast, cytochrome *cd*, isolated from anaerobically nitrate-grown *Pseudomonas aeruginosa*, shows hardly any spectral change upon

Abbreviations: TMPD, *N,N',N,N'*-tetramethyl-*p*-phenylenediamine; DCIP, 2,6-dichlorophenolindophenol.

addition of cyanide, and CO causes a flattening of the reduced (625 nm) absorption band[7].

In the branched respiratory chain as proposed by Jones and Redfearn[8], cyanide is an effective inhibitor of cytochrome a_1, but has less effect on cytochrome *d* in the particles of *A. vinelandii*. However, no spectroscopic studies were carried out on the oxidase to support their suggestion. We have, therefore, investigated the effect of cyanide on the absolute spectra of cytochrome *d* in particles of *A. vinelandii* and the kinetics of the spectral changes.

METHODS

Phosphorylating particles were prepared as described by Pandit-Hovenkamp[9] and stored in 40 mM phosphate buffer (pH 7.2)–0.25 M sucrose–40 mM KCl at 77 °K.

Spectra, recorded on a Perkin–Elmer spectrophotometer Model 356, were measured with particles suspended in 30 mM phosphate buffer (pH 7.6)–5 mM $MgCl_2$–1 mM EDTA. When absolute spectra were recorded the effect of light scattering was diminished by using a sonified clay–powder suspension in the reference cell[1]. The spectra were normalized as described in a previous paper[1] taking 600 and 725 nm as reference points. For calculating the contribution of the separate electronic transitions to the composite oxidized absorption band, spectra were analysed using a Dupont 310 Curve Resolver.

Chemicals were Analar grade, mainly obtained from British Drug Houses, except those used for the culture medium, that were less highly purified.

RESULTS

The effect of 2 mM cyanide on the spectrum of oxidized particles as a function of time is shown in Fig. 1. It is clear that cyanide causes a diminishing of the absorption band at 648 nm without the appearance of a band at 631 nm of reduced cytochrome *d*. In a previous paper[1] it was shown that the composite oxidized band can be resolved into three Gaussian curves with peak positions at 635, 648 and 670 nm. On resolving the spectra of Fig. 1 in this way it appears that the decrease of the composite band is largely due to a decrease of the Gaussian 648-nm absorption band of oxidized cytochrome *d*. The band at 670 nm also diminishes but more slowly, whereas the 635-nm band is hardly affected. Spectrum No. 5 of Fig. 1, remaining 46 min after addition of cyanide, has a peak at 645 nm. On resolution of this spectrum, it was found that 95% of the initial Gaussian band remains at 635 nm, 75% of that at 670 nm and only 10% of that at 648 nm. After 20 h incubation (spectrum No. 6), the two broad bands at 635 and 670 nm have further decreased.

In order to determine whether the decrease of the 635- and 670-nm bands is due to an effect of cyanide or to reduction, the reducing agent ascorbate *plus* TMPD was added to particles preincubated with 2 mM cyanide for 1 h, in which the residual broad absorption band consisted mainly of the Gaussian 635- and 670-nm bands. After the addition of the reducing agent, spectrum No. 7 of Fig. 1 is rapidly formed with a very broad and flat band centered around 650 nm. Therefore, it is likely that the decrease of the Gaussian absorption bands at 635 and 670 nm in the presence of

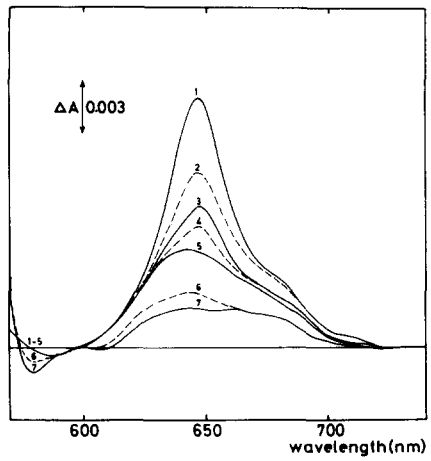

Fig. 1. The effect of cyanide on the spectrum of cytochrome *d* as a function of incubation time. Particles (0.7 mg protein/ml) were suspended in phosphate–$MgCl_2$–EDTA solution (pH 7.6) at 32 °C. The reference cell contained an appropriate clay-powder suspension to diminish the effect of light scattering. Spectra: 1, particles in the absence of cyanide; 2–6, after 11.5, 17.5, 26 and 46 min and 20 h incubation with 2 mM cyanide, respectively; 7, after 1 h cyanide (2 mM) pre-incubation and addition of ascorbate (7.5 mM), *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) (0.25 mM) followed by 1 min bubbling of oxygen. Scan speed 240 nm/min. The spectra are normalized (see Methods).

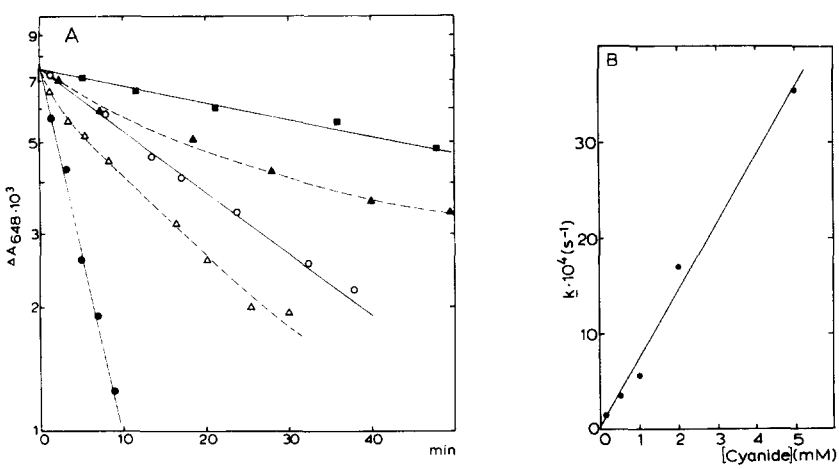

Fig. 2. Reaction of cyanide with oxidized cytochrome *d*. Particles (0.7 mg protein/ml) were diluted in phosphate–$MgCl_2$–EDTA solution *plus* variable cyanide concentrations. Intensities of the absorbance of cytochrome *d* at 648 nm were calculated by means of a Curve Resolver (see Methods). A. Time course of the reaction at 32 °C: ●—●, 5 mM cyanide; ○—○, 1 mM cyanide; ■—■, 0.1 mM cyanide, and at 22 °C: △----△, 5 mM cyanide; ▲----▲, 2 mM cyanide. B. Effect of cyanide concentration on the first-order rate constant at 32 °C.

cyanide is caused by reduction. This is supported by the observation that in the presence of cyanide, ferricyanide prevents the disappearance of these bands, but not that at 648 nm.

The disappearance of the Gaussian 648-nm band of cytochrome *d* follows first-order kinetics for different cyanide concentrations at 32 °C, as is shown in Fig. 2A, where the $\log \Delta A_{648\ nm}$ is plotted as a function of time. By plotting the observed pseudo first-order constants, which equal k_{on} [cyanide]$+k_{off}$, against cyanide concentrations (Fig. 2B), a second-order rate constant of 0.7 $M^{-1} \cdot s^{-1}$ can be calculated from the slope of the straight line. The k_{off}, estimated from the intersection point with the ordinate, has a value of approximately $5 \cdot 10^{-5}$ s^{-1}.

Fig. 2A shows that at 22 °C the decrease of the 648-nm band is no longer first order. Therefore, it must be concluded that a second reaction preceding or following the binding of cyanide is involved (see also Van Buuren *et al.*[10] and Van Buuren[11]). The convex lines in the plot of $\log \Delta A_{648\ nm}$ against time at 22 °C, can be explained by assuming that the second reaction approaches equilibrium. The marked effect of temperature on the deviation from first-order kinetics suggests the involvement of a conformational change in one of the reactions. On the basis of these data, combined with our observation[1] of two oxidized conformations of cytochrome *d*, we like to propose the following mechanism:

$$d_{648} \rightleftharpoons d_x \overset{CN}{\rightleftharpoons} d_x\text{–CN}$$

where d_{648} and d_x denote conformations of cytochrome *d* absorbing at 648 nm and hardly absorbing in the red, respectively (*cf.* also ref. 1).

It may be concluded that cyanide (2 mM) does not affect the ligand field of the heme of reduced cytochrome *d*, since after an incubation for 5 h under anaerobiosis no change in the 631-nm band is observed. This is in agreement with observations made by Negelein and Gerischer[2] with *Azotobacter chroococcum* and Tissières[5] with *Aerobacter aerogenes*. However, after opening the cuvette and aerating the solution, a rapid decrease of the 631-nm band is observed without the formation of the 648-nm absorption band. Therefore, it is concluded that cyanide can react rapidly with cytochrome *d* when oxygen is introduced.

Cyanide-liganded cytochrome *d* has little absorption in the red and its rate of reduction is inhibited, when compared to that in the absence of cyanide[2–6]. This makes it possible to study the relationship between the oxidized (648 nm) and reduced (631 nm) absorption bands of the non-liganded cytochrome. In Table I the intensities of both bands are compared as a function of the preincubation time with cyanide. It is clear that the decrease in intensity of the absorbance at 631 nm, measured directly after the addition of dithionite, corresponds with that at 648 nm. This demonstrates again that the two bands are intimately related, as was shown in a previous paper[1].

When dithionite is added to particles in which cytochrome *d* is present as cyanocytochrome *d*, prepared by the action of 2 mM cyanide in the presence of ascorbate *plus* TMPD and O_2, a slow appearance of the reduced band of cytochrome *d* (631 nm) is observed. This represents the decay of cyanocytochrome *d*, and is shown in a first-order plot in Fig. 3. From the slope of the straight line a first-order rate constant

($1.4 \cdot 10^{-3}$ s^{-1}) can be calculated, that is independent of the cyanide concentration used (not shown).

Under anaerobic conditions the reduction of cyanocytochrome *d* by NADH (Fig. 3) shows after a lag period, which is possibly due to traces of oxygen present,

TABLE I

THE PERCENTAGES OF THE OXIDIZED AND REDUCED ABSORPTION BANDS OF CYTOCHROME *d* AS A FUNCTION OF THE PREINCUBATION TIME WITH CYANIDE

Spectra were measured directly before ($A_{648\ nm}$) and after addition of dithionite ($A_{631\ nm}$). Conditions as described under Fig. 1.

Preincubation time (*min*)	[*Cyanide*] (*mM*)	$\%A_{648\,nm}$	$\%A_{631\,nm}$
0	0.1	100	100
40	0.1	76	73
86	0.1	59	54
160	0.1	44	43
55	5	0	0

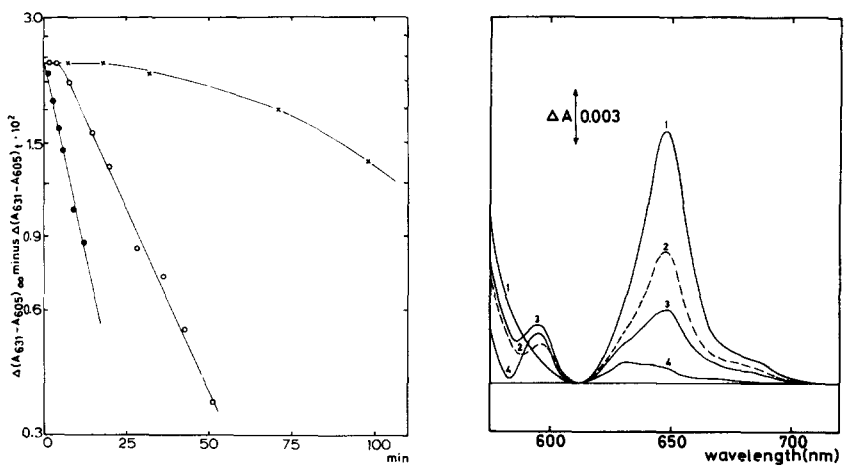

Fig. 3. Rate of reduction of cyanocytochrome *d* with different substrates. Cyanocytochrome *d* was prepared by aerobic incubation of particles (1.15 mg protein/ml) with cyanide (2 mM) for 10 min after which ascorbate (7.5 mM) *plus* TMPD (0.25 mM) were added. After evacuation (6 min) substrate was added from a side arm. The reference cuvette contained an appropriate clay–powder suspension. The degree of reduction of cytochrome *d* was calculated from spectra, normalized as described in Methods. The $\Delta(A_{631-605\ nm})_\infty$ value was obtained from a dithionite-reduced sample in the absence of cyanide. Spectra were scanned with a speed of 240 nm/min. ×—×, no addition; ●—●, *plus* dithionite; ○—○, *plus* 2 mM NADH.

Fig. 4. The effect of cyanide on the spectra of cytochrome *d* and cytochrome a_1 as a function of time. Particles (1.3 mg protein/ml) were suspended in phosphate–$MgCl_2$–EDTA solution at 21 °C. The reference cell contained an appropriate clay–powder suspension. Spectra were normalized as described in the previous paper[1] taking 610 and 710 nm as reference points. Scan speed 240 nm/min. Spectra indicated: 1, in the absence of cyanide; 2 and 3, after 5 and 12 min incubation with 10 mM cyanide, respectively; 4, 15 min preincubation with cyanide followed by the addition of ascorbate (7.5 mM) *plus* TMPD (0.75 mM) and bubbling with oxygen for 1 min.

also first-order kinetics with a k value of $7 \cdot 10^{-4}$ s^{-1}. The control experiment, with ascorbate *plus* TMPD, at anaerobiosis also shows reduction, but the lag period is substantially longer and no first-order kinetics are obtained. From this it may be concluded that cyanocytochrome *d* is reduced by substrates under anaerobic conditions either directly or *via* non-liganded heme in equilibrium with the cyanide compound.

In the particles used in the previously described experiments no clear absorption maximum of cytochrome a_1 could be detected. Nevertheless, the activity of these particles is as high as for other particles containing a substantial amount of cytochrome a_1 (3.1 and 4.1 μatoms O/min per mg protein for ascorbate *plus* 2,6-dichlorophenolindophenol (DCIP) and NADH, respectively, as substrates). Therefore, one wonders whether cytochrome a_1 is an integral part of the respiratory chain of *A. vinelandii*.

In the model of the chain as proposed by Jones and Redfearn[8] cytochrome a_1 functions as a cyanide-sensitive terminal oxidase. Therefore, we investigated the spectroscopic effect of cyanide on a batch of particles, isolated from bacteria cultured for 38 h, which contained a substantial amount of cytochrome a_1. As illustrated in Fig. 4, the addition of cyanide to oxidized particles causes a decrease of the absorbance at 648 nm (spectra Nos 2 and 3) in time, and a concomitant reduction of cytochrome a_1, as judged from the increase in absorbance at 596 nm. The maximal reduction of cytochrome a_1 is reached after addition of ascorbate *plus* TMPD (spectrum No. 4). Addition of potassium ferricyanide brought about a fast reoxidation of cytochrome a_1 (not shown), indicating that cyanide does not affect cytochrome a_1 since it remains easily oxidizable and reducible.

DISCUSSION

The disappearance of the oxidized (648 nm) band of cytochrome *d* without appearance of a reduced (631 nm) band after the addition of cyanide is in accordance with the observation[1] that these two bands are indirectly related. It was proposed that a second oxidized conformation of cytochrome *d*, termed cytochrome d_x, exists that scarcely absorbs in the red region. Furthermore, the 648-nm band of oxidized cytochrome *d* was ascribed to a charge transfer from a ligand to the iron[1]. The effect of cyanide on cytochrome *d* resembles the observation of Brill and Williams[12], that charge-transfer bands of ferric hemoproteins disappear in the presence of cyanide. Thus, the disappearance of the 648-nm band of cytochrome *d* can be explained as a binding of cyanide to cytochrome *d*, thereby preventing the charge transfer.

Based on the kinetics of the binding of cyanide to oxidized cytochrome *d*, and the effect of temperature on the rate of binding, a mechanism is proposed, in which a change of cytochrome *d* from the d_{648} to the d_x conformation is followed by the binding of cyanide to cytochrome d_x. This is supported by the observation that at turnover conditions, thereby promoting the d_x conformation[1], the binding of cyanide to cytochrome *d* was highly facilitated[13]. It is interesting to note that the kinetics of cyanide binding to oxidized cardiac cytochrome *c* oxidase[10,11,14–16] are of the same type as was found for cytochrome *d* at 22 °C. But, in contrast to cytochrome *c* oxidase[10,11], reduced cytochrome *d* shows no spectral change on the addition of cyanide.

Dithionite or NADH under anaerobic conditions are able to reduce cyanocytochrome *d*. For the decay of cyanocytochrome *d* first-order rate constants of $7 \cdot 10^{-4}$–$14 \cdot 10^{-4}$ s^{-1} at 32 °C could be calculated. The value of k is at least one order of magnitude larger than the k_{off} value ($5 \cdot 10^{-5}$ s^{-1}) which was calculated from the reaction of cyanide with oxidized cytochrome *d*. This difference can be explained in two ways. Firstly, reduction of the heme *d* occurs in cyanocytochrome *d*, after which the cyanide is split off from the reduced heme with a higher k_{off} value. Secondly, a conformational change of the cytochrome *d* molecule, brought about by the reduction of other components of the chain, and thus giving rise to a higher value for the dissociation rate constant of the oxidized heme *d*. The latter explanation is similar to that given by Van Buuren *et al.*[10] and Van Buuren[11] for the dissociation of cyanide from cyanocytochrome aa_3.

The reduction of cytochrome a_1 in the presence of cyanide and oxygen and its reoxidation after ferricyanide addition, showed that cyanide does not affect the heme of cytochrome a_1 and thus is unable to function as a cyanide-sensitive oxidase as was proposed earlier[8]. This is supported by the observation that a cyanide-sensitive ascorbate *plus* DCIP activity is found even in particles in which no cytochrome a_1 is detectable or that had been purified with Triton X-100[17].

The apparent effect of cyanide on oxidized cytochrome *d* in particles of *A. vinelandii* seems to be in contrast with the observations on isolated cytochrome *cd* from anaerobically nitrate-grown *Ps. aeruginosa* and *Micrococcus denitrificans*[18]. Although generally in literature[19,20] no distinction is made between cytochromes *d* from aerobically or anaerobically grown organisms, a comparison of the different properties of these two types of cytochromes, which are summarized in Table II, shows that the two enzymes differ considerably in physical and chemical properties. The apparent functional difference of the two enzymes, being an oxidase[8,21] or nitrite reductase[22,23], respectively, is expressed in different effects of ligand binding. The difference in peak positions of the pyridine hemochrome and alkaline heme *d* spectra[7] also indicate that the prosthetic groups are not identical, as was already proposed by Newton[18].

TABLE II

DIFFERENCES BETWEEN CYTOCHROMES *d* FROM AEROBICALLY AND ANAEROBICALLY NITRATE-GROWN ORGANISMS

Properties	*Cytochrome d*	*Cytochrome cd*
Function	Oxidase[8, 21]	Nitrite reductase[22, 23]
Growth condition	Aerobe	Anaerobe *plus* nitrate
Effect of oxygen during growth	Low $[O_2]$→ high [cyt. *d*] High $[O_2]$→ low [cyt. *d*][24, 25]	Enzyme is depressed, another oxidase is formed[26–28]
Oxidized absorption band	645–648 nm[1–6]	630–635 nm[7, 18, 22, 23, 29]
Reduced absorption band	630–632 nm[1–6]	625 nm[7, 18, 22, 23, 29]
Oxidized cytochrome *plus* cyanide	648-nm band disappears[1–6], this paper	Small spectral effects[7]
Reduced enzyme *plus* CO	634–636 nm[2–6]	625-nm band flattens[7]
Pyridine hemochrome	612–613 nm[1, 30]	618–620 nm[7, 29, 31]
Reduced alkaline haem *d*	618 nm[30]	626 nm[7, 29, 31]

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REFERENCES

1 Kauffman, H. F. and Van Gelder, B. F. (1973) *Biochim. Biophys. Acta* 305, 260–267
2 Negelein, E. and Gerischer, W. (1934) *Biochem. Z.* 268, 1–7
3 Keilin, D. (1934) *Nature* 133, 290–291
4 Fujita, A. and Kodama, T. (1934) *Biochem. Z.* 273, 186–197
5 Tissières, A. (1951) *Biochem. J.* 50, 279–288
6 Tissières, A. (1956) *Biochem. J.* 64, 582–589
7 Horio, T., Higashi, T., Matsubara, H., Kusai, K., Nakai, M. and Okunuki, K. (1958) *Biochim. Biophys. Acta* 29, 297–302
8 Jones, C. W. and Redfearn, E. R. (1967) *Biochim. Biophys. Acta* 143, 340–353
9 Pandit-Hovenkamp, H. G. (1967) in *Methods in Enzymology* (Estabrook, R. W. and Pullman, M. E., eds), Vol. 10, pp. 152–157, Academic Press, New York
10 Van Buuren, K. J. H., Nicholls, P. and Van Gelder, B. F. (1972) *Biochim. Biophys. Acta* 256, 258–276
11 Van Buuren, K. J. H. (1972) *The Binding of Cyanide to Cytochrome* aa_3, Ph. D. Thesis, University of Amsterdam, Gerja, Waarland
12 Brill, A. S. and Williams, R. J. P. (1961) *Biochem. J.* 78, 246–253
13 Eilermann, L. J. M. (1973) *Some Aspects of Energy Conservation in Azotobacter vinelandii*, Ph. D. Thesis, University of Amsterdam, Gerja, Waarland
14 Van Buuren, K. J. H., Zuurendonk, P. F., Van Gelder, B. F. and Muijsers, A. O. (1972) *Biochim. Biophys. Acta* 256, 243–257
15 Nicholls, P., Van Buuren, K. J. H. and Van Gelder, B. F. (1972) *Biochim. Biophys. Acta* 275, 279–287
16 Antonini, E., Brunori, M., Greenwood, C., Malmström, B. C. and Rotilio, G. C. (1971) *Eur. J. Biochem.* 23, 396–400
17 Mueller, T. J. and Jurtshuk, Jr, P. (1972) *Fed. Proc.* 31, 888 Abs
18 Newton, N. (1969) *Biochim. Biophys. Acta* 185, 316–331
19 Bartsch, R. G. (1968) *Annu. Rev. Microbiol.* 22, 181–200
20 Kamen, M. D. and Horio, T. (1970) *Annu. Rev. Biochem.* 39, 673–700
21 Castor, L. N. and Chance, B. (1959) *J. Biol. Chem.* 234, 1587–1592
22 Yamanaka, T., Ota, A. and Okunuki, K. (1961) *Biochim. Biophys. Acta* 53, 294–308
23 Yamanaka, T. and Okunuki, K. (1963) *Biochim. Biophys. Acta* 67, 379–393
24 Harrison, D. E. F. (1972) *Biochim. Biophys. Acta* 275, 83–92
25 Ackrell, B. A. C. and Jones, C. W. (1971) *Eur. J. Biochem.* 20, 29–35
26 Lam, Y. and Nicholas, D. J. D. (1969) *Biochim. Biophys. Acta* 180, 459–472
27 Sapshead, L. M. and Wimpenny, J. W. T. (1972) *Biochim. Biophys. Acta* 267, 388–397
28 Azoulay, E. and Couchoud-Beaumont, P. (1965) *Biochim. Biophys. Acta* 110, 301–311
29 Horio, T., Higashi, T., Yamanaka, T., Matsubara, H. and Okunuki, K. (1961) *J. Biol. Chem.* 236, 944–951
30 Barrett, J. (1956) *Biochem. J.* 64, 626–639
31 Yamanaka, T. and Okunuki, K. (1963) *Biochim. Biophys. Acta* 67, 407–416