The Occurrence of a Stepwise-Decreasing Respiration Rate during Oxidative Assimilation of Different Substrates by Resting *Klebsiella aerogenes* in a System Open to Oxygen

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The addition of an oxidizable substrate to a continuous culture of *Klebsiella aerogenes* is known to cause an increased respiration rate that decreases in discrete steps as the added substrate is being exhausted. We have used a simple new technique to show that this phenomenon is also produced by washed, resting cells harvested from batch or continuous growth culture. The stepwise-decreasing respiration rate is caused by the exhaustion of different pools of intermediates. Each plateau of respiration rate is a measure of the activity of one or more enzymes that are rate limiting in the exhaustion of pools of intermediates. If the identities of the enzymes that are rate limiting at the different plateaux are known, the method may allow the determination, in one experiment, of the activities of up to six different enzymes in the intact bacteria. Integration of the respiration-rate measurements yields the total amount of O₂ taken up. After the addition of glucose to the washed resting bacteria 37% of the amount of O₂ required for the complete oxidation of the glucose was taken up. Acetate, pyruvate and succinate were all oxidized to the extent of 51%.

Harrison & Maitra (1969) investigated the effect of adding different oxidizable substrates to aerated glucose-limited continuous cultures of *Klebsiella aerogenes*. The addition of a substrate caused the dissolved O₂ concentration to decrease as a result of the increasing respiration rate. As the added oxidizable substrate was used, the dissolved O₂ concentration returned to the original steady-state value in discrete steps, reflecting a stepwise decrease in the respiration rate. We have reproduced this phenomenon in batch culture as well as in small samples of washed cells harvested from batch or continuous cultures. Respiration measurements on samples of washed bacteria were made in a cell that is a slightly modified form of the conventional cell for polarographic O₂ concentration measurements. The only difference is that the surface of the liquid sample in our cell is in contact with the atmosphere. In this open system the respiration rate at a steady-state is proportional to the difference between the equilibrium concentration of O₂ and the steady-state concentration of O₂, whereas in the closed system the respiration rate is proportional to the negative slope of the plot of O₂ concentration versus time. The advantage of the open system is that the duration of the measurement is not limited by the amount of dissolved O₂ originally present in the liquid, as it is in the closed system. Some theoretical and technical details on respiration measurements in the open

system have been published by Degn & Wohlrab (1971).

Experimental

Apparatus

The cell used for the respiration measurements is shown in Fig. 1. It consists of a 12 mm × 12 mm × 35 mm sample compartment with a cylindrical well in the bottom serving as a bearing for a magnetic stirrer. In the top of the sample compartment there are two small holes serving as inlet and outlet for a stream of air or gas mixture of any desired composition. Through a hole in the side of the sample compartment is placed the sensor end of a Clark electrode. The O-ring holding the Teflon membrane seals the liquid sample off from the electrode compartment. The electrode used is manufactured by Radiometer Ltd., Copenhagen, Denmark. The apparatus is thermostatically maintained at 29°C by water circulation. The evaporation of water from the sample during the experiment is counteracted by bubbling the gas through water before passing it through the measuring cell. The temperature used is about the maximum at which this precaution is sufficient to prevent any significant loss of water from the cell during the experiment. The polarographic current is registered on a potentiometric

Fig. 1. Cell with O_2 electrode for respiration measurement in an open system

The sample compartment is not filled to the top. A stream of atmospheric air (or another gas) is led through the sample compartment above the liquid surface. The magnetic stirrer is cylindrical and placed in a well in the bottom of the sample compartment. The cell is kept at constant temperature by circulation of water from a thermostatically controlled container.

recorder equipped with an integrator (Servogor, Goerz Electro G.m.b.H., A-1101 Vienna, Austria).

Calculation of respiration rates from measurements of O_2 concentration in an open system

The open system we use for respiration measurements is essentially a miniature culture vessel. The theory for O_2 transfer into a suspension culture is well known and is applicable to our system. Provided that the liquid-gas interface surface area is constant and unobstructed by bubbles, the rate of transport of O_2 from the gas to the liquid is given by:

$$v_t = K(T_G - T_L) \quad (1)$$

where v_t is the rate of transport of O_2 , K is a constant that depends on the experimental volume, surface area and temperature, T_G is the molar concentration of O_2 in the buffer used, when it is equilibrated with the gas mixture and T_L is the O_2 concentration in the liquid. The rate of change of the O_2 concentration in the liquid is given by:

$$\frac{dT_L}{dt} = v_t - v_r \quad (2)$$

where v_r is the respiration rate.

Substituting eqn. (1) into eqn. (2) we obtain:

$$v_r = K(T_G - T_L) - \frac{dT_L}{dt} \quad (3)$$

From eqn. (3) the respiration rate can be determined if T_L is recorded as a function of time at constant T_G , provided that the O_2 -transfer constant, K , is known. This constant can easily be determined from experiments in which no respiring sample is added to the

liquid. The equilibration of the liquid with O_2 is then a first-order process with the rate constant, K . This rate-constant may be calculated from the observed half-time of the equilibration process. Eqn. (3) shows that the determination of the respiration rate at any specific time depends on the value of T_L as well as on the rate of change of T_L at that time. Both values can be read from the plot of T_L versus time. If the respiration rate is constant a steady-state value of O_2 concentration in the liquid is attained. In that case $dT_L/dt = 0$, and eqn. (3) is simplified to:

$$v_r = K(T_G - T_L) \quad (4)$$

The dimension of the rates in eqns. (1) and (4) is M/s. By multiplication by the volume of the sample, U , the rate is obtained in mol/s. The total amount of O_2 , A , that has entered the liquid phase is obtained by integration of eqn. (1).

$$A = UK \int_{t_0}^{\infty} (T_G - T_L) dt \quad (5)$$

This integral is the area under the curve for $(T_G - T_L)$ versus time. At a constant value of T_G it can be measured directly by means of a recorder equipped with an integrator. The curve of T_L versus time that would be expected if oxidizable substrate is added to a suspension of bacteria that do not exhibit any endogenous respiration is shown schematically in Fig. 2. Initially the liquid is equilibrated with the O_2 of the gas, i.e. T_L is equal to T_G . At time t_0 the oxidizable substrate is added and causes respiration to take place at a constant rate, resulting in a constant lowered O_2 concentration in

Fig. 2. Theoretical course of an experiment in which a substrate is added to a suspension of bacteria in the open system

The bacteria are assumed to have no endogenous respiration. Initially the suspension is saturated with O_2 from the gas ($T_L = T_G$). At the time t_0 a sample of an oxidizable substrate is introduced. This causes respiration to occur at a constant rate. The O_2 concentration in the liquid will decrease to a steady-state value that is determined by the rate of respiration and the rate of transport of O_2 from the gas into the liquid. When the substrate is exhausted respiration stops and the O_2 concentration in the liquid returns to the saturation value. The respiration rate at the steady-state is proportional to the difference between the equilibrium O_2 concentration and the steady-state O_2 concentration in the liquid ($T_G - T_L$).

the liquid. When the added substrate is exhausted, the respiration stops, and the O_2 concentration in the liquid returns to its initial value. The amount of O_2 used to oxidize the added amount of substrate is proportional to the area limited by the curve and the broken line (see Fig. 2) ($T_L = T_G$). In principle the upper integration limit is infinity, but experience shows that the time taken for the O_2 trace to return to its initial value is rarely very long.

The method for respiration measurement that we have developed for the present experiments is adapted from the technique of continuous culture. The reliability of the method depends on the constancy of the O_2 -transfer constant, K . This constant depends on the area of the gas-liquid interface, which, in the continuous culture, consists of the walls of small bubbles dispersed in the culture. The size and number of bubbles is very sensitive to changes in physical properties of the suspension, and therefore it is unlikely to be constant. In our system there are no bubbles and no foam. The area of the gas-liquid interface is determined by the geometry of the

cell and the rate of stirring. The stirring influences the surface area because of the formation of a vortex. At a moderate constant stirring rate the vortex is stable and K is constant.

Cultivation of bacteria

Identical batch cultures of *K. aerogenes* were inoculated simultaneously in Erlenmeyer flasks in a shaking incubator at 32°C. The cells were harvested at different points of the growth curve, established by extinction measurements. The growth medium contained (amounts in mg/l): $MgSO_4 \cdot 7H_2O$, 100; $ZnSO_4 \cdot 7H_2O$, 0.5; $MnSO_4 \cdot 4H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 2.5; $CuSO_4 \cdot 5H_2O$, 0.5; $CaCl_2 \cdot 6H_2O$, 2.5; EDTA, 125; KH_2PO_4 , 3000; $NaH_2PO_4 \cdot 2H_2O$, 530; $(NH_4)_2SO_4$, 1250; glucose, 2500.

The bacteria were harvested by centrifugation, washed twice with 0.1 M-potassium phosphate buffer, pH 6.5, containing 1 mM- Mg^{2+} , and stored in liquid N_2 . When used they were suspended in this phosphate buffer. Bacteria were also grown in continuous culture at pH 7.0, 32°C and a dilution rate of $0.03 h^{-1}$. The growth medium was as described above. With this medium the growth is limited by glucose. The cells were harvested and washed as described above. The apparatus used for continuous culture was also used to study the respiration of batch cultures, which were not harvested. In this case the apparatus was equipped with a sterilizable galvanic O_2 electrode (Harrison & Melbourne, 1970), and the CO_2 content of the exit gas was measured with a CO_2 analyser (Beckman model LB-7). Viability of the cells was determined by the method of Postgate *et al.* (1961). Glucose was assayed by using glucose oxidase and glucose 6-phosphate dehydrogenase supplied by C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, according to their procedure. Pyruvate was assayed by the method described by Bücher *et al.* (1965).

Results

We first observed the phenomena described in the present paper when studying the respiration of samples of bacteria grown in batch cultures. The results of experiments with bacteria harvested in the exponential growth-phase and the stationary phase are shown in Figs. 3(a) and 3(b) respectively. The sample of bacteria was suspended in a buffer saturated with atmospheric O_2 in the measuring cell. When the sample was added the O_2 content decreased owing to endogenous respiration. After having passed through a minimum the O_2 content became constant at about 90% saturation as a result of a constant endogenous respiration. Glucose was then added, causing an immediate fall in the O_2 concentration in the liquid.

After about 5 min the O_2 concentration in the liquid reached a sharp minimum, which indicates the maximum respiration rate. From this minimum value the O_2 concentration in the liquid returned to the initial value in discrete steps, indicating a stepwise

Fig. 3. Comparison of respiration properties of cells harvested from batch culture in (a) exponential growth phase and (b) stationary phase

At the arrow $8 \mu\text{mol}$ of glucose was added to a sample consisting of 1.5 ml of a 0.1 M -potassium phosphate buffer, $\text{pH } 6.5$, 1 mM-Mg^{2+} and $1.3 \text{ mg dry wt. of bacteria/ml}$. The temperature was 29°C . The liquid surface was in contact with atmospheric air. The letters indicate the transitions, as discussed in the text.

decrease in respiration rate. In Figs. 3(a) and (b), a letter is assigned to each transition to a higher steady-state value of O_2 concentration. The lettering indicates the transitions that we think correspond to each other in the two curves. Despite differences in the heights of the transitions and the durations of the plateaux, the two curves produced by the exponential-phase and the stationary-phase cells are essentially similar. With batch-grown cells we also found that the exhaustion of the endogenous substrate was associated with a stepwise decrease of the respiration rate. To study the stepwise pattern caused by different added substrates it was desirable to use bacteria that had the minimum endogenous respiration. These were obtained by growing the bacteria in a glucose-limited continuous culture at a low dilution rate. Organisms obtained from these continuous culture conditions were used to study the respiration supported by acetate, pyruvate, succinate and glucose. When acetate was used as a substrate (Fig. 4) the O_2 concentration kept decreasing slowly until it suddenly returned to the initial value. Some small humps were seen on the decreasing part of the O_2 concentration curve. However, no intermediate plateaux were observed during the return of the O_2 concentration to its initial value. With pyruvate (Fig. 5), succinate (Fig. 6) and glucose (Fig. 7) a number of steps were observed. In all cases where steps were observed these

Fig. 4. Acetate-supported respiration in cells harvested from continuous culture

At the arrows (a) 2, (b) 4 and (c) $8 \mu\text{mol}$ of acetate was added to samples of the same composition as in Fig. 3.

Fig. 5. Pyruvate-supported respiration of cells harvested from continuous culture

At the arrows (a) 2, (b) 4, (c) 8 and (d) $16 \mu\text{mol}$ of pyruvate was added to samples of the same composition as in Fig. 3.

Fig. 6. *Succinate-supported respiration in cells harvested from continuous culture*

At the arrows (a) 2, (b) 4, (c) 8 and (d) 16 μmol of succinate was added to samples of the same composition as in Fig. 3.

Fig. 7. *Glucose-supported respiration in cells harvested from continuous culture*

At the arrows (a) 2, (b) 4 and (c) 8 μmol of glucose was added to samples of the same composition as in Fig. 3.

only developed when the amount of substrate added exceeded a certain minimum value. The addition of substrate in greater amounts than those used in Figs. 4–7 did not lead to the development of more steps. The maximum number of transitions between plateaux observed in the different experiments is one with acetate, three with pyruvate, four with succinate and six with glucose. The step patterns in the curves produced by bacteria grown under different conditions were somewhat different as can be seen from Figs. 3 and 7. With some preparations we observed that the maximum number of transitions obtained with glucose was less than six. Also bacteria harvested from apparently identical runs of continuous cultures showed some variation.

To allow samples of the bacterial suspension to be taken for analysis a cell was used, which is essentially identical with that shown in Fig. 1, except that it can hold a sample of 150 ml. In this apparatus similar experiments to those described above, with addition of pyruvate (Fig. 8) or glucose (Fig. 9) were carried out. At a fixed time-interval, samples (1 ml) were taken for the determination of glucose and/or pyruvate. In both experiments the disappearance of the substrate coincided with a

Fig. 8. *Disappearance of pyruvate in an experiment with pyruvate-supported respiration*

At the arrow 250 μmol of pyruvate was added to a 150 ml suspension of 2.3 mg dry wt. of bacteria/ml in a large measuring cell. The O_2 concentration in the liquid was recorded (a) and the pyruvate was determined in samples (1 ml) (b).

Fig. 9. Disappearance of glucose and formation and disappearance of pyruvate in an experiment with glucose-supported respiration

At the arrow 250 μmol of glucose was added to a 150 ml suspension of 2.3 mg dry wt. of bacteria/ml in a large measuring cell. The O_2 concentration (a) in the liquid was recorded and glucose and pyruvate (b) were determined in samples (1 ml). $\square$, Pyruvate; $\bullet$, glucose.

transition to a higher step in the O_2 concentration curve. In neither case does the sharp minimum in the O_2 trace coincide with any event in the substrate concentration curve.

Simultaneously with the recording of the O_2 concentration in the liquid, we also recorded the area limited by the curve in some experiments. From this area the total amount of O_2 taken up in each case was calculated. This was plotted against the amount of O_2 theoretically required for the complete oxidation of the added substrate to CO_2 and H_2O . The results are shown in Fig. 10. The reproducibility of the method is illustrated by the close agreement between duplicate experiments. The amount of O_2 used is proportional to the amount of substrate added, but the oxidation is not complete. With acetate, pyruvate and succinate 51% of the theoretical amount of O_2 was taken up before the respiration caused by the addition of substrate ceased. With glucose, the amount of O_2 taken up was only 37% of the theoretical amount.

As we have used washed cells with no N source added, growth during our measurements could be ruled out. To see how growth would manifest itself we did some experiments with a N source added. Fig. 11 shows that the effect of adding NH_4^+ was to trans-

Fig. 10. Measured O_2 uptake versus theoretical O_2 uptake

The total amounts of O_2 taken up in the experiments shown in Figs. 4–7 are plotted against the amounts of O_2 theoretically required for complete oxidation of the added substrates to CO_2 and H_2O . $\circ$, Acetate; $\square$, pyruvate; $\diamond$, succinate; $\bullet$, glucose.

Fig. 11. Effect on respiration of adding an N source to washed bacteria

In (a) 8 μl of glucose was added to a sample of the same composition as in Fig. 7(c). In (b) the experiment was repeated with the further addition of 4 μmol of $(NH_4)_2SO_4$.

form all plateaux, except for the last one, into negative slopes. This indicates a slowly increasing respiration rate between the sudden stepwise decreases in the respiration rate. The bacteria used in all of the above experiments were kept in liquid N_2 after the harvesting and washing. In one experiment the O_2 trace obtained with washed bacteria respiring on glucose was compared with the O_2 trace produced by a similar sample after freezing in liquid N_2 and thawing. The curves were virtually identical. The viability of the bacteria before and after the freezing was determined to be 95 and 93% respectively.

We also studied the behaviour of bacteria that were not harvested. Bacteria were grown in batch culture in the same medium and apparatus that was used for the continuous culture. When the culture was

Fig. 12. Effect on respiration of the addition of glucose to a batch culture in the stationary phase

A batch culture of 1.8 litres was grown at an aeration rate of 2 litres of air/min in the apparatus used for continuous culture. When the culture was in the stationary phase 54mmol of glucose was added (time zero). The dissolved O_2 concentration, pH and the CO_2 content of the effluent gas were measured simultaneously.

in the stationary phase glucose was added. The O_2 concentration and the pH of the liquid as well as the CO_2 content of the exit-gas mixture were recorded simultaneously. The results (Fig. 12) show that the stationary-phase batch culture exhibited a stepwise decrease in respiration rate as found by Harrison & Maitra (1969) in continuous culture and by us in washed samples of bacteria from batch and continuous cultures. Each stepwise decrease in the respiration rate was accompanied by a stepwise decrease in the evolution of CO_2 . The pH trace indicates a production of acid until transition d, followed by a consumption of acid at a rate that decreased abruptly at transition c and ceased abruptly at transition b.

Discussion

We have developed a simple technique that enables us to follow the rate of O_2 uptake for a prolonged period of time in a small respiring sample. By utilizing this technique we have shown that the phenomenon of stepwise decreasing respiration rate after the addition of substrate, found by Harrison & Maitra (1969) in the continuous culture of *K. aerogenes*, is not peculiar to growing bacteria. Working with washed, resting bacteria harvested from a glucose-limited continuous culture we found that different substrates yielded different numbers of transitions to consecutively lower plateaux of nearly constant respiration rates. Increasing the added amount of substrate prolonged all plateaux. These findings agree well with the explanation proposed by Harrison & Maitra (1969) that each transition to a lower respiration rate is caused by the exhaustion of a pool of reducing equivalents. In

experiments where samples were taken and analysed for glucose and pyruvate we have shown directly that the disappearance of these two substances coincides with transitions to lower respiration rates.

If the different pools of reducing equivalents are oxidized in parallel pathways with additive rates it follows from the above explanation that the difference between two consecutive plateaux is a measure of the activity, in the intact bacteria, of the enzyme that is rate-limiting in the process of exhausting one particular pool. Other organizations of the oxidative processes may, however, lead to other interpretations. For example, if different pools are exhausted by pathways that compete at their junction, the total respiration rate at a plateau may be a measure of the activity of a single enzyme and the difference between plateaux may be the difference in activity between two enzymes. A general theory of rate limitations in multienzyme systems would aid the interpretation of our results, but such a theory does not seem to have been developed.

In the glucose curves in Figs. 3, 7, 9 and 11 we have assigned a letter to each transition to a lower respiration rate. In the pyruvate and succinate curves in Figs. 5, 6 and 8 we have lettered the transitions that we think are identifiable with those in the glucose curves. The clues used for the identification are, first, the sequence of the transitions and secondly the observed coincidence (Figs. 8 and 9) of the disappearance of pyruvate with transitions in the pyruvate and glucose curves. From the latter evidence it is clear that either transition a or b is absent in the pyruvate curve. By comparison of the heights of the transitions we conclude that transition a is absent in the pyruvate curve. The observation (Fig. 9) that transition c coincides with the disappearance of pyruvate is consistent with the finding (Fig. 12) that transition c also coincides with the deepest decrease in the CO_2 -evolution rate. This decline is caused by the cessation of oxidative decarboxylation of pyruvate.

In contrast with our findings Harrison & Maitra (1969) found that a sharp minimum in the O_2 concentration curve after the addition of glucose to the culture coincided with the disappearance of glucose. We find that neither with glucose nor with pyruvate does the sharp minimum of the O_2 trace coincide with any event in the substrate-concentration curve. In both cases the exhaustion of the substrate causes a transition to a higher plateau at a later time than the minimum. In the case of glucose two transitions, c and f, take place before the disappearance of glucose and in the case of pyruvate one transition takes place before the disappearance of pyruvate. It seems likely that the transitions that take place before the exhaustion of the added substrates are caused by the exhaustion of endogenous substances which facilitate respiration. These could be ADP and other receptors for high energy phosphate or phosphate itself.

Because acetate does not yield intermediary plateau values it may be suspected that the last transition, a, is due to the exhaustion of a pool of acetate. However, this does not agree with our finding that when acetate was added as a substrate the respiration rate was increasing slowly with time without reaching a well-defined plateau. Neither does it agree with the observation (Fig. 12) that there is no measurable change in pH after transition b. However, we found that the last plateau was not affected by the addition of an N source that caused all the previous plateaux to slant, owing to synthesis of the rate-limiting enzymes. This observation agrees with the assumption that the last plateau is caused by acetate because Hadjipetrou *et al.* (1964) have reported that respiration on acetate did not support growth in *K. aerogenes* that had been grown on glucose. In view of the above observations it seems likely that transition a is due to the exhaustion of a pool of a C_2 intermediate that is not extracellular acetate.

Siegel & Clifton (1950) studied the amount of O_2 taken up by *Escherichia coli* growing on glucose in batch culture. They found that about 40% of the theoretical amount of O_2 was taken up. Harrison & Maitra (1969) reported that 37% of the theoretical amount of O_2 was taken up by *K. aerogenes* growing on glucose in the continuous culture. In the present experiments we found that the degree of oxidation of glucose in washed, resting *K. aerogenes* is also 37%, indicating that the process of oxidative assimilation of glucose gives the same yield in growing bacteria as in non-growing bacteria. The substrates acetate, pyruvate and succinate all cause an O_2 uptake of 51% of the theoretical amount. The higher degree of oxidation of these substrates compared with glucose may be explained by a higher energy demand in converting these acids into storage materials.

The respiration rate per g dry wt. (Q_{O_2}) of bacteria is often determined from the slope of a polarographic O_2 trace obtained by measurements in a cell where the sample is not in contact with O_2 . Because of disturbing effects, such as the diffusion of O_2 into the sample from the outside, there is a limit to how low respiration rates can be measured with this technique. The slowest practically measurable respiration rate will exhaust the O_2 in the sample in about 10 min. The use of the closed system for Q_{O_2} determinations therefore depends on the assumption that a stable steady-state rate of respiration in bacteria is reached within a few min after the cells were exposed to O_2 or oxidizable substrate. One purpose of our developing the open-system technique for respiration measurements was to test this assumption, and our results show that the assumption cannot generally be relied on. In the experiments (Fig. 3) with batch-grown cells, a high

endogenous respiration rate was observed and the maximum respiration rate was reached within 5 min after the addition of glucose. In this case it would be practically possible to measure the maximum respiration rate in the closed system. However, in the experiments (Fig. 7) with cells grown at a very low dilution rate in continuous culture, a much lower endogenous respiration rate was observed and the maximum respiration rate caused by the addition of glucose occurred after about 45 min. In this case the maximum respiration rate would not be observable in a conventional closed system. We find that the maximum Q_{O_2} is about 8 mmol/h per g for the batch-grown cells as well as for the cells grown in continuous culture at the very low dilution rate of 0.03 h^{-1} . Harrison & Loveless (1971) measuring in a closed system found a 'potential' Q_{O_2} of about 8 mmol/h per g for *K. aerogenes* grown at a medium dilution rate in continuous culture, whereas their cells grown at the very low dilution rate of 0.025 h^{-1} showed a maximum Q_{O_2} of 4 mmol/h per g. This much lower value may be explained by the assumption that the slowly grown cells required such a long time to reach their maximum respiration rate that this did not occur in the closed measuring system.

Although the results reported here could have been obtained by the Warburg technique, this method suffers from practical difficulties when making measurements over long periods of time and, further, there is no simple way of recording the manometric readings continuously. To obtain a curve with the same resolution as Fig. 7 it would be necessary to do manometric readings with a very short time-interval for 5 h. Because of this we consider that the manometric technique is impractical in studies of the present type.

References

- Bücher, T., Czok, R., Lamprecht, W. & Latzko, E. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 253–259, Academic Press, New York and London.
- Degn, H. & Wohlrab, H. (1971) *Biochim. Biophys. Acta* **245**, 347–355.
- Hadjipetrou, L. P., Gerrits, J. P., Teulings, F. A. G. & Stouthamer, A. H. (1964) *J. Gen. Microbiol.* **36**, 139–150.
- Harrison, D. E. F. & Loveless, J. E. (1971) *J. Gen. Microbiol.* **68**, 35–43.
- Harrison, D. E. F. & Maitra, P. (1969) *Biochem J.* **112**, 647–656.
- Harrison, D. E. F. & Melbourne, K. U. (1970) *Biotechnol. Bioeng.* **12**, 633–634.
- Postgate, J. R., Crumpton, J. E. & Hunter, J. R. (1961) *J. Gen. Microbiol.* **24**, 15–24.
- Siegel, B. V. & Clifton, C. E. (1950) *J. Bacteriol.* **60**, 113–118.