Biochimica et Biophysica Acta, 347 (1974) 273–289

BBA 46732

ELECTRON-TRANSPORT PHOSPHORYLATION COUPLED TO FUMARATE REDUCTION IN ANAEROBICALLY GROWN *PROTEUS RETTGERI*

ACHIM KRÖGER

With the technical assistance of MARIANNE SCHIMKAT and SABINE NIEDERMAIER

Institut für Physiologische Chemie und Physikalische Biochemie der Universität München, D 8000 München 2, Pettenkoferstraße 14a (Germany)

(Received October 15th, 1973)
(Revised manuscript received December 27th, 1973)

SUMMARY

The metabolic role of the membrane-bound NADH– and formate–fumarate reductase which involves menaquinone as a redox carrier (A. Kröger et al. (1971) Eur. J. Biochem. 21, 322–333), is studied in the anaerobic growth of *Proteus rettgeri* with fumarate, citrate, glucose plus bicarbonate and pyruvate as the sole energy substrates. This is done especially with respect to the question of whether the reduction of fumarate is coupled to electron transport phosphorylation.

1. Pyruvate is fermented with a carbon and hydrogen recovery of about 85% according to the following reaction:

$$\text{pyruvate}^- + H_2O \rightarrow \text{acetate}^- + \text{formate}^- + H^+$$

The growth yield of this reaction is 6.3 g cells/mole acetate. This value is equal to the cell yield per mole of ATP, since the formation of acetate is coupled to substrate-level phosphorylation.

2. Fumarate is fermented with a recovery of about 95% of the carbon and the hydrogen according to the following reaction:

$$7\ \text{fumarate}^{2-} + 8\ H_2O \rightarrow 6\ \text{succinate}^{2-} + 4\ HCO_3^- + 2\ H^+$$

Since 5 moles of succinate are formed by fumarate reduction and 1 from isocitrate, only 1 mole ATP per 6 moles of succinate can be gained from substrate-level phosphorylation. The cell yield (5.5 g cells/mole succinate), however, corresponds to a greater gain of ATP as judged from the ATP requirement for cell synthesis (Y_{ATP}^{max}). A comparison of the cell yield to that measured with pyruvate indicates that almost 1 mole ATP per mole of succinate is formed. From this it is concluded that the electron transport with fumarate as the acceptor is coupled to the synthesis of ATP.

3. Citrate is mainly fermented according to the following reaction:

$$2\ \text{citrate}^{3-} + 2\ H_2O \rightarrow 2\ \text{succinate}^{2-} + \text{acetate}^- + 2\ HCO_3^- + H^+$$

1 mole of succinate is formed by fumarate reduction and the other from isocitrate.

The cell yield (7.8 g cells/mole citrate) indicates that ATP is also formed by fumarate reduction.

4. Glucose is fermented in the presence of bicarbonate with a carbon and hydrogen recovery of about 90% approximately according to the following reaction:

$$3\ \text{glucose} + 2\ HCO_3^- \rightarrow 4\ \text{formate}^- + 3\ \text{acetate}^- + 2\ \text{succinate}^{2-} + \text{ethanol} + 9\ H^+ + H_2O$$

From the cell yield (24 g cells/mole glucose) and the estimated gain of ATP (2.74 mole ATP/mole glucose) it is calculated that 8.8 g cells are synthesized per mole ATP with glucose as the growth substrate.

5. The metabolic pathways concluded from the growth reactions are confirmed by the enzyme equipment of the bacteria.

INTRODUCTION

In a previous paper [1] it was demonstrated that *Proteus rettgeri* grown anaerobically on complex medium contains a very active membrane-bound fumarate reductase with NADH and formate as hydrogen donating substrates. It was shown that the activity depends on the presence of menaquinone which participates in the electron transport from the donors to fumarate. These properties suggest that the function of this electron-transport system in the anaerobic metabolism is analogous to that of the respiratory chain under aerobic conditions.

In this communication the role of the fumarate reductase of *P. rettgeri* during the anaerobic growth on minimal media is studied. The question of whether the reduction of fumarate is coupled to phosphorylation is answered by measuring the cell yields of the growing bacteria [2–8]. This method requires that the energy metabolism sustaining the growth is well-known. Due to the limited accuracy of the method it is also necessary that the reduction of fumarate plays a quantitatively important role in the metabolism. These prerequisites are met by growing the bacteria on fumarate [9] and on citrate.

RESULTS

Growth on fumarate

In the experiment of Fig. 1 *P. rettgeri* is grown anaerobically on a minimal medium containing about 50 mM fumarate as the only carbon and hydrogen source. At the time after inoculation indicated (Fig. 1A) the cell density and the concentrations of fumarate plus L-malate, succinate and carbonate were determined. After a lag period of less than 10 h the cell density increases linearly rather than exponentially and parallel with the growth fumarate is consumed and succinate and carbonate are formed. The growth stops after about 40 h, when about 85% of the fumarate is consumed. During the growth an increasing part of the fumarate is converted to L-malate and the pH of the medium rises from 6.5 to about 6.8. About 95% of the carbon of the fumarate (plus malate) consumed is recovered as succinate and bicarbonate. The growth of the bacteria is sustained by Reaction a, according to which

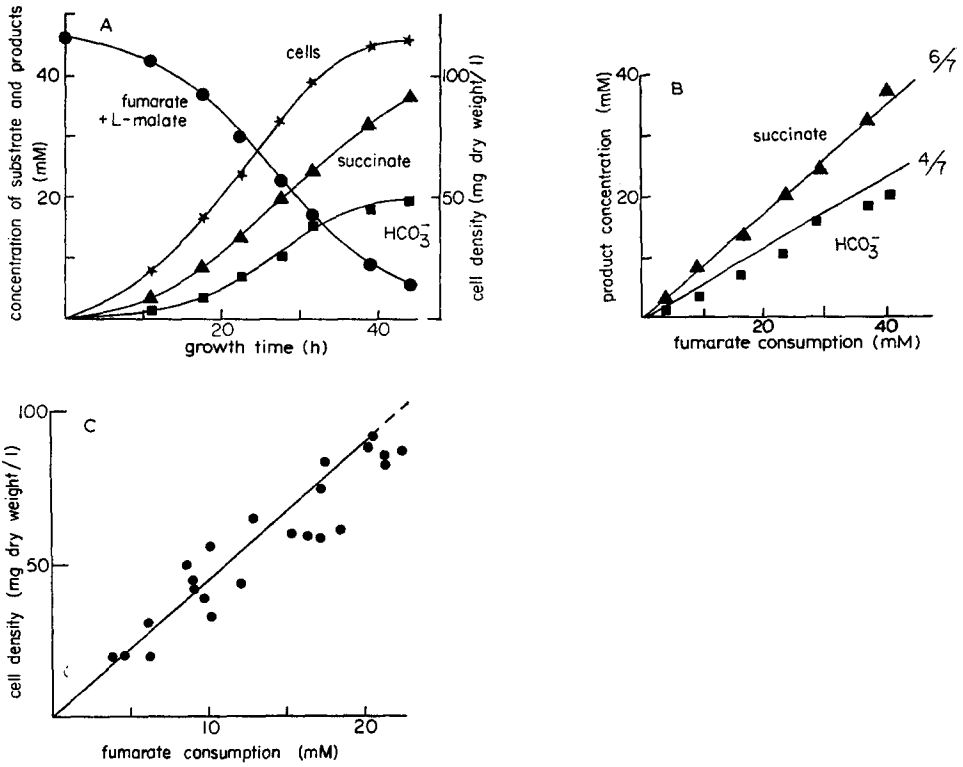


Fig. 1. Anaerobic growth of *P. rettgeri* on fumarate as the only carbon source. The growth medium, the inoculation and maintenance of the culture is described in Methods. (A) Samples were taken from the culture at the times indicated and analysed for the cell density and the concentrations of fumarate plus malate, succinate and carbonate as described in Methods. (B) The concentration of the products given in (A) is plotted against the corresponding decrease of the concentration of fumarate plus malate. The experimental data are compared to the theoretical lines with the slopes 6/7 and 4/7 which according to Reaction a give the relation to fumarate of succinate and carbonate, respectively. (C) Relation of the cell density to the corresponding decrease of the fumarate (plus malate) concentration. The data are taken from (A) and from five equivalent experiments. The slope of the straight line represents the average of the parameters up to the cell density of about 80 mg dry weight/l.

all the hydrogen derived from fumarate is recovered in the succinate.

$$7\ \text{fumarate}^{2-} + 8\ H_2O \rightarrow 6\ \text{succinate}^{2-} + 4\ HCO_3^- + 2\ H^+ - 106\ \text{kcal} \qquad \text{(a)}$$

This is demonstrated in Fig. 1B where the concentrations of succinate and carbonate given in Fig. 1A are plotted against the corresponding decrease of the concentration of fumarate (plus malate). The experimental points are compared with two theoretical lines with the slopes 6/7 and 4/7 which, according to Reaction a, give the relations of the amount of fumarate consumed to those of succinate and carbonate formed, respectively. The good fit obtained for succinate confirms the validity of Reaction a for the growth on fumarate. The concentrations measured for HCO_3^- are about 20% lower than expected from Reaction a.

The molar growth yield (Y_{fumarate}) is obtained from the slope of the plot of the cell yield against the corresponding consumption of fumarate. In Fig. 1C the values measured for six different cultures under the conditions of the experiment of Fig. 1A are given. An approximately linear increase of the cell density with the fumarate consumption is found for the fast growth phase up to 80 mg cells/l. In this range 4.5 g cells/mole fumarate are formed as the mean value. Since the cell mass accounts for 4% of the fumarate consumed, the $Y_{\text{fumarate}} = 4.7$ g cells/mole fumarate.

Growth on citrate

The growth curve with citrate in a minimal medium under anaerobic conditions is shown in Fig. 2A. Parallel with the increasing cell density the concentration of

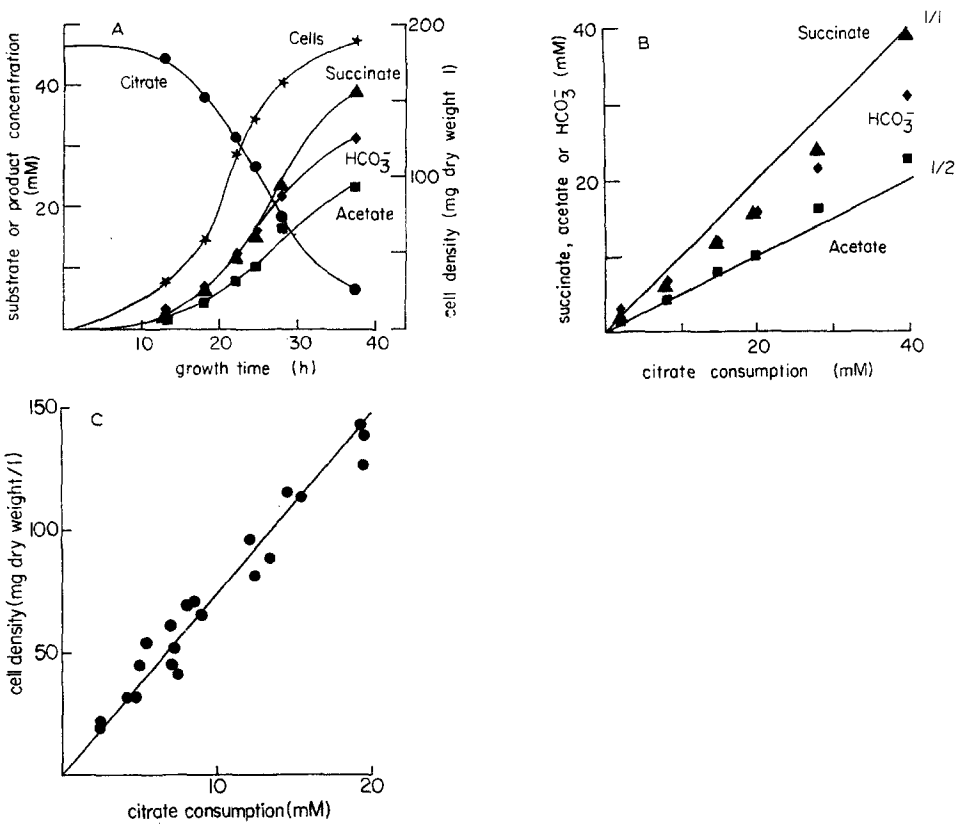


Fig. 2. Anaerobic growth of *P. rettgeri* on citrate as the only carbon source. The growth medium, the inoculation and maintenance of the culture is described in Methods. (A) Samples were taken at the times indicated and analysed for the cell density and the concentrations of citrate, succinate, carbonate and acetate as described in Methods. (B) The concentration of the products given in (A) is plotted against the corresponding decrease of the concentration of citrate. The experimental data are compared with two theoretical lines which according to Reaction b give the relation to citrate of succinate and carbonate (slope 1/1) and of acetate (slope 1/2). (C) Relation of the cell density to the corresponding decrease of the citrate concentration. The data are taken from (A) and from five equivalent experiments. The straight line represents the average of the parameters up to a cell density of about 100 mg dry weight/l.

citrate decreases and succinate, acetate and bicarbonate are formed. The pH of the medium remains at 7.0 during the growth. About 88% of the carbon and hydrogen of the citrate consumed is recovered as succinate, acetate and carbonate. 86–95% of these products are formed according to Reaction b.

$$2\ \text{citrate}^{3-} + 2\ H_2O \rightarrow 2\ \text{succinate}^{2-} + \text{acetate}^- + 2\ HCO_3^- + H^+ - 37\ \text{kcal} \qquad \text{(b)}$$

This is shown in Fig. 2B where the concentrations of succinate, acetate and bicarbonate are plotted against the corresponding decrease of the concentration of citrate. The experimental points are compared with two straight lines with the slopes 1/1 and 1/2 which, according to Reaction b, indicate the theoretical relations to citrate of succinate and bicarbonate and of acetate, respectively. The concentrations of succinate and of bicarbonate are slightly lower than expected from Reaction b, and those of acetate are slightly higher. This can be explained by the formation of 5–14% of the products according to Reaction c.

$$3\ \text{citrate}^{3-} + 5\ H_2O \rightarrow \text{succinate}^{2-} + 5\ \text{acetate}^- + 4\ HCO_3^- + 2\ H^+ - 69\ \text{kcal} \qquad \text{(c)}$$

The two reactions differ in the pathway of oxidation of citrate. This is done via isocitrate in Reaction b and via pyruvate in Reaction c. In both reactions the reducing equivalents are used for the reduction of oxaloacetate to succinate (see below).

The molar growth yield referred to citrate (Y_{citrate}) is obtained from the slope of the plot of the cell density against the corresponding decrease of the concentration of citrate (Fig. 2C). In the fast growth phase where the cell density is linearly related to the citrate consumption, 7.5 g cells are synthesized per mole of citrate consumed. Since the cell mass corresponds to 4% of the citrate consumed, the Y_{citrate} is 7.8 g cells/mole citrate.

Growth on pyruvate

In the experiment of Fig. 3 *P. rettgeri* is grown anaerobically on a minimal medium with pyruvate. The cell density increases in a parallel manner to the formation of acetate and formate from pyruvate. Although 0.1 M Tris is present, the pH of the medium decreases during the growth from 8.0 to values below 6. Reaction d is responsible for the growth of the bacteria.

$$\text{pyruvate}^- + H_2O \rightarrow \text{acetate}^- + \text{formate}^- + H^+ - 12\ \text{kcal} \qquad \text{(d)}$$

This is demonstrated by plotting the concentrations of acetate and of formate against the corresponding decrease of the concentration of pyruvate and by comparing the experimental points to the theoretical straight line with the slope 1/1 (Fig. 3B). In the fast growth phase, where the carbon recovery is at least 85%, the experimental points differ only slightly from the theoretical line, and this confirms that Reaction d is valid. The deviation (maximally 20% at the last point) is partly due to the formation of succinate. In an experiment similar to that of Fig. 3 the concentration of succinate was about 5% of that of the acetate and formate in the fast growth phase, and about 10% in the stationary phase. The cell mass corresponds to 7% of the pyruvate consumed.

It is clear from Reaction d that the ATP required for the growth of the bacteria is generated only by substrate-level phosphorylation from acetyl-CoA [10]. Therefore, the molar growth yield referred to acetate is equal to the amount of cells synthesized per mole of ATP (Y_{ATP}). This value is determined in Fig. 3C by plotting

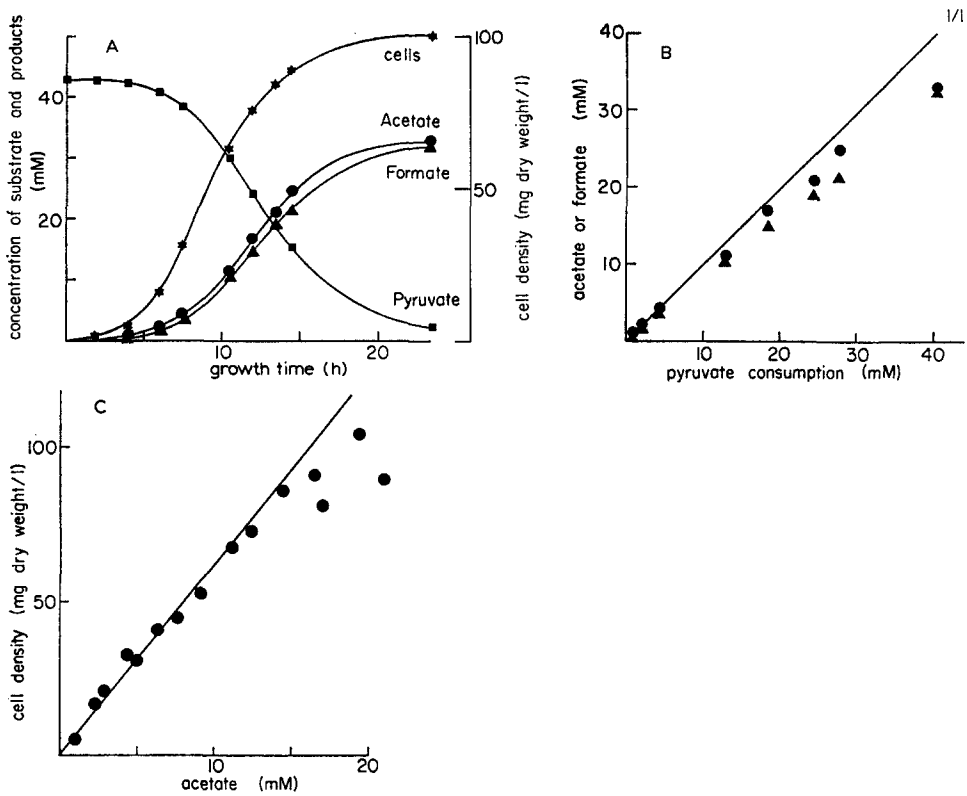


Fig. 3. Anaerobic growth of *P. rettgeri* on pyruvate as the only carbon source. The growth medium, the inoculation and maintenance of the culture is described in Methods. (A) Samples were taken at the times indicated and analysed for the cell density and the concentration of pyruvate, acetate and formate as described in Methods. (B) The concentration of the products given in (A) is plotted against the corresponding decrease of the concentration of pyruvate. The experimental data are compared to the theoretical line with the slope 1/1 which gives the relation of acetate and formate to pyruvate according to Reaction d. (C) Relation of the cell density to the corresponding acetate concentration. The data are taken from (A) and from two equivalent experiments. The slope of the straight line is the average of the parameters up to about 80 mg dry weight/l.

the cell density against the corresponding concentrations of acetate. The values are taken from the experiment of Fig. 3A and from two other equivalent experiments. A linear relation is obtained up to a cell density of about 80 mg/l with a slope of 6.3 g dry cells/mole acetate.

Growth on glucose

In the experiment of Fig. 4A *P. rettgeri* is grown anaerobically in a minimal medium with glucose. 50 mM HCO_3^- is added and the pH is 7.3 after the inoculation and 7.0 at the end of the exponential phase. The culture grows exponentially until the glucose is exhausted. As shown in Fig. 4B the concentrations of formate, acetate, succinate and ethanol increase linearly with the consumption of glucose and 1.24, 0.95, 0.54 and 0.30 moles/mole of glucose are formed, respectively. The products (5.90 moles carbon/mole glucose) amount to 90% of the carbon metabol-

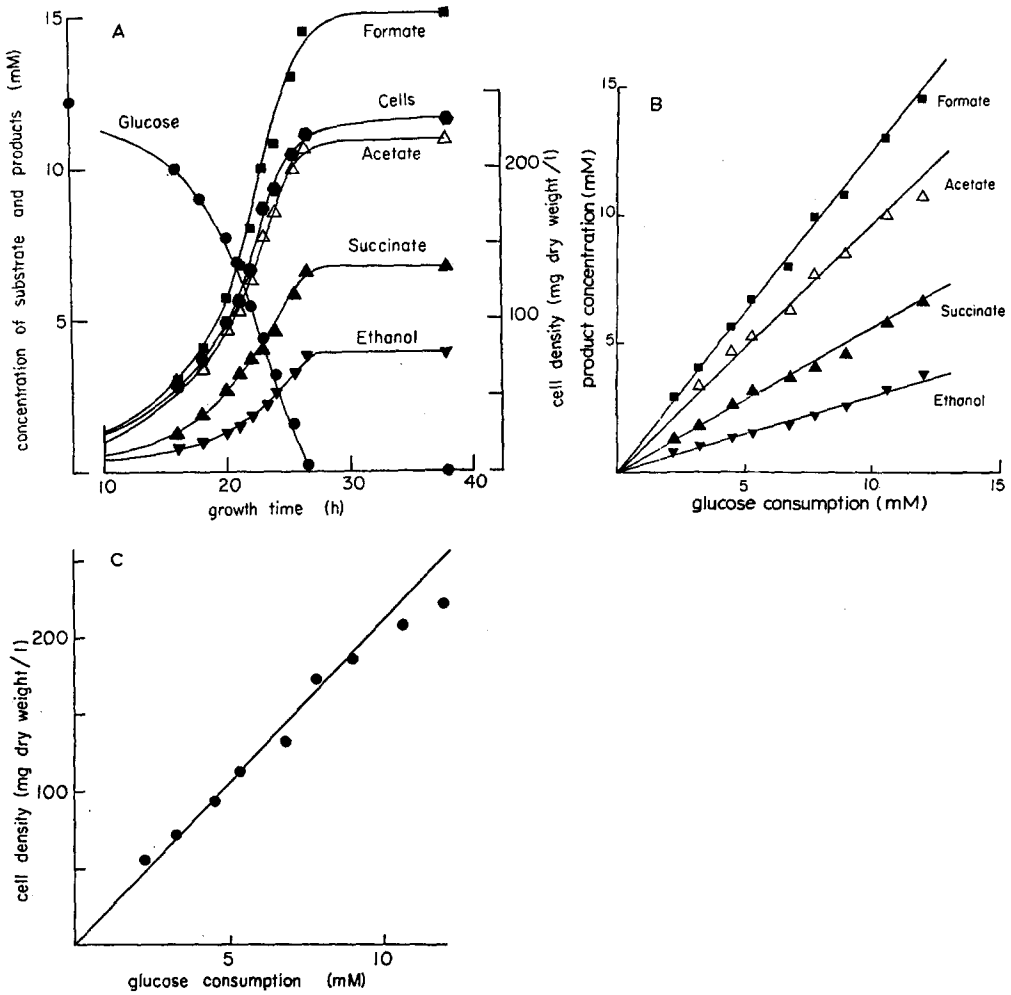
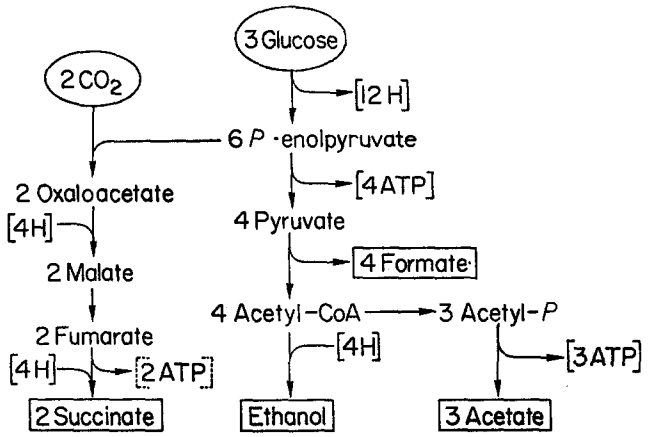


Fig. 4. Anaerobic growth of *P. rettgeri* on glucose in the presence of bicarbonate. The growth medium, the inoculation and maintenance of the culture is described in Methods. (A) Samples were taken at the times indicated and analysed for the cell density and the concentration of glucose, formate, acetate, succinate and ethanol. (B) The concentration of the products given in (A) is plotted against the corresponding decrease of the concentration of glucose. (C) The cell density given in (A) is plotted against the corresponding decrease of the glucose concentration.

ized (6.54 moles carbon/mole glucose), since in addition to the glucose consumed the uptake of 0.54 mole of HCO_3^- per mole of glucose according to Reaction g has to be taken into account (Scheme 1).

The fermentation of glucose can be described approximately by Reaction e. This reaction can be resolved into three part-reactions, one of which (f) produces, and the other two (g and h) consume, four reducing equivalents. The reactions f, g and h participate according to the relation 3 : 2 : 1, and this shows that the hydrogen of the glucose is recovered in the products.



Scheme 1. Assumed metabolic pathway of glucose as the substrate of the anaerobic growth of *P. rettgeri* according to Reaction e.

$$3\ \text{glucose} + 2\ HCO_3^- \rightarrow 4\ \text{formate}^- + 3\ \text{acetate}^- + 2\ \text{succinate}^- + \text{ethanol} + 9\ H^+ + H_2O - 180\ \text{kcal} \quad \text{(e)}$$

$$3 \times (\text{glucose} + 2\ H_2O \rightarrow 2\ \text{acetate}^- + 2\ \text{formate}^- + 4\ H^+ + [4\ H]) \quad \text{(f)}$$

$$2 \times (\text{glucose} + 2\ HCO_3^- + [4\ H] \rightarrow 2\ \text{succinate}^- + 2\ H^+ + 4\ H_2O) \quad \text{(g)}$$

$$1 \times (\text{glucose} + [4\ H] \rightarrow 2\ \text{ethanol} + 2\ \text{formate}^- + 2\ H^+) \quad \text{(h)}$$

From the metabolic pathways of the Reactions f, g and h [6, 11] the amount of ATP formed in the fermentation of glucose can also be estimated. The assumed pathway for the overall Reaction e is illustrated in Scheme 1. ATP is gained by the pyruvate kinase reaction in the pathways of the production of ethanol and acetate. In the latter pathway additional ATP results from the acetate kinase reaction. The pathway of succinate formation is assumed to provide ATP only by the reduction of fumarate (see Discussion). This pathway is assumed to branch from the others at the level of phosphoenolpyruvate which by carboxylation gives oxaloacetate [12]. Thus, Reaction e should give 3 moles ATP/mole glucose. The more accurate value which is calculated directly from the products is 2.74 moles ATP/mole glucose.

The cell yield is determined by plotting the cell density against the glucose consumption (Fig. 4C). A straight line is obtained for the exponential growth phase with a slope of 21 g cells/mole of glucose. Since the cell mass corresponds to 12% of the glucose, the $Y_{glucose}$ is 24 g cells/mole glucose.

Enzyme equipment of P. rettgeri

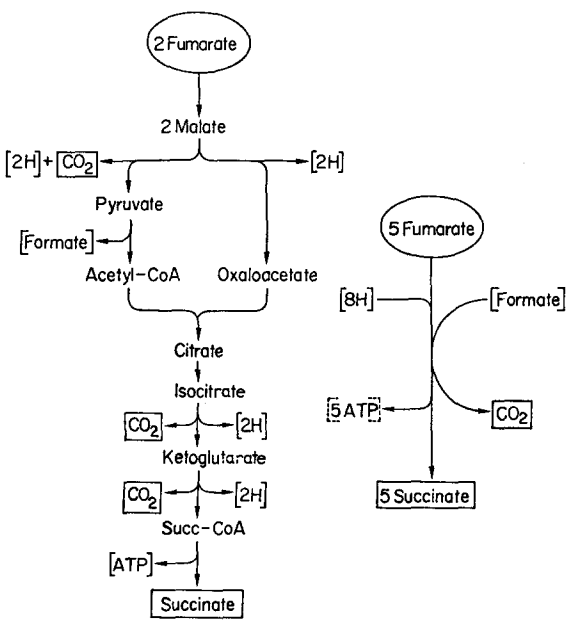
The enzyme activities measured in the sonic homogenates of *P. rettgeri* grown on the different substrates are compared in Table I. This experiment confirms the metabolic pathways of fumarate (Scheme 2) and of citrate (Scheme 3) which can already be concluded from the overall growth Reactions a and b, respectively. The activities measured in the bacteria grown on pyruvate serve as the control values. With pyruvate as the growth substrate (Reaction d) the enzymes necessary for the

TABLE I

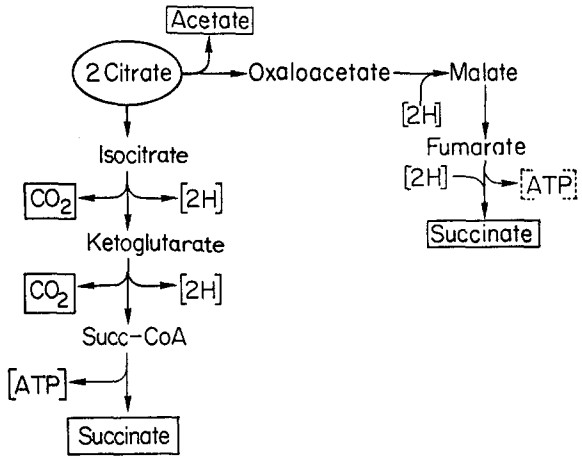
ENZYME EQUIPMENT OF *P. RETTGERI* GROWN ANAEROBICALLY ON VARIOUS CARBON SOURCES

The growth and the rupture of the bacteria as well as the assays for the enzymes are given in Methods. The data are obtained at 35 °C and referred to the total protein of the "sonic homogenate" (see Methods).

	Enzymic activity from cells grown on (μmoles/min per g protein)			
	Fumarate	Citrate	Glucose	Pyruvate
NADH–fumarate reductase	320	245	270	22
Formate–fumarate reductase	303	84	—	42
Fumarase	1230	930	1170	262
Malate dehydrogenase	252	720	850	54
Malic enzyme	253	138	80	50
Citrate synthase	227	17	73	15
Citrate lyase	14	262	0	0
Aconitase	260	480	10	30
Isocitrate dehydrogenase	1970	1740	97	102



Scheme 2. Metabolic pathway of fumarate as the substrate of the anaerobic growth of *P. rettgeri* according to Reaction a.



Scheme 3. Metabolic pathway of citrate as the substrate of the anaerobic growth of *P. rettgeri* according to Reaction b.

fermentation of fumarate and citrate are not required with the exception of the pyruvate–formate lyase which is involved in the fermentation of both pyruvate and fumarate.

The anaerobic metabolism of fumarate is illustrated in Scheme 2. 1 mole of fumarate is oxidized to give oxaloacetate which reacts with the acetyl-CoA resulting from a second mole of fumarate to give citrate. The citrate is metabolized via isocitrate dehydrogenase to give carbonate and succinate. Thus two moles of fumarate give 1 mole of succinate and ten reducing equivalents which further reduce 5 moles of fumarate to succinate. It is suggested that two reducing equivalents appear intermediarily as formate [10], whereas the residual eight are transferred by the pyridine nucleotides.

In the bacteria grown on fumarate, fumarate reductase with NADH and formate as donors, fumarase, malate dehydrogenase, citrate synthase, aconitase and isocitrate dehydrogenase are found in 5–20 times greater activities than in those grown on pyruvate. This indicates that these enzymes are required for the growth on fumarate and confirms that fumarate is metabolized according to Scheme 2. The suggestion that pyruvate–formate lyase rather than pyruvate dehydrogenase [10] participates in the fermentation of fumarate is confirmed by the increased activity of the formate–fumarate reductase compared to the activities of the bacteria grown on citrate. Pyruvate is probably formed via malic enzyme, since this enzyme exhibits the greatest activity with fumarate as the growth substrate.

The metabolic pathway of citrate according to Reaction b is illustrated in Scheme 3. As in the fermentation of fumarate, citrate is oxidized via isocitrate dehydrogenase to give bicarbonate and succinate. The reducing equivalents liberated in this reaction are used for the reduction of the oxaloacetate which is formed from the second half of the citrate consumed via the citrate lyase reaction.

Scheme 3 is confirmed by the enzymes found in the sonic homogenate of *P. rettgeri* grown on citrate. Citrate lyase which is specifically required, is induced only with citrate as the growth substrate. NADH–fumarate reductase, fumarase, malate

dehydrogenase, aconitase and isocitrate dehydrogenase which are needed for growth on both citrate and fumarate are about as active as on growth with fumarate. On the other hand, formate–fumarate reductase and citrate synthase which are involved only with fumarate as the growth substrate, are found with distinctly lower activities. The finding that malic enzyme and formate–fumarate reductase are more active with citrate than with pyruvate may be explained by the metabolization of a small part of the citrate according to Reaction c.

In *P. rettgeri* grown on glucose only the enzyme activities involved in the pathway of succinate formation are measured (Scheme 1). NADH–fumarate reductase, fumarase and malate dehydrogenase are present in activities commensurable with those found with fumarate and citrate as the growth substrates, whereas the other enzymes tested are only about as active as in the bacteria grown on pyruvate. This indicates that succinate is formed from oxaloacetate. Oxaloacetate is probably formed by carboxylation of phosphoenolpyruvate [12] and not from pyruvate. This is suggested by the finding, that malic enzyme is not induced by glucose as the growth substrate and that only a small amount of succinate is formed during the growth on pyruvate.

The enzyme activities given in Table I are consistent with those estimated for the bacteria in vivo. The latter values can be obtained from the rates of substrate consumption (Figs 1A, 2A and 4A) on the basis of Schemes 1, 2 and 3. Thus it is estimated that the activities of the enzymes of the bacteria in the middle of the fast growth phase range between about 100 and 300 μmoles substrate/min per gram of protein. The measured values are of the same order of magnitude and, therefore, confirm the participation of the enzymes of Table 1 in the fermentation of fumarate, citrate and glucose.

DISCUSSION

Fumarate as a hydrogen acceptor

Fumarate serves as an acceptor of the reducing equivalents liberated in the anaerobic metabolism of *P. rettgeri* not only when offered as the growth substrate (Reaction a, Scheme 2), but also after the intermediate formation from citrate (Reaction b, Scheme 3) and from glucose plus bicarbonate (Reaction e, Scheme 1). This is evident already from the amounts of succinate formed in the growth Reactions a, b and e, according to which all the hydrogen of the growth substrate is recovered in the products. The total hydrogen originating from the oxidation of fumarate as the growth substrate is used for the simultaneous reduction of an additional equivalent amount of fumarate to succinate. With citrate and glucose as the growth substrate 1/2 and 1/3, respectively, of the hydrogen is transferred to fumarate.

The membrane-bound NADH– and formate–fumarate reductase which involves menaquinone as a redox carrier [1] is responsible for the reduction of fumarate. This is shown by the induction of this electron-transport system during the growth on the three different substrates (Table I). With fumarate as the growth substrate both NADH and formate are used as the donors, whereas with citrate and glucose plus bicarbonate the hydrogen is transferred only by NAD^+.

In the previous communication [1] it was shown that the NADH– and formate–fumarate reductase is also induced during the anaerobic growth of *P. rettgeri* on

a complex medium. Under these conditions fumarate is probably formed from amino acids, and formate may be formed from pyruvate via the pyruvate–formate lyase as with fumarate as the growth substrate (Scheme 2).

Evidence for the occurrence of a fumarate reductase functioning with NADH or other donors is presented in the literature for many other bacteria [13–25]. The activity was demonstrated to be localized in the membrane [14, 17–24] which was shown to contain MK in most cases [17, 18, 20, 25–27]. Therefore, it is likely that a variety of bacteria ranging from obligate aerobes to obligate anaerobes contain a similar system of electron transport with fumarate as the acceptor. It is feasible that in some bacteria fumarate serves only as a high-potential sink for hydrogen, the function of which is to drive substrate-level phosphorylation reactions [15] or reactions necessary for the cell synthesis [20].

Phosphorylation coupled to fumarate reduction

Evidence for phosphorylation coupled to the reduction of fumarate has been presented with other bacteria, either by directly measuring phosphorylation in cell-free extracts [19, 24] or by determining the growth yields [16, 23, 28]. The latter method is applied to *P. rettgeri* since a decisive result can be expected especially with fumarate as the growth substrate.

With fumarate as the growth substrate per 7 moles of fumarate consumed 1 mole of ATP can be formed by substrate-level phosphorylation and 5 moles by fumarate reduction (Scheme 2). Thus n in Eqn 1 is expected to be either 1/7 or 6/7.

$$n = \frac{\text{mole ATP}}{\text{mole substrate or product}} = \frac{Y}{Y_{ATP}} \tag{1}$$

The estimation of n from the experimentally determined Y_{fumarate} (4.7 g cells/mole fumarate) and Y_{ATP} should, therefore, decide whether ATP is formed only by substrate-level phosphorylation or also by fumarate reduction. Since the Y_{ATP} valid for the growth of *P. rettgeri* on fumarate cannot be determined independently, the value of $Y_{ATP}^{max} = 15.4$ g cells/mole ATP is used. This was calculated from the ATP requirement of the cell synthesis for the growth on malate [29]. The resulting $n = 0.31$ is more than twice that expected for substrate-level phosphorylation alone, and this indicates that ATP is formed also by electron-transport phosphorylation coupled to fumarate reduction.

The difference between the expected and the calculated value for n corresponds to the discrepancy between the experimental Y_{ATP} and the theoretical Y_{ATP}^{max}. This discrepancy is usually observed and cannot quantitatively be explained so far [3–8, 29]. For further confirmation of the electron-transport phosphorylation it is, therefore, appropriate to calculate Y_{ATP} from Y_{fumarate} and the expected value of n and to compare this value with the Y_{ATP} obtained with the other substrates (Table II). Thus $Y_{ATP} = 5.5$ g cells/mole ATP ($n = 6/7$) for fumarate is obtained.

As indicated by the values of Y_{ATP}^{max}, pyruvate is nearly equivalent to fumarate with respect to cell synthesis. The Y_{ATP} for pyruvate as the growth substrate is obviously equal to Y_{acetate} ($n = 1$), and this value is only 15% greater than the Y_{ATP} for fumarate. This confirms the value of n assumed for calculating the Y_{ATP} with fumarate.

TABLE II

COMPARISON OF THE GROWTH YIELDS (Y) AND THE CELL YIELDS PER MOLE OF ATP (Y_{ATP}) OF *P. RETTGERI* GROWN ANAEROBICALLY ON VARIOUS CARBON SOURCES

The growth yields with fumarate, citrate, pyruvate and glucose are obtained from the slopes of the lines of Fig. 1C, 2C, 3C and 4C, respectively, after correction for the amounts of substrate incorporated into the cell mass (see Results). The values of Y_{ATP} for glucose, fumarate and citrate are calculated on the basis of the metabolic routes given in Scheme 1, 2 and 3, respectively, assuming that also the reduction of fumarate to succinate results in the formation of the equivalent amount of ATP. The Y_{ATP} with pyruvate implies that equimolar amounts of ATP and acetate are formed in the growth Reaction d.

Growth substrate	Y_{ATP}^{max} (g cells/mole ATP)	Y and ATP formation referred to	Y (g cells/mole)	Assumed ATP formation n (moles ATP/mole)	Y_{ATP} (g cells/mole ATP)	Fraction of ATP formed by	
						Electron-transport phosphorylation	Substrate-level phosphorylation
Fumarate	15,4 (29)	Fumarate	4.7	0.86	5.5	5/6	1/6
Pyruvate	13,4–15	Acetate	6.3	1	6.3	–	1/1
Citrate	16–20	Citrate	7.8	1	7.8	1/2	1/2
Glucose	28,8 (29)	Glucose	24	2.74	8.8	7/9	2/9

With citrate as the growth substrate $n = 1$ is assumed. This holds for Reaction b as well as for Reaction c. In the main pathway (Reaction b and Scheme 3) half the ATP is synthesized by substrate-level phosphorylation and the second half by fumarate reduction. The resulting Y_{ATP} (7.8 g cells/mole ATP) appears to be consistent with that for fumarate and pyruvate in view of the greater Y_{ATP}^{max} for citrate and, therefore, confirms that twice the amount of ATP resulting from substrate-level phosphorylation is gained. It is concluded that ATP is also formed by fumarate reduction with citrate as the growth substrate.

As expected from the Y_{ATP}^{max}, the greatest Y_{ATP} (8.8 g cells/mole ATP) is obtained for glucose. This number is in agreement with the generally accepted value (10.5 ± 2 g cells/mole ATP). The induction of the NADH–fumarate reductase suggests that ATP is also formed by fumarate reduction with glucose as the growth substrate.

METHODS

Growth of P. rettgeri

P. rettgeri (Strain No. 167-3, Dept of Bacteriology, University of Marburg, Germany) was grown on defined media, the mineral basis of which contained per liter 2.5 g NaCl, 0.5 g $(NH_4)H_2PO_4$, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.5 mM $NaHCO_3$ and 0.25 ml of the solution of trace elements. This solution contained per liter 25 g $Na_2EDTA \cdot 2\,H_2O$, 21 g $ZnSO_4 \cdot 7\,H_2O$, 28 g $FeSO_4 \cdot 6\,H_2O$, 0.34 g $Na_2B_4O_7 \cdot 10\,H_2O$ and 0.40 g $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$.

For growth on fumarate about 50 mM disodium fumarate was added to the mineral basis and the pH was adjusted to 6.5 by the addition of Tris. For growth on citrate about 50 mM citric acid and 20 mM $MgSO_4$ were added and the pH adjusted to 7.0 by KOH. The pyruvate medium contained about 50 mM sodium pyruvate and 0.1 M Tris at pH 8. For growth on glucose about 12 mM glucose and 50 mM $NaHCO_3$ were added and the pH was adjusted to 7.3.

These media were inoculated with 1–2% (v/v) of precultures which were grown for 24 h in test tubes containing 2% Proteose Peptone (Difco) and 0.6% NaCl at pH 7.6. After inoculation N_2 was bubbled for 5 min through the stirred cultures (0.2–2-l). The N_2 was freed from O_2 by passing it over a copper catalyst (BTS-catalyst, BASF, Ludwigshafen, Germany) which was heated to about 150 °C. The cultures were kept under an atmosphere of N_2 at 35 °C without stirring. Samples were taken through a glass tubing by pressing N_2 on the surface of the cultures. Substrates and products with the exception of carbonate were determined in 1-ml samples after removing the bacteria by centrifugation and the addition of 0.5 M HCl.

Analytical procedures

Cell density

The cell density of the cultures was determined photometrically using the Eppendorf photometer at 578 nm with a sample holder designed for measurements in test tubes. The absorbance increases linearly with the cell density up to about 0.4. The same relation is followed by cells grown on fumarate, pyruvate, citrate and glucose and the relation is independent of the growth phase of the cultures. The dry weight was determined after centrifugation, washing and drying of the bacteria in vacuo over P_2O_5 [30].

Protein

Protein was determined after sonication of the bacteria with the biuret method using KCN [31].

Determination of metabolites

Carbonate. Carbonate was measured gravimetrically after evaporation and absorption as CO_2. Samples (50–100 ml) of the cultures were pressed by N_2 in a stoppered vessel equipped with glass tubings and containing KOH (final concentration about 0.1 M) and 10 mM $AgNO_3$. After acidification by the addition of H_2SO_4 through a funnel with a stopcock, the CO_2 was evaporated by passing a stream of N_2 through the mixture at room temperature. The gas was dried by conc. H_2SO_4 and $CaCl_2$ and the CO_2 subsequently absorbed on soda asbestos.

Fumarate and L-*malate.* Fumarate and L-malate were determined by the absorption increase at 366 nm caused by the reduction of APAD in the following test mixture: 0.1 Tra, pH 7.4, 0.5 mM acetylphosphate, 50 μM coenzyme A, 1 mg/l phosphotransacetylase, 10 mg/l citrate synthase, 2 mg/l fumarase. The reaction was started by the addition of 5 mg/l malate dehydrogenase and ceased after 10–15 min. For the separate determination of malate and fumarate, the fumarase was added after the malate dehydrogenase. The extinction coefficient of reduced APAD is 9.1 $mM^{-1} \cdot cm^{-1}$ [32].

Succinate. Succinate was determined by the absorption decrease at 436 nm caused by the reduction of ferricyanide (1 mM) in 50 mM Tris, pH 7.6, on the addition of succinate dehydrogenase which was prepared according to Neufeld et al. [33]. The extinction coefficient of ferricyanide at 436 nm is 0.70 $mM^{-1} \cdot cm^{-1}$. The test requires about 30 min. The enzyme preparation was kept for several months in liquid N_2.

Acetate. Acetate was measured gas chromatographically as acetic acid as described by Fröhlich and Wieland [34]. The concentration of acetate was calculated from the peak area on the basis of a calibration curve obtained with acetic acid.

Formate. Formate was determined as the equivalent amount of O_2 consumed in the presence of a sonic homogenate of *P. rettgeri* according to Reaction i.

$$HCOO^- + 1/2\ O_2 \rightarrow HCO_3^- \qquad \text{(i)}$$

The O_2 consumption was monitored using a micro Clark-type O_2 electrode of our own design which was calibrated with air-saturated buffer at 25 °C (0.48 matom O/l). The homogenate was obtained by sonication at 0–5 °C (6 times for 5 s) of *P. rettgeri* grown for about 24 h at 35 °C on 2% Proteose Peptone and 0.6% NaCl, pH 7.6 (1-l), in shaken 2-l Fernbach flasks. About 0.5 mg protein/ml of the homogenate was incubated in 50 mM sodium/potassium phosphate, pH 7.2, and 10 mM malonate. On the addition of maximally 0.25 mM formate O_2 is taken up until the formate is consumed after less than 1 min.

Citrate, glucose, ethanol and pyruvate. Citrate, glucose, ethanol and pyruvate were determined as described by Bergmeyer [35] using citrate lyase, hexokinase, alcohol dehydrogenase and lactate dehydrogenase, respectively.

Enzyme activities

For measuring the enzyme activities the bacteria were harvested in the fast growth phase and sonicated at 0–5 °C (6 times for 5 s) in 0.5 M sucrose at a concentration of about 20 g/l protein. Part of the "sonic homogenate" so obtained was

centrifuged for 1 h at $10^5 \times g$. The sediment was separated from the supernatant and the fraction of the total protein determined for both parts.

NADH–fumarate reductase. NADH–fumarate reductase was measured photometrically as the oxidation of NADH under anaerobic conditions in the sonic homogenate as described earlier [1].

Formate–fumarate reductase and fumarase. Formate–fumarate reductase and fumarase were measured by recording the absorption of fumarate at 265–289 nm. The extinction coefficient of fumarate at these wavelengths is 0.57 $mM^{-1} \cdot cm^{-1}$. Fumarase was assayed in the supernatant incubated in 50 mM Tris, pH 7.6, on the addition of either 1 mM fumarate or 10 mM L-malate. Formate–fumarate reductase was measured in the sediment under anaerobic conditions as described earlier [1]. The results were corrected for the interference of fumarase.

Malate dehydrogenase. Malate dehydrogenase was measured in the supernatant as the rate of oxidation of NADH by oxaloacetate as described by Bergmeyer [35]. The results were corrected for the interference of the respiration with NADH.

Malic enzyme. Malic enzyme was measured in the supernatant as the rate of reduction of $NADP^+$ caused by the addition of L-malate in the presence of Mn^{2+} [36].

Citrate synthase. Citrate synthase was measured in the supernatant as the rate of reduction of APAD in the following test mixture: 0.1 M Tra, pH 7.6, 5 mg/l malate dehydrogenase, 6 mM L-malate, 0.5 mM APAD, 1 mg/l phosphotransacetylase and 2 mM acetylphosphate. The reaction was started by the addition of 2 mM coenzyme A.

Citrate lyase. Citrate lyase was measured in the supernatant as the rate of oxidation of NADH in the following test mixture: 0.1 Tra, pH 7.6, 5 mg/l malate dehydrogenase, 5 mg/l lactate dehydrogenase, 1 mM $MgCl_2$, 0.5 mM NADH. The reaction was started by the addition of 2 mM citrate. The results were corrected for the interference of the respiration with NADH.

Isocitrate dehydrogenase. Isocitrate dehydrogenase was tested as the rate of $NADP^+$ reduction in the following test mixture: 0.1 M Tra, 50 mM NaCl, pH 7.5, 4 mM $MnSO_4$ and 1 mM $NADP^+$. The reaction was started by the addition of 2 mM isocitrate.

Aconitase. Aconitase was tested as described above for isocitrate dehydrogenase, except that 0.1 g/l isocitrate dehydrogenase was present and that 2 mM citrate was used instead of isocitrate.

ACKNOWLEDGEMENTS

The authors thank Dr K. Jungermann and Dr R. K. Thauer for their stimulating discussions. This work was supported by a grant of the Deutsche Forschungsgemeinschaft.

REFERENCES

1 Kröger, A., Dadak, V., Klingenberg, M. and Diemer, F. (1971) Eur. J. Biochem. 21, 322–333
2 Bauchop, T. and Elsden, S. R. (1960) J. Gen. Microbiol. 23, 457–469
3 Senez, J. C. (1962) Bacteriol. Rev. 26, 95–107

4 Decker, K., Jungermann, K. and Thauer, R. K. (1970) Angew. Chem. 82, 153–173
5 Payne, W. J. (1970) Annu. Rev. Microbiol. 24, 17–52
6 Forrest, W. W. and Walker, D. J. (1971) Adv. Microbiol. Physiol. 5, 213–274
7 Stouthamer, A. H. (1969) Methods Microbiol. 1, 629–663
8 Stouthamer, A. H. and Bettenhausen, C. (1973) Biochim. Biophys. Acta 301, 53–70
9 Kröger, A. (1973) Z. Physiol. Chem. 354, 222
10 Henning, U. (1963) Biochem. Z. 337, 490–504
11 Wood, W. A. (1961) in The Bacteria (Gunsalus, J. C. and Stanier, R. Y., eds), Vol. 2, pp. 59–149, Academic Press, New York
12 Kornberg, H. L. (1965) Angew. Chem. 77, 601–632
13 Wolin, M. J., Wolin, E. A. and Jacobs, N. J. (1961) J. Bacteriol. 81, 911–917
14 Wolin, M. J. and Jacobs, N. J. (1963) Biochim. Biophys. Acta 69, 18–28
15 Deibel, R. H. and Kvetkas, M. J. (1964) J. Bacteriol. 88, 858–864
16 Buchanan, B. B. and Pine, L. (1967) J. Gen. Microbiol. 46, 225–236
17 Bogin, E., Higashi, T. and Brodie, A. F. (1969) Arch. Biochem. Biophys. 129, 211–220
18 Kröger, A. and Dadak, V. (1969) Eur. J. Biochem. 11, 328–340
19 Faust, P. J. and Vandemark, P. J. (1970) Arch. Biochem. Biophys. 137, 392–398
20 Newton, N. A., Cox, G. B. and Gibson, F. (1971) Biochim. Biophys. Acta 244, 155–166
21 Sone, N. (1972) J. Biochem. Tokyo 71, 931–940
22 Hatchikian, E. C. and Le Gall, J. (1972) Biochim. Biophys. Acta 267, 479–484
23 De Vries, W., von Wyck-Kapteyn, W. and Stouthamer, A. H. (1973) J. Gen. Microbiol. 76, 31–41
24 Barton, L. L., Le Gall, J. and Peck, H. D. (1970) Biochem. Biophys. Res. Commun. 41, 1036–1042
25 Sinclair, P. R. and White, D. C. (1970) J. Bacteriol. 101, 365–372
26 Baum, R. H. and Dolin, M. I. (1965) J. Biol. Chem. 240, 3425–3433
27 Maroc, J. R., Azerad, R., Kamen, M. D. and Le Gall, J. (1970) Biochim. Biophys. Acta 197, 87–89
28 Hobson, P. N. and Summers, R. (1972) J. Gen. Microbiol. 70, 351–360
29 Stouthamer, A. H. (1973) Antonie van Leeuwenhoek 39, 545–565
30 Thauer, R. K., Jungermann, K., Henninger, H., Wenning, J. and Decker, K. (1968) Eur. J. Biochem. 4, 173–180
31 Kröger, A. and Klingenberg, M. (1966) Biochem. Z. 344, 317–336
32 Holzer, H. and Söhling, H. D. (1962) Biochem. Z. 336, 201–214
33 Neufeld, H. A., Scott, C. R. and Stotz, E. (1959) J. Biol. Chem. 210, 869–876
34 Fröhlich, J. and Wieland, O. (1968) Z. Klin. Chem. 6, 277–280
35 Bergmeyer, H. U. (1970) Methoden der Enzymatischen Analyse, Verlag Chemie, Weilheim, Bergstraße
36 Takeo, K. (1969) J. Biochem. Tokyo 66, 379–387