The Influence of Growth Rate and Aeration on the Respiratory and Cytochrome System of a Fluorescent Pseudomonad Grown in Continuous Culture

By R. F. ROSENBERGER AND MARGOT KOGUT*

*Agricultural Research Council, Unit for Microbiology,
University of Sheffield*

SUMMARY: A continuous culture apparatus was used to study the effects of air supply and of growth rate on the respiratory activities and the cytochrome content of a strictly aerobic fluorescent pseudomonad. The oxidizing capacity, measured as Q_{O_2} (succinate), of organisms grown with air as the growth-limiting factor was lower than that of organisms grown with succinate as growth-limiting factor, and with air supply in excess. There was no significant correlation between oxidizing capacity and growth rate under either of the two conditions. Estimation of the cytochrome content of whole organisms grown under these different conditions showed that this tended to decrease with increase in growth rate. Organisms grown with air as the growth-limiting factor always had about double the cytochrome content of cells grown at the same rates but with succinate as the growth-limiting factor.

The effects of aeration on the development and composition of microbial respiratory systems have been studied by a number of workers (Chin, 1950; Ephrussi & Slonimski, 1951; Chaix & Roncoli, 1950; Chaix & Petit, 1956, 1957; Hollman & Thofern, 1955; Lenhoff & Kaplan, 1953; Lenhoff, Nicholas & Kaplan, 1956). These studies have suggested that oxygen supply is one of the factors which govern the quantitative and qualitative composition of cytochrome systems in several facultative and obligate aerobic micro-organisms. In some of these systems oxygen appeared to act as an inducer of cytochrome formation (Chin, 1950; Ephrussi & Slonimski, 1951); in others, maximum cytochrome formation occurred only under conditions where the air supply was severely restricted (Lenhoff & Kaplan, 1953; Lenhoff *et al.* 1956). However, difficulties of interpretation arise since in most of the work cited the organisms were grown in batch culture, where cultural conditions could not be adequately controlled. Thus, in order to study the effects of oxygen supply on bacterial growth and metabolism, it is necessary to ensure that oxygen concentration is kept constant throughout the experiment. In batch culture, excess of oxygen or its complete absence may be easily ensured, but an exponentially increasing oxygen supply to keep pace with the increase in numbers of organisms is required to investigate the effects of concentrations between these limits. Otherwise, organisms at the beginning of the experiment are likely to be growing under different conditions of aerobiosis from those at the end of the experiment. Moreover, in batch culture, the rate of oxygen supply, if not in excess, will control the growth rate, at least for obligate

* Present address: National Institute for Medical Research, Mill Hill, London, N.W. 7.

aerobes. Differences obtained by varying the oxygen supply cannot, therefore, be analysed into direct effects due to changed oxygen concentration and indirect ones due to the altered growth rate.

By means of the continuous culture technique (Monod, 1950; Novick & Szilard, 1950; Herbert, Elsworth & Telling, 1956) these difficulties can be overcome. Once a steady state has been reached during growth in continuous culture, the organisms are growing exponentially in a constant concentration of nutrients (including oxygen) and metabolic products, and at predetermined rates. The effects of limiting concentrations of metabolites or air supply and of growth rate can, therefore, be investigated independently.

In the present work, the production of cytochromes and the oxidizing abilities of a strictly aerobic fluorescent pseudomonad were studied under conditions where the organism was growing exponentially, at different rates, and with either air supply or carbon supply limiting growth.

METHODS

Continuous culture apparatus. The apparatus employed was designed by one of us (R. F. R.) and will be described in detail in a later publication. It consists of a stirred and baffled growth vessel of approximately 300 ml. working capacity, which is fitted with a heater, thermostat and overflow. Stirring is provided by a magnet which is mounted in a nylon jacket and held in position by a shaft projecting from the centre of the lid. Air is passed to the bottom of the vessel and ejected beneath the stirrer, by means of a stainless steel syringe needle with a bent and flattened tip. Nutrient medium is supplied to the culture from a reservoir by means of a rubber tubing pump and Revco Zeromax speed changer (Sigmamotor Inc. Middleport, N.Y., U.S.A.). At the concentrations of organism and degrees of aeration used in these experiments, some foaming occurred in the growth vessel. To decrease this, small amounts (c. 0.1–0.3 ml.) of a 25 % (w/v) suspension of silicone Antifoam 'B' in distilled water, sterilized by autoclaving, were added to the culture as required.

The theory of this type of continuous culture apparatus has been extensively discussed by Monod (1950), Novick & Szilard (1950) and Herbert *et al.* (1956), who have derived the mathematical equations for the relationships between concentration and rate of supply of nutrients and the concentration and rate of growth of organisms in the culture. They have shown that in a steady state, the specific growth rate (μ) of the organisms is equal to the dilution rate (D = rate of medium flow/volume of culture). This results because the specific growth rate is a function of the concentration, in the culture, of the growth-limiting factor. The growth rate of the organism can, therefore, be controlled by the rate of flow of medium, whilst the concentration of organisms in the culture at any given growth rate is controlled by the initial concentration of the growth-limiting factor.

Organism. The organism used was a strictly aerobic fluorescent pseudomonad, strain KB1 (Kogut & Podoski, 1953) which produces a green fluorescent, water-soluble pigment and does not reduce nitrate. It was maintained on

10 % (w/v) yeast autolysate (Barker & Beck, 1942) agar slopes by monthly transfer. For inocula a loopful from the slope was transferred to 10 ml. of 10 % (w/v) yeast autolysate in a 100 ml. conical flask and grown for 6–8 hr. at 30° with shaking; 5–10 ml. of such a culture were used to inoculate 300 ml. of the continuous culture medium.

Medium. The composition of the medium was as follows: 1 % (w/v) sodium succinate, 6H₂O; 0.25 % (w/v) NH₄Cl; 0.125 % (w/v) Na₂HPO₄; 0.875 % (w/v) KH₂PO₄; the following trace elements (μg./ml.): 61 Mg as MgO; 8 Ca as CaCO₃; 11.2 Fe as FeSO₄.7H₂O; 3.27 Zn as ZnSO₄.7H₂O; 2.75 Mn as MnSO₄.4H₂O; 0.64 Cu as CuSO₄.5H₂O; 0.59 Co as CoSO₄.7H₂O; 0.10 B as H₃BO₃. The medium was made up in 9 l. batches and sterilized by autoclaving at 15 lb./sq.in. The pH value of the sterilized medium was *c.* 6.0. When the organism grew and succinate was consumed, the pH value increased and the high buffer concentration kept it at pH 7.0–7.25 under conditions where succinate was the limiting factor, and at about pH 6.6–6.8 under conditions where oxygen was limiting.

Air supply. Air was supplied to the culture from a compressed air cylinder and was passed through a heated furnace and close-packed glass-wool filter to ensure sterility. The air flow into the vessel (in ml./min.) was measured by a Venturi meter. Since only these rates of air flow and not the amount of dissolved oxygen in the culture could be measured, we satisfied ourselves that carbon and not oxygen was the limiting factor under conditions of 'full aeration' by the following criteria. (a) In preliminary experiments we found that under similar conditions of aeration, but with a richer medium, this organism grew at a similar rate, but to *c.* 4 times the concentration of organisms here achieved. (b) Increase of air supply at constant rate of medium flow did not cause an increase in the concentration of organisms. (c) The concentration of organisms did not fall appreciably when the flow-rate of the medium was increased, indicating that air supply was not limiting the growth rate of the organisms. (d) The oxygen solution rate in the culture vessel, under the conditions of growth, was measured by the sulphite oxidation method of Cooper, Fernstrom & Miller (1944) and was found to be 504 mmole O₂/hr./l. As has been pointed out by Pirt (1957), this represents the maximum amount of available oxygen, and Wise (1951) has reported that measurement of oxygen solution rates by the sulphite method gives higher values than do polarographic measurements. However, the measured oxygen solution rate was at least 10 times in excess of the probable oxygen demand of the highest concentration of organisms in the culture, growing at the fastest rate which we used. This could be estimated, either from the maximum rate of succinate oxidation by washed suspensions, or from the growth rate and succinate concentration supplied in the medium, according to the equation given by Pirt (1957), assuming that the succinate is oxidized to completion with the uptake of 3.5 mole oxygen/mole of succinate. (If only 2.5 mole oxygen are consumed/mole succinate utilized, then the oxygen solution rate is 14 times in excess of the oxygen demand of the culture.)

Determination of amount of growth. For routine determinations of concen-

tration of organisms, the optical densities of samples were measured, after suitable dilution in distilled water, in a Hilger Spekker absorptiometer with neutral grey filter, against a water blank. The dry weight of the sample in mg./ml. was then calculated from a calibration graph, prepared from measurements on a sample of the organism growing in the logarithmic phase in batch culture. It has been suggested (Pirt, 1957) that the size (and hence the optical density for a given mass concentration) of an organism growing in continuous culture, varies somewhat with growth rate. We, therefore, also carried out 'direct' dry-weight determinations on samples growing under the various conditions. For this purpose, samples were centrifuged, after filtration through a coarse fluted filter-paper to remove precipitates, and the deposit

Table 1. *Concentrations of pseudomonad KB1 in continuous culture growing under different conditions*

Dilution rate (<i>D</i> (hr. ⁻¹))	(a) Concen- tration deter- mined turbidi- metrically (mg. dry wt. organism/ml.)	(b) Concen- tration deter- mined gravi- metrically (mg. dry wt. organism/ml.)	Difference (%)	Ratio <i>a/b</i>
Carbon-limited states				
0.142	1.28	1.36	5.9	0.94
0.142	1.09	1.44	24.3	0.75
0.228	1.39	1.48	6.0	0.94
0.228	1.35	1.45	6.8	0.93
0.396	1.41	1.47	4.1	0.96
Air-limited states				
0.072	0.70	0.68	2.9	1.03
0.072	0.70	0.79	11.4	0.89
0.144	0.53	0.56	5.4	0.95
0.144	1.03	0.97	6.2	1.06
0.216	0.61	0.59	3.4	1.03
0.216	0.46	0.46	0	1.00
0.378	0.71	0.68	4.4	1.04

washed 3 times with distilled water. The washed organisms were then dried at 105° to constant weight. Table 1 shows the values for concentrations of organisms determined turbidimetrically and gravimetrically on the same samples taken from the various steady states.

It can be seen that they do not differ by more than 7 % (except for two of the samples), and that the ratios of the dry weights obtained by the two methods do not appear to vary with growth rate. There does appear to be a difference in the average of the ratios (of the two dry-weight values) obtained from the carbon-limited and the air-limited steady states, but this was too small to affect the results of measurements (such as Q_{O_2} , cytochrome content, etc.) based on dry weights of samples determined turbidimetrically. For the two samples which gave differences for the two dry-weight determinations of 24 and 11 % respectively, duplicate determinations were carried out on further samples from the same steady states, and these agreed well with the rest of the values obtained.

Measurement of oxidizing ability as rate of succinate oxidation. The oxidizing abilities of organisms grown under the various conditions described were determined by measuring the rate of oxygen uptake by washed suspensions with succinate as substrate. For this purpose, samples of culture were collected, filtered through coarse fluted paper to remove any precipitates, centrifuged and the deposit washed once with distilled water. The organisms were then suspended in distilled water to concentrations equivalent to 2–3 mg. dry weight organism/ml. and the rate of oxygen uptake in $\mu\text{l./hr./mg. dry wt. of organism}$, in the presence of excess succinate ($=Q_{O_2}$ succinate) determined manometrically in the usual manner at 30° and pH 7.4. Duplicate determinations were made on each sample and the results averaged. The rate of endogenous respiration of each sample was also measured, but this was invariably very small compared to the rate of succinate oxidation (less than 5%) and was ignored.

Chemical estimations

Samples for chemical estimations were immediately acidified by collecting in tubes containing 0.5 ml. 10N- H_2SO_4 .

Succinate was estimated by the method of Krebs (1937).

Steam-volatile acids were estimated by distillation in the apparatus of Markham (1942), and titration with standard NaOH in a stream of CO_2 -free air.

Keto-acids were estimated by a method for which we are indebted to Dr E. R. Stadtman (personal communication, 1957). A portion of an acidified sample was centrifuged and to a volume of the supernatant fluid, containing from 0.1 to 0.25 μmole keto-acids, was added 1 ml. of M-acetate buffer (pH 4.9) and 0.5 ml. of 0.05M-thiosemicarbazide, and the volume made up to 5.0 ml. with distilled water. The solutions were incubated at room temperature for 30 min. and the optical density at 290 $\text{m}\mu$ read in the Unicam SP 500 spectrophotometer against a reagent blank containing water instead of the sample. The amounts of keto-acids expressed as $\mu\text{mole pyruvate/ml.}$ were then determined from a calibration graph prepared with a standard solution of sodium pyruvate.

Estimation of cytochrome content of whole organisms

The absorption spectrum of whole organisms, when measured in a spectrophotometer in the conventional way, with a water blank in the second cuvette, is distorted by light scattering. Shibata, Benson & Calvin (1954) (see also Keilin & Hartree, 1955) found that the effect of scattering could be minimized by inserting a diffusion screen between the cuvette and the photocell of the spectrophotometer. They found that either opal glass or a strip of filter-paper soaked in medicinal paraffin proved satisfactory for the purpose.

Fig. 1 shows the absorption spectrum of a suspension of pseudomonad KB1 which had been treated with a few crystals of dithionite to ensure complete reduction of the cytochrome. This spectrum was obtained with a Quartz prism Hilger Uvispec spectrophotometer fitted with a diffusion screen

made from Whatman No. 3 filter-paper soaked in medicinal paraffin and mounted between two glass slides. The slit width was 0.5 mm. and the band width in the range 410–430 $m\mu$ was 3–4 $m\mu$ and in the range 540–600 $m\mu$ was 5–7 $m\mu$. This absorption spectrum shows α -, β - and γ -bands, similar to those of crude and purified extracts of pseudomonad KB1 (Kogut, 1957). The γ -band (which was unsymmetrical) had a maximum at 418 $m\mu$. The optical density (OD) at 418 $m\mu$ of a suspension equivalent to 5 mg. dry wt. organism/ml. was of a magnitude, such that it seemed possible to use this method to

Fig. 1. Reduced absorption spectrum of a washed suspension of pseudomonad KB1. Suspension concentration equivalent to 5.6 mg. dry wt. organism/ml. Absorption measured with a quartz prism Hilger Uvispec Spectrophotometer fitted with a diffusion screen of Whatman No. 3 filter-paper soaked in medicinal paraffin. A few crystals of sodium dithionite were added to the suspension to ensure complete reduction.

obtain an estimate of the cytochrome content of whole organisms. The OD at 418 $m\mu$ is the sum of three components: (a) the absorption of the cytochrome pigments; (b) the absorption due to components other than cytochromes; (c) the scattering not compensated for by the diffuser. Both the non-specific absorption and scattering may be evaluated approximately by measuring the OD at 500 $m\mu$, a wavelength where the absorption due to cytochrome is least. The difference between the OD at 418 $m\mu$ and the OD at 500 $m\mu$ which we call Δ , will approximate to the OD of the cytochrome of the suspension.

Chance (see Chance & Williams, 1956) used a similar method for calculating the cytochrome content of particulate preparations. Figure 2 is a plot of Δ against mg. dry wt. organism/ml.; it will be seen that the curve is approximately linear over a limited range of concentrations of organism. By measuring OD (418) and OD (500) at a constant concentration of organisms, the correction factor is made more precise and the values of Δ so obtained are likely to represent more accurately the cytochrome content of the various suspensions. To test the procedure further, varying amounts of horse heart cytochrome *c* were added to a suspension of pseudomonad KB1 (equiv. 0.8 mg. dry wt.

organism/ml.) and OD (415) and OD (500) measured. Figure 3 is a plot of OD (415)–OD (500) against concentration of cytochrome *c* added; it will be seen that the curve is linear over a tenfold range of added cytochrome *c*.

Fig. 2

Fig. 3

Fig. 2. Estimation of cytochrome content of washed suspensions of pseudomonad KB1. Δ = optical density at wavelength of maximum absorption (418 $m\mu$) minus optical density at wavelength of minimum absorption (500 $m\mu$) as a function of concentration of organisms. Optical densities measured as for Fig. 1.

Fig. 3. Estimation of cytochrome content of washed suspensions of pseudomonad KB1. Proportionality of Δ (= absorption at Soret maximum of mammalian cytochrome *c* minus absorption at minimum) to concentration of added cytochrome *c* in the presence of washed suspensions of pseudomonad KB1 (equivalent to 0.8 mg. dry wt. organism/ml.). Optical densities measured as for Fig. 1.

Because it is uncertain whether the absorption at 418 $m\mu$ is due to a single cytochrome or to several, and because we do not know the extinction coefficients of the cytochromes of pseudomonad KB1, it is impossible to calculate from Δ the weight of cytochrome in a suspension. Consequently, all our results are given as optical densities and we propose to speak only of the relative cytochrome content of a suspension.

The relative cytochrome content of a suspension was measured as follows. Samples of culture were collected in a tube immersed in ice in a thermos flask. The sample was then filtered through a coarse fluted filter-paper, centrifuged and the deposit washed once in distilled water. The washed organisms were suspended in distilled water, the concentration measured and adjusted to equiv. 5.0 mg. dry wt. organism/ml. A sample was then placed in a 1 cm. cuvette, a few crystals of dithionite added and Δ was calculated from the OD measured at 418 and 500 $m\mu$.

RESULTS

Growth in steady states

Growth was started by transferring 5–10 ml. of inoculum, prepared as described in the section on Methods, to 300 ml. of medium in the culture vessel, and allowing the growth to reach the maximum with full aeration but

no medium flow. Continuous culture was then begun by starting the medium flow and adjusting to the required rate. The growth of the culture was followed by frequent readings of the optical density. For each combination of the two variables studied (growth rate and air supply) the concentration of organisms would adjust itself to a practically steady value, and samples for examination of oxidizing capacity, cytochrome content and metabolic products were taken from such 'steady states'.

In order to study the effect of oxygen supply on the organism, we arranged conditions so that at any growth rate the carbon source and the air supply were alternately the growth-limiting factors. In the first case, air supply was in excess (as judged by the criteria enumerated in the Methods section) and the amount of organism was governed by the concentration of succinate. In order to reach the second state, the air supply was decreased, keeping the medium composition constant. As the oxygen supply was decreased, the growth rate of the organism fell to a value below that of the dilution rate, and the amount of organism in the culture decreased until the steady-state concentration, in the culture, of the growth-limiting factor again allowed the organism to grow at a rate which was equal to the dilution rate. In this way a new steady state was reached, in which oxygen was the growth-limiting factor. The actual quantities of organism achieved for the different air-limited states varied, but since all our measurements of oxidizing capacity, cytochrome content, etc., were referred to equiv. dry wt. of organisms and not to volume of culture, these variations did not affect the results.

Fig. 4. Concentrations of pseudomonad KB1 growing in continuous culture under various steady-state conditions, as a function of time. (A): $\circ-\circ$, carbon-limited 'steady state'; dilution rate $D=0.142 \text{ hr}^{-1}$; $\bullet-\bullet$, air-limited 'steady state'; dilution rate $D=0.144 \text{ hr}^{-1}$; (B): $\circ-\circ$, carbon-limited 'steady state'; dilution rate $D=0.228 \text{ hr}^{-1}$; $\bullet-\bullet$, air-limited 'steady state'; dilution rate $D=0.216 \text{ hr}^{-1}$. The approximate times of sampling are indicated by the arrows on each graph. Measurement of pH values during each of the steady states showed these to be in the range 7.2–7.5 for the carbon-limited steady states, and in the range 6.6–6.9 for the air-limited steady states.

Figure 4 shows plots of concentration of organisms against time for a number of representative steady states. It can be seen that the variations in culture density were greater in the air-limited than in the carbon-limited steady states. This seems to be due to the fact that it is far easier to keep the concentration of carbon source constant, than to keep the air supply steady. In particular, we noticed that for a given setting, the actual speed of the stirrer motor often increased at night, and in the air-limited steady state the concentrations of

organisms in the culture tended to increase with it. We tried to overcome this by resetting the stirrer speeds late at nights, but were not wholly successful, because they tended to fluctuate again during the early hours of the morning. As far as possible we endeavoured to take samples only when the culture density was constant over several hours. The approximate times of sampling are shown on the graphs.

*Yield of organism with oxygen or succinate as
growth-limiting factor*

The range of pH values observed for the various steady states were from pH 7.2 to 7.5 for the carbon-limited steady states, and from pH 6.6 to 6.9 for the air-limited steady states. The relatively low pH values when air was the growth-limiting factor might be due either to incomplete breakdown of succinate with accumulation of acidic intermediates, or to succinate itself remaining in the medium.

Table 2. *Succinate consumed and intermediates found during growth of
pseudomonad KB1 in continuous culture*

Sample no.	Dilution rate (<i>D</i> (hr. ⁻¹))	Dry wt. organism (mg./ml.)	Succinate consumed (μmole/ml.)	Steam volatile acids found (μequiv./mg. dry wt.)	Keto-acids found (μmole/mg. dry wt.)	Yield constant (mg. organism/ mg. succinate consumed)
Carbon-limited states						
A 10	0.082	1.09	34.8	0.05	0.16	0.27
A 21	0.142	1.31	34.7	0.28	0.06	0.32
A 21	0.142	1.30	35.0	0.36	0.07	0.32
A 22	0.142	1.25	35.0	0.17	0.03	0.30
A 17	0.228	1.38	34.9	0.14	0.04	0.34
A 7	0.314	1.70	34.2	—	0.15	0.42
A 26	0.401	1.81	34.6	—	0.14	0.44
A 27	0.401	1.53	34.3	—	0.18	0.38
Air-limited states						
A 11	0.072	0.87	26.0	0.03	0.22	0.28
A 23	0.144	0.77	20.3	—	0.07	0.32
A 24	0.144	0.96	27.2	0.02	0.29	0.30
A 18	0.216	0.84	19.7	0.04	0.09	0.36
A 19	0.216	0.66	15.0	0.27	0.16	0.37
A 8	0.293	1.12	23.3	0.19	0.28	0.41
A 9	0.293	0.96	20.0	0.40	0.25	0.41
A 28	0.383	0.42	10.3	0	0.40	0.35
A 29	0.383	0.70	15.6	0	0.21	0.38

Table 2 shows the amounts of succinate consumed, the steam-volatile and keto-acids which accumulated and the yield constants (=mass of organisms formed/unit mass of succinate consumed) at different growth rates for the carbon-limited and air-limited steady states. It can be seen that for both the growth-limiting factors, the yield constants were practically the same, and that no significant amounts of steam volatile and keto-acids accumulated in the growth medium. Although under certain conditions pseudomonad KB1 can accumulate partially oxidized products from succinate (Kogut & Podoski,

1953), this does not appear to happen when oxygen concentration becomes growth limiting. Given growth-limiting concentrations of oxygen, it appears that part of the succinate is oxidized to the same extent as when oxygen is in excess, whilst part is not attacked at all.

*Variation in oxidizing capacity of organisms with
limiting factor and growth rate*

Measurement of the oxidizing capacities of organisms (expressed as Q_{O_2} , succinate) were carried out on two or three samples from each steady state. Figure 5 shows these values plotted against dilution rate.

Fig. 5. Oxidizing capacity, measured as Q_{O_2} (succinate) of washed suspensions of pseudomonad KB1 grown under different conditions, as a function of dilution rate. Each circle represents the mean of duplicate determinations carried out on a separate sample of organisms grown under the indicated conditions at the indicated rate. The separate samples for each set of growth conditions were taken at intervals of several hours. Endogenous values, which were never more than 5% of the substrate respiration rates, have been ignored.

Warburg cups contained organisms 1–2 mg. dry wt.; 30 μmole phosphate buffer (pH 7.4); 50 μmole sodium succinate in total volume 2.5 ml. The centre wells contained strips of folded filter-paper moistened with 10% (w/v) KOH. Gas phase air. Measurements were made at 30° and were started by tipping the suspensions from the side arms. Readings were taken over the period 5–35 min. after tipping, when the oxygen uptake was linear with time; the Q_{O_2} values were calculated from these. A: organisms grown with succinate as growth-limiting factor; B: organisms grown with air as growth-limiting factor.

Each point represents the measurement, carried out in duplicate, on one separate sample from a given steady state. Statistical analysis of the values obtained from the carbon-limited and air-limited steady states by the t -test shows that the differences are significant at the 0.1% level. On the other hand, the product-moment coefficients of correlation between Q_{O_2} (succinate) and dilution rate for either of the two groups were not significant at the 5% level; there were insufficient data to determine whether the rise in Q_{O_2} at the fastest dilution rate observed in the air-limited condition was significant. It

might be argued that for any given growth rate there must be a corresponding minimum respiration rate since the organism is an obligate aerobe. Pirt (1957) has shown that the oxygen uptake rate of populations of organisms growing in a steady state in continuous culture can be calculated from the dilution rate, the amount of substrate utilized and the 'oxygen demand constant' P =mole oxygen consumed/mole substrate utilized. Washed suspensions of pseudomonad KB1, harvested in the logarithmic phase of

Fig. 6. Calculated oxygen uptake rates of pseudomonad KB1 growing in continuous culture with succinate as sole source of carbon. The values for oxygen uptake rates in $\mu\text{l./hr./mg. dry wt. organism}$ have been calculated according to the equation of Pirt (1957)

$$\frac{-dc}{dt} = PD(s_0 - s),$$

where P =oxygen demand constant (mole oxygen consumed/mole substrate utilized), D =dilution rate, and $s_0 - s$ =amount of substrate consumed. The oxygen uptake rates have been calculated for complete oxidation of succinate ($P=3.5$) and for partial oxidation of succinate by washed suspensions of pseudomonad KB1 according to Kogut (1952) ($P=1.5$). A: organisms grown with succinate as growth-limiting factor; B: organisms grown with air as growth-limiting factor.

growth, oxidize succinate with the consumption of 1.5 mole oxygen/mole succinate (Kogut, 1952; Kogut & Podoski, 1953). Unfortunately, we do not know the oxygen demand constant for succinate utilization by growing cultures of pseudomonad KB1. However, we have calculated the oxygen uptake rates in $\mu\text{l./hr./mg. dry wt. organism}$ for pseudomonad KB1 growing in continuous culture at the different growth rates used, assuming complete oxidation of succinate ($P=3.5$) and for $P=1.5$. These values are shown in Figs. 6a and 6b.

It can be seen from a comparison of Figs. 5 and 6 that at low dilution rates the Q_{O_2} values of washed suspensions are in excess of the calculated oxygen uptake rates of the growing organisms. At the higher dilution rates, the oxygen uptake values of the growing organisms calculated on the assumption that $P=3.5$ correspond fairly closely to the measured Q_{O_2} values of washed suspensions.

*Variation in relative cytochrome content of organisms with
limiting factor and growth rate*

The relative cytochrome content of organisms grown under conditions where either succinate or air supply were the growth-limiting factors, and at different growth rates, was determined as described in the section on Methods. Figure 7 shows the values obtained plotted against dilution rate. Two or three samples were taken from each steady state and each point represents the value obtained for a single separate sample.

Fig. 7. 'Relative cytochrome content', measured as $\Delta = \text{OD at } 418 \text{ m}\mu - \text{OD at } 500 \text{ m}\mu$ of washed suspensions of pseudomonad KB1 at constant concentrations (equivalent to 5.0 mg. dry wt. organism/ml.) as a function of dilution rate. Each point represents the measurement, carried out on a separate sample of organisms grown under the indicated conditions. Separate samples for each set of growth conditions were taken at intervals of several hours. Measurements were carried out on washed organisms suspended in water containing a few crystals of sodium dithionite, in a 1 cm. cuvette, placed in a quartz prism Hilger Uvispec Spectrophotometer, fitted with a diffusion screen of Whatman No. 3 filter-paper, soaked in medicinal paraffin, between the cuvettes and the photocell. A: organisms grown with succinate as growth-limiting factor; B: organisms grown with air as growth-limiting factor.

Statistical analysis of the relation between relative cytochrome content and dilution rate shows a product-moment correlation of -0.783 , which is significant at the 1 % level, for the carbon-limited states and a correlation of -0.517 , which is barely significant at the 5 % level, for the air-limited steady states. Thus, the relative cytochrome content tended to decrease with increasing growth rate. At all the growth rates used, the relative cytochrome content of organisms which had been grown under air-limited conditions was more than double that of organisms grown with air supply in excess.

DISCUSSION

It would appear that with pseudomonad KB1, growth under conditions of limiting air supply, at a low rate, gives organisms with increased light absorption typical of cytochromes. Comparing changes at the same growth rates for the two different factors which limited growth, it further appears that decrease of the air supply alone is sufficient to bring about increased light absorption. It is not possible, unfortunately, to determine by the above technique whether this increased absorption can be ascribed to any particular cytochrome component. Washed suspensions and crude extracts of this organism showed two broad absorption bands in the α -region, at 551–556 m μ and at 560–565 m μ , and only one (unsymmetrical) peak in the Soret region (see Fig. 1) which probably represents the Soret bands of at least two cytochromes, one of which was isolated and purified by Kogut (1957). This cytochrome has its reduced absorption peak located at 417 m μ . What cannot be determined is the quantitative composition of the cytochrome system, since the specific extinction coefficients for the various cytochromes are not known. It is, therefore, also impossible to decide whether an increase in light absorption with different growth conditions represents an increase in the total amount of cytochromes produced, or a change to a predominant cytochrome with higher extinction coefficient.

A second question which cannot be answered by the present work is whether the change in relative cytochrome content is either a phenotypic change, or is due to the selection of variants. In these experiments we were particularly interested in establishing the connexion between cytochrome content and growth rate, physiological state of the organism and oxygen supply. Data were, therefore, obtained for conditions in steady states, and not for the transition periods before the steady states were reached. Moss (1956), with *Aerobacter aerogenes*, found increases in cytochromes a_2 and a_1 as the oxygen concentration was decreased from 10^{-3} to 10^{-6} M, and these changes could not be accounted for by selection of variants. Moss made his observations from the time of inoculation, over a total growth period, for any one oxygen concentration, of not more than 9 hr.; 'in no case was cytochrome increased after 5 hr.'.

Our findings show general agreement with the data of Moss (1956) for *Aerobacter aerogenes*. He found that when this organism was grown with different steady-state concentrations of oxygen in continuous culture (the steady states being of less than 9 hr. duration) the content of cytochrome a_2 in the organism increased as the oxygen concentration decreased from 10^{-3} to 10^{-6} M, whilst the cytochrome b_1 content remained constant. As the oxygen concentration was decreased further from 10^{-6} to 10^{-8} M, the concentration of cytochrome a_2 and b_1 in the organisms fell. The absorption due to cytochrome a_1 (which was always small relative to the absorption due to the other cytochromes) showed a slight increase with decreasing oxygen concentration over the range 10^{-3} to 10^{-6} M. At the same time, the growth rates of *A. aerogenes* in Moss's experiment also varied with the oxygen concentration in the medium. *A. aerogenes* is, of course, a facultative aerobe, with a respiratory and cytochrome system very different from that of pseudomonad KB1. In fact, the

only component of the pseudomonad KB1 cytochrome spectrum similar to one seen in *A. aerogenes* is the α -band at 560 m μ which Moss ascribes to cytochrome b_1 . But even this similarity in spectroscopic properties does not mean that the same substance is present in the two different organisms.

Observations on the production of cytochrome in *Pseudomonas fluorescens* have been reported by Lenhoff & Kaplan (1953), Lenhoff (1954) and Lenhoff *et al.* (1956). They found that when their organism was grown in batch culture without aeration or agitation, it produced much more of a cytochrome spectroscopically similar to animal cytochrome c , and a cytochrome peroxidase which could not be separated from it, than it did when grown with aeration. Their medium contained 0.5 % (w/v) sodium nitrate (in addition to 0.4 % of Difco Yeast Extract), so that anaerobic growth might have occurred at the expense of nitrate reduction. It is known from the work of Baalsrud & Baalsrud (1954) and of Sato & Egami (1949) that high values of cytochrome content are associated with growth under such conditions, i.e. with the development and operation of electron transport mechanisms in which nitrate is the final acceptor. In other words, this might be regarded as a special instance of a facultative system in which a cytochrome is part of a particular electron transport mechanism which may be inducible by its final electron acceptor, in this case nitrate. Other cases in which the production of cytochromes appears to be induced by the final electron acceptor are those observed by Chin (1950) and by Ephrussi & Slonimski (1951) where the cytochromes a , b and c of yeast appeared in response to aeration. Pseudomonad KB1 does not show any nitrate-reductase activity and does not grow under anaerobic conditions, either with or without nitrate (unpublished observations). The mechanism by which its relative cytochrome content is increased when it is grown on a simple defined medium with oxygen as the growth-limiting factor may therefore be an entirely different one.

Increase in haem respiratory pigments under conditions of low environmental oxygen concentration appears to be a general phenomenon and can occur in cases where selection of variants must be ruled out (Moss, 1956). Moreover, it has been shown by Moss that it can occur in a system (the cytochrome a_2 of *Aerobacter aerogenes*) which also shows an inductive response of increased cytochrome content to increased oxygen, over a different, lower range. Possible mechanisms to account for the apparent response of increased synthesis to decreased oxygen concentrations have been suggested by various authors (Moss, 1956; Lenhoff *et al.* 1956). Under the conditions of our experiment, the question of the possible effect of oxygen concentration on cytochrome content can be approached in the following way.

Longmuir (1954) has shown that the relation of the respiration rates of bacteria to oxygen concentration can be approximately described by the Michaelis-Menten equation. If it is assumed that a certain fixed fraction of the maximum possible respiration rate is required to allow bacteria to grow aerobically at a given rate, then it follows from Longmuir's data that for growth at any given rate, there exists a definite, minimum steady-state oxygen concentration. Under the experimental conditions here reported, the growth

rates were controlled by the rates of medium flow. On the other hand, in such a system, the growth rate is a function of the steady-state concentration, in the culture of the growth-limiting factor. It follows from the theory of continuous culture (Herbert, Elsworth & Telling, 1956) that under the air-limited conditions, the steady-state concentrations of oxygen in the culture must have been exactly those which allow the organisms to grow at rates equal to the dilution rates with all other nutrients, including carbon source, in excess. In the carbon-limited steady states, on the other hand, the oxygen concentrations in the culture must have been in excess of those required to allow growth at the given rates. It might be asked, therefore, 'can excess of carbon source stimulate cytochrome formation, or can excess of oxygen, either directly or indirectly inhibit cytochrome production?' A possible mechanism of inhibition by excess oxygen might be the production of an inhibitory substance or substances by an alternative electron transport system functioning at high oxygen concentrations. Such a changeover of the respiratory mechanism to an alternative pathway, possibly of a flavin nature, has been suggested by Lenhoff *et al.* (1956). Herbert (1955) has shown that inhibition due to peroxide formation in the medium under aerobic conditions accounted for the failure of small inocula of *Pasteurella pestis* to grow aerobically on agar plates in the absence of haematin or catalase.

The negative correlation between relative cytochrome content and growth rate in the carbon-limited steady states would follow from the assumption that the rate of cytochrome synthesis in these was determined by a factor—such as oxygen concentration—not governing the rate of general cell synthesis; variation of the latter, i.e. growth rate would, then, alter the relative cytochrome content of organisms in the observed direction. An alternative mechanism for the control of the rate of cytochrome synthesis might be the degree of oxidation of the electron-carrier system, in a manner analogous to the self-regulatory mechanism for the synthesis of photosynthetic pigments in *Athiorhodaceae* suggested by Stanier & Cohen-Bazire (1957).

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