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Control of Respiration and Metabolism in Growing *Klebsiella aerogenes*

THE ROLE OF ADENINE NUCLEOTIDES

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1. A rapid-sampling technique was used to obtain perchloric acid extracts of cells growing in a chemostat culture, so that meaningful values for ATP content could be obtained in spite of the fact that the turnover time for the total ATP content was about 1 sec. 2. For steady-state growth, it was found that, in a glucose-limited chemostat culture, the ATP/ADP concentration ratio was approximately constant with changes in dissolved-oxygen tensions above the critical value, but fell when the culture was grown under oxygen-limited conditions and was at a minimum in anaerobically grown cultures. The steady-state ATP content was lower in cells growing under nitrogen-limited conditions with glucose in excess than in glucose-limited cells. The steady-state ATP content was independent of growth rate at growth rates over 0·1 $hr.^{-1}$. 3. When the respiration rate of the cells was stimulated by lowering the oxygen tension the ATP content did not increase, indicating either an increased turnover rate of ATP or a fall in the P/O ratio. The sudden addition of extra glucose or succinate to a glucose-limited culture increased the respiration rate of the cells, but the ATP content quickly returned to the steady-state value after initial perturbations. This control over ATP content is explained in terms of regulation by adenine nucleotides of the catabolism and anabolism of glucose. An exception to this control over ATP content was found when the respiration rate was stimulated by addition of an antifoam.

Growing cultures of *Klebsiella aerogenes* show an interesting response to low dissolved-oxygen tension. Sustained oscillations in respiration rate can be obtained under these conditions (Harrison & Pirt, 1967), and it was shown that the q_{O_2} of the cells increases at low dissolved-oxygen tensions. No such phenomena have been reported for resting cells. A mechanism for the oscillations in respiration rate was proposed by Degn & Harrison (1969) based on the existence of a region of negative slope in the oxygen uptake–oxygen tension curve at low dissolved-oxygen tensions.

In the present work an attempt was made to determine the control properties of growing bacteria that enable them to respond to changes in oxygen tension in the observed manner. In recent years much has been published about glycolytic and respiratory control systems. The broad conclusion to be drawn from these studies is that adenine nucleotides play a central role in the control of respiration (Chance & Williams, 1956) and glycolysis (Chance, Ghosh, Higgins & Maitra, 1964). This seems to be true for resting suspensions of mammalian cells (Chance, 1964), yeast cells (Maitra, Estabrook & Chance, 1963; Pye, 1965) and bacteria (Hempfling, Hofer, Harris & Pressman, 1967). However, it is not known to what extent mechanisms described for resting cells function in the growing system. Clearly, there is a profound difference between the regulatory properties of actively growing cells and of resting cells, the latter lacking the adaptability of a growing culture.

Several workers have measured coenzyme concentrations in growing cells, but the results are contradictory, some finding that the ATP content was dependent on growth rate (Forrest, 1965; Cole, Wimpenny & Hughes, 1966) and others that it was independent of growth rate (Franzen & Binkley, 1961; Damoglou & Dawes, 1967; Smith & Maaløe, 1964). Such discrepancies may have arisen because in some cases the time between sampling of the cells and extraction of the ATP was long compared with the turnover time of the ATP content, and because the metabolic states of the organisms were

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not strictly comparable. So far no attempt appears to have been made by other workers to relate changes in ATP content of growing bacteria to specific control mechanisms.

In the present work, studies were made of the changes in concentrations of metabolic intermediates and coenzymes associated with changes in growth conditions in a chemostat culture of *K. aerogenes*, in order to relate these to possible regulatory systems.

Throughout this paper, dissolved oxygen in the culture is expressed as 'oxygen tension' in mm. Hg rather than as oxygen concentration. This is preferred because measurements of dissolved oxygen were made with membrane electrodes, which respond to oxygen tension and not to its concentration directly (Kinsey & Bottomley, 1963). An operational definition of the oxygen tension of a liquid would be 'the partial pressure of oxygen in the gas phase that, when in equilibrium with the liquid, would elicit the same output of current from an oxygen electrode'.

METHODS

The organism used was *K. aerogenes* (N.C.I.B. 8017). The medium was basically the synthetic medium described by Harrison & Pirt (1967) except that, to support a higher density of organisms, the $MgSO_4$ concentration was increased to 0·3g./l. For glucose-limited growth, the glucose concentration was increased to 10g./l. and the $(NH_4)_2SO_4$ concentration to 6·0g./l. For nitrogen-limited growth the glucose concentration was 20g./l. and the $(NH_4)_2SO_4$ concentration was 3·45g./l.

A magnetically stirred vessel (1l.) was used, which consisted of a Pyrex glass cylinder (6in. diam. × 6in. height) sealed by 'O' rings at the top and bottom to stainless-steel plates containing ports for electrodes, sampling etc. A Teflon-coated magnet was pivoted on the inside of the base plate and was rotated by means of a horseshoe magnet, situated under the vessel, which was coupled to a $\frac{1}{8}$ h.p. motor. Magnet rotation rates of 800 rev./min. could be maintained when the vessel was filled with medium. The culture volume was kept constant at 1l. by means of a stainless-steel overflow tube ($\frac{1}{2}$in. diam.) set into the base plate. Samples could be taken from a port in the base of the vessel. The temperature of the culture was maintained at 30±0·05° by controlling the temperature of water circulated continuously through a channel in the base plate of the vessel. The pH was controlled at 6·0 ±0·02 by the addition of 2M-NaOH. Aeration was achieved by means of a vortex, and the dissolved-oxygen tension in the vessel was altered by changing the partial pressure of oxygen in the gas supply. Test materials, e.g. glucose and succinate, were added by injection from a syringe through a vaccine cap in a port at the top of the vessel.

Dry weights of cells were determined gravimetrically with 10ml. samples of the culture. The cells were centrifuged, washed twice and dried to a constant weight at 98°.

Mackereth (1964) electrodes, made by Electronic Instruments Ltd., Richmond, Surrey, were used to record the dissolved-oxygen tension and the partial pressure of oxygen in the effluent gas.

Evolution of carbon dioxide was measured by bubbling the effluent gas through 5mM-$KHCO_3$–5% (w/v) KCl, kept at constant temperature, in which a pH electrode was placed. The pH reading obtained was a function of the CO_2 concentration in the gas. This method could be used with confidence over periods of several hours provided that calibration was carried out before and after use, but there was a drift in the readings over a period of days.

Sampling procedure. To obtain values of intermediates that have high turnover rates, it is essential to ensure that the cells are killed and extracted as rapidly as possible after leaving the culture. Usually, samples (10ml.) were collected in $\frac{3}{4}$in.-diam. test tubes containing 0·86ml. of 72% (w/v) $HClO_4$. Before collection of the sample, 5ml. of culture, a volume several times greater than the dead space in the sampling port, was allowed to flow to waste, and then the sampling tube was placed under the port and approx. 10ml. of culture was collected. By taking care that the jet of sample was directed down the centre of the tube and did not touch the sides, turbulence was maintained in the sample throughout the sampling time.

To estimate the sampling and mixing times involved in the above procedure, a model of the system was set up by sampling 9ml. of 10M-alkali into 1ml. of conc. HCl in a $\frac{3}{4}$in.-diam. test tube to which Thymol Blue indicator was added. The concentrations of the acid and alkali were arranged so that the indicator would change colour from red to blue after about 8ml. of alkali had been collected. The sampling procedure was filmed with a cine-camera at 67 frames/sec. It was found that both red and blue colours were present together in the liquid for only five frames, equal to about 0·08sec., which, it is assumed, is approximately the time required for mixing. The total time for collection of a sample was 0·5sec. and the time for the first drop to reach the acid after leaving the vessel was less than 0·1sec. Therefore the maximum time-interval between a cell leaving the culture vessel and being treated with acid was 0·2sec. The time required to inactivate most of the enzyme system is not known, although in yeast it is less than 0·5sec. (P. K. Maitra, unpublished work). However, even though the enzymes may not be completedly destroyed within 0·2sec., they will have little activity at the very low pH.

The acid-extracted sample was left on ice for 5min., then placed in water at 40° for 2min., and then kept on ice for a further 5min. This procedure was found to maximize the extraction of metabolic intermediates. The precipitate formed was spun down in a refrigerated centrifuge and a sample of the supernatant was carefully neutralized. Neutralized samples were immediately frozen and stored at −10° for a maximum period of 2 weeks, while analyses were performed. In fact, no significant fall in the concentrations of intermediates was observed in samples stored frozen for over 4 weeks.

Determination of intermediates. Metabolic intermediates in the $HClO_4$ extracts were determined enzymically by coupling reactions involving the intermediate to reactions of appropriate enzymes resulting in the oxidation or reduction of nicotinamide nucleotides, as described by Estabrook & Maitra (1962) and Maitra & Estabrook (1964). Glucose, G6P* and ATP were determined by using the glucose

* Abbreviation: G6P, glucose 6-phosphate.

6-phosphate dehydrogenase (EC 1.1.1.49) plus hexokinase (EC 2.7.1.1) system. Fructose 1,6-diphosphate, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate were determined by coupling with aldolase (EC 4.1.2.13), triose phosphate isomerase (EC 5.3.1.1) and α-glycerophosphate dehydrogenase (EC 1.1.1.8) respectively. The results were expressed as total C_3 units of fructose diphosphate plus triose phosphate, because some residual aldolase activity reappeared in the $HClO_4$ extract after neutralization. ADP, AMP, pyruvate and phosphoenolpyruvate were determined by using pyruvate kinase (EC 2.7.1.40), adenylate kinase (EC 2.7.4.3) and lactate dehydrogenase (EC 1.1.1.27). For the AMP assays it was essential to pretreat the NADH with phosphatase (EC 3.1.3.1) to remove contaminating AMP. Since ADP, AMP and phosphoenolpyruvate are determined in the neutralized supernatant as pyruvate, prior removal of this substance was required. This was normally achieved by the addition of NADH and lactate dehydrogenase. However, samples from nitrogen-limited cultures contained very high concentrations of pyruvate and, as the addition of very large quantities of NADH was undesirable, the pyruvate was chemically reduced to lactate as follows. The extract was adjusted to pH 8, and KBH_4 crystals were added to give the molar equivalent of 2–3 times the pyruvate present. After incubation at 23° for 5 min., the excess of KBH_4 was decomposed by adjusting the pH to 6·0 with HCl. Finally, the pH was readjusted to 7·2. Citrate was determined by using citrate lyase (EC 4.1.3.6) coupled with malate dehydrogenase (EC 1.1.1.37).

For all these determinations samples were diluted in 50 mM-triethanolamine–HCl buffer, pH 7·2, containing 5 mM-$MgCl_2$ (usually 0·2 ml. of sample in 2 ml. of buffer). It was necessary to keep the Mg^{2+} concentration low, to avoid precipitation of magnesium phosphates, as the samples had a high phosphate content. All these assays gave good duplication of results, the deviation between duplicates never being more than ±2·5%.

All the enzymes, intermediates and coenzymes used in these assays were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

RESULTS

For the same culture, under the same steady-state conditions, the concentrations of metabolic intermediates and coenzymes obtained were quite reproducible: S.D. ± 0·4 nmole/mg. dry wt. was obtained for the ATP content of 20 samples taken over a 2 hr. period. However, concentrations of ATP obtained in different cultures, grown under similar conditions, varied considerably. For instance, the aerobic steady-state ATP content reported in Table 1 was approx. 6·3 nmoles/mg. dry wt., whereas that reported in Fig. 4 was approx. 9·0 nmoles/mg. dry wt. Similar effects were obtained with other intermediates. The reason for this is not known, but possibly the past history of a culture affects the enzyme systems present and thus the steady-state content of intermediates.

Steady-state conditions. Table 1 shows the concentrations of G6P and adenine nucleotides at various dissolved-oxygen tensions and a constant growth rate of 0·18 $hr.^{-1}$. The G6P content varied considerably even at dissolved-oxygen tensions above the critical value, but there was a significantly higher G6P content under anaerobic conditions. ATP content and ATP/ADP concentration ratios were more or less constant as long as the rate of oxygen uptake was unaffected by dissolved-oxygen tension, but at low dissolved-oxygen tensions there was a fall in ATP content and a fall in ATP/ADP concentration ratio. In the anaerobic steady state the ATP/ADP concentration ratio was at a minimum, although the ATP content was the same as that obtained for oxygen-limited conditions. This emphasizes the advisability of determining all adenine nucleotides rather than ATP alone.

Table 1. *Steady-state concentrations of G6P and adenine nucleotides in cells grown at different dissolved-oxygen tensions*

Growth was glucose-limited at a growth rate of 0·18 $hr.^{-1}$. The values given are the means of at least two samples determined in duplicate. Details are given in the Methods section.

% of O_2 in gas phase	Dissolved-O_2 tension (mm. Hg)	Dry wt. (mg./ml.)	Concn. (nmoles/mg. dry wt.) G6P	ATP	ADP	AMP	Total adenine nucleotides	ATP/ADP concn. ratio
100	420	3·8	0·9	8·3	—	—	—	—
50	220	4·2	0·5	6·6	—	—	—	—
21	57	4·3	—	5·9	—	—	—	—
15	5·3	4·3	2·8	6·1	2·1	0·4	8·6	2·9
12	3·0	4·3	2·6	6·5	2·2	0·4	9·1	3·0
9	<0·2–2·5*	4·2	0·2	6·2	2·3	0·5	9·0	2·7
2·4	<0·2	2·5	1·2	3·7	2·9	0·8	7·4	1·3
0·0	0·0	0·7	12·4	3·7	3·9	1·1	8·7	0·9

* Oscillating transition state.

Table 2. *Comparison of steady-state concentrations of intermediates in nitrogen-limited and glucose-limited cells*

The growth rate was 0·21 hr.$^{-1}$, and cell concentrations were 4·5 and 5·0 mg. dry wt./ml. for glucose-limited and nitrogen-limited cultures respectively. The values given are the means of at least two samples determined in duplicate. Details are given in the Methods section.

	Concentrations of intermediates (nmoles/mg. dry wt.)							
Growth conditions	G6P	ATP	ADP	AMP	Total adenine nucleotides	ATP/ADP concn. ratio	Oxalo-acetate	Citrate
Glucose-limited								
Excess of O_2	2·3	8·0	1·7	0·4	10·1	4·7	0	1·49
Limited O_2	1·7	7·0	2·3	0·4	9·7	3·0	0	2·00
Nitrogen-limited								
Excess of O_2	5·8	4·5	2·7	1·0	8·8	1·7	1·7	9·0
Limited O_2	1·7	5·5	—	—	—	—	5·6	58·0

It is noteworthy that there was little apparent toxic effect when 100% oxygen was supplied to the culture. The dry weight yield was a little lower (Table 1) and a large amount of yellow pigment was formed. Also, 100% oxygen elicited the highest steady-state content of ATP.

Table 2 shows a comparison between glucose-limited and nitrogen-limited steady states at a constant growth rate of 0·21 hr.$^{-1}$. The ATP content under nitrogen-limited conditions was lower than that under glucose-limited conditions, although the energy source, glucose, was in excess. Any extra production of ATP when glucose is in excess is probably offset by its utilization for the build-up of polysaccharide reserves. A noteworthy aspect of the results shown here was the accumulation of citrate and oxaloacetate under nitrogen-limited conditions. The accumulation of tricarboxylic acid-cycle intermediates was probably caused by the lack of amino donors for the removal of oxaloacetate and α-oxoglutarate during nitrogen-limited growth.

The effect of growth rate on the steady-state content of adenine nucleotides is shown in Fig. 1. The ATP content was independent of growth rates above 0·1 hr.$^{-1}$, even up to values above the 'diluting out' phase, which was indicated by the fall in dry weight at a growth rate of 0·5 hr.$^{-1}$. At low growth rates the ATP content appeared to fall but so did that of ADP, so that the ATP/ADP concentration ratio was approximately the same as that at higher growth rates. Postgate & Hunter (1962) reported that at growth rates of 0·05 hr.$^{-1}$ 30% of the cells in a chemostat culture were non-viable. This may explain the low values for ATP content obtained at growth rates of 0·03 hr.$^{-1}$, if a loss of viability is accompanied by a loss of adenine nucleotides. The other experiments reported here were carried out at growth rates of about 0·2 hr.$^{-1}$,

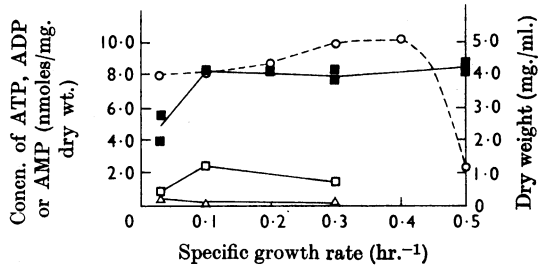

Fig. 1. Effect of growth rate on steady-state concentration of adenine nucleotides. Growth was glucose-limited and oxygen was in excess (dissolved-oxygen tension, 30–80 mm. Hg). ○, Dry weight; ■, ATP; □, ADP; △, AMP. Details are given in the Methods section.

so that small changes in the growth rate would not affect the adenine nucleotide content of the cells.

Stimulation of respiration at low dissolved-oxygen tensions. Lowering of the dissolved-oxygen tension below a certain value causes an increase in the respiration rate of a growing culture of *K. aerogenes* (Harrison & Pirt, 1967). This phenomenon was investigated by obtaining a steady-state respiration and dissolved-oxygen tension just above the 'critical' value, cutting off the oxygen supply for a short time, so that the tension fell below the critical value, and then resuming the oxygen supply. In response to this procedure, the respiration rate of the culture increased, indicated by a low dissolved-oxygen tension reading (Fig. 2) and high carbon dioxide production (not plotted). The respiration rate remained high for a time, well after the partial pressure of oxygen in the gas phase had been restored to its previous value, and then spontaneously reverted to its previous steady-state value (Fig. 2). During this cycle samples were taken for analysis for intermediates and coenzymes.

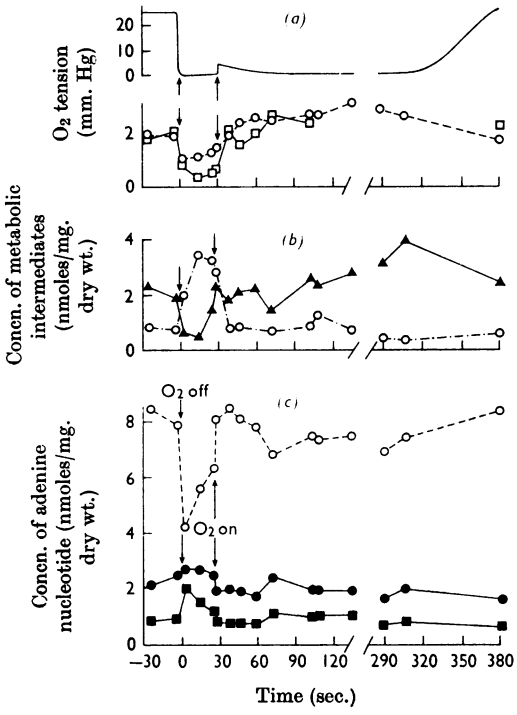

Fig. 2. Metabolic-intermediate and adenine-nucleotide concentrations of cells during the stimulation of respiration caused by interruption of the oxygen supply. Growth was glucose-limited at a growth rate of 0·2hr.$^{-1}$. ——, Dissolved-oxygen tension; (*a*) ○, phosphoenolpyruvate; □, citrate; (*b*) ▲, G6P; ○, fructose diphosphate plus triose phosphate, expressed as C_3 units; (*c*) ○, ATP; ●, ADP; ■, AMP. Arrows mark times of cutting off and resumption of oxygen supply. The low dissolved-oxygen tension reading after resumption of the oxygen supply indicates the period of stimulated respiration, and the rise in dissolved-oxygen tension after 300sec. indicates the spontaneous reversion to the steady-state respiration rate. Details are given in the Methods section.

The results are shown in Fig. 2. The extremely rapid fall in ATP content as the dissolved-oxygen tension fell to a low value emphasizes the need for the immediate extraction of the samples. The total adenine nucleotide content, as calculated from the sum of ATP, ADP and AMP concentrations, appeared to fall with the fall in ATP content on cutting off the oxygen. This was possibly because the ADP and AMP concentrations were underestimated, owing to incomplete extraction. After the initial fall, the ATP content recovered a little. Other experiments were carried out in which the culture was left anaerobic for over 10min., in which case the ATP content recovered to a steady-state value about half of that in the aerobic steady state. The rapid fall in ATP content on cutting off the oxygen supply is caused, no doubt, by the sudden imbalance between ATP production and turnover rate as the respiration rate of the cells abruptly falls. The subsequent establishment of a steady ATP content indicates that the cell corrected this imbalance. On resumption of the oxygen flow the ATP content rose rapidly, overshot and then fell to a steady state just below the fully aerobic steady-state value. When the dissolved-oxygen tension rose again, representing the spontaneous reversion to the lower respiration rate, the ATP content increased slightly to the previous fully aerobic steady-state value. The changes in ATP content were accompanied by changes in those of AMP and ADP in the opposite direction. The way in which AMP-concentration changes accompanied the changes in that of ATP, and the 'buffering' tendency of ADP concentration, are indicative of an active adenylate kinase reaction. This experiment was repeated and the same pattern of results was obtained, including the overshoot in ATP content on resumption of supply of oxygen.

The fact that, although the respiration rate had increased on resumption of the supply of oxygen to the culture, the ATP content did not rise above the previous aerobic steady-state value, but was slightly lower, requires either that the ATP turnover rate had increased over that of the steady-state condition or that the number of ATP molecules produced per molecule of oxygen taken up had decreased during the stimulation of respiration. An increased respiration rate in the absence of an elevated ADP content argues against direct control by ADP of respiration of the type demonstrated by state 4 mitochondria (Chance & Williams, 1956). However, these results cannot distinguish between the roles of ATP, ADP and AMP in feedback mechanisms, as the concentrations of all three are interrelated owing to the high adenylate kinase activity present in the cells.

The response of G6P concentration to the fall in dissolved-oxygen tension was to fall rapidly and then recover. On resumption of the supply of oxygen the G6P concentration returned to approximately its initial value. This pattern was reversed for the fructose diphosphate plus triose phosphate concentrations; these rose when that of G6P fell, and fell, after a short delay, when that of G6P rose. This is the well-known crossover pattern reported for the Pasteur effect in yeast (Ghosh & Chance, 1964) and is interpreted as indicating a site of glycolytic control at the phosphofructokinase reaction.

Concentrations of phosphoenolpyruvate and citrate changed in the opposite direction to those of fructose diphosphate and triose phosphate when oxygen was turned off and on. This would indicate

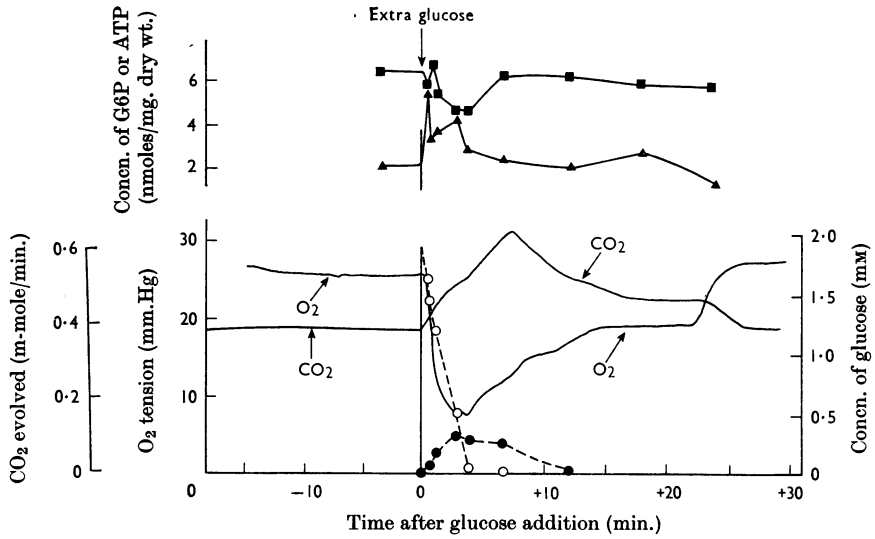

Fig. 3. Effect of addition of extra glucose on dissolved-oxygen tension and content of metabolic intermediates in a glucose-limited culture. The steady-state growth rate was 0·2 hr.$^{-1}$. ■, ATP; ▲, G6P; ○, glucose; ●, pyruvate. The arrow marks the time of adding 2 m-moles of glucose. A fall in dissolved-oxygen tension denotes an increase in respiration rate and a rise in dissolved-oxygen tension denotes a decrease in respiration rate. Details are given in the Methods section.

a reverse crossover between fructose diphosphate, triose phosphate and phosphoenolpyruvate that in itself cannot be interpreted as a site of interaction (Chance, Holmes, Higgins & Connelly, 1958) but does indicate that there is another positive cross-over further down the pathway. The flux of glucose moieties through phosphoenolpyruvate increases on a change to anaerobiosis because of an increased rate of reaction at pyruvate kinase or at a stage beyond, or both. These interpretations assume that the net flux of glucose through the system is in fact increased when it becomes anaerobic, i.e. that the Pasteur effect holds for growing systems. Evidence for this was given by Harrison (1965). Also, it is assumed that glycolysis is by far the most important pathway of glucose metabolism under these conditions.

Effect of additions of substrates. The most significant aspect of these results is the control over ATP content that was demonstrated. To investigate the control of ATP content further, the rate of production of ATP were altered by suddenly providing more energy source. To this end, 2 m-moles of extra glucose was added to the culture in a steady state. The respiratory response to glucose passed through three phases (Fig. 3). On addition of the glucose, the dissolved-oxygen tension fell rapidly, indicating a rise in respiration rate. When most of the glucose had disappeared the respiration rate fell again, but returned to the steady-state value in three steps, the dissolved-oxygen tension passing through two plateaux. The first of these plateaux, at 15 mm. Hg, seemed to be caused by the oxidation of the pyruvate that was built up during the period of rapid disappearance of glucose. The cause of the second plateau, at 20 mm. Hg, is unknown. Immediately after the addition of glucose, the G6P content rose rapidly, then fell again, but to a value higher than the steady-state value. ATP content, after going through an oscillation, fell to a value below that of the steady state. At first consideration this fall in ATP content with an increased availability of substrate and increased respiration rate seems anomalous, but it can be explained by an increased utilization of ATP for the synthesis of polysaccharides and other cell components that might be stimulated by the increased G6P content (Segel, Cattaneo & Sigal, 1963). In fact, by integrating the oxygen-uptake and carbon dioxide-production curves it was found that only about one-sixth of the excess of glucose added was oxidized, with an overall respiratory quotient of about 1·0. The rest may have been incorporated into cell material or converted into a non-oxidizable product. The former seems more likely. When the excess of glucose was exhausted, the G6P content fell and the ATP content immediately returned to its previous steady-state value. The return of the ATP content to its previous value after addition of glucose, and the small extent of

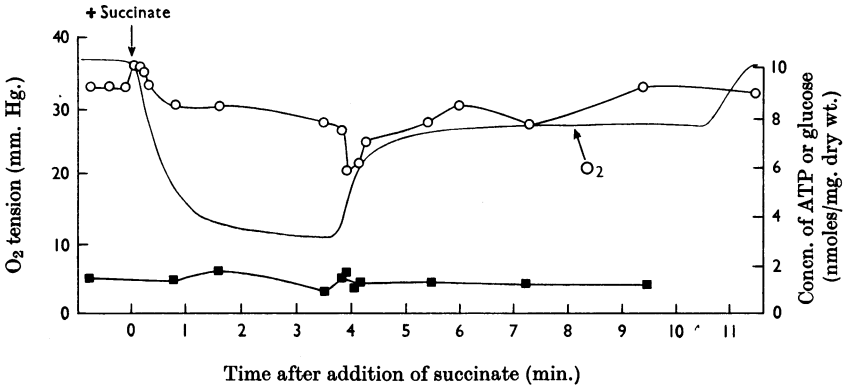

Fig. 4. Effect of addition of succinate on dissolved-oxygen tension, ATP content and glucose concentration, in a glucose-limited culture. The growth rate was 0·2 hr.$^{-1}$. ——, Dissolved-oxygen tension; ○, ATP; ■, glucose. The arrow marks the time of adding 1 m-mole of succinate. A fall in dissolved-oxygen tension denotes an increase in respiration rate and a rise in dissolved-oxygen tension denotes a decrease in respiration rate. Details are given in the Methods section.

its excursion while respiration was increased by addition of glucose, is a further example of a high degree of control over ATP content of the cell.

A similar experiment was carried out with the addition of 1 m-mole of succinate, which, it was thought, might be less readily converted into storage products than is glucose. Fig. 4 shows the results obtained. On addition of succinate, the respiration rate immediately increased, causing a fall in dissolved-oxygen tension, then maintained a new steady rate, and then it decreased abruptly, presumably because the succinate was exhausted. An intermediate plateau was obtained before the dissolved-oxygen tension finally returned to the aerobic steady-state value. The increased respiration rate on addition of succinate was accompanied by a small increase in ATP content; then the ATP content fell to a value just below that of the steady state. When the respiration rate fell again there was a sharp fall in ATP content, but this quickly recovered. The glucose concentration in the culture did not rise after addition of succinate, although the supply of glucose was maintained throughout. Thus glucose utilization was not affected by the presence of succinate.

It seems that addition of succinate causes increased production of ATP, but this is rapidly followed by a higher turnover of ATP, which serves to lower the ATP content again. As with the addition of glucose, the higher turnover of ATP is probably caused by energy utilized to incorporate more carbon into cell material, as in this case also only one-sixth of the added succinate was oxidized and the remainder was presumably incorporated into the cell material. The fall in ATP content when the succinate was exhausted occurred because the turnover of ATP was still high, but this was quickly decreased again to bring the ATP content back to its previous value.

Thus it would seem that the ATP content is controlled in such a manner that any change in its value causes reactions that tend to bring the value back to the steady-state condition, but there are time delays in the system that allow overshoots to occur.

These results are a further indication that the respiration rate of these cells is not ADP-limited, as the increase in respiration rate is accompanied by a rise in ATP content, and therefore, presumably, by a decrease in ADP content.

Fig. 5 shows the dissolved-oxygen-tension traces obtained after addition of 1 m-mole of succinate, 1 m-mole of fumarate or 1 m-mole of acetate to a steady-state culture, and also the effect of turning off the stirring for a short while to cause anaerobiosis. In all cases, except on addition of acetate, there was a two-stage recovery of dissolved-oxygen tension, with a plateau that was at the same level in each. This was also obtained by addition of citrate, oxaloacetate and pyruvate. This plateau might represent the oxidation of a substrate that built up during the oxidation of the other substrates. The fact that this plateau was at the same dissolved-oxygen tension as that obtained by adding acetate suggests that it may be acetate that builds up during the oxidation of other substrates. However, such a plateau was also obtained when a nitrogen-limited cultures recovered from an anaerobic shock, but when 1 m-mole of acetate was added to such a culture the respiration was slightly inhibited. The substance that built up might be a product of oxidation of acetate, or the

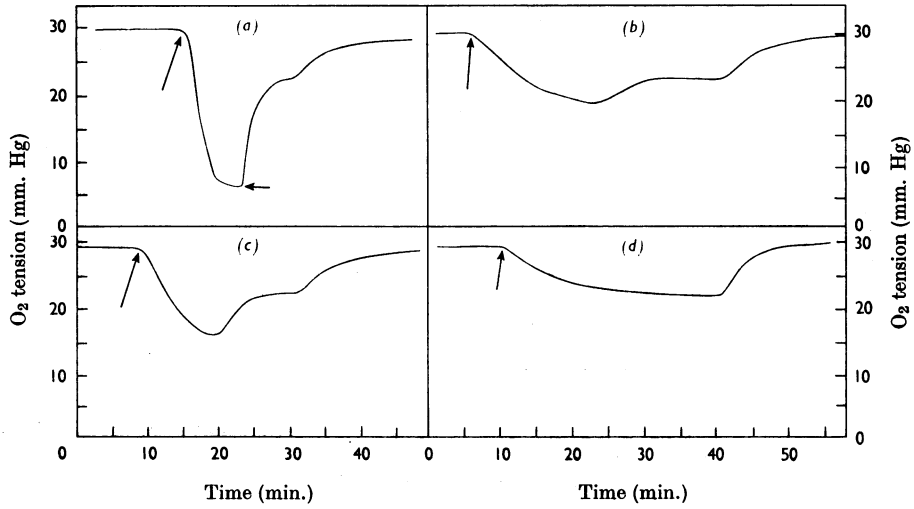

Fig. 5. Effect of interruption of the oxygen supply and the addition of various substrates on dissolved-oxygen tension. Growth was glucose-limited, at a growth rate of 0·2 hr.$^{-1}$. (*a*) Arrows indicate times of turning stirring off and on. The dissolved-oxygen-tension reading did not fall all the way to zero on turning off the stirring because a small area of membrane on the oxygen probe became exposed to the gas phase. (*b*), (*c*) and (*d*) Arrows indicate addition of 1 m-mole of fumarate, 1 m-mole of succinate and 1 m-mole of acetate respectively. A fall in dissolved-oxygen tension denotes an increased respiration rate, and a rise in oxygen tension denotes a decreased respiration rate. Details are given in the Methods section.

plateau might represent an energy deficit that must be made up. For instance, if the uptake of the added substrates were accompanied by the loss of ions, e.g. K^+, from the cell, then the plateau might represent an increase in respiration rate required to correct such a deficit.

Effect of antifoam. The high degree of control over ATP content seemed to be a constant feature of the culture, but an exception was found when antifoam was used to stimulate respiration. The addition of antifoam to a culture of *K. aerogenes* had been observed to cause a change of dissolved-oxygen tension (Harrison, 1965). On closer investigation it was found that addition of silicone antifoam (Antifoam 60, silicone emulsion; General Electric Co., Waterford, N.Y., U.S.A.) to the culture caused a rapid fall in dissolved-oxygen tension and a large increase in carbon dioxide production that lasted only a few minutes before recovering. The size of the response depended on dose size up to doses of about 1 part of antifoam in 10^4 of culture, but the increase in oxygen uptake was much too large to be caused only by the oxidation of a substance contaminating the antifoam. There is no doubt that the decrease in dissolved-oxygen tension was, in fact, a reflection of increased respiration rate, and this occurred under conditions when diffusion of oxygen from gas to liquid was far from limiting. This phenomenon is unusual, particularly in relation to the ATP content. Fig. 6 shows that the increased respiration rate was accompanied by a rise in ATP content. The ATP content remained high for some time before gradually falling again, and reached the previous steady-state value after about 30 min.

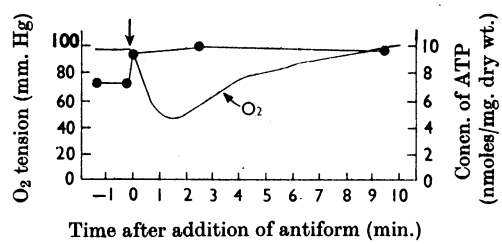

Fig. 6. Effect of the addition of antifoam (Antifoam 60, silicone emulsion) to the culture on the dissolved-oxygen tension and ATP content. Growth was glucose-limited at a growth rate of 0·2 hr.$^{-1}$. ——, Dissolved-oxygen tension; ●, ATP. The arrow indicates the addition of antifoam to give a concentration of 10 mg./l. in the culture. Details are given in the text.

DISCUSSION

Although it has been suggested by some workers that the efficiency of energy-coupling in aerobically growing bacteria is low (Gunsalus & Schuster,

1961), Hadjipetrou, Gerrits, Teulings & Stouthamer (1964), applying the concept of molar growth yields of ATP propounded by Bauchop & Elsden (1960), found energy-coupling in aerobically growing *K. aerogenes* under glucose-limited conditions to be as efficient as that in mitochondria. Calculations (D. E. F. Harrison, unpublished work) based on growth yields and respiration rates of *K. aerogenes*, obtained under growth conditions identical with those used in the present work, support the findings of Hadjipetrou *et al.* (1964) that *K. aerogenes* grows with an efficiency that indicates a P/O ratio of 3.

A system in which a common source of carbon and energy is provided for growing organisms, such as that investigated here, presents a number of problems in metabolic control. The organisms have to allocate a proportion of the source for energy and a proportion for cell material. The coinage for energy in the cell is ATP and so it is to be expected that the ATP content of the cell will play an important role in the control of glucose metabolism. The extent of control required will depend to some degree on the rate of turnover of ATP in relation to its 'pool' size inside the cell.

Approximate turnover times for the ATP content of cells can be calculated from the glucose-oxidation rate. The rate of glucose utilization (R) in m-moles mg. dry wt.$^{-1}$ min.$^{-1}$ is given by:

$$R \simeq \frac{s_0 . F}{180 V . x} \qquad (1)$$

where s_0 is the substrate concentration in the medium in mg./ml., F is the flow rate of the medium in ml./min., V is the volume of the culture in ml., and x is the concentration of cells in mg. dry wt./ml.

For *K. aerogenes*, grown under conditions used in this work, 37·5% of the glucose supplied is oxidized (from data of Harrison & Pirt, 1967). Therefore, assuming that there are three sites of phosphorylation and that 38 ATP molecules are obtained/glucose molecule oxidized, ATP production in m-moles mg. dry wt.$^{-1}$ min.$^{-1}$ is given by:

$$\frac{s_0 . F \times 37{\cdot}5 \times 38}{V . x \times 180 \times 100} \qquad (2)$$

In the experiments considered here, i.e. at a growth rate of 0·2hr.$^{-1}$ with a concentration of cells of 5mg./ml., the ATP production, calculated from expression (2), was 0·5μmole.mg. dry wt.$^{-1}$ min.$^{-1}$. The ATP content of the cells under these conditions was approx. 10nmoles/mg. dry wt., so that the turnover time of the total ATP content was approx. 1·2sec. Expression (2) shows that this value will vary with growth rate. Even if the P/O ratio is 1 or 2, the turnover time for ATP will be only a few seconds.

This extremely high turnover rate indicates the need to kill and extract the cells very rapidly if meaningful values for concentrations of adenine nucleotides are to be obtained from growing cultures. Thus results obtained by methods that involve delays of several seconds (Wimpenny, 1967) and in some cases even several minutes (Forrest, 1965; Polakis & Bartley, 1965) must be treated with caution. In the work reported here the actual delay between a cell leaving the culture and being immersed in perchloric acid was less than 0·2sec. Even so, 20% of the ATP content could possibly turn over in this time. However, the environment of the organism probably changes little in the short time between leaving the culture and immersion in the acid, so that changes in ATP content during the sampling procedure used here should be small. The fact that rapid small changes in ATP content were detected suggests that the results reported here are, in fact, meaningful.

Clearly the fact that the turnover rate of ATP is very high compared with the cell content of ATP means that the steady-state content of ATP cannot be used as an indication of cellular energy requirements. The so-called 'pool size' (Forrest, 1965) under these conditions merely represents the steady-state value to which ATP content is regulated, and cannot be regarded as a reserve. Thus the changes in ATP 'pool' size during the growth cycle reported by Cole, Wimpenny & Hughes (1967) probably correlate with changes in the regulatory system rather than in the ATP-production rate. Also, the low ATP content obtained under anaerobic conditions in this work is not, in itself, evidence that the energy supply became limiting.

To obtain useful information on the regulatory systems in growing cells it is necessary to follow transient changes in the content of metabolic intermediates and coenzymes rather than the steady-state values. This is the principle embodied in the crossover theorem (Chance *et al.* 1968), which has been invoked in this work to suggest that phosphofructokinase is a regulator of glycolysis.

With the exception of antifoam addition, which is perhaps a special case, the experiments reported here demonstrate a high degree of control by the cell over the ATP content. That is to say changes in respiration rate are not accompanied by large changes in ATP content. This may be brought about in one of two ways: respiration may not be obligatorily linked to ATP production, i.e. the P/O ratio is not constant, or the ATP-turnover rate may alter to offset changes in the ATP-production rate.

The results obtained on addition of glucose and

succinate to a glucose-limited culture are most simply interpreted in terms of an increased ATP turnover brought about by utilization of ATP for the synthesis of cell material. From this it is suggested that a high ATP/ADP concentration ratio may feed back to stimulate anabolic pathways and a low ATP/ADP ratio may favour catabolic pathways. There is good evidence that the latter applies to the control of glycolysis in yeast (Pye, 1965; Maitra & Estabrook, 1967).

The same explanation cannot so easily be applied in the case of the stimulation of respiration at low dissolved-oxygen tensions, where an increased rate of glucose oxidation was associated with an ATP content no greater than that in the aerobic steady state, as there was no excess of glucose available for increased synthetic activity. In fact, under these conditions a decrease in yield of cells from glucose is obtained (Harrison & Pirt, 1967). It may be possible to have an increased ATP-turnover rate that is not associated with an increase in synthetic processes but merely represents a 'wastage' of energy. Perhaps a more likely explanation in this case would be that, at low dissolved-oxygen tensions, an alternative pathway for electrons to oxygen functions, that by-passes one or more sites of oxidative phosphorylation, so causing a decreased ATP-production rate.

The rise in ATP content and in respiration rate observed on addition of antifoam is not explicable in terms of the controls suggested above. Antifoam may cause a breakdown in the control processes of the cell, possibly by altering the permeability properties of the membrane.

Control properties of growing bacteria would be expected to differ from those of washed starved bacterial suspensions. Therefore, the results reported here are not directly comparable with results reported previously for resting cells in which much wider variations in ATP content have been reported (Hempfling *et al.* 1967). Also, in resting cells ADP content may become limiting for respiration rate (Maitra & Estabrook, 1967), whereas in the experiments reported here with growing cells this did not appear to be the case, as increases in respiration rate are not accompanied by an increased ADP content.

For the sake of clarity, in the foregoing arguments only ATP has been considered as effecting control of glucose metabolism and respiration. However, ADP and AMP concentrations both reflect any changes in ATP concentration, as the adenylate kinase reaction is rapid, so that either ADP or AMP could equally well be considered as the regulator.

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