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Energy Requirement for Maintenance of the Transmembrane Potassium Gradient in *Klebsiella aerogenes* NCTC 418: A Continuous Culture Study

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Abstract. With a glucose-limited culture of *Klebsiella aerogenes*, growing at a fixed dilution rate ($0.4\,h^{-1}$), the specific respiration rate varied progressively as a function of the transmembrane K^+ gradient. The latter was varied by changing the input K^+ concentration and, under these conditions, the specific respiration rate was linearly related to the electrochemical potential of the K^+ gradient. Increasing or decreasing the transmembrane K^+ gradient in putatively potassium-limited cultures elicited marked changes in respiration rate consistent with the conclusion that the exceptionally high respiration rates expressed by fully glucose-sufficient potassium-limited cultures (i.e., values in excess of 25 mmol O_2/g dry weight organisms · h, at $D = 0.4\,h^{-1}$) are necessary to scavenge traces of K^+ from the environment and hence maintain an exceptionally high transmembrane K^+ gradient.

Key words: Potassium-limitation – Potassium gradient – *Klebsiella aerogenes* – Continuous culture.

One of the principal factors constraining the proliferation of living organisms in natural ecosystems must be the availability of essential nutrient substances. Therefore it is reasonable to suppose that, in the course of evolution, organisms will have been selected that are highly adapted, metabolically, to cope with these ubiquitous and extreme conditions. In this connection, it is clear that the capacity of organisms to scavenge traces of nutrient from their environment is not only important with respect to sustaining growth at a finite rate, but also in allowing them to compete effectively with other species for the limited supply of essential nutrient. Thus it is to be expected that a substantial part of the organisms' metabolic potential may be directed towards effecting the uptake of low concentrations of all those essential nutrients that will, from time to time, be limiting their rate of growth.

Recently, several possible mechanisms by which microorganisms may adapt to low-nutrient environments have been identified and discussed (Tempest and Neijssel, 1976, 1978; Neijssel and Tempest, 1979), and two aspects, in particular, have commanded attention; namely (i) the bioenergetic consequences of nutrient-limited microbial growth, and (ii) product formation as a consequence of growth limitation by nutrients other than the carbon source (i.e., "overflow" metabolism). With regards to the first of these, it is evident from thermodynamic considerations that much energy may be consumed in the uptake of low concentrations of some nutrients; particularly those (like K^+ and Mg^{2+}) that are subsequently concentrated within the cell in an unchanged state. Here, the energy required for the maintenance of a substantial transmembrane gradient, necessarily established under specific cation-limiting conditions, will be markedly influenced by the magnitude of that gradient, and by the intrinsic permeability of the membrane towards the substance that has to be accumulated. Indeed, it is possible that in some extreme cases growth rate may be limited not so much by the extracellular concentration of nutrient per se (i.e., by $\bar{s}$) but by the supply of energy needed to maintain the transmembrane gradient. In this paper, experiments are described which quantify the energetic requirements of growth of *Klebsiella aerogenes* NCTC 418 in potassium-limited environments, and which point to the substantial energetic losses associated with the maintenance of a large transmembrane K^+ gradient.

In the absence of rubidium, microbes seemingly have an absolute requirement for potassium (Tempest, 1969; Aiking and Tempest, 1977), and the concentration extant in the cytoplasm of growing cells is surprisingly high; that is, at least 50–100 mM for Gram-negative bacteria and 100–300 mM for Gram-positive bacteria (Tempest et al., 1966). Considering the

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relatively low concentrations of K^+ in most natural environments (less than 10 mM in sea-water, for example) and in many laboratory culture media (see Evans et al., 1970), one might expect that, even with cultures that are not overtly K^+-limited, the transmembrane K^+ gradient often will be substantial; hence, significant amounts of energy might be dissipated in maintaining this gradient. Moreover, since the potassium content of bacteria generally, if not invariably, varies with growth rate (Tempest, 1969), so too must the magnitude of the K^+ gradient; thus the associated maintenance energy losses also might vary with growth rate. Evidence consistent with this conclusion is included in this paper.

Materials and Methods

Organism. *Klebsiella aerogenes* NCTC 418 was maintained by monthly subculture on tryptic meat-digest agar slopes.

Growth Conditions. Organisms were grown in a 500 ml Porton-type chemostat (Herbert et al., 1965) in defined simple salts media as specified by Evans et al. (1970). The concentrations of the potassium source (KCl) and carbon source (glucose or glycerol) were varied, as indicated in the Results section, such as to maintain the steady state bacterial concentration at about 2.5 mg equivalent dry weight cells/ml. The pH of the culture was controlled, automatically, at 6.9 $\pm$ 0.1 by the addition of 4 M-NaOH, and the temperature was held at 35° C throughout. The culture was stirred mechanically with an impeller rotating at about 2,800 rev/min through which air was injected at a rate of 0.6 l/min. This ensured both good mixing of the culture and a high oxygen solution rate.

Substrate Analyses. Glucose in the medium and extracellular fluids was determined using the GLOX reagent of AB Kabi (Stockholm, Sweden) and glycerol by the enzymatic procedure described previously (Neijssel et al., 1975). The potassium concentration was determined by flame photometry as described by Aiking and Tempest (1976).

Other Measurements. The specific rate of oxygen consumption was calculated from the difference between the oxygen concentration in the influent and effluent air, the latter being determined by means of an oxygen analyser (Taylor Servomex Type OA 272; Crowborough, Sussex, England), and the steady state bacterial dry weight (measured gravimetrically; Herbert et al., 1971).

Results and Discussion

Assuming the free water content of a microbial cell to be about 4 times the weight of dry material (Schlegel, 1976), and that all the intracellular potassium is free in solution, then the concentration of K^+ within actively growing *Klebsiella aerogenes* organisms must be of the order of 50 – 100 mM. This value seemingly is independent of the nature of the growth limitation, but has been found to vary progressively with both growth rate and the osmolarity of the medium (Tempest et al., 1966; Tempest and Meers, 1968). It follows, therefore, that under many growth conditions organisms are obliged to maintain a substantial transmembrane potassium gradient which, presumably, requires the expenditure of energy. If this is so, then the actual concentration of potassium in the culture extracellular fluid ought to exert a detectable effect on the energetics of growth, as indicated by the respiration rate (q_{O_2} value) expressed at any particular growth rate. That this is indeed the case could be shown by varying the potassium content of medium used to supply a chemostat culture of *K. aerogenes* growing at a fixed dilution rate (Fig. 1A).

In this experiment, organisms were grown under fully aerobic conditions with glucose (5 g/l input) as the growth-limiting nutrient and the dilution rate set at $0.40 \pm 0.01\ h^{-1}$. The input potassium concentration was varied progressively from that which provided a substantial excess (10 mM) to 1 mM, which was barely sufficient to meet the requirements of a culture containing 2.5 g equivalent dry weight organisms/l. This provoked a progressive increase in the steady state rate of oxygen consumption from about 8.5 – 12 mmol/g dry weight organisms · h. At the lowest potassium input concentration (1 mM) the steady state extracellular K^+ concentration was less than 0.02 mM (the limit of measurement by flame photometry) and the bacterial concentration was close to that extent in potassium-limited (glucose-sufficient) cultures growing at an identical rate in media containing 1 mM K^+. Thus, in order to lower the extracellular K^+ concentration further, whilst maintaining the population density almost constant, the input K^+ concentration was held

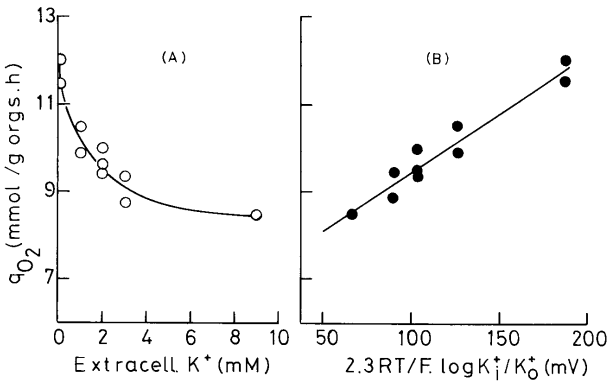
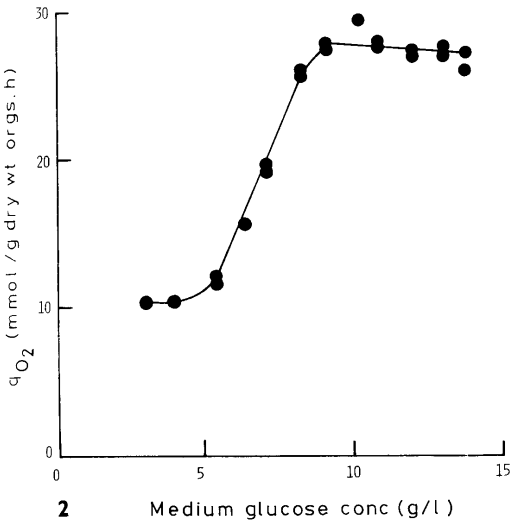


Fig. 1A. Relationship between the specific rate of oxygen uptake and the extracellular concentration of potassium extant in a glucose-limited culture of *Klebsiella aerogenes* growing at a dilution rate of $0.4\ h^{-1}$ (35° C; pH 6.9). At the lowest input K^+ concentration (1 mM) the extracellular K^+ concentration was close to the limit of measurement by flame photometry

Fig. 1B. Plot of the specific respiration rate as a function of the electrochemical potential of the K^+ gradient. Glucose-limited culture of *K. aerogenes* growing in the presence of a graded excess of K^+ from less than 0.05 – 9 mM. Over the range of extracellular K^+ concentrations studied the calculated electrochemical potential varied from 63 – 183 mV



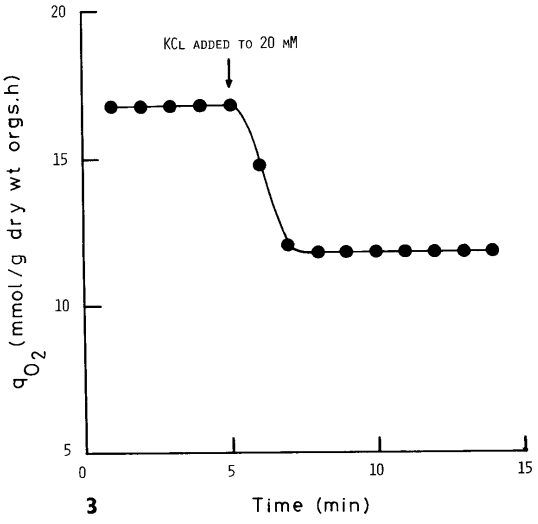


Fig. 2. Influence of increasing glucose input concentration on the respiration rate expressed by chemostat cultures of *K. aerogenes* growing in a medium containing 1 mM KCl. Other conditions as in Fig. 1. Over the range of input glucose concentrations studied, the extracellular glucose concentration was undetectably low; and below an input concentration of 8 g glucose/l, the sole products were cells and CO_2

Fig. 3. Effect of a sudden increase in the extracellular K^+ concentration on the specific rate of oxygen consumption by *K. aerogenes* cultures previously growing, in a steady state, in a putatively K^+-limited culture ($D = 0.4\,h^{-1}$; 35°C; pH 6.9; input glucose concentration of 7 g/l)

at 1 mM and the input glucose concentration progressively increased. As shown in Fig. 2, the specific rate of oxygen consumption then increased linearly up to a value of about 27 mmol/g dry weight organisms · h; the sole products being cells and CO_2 (see Hueting and Tempest, 1979). This steep increase in energy expenditure, with a concomitant decrease in the Y_O value from about 19 to about 7 (g cells formed/g atom oxygen consumed) correlated with a presumptive lowering of the extracellular potassium concentration, and with a corresponding increase in the transmembrane K^+ gradient.

Though, in this experiment, the actual level of extracellular potassium was beyond that which could be measured by techniques available to us, the proposition that it was the maintenance of a progressively increasing transmembrane K^+ gradient that provoked the increasing respiration rate could be tested by studying transient changes in respiration rate following a sudden lowering of this gradient. This was effected by transiently increasing the extracellular K^+ concentration. In this experiment, *K. aerogenes* was again grown at a dilution rate of $0.4\,h^{-1}$ in a medium containing 1 mM K^+ and 7 g glucose/l (i.e., the culture was essentially potassium-limited but the extracellular glucose concentration was very low). Once a steady state had been attained, the specific rate of oxygen consumption was monitored prior to, and immediately following injection into the culture of sufficient KCl solution to raise the extracellular K^+ concentration to 20 mM. A sharp and substantial decrease in respiratory activity was found (Fig. 3) which must have been due largely, if not entirely, to the lowering of the transmembrane K^+ gradient since all other conditions were maintained constant.

The large amount of energy apparently needed to take up low concentrations of potassium, and to maintain a high transmembrane K^+ gradient, presumably reflects, and must be exacerbated by, the fact that the membrane is rather permeable to potassium ions. This is clearly evident when cells of *K. aerogenes* are exposed to low osmotic environments; thus, washing the cells once in distilled water released 40% of the cell-bound potassium (Tempest et al., 1966). Moreover, K^+ may be rapidly lost from cells when cultures undergo transition from the exponential growth phase to the stationary phase (Zarlengo and Schultz, 1966). With glucose-limited cultures of *K. aerogenes* growing in a chemostat in the presence of a small excess of potassium, or vice versa, a sudden step-down to zero dilution rate was found to elicit an immediate efflux of K^+, thereby effecting a substantial rise in the culture pH value (due, presumably, to a concomitant uptake of protons; Harold, 1972). With a glucose-limited culture, this efflux of K^+, and associated rise in pH, was only observed when the extracellular K^+ concentration was less than 20 mM. In a typical experiment ($D = 0.4\,h^{-1}$; input glucose concentration of 7 g/l; input KCl con-

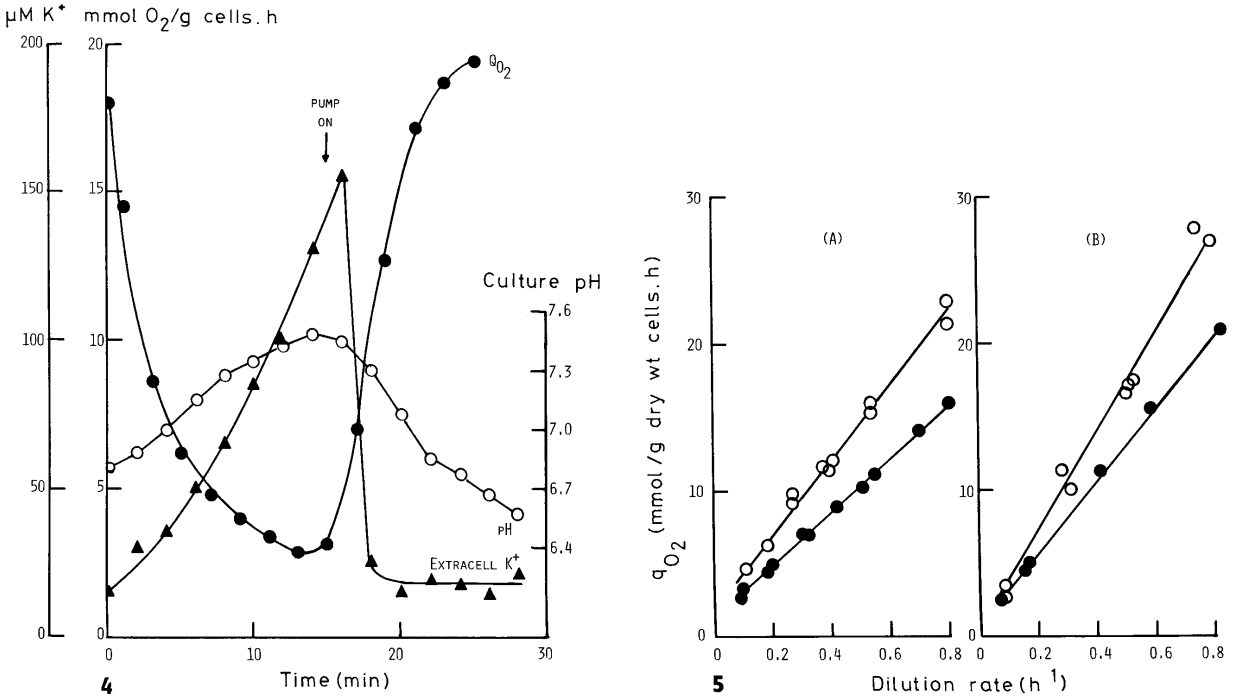


Fig. 4. Influence of a step-down to zero dilution rate on the respiration rate (●), culture pH value (○) and extracellular K^+ concentration (▲). Prior to turning off the medium supply, the automatic pH controller was disconnected. After 15 min, the pump was turned on and changes in respiration rate, pH and extracellular K^+ concentration followed for a further 15 min

Fig. 5. Specific rate of oxygen uptake of (**A**) glucose-limited and (**B**) glycerol-limited chemostat cultures of *K. aerogenes* growing in media containing (○) 1 mM KCl, and (●) 10 mM KCl. Respiration rate was determined at each of a number of steady state growth rates (35° C; pH 6.9)

centration of 1 mM), step-down to zero dilution rate caused the (uncontrolled) culture pH value to rise from 7.07 to 7.52 within 12 min, during which time the extracellular K^+ concentration rose from $<0.02-0.15$ mM (Fig. 4). After switching on the pump, there was an immediate increase in respiration rate and the culture pH value fell back towards its initial value as potassium ions were rapidly taken back into the cells.

Thus it can be concluded that, in order to maintain a transmembrane K^+ gradient, organisms need to expend energy which, presumably, is related quantitatively to the electrochemical potential of that gradient. This electrochemical potential can be calculated according to the Nernst equation:

$$\Delta\tilde{\mu}_{K^+} = \frac{2.303\,RT}{F}\log\frac{[K_i^+]}{[K_o^+]}$$

where K_i^+ and K_o^+ are the concentrations of K^+ inside and outside the cell, respectively, and the constants R, T and F have their usual meaning (activity coefficients are neglected). Here it can be seen that a logarithmic increase in the transmembrane K^+ gradient elicits a linear increase in the electrochemical potential ($\Delta\tilde{\mu}_{K^+}$). Moreover, an exponential increase in this gradient seemingly effected a linear increase in the specific respiration rate (q_{O_2}) since, with cultures growing at a fixed dilution rate, the latter was found to be directly proportional to the electrochemical potential of the K^+ gradient (Fig. 1B). Thus, a ten-fold increase in the transmembrane K^+ gradient evoked, and presumably must be supported by, an increase in the specific respiration rate of 1.7 mmol O_2/g · dry weight cells · h.

At this point, it is relevant to draw attention to the fact that the polarity of the electrochemical gradient of K^+ is opposite to that of protons. It follows, therefore, that an efflux of K^+ and influx of H^+, such as occurs following a step-down to zero dilution rate (Fig. 4) could, at least in theory, drive ATP synthesis. But to determine whether this does, in fact, occur would require a careful study to be made of the changes in the adenine nucleotide pool following transition to zero dilution rate (and anaerobiosis), and this has not been attempted. Nevertheless, it has been shown both with whole cells (Kashket and Wilson, 1972) and with bacterial membrane vesicles (Altendorf et al., 1974) that a flux of K^+, mediated by valinomycin, can generate a membrane potential, and that the efflux of K^+ can drive ATP synthesis in both mitochondria (Cockrell et al., 1967; Rossi and Azzone, 1970) and in bacterial membrane vesicle preparations (Tsuchiya and

Rosen, 1976). It seems possible, therefore, that ATP synthesis might accompany the efflux of K^+ that occasionally occurs from cells in growing cultures (e.g., during the transition from exponential phase to stationary phase in a batch culture).

As a consequence of the fact that the extracellular K^+ concentration markedly affects respiration rate, and hence the energy demands for growth at a fixed dilution rate, it is clear that the relationship that exists between respiration rate and growth rate also may be substantially affected by the ratio of carbon substrate to K^+ in the feed medium. Two examples are shown in Fig. 5. Here, *K. aerogenes* was cultured under putatively carbon substrate-limited conditions with an input concentration of either glucose or glycerol of 5 g/l and with 1 mM KCl. The specific rates of oxygen consumption were determined as functions of the dilution rate, and the data obtained compared with those of similarly-limited cultures growing in the presence of 10 mM KCl. Clearly, the low-potassium cultures expressed an increased respiration rate at all growth rates, the magnitude of which increased with growth rate. Again, from assessments of the residual K^+ in the extracellular fluids of the low-potassium cultures, and from a knowledge of the intracellular K^+ contents of the growing cells, the transmembrane potassium gradient could be calculated at each growth rate. Thus it could be shown that, with the low-potassium culture, the magnitude of the K^+ component of the electrochemical potential increased from about 60 mV (positive inside), at $D = 0.1\,h^{-1}$ to over 200 mV at $D = 0.8\,h^{-1}$. And when corrections are made to the expressed oxygen consumption rates, to take account of that increment of respiration rate associated with the maintenance of the K^+ gradient (i.e., 2.8 mmol O_2 consumed/g · dry weight organisms · h · 100 mV equivalent), values are obtained, at each growth rate, that correspond closely with those expressed by cultures growing in the presence of excess (10 mM) potassium.

Significantly, though glycerol-limited cultures expressed at each comparable growth rate, a higher respiration rate than glucose-limited cultures (cf. Neijssel and Tempest, 1975), the differences found between respiration rate in cultures growing with 1 mM K^+ and 10 mM K^+ were virtually identical for the different carbon substrate limitations. Thus, at a dilution rate of $0.4\,h^{-1}$, the differences were 3.4 and 3.2 mmol oxygen/g dry weight organisms · h, for glycerol- and glucose-limited cultures, respectively; at $D = 0.8\,h^{-1}$, the corresponding values were 6.8 and 6.5 (Fig. 5). Clearly, in the low-potassium environments the growth-unassociated energy losses (i.e., "maintenance" requirements) varied with growth rate by virtue of the fact that the transmembrane K^+ gradient varied. This supports the conclusion drawn previously by Neijssel and Tempest (1976) that, at least under some conditions, energy expenditure in growth-unassociated "maintenance" functions is not independent of the growth rate.

As found earlier with potassium-limited cultures of the yeast *Candida utilis* (Aiking and Tempest, 1976), transitions from conditions of K^+-limitation to glucose-limitation, and vice versa, is not a steep step function; just as with *K. aerogenes* cultures, there is a gradual transition during which both the Y_O and $Y_{glucose}$ values change progressively as the transmembrane K^+ gradient changes. The two situations are only different in that, with *K. aerogenes* cultures, the K^+ content of fully potassium-limited cells is closely similar to that of fully carbon substrate-limited (K^+-sufficient) cells (Tempest and Meers, 1968), whereas with *C. utilis* cultures they are very different, particularly at low dilution rates. Nevertheless, in *K. aerogenes* cultures, as in cultures of *C. utilis*, the transmembrane K^+ gradient varies substantially during this transition, and seemingly affects markedly respiration rate, and thereby the overall energetics of growth. Clearly, in steady state K^+-limited cultures, growing in the presence of suboptimal concentrations of carbon substrate, growth is essentially energy-limited and changes in the availability of carbon substrate serve, within limits, solely to change the transmembrane K^+ gradient. It follows, therefore, that under these conditions, the relationship between growth-limiting substrate concentration ($\bar{s}$) and growth rate (μ) is more complex than that specified by Michaelis-Menten-type kinetics (see Monod, 1942).

In eco-physiological terms, the value to the organism in not tightly regulating carbon substrate uptake and catabolism, when exposed to low-potassium environments, is abundantly obvious. The capacity to scavenge K^+ from the environment demands the expenditure of much energy since the transmembrane K^+ gradient necessarily must be maximized. Hence, when growing in such environments in the presence of other species, those that can express the highest respiration rate ought to succeed. The validity of this hypothesis is currently being tested.

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