MICROCALORIMETRIC MEASUREMENTS OF HEAT EVOLUTION AND THEIR CORRELATION WITH OXYGEN UPTAKE IN *ESCHERICHIA COLI* WITH GENOTYPICALLY- AND PHENOTYPICALLY-MODIFIED ELECTRON TRANSPORT CHAINS

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1. Introduction

The use of microcalorimetry for investigation of microbial metabolism is of considerable and growing interest [1]. Recently, Long et al. [2] described the heat evolution associated with active transport of non-metabolisable compounds by *Escherichia coli.* Previously, a direct correlation between heat evolution and oxygen uptake during growth of a variety of micro-organisms (including *E. coli*) had been reported [3]. This proportionality was independent of growth rate, but a dependence on growth substrate and type of organism was proposed. We have used a sensitive microcalorimeter to measure heat evolution following addition of fermentable and non-fermentable substrates to various strains of *E. coli,* possessing modified electron-transport chains. The results demonstrate a close correlation between heat evolution and the rate of oxygen uptake.

2. Materials and methods

All strains were derived from *E. coli* K12; the genotype and phenotypic modifications which characterize them are shown in table 1. Strain A1002 is a parental strain for mutants A103c and A1004a and was kindly supplied by Dr H. U. Schairer, Max Planck Institut für Biologie, Tübingen, W. Germany. In this strain and in glycerol-limited strain C-1, oxidation of NADH-linked substrates is probably accompanied by ATP synthesis at each of two sites. These are proposed to correspond to two functional protontranslocating segments of the respiratory chain, one between NADH and the junction of the flavin-linked dehydrogenases (Site I) and one between this junction and O_2 (Site II) [10].

All strains, except C-1, were grown in a mineral salts medium containing 0.5% glucose and supplemented with amino acids [11] in baffled shake flasks. Strain C-1 was grown in a similar medium, but containing 0.5% glycerol as carbon source and lacking amino acids. Growth of this strain was in a chemostat; glycerol-limited growth was obtained in the presence of 15 mM K_2SO_4 whereas sulphate-limited growth was achieved at an entering K_2SO_4 concentration of 50 μM [8]. Cells from the exponential phase of batch growth were harvested, washed twice in medium lacking glucose and amino acids, and were starved of endogenous substrates by shaking in the same medium for 2 h at 37°C. Cells were again collected by centrifugation and finally resuspended in the same medium. Sulphate-limited cells were harvested as described above and washed in the same medium but lacking K_2SO_4; they were finally resuspended to a fifth of the original volume harvested. This procedure removed residual glycerol from the chemostat effluent medium and brought the cell suspension to a concentration suitable for analysis. Glycerol-limited cells were used direct from the chemostat without further

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Table 1
Genotypic and phenotypic modifications of the respiratory chain in *Escherichia coli* K12

Cell type	Primary lesion	Presence (+) or absence (−) of respiration-driven proton-translocating oxidoreduction segments at: Site I	Site II	Postulated P/O ratio for NADH oxidation	References
A1002	none	+	+	2	[5]
A103c	ATPase	+	+	0	[5,6]
A1004a	cytochromes	(+)[a]	−	0	[7]
Glycerol-limited C-1	none	+	+	2	[8]
Sulphate-limited C-1	Fe/S proteins?	−	+	1	[8]

[a] Respiration-dependent proton translocation can be demonstrated in the cytochrome-independent span of the respiratory chain between NADH and added ubiquinone analogues [9]; it is unlikely that energy conservation is associated with this region in vivo.

treatment. The effluent medium from glycerol-limited cultures contained no detectable glycerol.

Heat evolution was measured using an LKB 10700-1 heat conduction flow microcalorimeter [12]. Cell suspension (3–6 ml), substrate (as indicated) and growth medium (lacking carbon source and amino acids) were aerated (total volume 30 ml) at 37°C. Final concentrations of substrate used were: glucose and glycerol (0.5%), L-malate (10 mM) and succinate and D-lactate (20 mM). Unless otherwise specified, the final cell concentration was 0.1–0.2 mg dry wt/ml. After 1 min, 5 ml of cell suspension were removed for determinations of oxygen uptake rate at 37°C [13] and cell concentration [14]. The remaining suspension was pumped at 60 ml/h to a T-piece where it was mixed with a constant flow of air (60 ml/h) and then to the flow cell of the calorimeter. Temperature of the thermostatic air bath was 37°C and the amplification range 30 or 100 μV. Calorimetric response was recorded on a potentiometric recorder (full scale deflection 100 mJ/min). The instrument was calibrated before each experiment by applying an internal current of 2.0, 3.0 or 4.0 mA for 16.7 min to achieve a steady state heat evolution. All reagents were from commercial sources and of the highest purity available.

3. Results and discussion

3.1. *Performance of the calorimeter*

The flow of aerated medium through the instrument established a stable baseline. After transferring the sampling tube from medium to the cell suspension, a lag of approx. 2 min preceded a calorimetric response (fig.1). A steady state level of heat evolution was established after a further 10–12 min. Addition of FCCP* (5–10 μg/g dry wt cells) resulted in a small stimulation of heat evolution and attainment of a second higher steady state. Replacement of the cell suspension by medium resulted in a slight irregularity in the trace (caused by entry of air) followed by a decreased calorimetric response to the baseline level. Heat evolution is the measured displacement of the steady-state level from the baseline. Measurement of cell concentrations of suspensions entering and leaving the flow vessel showed that measurable growth did not occur during the period in which heat evolution was measured. Calorimetric response (up to 60 mJ/min) was linearly dependent on cell concentration.

3.2. *Heat evolution and O_2 uptake in glucose-grown cells*

The following conclusions may be drawn from the results presented in table 2. (1) In the absence of added substrate, rates of oxygen uptake are low, particularly in strain A1004a. Heat evolution is also low but measurable (e.g. fig.1) for strains A1002 and A103c, but is too low for accurate measurements

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* *Abbreviations:* ATPase, Mg^{2+}-dependent proton-translocating adenosine triphosphatase activity (EC 3.6.1.3) [4]. FCCP, carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone.

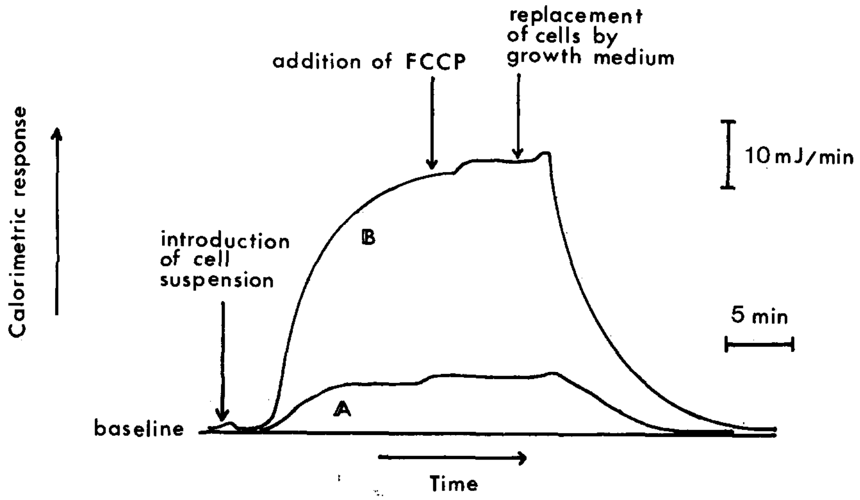

Fig.1. Typical recorder trace, showing rate of heat evolution associated with oxidation of endogenous substrates (A) and added succinate (B) by cells from a glycerol-limited chemostat. Cell concentrations in both experiments were 0.13 mg dry wt/ml. FCCP concentration was about 10 µg/mg dry wt cells.

with suspensions of strain A1004a containing up to 0.6 mg dry wt/ml. (2) Addition of either D-lactate or succinate stimulated rates of both oxygen uptake and heat evolution in all three strains. For either substrate the value of mJ heat evolved per ng atom O consumed is similar in all strains, despite the low oxidase activities of strain A1004a. Comparison of the values for strain A103c and for parental A1002 suggest that neither ATP hydrolysis nor ATP synthesis mediated by the Mg^{2+}-dependent ATPase contribute markedly to the observed heat evolution. (3) L-Malate is oxidized only slowly by glucose-grown cells and, therefore, was not used routinely as substrate. However, for strain A103c the heat evolution associated with malate oxidation is considerably higher than that measured during oxidation of other non-fermentable substrates by the various strains tested. (4) Oxygen uptake is rapid on adding glucose to a sus-

Table 2
Oxygen uptake and heat evolution of parental and respiratory-deficient mutant strains of *Escherichia coli*

	Strain A1002		Strain A103c		Strain A1004a	
Substrate	O_2 uptake (ng atom O/ min/mg dry wt)	mJ evolved / ng atom O	O_2 uptake (ng atom O/ min/mg dry wt)	mJ evolved / ng atom O	O_2 uptake (ng atom O/ min/mg dry wt)	mJ evolved / ng atom O
Endogenous	33.3 (40.6)	0.19 (0.16)	27.7 (20.9)	0.24 (0.33)	5.08 (5.33)	not measurable
D-Lactate	78.0 (85.2)	0.39 (0.43)	120 (126)	0.31 (0.31)	20.0 (16.2)	0.37 (0.51)
Succinate	119 (119)	0.27 (0.27)	124 (100)	0.28 (0.36)	11.33 (11.86)	0.29 (0.22)
L-Malate	n.t.	n.t.	28.7	0.56	n.t.	n.t.
Glucose	382 (316)	0.27 (0.30)	985 (913)	0.28 (0.29)	42.7 (44.1)	1.01 (0.96)

Cells were grown in batch culture with glucose as sole carbon source. Strain A1004a was grown in the absence of 5-aminolaevulinic acid; under these conditions no functional cytochromes are formed. After harvesting, washing and starving, rates of heat evolution and O_2 uptake were measured with cell suspensions oxidizing endogenous or added substrates as described in Materials and methods. Values of oxygen uptake rates and of heat evolved per unit of oxygen consumed refer to a single experiment, with results from another experiment given in parentheses to indicate variation of values between experiments. n.t., not tested.

pension of either strain A1002 or A103c, but is slow in the case of strain A1004a grown in the absence of 5-aminolaevulinic acid. The heat evolution per ng atom O consumed is similar in both A1002 and A103c but is 3.5-fold higher in strain A1004a. If glucose metabolism is assumed to be wholly oxidative, it may be calculated [15], from published values of ΔG for glucose oxidation [16], that the rate of oxygen uptake by the respiratorycompetent strain A1002 in the presence of glucose (349 ng atom O/min/mg) should be accompanied by heat evolution of approximately 840 mJ/min/mg dry wt. This value accounts for 86% of the measured heat evolution (980 mJ/min/mg dry wt). In contrast, the measured heat evolution by strain A1004a (grown in the absence of 5-aminolaevulinic acid and lacking detectable cytochromes) exceeds by a factor of 4 the theoretical amount and suggests that, in this case, glucose metabolism is largely fermentative.

The application of similar calculations to the few published results on heat evolution by other microbial systems shows that glucose metabolism in a variety of micro-organisms [3] and in the alga *Scenedesmus obtusiusculus* [15] is oxidative, but is largely fermentative in the yeast *Schizosaccharomyces pombe* [12]. Thus, combined polarographic and calorimetric measurements may be of value in the rapid assessment of the mode of glucose metabolism and perhaps in the initial recognition of respiratory-deficient strains.

In a control experiment, the measured rates of heat evolution (161 mJ/min/mg) and oxygen uptake (690 ng atoms O/min/mg) on adding glucose were higher for mutant A1004a grown in the presence of 5-aminolaevulinic acid, such that heat evolution per ng atom O consumed (0.23 mJ/ng atom O) was similar to the ratio determined for strains A1002 and A103c (table 2). Partial reconstitution of functional cytochromes was also achieved by incubating cells grown in the absence of 5-aminolaevulinic acid with 30 μM 5-aminolaevulinic acid, chloramphenicol (25 μg/ml) and glucose (0.5%) [7,13]. This resulted in a 4-fold increase in oxygen uptake rate and a less marked stimulation of heat evolution such that heat evolved with respect to the amount of oxygen consumed (0.29 mJ/ng atom O) was close to that observed for strain A1004a grown in the presence of 5-aminolaevulinic acid, and for strains A1002 and A103c.

3.3. *Heat evolution and oxygen uptake in glycerol-grown cells*

The heat evolution associated with substrate oxidation by chemostat-grown cells was only slightly stimulated by the addition of FCCP (5–10 μg/g dry wt cells; fig.1). A similar proportional increase was observed in the apparent rate of substrate oxidation. Higher concentrations of FCCP inhibited both heat evolution and oxygen uptake. Therefore, the addition of FCCP had no overall effect on the value for mJ heat evolved per ng atom O consumed presented above, though the small effect of FCCP on both the heat evolution and rate of substrate oxidation indicates the lack of significant respiratory control in these cells. This observation, together with data obtained with strain A103c, suggests that, under the experimental conditions described here, electron transport through the respiratory chain during substrate oxidation results in heat evolution rather than ATP synthesis.

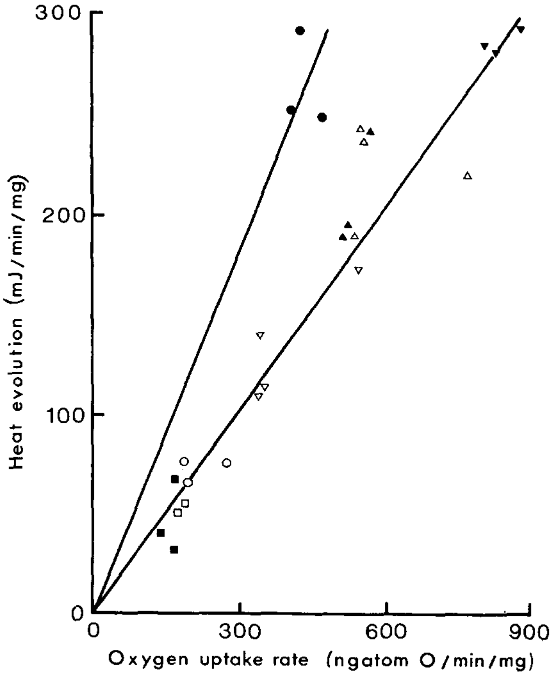

Fig.2. Rate of heat evolution as a function of oxygen uptake rate for cells grown in a chemostat with glycerol as sole carbon source, under conditions of glycerol-limitation (filled symbols) or sulphate-limitation (open symbols). Substrates used were endogenous (■, □), or added glycerol (▲, △), succinate (▼, ▽), or L-malate (●, ○).

For sulphate-limited cells, heat evolution was proportional to oxygen uptake rate, irrespective of the substrate (fig.2). The heat evolved per ng atom O consumed is represented by the slope of the fitted line (0.34 mJ/ng atom O). For glycerol-limited cells, similar ratios for heat evolution per ng atom O consumed were obtained for the oxidation of endogenous substrates and added glycerol or succinate, but the oxidation of L-malate was accompanied by relatively greater heat evolution, such that the data in figure 2 are fitted by a line of slope 0.60 mJ/ng atom O. It is of particular interest that the loss of Site I oxidative phosphorylation in sulphate-limited cells is accompanied by decreased heat evolution per ng atom O consumed. These results are in contrast to those expected if it is assumed that the inability to conserve energy in the form of ATP would result in the liberation of such energy as measurable heat. Whether the observed heat evolution is a direct consequence of the number of functional proton-translocating oxidoreduction segments of the respiratory chain or is a reflection of the increased contribution by non-oxidative (i.e. fermentative) metabolism by L-malate or its oxidation products remains to be determined.

From this study it is clear that microcalorimetry is a novel and potentially powerful technique in investigations of respiration and energy-linked biochemical reactions. We conclude that the measured heat evolution arises largely from substrate oxidation or, in the case of glucose metabolism by a cytochrome-deficient mutant, from reactions involved in fermentation. The ineffectiveness of FCCP in stimulating heat evolution and the use of an ATPase-deficient mutant shows that ATP synthesis or hydrolysis by the enzyme did not contribute markedly to the heat evolved. The similarity between the values of heat evolution per unit of oxygen consumed for endogenous substrates and for a variety of added substrates suggests that, in contrast to the results of Long et al. [2], active transport processes do not contribute markedly to the observed heat evolution in our experiments. Further work with membrane particles is required to eliminate other possible sources of heat evolution (e.g. non-oxidative turnover of cellular components) observed in intact cells.

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