APPENDIX

Growth Yields of *Torulopsis utilis* Grown in Continuous Culture with Glycerol or Iron as the Growth-Limiting Nutrient

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The extent of growth in a microbial culture, measured as dry weight of cellular material, has been found to be proportional to the quantity of carbohydrate added to the culture provided that this is the growth-limiting nutrient (Monod, 1942). Other growth conditions, for instance temperature (Senez, 1962) and growth rate (Herbert, 1958), must remain constant to preserve this proportionality. If a nutrient other than carbohydrate is growth-limiting, then the utilization of the carbohydrate energy source may be uncoupled, i.e. not associated with a proportional increase in dry-cell mass. Such uncoupled carbohydrate utilization may result in an increased rate of heat production or an intracellular accumulation of carbohydrate storage compounds (for reviews of these topics see Stouthamer, 1969; Payne, 1970).

The simplest mathematical treatments of the behaviour of chemostat cultures (e.g. that of Herbert, Elsworth & Telling, 1956) explicitly assume that a proportional relationship exists between the quantity of growth-limiting nutrient consumed, regardless of its chemical identity, and the steady-state culture cell density (x). Under most conditions, the entering concentration of growth-limiting substrate (S_0) is large compared with the concentration within the culture vessel (s). Thus the relationship $x \propto (S_0 - s)$ may be approximated to $x \propto S_0$, or

$$x = YS_0$$

The proportionality constant (Y) is conveniently expressed as the molar growth yield coefficient, whose dimensions are g dry cell mass produced/mol of substrate consumed (Bauchop & Elsdén, 1960). A subscript defines the substrate in question.

In this appendix we describe studies made of Y_{Fe} and $Y_{glycerol}$ for *Torulopsis utilis* grown in continuous culture under a variety of conditions of growth where the limiting nutrient was glycerol or iron. The results for Y_{Fe} are novel and describe for the first time the minimal amount of iron required by a cell for growth. Different values for $Y_{glycerol}$ provide indirect evidence, although *in*

vivo, for the changes in efficiency of mitochondrial energy conservation caused by iron deficiency (Light, Ragan, Clegg & Garland, 1968; Clegg & Garland, 1971).

RESULTS AND DISCUSSION

Relationship between culture growth and iron utilization. As shown in the main paper (Clegg & Garland, 1971) Y_{Fe} was constant in iron-limited cultures, having a mean value of $8.9 \times 10^6 \pm 0.4 \times 10^6$ (s.d. for 24 observations).

When iron was not the growth-limiting nutrient, its utilization was approximately proportional to its entering concentration and was unrelated to the culture cell density, i.e. Y_{Fe} was not constant under iron-non-limiting growth conditions. Results in the main paper (Clegg & Garland, 1971) show that concentrations of haem and non-haem iron in iron-non-limited cells and their mitochondria varied markedly with the entering iron concentration.

When the growth-yield coefficient of a component of the medium that becomes a constituent of cells (i.e., any component of the medium other than the energy source) is found to be constant, this implies a constancy of composition of these cells with respect to this nutrient or the sum of its metabolic derivatives. We have found that intact cells, and mitochondria prepared from them, produced under iron-limited growth conditions, always contained constant quantities of haem and non-haem iron (per mg of dry cells or per mg of mitochondrial protein) regardless of the absolute value of the entering iron concentration.

Relationship between culture growth and glycerol utilization. Glycerol utilization was measured over a period of 6 months in chemostat cultures whose growth was iron-limited, i.e. the cell density in the culture was determined not by the entering glycerol concentration but by the entering iron concentration. The mean value of $Y_{glycerol}$ obtained was 39.8 ± 4.6 (30), measured at a wide variety of cell densities and entering glycerol concentrations. In such iron-limited cultures then there was no

uncoupled degradation of carbon source of the kind that has been observed in the stationary phase of batch cultures (Dawes & Ribbons, 1964).

Similar measurements were performed on chemostat cultures whose growth was limited by glycerol concentrations. Y_{glycerol} was constant over a range of entering glycerol concentrations and hence (since these cultures were glycerol-limited) cell densities. The mean value of Y_{glycerol} obtained was 53.8 ± 1.5 (15).

It has been demonstrated (Clegg & Garland, 1971) that this increase in Y_{glycerol} may be induced in an

initially iron-limited culture within 24 h of increasing the input iron concentration to a value at which it is no longer growth-limiting. This time-course is incompatible with the possibility of the change being genotypic in origin. As illustrated in Fig. 1, a downward transition in the value of Y_{glycerol} may, as anticipated, be produced in an initially glycerol-limited culture (if the concentration of iron is only just supra-limiting) by increasing the glycerol concentration in the ingoing medium to a point where the iron concentration becomes growth-limiting.

There are several possibilities for the mechanism whereby Y_{glycerol} decreased when growth was iron-limited. The most likely was the loss of site 1 energy conservation, since this was demonstrable in isolated mitochondria (Clegg & Garland, 1971). A lowered value of Y_{glycerol} could also occur if iron limitation altered metabolic pathways resulting, for example, in the incomplete oxidation of that glycerol not used biosynthetically. This possibility was readily open to investigation by following the overall metabolic fate of radioactive glycerol introduced into the medium and these studies are described below.

Carbon-balance studies of iron- and glycerol-limited T. utilis cultures. Uniformly labelled [^{14}C]glycerol was added to 15 litres of medium to a final concentration of approx. $3 \mu\text{Ci/litre}$. Growth was iron-limited initially. When measurements (shown in Table 1) had been made under these conditions, iron was added to the medium to a concentration such that growth became glycerol-limited and, when a steady state had been achieved, the measurements taken during iron-limited growth were repeated. The results of this experiment are summarized in Table 1 and show that, apart from the different proportions of glycerol either oxidized to CO_2 or incorporated into cellular material, the overall metabolic balance was unaffected by iron limitation. The nature of the water-soluble extracellular radioactive material in the culture is not

Fig. 1. Transition from glycerol-limited to iron-limited growth in a *T. utilis* chemostat culture. The culture was glycerol limited on days 1 to 3. The input glycerol concentration was increased, at the time indicated by the arrow, from 166 mm to 338 mm. The entering iron concentration was $0.86 \mu\text{M}$ throughout the experiment. The average Y_{glycerol} before transition was 52, and after transition, 32. $\blacktriangle$, Cell density in culture; $\bullet$, concn. of glycerol in culture; $\circ$, glycerol consumed by culture.

Table 1. Carbon balance of iron-limited and glycerol-limited *T. utilis* chemostat cultures

Uniformly labelled [^{14}C]glycerol was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Growth-limiting nutrient	...	Glycerol	Iron
Culture density (g of dry cell mass/l)		3.7	3.0
Input glycerol concn. (mm)		70	70
Unused glycerol concn. remaining in culture (mm)		0	1
Y_{glycerol} (g dry cell mass/mol of glycerol consumed)		53	44
Quantity of total input ^{14}C assimilated into cellular material (%)		72	60.3
Quantity of total input ^{14}C in spent medium (%)		5.7	8.8
Quantity of total input ^{14}C evolved as CO_2 (%; derived by difference)		22.3	30.9
Quantity of total input ^{14}C assimilated per g of dry cell mass (%)		19.5	20
Elemental carbon content of cells (as % of dry wt.)		48.3	49.5
Y_{CO_2} (g of dry cell mass produced/mol of CO_2 evolved)		79	46

Table 2. *Calculated values of Y_{oxygen} and Y_{ATP} for iron-limited and glycerol-limited *T. utilis* chemostat cultures*

In the calculation of these values a correction has been made for the presence of non-cellular carbon compounds other than CO₂ in both types of culture, by assuming that their production entailed neither the production nor the consumption of energy. Y_{ATP} (column 1) is calculated from Y_{oxygen} by assuming P/O ratios of 3.0 and 2.0 for glycerol-limited and iron-limited cells respectively. Y_{ATP} (column 2) is calculated from Y_{oxygen} by assuming P/O ratios of 1.6 and 1.1 for glycerol-limited and iron-limited cells respectively. Y_{ATP} (column 3) is calculated directly from the carbon-balance data as described in the text.

Method used to calculate Y_{oxygen}	Growth-limiting nutrient	Y_{oxygen} (g of dry cell mass/ g-atom of O ₂ consumed)	Y_{ATP} (g of dry cell mass/mol of ATP derived from catabolism)		
			(1)	(2)	(3)
Carbon-balance data applied to the formula of Johnson (1964)	Glycerol	23.8	7.9	14.9	—
	Iron	15.0	7.5	13.6	—
Carbon-balance data and the assumption of Von Meyenburg (1969) about the degree of oxidation of cellular carbon	Glycerol	20.8	6.9	13.0	7.7
	Iron	14.5	7.25	13.2	6.2

known, but its amount was similar during both iron- and glycerol-limitation.

Calculations of Y_{ATP} . Values of Y_{ATP} (g dry cell mass produced/mol of ATP generated by catabolism of energy sources) may be calculated from the theoretical ATP yield from complete substrate oxidation (Kormančiková, Kováč & Vidová, 1969). The extent of complete substrate oxidation may be estimated from values of Y_{CO_2} . In addition, since glycerol serves as both carbon and energy source, some ATP is derived from partial oxidation of substrate carbon atoms incorporated into the cells themselves. Our results provide no direct measure of the degree of oxidation of cellular carbon; we have assumed that two-thirds of the cell carbon is derived from pyruvate (Von Meyenburg, 1969) and the remaining one-third undergoes no energy-yielding transformations before biosynthetic usage. Y_{ATP} values of iron-limited and glycerol-limited cells calculated by this method are shown in column (3) of Table 2.

Columns (1) and (2) in Table 2 list values of Y_{ATP} derived by dividing calculated values of Y_{oxygen} (g dry cell mass produced/g-atom of oxygen consumed) by various estimates of the P/O ratio of intact glycerol-limited and iron-limited cells. Hernandez & Johnson (1967) have assumed a P/O ratio of 3 for *T. utilis* cells; Von Meyenburg (1969) has calculated the more realistic value 1.6. In both cases, iron-limited cells are assumed to phosphorylate with two-thirds the efficiency of glycerol-limited cells. The Y_{oxygen} values calculated for glycerol-limited cells are in good agreement with the value of 22.4 measured by Button & Garver (1966) for oxygen-limited growth of *T. utilis*.

The similarity of Y_{ATP} values, calculated by any one method, in iron-limited and glycerol-limited

cells implies that the observed difference in Y_{glycerol} value between them is adequately accounted for by an oxidative-phosphorylation efficiency of iron-limited cells which is only two-thirds that of glycerol-limited cells.

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