Carbon Dioxide Fixation by Baker's Yeast in a Variety of Growth Conditions

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Fixation of CO₂ by *Saccharomyces cerevisiae* growing under ¹⁴CO₂ in a chemostat was investigated. Under anaerobic conditions, CO₂ provided 6.5 ± 1 % of the total carbon of yeast grown on glucose and 1.6 % of the total carbon of yeast grown on glucose plus excess aspartate. Under aerobic conditions, 2.6 % of the yeast carbon was derived from exogenous CO₂ during growth on glucose or glycerol, and 3.3 % during growth on pyruvate or ethanol.

The distribution of the fixed carbon among chemical components of the yeast, including some individual amino acids, was determined. Equilibration of CO₂ across the cell membrane was probably not quite complete. Under anaerobic conditions, the similar molar radioactivities of aspartate and glutamate indicated that oxaloacetate was not metabolically compartmented. The unequal labelling of aspartate and glutamate during aerobic growth was consistent with operation of the glyoxylate bypass and/or compartmentation of oxaloacetate. Increased CO₂ fixation and labelling of carbohydrate during growth on pyruvate or ethanol are ascribed to the activity of phosphoenolpyruvate carboxykinase.

INTRODUCTION

Rockwell & Highberger (1927) showed that *Saccharomyces cerevisiae* requires CO₂ in order to grow. Several essential biosynthetic steps are now known that incorporate CO₂ (or HCO₃⁻) into cell constituents, such as amino acids and nucleotides, while other reactions, such as the formation of malonyl-CoA via acetyl-CoA carboxylase, involve CO₂, although they do not lead to its permanent fixation. However, there are few experimental results on the extent of utilization of CO₂ by growing yeast. Apparently, growing *S. cerevisiae* and *Candida utilis* can obtain 4.8 % and 3.5 %, respectively, of their total cell carbon from CO₂ (Liener & Buchanan, 1951). Sorokin (1965) reported a smaller value, 1.8 %, for *Candida utilis*. Oura (1974a) has calculated that, in theory, 6.3 % of the carbon of a yeast of average composition is derived from CO₂ when all oxaloacetate regeneration proceeds through pyruvate carboxylase, but only 1.3 % when the glyoxylate bypass fully replaces the pyruvate carboxylase reaction. The present study is a quantitative investigation of the fixation of CO₂ by baker's yeast during growth on different carbon sources under aerobic or anaerobic conditions.

METHODS

Organism and growth conditions. A baker's yeast, *Saccharomyces cerevisiae* strain no. 306 from the collection of our laboratories, was used. The yeast was cultivated continuously at a maintained pH of 4.5, 28 °C and a dilution rate of 0.1 h⁻¹ in a 2.7 l Biotec fermenter, type FL103 (LKB, Sweden) with the carbon source as the growth-limiting factor. Except for some special arrangements described below, the

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culture conditions and the composition of the synthetic medium were as described by Oura (1974*b*). The carbon sources were 5% (w/v) glucose, 5% glucose plus 0.5% aspartate, 2% glycerol, 5% pyruvic acid, or 2.5% ethanol. To obtain a satisfactory growth rate with glycerol, the medium was supplemented with an ethanolic extract of dried egg yolk (the part of 0.3 g dried egg yolk extracted with 3 ml hot ethanol was added to 1 l medium: personal communication from Dr C. Gancedo, Madrid, Spain). Pyruvic acid was purified by vacuum distillation and neutralized with KOH. The growth medium was gassed with air (aerobic cultivations) or O₂-free N₂ (anaerobic cultivations) at 70 l h⁻¹ through a filter. A solution containing 50 mM-KHCO₃ in 1 M-KOH, with or without 12.5 mCi NaH¹⁴CO₃ l⁻¹, was continuously added at 7 ml h⁻¹. The culture vessel, medium, pH adjusting solution and an anti-foaming device were sterilized in steam at 120 °C for 30 min, and the bicarbonate solutions were sterilized using a Millipore filter.

Labelling of yeast with ¹⁴CO₂. When the dry matter content of the outflowing culture became constant, the unlabelled bicarbonate solution was replaced by the labelled solution. Exhaust gas and outflowing medium were led separately into NaOH solution. Yeast in the fermenter was harvested by centrifugation and washed with water 50 h after starting the input of ¹⁴CO₂. The spent medium and lyophilized yeast were stored at -18 °C until analysed.

Determination of the radioactivities of evolved CO₂ and yeast carbon. Several (up to 50) 100 ml samples of exhaust gas were analysed with an Orsat gas analyser. The radioactivity of the CO₂ absorbed by the NaOH solution of the analyser was determined by liquid scintillation, using 50 to 250 µl samples, 5 ml methanol and 10 ml Insta-Gel (Packard Instrument Co., Downers Grove, Ill., U.S.A.). An internal standard of NaH¹⁴CO₃ was used.

Acid-stable total carbon and ¹⁴C contents of the yeast were determined after hydrolysis of samples containing 1 mg dry wt of yeast ml⁻¹ in 3 M-HCl at 110 °C for 4 h. Portions of the hydrolysate were analysed for total carbon and nitrogen with a CHN Analyser (Model 1104; Carlo Erba, Milan, Italy) or evaporated to dryness, dissolved in water, and counted by liquid scintillation in the presence or absence of DL-[1-¹⁴C]-glutamic acid internal standard.

Chemical fractionation of the yeast. Lipids were extracted from 500 mg dry wt of lyophilized yeast with chloroform/methanol (2:1, v/v) (Folch *et al.*, 1957) and their radioactivity was determined after evaporation of the solvent. The lipid-free yeast was then hydrolysed and fractionated into a cationic fraction (amino acids, and adenine, guanosine, cytosine and their derivatives), an anionic fraction (uracil, thymine and their derivatives, and non-amino organic acids) and a neutral fraction (mainly carbohydrate). Samples of 25 mg dry wt of lipid-free yeast were hydrolysed in 5 ml 3 M-HCl for 5 h at 110 °C in a sealed glass tube. The HCl was then evaporated and a slurry containing 50 ml Dowex 50W-X8 (H⁺ form) cation exchange resin in 50 ml water was added, and the pH was adjusted to about 2 with HCl. Anionic and neutral compounds were removed by four washes with 100 ml water, and cationic compounds were then eluted with three lots of 75 ml 1 M-NH₄OH. Anionic compounds were adsorbed from the water washes by addition of 50 ml Dowex 1-X8 (formate form) anion exchange resin suspended in 50 ml water. The pH was adjusted to about 10 with NH₄OH. Neutral compounds were removed by four washes with 100 ml water, and anionic compounds then eluted with four lots of 100 ml 6 M-formic acid. The radioactivity of these fractions was determined after concentration and removal of NH₃ and formic acid by evaporation.

Determination of the molar radioactivities of amino acids. A portion of the cationic fraction corresponding to about 7.5 mg dry wt of yeast was further hydrolysed in 6 M-HCl for 24 h at 110 °C, and then dried *in vacuo* and dissolved in water. The amino acid composition of one portion was determined with a Beckman Spinco 120B Amino Acid Analyser (Beckman Instruments, Palo Alto, Calif., U.S.A.) (Spackman *et al.*, 1958). A second portion, containing not more than 250 nmol amino acids, was fractionated by high voltage electrophoresis (Ryle *et al.*, 1955): after a first run at pH 6.5 (5 min at 1 kV, and 45 min at 3 kV) on Whatman 3MM paper, the zone of neutral amino acids was cut off, and sown to a second sheet of paper for electrophoresis at pH 1.9 (40 min at 3 kV). Amino acid zones, identified by comparison with standard and duplicate samples run in parallel and stained with ninhydrin, were cut out, and the acids were eluted at pH 6.5, dried *in vacuo*, dissolved in water and their radioactivity was determined.

Radioactivity measurements. Unless otherwise stated, samples were suspended in Insta-Gel and counted with a Packard Tri-Carb 2420 liquid scintillation spectrometer.

Other determinations. Carbohydrate (Pfäffli & Suomalainen, 1960), RNA (Mejbaum, 1939), DNA (Burton, 1956) and lipid (Folch *et al.*, 1957) were determined by standard methods. Total protein was taken to be the difference between the nucleic acids and 6.25 times the total nitrogen. Phosphoenolpyruvate carboxykinase (EC 4.1.1.49), pyruvate carboxylase (EC 6.4.1.1) (Haarasilta & Oura, 1975), malate dehydrogenase (EC 1.1.1.37) (Ochoa, 1955) and isocitrate lyase (EC 4.1.3.1) (Dixon & Kornberg, 1959) were determined by the methods indicated in cell-free extracts prepared (Haarasilta & Oura, 1975) from samples taken on at east two different days from steady-state cultures. The activities of enzymes are expressed per mg protein in the cell-free extracts determined by the Lowry method.

Fig. 1. The acid-stable ^{14}C activity of the culture suspension after exposure to $^{14}\text{CO}_2$. Yeast was cultivated aerobically on 5% glucose, 0.2 ml samples from outflowing culture suspension were added to 5 ml 3 M-HCl and hydrolysed in sealed glass tubes at 110°C for 4 h. ^{14}C activity was estimated after dissolving the evaporated samples in water.

Chemicals. DL-[1- ^{14}C]Glutamic acid (29 mCi mmol $^{-1}$; 1.07 GBq mmol $^{-1}$) and $\text{NaH}^{14}\text{CO}_3$ (59 mCi mmol $^{-1}$; 2.18 GBq mmol $^{-1}$) were from The Radiochemical Centre, Amersham. Other reagents were from Boehringer, Fluka, Merck and Sigma.

RESULTS

The carbon contents of the yeast grown under anaerobic and aerobic conditions with different substrates (see Table 1) were between 44.5 and 45.6% of their dry weights. Their contents of carbohydrate and protein are given in Table 1. RNA varied from 7.4 to 9.4% of dry weight, DNA from 0.4 to 0.6%, and lipid from 2.5 to 4.5%.

The acid-stable radioactivity of all the cultures became constant about 30 h after the start of the labelled bicarbonate feed (e.g. Fig. 1). To ensure that the label was distributed according to steady-state conditions, cultivations were continued for another 20 h before harvesting the yeast. The amounts of CO_2 fixed in the yeast and the distribution of label among various chemical fractions of the yeast are shown in Table 1. The values given are from individual steady-state cultures. The radioactivities of the evolved CO_2 and of the yeast [as d.p.m. (mg dry wt) $^{-1}$] were reproducible to $\pm 7\%$. The distribution of label among the chemical fractions of yeast grown aerobically on glucose or ethanol in two pilot experiments (not shown) agreed with the values in Table 1 within $\pm 10\%$ (i.e. ± 8 at 80%, and ± 0.8 at 8%). In calculating the amount of yeast carbon derived from CO_2 , it has been assumed that there is no isotope effect between ^{12}C and ^{14}C (see O'Leary, 1976) and that the molar radioactivity of the cellular CO_2 pool available for carboxylation reactions is equal to that of the evolved CO_2 in the exhaust gas. This second assumption is discussed later.

Under all growth conditions very little label was incorporated into lipids and only during growth on ethanol and pyruvate was a significant amount of label found in the non-lipid neutral fraction, which presumably consists mainly of carbohydrate. The anionic fraction contained 11 to 19% of the fixed CO_2 . The bulk of the fixed radioactivity (70 to 90%) was always found in the cationic fraction. On the basis of their pK values, amino acids and adenine, guanosine, cytosine and their derivatives are expected to be in this fraction. Aspartate and glutamate together accounted for 30 to 50% of the total radioactivity found in amino acids. The molar radioactivities of six amino acids are shown in Table 2. The values have been normalized to the same molar radioactivity (683 d.p.m. μmol^{-1}) of evolved CO_2 by multiplying the observed radioactivity of the amino acids by 683 divided by the observed molar radioactivity of evolved CO_2 from column 4 of Table 1). It is notable that the molar radioactivity of threonine differed from that of its precursor, aspartate, by only 3 to

Table 1. *The amount of CO₂ fixed in yeast grown under different conditions and its distribution among different chemical fractions of the yeast*

Yeast grown under ¹⁴CO₂ (input radioactivity: 555 d.p.m. nmol⁻¹) in a chemostat either anaerobically or aerobically with the carbon sources indicated was fractionated and analysed as described in Methods. The proportion of yeast carbon derived from CO₂ was calculated by dividing the results in column 5 by those in column 4. The distribution of ¹⁴C among different chemical fractions and the acid-stable ¹⁴C content of the medium are both expressed as percentages of the total amount of ¹⁴C in yeast.

Growth conditions	Chemical composition of yeast		Radio-activity of evolved CO ₂ (d.p.m. μmol ⁻¹)	Radio-activity of yeast carbon (d.p.m. μmol ⁻¹)	Proportion of yeast carbon derived from CO ₂ (%)	Distribution of radioactivity among fractions (%)				Acid stable ¹⁴ C in spent medium (%)	
	Protein (% dry wt)	Carbo-hydrate (% dry wt)				Cationic	Neutral		Lipid		
							Anionic				
Anaerobic											
Glucose	42	36	683	37.8	5.5	90	11	0.1	0.5	28	
Glucose + Aspartate	48	30	840	13.4	1.6	86	13	0.2	0.8	10	
Aerobic											
Glucose	46	36	632	16.6	2.6	86	14	0.2	< 0.1	1.5	
Glycerol	42	32	1240	30.5	2.5	85	13	0.3	1.5	1.9	
Pyruvate	50	27	604	19.5	3.2	75	17	0.3	8	6.7	
Ethanol	48	21	1640	55.1	3.4	70	19	0.3	11	1.6	

Table 2. *Molar radioactivities of six amino acids and activities of four enzymes in yeast grown under different conditions*

The yeast cultures were the same as in Table 1. Amino acids and enzymes were isolated and assayed as described in Methods. The molar radioactivities of the amino acids (d.p.m. μmol^{-1}) are the results of single determinations and are normalized to the same molar radioactivity (683 d.p.m. μmol^{-1}) of evolved CO_2 , as described in the text. Enzyme activities [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$] are means of at least two determinations.

	Growth conditions					
	Anaerobic		Aerobic			
	Glucose	Glucose + Aspartate	Glucose	Glycerol	Pyruvate	Ethanol
Amino acid						
Aspartate	498	118	309	244	467	ND
Threonine	482	107	330	218	441	ND
Glutamate	564	128	195	148	336	ND
Proline	523	124	213	127	336	ND
Glycine	24	10	40	52	320	ND
Alanine	24	12	19	31	227	ND
Enzyme						
Pyruvate carboxylase	116	19	56	32	33	42
Isocitrate lyase	0.9	0.3	11	86	126	302
Phosphoenolpyruvate carboxykinase	< 0.1	< 0.1	0.1	0.9	4.2	7.3
Malate dehydrogenase	1200	1100	3300	4300	5700	7100

ND, Not determined.

12% over a nearly fivefold range of molar radioactivities. Similarly, the molar radioactivity of proline was always equal to that of its precursor, glutamate. These identities are evidence that the observed labelling patterns reflect steady-state conditions.

The amount of acid-stable radioactivity found in the medium was rather small under aerobic conditions (except after growth on pyruvate) but was 28% and 10%, respectively, of that found in the cells during anaerobic growth on glucose or glucose plus aspartate. The chemical nature of this extracellular fixed CO_2 was not investigated in the present work.

The amounts of pyruvate carboxylase and phosphoenolpyruvate carboxykinase, isocitrate lyase (as a marker for the glyoxylate bypass) and malate dehydrogenase determined by *in vitro* assays of the yeast samples are shown in Table 2.

DISCUSSION

The proportion of yeast carbon derived from CO_2 was largest during anaerobic growth on glucose alone being then 5.5% (Table 1). The calculation in Table 1 assumes that the molar radioactivity of the cellular pool of CO_2 was the same as that of the CO_2 in the exhaust gas, whereas it may have been only 73% of this, as was that of aspartate (see below). If so, the proportion of yeast carbon derived from CO_2 would be higher by a factor of 1.35, i.e. it may be as large as 7.4%. Thus, $6.5 \pm 1\%$ of the yeast carbon was derived from CO_2 . Liener & Buchanan (1951) found 4.8% during batch growth of *S. cerevisiae*. Oura (1974a) obtained a theoretical value of 6.3% for a model yeast containing a similar proportion of protein (39%) to the present yeast (about 42%).

The smallest proportion of yeast carbon derived from CO_2 was 1.6%, during anaerobic growth on glucose plus aspartate (Table 1). This is not much more than the theoretical value of 1.3% for growth in the absence of oxaloacetate synthesis via pyruvate carboxylase (Oura, 1974a). The amount of pyruvate carboxylase in this yeast was less than under any other condition (Table 2). However, the molar radioactivity of aspartate was still 17% of

that of the evolved CO_2 so that aspartate continued to be synthesized via pyruvate carboxylase even when excess aspartate was available (the steady-state concentration of aspartate in the medium was 0.2%).

During aerobic growth on glucose both CO_2 fixation and pyruvate carboxylase activity were about half the values obtained during anaerobic growth (Tables 1 and 2). The aerobic yeast contained isocitrate lyase (Table 2) so that the glyoxylate bypass could lessen the need for oxaloacetate formation via pyruvate carboxylase. The proportion of yeast carbon derived from CO_2 during growth on glycerol was similar to that during aerobic growth on glucose. Yeast grown on pyruvate or ethanol contained larger amounts of radioactivity including considerable amounts in carbohydrate. Both observations probably reflect the appearance of phosphoenolpyruvate carboxykinase in these yeasts (see below). The CO_2 requirement for purine and pyrimidine synthesis accounts for only a small amount (about 0.8% of the total yeast carbon; E. Oura, unpublished results) of CO_2 fixation, so that the variation in RNA plus DNA content of the different yeasts does not cause a significant variation in CO_2 fixation. However, the decreased proportion of carbohydrate and increased proportion of protein is partly responsible for the relatively high proportion of yeast carbon derived from CO_2 during growth on pyruvate and ethanol compared with aerobic growth on glucose and glycerol.

Pathways of CO_2 fixation during anaerobic growth. The distribution of label from $^{14}\text{CO}_2$ among the amino acids from yeast grown in steady-state conditions throws some light on the actual metabolic pathways operating *in vivo*. Pyruvate carboxylase introduces labelled carbon from $^{14}\text{CO}_2$ into the C-4 position of oxaloacetate. This label is transferred by citrate synthase, aconitase and isocitrate dehydrogenase to the C-1 position of 2-oxoglutarate (and glutamate). If the complete tricarboxylic acid cycle is functioning, the label will then be lost during decarboxylation of 2-oxoglutarate, so that unlabelled oxaloacetate will be formed and will dilute the radioactivity of the oxaloacetate pool, unless there are separate metabolic pools of this compound. During anaerobic growth on glucose alone, the molar radioactivity of aspartate, and therefore of its precursor, oxaloacetate, was 73% of the molar radioactivity of CO_2 in the exhaust gas (Table 1). The tricarboxylic acid cycle functions only slowly, if at all, under these conditions, because succinate dehydrogenase and 2-oxoglutarate dehydrogenase are virtually absent (Chapman & Bartley, 1968; Machado *et al.*, 1975). It follows that the molar radioactivity of the CO_2 pool available to pyruvate carboxylase was probably only 73% of that of the evolved CO_2 , so that equilibration of CO_2 across the cell membrane only reached 73%. This is perhaps not surprising, since the amount of unlabelled CO_2 produced inside the cells was 900-fold greater than the supply of $^{14}\text{CO}_2$.

The molar radioactivities of the glutamate and aspartate families of amino acids were close during anaerobic growth on glucose, and both fell by 80% when exogenous aspartate was supplied. Under these conditions, the oxaloacetate available for glutamate synthesis via citrate synthase must, therefore, be the same oxaloacetate available to pyruvate carboxylase and transaminase, which are both cytosolic enzymes in yeast (Haarasilta & Taskinen, 1977, and unpublished results). Furthermore, the activity of transaminase is evidently sufficient to equilibrate the carbon skeletons of aspartate and oxaloacetate. The location of citrate synthase in anaerobic yeast is not certain but under some conditions it may be partly in the cytosol (cf. Duntze *et al.*, 1969).

In contrast, interconversion of oxaloacetate and fumarate, leading to partial randomization of the label between C-4 and C-1 of oxaloacetate (cf. Heath, 1968), appears to be very slow in these yeasts, although substantial amounts of malate dehydrogenase were present (Table 2; fumarase levels were not determined). Since both C-4 and C-1 of oxaloacetate are transferred to aspartate, but only C-4 is transferred to glutamate (C-1 is lost in the decarboxylation catalysed by isocitrate dehydrogenase), partial randomization of C-4 and C-1 of oxaloacetate would lead to the molar radioactivity of glutamate being less than that of aspartate. In fact, the molar radioactivities of both proline and its precursor, glutamate,

were consistently about 10% greater than those of threonine and its precursor, aspartate. We have no satisfactory explanation for this observation. Exchange between CO_2 and 2-oxoglutarate is catalysed very slowly by bacterial 2-oxoacid dehydrogenase complexes (Strecker & Ochoa, 1954), and aspartase, which might form unlabelled aspartate from fumarate, has not been demonstrated in yeast.

Pathways of CO_2 fixation during aerobic growth. The distribution of label during aerobic growth differed from that during anaerobic growth in two important respects. Firstly, the molar radioactivity of glutamate was now much less than that of aspartate (Table 2). This could result either from partial randomization of C-4 and C-1 of oxaloacetate, via fumarase (see above), or via the glyoxylate bypass (which transfers C-4 of oxaloacetate sequentially to the C-1 atoms of glyoxylate, malate and oxaloacetate), or by metabolic compartmentation of oxaloacetate. The relative importance of these three mechanisms cannot be determined by the present results alone: other kinds of information, such as the distribution of label between C-4 and C-1 of aspartate, are also required. Secondly, significant amounts of label now appeared in carbohydrate. The labelling of carbohydrate ('neutral fraction', Table 1) correlated closely with phosphoenolpyruvate carboxykinase activity (Table 2), as expected, since this enzyme is believed to be the main gluconeogenic route from compounds such as pyruvate and ethanol. The C-4 of oxaloacetate is lost during decarboxylation to phosphoenolpyruvate, so that incorporation of label from $^{14}\text{CO}_2$ into carbohydrate by this route requires partial randomization of C-4 and C-1 of oxaloacetate by one of the mechanisms mentioned above.

The large molar radioactivities of glycine (derived from phosphoenolpyruvate) and alanine (derived directly from pyruvate) during growth on pyruvate are remarkable. They probably result from the CO_2 /oxaloacetate and pyruvate/oxaloacetate exchanges, catalysed, respectively, by phosphoenolpyruvate carboxykinase (Cannata & Stoppani, 1963) and pyruvate carboxylase (Gailiusis *et al.*, 1964), becoming rapid compared with the turnover of pyruvate under these conditions (cf. Cazzulo *et al.*, 1968). These exchange reactions probably also contribute to the labelling of carbohydrates in yeast grown on ethanol and pyruvate.

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