Origins of Fermentation Products Formed during Growth of *Bacteroides ruminicola* on Glucose

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Bacteroides ruminicola grown on complex medium with glucose as carbon source gave acetate, CO₂, formate and succinate as main fermentation products. No evidence was found for significant glucose catabolism by pathways other than the Embden–Meyerhof sequence. However, [U-¹⁴C]glucose fermentation gave products whose specific radioactivities were much lower than expected. There appear to be two main causes. Firstly, a rapid exchange occurred between metabolic intermediates and CO₂, probably due to reversibility of the pathway between phosphoenolpyruvate and fumarate. Secondly, non-glucose precursors, mainly peptides and acetate, added to the medium as growth factors, also gave rise to the above end-products. The distortions that such reactions introduce into measurements of ATP molar growth yields based on product analyses and measurements of carbon flux based on radioactivity recovered in products are discussed.

INTRODUCTION

Bacteroides ruminicola grown on glucose gives very high molar growth yields (Howlett *et al.*, 1976) as does *Bacteroides fragilis* grown on glucose (Macy, Probst & Gottschalk, 1975) and *Bacteroides amylophilus* grown on maltose (Hobson & Summers, 1967). Calculations of ATP molar growth yields from such data depend on a knowledge of the pathway(s) of substrate catabolism, accurate estimates of ATP yields from substrate level phosphorylation, and unmeasured (and thus uncertain) ATP yields from electron transport-linked phosphorylation (Mountfort & Robertson, 1977). The first two factors are usually ascertained from measurements of fermentation products and, where possible, from studies on enzymes and radioactive substrates. Where bacteria are grown in complex medium, difficulties in interpretation arise when a portion of the 'products' is made from compounds other than the major supplied energy source, even when growth is proportional to the quantity of energy-substrate added. In addition, the transfer of radioactive material from substrate into products can be complicated by unexpected exchange reactions.

In *Bacteroides*, hexoses and disaccharides are assumed to be fermented by the Embden–Meyerhof pathway as far as phosphoenolpyruvate (PEP). However, Caldwell, Keeney & van Soest (1969) have shown that the radioactivity incorporated into succinate, the main product from [U-¹⁴C]maltose fermentation by *B. amylophilus* growing in a defined medium, was less than expected. They concluded that a pathway involving CO₂ fixation could account for 60% of the succinate formed, but further study was required to decide whether this or another pathway accounted for the remaining 40%. In this paper we have studied glucose fermentation by *B. ruminicola* in complex medium to see how much of the fermentation products arises from glucose carbon, and whether all glucose fermented can be accounted for by the Embden–Meyerhof pathway.

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METHODS

Bacterium. *Bacteroides ruminicola*, strain B₁₄, was maintained as previously described (Howlett *et al.*, 1976). The strain was kindly supplied by Professor M. P. Bryant of the University of Illinois. Its growth and nutrition were reported by Bryant *et al.* (1958) and Bryant & Robinson (1962).

Medium for growth of liquid cultures. In experiments using radioactive materials, the medium contained (per litre): 75 ml mineral one [0.6 % (w/v) K_2HPO_4], 75 ml mineral two [0.6 % (w/v) KH_2PO_4 , 0.6 % (w/v) $(NH_4)_2SO_4$, 1.2 % (w/v) NaCl, 0.245 % (w/v) $MgSO_4 \cdot 7H_2O$ and 0.159 % (w/v) $CaCl_2 \cdot 2H_2O$] (Latham & Sharpe, 1971), 10 g Trypticase (Baltimore Biological Laboratories), 2 g yeast extract, 2.5 mg haemin, 1.0 mg resazurin, 4 g CO_2 -equilibrated sodium carbonate, 5 g cysteine hydrochloride, 1 g glucose, and 100 % CO_2 gas phase, prepared and made anaerobic as previously described (Howlett *et al.*, 1976), unless otherwise indicated. The inoculum was grown to two-thirds of full growth in 5 ml of non-radioactive medium; it was then centrifuged anaerobically (1700 g, 10 min) and resuspended in 2 ml of radioactive medium, and 0.1 ml portions were inoculated into 10 ml of radioactive medium in 16×150 mm tubes stoppered with butyl rubber bungs. Growth at 37 °C was followed spectrophotometrically at 600 nm.

Chemical analyses. Determinations were carried out on medium after bacteria had reached full growth and on uninoculated medium, where appropriate. Except for CO_2 estimations, bacteria were removed by centrifugation (5000 g, 10 min) before analysis. Glucose was determined using the toluidine reagent as previously described (Howlett *et al.*, 1976). Substances in uninoculated medium reacting with toluidine gave less than 1 % of the colour given by added glucose. Organic acid fermentation products were separated by celite column chromatography (Swim & Utter, 1957), and the acidity of fractions was measured by titration against 0.01 M-NaOH. Recoveries of standard acids added to uninoculated medium were, acetic 94.5 %, formic 93 %, and succinic 96 %, and experimental values were corrected accordingly where indicated. Succinic acid isolated by this method was shown to be pure by gas-liquid chromatography of the methylated derivative as previously described (Howlett *et al.*, 1976), and by thin-layer chromatography on cellulose plates using 1-butanol/acetic acid/water (50 : 25 : 25, by vol.) and amyl alcohol/formic acid/water (40 : 40 : 2, by vol.) as solvents, and on silica-gel plates using ethanol/ NH_3 /water (80 : 20 : 10, by vol.) as solvent.

Radiochemicals. ^{14}C -Labelled compounds were obtained from The Radiochemical Centre, Amersham. [U - ^{14}C]Peptides were prepared either from a mixture of casein and commercial algal [U - ^{14}C]protein (99 : 1, w/w) or from [U - ^{14}C]protein extracted by the method of Roberts *et al.* (1957) from *Chlorella* cells grown in the presence of $^{14}CO_2$. The protein was digested with pancreatin in 2 M-urea (pH 7.5) for 3 h at 25 °C, and then urea was removed by absorption of the peptides on a Dowex 50 (H^+) column and elution with 4 M- NH_3 (Bennett, 1967). The carbon content of tryptone and algal peptides was kindly determined by Professor A. D. Campbell and associates, University of Otago, New Zealand.

Radiochemical measurements. A Packard Tricarb scintillation spectrometer (model 3320) was used for liquid scintillation counting, and counts were corrected for quenching by the channels ratio method. To estimate the specific radioactivity of $^{14}CO_2$, the CO_2 was trapped in 3 M-NaOH, as previously described (Howlett *et al.*, 1976), and precipitated by adding 12 % (w/v) $BaCl_2$. The $Ba^{14}CO_3$ was separated by centrifuging, then washed, collected on tared filter discs, dried and weighed. Samples were scraped from filter discs, weighed, and counted in 10 ml scintillant [1 vol. ethanol/1 vol. toluene containing 0.4 % (w/v) 2,5-diphenyloxazole (PPO) and 0.01 % (w/v) 1,4-di-2-(5-phenyloxazolyl)benzene (POPOP)]. Loss of counting efficiency due to self-absorption of $BaCO_3$ was estimated using $Na_2^{14}CO_3$ standards, and was less than 5 %; samples were corrected accordingly. When the radioactivity, but not the mass, of CO_2 was required, the gas was trapped in 10 ml ethanolamine/methoxyethanol (2 : 1, v/v) and portions were counted by the method of Jeffay & Alvarez (1961). Radioactivity in uninoculated medium (except medium containing $^{14}CO_2$) was determined using duplicate samples (0.2 ml) to which 0.8 ml water and 10 ml scintillant [1 vol. Triton X-100/2 vol. toluene containing 0.4 % (w/v) PPO and 0.01 % (w/v) POPOP] were added. Radioactivities of organic acids fractionated by chromatography on celite were also determined in this scintillant. Incorporation of radioactivity into bacteria was measured after centrifugation, a single wash with distilled water, collection on a Millipore filter (0.8 μm pore diameter), drying, and counting in the Triton X-100/toluene scintillant described above.

Degradation of [^{14}C]succinate. Succinate, separated by celite column chromatography, was dried under reduced pressure and mixed with 0.5 mmol of unlabelled sodium succinate. A portion (0.2 mmol) was degraded to propionate and CO_2 by the method of Swim (Swim & Utter, 1957) using *Veillonella alcalescens* (ATCC 17745). The specific radioactivity of CO_2 was determined as described above, and that of propionate was measured after celite column chromatography.

Table 1. *Distribution of radioactivity after fermentation of [U-¹⁴C]glucose*

	Radioactivity present initially in substrates (S) and in products (P) after fermentation	
	$10^{-6} \times \text{d.p.m.}^*$	% of total
Glucose (S)†	11.35	100
CO ₂ (S)	0	0
Acetate (P)‡	2.75 ± 0.01	24.2
Formate (P)‡	0.22 ± 0.03	1.9
Succinate (P)‡	3.65 ± 0.07	32.0
CO ₂ (P)‡	1.88 ± 0.09	16.5
Glucose not utilized§	0.42 ± 0.06	3.7
Non-volatile unknown	0.70 ± 0.06	6.2
Bacteria	1.47 ± 0.07	13.0
Total recovery	11.09 ± 0.28	97.5

* Values are the mean of two experiments, with standard errors.

† Estimated from uninoculated medium.

‡ Corrected for recovery losses during separation.

§ Estimated from toluidine assay.

|| See text.

Table 2. *Specific radioactivities of products* formed during fermentation of [U-¹⁴C]glucose*

	Amount† (μmol)	$10^{-4} \times$ Sp. radioactivity† [d.p.m. ($\mu\text{g-atom C}^{-1}$)]	Ratio of sp. radioactivity of product to glucose‡
Initial glucose	55.67	3.35	1.00
Acetate	40.54 ± 0.88	3.15 ± 0.06	0.94
Formate	21.46 ± 2.49	0.99 ± 0.03	0.30
Succinate	51.54 ± 3.40	1.61 ± 0.06	0.48

* Quantities of fermentation products and radioactivity recovered from chromatography columns were not corrected for losses during separation.

† Values are the mean of three experiments, with standard errors.

‡ Specific radioactivities expressed as d.p.m. ($\mu\text{g-atom C}^{-1}$).

RESULTS

Fermentation of [U-¹⁴C]glucose

At full growth, the distribution of radioactivity in products of [U-¹⁴C]glucose fermentation was 16.5% in CO₂, 32% in succinate, 24% in acetate, and 2% in formate (Table 1). The bacteria contained 13% of the added radioactivity, most of which was probably located in internal polysaccharide (Howlett *et al.*, 1976). Uncharacterized material was present in the supernatant, accounting for 6.2% of the original radioactivity. This material was not glucose, but was anthrone-positive (5% of the original glucose when assayed chemically), toluidine-negative, insoluble in ether in acidic conditions and non-volatile, suggesting that at least part of it was polysaccharide. The overall recovery of radioactivity was 97.5%. The specific radioactivities of the products [expressed as d.p.m. ($\mu\text{g-atom C}^{-1}$)] are presented in Table 2. The ratios of the specific radioactivities of products to glucose were: succinate 0.48, acetate 0.94 and formate 0.30. Theoretical ratios, assuming that (i) succinate is made by CO₂ fixation into PEP, (ii) the Embden-Meyerhof pathway operates, and (iii) no exchange reactions occur, are 0.75, 1.00 and 1.00.

Metabolism of ¹⁴CO₂ during growth on unlabelled glucose

Although over 90% of the initial radioactivity remained in the CO₂, there was significant incorporation into succinate (6.1%), formate (0.75%) and bacteria (0.5%). The specific

Table 3. *Specific radioactivities of products* formed during fermentation of unlabelled glucose in the presence of $^{14}\text{CO}_2$*

	Amount (μmol)	$10^{-4} \times$ Sp. radioactivity (d.p.m. μmol^{-1})	Ratio of sp. radioactivity of product to CO_2 †
Initial glucose	58.5	—	—
Initial $^{14}\text{CO}_2$ ‡	1200	1.65	1.00
Acetate	42.0	0.03	0.02
Formate	18.0	0.70	0.42
Succinate	56.0	2.25	1.36

* Quantities of fermentation products and radioactivity recovered from chromatography columns were not corrected for losses during separation.

† Specific radioactivities expressed as d.p.m. μmol^{-1} .

‡ Estimated from uninoculated duplicate tube: $^{14}\text{CO}_2$ was added as sterile Na_2CO_3 solution.

Table 4. *Distribution of radioactivity after fermentation of $[1-^{14}\text{C}]$ glucose*

	Radioactivity present initially in substrates (S) and in products (P) after fermentation	
	$10^{-6} \times \text{d.p.m.}^*$	% of total
Glucose (S)†	4.5	100
CO_2 (S)	0	0
Acetate (P)‡	1.49 ± 0.004	33
Formate (P)‡	0.06 ± 0.01	1.2
Succinate (P)‡	1.53 ± 0.004	34
CO_2 (P)‡	0.09 ± 0.02	2.0
Glucose not utilized§	0.12 ± 0.005	2.8
Non-volatile unknown	0.12 ± 0.007	2.7
Bacteria	0.69 ± 0	15.4
Total recovery	4.09 ± 0.025	91.2

Footnotes as in Table 1.

radioactivities (d.p.m. μmol^{-1}) of CO_2 added initially and end-products were: CO_2 1.65×10^4 , succinate 2.25×10^4 , acetate 0.03×10^4 , formate 0.7×10^4 (Table 3). The ratio of the specific radioactivities of succinate to CO_2 was 1.36, i.e. 0.36 greater than expected if 1 mol CO_2 were fixed per mol succinate formed in the absence of CO_2 exchange. When the succinate was degraded, using *V. alcalescens* cells, to propionate and CO_2 , these compounds showed 48.7 and 48%, respectively, of the specific radioactivity (expressed as d.p.m. μmol^{-1}) of the succinate (results not shown). These results indicated that the extra ^{14}C in succinate resulted from $^{14}\text{CO}_2$ incorporation exclusively into the carboxyl carbon atoms of succinate as the specific radioactivities of the degradation products, propionate and CO_2 , were equal. Formate had a specific radioactivity 0.4 times that of CO_2 , i.e. 0.4 μg -atom more radioactivity per mol formate than expected, indicating that a CO_2 -exchange reaction was indeed operating. The acetate formed during $^{14}\text{CO}_2$ metabolism had a negligible specific radioactivity.

A possible explanation for the higher than expected specific radioactivities of succinate and formate is that the pathway between fumarate and PEP (or pyruvate) is reversible. When fumarate is formed from PEP, the first carboxyl group will become labelled, and when fumarate is metabolized back to the C_3 compound, theoretically only 50% of the label will be lost. Further metabolism to formate or back to succinate will then give higher than expected counts in these products. This hypothesis of fumarate exchange with C_3 intermediates was tested in the following experiment.

Table 5. *Specific radioactivities of products* formed during fermentation of [1-¹⁴C]glucose*

	Amount† (μmol)	$10^{-4} \times$ Sp. radioactivity† (d.p.m. μmol^{-1})	Ratio of sp. radioactivity of product to glucose‡
Initial glucose	49.5	9.28	1.00
Acetate	37.7 ± 1.99	4.10 ± 0.15	0.44
Formate	19.9 ± 0.08	0.28 ± 0.05	0.03
Succinate	49.2 ± 0.40	3.13 ± 0.04	0.34

* Quantities of fermentation products and radioactivity recovered from chromatography columns were both corrected for losses during separation.

† Values are the mean of two experiments, with standard errors.

‡ Specific radioactivities expressed as d.p.m. μmol^{-1} .

Metabolism of [2,3-¹⁴C]fumarate during growth on unlabelled glucose

When 19 μmol [2,3-¹⁴C]fumarate was added to bacteria fermenting 53 μmol unlabelled glucose, some ¹⁴C from fumarate was incorporated into both acetate and succinate (results not shown). The ratios of the specific radioactivities (expressed as d.p.m. μmol^{-1}) of products to added fumarate were: acetate 0.013, succinate 0.070. During separation on the celite column, the formate and fumarate peaks overlapped, and though the formate appeared to be unlabelled no definitive results were obtained. The specific radioactivities of acetate and succinate were lower than that of added fumarate when expressed as d.p.m. ($\mu\text{g-atom C}$)⁻¹. This would be expected due to incomplete utilization of labelled fumarate and dilution of radioactive products by glucose-derived products. The specific radioactivity of succinate was higher than that of acetate, probably because carbon flux would be towards succinate. The results show that an exchange occurred between fumarate and a C₃ intermediate, despite the permeability barrier slowing transport into the cell.

Fermentation of [1-¹⁴C]glucose

If CO₂-exchange, caused by PEP and fumarate interconversion, is solely responsible for the low specific radioactivities of succinate and formate formed during [U-¹⁴C]glucose fermentation, then no dilution of label should occur when [1-¹⁴C]glucose is fermented. Also, with the latter substrate, extensive formation of ¹⁴CO₂ would indicate operation of the Entner–Doudoroff, hexose monophosphate and/or phosphoketolase pathways rather than the Embden–Meyerhof pathway.

The results (Table 4) show that only 2% of the radioactivity from [1-¹⁴C]glucose was metabolized to CO₂ and the carbon recovery was 91%. Recoveries of the total added radioactivity in acetate and succinate were nearly equal, suggesting that nearly equimolar concentrations of these acids were derived from [1-¹⁴C]glucose. In contrast, equal amounts were not found by titration of the supernatant (see Tables 2 and 3, and Howlett *et al.*, 1976). When specific radioactivities (d.p.m. μmol^{-1}) were calculated (Table 5), acetate, formate and succinate had values 0.44, 0.03 and 0.34 times that of glucose; theoretical values are 0.50, 0 and 0.50. Degradation of succinate to propionate and CO₂ by *V. alcalescens* (results not shown) indicated that 96% of the label was in the methylene carbon atoms.

Metabolism of [U-¹⁴C]peptides during growth on unlabelled glucose

Since [U-¹⁴C]Trypticase was not commercially available, [U-¹⁴C]peptides were prepared from a mixture of commercial algal [U-¹⁴C]protein and casein, (experiment 1, Table 6) and from algal [U-¹⁴C]protein from cells grown in this laboratory (experiment 2, Table 6). These peptides were used in growth studies. The bacteria reached full growth after 7.5 h and 12 h, respectively. The slower growth in experiment 2 compared with experiment 1 could have been due to the lower peptide concentration (35 mg and 50 mg peptide, respectively, per 5 ml) and/or to a lower acetate concentration in experiment 2 (Trypticase contains acetate).

Table 6. *Specific radioactivities of products* formed during fermentation of unlabelled glucose in the presence of [U-¹⁴C]peptides*

	Experiment 1			Experiment 2		
	Amount (μ mol)	$10^{-3} \times$ Radioactivity (d.p.m.)	$10^{-2} \times$ Sp. radioactivity [d.p.m. (μ g-atom C) ⁻¹]	Amount (μ mol)	$10^{-5} \times$ Radioactivity (d.p.m.)	$10^{-3} \times$ Sp. radioactivity [d.p.m. (μ g-atom C) ⁻¹]
Initial glucose	28.0	—	—	29.0	—	—
[U- ¹⁴ C]Peptide	(50 mg)†	550	3.1	(35 mg)‡	185	15.1
Acetate	21.0	2.3	0.55	22.0	1.13	2.5
Formate	ND	ND	ND	9.0	0.415	4.5
Succinate	26.0	3.56	0.34	26.0	1.44	1.39

ND, Not determined.

* Quantities of fermentation products and radioactivity recovered from chromatography columns were not corrected for losses during separation.

† Peptide made by digestion of mixture of casein and commercial algal [U-¹⁴C]protein; measured C content 41.9%.

‡ Peptide made by digestion of [U-¹⁴C]protein extracted from *Chlorella*; measured C content 42.5%.

The ratio of the specific radioactivities [expressed as d.p.m. (μ g-atom C)⁻¹] of succinate to peptides was 0.11 in experiment 1, and 0.093 in experiment 2. These are minimum values. A correction of unknown magnitude needs to be made for label dilution due to possible CO₂ fixation into C₃ precursors and carboxyl group exchange with CO₂. The position of the label in the succinate was not determined. The ratio of specific radioactivities of acetate formed to peptides was 0.177 in experiment 1 and 0.165 in experiment 2. These values were higher than predicted from the studies with [U-¹⁴C]- and [1-¹⁴C]glucose but could have been due to the difference in amino acid constituents of Trypticase and algal peptides. The ratio of the specific radioactivities of formate to peptides was 0.3.

Metabolism of [2-¹⁴C]acetate during growth on unlabelled glucose

Since acetate was the major volatile fatty acid present in uninoculated medium and was required for optimal growth, the incorporation of radioactive acetate into other products was studied. Unlabelled glucose (53 μ mol) was fermented in the presence of 119 μ mol [2-¹⁴C]acetate (results not shown). About 95% of the added acetate was not metabolized. However, 4.5% was transformed into succinate, and the specific radioactivity of succinate (expressed as d.p.m. μ mol⁻¹) was 0.11 times that of the initial acetate. This is a minimum value since acetate radioactivity would have been diluted by acetate production during growth. Little significant incorporation of acetate into formate occurred, this product having 0.01 times the specific radioactivity of added acetate.

Metabolism of other ¹⁴C-labelled compounds during growth on unlabelled glucose

L-[U-¹⁴C]Cysteine and [2-¹⁴C]propionate were tested for incorporation into products (data not shown). In the presence of [U-¹⁴C]cysteine, the ratios of the specific radioactivities [expressed as d.p.m. (μ g-atom C)⁻¹] of products to cysteine were: formate 0.03, acetate 0.03, succinate 0.005. The corresponding values in the presence of [2-¹⁴C]propionate (with specific radioactivities expressed as d.p.m. μ mol⁻¹) were: acetate 0.005, succinate 0.008. Thus, incorporation from these compounds was small.

DISCUSSION

Pathways of glucose catabolism. Studies on [1-¹⁴C]glucose catabolism indicate that the Entner-Doudoroff, phosphoketolase and hexosemonophosphate shunt pathways are not major routes of glucose utilization, because radioactivity incorporated into CO₂ was too

low (Table 4). Labelling patterns of other products formed from [1- ^{14}C]glucose (Table 5 and text) were consistent with a high proportion of glucose being metabolized via the Embden–Meyerhof pathway, the methylene carbon atoms containing 96% of the label in succinate, and CO_2 and formate being virtually unlabelled. Acetate, formate and CO_2 are thought to be formed in *B. ruminicola* from pyruvate catabolism, and succinate via PEP carboxykinase-mediated CO_2 -fixation to oxaloacetate (Scardovi & Chiappini, 1966). Aldolase (Joyner & Baldwin, 1966), hexosephosphate isomerase and phosphofructokinase (P. G. Glucina & A. M. Robertson, unpublished results) are known to be present. However, although the labelling patterns and enzyme studies are consistent with operation of the Embden–Meyerhof pathway for glucose catabolism, it is still necessary to explain the unexpectedly low specific radioactivities of some products formed during [U- ^{14}C]glucose and [1- ^{14}C]glucose catabolism.

Studies on [U- ^{14}C]maltose fermentation in *B. amylophilus* by Caldwell *et al.* (1969) using a defined medium showed that the specific radioactivity of succinate, when expressed as d.p.m. ($\mu\text{g-atom C}^{-1}$), was lower than expected. They also showed that $^{14}\text{CO}_2$ was incorporated into the carboxyl groups of succinate. We find similar results with *B. ruminicola* fermenting [U- ^{14}C]glucose, and have made further studies to be certain that such results are consistent with the Embden–Meyerhof pathway. Several factors appear to contribute to the unexpected specific radioactivities of products from labelled glucose. (i) CO_2 exchange (in addition to CO_2 fixation) occurred into the carboxyl groups of succinate and formate. This was catalysed by a reversible pathway between PEP and fumarate. The extent can be estimated from $^{14}\text{CO}_2$ incorporation measurements (Table 3) in which succinate and formate had, respectively, 0.36 and 0.42 $\mu\text{g-atom}$ more ^{14}C than expected. (ii) Non-glucose precursors contributed to formation of succinate, acetate and formate. Their contribution to succinate production can be deduced from experiments using [1- ^{14}C]glucose, which does not exchange label with CO_2 . The data in Table 5 suggest that a minimum of 68% of succinate was derived from glucose, and a maximum of 32% was derived from non-glucose precursors. A theoretical calculation of the expected specific radioactivity of succinate, produced from [U- ^{14}C]glucose in the presence of CO_2 exchange and non-glucose precursors, can be made by multiplying the fraction of unlabelled carbon atoms in succinate formed in the presence of $^{14}\text{CO}_2$ [(4.00–1.36)/4] by the fraction of succinate labelled from [1- ^{14}C]glucose catabolism [0.34/0.50]. Thus a minimum of 0.45 of the carbon atoms in succinate formed during [U- ^{14}C]glucose fermentation should be labelled. This is in good agreement with the experimentally determined value of 0.48 (Table 2).

Experiments using [^{14}C]peptides and [2- ^{14}C]acetate suggest that these compounds are the major non-glucose precursors of fermentation products. [^{14}C]Peptides contributed a minimum of 10% of the succinate formed during glucose catabolism (Table 6). This value may have depended on the amino acid content of the peptides used, and could have been greater if CO_2 exchanges with precursor carboxyl groups. Peptides also contributed to acetate and formate formation, values of 17 and 30% being found, respectively (Table 6). The ratio of the specific radioactivities [expressed as d.p.m. ($\mu\text{g-atom C}^{-1}$)] of formate to peptides was higher than expected. Perhaps formate may have been formed directly from such peptide constituents as glycine and serine, in addition to the pathways from C_3 precursors common to acetate production; more acetate than formate is formed from glucose. Finally [2- ^{14}C]acetate gave rise to labelled succinate (a minimum of 11%) and this could indicate either exchange and/or net incorporation.

Stoichiometry of glucose fermented to products derived from glucose. Data in Tables 1, 2, 4 and 5 show a measured stoichiometry of glucose disappearing from the medium to products of 1 mol glucose : 1.02 mol succinate, 0.80 mol acetate (and 0.41 mol formate). To find the portion of glucose fermented to products, one must subtract 19.2% (glucose converted to cell material and non-volatile unknown, see Table 1). To estimate the products actually formed from fermented glucose, a correction can be made on the basis of lowered

specific radioactivity values (Table 5); i.e. 68% of 1.02 mol succinate per mol glucose and 88% of 0.80 mol acetate per mol glucose. Formate can be ignored in energy calculations.

In essence, for every 1 mol glucose disappearing from the medium, only 0.808 mol was fermented to give energy, and 0.695 mol succinate and 0.705 mol acetate were produced from this. From non-glucose sources, 0.32 mol succinate and 0.10 mol acetate were produced.

ATP yields. Assuming zero net ATP yield during glucose transformation to PEP, the substrate level phosphorylation reactions giving rise to ATP are PEP carboxykinase, pyruvate kinase and acetokinase. The ATP yield from 0.808 mol glucose will be 2.1 to 2.3 ATP by such reactions, i.e. 2.6 to 2.8 ATP per mol glucose. The reactions yielding unknown amounts of ATP are those from peptides to succinate and acetate, anaerobic electron transport-linked phosphorylation, and acetate to succinate (energy requiring). The data in this paper clearly show the difficulties inherent in estimating ATP yields from product analysis. A substantial portion of 'products' was not made from the energy source in complex medium; corrections had to be made for assimilation of substrate into cell material and extracellular products, and the use of radioactive substrates shows that exchange reactions obscured the true recovery of products. Only when such factors are assessed will growth yield experiments be meaningful. Such considerations will also be important in studies on pure or mixed cultures in which the radioactivity found in products is used as a measure of the breakdown of an added radioactive substrate.

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