Reprod Nutr Dev (1992) 32, 129-133

Original article

Distribution of radioactivity of ^{14}C-amino acids added to the medium in cells and metabolites in cultures of rumen fungi

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(Received 12 August 1991; accepted 11 February 1992)

Summary — A mixture of L-(U-^{14}C) amino acids was added to cultures of 11 strains of rumen anaerobic fungi belonging to *Neocallimastix frontalis*, *Neocallimastix joyonii*, *Sphaeromonas communis* and *Piromonas communis*. Fungi were grown in a complex medium with glucose for 4 days. The radioactivity was found in cellular protein (27.7–65.3% of the total radioactivity recovered), lactate (16.9–41.8%), volatile fatty acids (7.4–25.7%) and ethanol (4.6–10.5%). A small amount of radioactivity was recovered in lipids (0.2–1.8%) and CO_2 (0.3–1.0%). The results suggest that the assimilation of amino acids by growing fungal cells was quantitatively comparable with their dissimilation to metabolites.

rumen / amino acid / fungus

Résumé — Distribution de la radioactivité d'acides aminés marqués par ^{14}C dans les cellules et les métabolites de champignons du rumen en culture. *Un mélange de* L-*(U-^{14}C) acides aminés a été ajouté à des cultures de champignons anaérobies du rumen appartenant aux espèces* Neocallimastix frontalis, Neocallimastix joyonii, Sphaeromonas communis *et* Piromonas communis. *Après 4 j d'incubation dans le milieu complet glucosé, la radioactivité a été retrouvée dans les protéines cellulaires (27,7–65,3% du taux total), le lactate (16,9–41,8%), les acides gras libres (7,4–25,7%) et l'éthanol. Un taux faible de radioactivité a été mesuré dans les lipides (0,2–1,8%) et le dioxyde de carbone (0,3–1,0%). Les résultats obtenus ont révélé que l'assimilation des acides aminés par les cellules des champignons en croissance était quantitativement comparable à leur dissimilation dans les métabolites.*

rumen / acide aminé / champignon

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INTRODUCTION

In the process of rumen digestion, proteins are converted to amino acids and eventually degraded to ammonia, carbon dioxide, volatile fatty acids and hydrogen sulphide or incorporated into microbial cells. The studies of Wallace and Joblin (1985) and Wallace and Munro (1986) have shown that anaerobic rumen fungi participate in protein breakdown. The metabolic fate of the amino acids released, however, has been omitted from systematic studies of fungal metabolism. A search in the literature revealed only one paper that dealt with this problem. Gulati *et al* (1989) in experiments with the rumen fungus *Neocallimastix* sp LM1 found that ^{14}C-lysine, ^{14}C-methionine and ^{3}H-tyrosine were incorporated in largely unaltered form into fungal protein, with little or no degradation. It is known that rumen fungi require amino acids for good growth (Orpin, 1988). Growth of *Neocallimastix patriciarum* in the presence of ammonium ions in media lacking amino acids was poor (Orpin and Greenwood, 1986).

The purpose of the present study was to gain an insight into amino acid metabolism in these important organisms. The experiments reported here were designed to study the distribution of radioactivity of ^{14}C-amino acid mixture into major fungal cellular constituents (proteins and lipids) and metabolites (lactate, volatile fatty acids, ethanol and carbon dioxide).

MATERIALS AND METHODS

Cultures and media

Neocallimastix frontalis strains PC3 and CB3, *Neocallimastix joyonii* strains PNg, PM and P1 and *Piromonas communis* strains G3 and PC2 were isolated from the rumen of fistulated cows fed concentrate, hay and silage. *Piromonas communis* strain PC1 and *Sphaeromonas communis* strain SC3 were isolated from the rumen of sheep fed a hay diet. *Sphaeromonas communis* strain DSC3 originated from a fallow-deer and *Neocallimastix joyonii* strain AA1 from a camel fed a hay diet. The cultures were assigned to the given genera of anaerobic fungi on the basis of distinctive morphological criteria, as described by Orpin (1975, 1976, 1977), Heath *et al* (1983) and Breton *et al* (1989).

Complex medium used for growth of fungi was based on medium 10 of Caldwell and Bryant (1966) except that glucose (4 g/l) was the only sugar present and 10% (v/v) of clarified rumen fluid was added. The medium contained enzymatically digested casein (2 g/l), yeast extract (2 g/l), inorganic salts, growth factors and cysteine / Na_2S (0.5 g/l) as reducing agents. The pH of the medium was adjusted to 7.0. L-(U-^{14}C) Amino acid mixture (1.45 GBq/mg-atom C) was purchased from the Institute for Research, Production and Application of Radioisotopes (Prague, CSFR). The mixture contained 14 amino acids (table I). A 0.5-ml portion of the mixture (1.5 MBq) was injected into 40 ml of sterile medium in 50-ml hermetically closed flasks. The medium was inoculated by transferring 2 ml of 72-h-old culture of a fungus. Inoculated triplicate cultures were grown anaerobically under CO_2 atmosphere at 39 °C for 4 days. Glucose was completely utilized during this period. The pH fell to about 5.4–5.7 after incubation.

Measurement of radioactivity

Incubation flasks were degassed and CO_2 trapped in 5 M NaOH (practically all CO_2 is present as free CO_2 at pH ≈ 5.5). Cultures were centrifuged (4 000 *g* for 20 min) and the cells washed twice with rinsing solutions (Jenkinson *et al,* 1979). Washed cells were freeze-dried and lipids were extracted with chloroform/methanol (Folch *et al,* 1957). The remaining fungal mass was extracted with 5 M NaOH (90 °C for 3 h) to dissolve proteins. The alkaline extract was neutralized with 5 M acetic acid.

The culture supernatants were used to measure radioactivity of ethanol and fermentation acids. Ethanol, volatile fatty acids (VFA) and lac-

Table I. Concentration of amino acids in the medium M10 and amount of radioactivity added.

Amino acid	*Concentration (µg/ml)*		*Radioactivity added (kBq/ml)*
	Free form	*Total amount* [1]	
Aspartic acid	20	366	3.33
Threonine	22	197	2.22
Serine	30	221	1.85
Glutamic acid	78	829	4.63
Proline	traces [2]	452	2.22
Glycine	11	136	1.85
Alanine	38	156	3.71
Cysteine	74	463	–
Valine	46	272	2.59
Methionine	21	118	–
Isoleucine	32	201	1.85
Leucine	96	366	4.45
Tyrosine	53	175	1.30
Phenylalanine	56	193	2.59
Histidine	19	110	–
Lysine	85	371	2.04
Arginine	44	157	2.41
Total	725	4 783	37.04

[1] After acid hydrolysis (6 M HCl, 105 °C/20 h); [2] less than 5 µg/ml.

tate (converted to acetaldehyde) were trapped in solutions of 0.4 N potassium biochromate, 1 M NaOH and 0.01 M semicarbazide–HCl, respectively using microdiffusion chambers (Conway, 1957). We found in experiments with nonlabelled substrates that the efficiency of the microdiffusion process was 86.0, 40.0 and 88.6 for ethanol, VFA and lactate, respectively.

Aliquots of radioactive extracts and solutions were dissolved in aqueous-compatible scintillation fluid (Bray solution, Lachema, CSFR) and counted in Nuclear Chicago liquid scintillation spectrometer. The findings were corrected for different efficiencies of the microdiffusion process. Also the fact that only 2 out of 3 lactate carbons were trapped in semicarbazide solution was taken into account. Total radioactivity recovered in cells was compared with initial radioactivity of cultures before incubation.

Chemical analyses

Amino acid composition of the growth medium was estimated by ion-exchange chromatography using an automatic amino acid analyser (model T 339, Mikrotechna, Prague). Free amino acids were estimated in TCA–deproteinized medium. Total amounts of amino acids were estimated after acid hydrolysis (6 M HCl, 105 °C/20 h).

Concentrations of cellular protein were estimated in quadruplicate cultures of *Neocallimastix frontalis* CB3 before incubation and after 4 days of growth. Fungus cells were harvested by centrifugation (5 000 *g* for 15 min), washed with rinsing solutions, dried at 105 °C and digested with 1 N NaOH (1 h/100 °C). Protein was measured by the method of Lowry with BSA as standard.

RESULTS

The results have been summarized in tables I and II. The data in table I show that free amino acids represent only a portion of the total amount of amino acids present in the medium M 10 (0.73 and 4.78 g/l, respectively). Production of cellular protein in 4-day-cultures of strain CB3 was 0.21 g/l on average.

Table II shows the distribution of radioactivity in main metabolites and cellular components in cultures of rumen fungi. The principal fraction of the radioactivity recovered, 46.2 ± 13.4% was found in the alkaline extract of fungal cells, which contains mainly radioactive proteins, contaminated with nucleic acids. A small amount of radioactivity was distributed in the lipids (0.2–1.8%) and free CO_2 (0.3–1.0%). An appreciable amount of the label was recovered in lactate (16.9–41.8%), volatile fatty acids (7.4–25.7%) and etha-

Table II. Distribution of radioactivity from ^{14}C-amino acids added[1] to cultures of rumen fungi.

	Strain	Distribution of label[2] (10^3 cpm/ml)						Total radioactivity recovered[3] (%)	
		Cells		Metabolites					
		NaOH extract	Lipids	Lactate	VFA	Ethanol	CO_2		
N frontalis	CB3	110.2	1.2	30.6	12.1	8.7	1.0	12.1	(1.4)
	PC3	32.0	0.9	25.0	20.8	6.8	0.8	6.4	(1.6)
	PNg	32.4	1.2	20.2	20.2	5.5	0.8	5.9	(0.2)
N joyonii	PM	170.5	2.6	44.1	30.6	12.0	1.3	19.3	(2.6)
	P1	133.6	1.3	45.9	19.5	13.1	1.1	15.9	(1.7)
	AA1	56.6	0.5	31.8	15.1	8.6	0.7	8.4	(0.6)
	G3	38.9	1.6	28.3	14.2	6.6	0.8	6.7	(0.5)
P communis	PC1	22.2	0.1	28.3	10.8	5.5	0.3	5.0	(0.1)
	PC2	49.6	0.6	32.9	20.2	11.9	0.7	8.6	(0.9)
S communis	DSC2	22.7	0.4	29.1	21.1	8.1	0.7	6.1	(0.4)
	SC3	28.4	0.3	26.0	11.5	7.8	0.2	5.5	(0.6)

[1] 1 350.10^{13} cpm/ml; [2] results are means from 3 cultures; [3] percentage of radioactivity added. SD is given in parentheses.

nol (4.6–10.5%). Distribution of radioactivity was rather similar in different strains, despite their different origin and taxonomic position.

DISCUSSION

The complex medium used in this study was rich in nitrous substances. The amount of amino acids available in the medium would exceed nutritional requirements even if we assumed that all fungal protein was derived from this source of nitrogen. The growth of fungi was limited by concentration of glucose rather than nitrogen and the utilization of ^{14}C-amino acids was therefore incomplete. The recovered ^{14}C varied between 5.0 and 19.3% of the administered label. A certain amount of the radioactivity was probably present in compounds not included in our experimental scheme. These include non-volatile end-products of phenylalanine and tyrosine metabolism (10.5% of radioactivity added) and chitin. Incorporation of amino acid carbon into chitin was probably of minor significance, as this structural polysaccharide is synthesized from glucose, which was available in the medium. Low radioactivity in CO_2 indicates the absence of amino acid decarboxylation. Results obtained in this study therefore more or less reflect real fungal metabolism of amino acids.

There is ample evidence in the literature that within the rumen only limited incorporation of amino acids into microbial proteins occurs. The fate of amino acids in this part of the gastrointestinal tract is predominantly to be deaminated rather than to be assimilated into the microbial biomass (Blackburn, 1965). The principal rumen or-

ganisms involved in the protein and amino acid breakdown are bacteria, which usually incorporate amino acids only to a limited extent. Rumen fungi differ in this respect. In cultures of 11 strains tested, the amino acid carbon was almost equally distributed between cells and metabolites. In addition to the direct incorporation of amino acids into fungal protein observed by Gulati *et al* (1989), we found a significant production of lactate, volatile fatty acids and ethanol in this substrate.

ACKNOWLEDGMENT

The authors are grateful to B Hodrová for providing the cultures.

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