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Production of tyrosine and other aromatic compounds from phenylalanine by rumen microorganisms

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Summary. Rumen contents from three fistulated Japanese native goats fed Lucerne hay cubes (*Medicago sativa*) and concentrate mixture were collected to prepare the suspensions of mixed rumen bacteria (B), mixed protozoa (P) and a combination of the two (BP). Microbial suspensions were anaerobically incubated at 39°C for 12 h with or without 1 mM of L-phenylalanine (Phe). Phe, tyrosine (Tyr) and other related compounds in both supernatant and microbial hydrolysates of the incubations were analyzed by HPLC. Tyr can be produced from Phe not only by rumen bacteria but also by rumen protozoa. The production of Tyr during 12 h incubation in B (183.6 μmol/g MN) was 4.3 times higher than that in P. One of the intermediate products between Phe and Tyr seems to be *p*-hydroxyphenylacetic acid. The rate of the net degradation of Phe incubation in B (76.0 μmol/g MN/h) was 2.4 times higher than in P. In the case of all rumen microorganisms, degraded Phe was mainly (>53%) converted into phenylacetic acid. The production of benzoic acid was higher in P than in B suspensions. Small amount of phenylpyruvic acid was produced from Phe by both rumen bacteria and protozoa, but phenylpropionic acid and phenyllactic acid were produced only by rumen bacteria.

Keywords: Amino acids – Tyrosine – Phenylalanine – Aromatic compounds – Rumen microorganisms

Introduction

It has been reported that certain species of bacteria can form tyrosine (Tyr) from phenylalanine (Phe) under proper conditions (Dagley et al., 1953; Kunita, 1956; Guroff and Ito, 1965; Fazel and Jensen, 1979). There is few information about the production of Tyr from Phe by rumen microorganisms. In the study of Phe metabolism by rumen bacteria and protozoa, a relatively large amount of an unknown compound was detected at the same retention time as Tyr on the chromatograms of HPLC (Amin and Onodera, 1997a).

However, the compound was not identified as Tyr because the peak of Tyr overlapped with *p*-hydroxyphenylacetic acid (HPA) by the HPLC method (Amin et al., 1995). Kristensen (1974) observed that a large amount of Tyr and a small amount of Phe from HPA, and a large amount of Phe and a trace of Tyr from phenylacetic acid (PAA) were produced by mixed rumen bacteria. Moreover, Chalupa (1976) and Smith (1979) suggested that Tyr could be synthesized from Phe by the rumen bacterial population although without any substantiation by experiment, but many researchers found no synthesis of Tyr from Phe or its immediate precursors (Scott et al., 1964; Allison, 1965; Patton and Kesler, 1967) by rumen microorganisms. No information is available about the production of Tyr or HPA from Phe by rumen protozoa. Thus, it is necessary to clarify whether Tyr or its precursor (HPA) can be produced from Phe by rumen microorganisms. Phe has been shown to be deaminated and converted to PAA by rumen bacteria (Van Den Hende et al., 1964; Amin and Onodera, 1997a), rumen protozoa (Coleman, 1967; Amin and Onodera, 1997a) and mixed rumen microorganisms (Patton and Kesler, 1967; Martin, 1973; Amin and Onodera, 1997a). During the studies with [U-^{14}C]L-Phe, Scott et al. (1964) observed that most of the radioactivity was present in PAA and slight activity was found in phenylpropionic acid (PPA) and benzoic acid (BZA). However, no quantitative data for the production of PAA, PPA or BZA were presented by them. In a recent study, Amin and Onodera (1997a) reported the quantitative productions of PAA and PPA from Phe. They first reported that BZA was produced from Phe by rumen bacteria and also by rumen protozoa, but PPA and phenyllactic acid (PLA) were produced from Phe not by rumen protozoa but by only mixed rumen bacteria. There is evidence that *Clostridium sp.* can produce PPA (Moss et al., 1970) and PLA (Elsden et al., 1976) from Phe.

Recently we established a HPLC method for the quantitative determination of the aromatic amino acids and their related eleven compounds with which Tyr can clearly be separated from HPA (Khan et al., 1998). The present research was conducted to verify whether Tyr is produced from Phe by mixed rumen bacteria and protozoa, and simultaneously to make a quantitative investigation of other metabolites produced during the *in vitro* metabolism of Phe. Though the number and species of microorganisms in rumen may vary from batch to batch, the present research has been conducted with mixed rumen bacteria and protozoa in order to know the fact in absolute rumen condition (Hungate, 1955). A quantitative study was also carried out to investigate and compare the production of these compounds in the actual rumen microbial ecosystem, important for the nutrition and physiology of the host animal (Hungate, 1950). All the quantitative analyses were done using the HPLC method (Khan et al., 1998).

Materials and methods

Collection of rumen contents and preparation of microbial suspensions

Rumen contents were collected before morning feeding of three fistulated goats (Japanese native breed, mature, live weight 35 ± 5 kg), fed on a daily ration consisting of Lucerne hay cubes (23 g DM/kg $BW^{0.75}$) and concentrate mixture (8 g DM/kg $BW^{0.75}$)

provided in two equal portions at 9 am and 5 pm. The goats were housed in individual pens under approximately constant environmental conditions with a good ventilation system. Ad libitum supply of fresh water was ensured for every goat.

Rumen contents were strained through four layers of surgical gauze into a separating funnel which was gassed with a mixture of 95% N_2 and 5% CO_2. The strained contents were then incubated at 39°C for about 60 min to allow feed debris to float. The lower liquid portion was separated, mixed properly and a part of it was used as bacteria plus protozoal suspension (BP). The suspensions of mixed bacteria (B) and mixed protozoa (P) were prepared from the rest of the lower liquid portion according to Onodera et al. (1992).

The P suspensions always included 0.1 mg/ml each of chloramphenicol, streptomycin sulfate and penicillin G potassium to suppress the biochemical activities of the contaminating bacteria if any.

Incubation and sample treatments

Microbial suspensions (20 ml) were anaerobically incubated with and without 1 mM L-Phe (Nacalai Tesque Inc., Kyoto, Japan) as a substrate in an artificial rumen at 39°C for 12 h. All incubations contained 0.5 mg/ml of rice starch. Samples were collected (0.5 ml) at 0, 6 and 12 h into 1.5 ml tubes each of which contained 0.5 ml of 5% (v/v) perchloric acid for deproteinization (Necker et al., 1981) and kept over night at 4°C. The deproteinized samples were centrifuged at 27,000 g for 20 min. The supernatant fluids were filtered through a membrane filter (0.45 μm) and stored at 4°C. Pellets were hydrolysed with 6 M HCl at 110°C for 20 h (Amin and Onodera, 1997a). The hydrolysates were filtered through filter paper (Whatman, No. 2), and washed three times with distilled water. The filtrates were evaporated to dryness under reduced pressure at 45°C, washed with distilled water and again evaporated. Three times evaporations were practiced to remove the HCl. The remaining portion was then dissolved in ultra pure water (5 ml) made with Milli-Q Labo (Nihon Millipore, Tokyo, Japan), filtered with membrane filter (0.45 μm porosity) and stored at 4°C.

Analytical method

All the supernatant fluids of the incubations and hydrolysates of the pellets were analyzed by HPLC (Khan et al., 1998). The net increases of Phe and Tyr in the microbial pellets in 6 h and 12 h of incubations were assumed as incorporation and production of these compounds at respective hours. Net degradation of Phe was calculated by subtracting the microbial body incorporated Phe (Fig. 2) from the apparent disappearance (Fig. 1). The values of all the components found in the supernatants and hydrolysates were expressed as the means of nine determinations and standard deviations of the differences between incubation with and without substrates.

In order to determine the protozoal numbers of P and BP suspensions, 0.5 ml portions from those suspensions were collected, mixed with 4.5 ml of methylgreen-formalin-salt solution (Onodera et al., 1977), kept at room temperature and then counted with the aid of a Fuchs-Rosenthal hemocytometer. Protozoal compositions ($\times 10^4$) in P and BP were 152.1 and 113.7, 16.8 and 7.3, and 3.1 and 0.9 for Entodiniiae, Diplodiniiae, Dasytricha, respectively.

The microbial nitrogen (MN) of B, P and BP suspensions were determined by Kjeldahl method (A O A C, 1990) after taking 1 ml (triplicate of each) of microbial suspensions and the values were 0.83 ± 0.21, 1.08 ± 0.11 and 1.51 ± 0.17 mgN/ml, respectively.

Results

The apparent disappearances of Phe in B, P and BP suspensions during 12 h incubation period are shown in Fig. 1. Phe was apparently degraded by 69 and

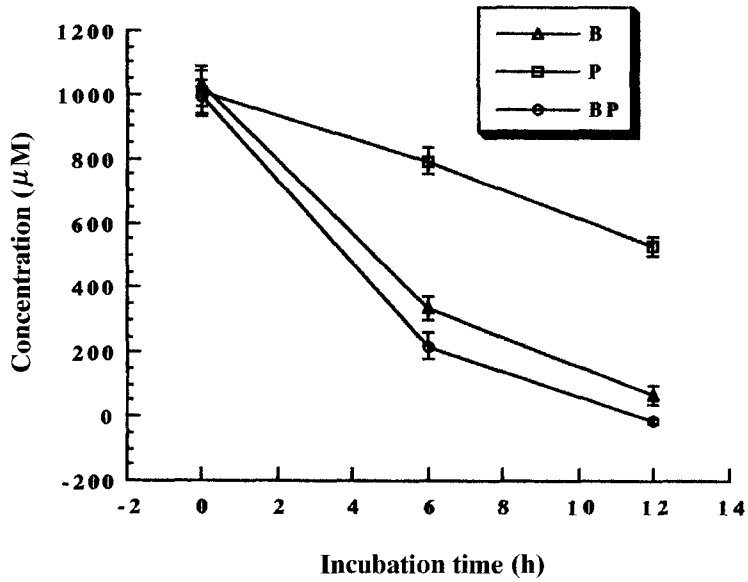

Fig. 1. Apparent disappearance of phenylalanine (Phe) by mixed rumen bacteria (*B*), protozoa (*P*) and B plus P (*BP*) during 12 h incubation

96%, 21 and 47%, and 77 and 103% of added Phe during 6 and 12 h incubation of B, P, and BP, respectively. When the degradation rate was expressed with a unit of per MN, Phe degradation was more in B (97.2 μmol/g MN/h) followed by BP (56.9 μmol/g MN/h) and P (36.3 μmol/g MN/h) in a 12-h incubation. A portion of degraded Phe actually was incorporated into the amino acid pool of the microbial body (Chalupa, 1976; Coleman and Sandford, 1980; Broderick et al., 1991; Armstead and Ling, 1993; Ling and Armstead, 1995) and the microbial body protein (Fig. 2). So, the net degradation was calculated and were 56.5 and 75.4%, 19.6 and 40.7%, and 67.8 and 90.8% at 6 and 12 h incubation in B, P and BP, respectively (Fig. 3). The rate of net degradation of Phe in 12 h incubation in B (76.0 μmol/g MN/h) was 2.4 times higher than in P.

After 12 h incubation with Phe it was observed that Tyr increased 19.7, 84.3 and 71.0 μM in the supernatant of B, P and BP suspensions, respectively (Table 1). The increment of Tyr was higher in P followed by BP and B suspension, and in all cases Tyr increased with the incubation period up to 12 h. Most of Tyr increased in the supernatant of P suspension was not produced from Phe, but released from the protozoal cells during the incubation time (Fig. 2). It was also observed that most of the Tyr produced from Phe in the B suspension was incorporated with bacterial cells (Fig. 2).

The net productions of Tyr (Fig. 3) by B, P and BP during incubation for 6 h and 12 h were calculated considering the increment of Tyr into the supernatant (Table 1) and simultaneous incorporation with microbial cells or losses from the cells (Fig. 2). As shown in Fig. 3, it was observed that Tyr could be produced from Phe by rumen bacteria and also by protozoa. When expressed by per MN, the production of Tyr in B during a 12 h-incubation period

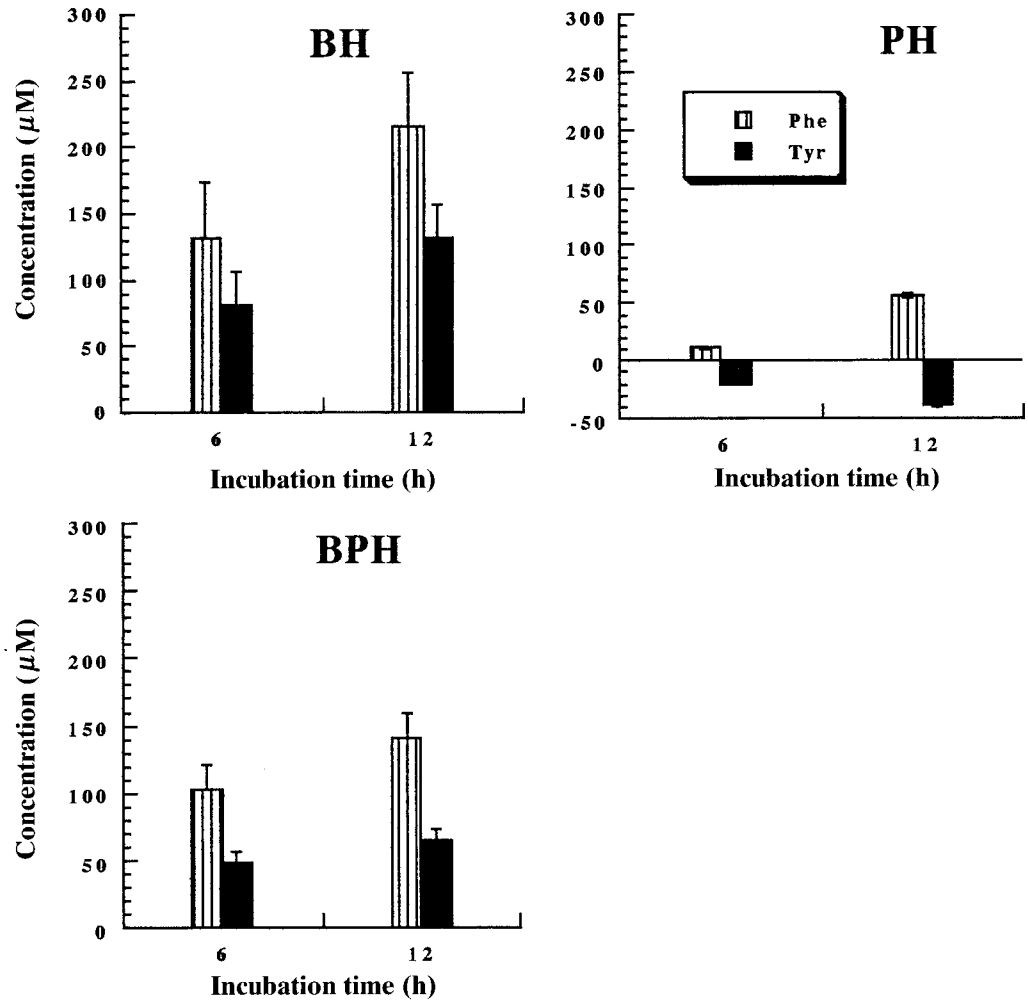

Fig. 2. Incorporation of phenylalanine (*Phe*) and production of tyrosine (*Tyr*) in hydrolysates (*H*) by mixed rumen bacteria (*B*), protozoa (*P*), and B plus P (*BP*) during 12 h incubation of Phe

(183.6 μmol/g MN) was 4.3 and 2.0 times higher than those in P and BP, respectively. It was also observed that about 14.8 and 20.1%, 10.6 and 11.3%, and 14.2 and 15.2% of the disappeared Phe was converted to Tyr during 6 and 12 h incubation in B, P and BP suspensions, respectively.

The production of related phenyl compounds are given in Table 1. HPA increased at 6 h incubation time, but decreased at 12 h incubation in all microbial suspensions (B, P and BP). The conversion of disappeared Phe into HPA at 6 h incubation was higher in P (15.7%) than that in B (6.0%) and BP (6.4%), but the net production of HPA in B (40.9 μmol/g MN) was 1.4 times higher than that of P (28.3 μmol/g MN) and BP (28.7 μmol/g MN) at 6 h incubation. In B suspension HPA produced from Phe was more rapidly degraded than in P and BP (Table 1) from which other products such as Tyr, PAA might be produced (Scott et al., 1964; Kristensen, 1974).

Table 1. Production of tyrosine and related compounds in the supernatant of the incubations of rumen bacteria (B), protozoa (P) and B plus P(BP)

Compound (μM)	B		P		BP	
	6h	12h	6h	12h	6h	12h
Phenylacetic acid	399.6 ± 32.0	436.6 ± 55.4	96.6 ± 14.1	218.1 ± 34.6	354.0 ± 32.3	506.8 ± 27.6
Tyrosine	2.3 ± 7.6	19.7 ± 14.0	41.0 ± 4.1	84.3 ± 6.7	27.7 ± 8.7	71.0 ± 11.2
p-Hydroxyphenylacetic acid	33.8 ± 4.0	−1.6 ± 3.6	30.7 ± 3.0	11.7 ± 2.3	43.3 ± 7.6	10.2 ± 4.3
Benzoic acid	18.5 ± 6.1	28.3 ± 8.9	22.6 ± 5.9	48.7 ± 7.1	28.1 ± 8.3	43.4 ± 12.4
Phenylpyruvic acid	16.7 ± 13.5	–	8.2 ± 5.3	–	15.4 ± 9.7	–
Phenylpropionic acid	14.1 ± 3.4	26.9 ± 10.5	–	–	10.4 ± 4.3	18.0 ± 5.5
Phenyllactic acid	8.6 ± 2.3	13.5 ± 4.4	–	–	–	–

± standard deviation of six determinations.

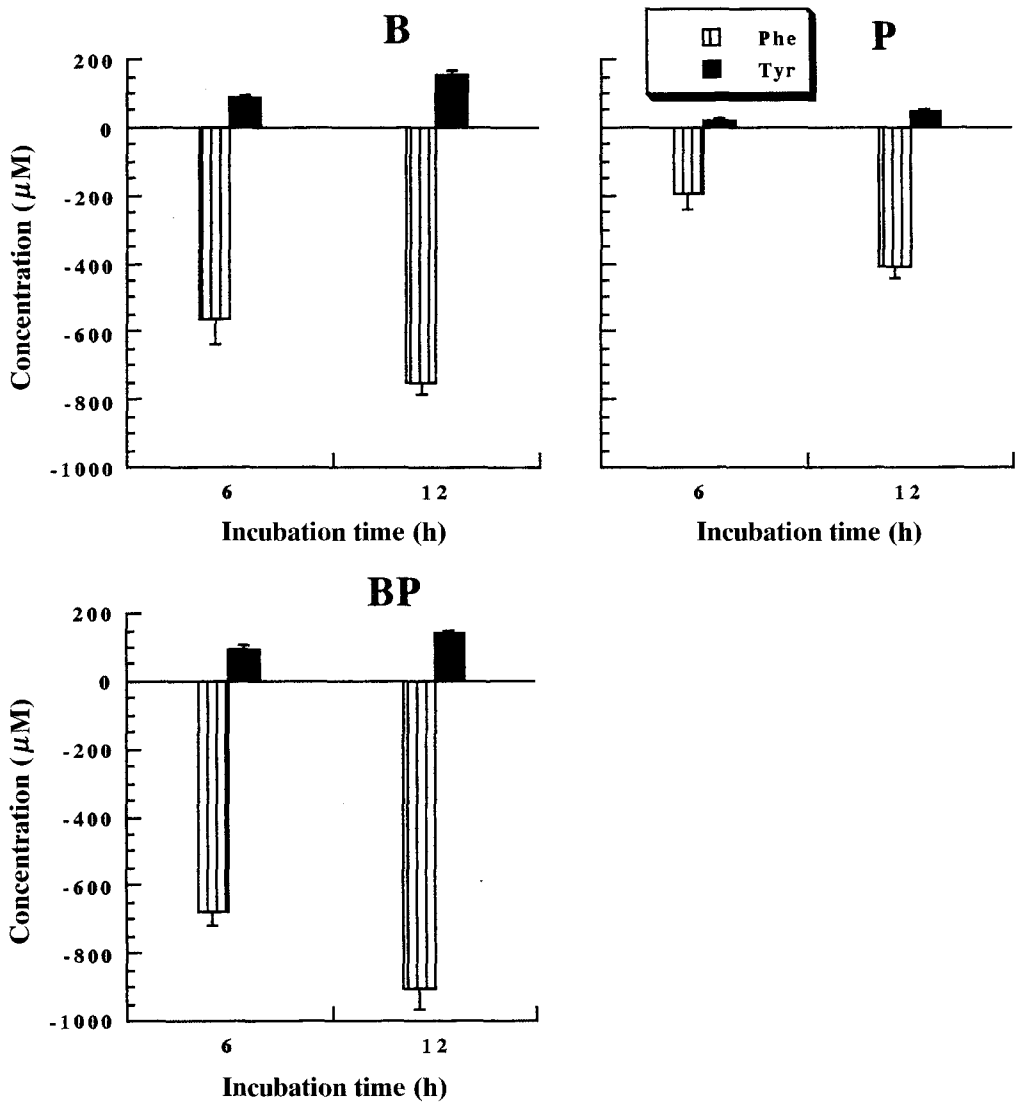

Fig. 3. Net degradation of phenylalanine (*Phe*) and total production of tyrosine (*Tyr*) by mixed rumen bacteria (*B*), protozoa (*P*), and B plus P (*BP*) during 12 h incubation

In all rumen microbial suspensions, the disappeared Phe mainly converted to PAA (Table 1), namely 70.7 and 57.9% in B, 49.4 and 53.6% in P, and 52.2 and 55.8% in BP at 6 and 12 h incubation, respectively. In 12 h incubation the production of PAA in B (527.9 μmol/g MN) was 2.6 and 1.6 times higher than those in P and BP, respectively.

As shown in Table 1, a small amount of phenylpyruvic acid (PPY) was found to be produced from Phe at a 6-h incubation, and it seemed to be very labile. PPY was not detected in any of those microbial suspensions at 12 h incubation period. As far as we are aware this is the first report of the quantitative determination of PPY from Phe by rumen bacteria, protozoa and their mixture. Although PPY is an unstable compound specially in acid me-

dium and in the present analytical method the recovery of PPY of one day variation was 80%.

In B, P and BP suspensions, 3.8, 12.0 and 4.8% of the Phe, respectively, that degraded during 12 h incubation, were converted to BZA. The production of BZA in P in a 12-h incubation (45.0 μmol/g MN) was 1.3 and 1.6 times higher than those in B and BP, respectively.

A small amount of PPA was produced from Phe by B (26.9 μM) and BP (18.0 μM), but not by P during 12 h incubation (Table 1).

It was observed that 3.1% of the disappeared Phe was converted to PLA (13.5 μM) during 12 h incubation only by B but neither by P nor BP (Table 1).

Discussions

1. Degradation of Phe

In the present experiment the apparent degradation rate of Phe in B (0.080 mM/h) (Fig. 1) was a bit higher than the value (0.063 mM/h) reported by Amin and Onodera (1997a), but similar to the value (0.083 mM/h) of Scheifinger et al. (1976). A relatively higher (0.290 mM/h) apparent disappearance rate *in vitro* by rumen bacterial population of cattle was observed by Chalupa (1976).

The Phe degradation by BP (0.086 mM/h) (Fig. 1) agreed with that of the value (0.082 mM/h) of Amin and Onodera (1997a), though it is very low compared to the value (0.540 mM/h) found by Prins et al. (1979) after incubation of Phe with mixed rumen fluid of dairy cow.

The protozoal degradation ability of Phe found during this study (0.039 mM/h) is like that of previous report (Amin and Onodera, 1997a) and also lower than the ability of rumen bacteria.

2. Production of Tyr from Phe

In the present experiment a large quantity of Tyr was produced from Phe by bacteria. More than 20% of the disappeared Phe was converted to Tyr in 12 h incubation. Most of the Tyr produced by rumen bacteria can be efficiently utilized for growth and reserved as body protein. In P, a large quantity of Tyr was released from the cell protein because protozoal cells could not grow in this medium. When protozoal suspensions are incubated *in vitro* in a buffer solution, they usually liberate endogenous amino acids into the medium (Onodera and Kandatsu, 1970; Morgavi et al., 1993). The net production of Tyr from Phe by rumen protozoa was 46.13 μM (42.60 μmol/g MN) in a 12-h incubation which was about 11.3% of the disappeared Phe. It is the first finding for the production of Tyr from Phe by mixed rumen bacteria, protozoa and their mixture (BP).

Recently Amin and Onodera (1997a) detected a large amount of an unknown compound during the incubation of Phe with B, P and BP. In the chromatography they used (Amin et al., 1995), however, the retention time of

Tyr overlapped with that of HPA and expressed with the word "unknown compound" for the peak. They demonstrated that more than 30% of the disappeared Phe was converted to the unknown compound in B and P suspensions. However, in the present experiment 20.1 and 11.3% of the disappeared Phe were shown to be converted to Tyr in B and P during 12h incubation, respectively. The proportions of the total amount of Tyr and HPA to the disappeared Phe in B and P were similar to the trend of previous data (Amin and Onodera, 1997a). The present study clarified that the production of Tyr from Phe in B is greater than that in P and BP. Amin and Onodera (1997a) also observed that a trace amount of PPA produced from Phe at 6h after incubation which was increased at 12h. It may be due to the degradation of Tyr (Scott et al., 1964) produced from Phe, because PPA could not be produced from Phe by rumen bacteria or protozoa (Chen et al., 1994).

Chalupa (1976) observed increased pool size of Tyr in cattle providing with a diet of a mixture of essential amino acids including Phe. In the study of [U-^{14}C]L-Phe metabolism by mixed rumen microorganisms, Scott et al. (1964) observed no radioactive BZA after 1h incubation which was found at 2h incubation. They also observed a large amount of radioactive BZA in urine after administration of radioactive Tyr into the rumen. So the late production of BZA may be due to the break down of Tyr produced from Phe.

3. HPA production from Phe

Kunita (1955) demonstrated that HPA can be produced from PAA by *Pseudomonas fluorescens*. Rumen bacteria can produce HPA from Tyr (Scott et al., 1964). The present study revealed for the first time that HPA was also produced from Phe by rumen bacteria, protozoa and their mixture. In the present study the production of HPA increased at 6h incubation, but decreased at 12h incubation in all microbial suspensions. It indicates that HPA may be primarily produced from Phe, then converted to produce other products. The most probable product from HPA is Tyr (Kristensen, 1974).

4. PAA production from Phe

The production of PAA from Phe by mixed rumen microorganisms was observed by many researchers (Scott et al., 1964; Patton and Kesler, 1967; Amin and Onodera, 1997a). Except for a report by Amin and Onodera (1997a), however, all researchers reported about only the qualitative production of PAA from Phe. In the present study the percent of degraded Phe which converted to PAA in B was slightly higher than the previous study (Amin and Onodera, 1997a). We also observed that the percent conversion of degraded Phe to PAA was higher at 6h (70.7%) than that of 12h (57.9%). It indicates that the higher concentration of PAA prevents its production rate or

it might be participate to produce other metabolites such as Tyr and HPA (Kristensen, 1974; Amin and Onodera, 1997b). In this study the percent of conversion of degraded Phe into PAA was similar in P but little lower in BP than the previous study (Amin and Onodera, 1997a). It may be due to an interaction of B and P or due to the differences of microbial species (Scheifinger et al., 1976). When we expressed the production of PAA with per MN, the productions of PAA in P and BP were similar to the previous study (Amin and Onodera, 1997a) but the production of PAA in B was high in the present experiment. It also can be possible due to differences of bacterial species (Scheifinger et al. 1976). The differences in bacterial number and ecological condition of the rumen (Hungate, 1955) may be additional causes of this variation.

5. Production of PPY from Phe

During this study a small amount of PPY was found at 6 h incubation with Phe by B, P and BP, but no PPY was detected at 12 h incubation period of any of those microbial suspensions. It indicates that Phe is deaminated or transaminated to produce PPY, and then rapidly decarboxylated to form PAA by mixed rumen microorganisms (Van Den Hende et al., 1964; Amin and Onodera, 1997a). After incubation of Phe by concentrated washed bacterial suspension Van Den Hende et al. (1964) found no PPY, but they observed a small amount by using diluted washed suspensions. Amin and Onodera (1997a) did not find any PPY after incubation with Phe by B, P and BP, as the minimum detectable limit of PPY was 20μM in their HPLC method (Amin et al., 1995). Fazel and Jensen (1979) also demonstrated that anaerobic coryneform bacteria can produce PPY from Phe.

6. Production of BZA, PPA and PLA from Phe

The present study confirms the results of Amin and Onodera (1997a) which is the first report of the quantitative production of BZA from Phe by ruminal B, P and BP.

A small amount of PPA was produced only in B but not in P found in the present study. This also agreed with the results of previous studies (Scott et al., 1964; Amin and Onodera, 1997a), though Chen et al. (1994) found no PPA from Phe by rumen microorganisms.

During the present study a trace amount of PLA was produced from Phe only by rumen bacteria but not by protozoa, which confirms the previous study (Amin and Onodera, 1997a). Though the amount of produced PLA in this experiment is higher than the previous one.

It may be concluded that Phe can be degraded and converted to Tyr, HPA, PAA, PPY and BZA by mixed rumen bacteria and also by protozoa, and PPA and PLA can be produced only by mixed rumen bacteria. Further research is necessary to investigate the Phe metabolism by isolated rumen microorganisms.

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