The effect of a high dose of 3-hydroxy-3-methylbutyrate on protein metabolism in growing lambs

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The effect of a high dose of 3-hydroxy-3-methylbutyrate (HMB, a leucine catabolite) on protein metabolism was investigated in growing male lambs fed on hay and concentrate. Concentrate was supplemented with either $Ca(HMB)_2$ (4 g/kg) or $Ca(CO_3)_2$ in experimental (HMB) and control groups respectively. Both groups consisted of six 2-month old lambs. Three complementary methods to study protein metabolism were carried out consecutively 2·5 months after beginning the dietary treatment: whole body phenylalanine fluxes, postprandial plasma free amino acid time course and fractional rates of protein synthesis in skeletal muscles. Feeding a high dose of HMB led to a significant increase in some plasma free amino acids compared with controls. Total, oxidative and non-oxidative phenylalanine fluxes were not modified by dietary HMB supplementation. Similarly, an acute infusion of HMB, in the control group, did not change these fluxes. In skeletal muscles, fractional rates of protein synthesis were not affected by long-term dietary supplementation with HMB. Taken together our results showed that administration of a high dose of HMB to lambs was able to modify plasma free amino acid pattern without any effect on whole-body protein turnover and skeletal muscle protein synthesis.

3-Hydroxy-3-methylbutyrate: Amino acids: Protein turnover: Protein synthesis

Metabolic activity of the branched-chain amino acid, leucine, includes regulation of protein turnover (May & Buse, 1989). Leucine has been shown specifically to stimulate protein synthesis in isolated and incubated muscle of young rats by mechanisms other than its role as a precursor for protein. It also inhibits protein degradation (Buse & Reid, 1975; Li & Jefferson, 1978; Chua *et al.* 1979). These metabolic properties of leucine are not shared by the other two branched-chain amino acids, valine or isoleucine. Chua (1994) suggested that the effect of leucine on protein degradation in perfused rat heart is mediated by plasma membrane regulatory sites. Similar findings indicate the presence of inhibitory recognition sites for hepatic proteolysis on which leucine acts (Miotto *et al.* 1992). Based on the metabolic activity of leucine on protein turnover numerous *in vivo* studies have been performed to evaluate the ability of leucine to increase muscle mass or protein balance in animals, healthy human subjects and patients (Walser, 1984; May & Buse, 1989).

Branched-chain amino acids are transaminated to branched chain 2-oxoacid by the branched-chain amino acid aminotransferase (*EC* 2.6.1.42) then decarboxylated to the corresponding acylSCoA by the branched-chain oxo acid dehydrogenase (*EC* 1.2.4.4) (Harper *et al.* 1984). Alternatively 2-oxoisocaproate (KIC) the 2-oxoacid of leucine can be

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oxidized by the cytosolic KIC-oxygenase to produce 3-hydroxy-3-methylbutyrate (HMB) (Sabourin & Bieber, 1981).

Because leucine is unique among branched-chain amino acids with respect to both its metabolic activity and catabolic pathway it has been speculated that the specific metabolic activity of leucine could be mediated through its particular metabolite, HMB (Sabourin & Bieber, 1981). Dietary supplementation with HMB decreased the incidence of sudden-death syndrome in broilers (Nissen *et al.* 1994*b*). While not affecting growth or the immune response in weanling pigs (Nissen *et al.* 1994*a*), it did increase the percentage of fat in sows' milk and consequently the weight gain of piglets (Gatnau *et al.* 1995). HMB administration modified muscle composition towards higher carcass quality in steers (Van Koevering *et al.* 1994). Its effect on protein metabolism has been poorly studied. HMB can increase muscle mass strength in human subjects by decreasing exercise-induced proteolysis and muscle damage (S. Nissen, unpublished results).

The present study was carried out to address the question of whether a high dose of HMB can modulate protein metabolism in growing lambs. Long-term (diet supplementation for 2·5 months) and acute (infusion) effects of HMB were tested in order to distinguish any metabolic adaptation with respect to HMB. In order to study more closely the possible effect of HMB on protein turnover, whole-body protein kinetics and muscle protein synthesis were investigated using tracer infusion and flooding dose methods respectively. Lambs are large convenient animals, in that multiple blood sampling can be easily performed within the 6 h infusion period without inducing a shock state and sufficient amounts of tissues for metabolite analysis can be sampled at slaughter.

MATERIALS AND METHODS

Animals and diets

Studies were performed in twelve male lambs (Ile-de-France × Romanov) born in November 1991 from a herd at Institut National de la Recherche Agronomique (INRA) Clermont-Ferrand Theix Centre. When lambs were 2 months old, they were transferred into individual pens in a controlled environment. They were fed on a hay (7·5 MJ metabolizable energy (ME), 77 g crude protein/kg) and a ground commercial concentrate (11·1 MJ ME, 175 g crude protein/kg, UCABEC, Lapeyrouse, France) in the proportion 1 : 4, w/w. The total amount of food provided was 35 g/kg body weight. The lambs were fed once daily at 08.30 hours except when mentioned. No refusal was registered. They had free access to nipple drinkers.

Lambs were divided into two groups: experimental (HMB) and control. The HMB group received a high dietary supplement of $Ca(HMB)_2$ for a 14-week period. This compound represented 1 g/kg concentrate (i.e. about 0·5 g HMB/d) for the first 2 weeks and 4 g/kg thereafter (i. e. there was a graded increase in the daily amount from 2·0 to 3·5 g as concentrate intake increased in relation to body weight). $Ca(HMB)_2$ can be added to the diet without any protection since it is not degraded in the rumen (S. Nissen, unpublished results). The control group received $Ca(CO_3)_2$ in order to equalize the amount of Ca in each diet. Body weight did not differ between the two groups throughout the experiment (Fig. 1). There was no effect of dietary HMB on growth rates which were 232 (SE 13) and 219 (SE 18) g/d in control and HMB groups respectively during the 10–14 weeks after the experiment started (i. e. when protocols were carried out). Because both groups consumed the same amount of food, there was no difference in the feed efficiency.

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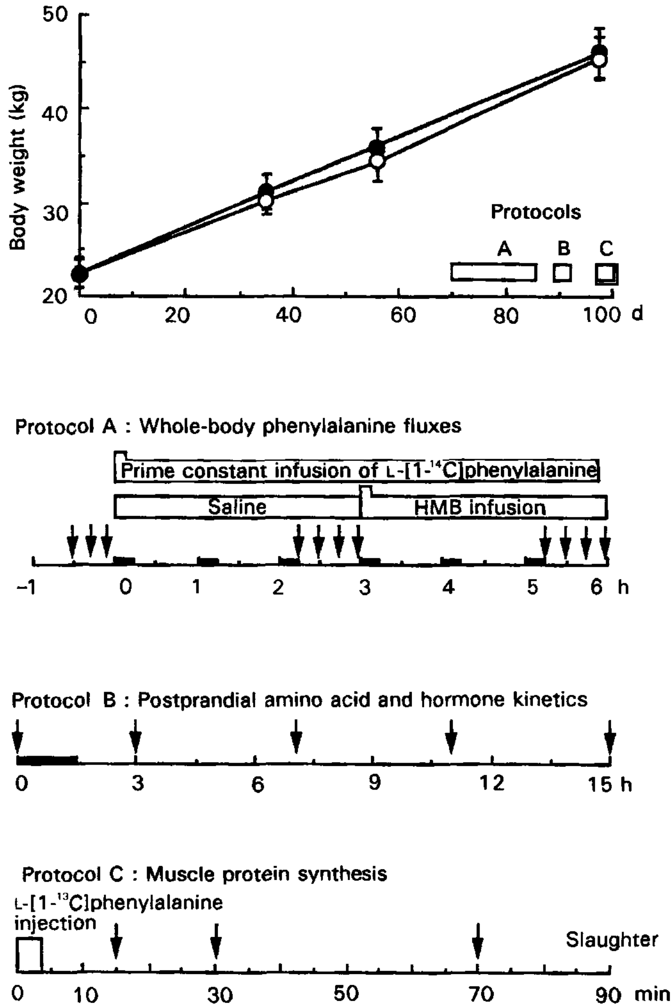

Fig. 1. Description of Protocols A, B and C. The graph (top) shows when the protocols were carried out in relation to time and changes in body weight. (○), Control group; (●), group receiving a dietary supplement of 3-hydroxy-3-methylbutyrate (HMB). The subfigures give detailed information for each protocol, including times of injections, infusions, food intake (■) and sampling (↓). In Protocol A infusion was performed for 6 h in the control group as shown, but only for the first 3 h in the HMB group.

Protocols

Three protocols (A, B and C) were carried out in lambs: protocol A was performed during weeks 10–12 of the experimental period (body weight about 40 kg), protocol B during the 13th week and protocol C during weeks 13–14 (body weight about 46 kg; Fig. 1). Protocol A was performed to investigate whole-body protein turnover by using a constant infusion of labelled phenylalanine. In this protocol the chronic effect of HMB was analysed by comparing phenylalanine fluxes in the two groups. In addition, the acute effect of HMB was investigated in the control group by comparing phenylalanine fluxes in a basal state to

those during a 3 h period of HMB infusion. Protocol B was performed to compare postprandial plasma free amino acid and hormone concentrations in the two groups. Finally, in protocol C, fractional rates of protein synthesis were measured in skeletal muscles of lambs from both groups using the flooding dose injection of labelled phenylalanine. Whenever needed, one or both external jugular veins were catheterized under local anaesthesia on the previous day.

In protocol A, lambs received a primed constant infusion of L-[1-^{14}C]phenylalanine (98 % 2·0 GBq/mmol, Dositek, France) in a jugular vein for 6 h (control group) or 3 h (HMB group) (148 kBq prime, 8·1 kBq/min in saline at 0·1 ml/min) beginning at 09.30 hours in both groups. The bicarbonate pool was primed with 27·7 kBq [^{14}C]NaHCO$_3$ (2·1 GBq/mmol, Dositek). To study the acute effect of HMB, lambs from the control group received a primed constant infusion of HMB (14·4 μmol/kg prime, 0·8 μmol/min per kg body weight at the rate of 0·1 ml/min) for 3 h starting 3 h after the beginning of the tracer infusion (Fig. 1). On that infusion day, the total daily food intake was consumed as six equal portions at 1 h intervals. Blood (20 ml) was collected before infusion commenced and at 130, 145, 160 and 175 min after the tracer infusion started in both groups, and also at 310, 325, 340 and 355 min only in the control group. Plasma was immediately separated by centrifugation and stored at $-20°$ until analysis. Expired air was collected for a 5·5 min period with an appropriate mask at the same times as blood withdrawals. This was dried on $CaCl_2$ absorbers and its volume measured by passage through a gazometer (Slonic, Schlumberger, France). The CO_2 content of a portion of dry air was measured by gravimetry after trapping it in soda lime–NaOH (1 : 4, w/w). The remainder of the dry air was bubbled into 0·8 M-NaOH in order to trap and measure $^{14}CO_2$. Radioactivity was measured by liquid scintillation spectrometry (Packard Tricarb 460 CD, Packard Instruments, Downers, IL, USA) using Quick Safe A (Zinsser Analytic, Maidenhead, Berks.) as scintillation liquid.

In protocol B, blood was collected before and at various times after the meal for insulin, insulin-like growth factor-1 (IGF-1) and free amino acids measurements (Fig. 1). Plasma was immediately separated by centrifugation and stored at $-20°$ until analysis.

In protocol C, five lambs per group were injected over 6 min into a jugular vein with a flooding dose of L-[1-^{13}C]phenylalanine (0·53 mmol/kg body weight, 25 atoms %, Tracer Technologies, Somerville, MA, USA) at 12.30 hours (i. e. 4 h after feeding; Fig. 1). Blood samples were taken from the other jugular vein before and at several post-injection times. Plasma was separated by centrifugation and stored at $-20°$ until analysis. Lambs were killed under sodium pentobarbitone anaesthesia 90 min after the injection. Muscle (diaphragm, *longissimus dorsi*, *tensor fasciae latae*) samples were excised, immediately frozen in liquid N_2 and stored at $-20°$. One lamb per group was used to determine the basal enrichment of L-[1-^{13}C]phenylalanine in proteins. This lamb was treated exactly as the others except that labelled amino acid injection was omitted.

Analytical methods

Specific radioactivity of plasma free L-[1-^{14}C]phenylalanine (Protocol A) was determined from 10 ml plasma samples. After deproteinization with TCA (100 g/l) amino acids were purified using the cationic resin Dowex AG 50. Amino acids were separated with an automatic amino acid analyser and effluent from the column was collected to measure the radioactivity in the phenylalanine and tyrosine peaks by liquid scintillation spectrometry. Phenylalanine and tyrosine were quantified using an amino acid analyser (4151 Alpha Plus

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Pharmacia, Saint-Quentin-Yvelines, France) and specific radioactivity was calculated as the radioactivity : concentration ratio measured on the same sample.

Plasma amino acid concentrations (Protocol B) were quantified as described above using norleucine as internal standard.

Plasma and muscle free L-[1-^{13}C]phenylalanine enrichments (Protocol C) were measured by formation of the tertiary isobutyldimethylsilyl derivative as previously described for L-[1-^{13}C]valine (Breuillé *et al.* 1993). This derivative was injected into a gas chromatograph–mass spectrometer (Nermag R10-10C quadripole spectrometer coupled to a Delsi gas chromatograph Delsi-Nermag, Argenteuil, France) with chemical ionization and selected ion monitoring of ions 336 and 337. To measure the enrichment of protein-bound L-[1-^{13}C]phenylalanine the pure amino acid was decarboxylated by the ninhydrin reaction and the $^{13}CO_2$ produced was injected into a gas isotope ratio mass spectrometer VG 903 coupled with a Roboprep-G gas chromatograph (Europa Scientific) (Breuillé *et al.* 1993). The protein hydrolysate was cleaned onto cationic resin Dowex AG 50 before the purification of [1-^{13}C]phenylalanine. The latter was performed using HPLC (Kontron Montigny-le-Bretonneux, France). Each sample was injected twice on to a Spherisorb ODS2 5 μ, 250 × 10 mm column (Interchrom, Interchim, Montluçon, France). The phenylalanine peak appeared at 10 min post-injection using an isocratic elution with potassium phosphate 0·01 M, pH 7, flow rate 4·25 ml/min. The purity of the phenylalanine peak was checked on the amino acid analyser and external standards of known enrichments were processed in the same way as the samples.

Plasma insulin (Protocols B and C) was determined by radioimmunoassay (RIA) with a commercial kit (INSI-PR, Oris Industrie, Gif-sur-Yvette, France) containing human insulin as a standard. Plasma IGF-1 (Protocol B) underwent a 0·5 M-HCl extraction process and a C_{18} Sep Pak (octadecylsilyl silica cartridge) purification before RIA (IN-SOM-C, Oris Industrie).

Calculations and statistics

Plasma phenylalanine kinetics were calculated from steady-state isotope dilution equations according to the primary pool model. Total flux (μmol/min) equals the ratio of the L-[1-^{14}C]phenylalanine infusion rate (Bq/min) to plasma free L-[1-^{14}C]phenylalanine specific radioactivity (Bq/μmol). Oxidative flux was calculated by dividing the rate of expired $^{14}CO_2$ (Bq/min) by the plasma free L-[1-^{14}C]phenylalanine specific radioactivity (Bq/μmol). A correction was made to take into account the fixation of $^{14}CO_2$ in the body, assuming that it would have been the same in both groups and reached 20 %. Non-oxidative flux (an estimate of protein synthesis) was obtained by subtracting oxidative flux from total flux.

Fractional rate of protein synthesis (*Ks*) was calculated as $Ks = (P_t - P_b) \times 100/AUC$ where P_b is basal enrichment of [1-^{13}C]phenylalanine (atom %) from muscle protein before the injection, P_t is enrichment of [1-^{13}C]phenylalanine (atom %) from muscle protein at the end of the incorporation of the tracer and AUC is the area under the curve of muscle free [1-^{13}C]phenylalanine enrichment (atom % excess) during the incorporation duration. It was assumed that the decay in muscle free [1-^{13}C]phenylalanine enrichment was parallel to that of plasma free [1-^{13}C]phenylalanine enrichment. In the two extra lambs which were not injected with the flooding dose, enrichments of [1-^{13}C]phenylalanine in proteins from muscles had been shown to be similar to the one of plasma proteins. So the basal enrichment of [1-^{13}C]phenylalanine from plasma proteins measured in each lamb before the injection was used to calculate fractional rates of protein synthesis in muscles.

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ANOVA for two test factors with repeated measures was employed to analyse the effects of time (within-group factor) and HMB feeding (between-group factor) on plasma hormone and amino acid concentrations. The effect of HMB dietary supplementation on phenylalanine fluxes and fractional rates of protein synthesis was assessed by Student's two-tailed t test. The effect of HMB infusion in the control group was analysed using the paired two-tailed t test. For all statistical tests, differences were considered significant at $P < 0.05$.

RESULTS

Postprandial plasma hormones and amino acids

Food ingestion led to a significant increase in plasma insulin, but not IGF-1, concentration (Table 1). HMB feeding did not significantly modify these hormone concentrations.

Plasma concentrations of most free amino acids were higher in the HMB group than in the control group (Table 2). This increase was significant ($P < 0.05$) for methionine, phenylalanine, lysine, histidine, proline and citrulline. The same tendency ($P < 0.1$) was registered for three additional amino acids (serine, arginine, and ornithine). We had checked that there was no difference between the two groups for any amino acid before the beginning of the experiment (results not shown). Food ingestion led to significant changes ($P < 0.05$) in plasma levels of threonine, serine, lysine, methionine, arginine, tyrosine, the sum of glutamate and glutamine, glycine, proline, citrulline, ornithine, 1-methylhistidine and 3-methylhistidine.

Phenylalanine fluxes

The specific radioactivity of plasma free [1-^{14}C]phenylalanine plateaued at 13–15 disintegrations/min (dpm)/nmol and expired $^{14}CO_2$ at 35–45 dpm/min (Table 3). Plasma free tyrosine was found to be labelled. Its specific radioactivity was about 5 % of the specific radioactivity of [1-^{14}C]phenylalanine.

The basal whole-body phenylalanine flux was 0·9 μmol/min per kg body weight in the control group (Table 3). It increased slightly, but not significantly, after HMB infusion. Similarly whole-body phenylalanine flux tended to be higher (also not significantly) in lambs receiving the dietary HMB supplement compared with controls under basal

Table 1. *Protocol B. Changes in the concentrations of insulin and insulin-like growth factor-1 (IGF-1) with time after feeding in sheep receiving a dietary supplement of 3-hydroxy-3-methylbutyrate (HMB) and controls*

(Mean values for five lambs with their pooled standard error)

		Postprandial time (h)					
Hormone	Group	0	3	7	11	15	SE
Insulin (μU/ml)	Control	10	17	24	20	17	7
	HMB	16	19	34	25	25	2*
IGF-1 (nmol/ml)	Control	42	52	45	58	59	6
	HMB	52	52	52	60	48	5

*Difference within the group (effect of time) was significant, $P < 0.05$. There was no significant interaction between the factors for any hormone.

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Table 2. *Protocol B. Changes in the concentrations of plasma free amino acids (nmol/ml) with time after feeding in sheep receiving a dietary supplement of 3-hydroxy-3-methylbutyrate (HMB) and controls*

(Mean values for five lambs with their pooled standard error)

Amino acid	Group	Postprandial time (h) 0	3	7	11	15	SE
Thr	Control	93	85	80	80	72	39
	HMB	127	127	115	108	103	6†††
Ser	Control	43	50	47	56	55	9
	HMB	56	64	63	64	59	3†
Glu + Gln	Control	179	206	194	183	165	33
	HMB	171	216	197	177	164	8†††
Gly	Control	538	445	399	406	435	97
	HMB	631	563	483	484	511	18†††
Ala	Control	164	172	167	172	156	32
	HMB	192	195	196	178	176	10
Cit	Control	186	146	112	118	128	42*
	HMB	254	211	170	177	190	7†††
Val	Control	148	133	127	139	134	57
	HMB	195	194	190	185	177	9
Met	Control	23	26	21	20	18	4*
	HMB	31	31	29	25	24	3††
Ile	Control	68	60	67	69	56	18
	HMB	86	81	84	78	72	5
Leu	Control	94	77	87	100	87	32
	HMB	131	120	133	133	121	8
Tyr	Control	90	94	84	82	83	10
	HMB	102	109	96	86	99	4††
Phe	Control	52	53	52	55	50	6**
	HMB	66	68	69	64	65	3
Orn	Control	79	95	93	86	79	33
	HMB	137	154	138	121	104	9††
Lys	Control	134	123	112	98	78	24*
	HMB	197	181	131	110	110	10†††
1met His	Control	159	180	157	141	169	34
	HMB	167	185	150	139	174	5†††
His	Control	62	63	68	68	61	9*
	HMB	78	76	83	79	75	3
3met His	Control	68	65	63	61	62	21
	HMB	65	63	57	55	58	1†††
Arg	Control	134	156	146	123	108	30
	HMB	181	203	191	143	143	9†††
Pro	Control	106	109	109	105	94	14*
	HMB	144	140	126	116	113	6††

Differences between control and HMB groups were significant: * $P < 0.05$, ** $P < 0.01$.
Differences within groups (effect of time) were significant: † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$.
There was no significant interaction between the factors for any amino acid.

conditions. These tendencies, although not significant, existed with respect to both oxidative and non-oxidative phenylalanine disposals.

Muscle fractional rates of protein synthesis

The flooding dose injection of [1-^{13}C]phenylalanine led to a large increase in plasma free phenylalanine level. At the end of the 90 min incorporation values were 762 (SE 159) and

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Table 3. *Protocol A. Phenylalanine and tyrosine specific radioactivities and expired $^{14}CO_2$, and total, oxidative and non-oxidative phenylalanine fluxes in sheep receiving a dietary supplement of 3-hydroxy-3-methylbutyrate (HMB) infused with saline for 3 h (basal), and controls infused sequentially with saline and HMB**

(Mean values with their standard errors for six sheep)

Group...	Control				HMB	
Condition...	Basal		HMB-infused		Basal	
	Mean	SE	Mean	SE	Mean	SE
^{14}C phe (dpm/nmol)	14·8	1·7	13·5	1·7	12·6	1·7
^{14}C tyr (dpm/nmol)	0·67	0·03	0·74	0·03	0·71	0·05
$^{14}CO_2$ (dpm/min)	34·7	4·2	45·8	4·7	40·6	3·4
Flux (μmol/min per kg)						
Total	0·90	0·10	0·99	0·13	1·04	0·13
Oxidative	0·08	0·01	0·10	0·01	0·12	0·02
Non-oxidative	0·82	0·09	0·89	0·12	0·92	0·12

*For details of procedures, see Fig. 1 and pp. 886–888. No significant effect of HMB dietary supplementation or HMB infusion was observed.

838 (SE 178) nmol/ml in the control and HMB groups respectively, which corresponded to at least ten times the preinjection level. The enrichment of plasma free [1-^{13}C]phenylalanine decreased significantly from 21·3 (SE 0·2) and 21·4 (SE 0·1) atom % excess 15 min after the injection to 16·7 (SE 0·5) and 16·3 (SE 0·3) atom % excess 70 min post-injection in the control and HMB groups respectively. Because this decrease was significant, the slope of the linear curve of enrichment *v.* time was taken into account to calculate the area under the curve of the enrichment of muscle free [1-^{13}C]phenylalanine *v.* time. Muscle free [1-^{13}C]phenylalanine concentration and enrichment were 625–722 nmol/g and 15·9–17·5 atom % excess respectively. Mean fractional rates of protein synthesis were 4·1 (SE 0·7), 3·0 (SE 0·3) and 3·3 (SE 0·2) %/d in diaphragm, *tensor fasciae latae* and *longissimus dorsi* muscles respectively in control lambs. In the HMB group they were 4·4 (SE 0·5), 2·9 (SE 0·2) and 3·2 (SE 0·2) %/d, respectively. So, dietary supplementation of HMB did not significantly modify fractional protein synthesis rates in skeletal muscles. The flooding dose of phenylalanine resulted in a significant increase in plasma insulin level (Table 4). In addition plasma insulin concentration was significantly higher in the HMB group than in the control group.

Table 4. *Protocol C. Plasma insulin concentration (μU/ml) after a flooding injection of [^{13}C]phenylalanine in sheep receiving a dietary supplement of 3-hydroxy-3-methylbutyrate (HMB) and controls*

(Mean values for five lambs with their pooled standard error)

	Time (min)					
Group	0	15	30	50	70	SE
Control	16	26	18	19	22	6*
HMB	22	36	34	32	24	2†

* Difference between control and HMB groups was significant, $P < 0.05$.
† Difference within groups (effect of time) was significant, $P < 0.05$.
There was no significant interaction between the factors.

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DISCUSSION

The present study was designed to investigate the metabolic activity of HMB on protein metabolism in growing lambs. HMB administration was well tolerated by all lambs and no adverse effects were observed. The amount of HMB provided to lambs in the diet or by infusion was very high compared with the basal endogenous production. Indeed about 10 % of leucine is oxidized via the dioxygenase pathway (Van Koevering & Nissen, 1992), the equivalent of a daily endogenous production of HMB of about 120 mg in a 40 kg lamb. Further metabolism of HMB occurs, but it has not been characterized. The excretion of HMB into the urine has been estimated to be 34 % of the endogenous production in 40 kg lambs (Van Koevering & Nissen, 1992).

Only plasma free amino acid studies revealed a significant effect of dietary supplementation with a high dose of HMB in growing lambs. Plasma concentrations of free amino acids were generally higher in the HMB group than in the control group ($P < 0{\cdot}05$ for methionine phenylalanine, lysine, histidine, proline and citrulline, $P < 0{\cdot}1$ for serine, arginine and ornithine). The origin of these variations was not evident. The potential action of HMB-induced-hyperaminoacidaemia on protein metabolism is discussed later.

We analysed the effect of HMB on whole-body protein turnover using the constant infusion method. In ruminants, fluxes are usually quantified with the infusion of labelled leucine (Lobley, 1993). We chose phenylalanine instead of leucine as a tracer in order to avoid any eventual specific effect of HMB on the metabolism of its precursor, leucine. Unlike leucine, phenylalanine has no plasma metabolite that reflects its intracellular labelling, so total flux was calculated using the specific radioactivity of plasma free [1-^{14}C]phenylalanine. Phenylalanine is irreversibly converted to tyrosine in the liver, then tyrosine is transported out of the liver and delivered to the cells from the plasma. Specific radioactivity of plasma free [1-^{14}C]tyrosine was only 5 % of that of [1-^{14}C]phenylalanine due to its dilution by unlabelled tyrosine coming from the digestive tract and protein degradation. Similar labelling was observed in humans infused with stable labelled phenylalanine (Thompson *et al.* 1989; Zello *et al.* 1993). Oxidative flux was calculated according to Sanchez *et al.* (1995) also using plasma free [1-^{14}C]phenylalanine as the precursor pool. Plasma free [1-^{14}C]tyrosine can not be used as the precursor for oxidation of phenylalanine. It is likely that the absolute value of phenylalanine oxidation was underestimated since we did not have values for the intracellular phenylalanine labelling to use as the precursor for oxidation. Non-oxidative flux would have been a more accurate method of calculation, i.e. by subtracting the irreversible loss of phenylalanine via its hydroxylation to total flux. However hydroxylation can be quantified only by infusing both labelled phenylalanine and labelled tyrosine (Clarke & Bier, 1982; Zello *et al.* 1990). In the present study, neither acute infusion nor chronic dietary supplementation modified total, non-oxidative and oxidative fluxes of phenylalanine.

The fractional rate of protein synthesis was quantified in skeletal muscles which contribute a large part of whole-body protein synthesis (Attaix *et al.* 1988). The method of choice to investigate protein synthesis in tissues in animals is the flooding dose method initially used in rats (Garlick *et al.* 1980) and subsequently adapted to ruminants (Attaix *et al.* 1986; Lobley, 1993). The supplementation of the diet with HMB did not change the fractional rate of protein synthesis in skeletal muscles from growing lambs. We previously showed that a 4 h leucine infusion did not modify skeletal muscle protein synthesis in sucking lambs, even when the leucine-induced-hypoaminoacidaemia was compensated for by infusing an appropriate amino acid mixture (Papet *et al.* 1992). In man leucine infusion also failed to increase protein synthesis (Louard *et al.* 1990; Nair *et al.* 1992).

An effect of HMB on whole-body protein turnover and skeletal muscle protein synthesis would have been expected since aminoacidaemia was higher in HMB-fed than in control lambs. Indeed hyperaminoacidaemia has been shown to increase whole-body protein synthesis and oxidation in man (Bennet *et al.* 1989), pigs (Watt *et al.* 1992) and goats (Tesseraud *et al.* 1993). In the present experiment variation in aminoacidaemia was probably too small to stimulate whole-body protein turnover. Similarly, physiological hyperaminoacidaemia failed to increase skeletal muscle protein synthesis in fed goats (Tauveron *et al.* 1994) and only very high or long-term unphysiological hyperaminoacidaemia had a stimulatory effect in rats (Mosoni *et al.* 1993).

The HMB-induced increase in plasma amino acids suggests that whole-body proteolysis should have been higher than whole-body protein synthesis. Alternatively the increase in plasma amino acids induced by the dietary HMB supplementation could have resulted from an increase in amino acid absorption. In the present study no attempt was made to quantify amino acid absorption or whole-body proteolysis. In ruminants, amino acids absorbed by the intestine are derived from microbial proteins synthesized in the rumen (Vérité & Peyraud, 1989). It is well known that aminoacidaemia depends on ruminal fermentation but it is difficult to determine whether HMB, which is not degraded in the rumen, could have modified fermentations and consequently plasma amino acid levels. However, an increase in amino acid absorption in lambs given a high dose of HMB cannot be completely ruled out. We also questioned whether HMB could have inhibited amino acid transport from plasma to tissues, thereby leading to increased plasma amino acid concentrations. Each tissue exhibits various amino acid transport systems, e.g. A, ASC, L and y^+ (Christensen, 1990; Kilberg *et al.* 1993). Competition between amino acids sharing the same transport system can theoretically occur when the plasma level of one amino acid increases. However, the mechanism of amino acid transport regulation *in vivo* is not clear (Christensen, 1990). Based on the structural analogy between leucine and HMB, one can speculate that HMB could compete with amino acids transported by the L system. However, even with the high dose of HMB, the plasma HMB level was probably much lower than plasma amino acid levels; so it is unlikely that competition occurred in the present experiment.

In conclusion, administration of a high dose of HMB increased plasma levels of some amino acids. These variations were not associated with any change in whole-body protein turnover or skeletal muscle protein synthesis. Further studies are needed to determine mechanisms responsible for the positive effects of HMB administration in various farm animals.

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