Energy expenditure associated with sodium/potassium transport and protein synthesis in skeletal muscle and isolated hepatocytes from hyperthyroid sheep

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The object of the present study was to determine the effect of thyroxine (T_4) treatment of sheep on protein synthesis and associated energy costs in skeletal muscle and hepatocytes. Protein synthesis, and ouabain-sensitive and cycloheximide-sensitive respiration in isolated intercostal muscle and hepatocytes were determined in sheep after 5 weeks of daily injections of either saline or T_4. Plasma T_4 and total triiodothyronine (T_3) concentrations were doubled and free T_3 concentrations were quadrupled by T_4 injections. The fractional rates of protein synthesis increased in isolated external intercostal muscle and hepatocytes from hyperthyroid sheep. Fractional rates of protein synthesis in isolated external intercostal muscle and hepatocytes were linearly correlated with plasma free T_3 concentrations. Total oxygen consumption of muscle and hepatocytes was unaffected by T_4 injections. Ouabain-sensitive respiration increased in hepatocytes and muscle of T_4-treated animals. Cycloheximide-sensitive respiration was elevated in hepatocytes from hyperthyroid sheep. Cycloheximide-sensitive respiration in muscle was unaffected by T_4 treatment. The present experiment demonstrates that T_4 increases protein synthesis in ruminants. The energy expenditure in support of Na^+, K^+-ATPase and protein synthesis in skeletal muscle and hepatocytes may account for 34–60% of total cellular energy expenditure.

Energy expenditure: Protein synthesis: Sodium/potassium transport: Sheep

Thyroid hormones have a significant influence on metabolic rate and thermogenesis in animals (Ismail-Beigi & Edelman, 1970). In part, thyroid hormones enhance thermogenic capacity by increasing Na^+,K^+-ATPase (*EC* 3.6.1.3) synthesis and activity in a variety of tissues, including liver and skeletal muscle (Ismail-Beigi *et al.* 1979; Karin & Cook, 1983). Thyroid hormones also increase the fractional rate of protein synthesis in skeletal muscle, possibly through elevation of RNA synthesis (Brown & Millward, 1983; Jepson *et al.* 1988). However, the combined analysis of protein synthesis and Na^+,K^+-ATPase has not been done under thyroid-hormone induction. Therefore, the object of the present study was to determine the influence of thyroxine (T_4) treatment of sheep on the fractional rate of protein synthesis (K_s) and Na^+,K^+-ATPase activity and their associated energy costs in isolated hepatocytes and skeletal muscle.

EXPERIMENTAL

Animals, feeding and treatment

Ten Suffolk wethers (40 (SE 1·3) kg) were fed twice daily (08.00 and 16.00 hours) on equal amounts of a diet composed of 800 g chopped bromegrass hay (*Bromus* spp.) (901 g dry matter (DM)/kg, 110 g crude protein (CP)/kg and 18·9 MJ gross energy (GE)/kg), 300 g ground maize (823 g DM/kg, 103 g CP/kg, 23·2 MJ GE/kg) and 100 g soya-bean meal (899 g DM/kg, 655 g CP/kg, 19·7 MJ GE/kg). The daily intakes of DM, CP and GE for

the complete diet were 1058 g, 154 g and 21·2 MJ respectively. Water and trace-mineral salt were offered *ad lib*. Animals were randomly assigned to two experimental groups. One group received a daily subcutaneous injection of 1 mg L-thyroxine (Sigma Chemical Co., St Louis, MO) in 1 ml saline (9 g sodium chloride/l) and the other group an injection of 1 ml saline. Injections were administered over a 5 week period and body-weights were monitored weekly over the duration of treatment. Blood samples were taken via jugular venous puncture immediately before the animal was killed, and the plasma separated and stored at −20°. Plasma was later analysed for insulin, thyroxine, triiodothyronine (T_3) and free T_3 using commercial kits (Diagnostic Products Corporation TKIN1, TKT_4, TKT_3, $TKfT_3$) supplied by Intermedico (Willowdale, Ontario). After 5 weeks of daily injections, animals were killed and hepatocytes and external intercostal muscle bundles isolated by the procedures described below.

Tissue isolation

External intercostal muscle bundles (including part of the tendons) were carefully excised from the ribs by the method of Wijayasinghe *et al.* (1984). Muscle samples were kept in oxygenated incubation media at 37° until further processing. The incubation medium was a Krebs–Henseleit buffer (Dawson *et al.* 1969) containing 10 mM-D-glucose and sodium acetate, sodium propionate, sodium butyrate and amino acids at concentrations similar to those found in sheep plasma (Heitmann & Bergman, 1980). With the aid of a dissecting microscope, tissues were further dissected into six to eight muscle bundles (20–35 mg each). Tissues were attached to small supports to simulate resting muscle length, during incubations for the measurement of protein synthesis and oxygen consumption. Results by Wijayasinghe *et al.* (1984) have shown that intact external intercostal muscle preparations exhibit a high viability as evidenced by the maintenance of in vivo concentrations of ATP and creatine phosphate throughout 1–4 h of incubation. Furthermore, they showed that the rates of protein synthesis in external intercostal muscle preparations from sheep, as determined by phenylalanine incorporation into protein, remain linear during 1–4 h of incubation.

Hepatocytes were prepared as described by McBride & Milligan (1985*a*). Briefly, the caudate lobe of the liver was isolated by the procedure of Clark *et al.* (1976) and a catheter placed in a major vessel. The liver was first perfused with a calcium-chelating buffer which consisted of a gassed (O_2–carbon dioxide; 95:5, v/v) modified Hanks buffer (pH 7·4 (SE 0·1), 0·5 mM-EGTA, 26·1 mM-sodium bicarbonate; Moldeus *et al.* 1978). The liver was then perfused with a digestion buffer which consisted of a gassed (O_2–CO_2; 95:5, v/v) modified Krebs–Henseleit buffer (pH 7·4 (SE 0·01) Dawson *et al.* 1969) containing 5 mM-Ca^{2+}, 1 mg collagenase IV (*EC* 3.4.24.3) (Sigma Chemical Co.)/ml and 20 mM-Hepes buffer. Cells were freed from the tissue with a stainless-steel comb and then filtered, centrifuged (50 ***g***; 1 min) and resuspended in the incubation media as described by McBride & Milligan (1985*a*). Portions of the cell suspensions (100 μl) were then allotted to studies on either protein synthesis or O_2 consumption as described later. The viability of the hepatocytes averaged 95·6 (SE 1·2)% as determined by trypan blue exclusion (Seglen, 1976). Previous experiments with sheep hepatocytes showed that the rate of incorporation of phenylalanine into protein remained linear from 1 to 3 h of incubation (McBride & Early, 1987).

In vitro protein synthesis

Muscles were incubated at 37° in 4·0 ml of the incubation media that additionally contained 0·1 μg insulin and 1 μCi L-[ring-2,6-^{3}H(N)]phenylalanine in a final concentration of 2·4 mmol phenylalanine. The duration of incubation was 2 h. On completion of the incubation, the muscle bundles were immediately frozen in liquid nitrogen and stored at −20° until further processing.

Hepatocytes were incubated under the conditions described previously. The final density of cells in the incubation media was 1×10^5 cells/ml. After a 2 h incubation, the cells were separated from the incubation media by centrifugation (50 *g*; 1 min) at 4°, and then stored at −20° until further processing.

To determine tissue protein specific activity, tissues were homogenized in 2 ml of 2% ice-cold perchloric acid using an all glass Potter–Elvehjem apparatus. Centrifugation at 2400 *g* separated the precipitate and supernatant fraction (discarded). The protein precipitate was then washed twice with 2 ml methanol. Precipitates were hydrolysed at 100° for 24 h in 500 μl 6 M-hydrochloric acid under N_2. After hydrolysis, the acid was removed under vacuum at room temperature, reconstituted in exactly 2 ml 0·1 M-HCl that contained exactly 1 μmol norleucine as the internal standard, and filtered through a 0·22 μm filter. Norleucine and phenylalanine contents were determined on 50 μl filtrate by high-performance liquid chromatography (HPLC) as phenylthiocarbamyl derivatives (Bidlingmeyer *et al.* 1984). Radioactivity in exactly 375 μl filtrate in 5 ml ACS liquid-scintillation cocktail (Amersham Co., Oakville, Ontario, Canada) was counted using a LKB Rack Beta liquid-scintillation counter (model 1212; Turku, Finland). Counting efficiency was determined by the channels' ratio after adding 2000 counts of unquenched [^{3}H]phenylalanine to the scintillation vial. Counting efficiency was 25 %.

To determine the specific activity of phenylalanine in the medium, a 2 ml portion of medium was lyophilized, reconstituted in exactly 2 ml 0·1 M-HCl that contained 1 μmol norleucine as described previously, and filtered through a 0·22 μm filter. Norleucine and phenylalanine contents were determined using 300 μl filtrate with HPLC as described previously. Radioactivity in exactly 60 μl filtrate in 5 ml of ACS liquid-scintillation cocktail was counted as described previously. Counting efficiency was determined by the channels' ratio and averaged 36%.

The radioactive purity of the counted samples was determined by derivatizing sufficient amounts of medium and protein-precipitate hydrolysates to give at least 400 counts/min per injection with HPLC. The effluent from the HPLC detector was sequentially collected at 0·5 min intervals and each fraction subjected to liquid-scintillation counting. By this method, 99·0 (SE 1)% of the radioactivity associated with all media and muscle-protein precipitates was found in phenylalanine. In protein precipitates from hepatocytes, however, 38·9 (SE 0·6)% of the radioactivity was found in tyrosine and these samples have been corrected for this radioactivity.

O_2 consumption during protein synthesis and Na^+/K^+ transport. O_2 consumption was determined on muscles in 4·0 ml incubation media or on hepatocyte suspensions. O_2 consumption was monitored polarographically using a YSI model 5300 biological O_2 monitor (Yellow Springs Instrument Co. Inc., Scientific Division, Yellow Springs, Ohio). After 15–20 min of incubation, either cycloheximide (final concentration 10^{-4} M) or ouabain (final concentration 10^{-4} M) was added and the incubation continued for another 15–20 min. The difference between the initial O_2 consumption and the uninhibitable O_2 consumption was termed cycloheximide-sensitive or ouabain-sensitive respiration. Cycloheximide-sensitive respiration represented the energy costs associated with cytosolic-protein synthesis (Siems *et al.* 1984) and ouabain-sensitive respiration represented the energy costs associated with maintenance of Na^+,K^+-ATPase activity (McBride & Milligan, 1985*b*). O_2 consumption rates were expressed on a tissue dry weight basis. Tissue and cell weights were determined after oven-drying at 100° overnight.

Calculations and statistics

The fractional rate of protein synthesis, K_s, was calculated according to Smith *et al.* (1983) as follows:

$$K_s\ (\%/\mathrm{d}) = SA_B/SA_M \times 1440/t,$$

Table 1. *Plasma concentrations of thyroid hormones and insulin in saline (9 g sodium chloride/l)- and thyroxine-treated sheep*†

(Mean values with their standard errors for five sheep)

	Saline		Thyroxine	
	Mean	SE	Mean	SE
Thyroxine (ng/ml)	70	8	140*	5
Triiodothyronine (ng/ml)	1·06	0·14	2·28	0·09
Free triiodothyronine (pg/ml)	19	2	81*	16
Insulin (μUnits/ml)	23	3·9	19·7	0·9

* Mean values were significantly different from those for saline-tested sheep: $P < 0·05$.
† For details of treatments, see p. 674.

Table 2. *Ouabain-sensitive respiration in external intercostal muscle from saline (9 g sodium chloride/l)- and thyroxine-treated sheep*†

(Mean values with their standard errors for five sheep)

	Saline		Thyroxine	
	Mean	SE	Mean	SE
Total oxygen consumption (μl O_2/mg dry wt per h)	2·31	0·13	2·28	0·16
Ouabain inhibition (%)	18·1	1·6	26·5*	1·6
Ouabain-sensitive respiration (μl O_2/mg dry wt per h)	0·41	0·03	0·60*	0·05
Ouabain-insensitive respiration (μl O_2/mg dry wt per h)	1·90	0·14	1·68	0·12

* Mean values were significantly different from those for saline-treated sheep: $P < 0·05$.
† For details of treatment, see p. 674.

where SA_B and SA_M are protein-bound and free phenylalanine specific radioactivities in the incubation medium respectively, and t is time (min). A t test was used to determine significant ($P < 0·05$) differences between treatment means (Steel & Torrie, 1960). Relationships between variables were determined by Pearson correlation coefficients and linear regression. All analyses were performed using SAS (Statistical Analysis System Inc., 1982) statistical programs.

RESULTS

Daily T_4 injections approximately doubled plasma T_4 and T_3 concentrations and quadrupled free T_3 concentrations, whereas insulin concentration did not change (Table 1). Total O_2 consumption in external intercostal muscle was unaffected by T_4 treatment (Table 2). However, the percentage and total amount (μl O_2/mg dry weight per h) of O_2 consumption inhibited by ouabain increased ($P < 0·05$) in T_4-treated animals. Ouabain-insensitive O_2 consumption in the external intercostal muscle was unaffected by T_4. Total O_2 consumption in hepatocytes was also unaffected by T_4 treatment (Table 3). However, the total amount (μl O_2/mg dry weight per h), but not the percentage, of O_2 consumption inhibited by ouabain increased ($P < 0·05$) in the hepatocytes of T_4-treated animals. Ouabain-insensitive O_2 consumption in the hepatocytes was unaffected by T_4.

Table 3. *Ouabain-sensitive respiration in hepatocytes from saline (9 g sodium chloride/l)- and thyroxine-treated sheep*†

(Mean values with their standard errors for five sheep)

	Saline		Thyroxine	
	Mean	SE	Mean	SE
Total oxygen consumption (μl O_2/mg dry wt per h)	6·15	0·35	6·77	0·45
Ouabain inhibition (%)	28·6	3·0	35·7	2·9
Ouabain-sensitive respiration (μl O_2/mg dry wt per h)	1·77	0·22	2·36*	0·07
Ouabain-insensitive respiration (μl O_2/mg dry wt per h)	4·39	0·29	4·47	0·45

* Mean values were significantly different from those for saline-treated sheep: $P < 0·05$.
† For details of treatment, see p. 674.

Table 4. *Fractional rate of protein synthesis (%/d) in external intercostal muscle and hepatocytes from saline (9 g sodium chloride/l)- and thyroxine-treated sheep*†

(Mean values with their standard errors for five sheep)

	Saline		Thyroxine	
	Mean	SE	Mean	SE
External intercostal muscle	3·04	0·22	5·72*	0·7
Hepatocytes	28·6	3·7	50·1*	3·0

* Mean values were significantly different from those for saline-treated sheep: $P < 0·05$.
† For details of treatment, see p. 674.

Table 5. *Cycloheximide-sensitive respiration in external intercostal muscle from saline (9 g sodium chloride/l)- and thyroxine-treated sheep**

(Mean values with their standard errors for five sheep)

	Saline		Thyroxine	
	Mean	SE	Mean	SE
Total oxygen consumption (μl O_2/mg dry wt per h)	2·29	0·25	2·25	0·19
Cycloheximide inhibition (%)	17·5	1·4	22·6	3·0
Cycloheximide-sensitive respiration (μl O_2/mg dry wt per h)	0·39	0·06	0·53	0·10
Cycloheximide-insensitive respiration (μl O_2/mg dry wt per h)	1·81	0·22	1·71	0·11

* For details of treatment, see p. 674.

Values from both external intercostal muscle and hepatocytes indicated that energy expenditures associated with Na^+–K^+ transport increased in T_4-treated animals.

K_s values for both external intercostal muscle and hepatocytes were approximately doubled ($P < 0·05$) in T_4-treated animals (Table 4). However, the live-weight change during the experimental period in the T_4-treated animals (−40·0 (SE 3·6) g/d) was significantly ($P < 0·01$) different from that of the control animals (78·6 (SE 0·4) g/d).

Table 6. *Cycloheximide-sensitive respiration in hepatocytes from saline (9 g sodium chloride/l)- and thyroxine-treated sheep*†

(Mean values with their standard errors for five sheep)

	Saline		Thyroxine	
	Mean	SE	Mean	SE
Total oxygen consumption (μl O_2/mg dry wt per h)	5·52	0·53	6·37	0·49
Cycloheximide inhibition (%)	15·5	2·9	24·4*	1·6
Cycloheximide-sensitive respiration (μl O_2/mg dry wt per h)	0·88	0·19	1·50*	0·20
Cycloheximide-insensitive respiration (μl O_2/mg dry wt per h)	4·64	0·43	4·70	0·31

* Mean values were significantly different from those for saline-treated sheep: $P < 0·05$.
† For details of treatment, see p. 674.

In cycloheximide-inhibition experiments (Table 5), total O_2 consumption and cycloheximide-sensitive respiration in external intercostal muscle were unaffected by T_4 treatment. In cycloheximide-inhibition experiments on hepatocytes (Table 6), total O_2 consumption was unaffected by T_4 treatment. The total amount and percentage of O_2 consumption inhibited by cycloheximide increased ($P < 0·05$) in the hepatocytes of T_4-treated animals. Cycloheximide-insensitive O_2 consumption in the hepatocytes was unaffected by T_4.

DISCUSSION

The object of the present study was to determine the effect of T_4 on protein synthesis and associated energy costs in the ruminant. Daily T_4 injections induced approximately a twofold increase in T_4 and T_3 concentrations and a fourfold increase in free T_3 concentration. The magnitude of increase in T_3 was similar to that found in mildly-hyperthyroid rats (Brown & Millward, 1983). At this concentration, K_s (%/d) values were increased 13, 20 and 48% in the gastrocnemius, plantaris and soleus muscles respectively (Brown & Millward, 1983). Increasing the injected dose of T_3 by as much as fifty-fold, however, did not improve K_s (Brown & Millward, 1983). Mild hyperthyroidism in the present study increased external intercostal muscle K_s by 88%, but these values were still considerably less than those observed in the rat (Brown & Millward, 1983). K_s in hepatocytes showed a similar response (75% increase) to T_4 injections. Brown *et al.* (1981) have also shown that treatment with T_3 increases K_s and fractional rates of protein degradation in skeletal muscle of rats. Furthermore, the relative increase in the fractional degradation rate compared with the control animals was higher than the increase reported for protein synthesis, suggesting a negative N balance status in muscle. In the present study, the T_4-treated animals lost body-weight during the treatment period, suggesting that whole-body protein fractional degradation rates exceeded protein synthesis rates, even though the protein synthesis rates were elevated in skeletal muscle and hepatocytes. This is consistent with the known stimulation of lysosomal proteinases (De Martino & Goldberg, 1978) and Ca^{2+}-activated neutral proteinase (*EC* 3.4.24.4; Zeman *et al.* 1986) in skeletal muscle by free T_3.

Plasma free T_3 concentrations were linearly correlated with K_s of muscle (r 0·77, $P < 0·01$; Fig. 1) and hepatocytes (r 0·84, $P < 0·01$; Fig. 2). Similar results have been

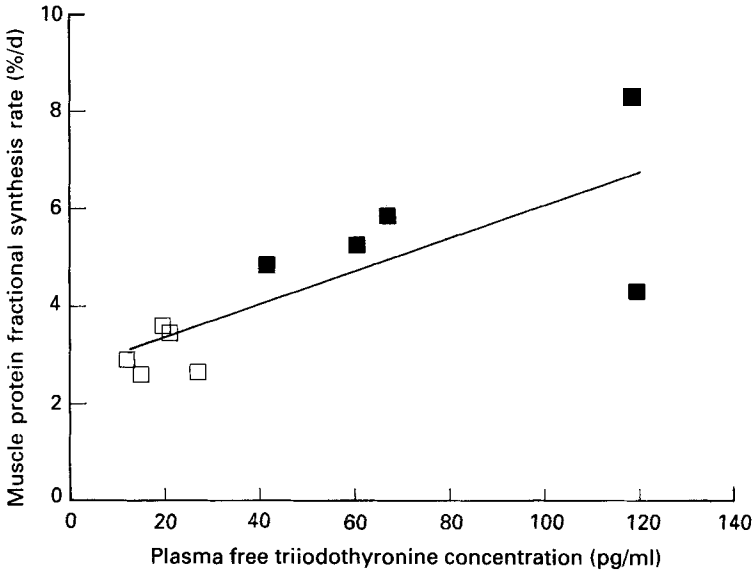

Fig. 1. Relation between muscle protein synthesis (%/d) and plasma free triiodothyronine (pg/ml) in control, saline (9 g sodium chloride/l)-treated (□) and thyroxine-treated (■) sheep. r 0·77, $P < 0·01$; $y = 0·034x + 2·69$. For details of treatments see p. 674.

demonstrated in rats by Jepson *et al.* (1988). Given that insulin status did not change in T_4-treated animals (Table 1), it is possible that free T_3 had a direct effect on muscle and hepatocyte protein synthesis, possibly through elevation of tissue RNA content, as has been previously reported for rats (Flaim *et al.* 1978; Jepson *et al.* 1988).

Ruminant skeletal muscle and hepatocytes appear insensitive to insulin induction of protein synthesis both in vivo and in vitro (Early *et al.* 1988*a*, *b*). Even treatment of ovine muscle cells, cultured in fetal calf serum, with pharmacological doses of insulin has no effect on protein synthesis (Harper *et al.* 1987). In contrast, insulin is a potent stimulator of skeletal muscle protein synthesis in rodents (Garlick *et al.* 1985). However, insulin-like growth factor 1 (IGF-1) and epidermal growth factor (EGF) do increase protein synthesis and decrease protein degradation in ovine muscle cells (Harper *et al.* 1987). Free T_3 may be another key hormone in the enhancement of protein synthesis in the ruminant. If free T_3 increases protein synthesis capacity by increasing RNA concentration then free T_3 and IGF-1 should act synergistically to increase protein synthesis, since IGF-1 increases amino acid uptake in isolated cells (Moses & Pilistine, 1985). Augmentation of free T_3 and peptide growth-factor concentrations (IGF-1 and EGF) by nutritional or hormone therapy, or both, may provide a means to enhance skeletal muscle growth in domestic ruminants.

In order to maintain greater rates of protein synthesis, energy expenditures associated with protein synthesis and related supportive functions would be expected to increase as well. Surprisingly, cycloheximide-sensitive respiration, which reflects energy costs associated with protein synthesis, was not significantly increased in the isolated external intercostal muscles of T_4-treated animals, although a trend was evident. However, cycloheximide-sensitive respiration was significantly increased in the hepatocytes of T_4-treated animals. Allowing for differences in O_2 consumption, the cost of protein synthesis was proportionally similar in muscle and hepatocytes (16–24% of total cellular respiration). These values were also similar to observations in non-ruminants (Reeds *et al.* 1985).

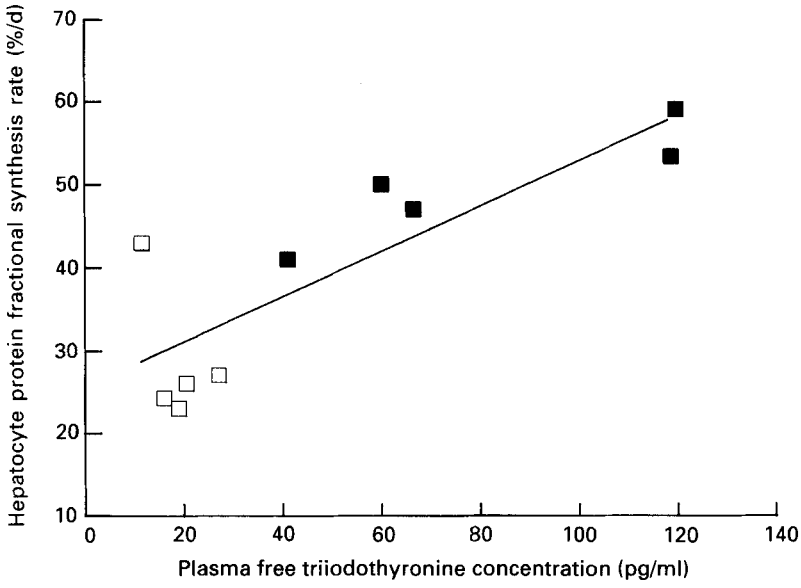

Fig. 2. Relation between hepatocyte protein synthesis (%/d) and plasma free triiodothyronine (pg/ml) in control, saline (9 g sodium chloride/l)-treated (□) and thryoxine-treated (■) sheep. r 0·84, $P < 0·01$; $y = 0·276x + 25·52$. For details of treatment see p. 674.

It was interesting that the energy cost not associated with protein synthesis was unaffected by T_4. But, certainly, there could be a cost within this fraction that could be affected by T_4 and supportive of protein synthesis. Of the cycloheximide-insensitive respiration fraction, Na^+/K^+ transport probably constitutes the greatest proportion. Increasing Na^+/K^+ transport is associated with an increase in protein synthesis in chick myotubes (Vandenburgh & Kaufman, 1981; Vandenburgh, 1984). The increase in protein synthesis may partially result from an increase in the transport of amino acids (Christensen, 1982) and glucose (Schultz & Curran, 1970) that are Na^+-dependent and required for protein synthesis. Karin & Cook (1983) have reported that T_3 increases Na^+-pump activity in the liver and muscles of non-ruminant species. Gregg & Milligan (1987) have also demonstrated this effect in ruminants with liver biopsies. The effect of T_4 administration on ouabain-sensitive respiration in the present study showed similar responses in isolated hepatocytes. Moreover, the present findings also demonstrated the same effect on isolated external intercostal muscle. Therefore, it seems probable that the increased energy expenditure to support protein synthesis in T_4-treated animals was in part due to additional energy expenditure associated with support processes such as Na^+/K^+ transport.

The present experiment demonstrates that T_4 increases protein synthesis and the energetic costs directly involved in, and supportive of, protein synthesis in ruminants. It also confirms the results from Siems *et al.* (1984) that the energy expenditure in support of Na^+,K^+-ATPase and protein synthesis can account for a significant percentage (50) of cellular energy expenditure. Support of these two processes totalled 34–49 and 44–60% of the total energy expenditure in skeletal muscle and hepatocytes of young sheep respectively. Furthermore, it is conceivable that manipulation of background processes such as Na^+/K^+ transport, associated with protein synthesis, could alter the energetic efficiency of growth. Further research to examine the relationship between activation of the Na^+-pump, protein synthesis and growth is in progress.

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