

Original article

Effect of protein deficiency on some hypothalamic and pituitary hormones in growing male lambs. An immunohistochemical study

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Summary — Growing male lambs were fed with diets containing 14.0, 10.8 and 7.6% protein for 3 months to determine their effects on the content of hypothalamic LHRH and SRIH and pituitary LH and GH, using immunohistochemical methods. Lowering the concentration of dietary proteins caused marked changes in the immunoreactivity of these hormones. The immunoreactive (IR) content of LHRH stored in the median eminence was enhanced, and the proportion of LH cells and their IR content increased. Opposite effects were observed in the SRIH/GH system; IR SRIH content stored in the median eminence markedly diminished, and, although hyperplasia of GH cells was observed, their IR content was diminished. This study indicates that prolonged restrictions of protein in the diet of growing male sheep affects the immunoreactive content of the investigated hormones. It seems that they suppress LHRH/LH release and augment GH release, possibly via suppression of hypothalamic somatostatin.

protein deficiency — LHRH — LH — SRIH — GH — sheep — immunohistochemistry

Résumé — Effet d'un régime hypoprotéique sur certaines hormones hypothalamiques et hypophysaires chez le bélier en croissance. **Etude immunohistochimique.** Afin d'examiner les effets de l'alimentation en protéines sur les contenus de LHRH et de SRIH dans l'hypothalamus, et de GH et LH, dans l'hypophyse, des béliers en croissance ont reçu pendant 3 mois des régimes contenant respectivement 14,0, 10,8 et 7,6% de protéines; les concentrations en différentes hormones ont été évaluées par des méthodes immunohistochimiques. Lorsqu'on diminue la proportion de protéines dans la nourriture, la quantité de LHRH immunoréactif accumulé dans l'éminence médiane (ME) augmente de façon importante, comme augmente dans l'hypolyse, la proportion de cellules lutéotropes et leur contenu en LH immunoréactive. Inversement, dans le cas de système SRIH/GH, on observe une diminution importante de la SRIH immunoréactive dans la ME et bien qu'on note l'hyperplasie des cellules somatotropes, leur contenu en GH immunoréactive est plus faible. Ces résultats montrent que, chez le bélier en croissance, un régime hypoprotéique prolongé affecte le contenu immunoréactif des hormones étudiées, d'une part en inhibant la libération de LHRH et de LH, et d'autre part, en augmentant la libération de GH, probablement par blocage hypothalamique de la somatostatine.

régime hypoprotéique — LHRH — LH — SRIH — GH — bélier — immunohistochimie

Introduction

The mechanisms mediating the effects of nutrition on the sexual development of mammals are not well understood, especially in relation to hypothalamic and pituitary hormones. Protein malnutrition during the postnatal period has been claimed to cause significant abnormalities in the developing central nervous system, as well as changes in the endocrine system (Morgane *et al.*, 1978; Primstone, 1976). Recent data, obtained from female rats, monkeys and sheep, confirm that the nutritional state of immature animals delays puberty by inhibiting gonadotropin secretion (*e.g.* Foster and Olster, 1985). The central mechanisms responsible for regulating the luteinizing-hormone releasing-hormone (LHRH) pulse generator in cases of malnutrition are unknown. In starved rats, LHRH stored in the median eminence (ME) increases without increasing LHRH release (Warnhoff *et al.*, 1983). Pubertal development in mammals seems to be directly related to whole body growth (*e.g.* Foster and Olster, 1985). One of the factors which plays an important role in the control of somatic growth, and the regulation of the growth hormone (GH) secretion, is somatostatin (SRIH) (Vijayan and McCann, 1977). The administration of SRIH decreases food intake in rats and baboons (Lotter *et al.*, 1981). GH is consistently increased in ruminants on a diet with a chronic energy deficiency (Hart *et al.*, 1978), but it is not known whether the activity of somatostatin is reduced during protein deficiency.

Thus, although an effect of the CNS on the secretion of pituitary hormones has been postulated in cases of under-nutrition, its mechanism of action is unknown. No data are available on changes in the hypothalamic activities of

LHRH and SRIH in growing male sheep during protein deficiency, or their possible interactions with pituitary hormones. In order to obtain such information, an immunohistochemical investigation of the hypothalamus and pituitary in growing male sheep was performed.

Materials and Methods

The experiment was carried out on twelve 3-mo-old male Merino sheep, divided into 3 groups shortly after weaning. The animals were kept in individual pens and fed with a standard diet (see composition in Table I). Rapeseed oil meal was used as the main source of protein. The composition of minerals was the same in all groups and the energy value of all diets was similar. Daily rations were divided into 2 equal feeds, given at about 08.00 and 14.00 h; water was available *ad libitum*. The protein content of the diet for group I (control), was 14.0%, for group II, 10.8% and for group III, 7.6%. The daily food allowance was 9.8% of metabolic body weight (BW—EO.75), adjusted every 2 wk for actual body weight. The lambs remained on experimental diets until 6 mo of age and were then slaughtered in the local abattoir. The daily body weight gain calculated for the whole experimental period in groups I, II and III, was 180 ± 34 , 159 ± 48 and 73 ± 17 g/day, respectively.

Immunohistochemical procedure

Immediately after decapitation, the brains were perfused *via* both internal carotid arteries with 0.1 M phosphate buffered saline (PBS), and subsequently, with PBS containing 4% (w/v) paraformaldehyde and 15% (w/v) water saturated with picric acid. The hypothalami and pituitaries were dissected 30 min after the start of perfusion and fixed for a further 72 h by immersion in the same fixative.

Preparations were washed with 0.01 M PBS, dehydrated in graded alcohols, embedded in paraplast and then cut in coronal planes at 5 μ m thickness. Hormones were localized by the peroxidase labelled antibody method (Nakane and Pierce, 1966) using a

procedure described by Polkowska (1986). The following antisera were used: anti-LHRH and anti-SRIH (ref. 19900 and ref. 19609, respectively) incubating at a dilution of 1% for 72 h at 4 °C; anti-beta LH and anti-GH incubating at dilutions of 1% and 0.5% for 24 h at 4°C. Methodological details, and the specificity of these antibodies, were as described by Dubois (1971), Dubois and Barry (1974) and Dubois and Dubois (1974). Some material was also stained by the intensification method of Liposits *et al.* (1983). The inhibition of anti-hormone serum, with its homologous antigen, was used as a control reaction. The immunoreactivities of antisera were blocked by 4–10 µg of antigen per 1 ml of a 1% dilution of antisera in the case of pituitary hormones, or a 2% dilution of antisera, in the case of hypothalamic hormones. These mixtures of antigen and antisera were incubated for 48 h at 4 °C before use.

Statistical analysis

The percentage of LH and GH cells in the pituitary glands was determined from populations of approximately 6,000 cells. Overall effects of treatments and the preparations of cells were initially analysed by

ANOVA, and, due to the difference in distribution of values found for 1 sheep from the control group, the nonparametric, Kruskal-Wallis test was applied, using the computer program described by Theodorsson-Norheim (1986).

Results

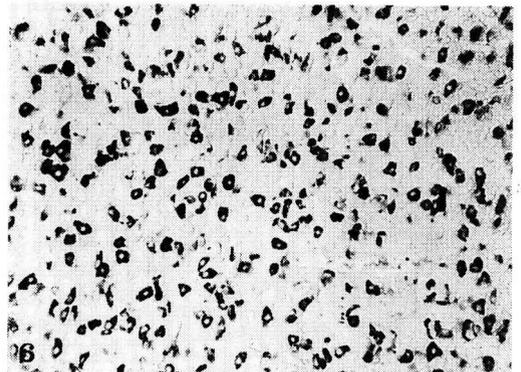
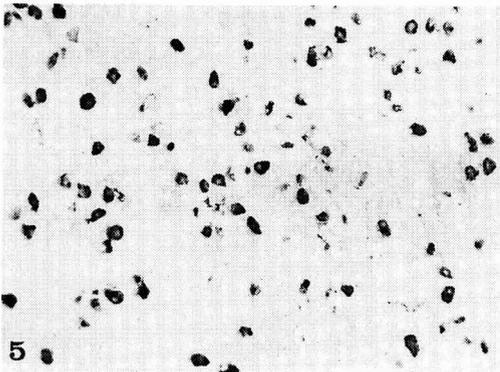
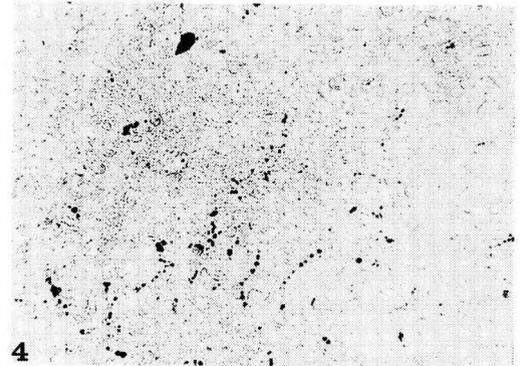
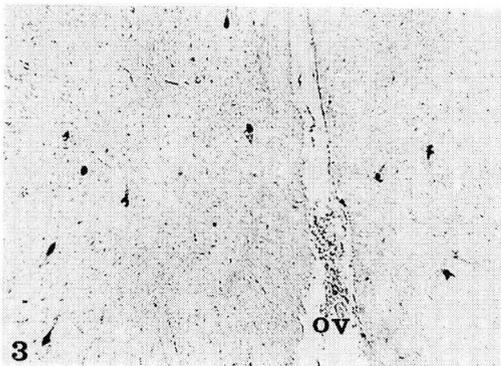
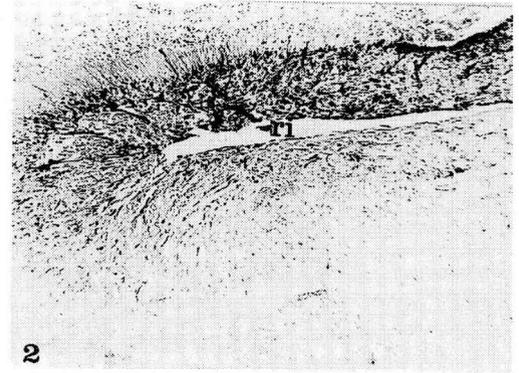
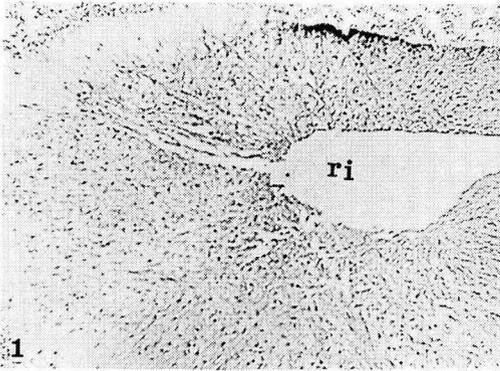
LHRH/LH axis

In control lambs (group I, 14% of dietary proteins), moderate amounts of IR LHRH material were situated in the lateral parts of the ME (Fig. 1). Numerous (\approx 15–25 cells per slide) IR LHRH perikarya, strongly stained for immunoreactive material, were typically localized in the anterior hypothalamo-preoptico-septal region (Fig. 3). Protein restrictions at 10.8% (group II), caused a visible increase of IR LHRH material stored in the nerve

Table I. Composition of diets (g/kg)

Feeds	Group No.		
	I	II	III
Meadow hay	200	200	–
Wheat straw	–	–	200
Rapeseed oil meal	70	–	–
Linseed oil meal	20	–	–
Dried sugar beat pulp	100	500	384
Ground barley	540	230	–
Ground rye	–	–	400
Ground oats	50	50	–
Mineral mixture "Microfos" *	20	20	20
Crude protein (g/kg DM)	136.6	107.9	76.3
Gross energy (MJ/kg DM)	18.7	18.8	17.7

* Composition of "Microfos" in g/kg: P, 124; Ca, 255; Na, 80; Cl, 93; Mg, 4; Mn, 0.164; Fe, 0.6; Cu, 0.21; Co, 0.04; Zn, 1; J, 0.02.



Figs. 1-6. IR LHRH material in the ME of representative lambs from groups I (Fig. 1) and III (Fig. 2). Note the conspicuous accumulation of IR LHRH material in the lamb fed with low protein diet. (x 28); ri-recessus infundibularis; Fig. 3. IR LHRH perikarya in the medial preoptic area of the representative lamb from group I. (x 28); ovorganum vasculosum of the lamina terminalis; Fig. 4. A dense network of IR LHRH axons in the periventricular part of the hypothalamus of the representative lamb from group III. (x 70); Figs. 5 and 6. IRLH cells in the adenohypophysis of representative lambs from groups I (Fig. 5) and III (Fig. 6). Note the increased number and staining intensity of the cells in the lamb fed with low dietary proteins. (x 70).

terminals of the ME, but did not affect the immunocytochemistry of LHRH perikarya. Further lowering of proteins (up to 7.8%) (group III), induced an even greater increase of IR LHRH stored in the ME (Fig. 2). However, the number of visible IR LHRH perikarya markedly diminished in the group. In the area surrounding the organum vasculosum of the lamina terminalis (OVLT), where these perikarya were generally most numerous in controls, only single and very lightly stained LHRH cell bodies were apparent. Also a dense network of IR LHRH axons appeared in some areas where they were not seen in the other groups; they were distributed mainly in the lateral parts of the preoptic area (AP), along lateral parts of the supraoptic recess, above the supra-chiasmatic nucleus and along the wall of the third ventricle (Fig. 4). Such distribution led to the assumption that they belonged to the LHRH pathways running from the LHRH perikarya of the AP to the ME. It should be noted that the IR LHRH network of axons and terminals located in the OVLT, did not show any changes in either experimental group, as compared to controls.

The population of the pituitary LH cells in group II, as well as in group III, doubled, as compared to control group I (Table II, Figs. 5 and 6) ($P < 0.001$), and

showed an intense immunocytochemical reaction. However, the lowering of protein content in the diet, below 10.8%, did not cause any further increase of LH cell population.

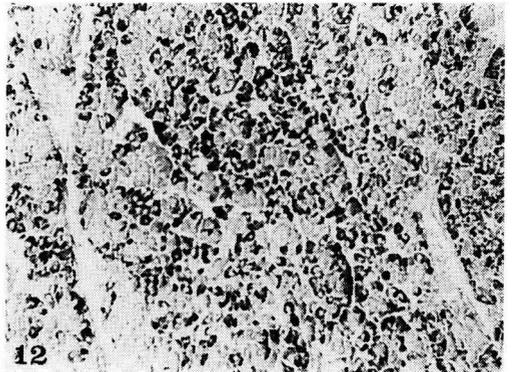
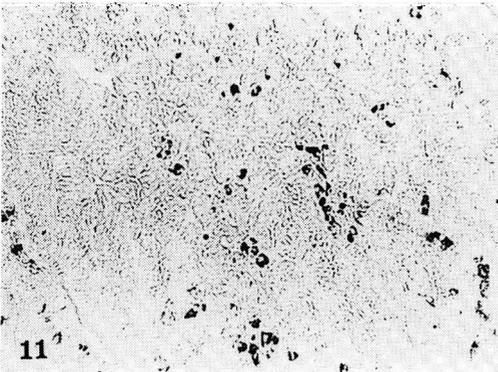
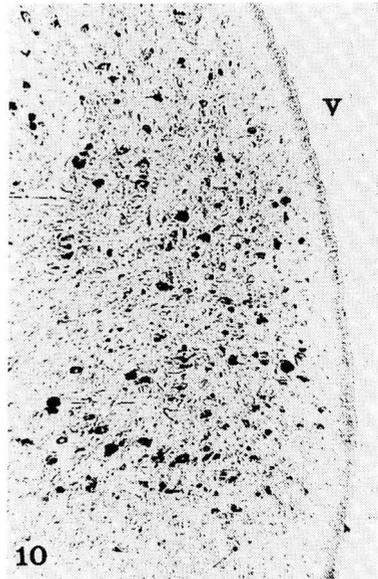
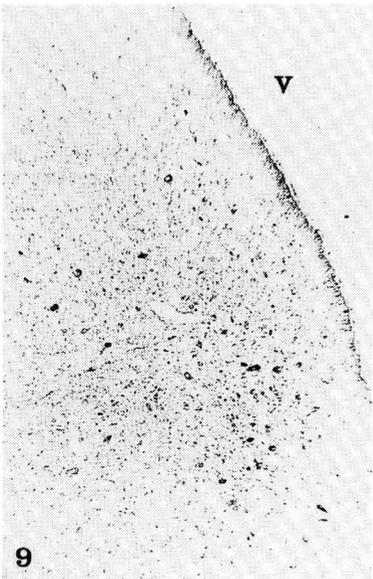
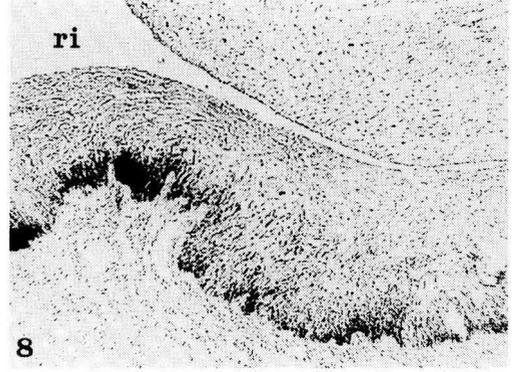
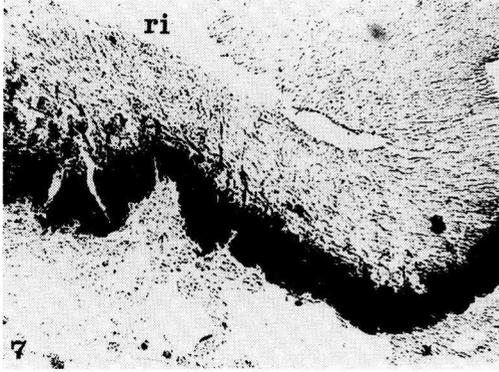
SRIH/GH axis

In the control lambs, a very abundant SRIH material was seen in the central part of the ME and pituitary stalk (Fig. 7). IR SRIH perikarya, typically localized in the periventricular area of the paraventricular and supra-chiasmatic nuclei, were rare and contained little immunoreactive material (Fig. 9). Reduction of proteins in the diet to 10.8% (group II), induced a visible decrease in the amount of IR SRIH material stored in the ME and the pituitary stalk, and an increase in the number of visible perikarya. In the tissue from group III, these changes were much more pronounced. IR SRIH material stored in the ME and the pituitary stalk was very scarce, when compared to the controls and group II (Fig. 8). The neuronal center producing SRIH, and situated in the periventricular area of the hypothalamus, was filled with a large number of strongly stained IR SRIH perikarya. This increase occurred in the same way, in both the supra-chiasmatic, and the paraventricular nuclei (Fig. 10). A very pronounced hyperplasia of pituitary IR GH cells was

Table II. Percentage of LH and GH cells in the pars distalis of pituitary gland (mean \pm SEM).

Group number	Dietary protein level (%)	N	LH cells (%)	GH cells (%)
I (control)	13.7	4	12.0 \pm 0.27 ^d	18.4 \pm 0.26 ^a
II	10.8	4	25.4 \pm 0.72 ^e	28.4 \pm 0.85 ^b
III	7.6	4	24.2 \pm 0.69 ^e	37.8 \pm 0.83 ^c

a, b, c, d, e = Values with different superscripts differ ($P < 0.001$).



observed in both protein-restricted experimental groups, and was inversely proportional to the protein content in the diet. The percentage of GH cells increased by 50% in group II, and doubled in the animals of group III, as compared to the control group (Table II, Figs. 11 and 12) ($P < 0.001$). No special morphological changes were seen in these cells, except for lower immunostaining intensity.

Discussion

Most studies dealing with the relationship between nutrition and the endocrine systems in mammals, have described the effects of acute, severe nutritional conditions like starvation, or generally undefined restriction of diet or dietary components. In such experiments, the animals were deprived, not only of sufficient proteins, but also of energy and other essential dietary components. The present data have demonstrated neuroendocrine changes in animals fed isocaloric diets, two of which had a chronic deficiency of protein, in comparison to rations known to produce a good growth response in fattening Polish Merino lambs (14% of crude protein) (Pajak and Zebrowska, 1985; Urbaniak, 1986). These protein restrictions affected the whole body growth of the male lambs studied, as well as, the endocrine axis controlling reproduction and growth.

However, the presented work should be treated as a preliminary study. In principle, this experiment was only nutritional (Pajak and Zebrowska, 1985), so accessible material, like the blood plasma for RIA estimation, was incomplete. It is difficult to interpret immunohistochemical data without correlation, with concentrations of hormones in blood. The present study describes only the changes in the cellular content of investigated hormones during protein deficiency conditions. The data concerning the LHRH/LH axis, suggest that, although the LHRH hypothalamic neuronal system was not impaired by a decrease of dietary protein, there were changes in the balance between the synthesis, axonal transport, storage and release of IR LHRH. The disappearance of the immuno product from the LHRH perikarya, and its appearance in the axons, indicates an acceleration of axonal transport of the hormone, from the perikarya located in the preoptic area, to the ME. The increase of IR LHRH in the ME, can be interpreted as an inhibition of this neurohormone release from nerve terminals to the capillaries of the portal blood. These observations are in agreement with data reported on starved male rats, showing an increase of LHRH in the ME (Pirke and Spyra, 1981; Warnhoff *et al.*, 1983). This LHRH material can be released *in vitro*, using a depolarizing agent (Warnhoff *et al.*, 1983). The effect of protein deficiency in inhibiting LHRH release, may be responsible for the observed changes in the immunoreactivity of LH cells in the

Figs. 7—12. IRSRIH material in the central part of the ME of representative lambs from groups I (Fig. 7) and III (Fig. 8). Note the sharp reduction of IRSRIH material in the lamb fed with a low protein diet (Fig. 8) (x 28). Figs. 9 and 10. IRSRIH perikarya in the nucleus paraventricularis of representative lambs from groups I (Fig. 9) and III (Fig. 10). Note the increased number and stain intensity of the cells in the lamb fed with low protein diet (Fig. 10) (x 28), v-third ventricule. Figs. 11 and 12. IRGH cells in the adenohypophysis of representative lambs from groups I (Fig. 11) and III (Fig. 12). Note the increased number of the cells in the lamb fed with a low protein diet (Fig. 12) (x 70).

pituitary gland. The increased proportion of LH cells, with a concomitant increase in IR LH material, can be interpreted as an impairment of LH release from the pituitary gland. This interpretation is supported by data obtained from experiments on underfed female lambs (Haresign, 1981; Foster and Olster, 1985), where restricted food intake reduced the concentration of circulating LH in blood, but the LH levels were immediately restored after a LHRH injection (Haresign, 1981). This suggests that the pituitary gland of underfed female sheep, contains enough LH to respond to the releasing action of LHRH. A similar observation was made in male rats, in which LH release from the pituitary gland was not impaired by starvation and responded to LHRH stimulation (Pirke and Spyra, 1981; Warnhoff *et al.*, 1983). It seems, therefore, that the major effect of the low protein diets was regulated at the hypothalamic, rather than at the pituitary level, and that the reduction of LH secretion, as a consequence of protein restriction, was primarily caused by a decrease in LHRH release, rather than by a suppression of LH secretion. The present data indicate the existence of a link between the activity of hypothalamic LHRH secretory neurons and dietary proteins. It is known that dietary deprivation causes abnormalities in the developing central nervous system, and also strongly influences neurotransmitter systems in the brain (Wiggins *et al.*, 1984). The lack of amino acids, acting as precursors for the synthesis of neurotransmitters, could change the activity of the neurotransmitters stimulating and inhibiting LHRH secretion.

Protein restrictions also induce a significant hyperplasia of GH cells in the pituitary gland, with simultaneous diminution of IR GH material. This observation, together with the evidence

that reduction of food intake in sheep causes an increase in GH in peripheral blood (Forbes *et al.*, 1979; Hart *et al.*, 1985), indicates hypersecretion of GH cells. This hypothesis is also supported by the results of our next experiment on female sheep. The lowering of dietary proteins (up to 7%) causes a significant elevation of plasma GH levels (Polkowska and Krejci, unpublished observation). This increase in GH cell activity could be consistent with the clear decrease in SRIH activity observed in the hypothalamic neuronal system of sheep deprived of proteins. Increasing numbers of visible IR SRIH perikarya in the periventricular area, of both the supra-chiasmatic and paraventricular nuclei, with a concomitant decrease of stored IR SRIH material in the ME, suggests suppression of the axonal transport of this hormone. This interpretation can be supported by the finding that colchicine inhibition of axonal transport is manifested by an unmasking of the neuronal perikarya, due to increase of their secretory product (Sétáló *et al.*, 1976). The presumable decrease of somatostatin might be responsible for the increase of GH secretion during protein deprivation. However, it is known that GH is regulated by the second hypothalamic factor : growth hormone releasing-hormone (GHRH). Somatostatin and GHRH are secreted tonically from the hypothalamus, stimulating integration of the ultradian rhythm of GH secretion (Tannenbaum and Ling, 1984). The role of these two factors, in some food restriction conditions, is very poorly understood. There is some evidence that, in these conditions, GHRH could be involved in the regulation of GH release. It has been found that, in steers, some nutritional factors alter GH response to GHRH (Moseley *et al.*, 1988). In humans, caloric restrictions enhance GH responsiveness to GHRH (Kelijman and

Frohman, 1988), but, in sheep, maintained on a negative energy balance, injections of different GHRH do not significantly affect plasma GH levels (Hart *et al.*, 1985). There are no previous reports describing the response of somatostatin to reduction of food intake in sheep or other ruminants. However, a clear relationship between GH and SRIH, involving hypersecretion by GH cells and a concomitant decrease of IR SRIH content in nerve terminals of the ME, was found in fishes (Olivereau *et al.*, 1984). Response of GH to low protein diets, varies with the species. In humans, sheep, cows and rabbits, blood concentrations of GH are elevated under conditions of malnutrition (*e.g.* Hart *et al.*, 1985). In rats, both short and long-term starvation induces the opposite effects by increasing pituitary GH and suppressing GH episodes, without changing the concentration of SRIH in the hypothalamus (Tannenbaum *et al.*, 1979). The data presented in this study on the diminishing activity of the SRIH hypothalamic neuronal system and increasing activity of GH pituitary cells, support the suggestion of Hart *et al.* (1985), that in ruminants subjected to a low dietary protein intake, the elevation of GH in blood is due to a suppression of the inhibitory activity of somatostatin. The increased activity of growth hormone helps to maintain metabolic homeostasis by conserving body proteins.

In conclusion, it may be postulated that prolonged restriction of protein in the diet, of growing male lambs, causes disturbances in the neuroendocrine systems, as evidenced by the changes of immunoreactive content of the studied hormones. Protein restrictions in the diet attenuate the release of LHRH and LH and activate the release of GH, possibly *via* suppression of hypothalamic somatostatin.

Acknowledgments

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