Variations through the day of hepatic and muscular cathepsin A (carboxypeptidase A; *EC* 3.4.12.2), C (dipeptidyl peptidase; *EC* 3.4.14.1) and D (endopeptidase D; *EC* 3.4.23.5) activities and free amino acids of blood in rats: influence of feeding schedule

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1. Growing rats were fed either *ad lib.* or with six (equal) meals offered every 4 h (from 10.00 hours). Rats of each group were killed at intervals of 4 h beginning at 11.00 hours. Activities of cathepsin A (carboxypeptidase A; *EC* 3.4.12.2), C (dipeptidyl peptidase; *EC* 3.4.14.1) and D (endopeptidase D *EC* 3.4.23.5) were measured in liver and muscle homogenates and free amino acids in blood were determined.

2. In the rats fed *ad lib.* activities of carboxypeptidase A and endopeptidase D in liver and muscle showed significant variation, with maximum activity in the light period. In general, meal-feeding only caused minor differences in cathepsin activities; although significant differences occurred for carboxypeptidase A. For the latter enzyme a peak in activity occurred in the dark as well as in the light period.

3. Irrespective of the feeding schedule, the lower concentration of free essential amino acids of blood occurred generally during the night period. With the controlled-feeding schedule there is an increase of essential amino acids and a slight decrease of non-essential amino acids of blood.

Lysosomes are involved in the breakdown of proteins in all tissues including muscle (Coffey & De Duve, 1968; Segal *et al.* 1974; Dayton *et al.* 1975; Dean, 1975). The endopeptidase cathepsin D (*EC* 3.4.23.5) splits proteins into large peptides (Young *et al.* 1969) and its action is complemented by exopeptidases such as cathepsin A (carboxypeptidase A; *EC* 3.4.12.2) and cathepsin C (dipeptidyl peptidase; *EC* 3.4.14.1) (McDonald *et al.* 1969; Young *et al.* 1969). It has been shown that lysosomal enzyme activity varies with a number of nutritional stimuli such as starvation (Filkins, 1970; Pontremoli *et al.* 1976), amino acid supply (Neely & Mortimore, 1973) and hormonal stimuli (Bird *et al.* 1968; Neely & Mortimore, 1973; Li *et al.* 1975). Moreover the intensity of protein metabolism in different tissues fluctuates throughout the day (Wurtman, 1970; Reinberg, 1974) and the activity of several enzymes such as liver acid phosphatase (*EC* 3.1.3.2) are not constant during the day (Bhattacharya & Mayersbach, 1975). This periodicity seems to allow the animals to regulate the nutrient flow between different tissues and organs according to their needs, and it also moderates the effect of the discontinuity of food intake. The importance of this factor can be studied using a controlled-feeding schedule.

In a previous work, we have shown that protein synthesis in various tissues followed daily variations irrespective of the feeding schedule (Obled *et al.* 1975). Controlled-feeding resulted in a reduction of protein synthesis in the liver but not in muscle. Following from these observations, the objective of the work described here was to establish if similar variations occurred in the activities of some lysosomal enzymes involved in protein catabolism. Furthermore we aimed to clarify the possible role of these enzymes in the short-term regulation of protein metabolism.

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MATERIALS AND METHODS

Two groups of sixty male Sprague-Dawley rats, with an average weight of 96 g, were housed at 21–22°, 60% relative humidity, under controlled lighting conditions (dark period 10.00–22.00 hours). The animals were given a semi-synthetic diet containing fish meal (140 g crude protein (nitrogen × 6·25)/kg) supplemented with methionine (Arnal *et al.* 1971). The control animals were fed *ad lib.* The experimental animals were fed using an automatic feeding schedule; they consumed in approximately 5 min each of the six equal meals given every 4 h, beginning at 10.00 hours. The average growth rate of animals in the two groups during the experimental period (12 d) was 6 g/d. There was no difference between the two groups (Obled *et al.* 1977).

Eight to twelve rats approximately 170 g live weight from each group were killed by decapitation, without anaesthesia, at intervals of 4 h beginning at 11.00 hours. Blood samples were mixed with 8 vol. ethanol (950 ml/l). The liver and the muscles of the hind-limbs were quickly removed and weighed. Samples, grouped by two, were chilled in aqueous Triton X-100 solution (2 ml/l) and homogenized using a Waring blender. The homogenate was left overnight at 4°. The muscle homogenates were centrifuged at 5000 *g* for 15 min. The pellets were resuspended in Triton X-100 solution and extracted three times during a 3 h period. The muscle supernatant fractions and the supernatant fraction from the liver homogenate were dialysed overnight in Triton X-100.

Total enzyme activities were measured in the dialysate. Carboxypeptidase A activity was determined at pH 5 and 37° with 35 mM-*N*-carbobenzoxy-α-L-glutamyl-L-tyrosine as the substrate (Iodice *et al.* 1966). After 2 h incubation for muscle and 1 h for liver, the reaction was stopped by adding trichloracetic acid (TCA; 100 g/l). The amount of tyrosine released was colorimetrically analysed in the supernatant fraction using ninhydrin. The cathepsin A activity was expressed as μmol tyrosine released/h per g wet weight of tissue or μmol tyrosine released/h per mg protein.

The dipeptidyl peptidase activity was measured in 0·1 M-phosphate buffer pH 6, at 37° with 2 mM-glycyl-L-phenylalanine-β-naphtylamide as the substrate and in the presence of mercaptoethylamine (McDonald *et al.* 1969). After incubating the system for 30 min for the muscles and 15 min for the liver, the reaction was stopped by addition of TCA. The precipitate was removed by centrifugation and the amount of β-naphthylamine released was measured (Goldbarg & Ruttenberg, 1958). The activity was expressed as mg β-naphthylamine/h per g wet weight of tissue or mg β-naphthylamine/h per mg protein.

Endopeptidase D activity was determined with acid-denatured haemoglobin. After incubating for 2 h for the muscle samples and 1 h for the liver samples, TCA was added to stop the reaction. After centrifugation, the extinction at 280 nm of the supernatant fraction was determined in order to measure the release of acid-soluble peptides containing tyrosine and tryptophan. The activity was expressed as the change in extinction at 280 nm/h per g wet weight of tissue or change in extinction at 280 nm/h per mg protein (Valin, 1967).

Free amino acids of blood were extracted with ethanol (820m l/l) (Pawlak & Pion, 1968) and estimated by ion-exchange chromatography (Moore *et al.* 1958) with an automatic apparatus.

The protein in the homogenates was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

The interpretation of results from this investigation depended on evaluating changes during the day. The plot of mean values with standard errors at different time-points presented the results with minimum deletion of information but the decision as to whether or not characteristics of a given rhythm have changed was highly subjective on the values. For this reason, when cathepsin activities exhibited one maximum and one minimum

throughout the day results were analysed according to the cosinor method (Halberg *et al.* 1972). This method fitted a 24 h cosine function to the values and the validity of the approximation can be assessed by an F test (Snedecor & Cochran, 1971).

RESULTS

Activity of liver cathepsins

The variations of the activities of liver carboxypeptidase A, dipeptidyl peptidase and endopeptidase D for the two feeding schedules are shown in Fig. 1 and Table 1. In the rats fed *ad lib.*, activities of liver carboxypeptidase A and endopeptidase D showed a signficant variation with time when expressed as units of activity/g wet weight. The activities for both these enzymes reached a maximum in the light period. The activity of liver dipeptidyl peptidase varied slightly throughout the day. Results expressed as units of activity/mg protein showed the same trend for carboxypeptidase A but not for endopeptidase D.

The trend for changes in activities of the three liver enzymes were not different between the meal-fed and *ad lib.*-fed rats. Although a notable exception occurred for carboxypeptidase A. At the end of the dark period, the activity of the enzyme was significantly higher in the meal-fed group than in the *ad lib.*-fed group. (The over-all mean values for the activities of the three enzymes did not differ among the two groups.)

When the results were expressed in terms of total activity of enzyme for liver (i.e. taking liver weight into account) the pattern of the rhythms of carboxypeptidase A and endopeptidase D in the two groups was the same. However, a significant variation during the day for the activity of dipeptidyl peptidase occurred for the two groups of rats with a peak value in the dark period.

Activity of muscle cathepsins

The variations in the activities of muscle cathepsins for the two feeding schedules are shown in Fig. 1 and Table 2. In the *ad lib.*-fed rats, the activities of endopeptidase D exhibited a significant variation during the day when expressed in units of activity/g wet weight, with a peak value in the light period. The activity of dipeptidyl peptidase showed no significant variation during the day. The mean activity of carboxypeptidase A was highest during the light period.

Meal-feeding did not significantly change the activities of dipeptidyl peptidase and endopeptidase D throughout the day. The activity of carboxypeptidase A showed two peaks of activity during the day, therefore the values were not analysed according to the cosinor method. A peak of activity occurred during the light period, the activity was very high during the dark period, and was significantly greater in the meal-fed rats than in the animals fed *ad lib.*

Free amino acids of blood

When the rats were fed *ad lib.*, the concentrations of the essential amino acids were low at the end of the light period or at the beginning of the dark period (Table 3). After the onset of darkness the amounts of some essential amino acids (valine, leucine, isoleucine, tyrosine, threonine) increased. For example the differences between the lowest and the highest values were between 40 and 70% for tyrosine, phenylalanine, methionine, histidine, arginine and approximately 30% for lysine and isoleucine.

For *ad lib.*-fed animals the content of most of the free non-essential amino acids of blood (e.g. proline, serine, glycine, ornithine) fluctuated less than did the essential amino acids. Also, in contrast to the essential amino acids, the amounts of non-essential amino acids were in general highest in the first 4 h of the dark period.

Meal-feeding did not substantially modify the variations observed for *ad lib.*-fed animals, although the fluctuations were smaller. In general meal-feeding decreased the amount of

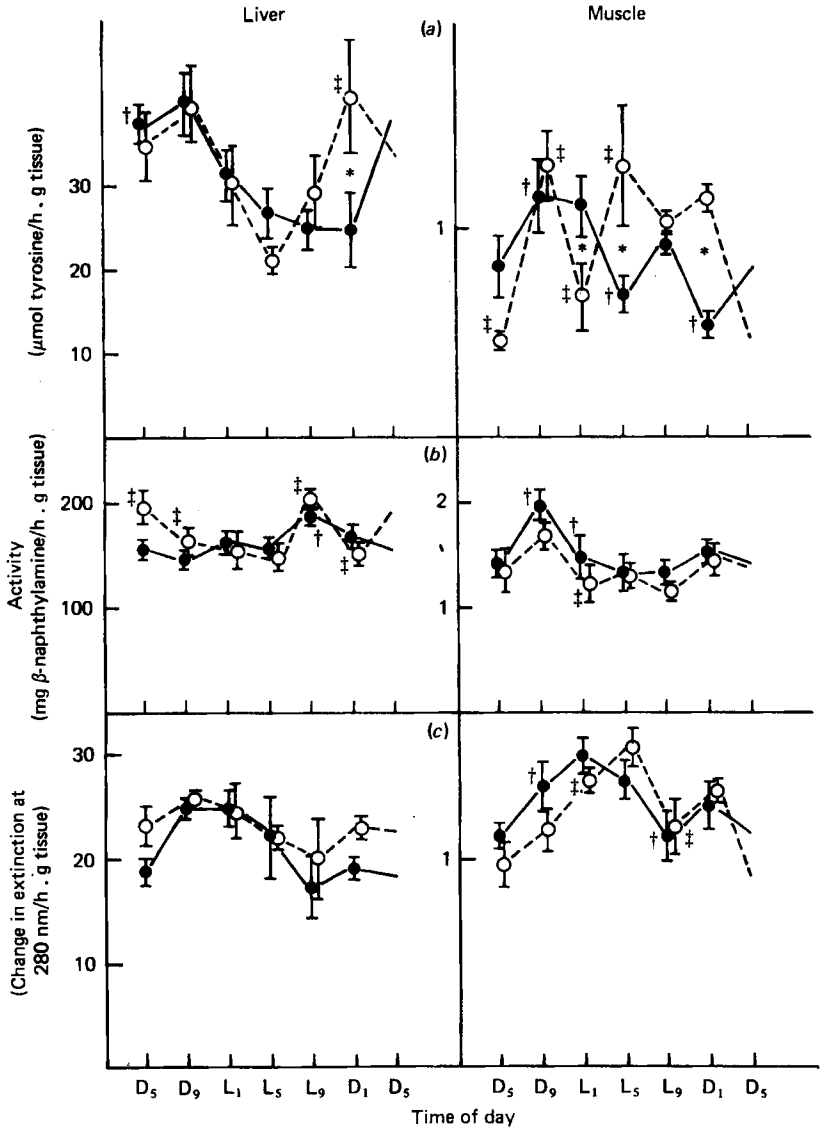

Fig. 1. Variations in activity of (*a*) cathepsin A (carboxypeptidase A; *EC* 3.4.12.2), (*b*) cathepsin C (dipeptidyl peptidase; *EC* 3.4.14.1), (*c*) cathepsin D (endopeptidase D; *EC* 3.4.23.15), expressed as units of activity/g wet weight, in liver and muscle of rats on two feeding schedules: *ad lib.* (●——●) or six equal meals offered every 4 h from 10.00 hours (meal-fed) (○----○). Units of activity were: carboxypeptidase A, μmol tyrosine released/h; dipeptidyl peptidase C, mg β-naphthylamine released/h; endopeptidase D, change in extinction at 280 nm/h. * , Values were statistically significantly different from the corresponding value for the *ad lib.*-fed group ($P \leqslant 0.005$); †, *ad lib.*-fed group; ‡, meal-fed group values were statistically significantly different from the value observed in the previous 4 h ($P \leqslant 0.05$). L_1, L_5, L_9, three successive 4 h periods of light period; D_1, D_5, D_9, three successive 4 h periods of dark period. Points are mean values with their standard errors represented by vertical bars.

Table 1. *Analysis according to the cosinor method (Halberg* et al. 1972*) of the activities of cathepsins A (carboxypeptidase A*; EC 3.4.12.2), *C (dipeptidyl peptidase*; EC 3.4.14.1) *and D (endopeptidase D*; EC 3.4.23.5) *in liver of rats fed either* ad lib. *or six equal meals given every 4 h*

(Mean values with their standard errors)

Cathepsin	Feeding schedule	Mesor		Amplitude		Acrophase: Period after lighting onset (h)	Acrophase: 95% confidence limits	Statistical significance of effect: P*
		Mean	SE	Mean	SE			
		Units†/g wet weight						
A	*ad lib.*-fed	31·8	1·8	8·2	7·4	1·6	0·7–2·5	< 0·005
	Meal-fed	33·0	2·2	8·3	6·7	1·0	23·7–2·2	0·025
C	*ad lib.*-fed	161·7	4·3	14·5	10·3	17·4	15·9–18·9	0·1
	Meal-fed	168·7	7·0	14·7	10·2	21·7	—	> 0·25
D	*ad lib.*-fed	21·6	1·1	4·0	1·4	7·9	6·6–9·3	0·025
	Meal-fed	23·3	1·0	2·3	3·9	5·4	—	0·25
		Units†/total wet weight of liver						
A	*ad lib.*-fed	264	17	78	—	3·4	2·4–4·3	< 0·005
	Meal-fed	265	22	81	5	0·5	23·2–1·8	0·025
C	*ad lib.*-fed	1310	50	168	54	21·6	20·4–22·9	0·025
	Meal-fed	1350	50	192	85	21·8	22·5–1·0	0·025
D	*ad lib.*-fed	172	8	32	36	4·7	3·4–6·1	0·025
	Meal-fed	188	8	30	—	3·7	2·2–5·1	0·05
		Units†/mg protein						
A	*ad lib.*-fed	0·21	0·01	0·06	—	4·4	3·3–6·7	< 0·01
	Meal-fed	0·20	0·01	0·05	—	2·4	—	0·025
C	*ad lib.*-fed	1·08	0·03	0·09	—	17·8	—	> 0·25
	Meal-fed	1·04	0·05	0·05	—	19·7	—	> 0·25
D	*ad lib.*-fed	0·14	0·01	0·02	0·01	7·8	—	0·15
	Meal-fed	0·14	0·01	0·02	0·02	6·7	—	0·25

* From F test: probability of obtaining F equal to or exceeding observed F if there were no 24 h sinusoidal variations.

† Carboxypeptidase A, μmol tyrosine released/h; dipeptidyl peptidase, mg β-naphthylamine released/h; endopeptidase D, change in extinction at 280 nm/h.

essential amino acids and increased the amount of the non-essential amino acids in blood. However, these slight differences may have been due to the high protein content of the diet used in this experiment.

DISCUSSION

The role of the different cathepsins in the regulation of protein breakdown

The activities of liver and muscle cathepsins underwent large fluctuations throughout the day in the *ad lib.*-fed animals.

Among the three cathepsins studied, the amplitude of the variation of dipeptidyl peptidase activity was the lowest, and its change was opposite to that of the other cathepsins in the liver. This suggests that the stimuli acting on the enzyme may be different from those acting on carboxypeptidase A and endopeptidase D in this organ. The variations of carboxypeptidase A and endopeptidase D were synchronized, even though there was a slight time shift. In muscle and liver, both enzymes had their acrophase during the light period, although it appeared a little sooner for carboxypeptidase A than for endopeptidase D. Carboxypeptidase A appeared to be the first enzyme involved in the process of protein breakdown.

Table 2. *Analysis according to the cosinor method (Halberg* et al. 1972*) of the activities of cathepsins C* (dipeptidyl peptidase; EC 3.4.14.1) *and D (endopeptidaseD* EC 3.4.23.5) *in muscle of rats fed either* ad lib. *or with six equal meals given every 4 h*

(Mean values with their standard errors)

Cathepsin	Feeding schedule	Mesor		Amplitude		Acrophase: Period after lighting onset (h)	Acrophase: 95% confidence limits	Statistical significance of effect: P*
		Mean	SE	Mean	SE			
		Units†/g wet weight						
C	*ad lib.*-fed	1·53	0·08	0·23	—	4·1	—	0·10
	Meal-fed	1·35	0·07	0·17	—	2·0	—	0·25
D	*ad lib.*-fed	1·30	0·05	0·17	0·06	9·3	5·7–10·6	0·05
	Meal-fed	1·25	0·05	0·21	0·13	12·7	11·6–14·0	0·025
		Units†/mg protein						
C	*ad lib.*-fed	0·046	0·002	0·005	—	1·8	—	> 0·25
	Meal-fed	0·045	0·002	0·005	—	23·6	—	0·25
D	*ad lib.*-fed	0·031	0·001	0·002	—	11·8	—	> 0·25
	Meal-fed	0·031	0·001	0·005	—	14·6	—	0·15

* From F test: probability of obtaining F equal to or exceeding observed F if there were no 24 h sinusoidal variations.

† Dipeptidyl peptidase, mg β-naphthylamine released/h; endopeptidase D, change in extinction at 280 nm/h.

Table 3. *Variations in the concentrations (mg/kg) of free amino acids in blood of rats fed* ad lib. *or with six equal meals given every 4 h*

Feeding schedule ...	*ad lib.*-fed					Meal-fed			
Time of day ...	L_9	D_1	D_5	D_9	L_1	D_5	D_9	L_1	L_5
Aspartic acid	6	9	5	7	6	5	6	5	4
Threonine	51	45	47	51	54	46	60	67	57
Serine	34	35	32	35	34	41	36	43	35
Glutamine and asparagine	21	26	23	24	66	80	74	68	55
Glutamic acid	53	61	56	63	36	40	38	32	28
Proline	28	30	28	30	33	29	31	34	27
Citrulline	12	18	18	19	—	18	20	15	13
Glycine	35	43	33	37	38	40	38	42	36
Alanine	73	92	67	73	63	74	68	79	63
Valine	23	20	22	25	25	21	22	26	21
Cystine	8	8	8	10	—	5	5	11	4
Methionine	8	17	13	16	14	15	17	9	12
Isoleucine	10	12	12	14	12	11	12	11	10
Leucine	14	15	17	18	17	13	16	18	15
Tyrosine	10	4	8	7	12	7	9	12	11
Phenylalanine	9	7	9	11	11	8	9	10	9
Ornithine	5	5	6	5	6	6	5	—	6
Lysine	79	68	87	81	104	84	86	—	102
Histidine	9	4	8	8	12	8	7	—	12
Arginine	25	26	51	22	38	34	36	—	55
Total essential amino acids	246	226	282	263	308	252	279	305	308
Total non-essential amino acids	267	319	268	293	298	333	316	323	267

L_1, L_5, L_9 three successive 4 h periods of light period; D_1, D_5, D_9 three successive 4 h periods of dark period.

It can be assumed that if the intermediate products of protein catabolism (peptides) were needed as the substrate for carboxypeptidase A, endopeptidase D may be required. These enzymes may belong to the same metabolic pathway or at least respond to the same stimuli.

In the meal-fed rats the food intake was reduced during the night compared to *ad lib.*-feeding (Obled *et al.* 1977) and did not seem to supply a sufficient flux of amino acids; therefore it was necessary for the animal to catabolize proteins. This catabolism of proteins was characterized by the higher activity of carboxypeptidase A in meal-feeding than in *ad lib.*-feeding, without significant change in endopeptidase D activity during the night in muscle, and at the end of the night in liver. It appeared that carboxypeptidase A played a particularly important role in the regulation of amino acid supply in relation to the needs of the body.

The regulation of protein metabolism throughout the day in the ad lib.*-fed rats*

Carboxypeptidase A was active mainly in the first hours of the light period and endopeptidase D later. The activity of dipeptidyl peptidase did not change significantly during the light period. These observations suggested that protein breakdown in these tissues was important at the beginning of the light period. This light period, which is known as a resting period for such nocturnal animals, has been characterized by a reduced food intake (Obled *et al.* 1977). Protein catabolism in the liver may not provide sufficient amino acids for the tissues' requirements and a similar catabolism was induced in muscles.

In the first part of the light period a set of factors, especially hormones which complete the effect of the feeding rhythm, were favourable to a greater lysosomal activity (Grizard *et al.* 1976; Obled *et al.* 1977). The levels of insulin and corticosterone have been found to be low and therefore favour an increase in cathepsin activity in the liver (Neely & Mortimore, 1973; Li *et al.* 1975). Later in the light period the variations in the hormonal level could explain the decrease in the liver and muscle cathepsin activity. First, corticosterone concentrations in the blood could have increased in the middle of the light period and induced an acceleration of protein synthesis in the liver (Munro, 1964); consequently, the protein catabolism in the liver would have decreased. Carboxypeptidase A activity was more affected than that of endopeptidase D. In the muscle, corticosterone increases the cathepsin activity (Canonico & Bird, 1970; Bird *et al.* 1968) (carboxypeptidase A and endopeptidase D activity were always high in the last 4 h of the light period). In the last hours of the light period blood insulin also could have increased and this hormone which is known to be an inhibitor of protein breakdown in the liver (Filkins, 1970) may have played a part in the decrease of cathepsin activity. At the same time this hormone acting as a stimulator of protein synthesis as well as an inhibitor of protein breakdown in muscle (Munro, 1964) could have produced the same variations in cathepsin activity as in the liver and the protein breakdown decreased. This latter phenomenon and the reduced food intake during the light period induced a decrease in several free essential amino acids of blood. This decrease was enhanced by the occurrence of higher protein synthesis in the muscle (Rebolledo & Gagliardino, 1971; Obled *et al.* 1975). The lowest concentration of essential amino acids took place during the first hours of the dark period as also reported by different authors (Wurtman *et al.* 1968; Fernstrom *et al.* 1971; Young *et al.* 1973). The blood concentration of some free non-essential amino acids were low indicating that many amino acids derived from protein breakdown were oxidized. This process could explain the high uraemia observed during the light period (Grizard *et al.* 1976). Among others the level of plasma alanine was low, presumably due to the glucose-alanine cycle, especially at the end of the light period, when corticosterone concentration was high and insulin was low. This gluconeogenesis could partly explain the hyperglycaemia observed a few hours before the night period by Grizard *et al.* (1976). During the night the protein breakdown was low in liver as well as in muscle.

The minimum activity of cathepsins occurred in this period, except for liver dipeptidyl peptidase which presented its acrophase in the first part of the night when muscle carboxypeptidase A exhibited a peak value.

High food intake occurred especially at the beginning of the night (Obled *et al.* 1977) and the animals utilized mainly the amino acids from the diet. The high level of insulin (Grizard *et al.* 1976) maintained a low protein breakdown in liver and the high corticosterone level (Obled *et al.* 1977) associated with the input of food amino acids induced a high rate of protein synthesis in this organ (Obled *et al.* 1975). In muscle, the hormones could have a determining influence (Munro, 1964). The effects of corticosterone, which may have reached its maximum of concentration sooner than insulin, in the first hours of the night, could have induced the transient peak of carboxypeptidase A activity. But later in the night the effects of insulin may have become the more important, thereby explaining the apparent low rate of catabolism. The high food intake during the night period was the principal cause of the increased level of almost all free essential amino acids of blood during the night.

Influence of feeding schedule

In meal-fed rats, activity of the three cathepsins showed the same trend as that seen in *ad lib.*-fed rats. During the light period the food intake was higher in meal-fed rats than in *ad lib.*-fed rats (Obled *et al.* 1977) and the amino acid catabolism had the same intensity in the two groups of rats (Grizard *et al.* 1976). At the end of this period, the meal-fed rats, which had a slightly lower need for protein synthesis than *ad lib.*-fed rats (Obled *et al.* 1975) presumably possessed sufficient gut amino acids to fulfil their needs. The supply of endogenous amino acids, derived from protein breakdown, would probably have been lower in meal-fed rats than in *ad lib.*-fed rats, at least as indicated by the decrease in muscle carboxypeptidase A activity. This decrease in protein breakdown was related to the significantly higher insulin levels at this period of day in meal-feeding than in *ad lib.*-feeding (Grizard *et al.* 1976).

In the night period, the opposite result was observed; the food intake was reduced in meal-fed rats as compared with *ad lib.*-fed rats, and the animals digested their meal more rapidly in the dark period than in the light period (Obled *et al.* 1975). The dietary amino acids could not fulfil the needs and the animals utilized endogenous amino acids produced as a result of the increased carboxypeptidase A activity in the muscle and liver. During the night, corticosterone and insulin levels were low and would allow this high protein catabolism. Most of the essential free amino acids were lower in the blood of meal-fed rats than in the blood of rats fed *ad lib.* The opposite situation existed for most of the non-essential amino acids of blood (Grizard *et al.* 1974).

In the first hours of the light period, the increase in food intake related to meal-feeding seemed to be the main factor that explained the high concentration of lysine, threonine and some non-essential amino acids (alanine, glycine) in meal-fed rats. During the night, the protein synthesis in the liver was lower in meal-fed rats than in *ad lib.*-fed rats but the protein synthesis levels were similar in muscle (Obled *et al.* 1975). The lower concentrations of several essential amino acids were most likely to be due to the lower food intake in meal-feeding than in *ad. lib.*-feeding. The lack of difference between the lysine and threonine in the two groups was the consequence of their known low oxidative rate. Some concentrations of non-essential amino acids were higher in meal-fed rats than in *ad lib.*-fed animals.

In addition to the changes known to occur in protein synthesis, the fluctuations in activities of cathepsins, which we observed, indicate that protein catabolism underwent marked variations throughout the day. Catabolism is therefore an important regulator of protein metabolism. Carboxypeptidase A appeared to play an especially important role in protein breakdown.

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REFERENCES

Arnal, M., Fauconneau, G. & Pech, R. (1971). *Annls Biol. anim. Bioch. Biophys.* **11**, 245.
Bhattacharya, R. & Mayersbach, H. (1975). *Chronobiologia* suppl. 1, 12 (Abstr).
Bird, J. W. C., Berg, T. & Leathem, J. L. (1968). *Proc. Soc. exp. Biol. Med.* **127**, 182.
Canonico, P. G. & Bird, J. W. C. (1970). *J. Cell Biol.* **45**, 321.
Coffey, J. W. & De Duve, C. (1968). *J. biol. Chem.* **243**, 3255.
Dayton, W. R., Goll, D. E. & Reville, W. J. (1975). *Proc. Meat Conf.* p. 214.
Dean, R. T. (1975). *Eur. J. Biochem.* **58**, 9.
Fernstrom, J. D., Larin, F. & Wurtman, R. J. (1971). *Life Sci.* **10**, 813.
Filkins, J. P. (1970). *Am. J. Physiol.* **219**, 923.
Goldbarg, J. A. & Ruttenberg, A. M. (1958). *Cancer* **11**, 83.
Grizard, J., Obled, C., Arnal, M. & Pion, R. (1976). *Compt. rend. Soc. Chim. Biol.* **170**, 1201.
Grizard, J., Prugnaud, J. & Pion, R. (1974). *C. r. Soc. Chim. Biol.* **168**, 738.
Halberg, F., Johnson, E., Nelson, W., Runge, W. & Sothern, R. (1972). *Physiol. Teacher* **1**, 1.
Iodice, A. A., Leong, V. & Weinstock, I. M. (1966). *Archs Biochem. Biophys.* **117**, 477.
Li, J. B., Rannels, S. R., Burkart, M. E. & Jefferson, L. S. (1975). *Fedn Proc. Fedn Am. Socs exp. Biol.* **34**, 654.
Lowry, O. H., Rosebrough, N. J. & Carr, A. L. (1951). *J. biol. Chem.* **193**, 265.
McDonald, J. K., Zeitman, B. B., Reilly, T. J. & Ellis, S. (1969). *J. biol. Chem.* **244**, 2693.
Moore, S., Spackmann, D. H. & Stein, H. H. (1958). *Analyt. Chem.* **30**, 1185.
Munro, H. N. (1964). In *Mammalian Protein metabolism*, vol. 1, p. 381 [H. N. Munro and J. B. Allison, editors]. New York: Academic Press.
Neely, A. N. & Mortimore, G. E. (1973). *Biochim. biophys. Acta* **338**, 458.
Obled, C., Arnal, M. & Fauconneau, G. (1975). *Annls Biol. anim. Bioch. Biophys.* **15**, 73.
Obled, C., Arnal, M. & Grizard, J. (1977). *C. r. Séanc. Acad. Sci., Paris* D **284**, 195.
Pawlak, M. & Pion, R. (1968). *Annls Biol. anim. Bioch. Biophys.* **8**, 517.
Pontremoli, S., Melloni, E., De Flora, A., Accorsi, A., Balestrero, F., Tsolas, O., Horecker, B. L. & Poole, B. (1976). *Biochimie* **58**, 148.
Rebolledo, O. R. & Gagliardino, J. J. (1971). *J. Interdisc. cycle Res.* **2**, 101.
Reinberg, A. (1974). *Chronobiologia* **1**, 22.
Segal, H. L., Winckler, J. R. & Miyagi, M. P. (1974). *J. biol. Chem.* **249**, 6364.
Snedecor, G. W. & Cochran, W. G. (1971). *Méthodes Statistiques.* Paris: Association de coordination technique Agricole.
Valin, C. (1967). *Annls Biol. anim. Biochem. Biophys.* **7**, 475.
Wurtman, R. J. (1970). In *Mammalian Protein metabolism*, vol. 4, p. 445 [H. N. Munro and J. B. Allison, editors]. New York: Academic Press.
Wurtman, R. J., Rose, C. M., Chou, C. & Larin, F. F. (1968). *New Engl. J. Med.* **279**, 171.
Young, J. O., Liao, F., Hanes, D. & Tappel, A. L. (1969). *Fedn. Proc. Fedn. Am. Socs exp. Biol.* **28**, 266.
Young, V. R., Vilaire, G., Newberne, P. M. & Wilson, R. B. (1973). *J. Nutr.* **103**, 720.

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