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Relationship between the Proteolytic Activity and the Nitrogen Content of the Liver[1]

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The changes in neutral and acid proteolytic activities as well as the changes in acid phosphatase activity in the liver of male albino rats were studied during the period of growth encompassing the sexual maturation of the animals. It was found that under normal growth conditions there is a direct relationship between the rate of increase of the liver weight and the rate of increase of the nitrogen content of the organ. The total nitrogen of the liver and the enzymic activities studied showed specific changes between the ages of 37 and 55 days that seemed to be related to the process of sexual maturation. During this period the ratio of the acid to the neutral proteolytic activities and the total nitrogen content of the liver became inversely related, suggesting a role for these enzymic activities in the catabolic phase of protein turnover. When groups of rats similar to the normal one were subjected to a 5% corn protein diet or to a nitrogen-free diet, the direct relationship between the rate of growth of the liver and the total nitrogen content of the organ, as well as the changes observed during the sexual maturation period, were lost. The inverse relationship between the ratio of the proteolytic activities and the nitrogen content of the liver could be repeatedly demonstrated. Similar results were obtained when a group of adult male rats were subjected to an 8-day period of starvation. As suggested by previous studies there seems to be a specific type of response of the proteolytic enzymes of the liver to each of the dietary conditions studied. The process of malnutrition does not follow a continuously deteriorating pattern but seems to be characterized by successive periods of adaptation in which the enzymic activities and the nitrogen content of the liver recover somewhat at certain times to fall again to even lower levels when the adaptation mechanisms become exhausted.

Intracellular proteases have been found widely distributed in all mammalian tissues so far examined (1). The biological role of these enzymes is not well understood. However, several reports in the literature suggest that they might play an important role in the catabolic phase of protein metabolism. Studies by Benz (2, 3) and Faulhaber *et al.* (4) have shown that organs with a high turnover of proteins also present high proteolytic activities. Maver *et al.* (5) have reported that regenerating liver and hepatic neoplasma have higher proteolytic activities at low pH than normal liver. Studies by Weber (6) and Weissmann (7) have shown that during the period of growth of the tail of the larvae of *Xenopus laevis* the catheptic activity of the tail decreases while the total nitrogen content is increasing. Shortly before the onset of metamorphosis the specific catheptic activity begins to increase and augments progressively during the period of resorption of the tail. The work of de

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Duve and co-workers (8) has shown that the acid proteolytic activity is contained within the lysosomes, and Weissmann (9, 10) and Mego and McQueen (11) have reported that these organelles play an active role in the process of intracellular digestion and focal destruction. Koszalka *et al.* (12) have also reported high proteolytic activities in the muscle of rats with muscular dystrophy produced by vitamin E-deficient diets.

The presence of high neutral proteolytic activity in the microsomal fraction, especially in the membrane component (13, 15), and the important role of these membranes in protein synthesis (16) certainly opens the possibility that this activity might be related to the catabolic aspect of protein turnover as suggested by Marks and Lajtha (17).

Protein deficient diets are known to produce a negative nitrogen balance with a secondary decrease in the nitrogen content of the liver (18). The experiments presented in this article were designed to determine the changes produced by different conditions of protein deficiency on the neutral and acid proteolytic activities of the liver, and to try to find some relationship between these activities and the nitrogen content of this organ. Our results have further supported the hypothesis that the intracellular proteolytic enzymes may be concerned with the catabolic phase of protein turnover. The ratio of the total acid proteolytic activity to the total neutral proteolytic activity was found to bear an inverse relationship to the nitrogen content of the liver.

METHODS

Three groups of 60 male rats of the Sprague-Dawley strain weighing about 50 gm were fed a 20% casein diet (control group), a 5% corn protein diet, and a nitrogen-free diet,[4] respectively. The composition of these diets has been published elsewhere (19). Five animals of each group were decapitated the first day of the experiment to serve as zero time controls, and subsequently, groups of four to five animals were sacrificed every 4 to 5 days during the following 40 days. Since preliminary studies had shown no significant difference between the proteolytic activities of perfused and nonperfused bled livers, the organ from bled rats was used for these studies. The liver was blotted, weighed, and homogenized for 3 minutes at top speed in a Waring Blendor fitted with a semi-micro jar; an appropriate volume of ice-cold 0.14 M NaCl was used so that the final concentration of the homogenate was 10%. In order to avoid any undue increase in the temperature of the homogenate during the homogenization, the semi-micro jar was kept in the freezer and was packed with cracked ice during the process. Preliminary experiments had shown that under these conditions the homogenization did not produce inactivation of the proteolytic activities studied.

A fourth group of adult male rats (250 gm) was starved but allowed free access to water. In this group five rats were decapitated at the beginning of the experiment (zero time control) and subsequently, a similar number every day for 8 days. After bleeding, the liver was removed and homogenized as previously described. Appropriate aliquots of the homogenate were taken for the determination of total nitrogen, and the assay for proteolytic activities and acid phosphatase. The latter activity was included in the study as a marker for intralysosomal enzymes not related to protein metabolism.

Total nitrogen determination. Total nitrogen was determined by the micro-Kjeldahl technique as described by the Association of Official Agricultural Chemists (20).

Assay of proteolytic activity at pH 4.5. The assay system used was basically the one described by Koszalka and Miller (21). The substrate used was a 2.5% solution of hemoglobin in 8 M urea, the pH being adjusted to 4.5 with HCl.

The composition of the assay mixture was carefully determined in order to obtain a rate of reaction that would follow zero-order kinetics with respect to the substrate. It was made of 5 ml of urea-hemoglobin substrate, 6 ml of water, and 4 ml of 10% liver homogenate. The cold mixture was allowed to equilibrate to 37° in a Dubnoff-type water bath for 5 minutes. Two milliliters of the mixture were taken at this time and delivered into 5 ml of 10% trichloroacetic acid. This was considered to be the zero time aliquot. Thirty minutes later a second aliquot of 2 ml was taken and the reaction was also stopped in 5 ml of 10% trichloroacetic acid. After standing for 30 minutes both aliquots were filtered and the absorbance of the filtrate was determined at 280 mμ. The prote-

[4] This diet was shown to contain less than 0.5% of nitrogen of undetermined origin. The term *N-free* will be used throughout the manuscript, despite its inaccuracy, to maintain the terminology used in previous publications related to protein deficiency.

olytic activity of the homogenate was calculated by subtracting the absorbance of the 5-minute aliquot from the absorbance of the 30-minute aliquot. One unit of enzyme activity was defined as the amount of enzyme necessary to produce a change in absorbance of 0.125 in 30 minutes at 37° in the described reaction mixture. This absorbance was found to correspond to the absorbance of a 100 μM solution of L-tyrosine.

Assay of Proteolytic activity at pH 7.0. The method used for the assay of this activity was similar to the one previously described for the activity at pH 4.5, except that the substrate used was a 2.5% solution of bovine serum albumin in 8 M urea instead of the 2.5% solution of hemoglobin. The composition of the assay mixture which provided a rate that followed zero-order kinetics was the following: albumin-urea substrate, 5 ml; dionized water, 8 ml; and liver homogenate, 2 ml. The time and temperature of incubation, the calculation of the proteolytic activity, and the definition of a unit were the same as those previously described for the acid protease activity.

Assay of acid phosphatase. The method employed (22) is based on the release of *p*-nitrophenol from *p*-nitrophenyl phosphate. The composition of the assay mixture was determined so that the rate of the reaction would follow zero-order kinetics. The composition of the mixture was the following. One hundred microliters of substrate solution (5.5×10^{-3} M *p*-nitrophenyl phosphate in 0.05 M citrate buffer, pH 4.8); and 15 μl of 10% liver homogenate diluted 1:25 with water. The control mixture contained 15 μl of diluted homogenate and 100 μl of water instead of the substrate solution. Both mixtures were incubated at 37° for 30 minutes. The reaction was stopped by adding 400 μl of 0.1 N NaOH. After centrifugation the absorbance of the supernatant fluid was determine at 400 mμ.

The activity of the homogenate was calculated by subtracting the absorbance of the control from that of the assay mixture. One unit of enzyme activity was defined as the amount of enzyme necessary to liberate 1.0 μmole of *p*-nitrophenol from *p*-nitrophenyl phosphate in 30 minutes at 37°. A 1 mM-solution of *p*-nitrophenol was found to have an absorbance of 0.255.

Expression of results. The results were calculated as the percentage change in weight or activity at each particular time (t) with respect to the zero time control (t_0).

Percentage change has been defined as follows:

$$\% \text{ change} = \frac{100(W_t - W_{t0})}{W_{t0}},$$

where W_t is the mean total weight or activity at time t, and W_{t0} is the mean total weight or activity at time t_0. According to this type of calculation, the first group of 5 rats studied (t_0 control) on each diet gave a percentage change of zero since at this time the values at t were equal to the values at t_0. Therefore, when the percentage change gave positive numbers, it was interpreted as an increase of weight or activity with respect to the first group studied (t_0 control), and when it gave negative numbers was interpreted as a loss of weight or activity with respect to the t_0 control.

The mean total activity of each group was used for the calculation of the ratio *Total proteolytic activity at pH 4.5/Total proteolytic activity at pH 7.0.*

The statistical significance of the difference between the mean values of the points on consecutive peaks and valleys of the curves was determined by the "t" test.

RESULTS

Animals fed the 20% casein diet. The curves in Fig. 1 represent the rate of growth of a normal rat as estimated by the change in body and liver weight and nitrogen content of the liver. It is evident that, under normal conditions, there is a direct relationship between the rate of increase of the liver weight and the total nitrogen of the organ. From the twelfth to the twenty-fifth day of the experiment the smoothness of the curves representing the change in liver weight and nitrogen content was altered by a well defined hump. This period of

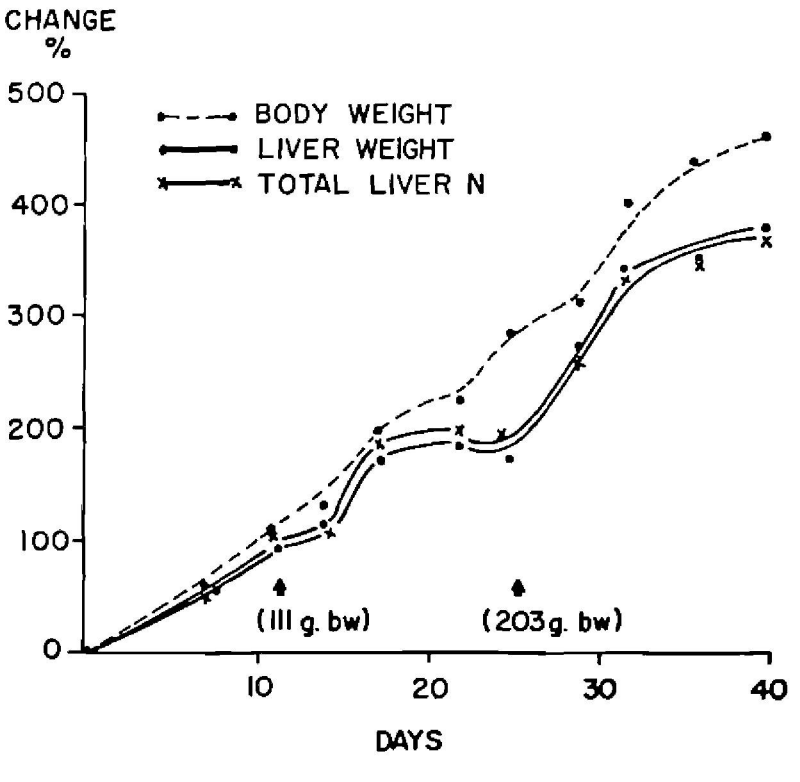

FIG. 1. Rate of growth of the normal male rat as judged by the increase in body and liver weight and total liver nitrogen with age. Diet: 20% casein.

time corresponds with the period of sexual maturation of the male rat (23).

The curves in Fig. 2 represent the change in the enzymic activities of the liver. The

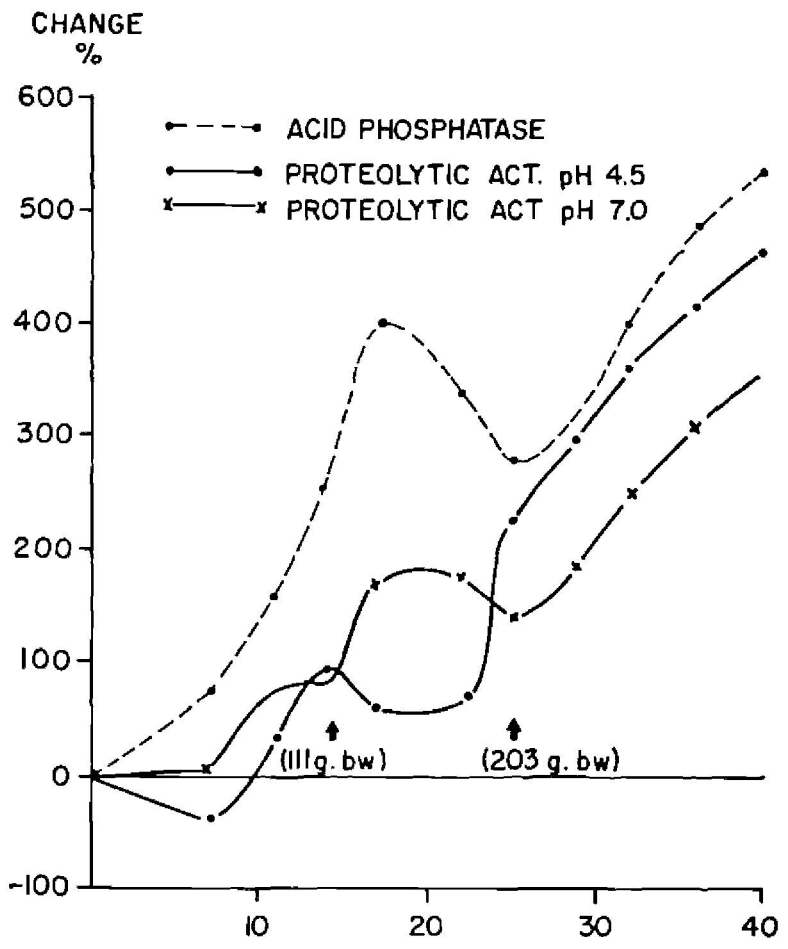

FIG. 2. Changes in the proteolytic activities and the acid phosphatase activity of the liver during the normal growth of the male rat. Diet: 20% casein.

largest change was shown by the acid phosphatase activity. The increase in neutral proteolytic activity and the decrease in acid proteolytic activity occurred simultaneously and suggested an inverse relationship between both activities. The absolute values of the total proteolytic activities of the liver are presented in Table I. The changes recorded during the sexual maturation period were shown to be statistically significant, but the difference between both activities, after the completion of this period, were shown to be nonsignificant.

Animals fed the 5% corn diet. Figure 3 shows the rate of growth of the animals in this group. It is evident that the increase in the liver weight no longer holds a direct relationship to the nitrogen content of the organ as in the case of the normal animals. It is of great interest to note the wavy pattern of these curves, as well as the absence of the hump showed by the normal animals during the period of sexual maturation (marked by arrows on Figs. 3 and 4).

The curves in Fig. 4 represent the change in the enzymic activities in the livers of these animals. Notice again the wavy pattern of the curves and the inverse relationship between the acid and the neutral proteolytic activities. It is interesting to note the great increment of the acid pro-

TABLE I

TOTAL PROTEOLYTIC ACTIVITY OF THE LIVER OF RATS FED THE CASEIN AND CORN DIETS (UNITS)

Casein[a]					Corn[a]				
Days	pH 4.5		pH 7.0		Days	pH 4.5		pH 7.0	
	$\bar{x}$	S.D.	$\bar{x}$	S.D.		$\bar{x}$	S.D.	$\bar{x}$	S.D.
0	19.4	2.2	25.5	5.0	0	23.0	2.0	26.0	1.7
7	13.0[b]	1.7	27.1	6.3	4	38.7[c]	4.6	23.5	4.4
11	26.6[b]	5.4	45.7[b]	11.3	8	29.6	3.2	19.9[c]	4.5
14	38.6[b]	2.0	46.7	8.7	11	27.0	3.6	24.8	1.4
17	32.1[b]	2.8	70.7[b]	15.2	15	15.6[c]	8.4	25.5[c]	2.2
22	33.7	3.5	71.6	24.1	18	24.4	6.5	20.3	5.8
25	65.7[b]	10.0	62.6	10.0	22	19.5	2.0	19.5	1.4
29	78.5	15.5	73.5	30.8	24	17.9	2.4	17.2	1.4
32	91.2	15.8	89.8	30.6	28	16.7[c]	2.8	21.5	2.0
36	102.1	8.4	107.5	21.2	31	20.9	2.6	21.8	4.6
40	111.8	15.5	117.6	21.8	34	21.9[c]	1.7	25.5[c]	2.8
44	93.7	7.7	92.2	16.4	37	17.0[c]	2.3	19.1	5.0

[a] Five rats per group.

[b] $P \ll 0.05$ with respect to the preceding value.

[c] $P \leq 0.05$ with respect to the point in the preceding peak or valley.

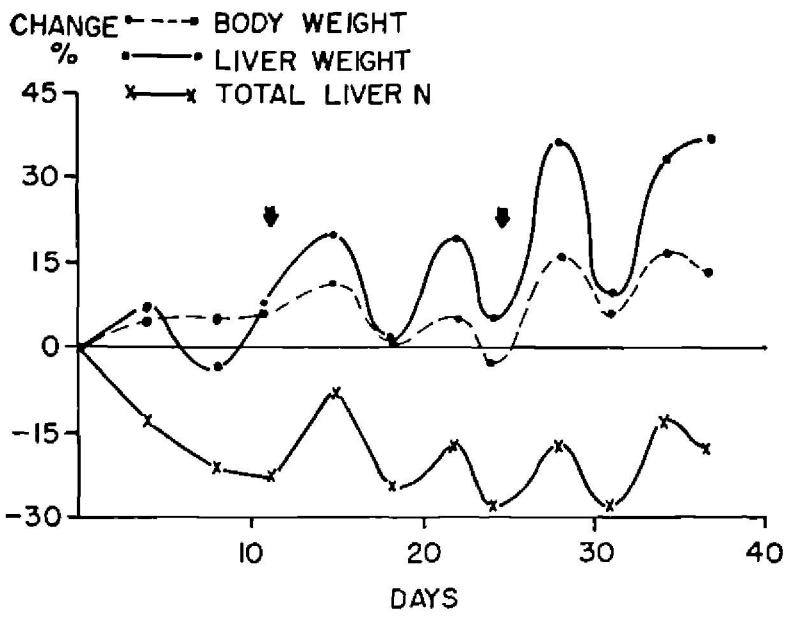

FIG. 3. Effect of a 5% corn protein diet on the rate of growth of the male rat.

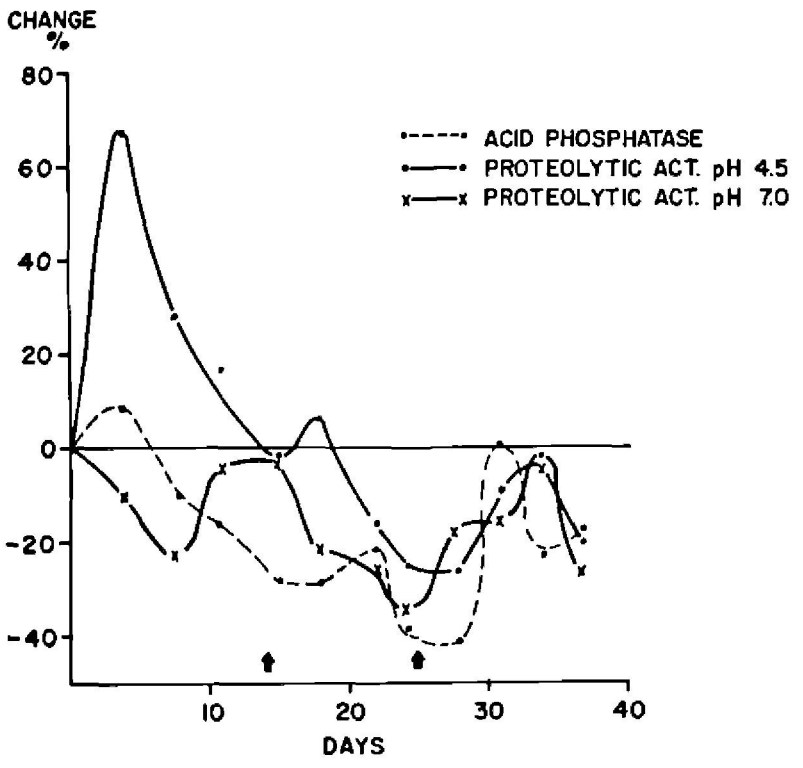

FIG. 4. Changes in the enzymic activities of the liver produced by the 5% corn protein diet.

teolytic activity during the first 4 days of the experiment. The study of the absolute values presented in Table I shows that the difference between the mean value of the points in consecutive peaks and valleys is statistically significant.

The changes in acid phosphatase activity, which is considered to be mainly intralysosomal, did not show any relationship to the changes observed in either of the proteolytic activities studied, despite the fact that the acid proteolytic activity has been also considered to be mainly intralysosomal.

Animals fed the nitrogen-free diet. The curves in Fig. 5 show the effect of this diet on the rate of growth of the rat. It is evident that the pattern of response is markedly different from the one showed by the rats fed the corn diet. Nevertheless, the wavy pattern previously described for the growth curve of the animals on the corn diet is also evident, as well as the dissociation of the relationship between the weight and the total nitrogen content of the liver. No definite changes could be detected during the sexual maturation period. Figure 6 represents the changes in the enzymic activities studied. As in the case of the previous experiment one of the outstanding features of this graph is the increment in the acid proteolytic activity during the first 4 days of the experiment, concomitant with a marked decrease in the neutral proteolytic activity. After the tenth day this pattern was lost and both curves followed similar course with their waves in phase. As in the case of the rats fed the corn diet, the study of the absolute values (Table II) showed the differences between peaks and valleys to be significant.

The changes in the activity of acid phosphatase did not show any relationship with the changes in the proteolytic activities, nor any pattern suggestive of the changes normally found during the period of sexual maturation.

Starved animals. Figure 7 represents the effect of starvation on the rate of growth

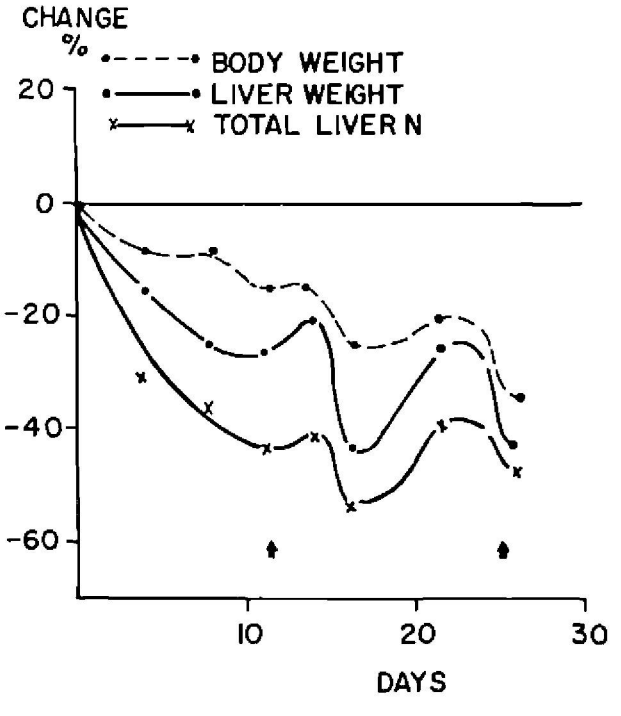

FIG. 5. Effect of a nitrogen-free diet on the rate of growth of the male rat.

of the adult animals (250 gm). Notice that the experimental period covers 8 days instead of the 4 weeks of the other experiments and that the interval between each point represents one day instead of 3 or 4 as in the other studies.

During the first day of the experiment there was a marked increase in the total liver nitrogen, in spite of the fact that the liver weight decreased about 6%. After the fourth day of starvation the change in the nitrogen content became directly related to the change in liver weight.

Figure 8 shows the change in the enzymic activities of these livers. It is noteworthy that during the first day of the experiment there was an increase in the neutral proteolytic activity and a decrease in the acid proteolytic activity which coincided with the increase in the nitrogen content of the liver. When the relationship between the proteolytic activities was reversed (second day) a marked decrease in the nitrogen content of the organ was detected. The inverse relationship between the two proteolytic activities was maintained only dur-

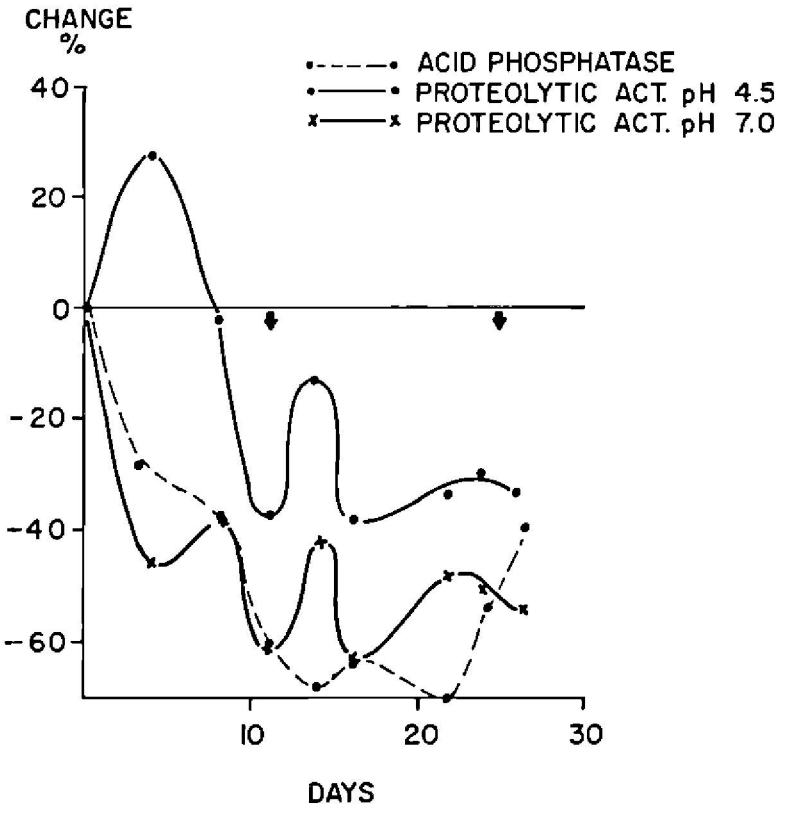

FIG. 6. Changes in the enzymic activities of the liver produced by the nitrogen-free diet.

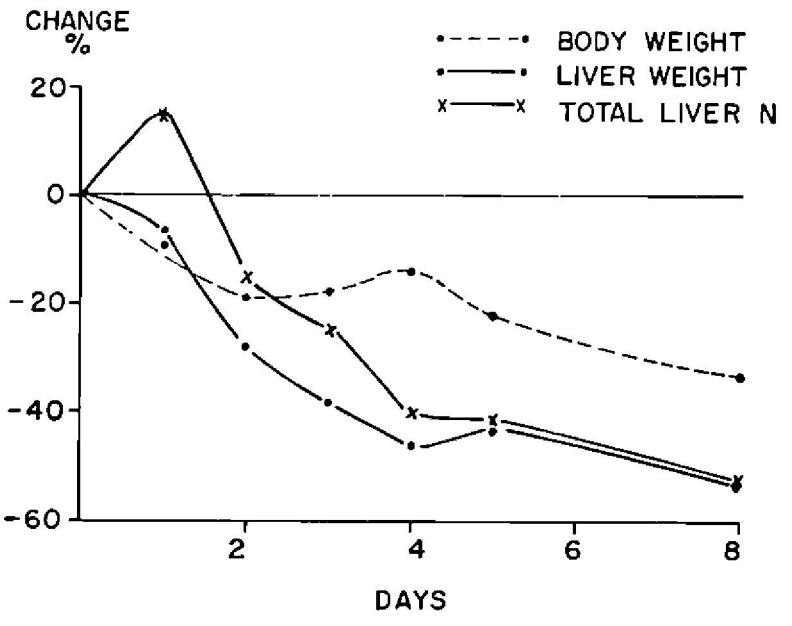

FIG. 7. Effect of acute starvation on the rate of growth of the male rat.

TABLE II

TOTAL PROTEOLYTIC ACTIVITY OF THE LIVER OF RATS FED THE N-FREE DIET AND OF STARVED RATS (UNITS)

N-Free[a]					Starvation[a]				
Days	pH 4.5		pH 7.0		Days	pH 4.5		pH 7.0	
	$\bar{x}$	S.D.	$\bar{x}$	S.D.		$\bar{x}$	S.D.	$\bar{x}$	S.D.
0	13.8	1.6	19.4	3.2	0	78.0	15.5	73.5	30.8
4	17.7[c]	2.2	10.5[c]	2.0	1	73.5	8.4	82.2	25.2
8	13.1	3.3	11.8	2.2	3	60.4[b]	2.5	53.4	10.7
11	8.5[c]	1.1	7.4[c]	1.9	4	51.9	12.5	48.1	8.7
14	12.1[c]	2.1	11.2[c]	2.2	5	39.5	7.2	32.9	5.5
16	8.6[c]	0.7	7.5[c]	0.6	8	43.9	1.3	31.5	11.9
22	9.2	1.7	10.0[c]	1.4					
24	9.8	1.7	9.5	1.7					
26	9.4	2.8	9.0	1.7					

[a] Five rats per group, except in the starvation experiment in which there were three rats per experimental point and five rats in the control group.

[b] $P < 0.05$ with respect to the preceding value.

[c] $P \leq 0.05$ with respect to the point in the preceding peak or valley.

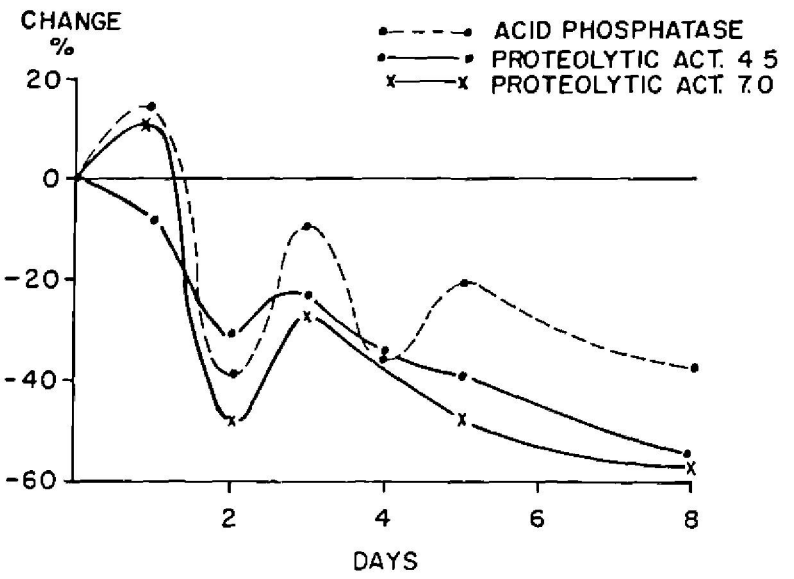

FIG. 8. Changes in the enzymic activities of the liver produced by 8 days of acute starvation.

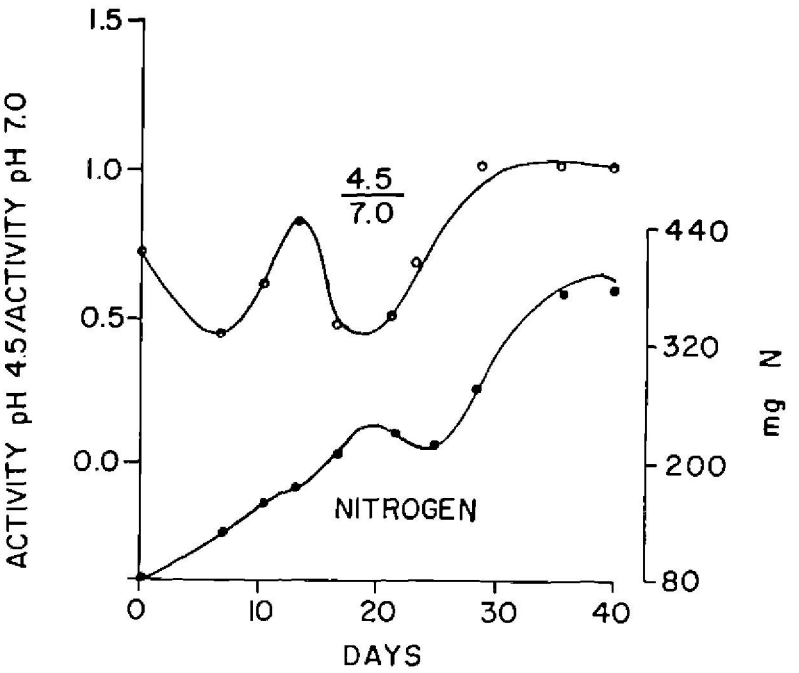

FIG. 9. Relationship between the nitrogen content of the liver and the ratio of proteolytic activities in the normal male rat. Diet: 20% casein.

ing the first 2 days of the experiment; the rest of the time both curves followed a similar course with their waves in phase. Due to the small number of animals per group and the large variance, statistical significance between peaks and valleys could not be demonstrated.

The activity of the acid phosphatase followed the neutral proteolytic activity during the first 4 days of the experiment. During the rest of the time it followed an independent course.

Ratio of the acid proteolytic activity to the neutral proteolytic activity. Since all the curves showing the changes in proteolytic activities under different dietary conditions have suggested the possibility of an inverse relationship between the acid and the neutral activities, the ratio of the total acid proteolytic activity to the total neutral activity was calculated and plotted together with the change in total liver nitrogen. Figures 9, 10, 11, and 12 show the result of this type of plot. Notice that, except for the control group, in all the other experiments the ratio of proteolytic activities bears

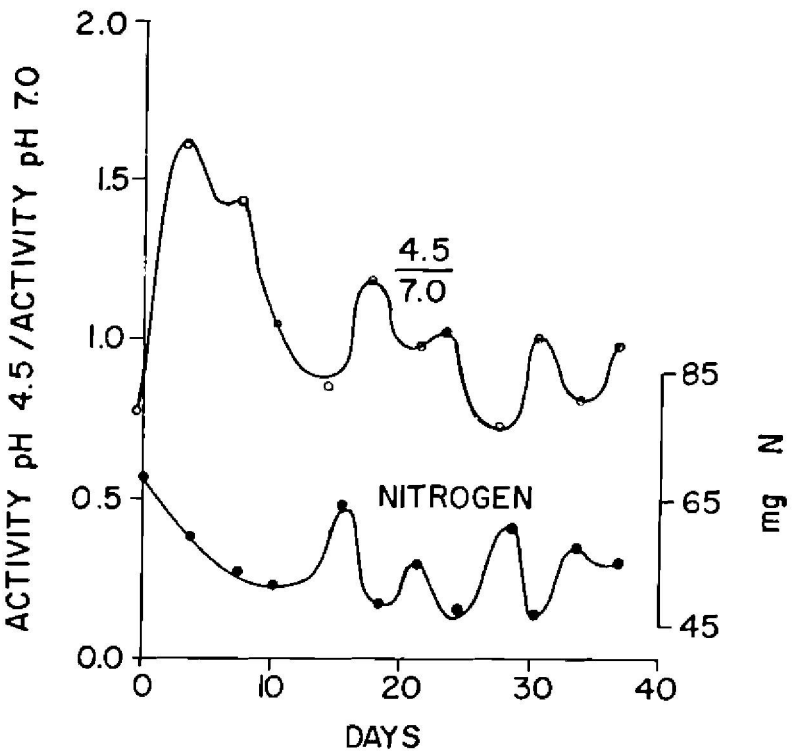

FIG. 10. Relationship between the nitrogen content of the liver and the ratio of proteolytic activities in the male rat subjected to a 5% corn protein diet.

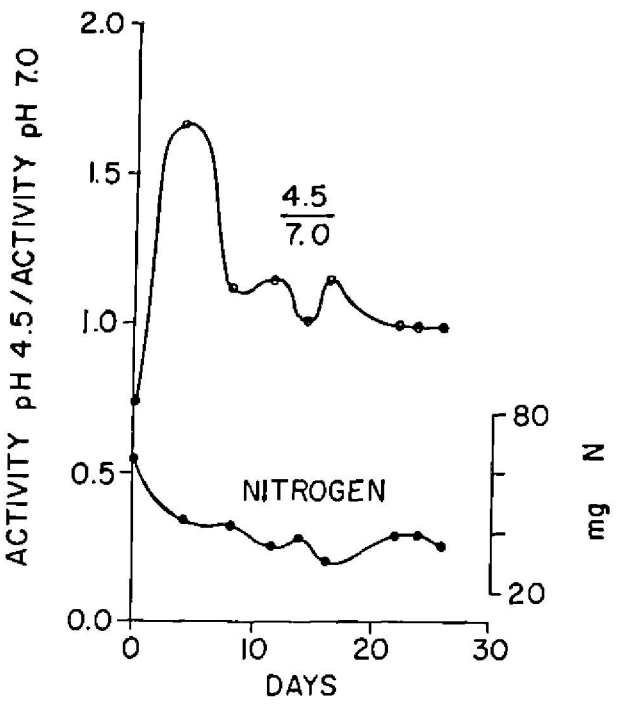

FIG. 11. Relationship between the nitrogen content of the liver and the ratio of proteolytic activities in the male rat subjected to a nitrogen-free diet.

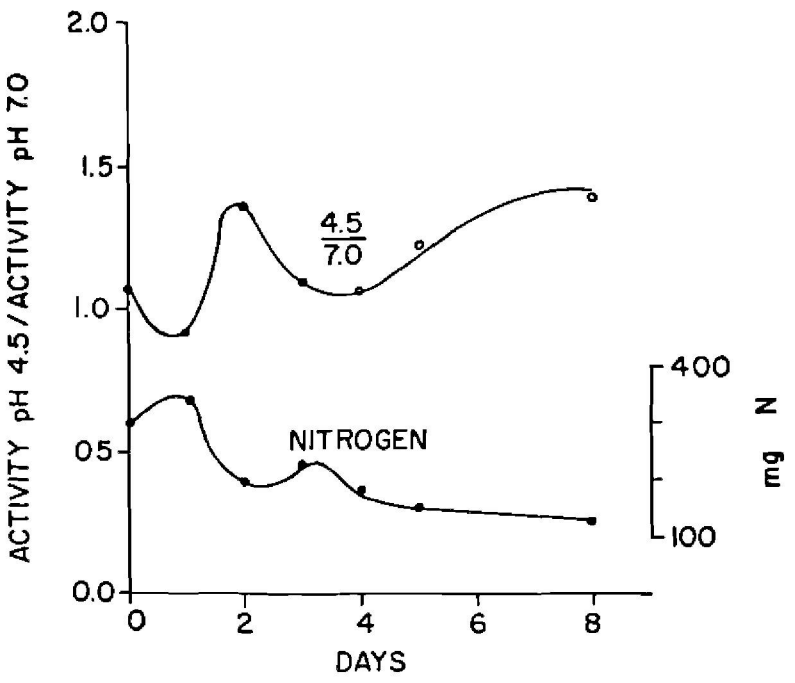

FIG. 12. Relationship between the nitrogen content of the liver and the ratio of proteolytic activities in the male rat subjected to an acute period of starvation.

an inverse relationship to the nitrogen content of the liver.

DISCUSSION

Before any attempt is made to interpret the results described in the previous section it is necessary to enumerate briefly some of the limitations of the methods used for the enzyme assays.

The use of total homogenates for the assay of proteolytic activities is mainly limited by the fact that there are several proteases in the liver cells that simultaneously hydrolize the proteins used as substrates (1). Therefore, the absorbance at 280 mμ of the trichloroacetic acid supernatant fractions does not measure the activity of a single enzyme, but the activity of several enzymes with a broad pH range of activity.

The acid phosphatase and the acid proteinase activities have been mainly located within the lysosomes (8), and the neutral proteolytic activity has been mainly located in the microsomal fraction (13, 15). However, the studies of Umaña (13), Umaña and Dounce (14), and Finkenstaedt (24) have shown that the acid proteolytic activity can also be detected in the nuclear, and microscomal fractions and the neutral proteolytic activity in the nuclear and mitochondria-lysosomal fractions. Both activities have also been found in the soluble phase of the homogenate.[5] However, it is impossible to decide at this time whether or not these are truly soluble activities or artifacts produced during homogenization. Palade (25) has found that the acid phosphatase activity can also be detected in the nuclear fraction. Therefore, the activity of the whole homogenate has to be considered as the combined expression of the activities of the different intracellular fractions, being impossible, under our experimental conditions, to ascribe the changes of the acid phosphatase and the acid proteolytic activity to alterations in the lysosomes, or the changes in the neutral proteolytic activity to alterations of the microsomal fraction.

It has been pointed out that in periods in which the total nitrogen of the liver increases (sexual maturation and the first day of starvation) there is an increase in the neutral proteolytic activity and a simultaneous decrease in the acid proteolytic activity. In contrast with this pattern, every time that the liver nitrogen is reduced, such as during the first few days on the protein-deficient diets, the opposite changes in these activities are observed. Considering the intracellular location of the neutral proteolytic activity, and the fact that rapidly growing organs like regenerating liver (11) and organs with a high rate of protein turnover also have a high proteolytic activity, it is possible to conclude that the neutral proteolytic activity may be mainly concerned with the catabolic aspect of protein turnover. On the other hand, the main intracellular location of the acid proteolytic activity within the lysosomes, and its great increase during the periods in which nitrogen is being lost from the liver, suggest that this activity may be mainly concerned with excess protein breakdown under conditions in which amino acids are required for energy production or for refilling partially depleted amino acid pools. Since the ratio of the acid to the neutral proteolytic activity was found to bear an inverse relationship with

[5] This fraction has been operationally defined as those components left in solution after centrifugation of the homogenate at 105,000*g* for 2 hours.

the nitrogen content of the liver, it seems possible that the nitrogen content of the liver could be regulated by the relationship between the overall rate of the concerted activity of the proteolytic enzymes and the rate of protein synthesis.

Miller (26, 27) and Schultz (28) have reported that rats fed a protein-deficient diet for 21–23 days or starved for 6–7 days showed a decrease in the catheptic activity of the liver. Nagel and Willig (29) reported that renal necrosis produced by complete ligation of the renal blood vessels resulted in inactivation of cathepsins B and D. From these results these authors concluded that intracellular proteolytic enzymes do not play a role in protein catabolism. Our results have shown that after a certain period of time there is indeed a reduction in the total proteolytic activity of the protein-deficient livers. However, as the relationship between the proteolytic activities ratio and the nitrogen content of the liver showed, it is not the specific or the total activity of a particular type of protease that reflects the metabolic status of the liver with respect to protein metabolism, but the relationship between the activity of the different proteases considered at a particular time during the process of protein malnutrition.

In the case of the necrosis produced by a complete ligation of the blood vessels it is evident that other factors besides the necrotic process itself might have contributed to the inactivation of the enzymes, since the occlusion of the renal vein produced an accumulation of metabolites that conceivably could have acted as inhibitors.

It is interesting to note that, even though the acid proteolytic activity and the phosphatase activity are mainly contained within the lysosomes, their pattern of response is independent of one another. It will be interesting to investigate whether the intralysomal enzymes are differently affected by protein deficiency and if, under the conditions used in our studies, the permeability of the lysosomal membrane is differently affected for each hydrolase, especially with respect to the acid proteolytic activity.

Another interesting feature of the curves presented in this article is their wavy pattern. This pattern is probably due to the existence of several periods of adaptation of the organism to the stress situation created by a qualitative and quantitative deficit of amino acids. The continuously decreasing ability of the organism to adapt to this situation might be measured by the decreasing amplitude of the waves. At this time no explanation can be given as to the mechanisms involved in this series of adaptations at different metabolic levels, even though hormonal influences should be considered among the best candidates to play an important role in this phenomenon.

The marked differences in the patterns obtained for each type of protein deficiency studied further support the hypothesis previously presented by the author (30) that different types of protein-deficient diets will result in different types of proteolytic response.

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