DIURNAL VARIATIONS IN ACTIVITY OF FOUR PYRIDOXAL ENZYMES IN RAT LIVER DURING METABOLIC TRANSITION FROM HIGH CARBOHYDRATE TO HIGH PROTEIN DIET

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SUMMARY

The diurnal variations in enzyme activities including tyrosine aminotransferase (TAT), ornithine decarboxylase (ODC), ornithine aminotransferase (OAT) and serine dehydratase (SDH) have been studied in rats trained to a 2 hour meal feeding schedule ("2+22") during metabolic transition from 12.5 to 60% protein diets over a period of 21 days. Although the maximal TAT activity on the first day was slightly lower compared with other days, both TAT and ODC activities adapted rapidly to the increased dietary protein from the first day. The responses of TAT and ODC to the food were so rapid that the maximal value was observed only 4 hrs after the onset of feeding. After each feeding ODC activity decreased rapidly after 4 hours, while TAT activity declined only after 6 hours had elapsed. No clear diurnal rhythm was observed in either OAT or SDH, though OAT activity tended to decrease from the beginning of the dark period and to resume a slow adaptation after about four hours. In contrast to ODC and TAT both OAT and SDH required about 7 days to fully adapt to the high protein diet. The activities of the four enzymes were also compared after 4 groups of rats had been adapted to the "2+22" feeding of 12.5, 30 and 60% protein diets and to 60% diet, ad libitum, respectively. The enzyme activities were not directly proportional to the protein content of the diets although higher activity was observed on the high protein diets. The diurnal variations in both TAT and ODC were observed in all "2+22" groups although the timing of the peak values were slightly different from each other. The maximal activities of TAT were found at earlier times in 12.5 and 30% protein groups than in the 60% protein group. The peak time for ODC activity was found at a later time in the 12.5% protein group than in rats fed 30% and 60% protein. Ad libitum rats fed 60% protein maintained relatively high levels of TAT activity compared to the rats on the "2+22" schedule. However, the maximal activity of ODC on the 60% diet was not observed until after about 7 days. The enzyme activities were not directly proportional to the protein content of the diets although higher activity was observed on the high protein diets. The diurnal variations in both TAT and ODC were observed in all "2+22" groups although the timing of the peak values were slightly different from each other. The maximal activities of TAT were found at earlier times in 12.5 and 30% protein groups than in the 60% protein group. The peak time for ODC activity was found at a later time in the 12.5% protein group than in rats fed 30% and 60% protein. Ad libitum rats fed 60% protein maintained relatively high levels of TAT activity compared to the rats on the "2+22" schedule. However, the maximal activity of ODC on the 60% diet was not observed until after about 7 days.

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protein diet ad libitum was so low that a diurnal rhythm was not clearly evident.

The study of enzyme regulation under in vivo conditions is rendered increasingly complicated by several new developments that seem to suggest the need for the protocol described below. First, in contrast to previous data that suggested a smooth circadian rhythm in the secretion of certain hormones Wetzman et al. have published a series of studies on the "episodic" secretion of ACTH, cortisol, and growth hormone based on the use of indwelling catheters in humans and taking of blood samples as frequently as every five minutes (1,2). Potter (2) has described these episodic secretions as "probabilistic" in that the individual episodes appear to be unpredictable, while their circadian probability can be seen only when a sufficient number of records is averaged. Second, many of the enzymes that can be readily induced by hormones have very short half-lives, and are subject to multiple controls at three or more levels, transcription, translation, and degradation (3-5). Thirdly, many enzymes are rapidly affected by food intake and diet composition (6). Although rats eat more during the hours of darkness they tend to eat during short unpredictable intervals in both light and dark periods under ad libitum conditions and these will vary from one laboratory to another (6). If enzyme regulation is to be studied in vivo it might be desirable to try to standardize the behavioral stimuli and the food intake so as to minimize the effect of individual variations in the multiple factors affecting enzyme level. An alternative approach is obviously the use of cell cultures (3,5,7) but ultimately it would be desirable to bring in vivo and in vitro findings into a rational relationship.

Previous reports have described the use of controlled feeding and lighting schedules for studies on enzyme regulation in rat liver in vivo (8-10). It was established that tyrosine aminotransferase (L-tyrosine:2-oxoglutarate transaminase, EC 2.6.1.5) in rat liver shows a marked daily oscillation associated with food intake and proportional to protein in the diet, while serine dehydratase (L-serine dehydratase (deaminating), EC 4.2.1.13) fails to show a significant daily shift in adapted animals (9). In a metabolic transition from a 12% protein diet to a 60% protein diet it was further shown that the response to the high protein diet was immediate in the case of the tyrosine enzyme, in intact or adrenalectomized animals, but inoperative during the first 12 hours in the case of the serine enzyme in both groups of animals (9). The delayed response was later confirmed and shown to occur over a period of several days (11). In a recent report (10) it was shown that when the controlled feeding period was shortened to 2 hours a sharp rise in ornithine decarboxylase (L-ornithine carboxylase, EC 4.1.1.17) as well as in tyrosine transaminase occurred and was more pronounced when food was given at the onset of the dark period than at a later time. Peraino (12,13) has studied the response to a shift from low protein to high protein diets in terms of both serine dehydratase and ornithine aminotransferase (L-ornithine:2-oxoacid aminotransferase, EC 2.6.1.13), showing a delayed onset of adaptation in both cases, and demonstrating that the response is increased by hydrocortisone in the case of the former but decreased by hydrocortisone in the case of the latter (13).

The present experiment was arranged to reveal the different responses to a shift in protein intake in rats that were meal-fed for 2 hours at the onset of the dark period, using as parameters two enzymes known to make a rapid response and two enzymes known to make a slow response, selecting critical time points in each 24 hour period, over a period of 3 weeks. Of possible additional interest is the fact that all 4 enzymes contain
pyridoxal phosphate (14-19), while two of them have the same substrate (ornithine). The results suggest that the biochemistry of enzyme synthesis and degradation may be more advantageously studied if diet and behavior are carefully controlled in in vivo experiments.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain were purchased from the Sprague-Dawley Co., Madison, Wisconsin at 28 days of age, fed 12.5% protein diet (9) (Teklad) according to the "8+16" regimen (9) in which the rats were fed from 8:30 to 16:30 for a week, then were shifted to the "2+22" regimen (10) in which rats were fed from 8:30 to 10:30. An inverted lighting schedule with the dark period from 8:30 to 20:30 was employed in all cases. After three weeks of adaptation to the "2+22" schedule, the rats were shifted from 12.5% to 60% protein diet (9). The rats were killed at 8:30, just prior to feeding and at 12:30, 14:30, 16:30 and 20:30 over a period of 3 weeks. A similar schedule was followed for one group of rats on the day preceding the shift, in order to establish the values in rats adapted to 12.5% protein. The livers of the rats killed by decapitation were quickly removed, washed in cold physiological saline, frozen in liquid nitrogen, weighed and homogenized with a Potter-Elvehjem homogenizer in an equal volume (w/v) of 0.1 M sodium phosphate buffer (pH 7.5) containing 0.8 mM pyridoxal phosphate and 2 mM ethylenediaminetetraacetic acid. The homogenates were centrifuged at 105,000 x g for 60 min and the resultant supernatant fractions were stored at -20°C until used.

Diurnal variations in the enzyme activities were also studied in livers of rats fed 12.5, 30 and 60% protein diets on the "2+22" schedule and of rats fed 60% protein diet ad libitum. Rats were purchased at 35 days of age, divided into 4 groups, maintained under these feeding conditions for 3 weeks and killed at the times described above.

The assay mixture for tyrosine aminotransferase activity consisted of 0.2 M potassium phosphate buffer (pH 7.6) containing 6.6 mM tyrosine, 0.04 mM pyridoxal phosphate and 10 mM a-ketoglutarate. The reaction was started by adding 0.1 ml of the enzyme solution which had been diluted appropriately with the homogenizing buffer. After 10 min reaction at 37°C the reaction was stopped by adding 0.2 ml of 10 N NaOH and the optical density at 331 nm was measured after 20 min. For the calculation of the activity a value of 19,9000 M⁻¹ cm⁻¹ was used for the molecular absorption coefficient of p-hydroxyphenylpyruvate (20).

The activity of ornithine decarboxylase was determined by measuring the liberation of 14CO₂ from DL-(1-14C)ornithine by the method of Pegg and Williams-Ashman (18), with some modifications. The assay reaction mixture contained 0.5 µCi radioactivity and was at the optimum pH 6.6 with optimum dithiothreitol concentration of 3 mM, ethylenediaminetetraacetate 1 mM, a saturating concentration of ornithine 2 mM, and of pyridoxal phosphate 0.4 mM as established for our samples and assay conditions. The composition of the initial assay mixture at room temperature was as follows: 0.8 ml 56 mM sodium phosphate pH 6.5 containing 3.8 mM dithiothreitol and 2.3 mM ethylenediaminetetraacetate. To this was added 0.1 ml of cold supernatant fraction. The reaction was started by adding 0.1 ml of 20 mM ornithine substrate solution and transferring to a 37°C water bath. The 20 mM ornithine substrate solution was prepared from reagent grade L-ornithine to which was added 5 µCi/ml radioactivity from DL ornithine, 1-14C monohydrate specific activity 61 mCi/mmol (Amersham Searle, Arlington Heights, Ill.) Low blank 14CO₂ values were obtained by storing
this acid solution in the frozen state in an assay reaction flask fitted with a rubber stopper suspending a centerwell carrying a pellet of sodium hydroxide.

The incubation was carried out in 25 ml Erlenmeyer flasks equipped with a rubber stopper suspending a small polypropylene centerwell (Kontes Glass Co.) (18). After 30 min reaction at 37°C the reaction was stopped by injecting 0.5 ml of 6 N citric acid. CO₂ evolved was trapped by 0.2 ml of a mixture of ethanolamine and ethyleneglycolmonomethylether (1:2) in the center well. The reaction flasks were further incubated at 37°C for 45 min and kept at room temperature overnight. The radioactivity was measured by a liquid scintillation spectrometer (Packard Model 3003).

The activity of OAT was determined by the method described by Peraino (21) and calculated using a value of $2.45 \times 10^3$ M⁻¹ cm⁻¹ for the molar absorption coefficient of pyrroline-5-carboxylate at 440 nm (14).

Reaction conditions for measuring SDH activity consisted of 0.1 M Tris-HCl buffer (pH 8.4) containing 0.025 M KCl, 0.2 mM pyridoxal phosphate, 0.15 M serine, 0.4 mM NADH, and 0.5 mg lactic dehydrogenase/100 ml of the assay mixture. The reaction was started by adding 0.1 ml of an appropriately diluted enzyme solution into 2.5 ml of the reaction mixture, and the decrease in the optical density at 340 nm was recorded automatically at 25°C (12).

The enzyme activities are expressed in μmol or nmol per minute per gram liver as indicated in the Figures.

RESULTS

Both ODC and TAT responded quickly to the nutritional changes as reported previously (9–11), but differences between the two enzymes were revealed. Fig. 1 shows that TAT activity attained a nearly maximal value 4 hours after the beginning of feeding, but the maximal activity was usually obtained 6 hrs after the feeding, especially when the activity was calculated in umoles/liver/100 g body weight (data not shown). The highest level of the activity on the first day was about two thirds of the peak values on the later days. From the second to the 7th day the highest values were in remarkably good agreement. Each day the induced activity of TAT decreased by 12 hrs after the feeding to low levels which were little higher than before the diet change. However, some rats on the 2nd day were found to have fairly high activity even 12 hrs after the feeding (10 hrs after removal of the diet) (Fig. 1).

The activity of ODC showed very sharp increases after feeding beginning from the very first contact with the 60% protein diet (Fig. 2). The activity reached its highest level 4 hrs after the beginning of feeding and decreased to less than half of the maximum value 6 hrs after feeding, and the lowest level was maintained until the next feeding. The enzyme activity was fully induced on the first day, but fairly large differences were observed among the peak values for subsequent days, which may be a reflection of the short half life of this enzyme (18).

The activity of OAT responded very slowly to the increased level of dietary protein, confirming earlier reports by Peraino (12,13). The activity decreased slightly after the beginning of the dark period, while ODC and TAT were increasing, and began to increase again during the dark period while ODC and TAT were decreasing but the activity was found to
Tyrosine aminotransferase activity during metabolic transition from 12.5% to 60% protein diets. Shaded areas at the top of the chart show the dark periods. Rats were fed for two hours in the dark period as shown by black bars. Each different symbol represents a different experiment. Each point represents the mean of 3 rats, with standard error indicated if it exceeded the diameter of the symbol. Transition began at 8:30 on DAY 1.

Ornithine decarboxylase activity during metabolic transition from 12.5% to 60% protein diets. Symbols as in Fig. 1.
Ornithine aminotransferase activity during metabolic transition from 12.5% to 60% protein diets. Symbols as in Fig. 1.

Serine dehydratase activity during metabolic transition from 12.5% to 60% protein diets. Symbols as in Fig. 1.
Increase mainly in the light period (Fig. 3). Thus, as a whole, the activity seems to increase in a stepwise fashion each day.

A slight increase in SDH activity was observed on the 2nd day of the shift but on the whole the response was very slow and similar to that of OAT (Fig. 4). The activity showed no diurnal changes related to either dark-light nor feeding-fasting rhythms. The highest activity which was observed on the 7th day after the shift was more than 40 times as high as that of 0 day. The high activity was maintained up to 3 weeks in the present study.

The diurnal changes in the enzyme activities were also studied in the livers of rats that had been fully adapted to the various feeding conditions, that is, for a period of 3 weeks (see Figs. 1-4). Since the activities of TAT and ODC are closely related to the food intake, the dry weights of the stomachs are shown in Figure 5. Rats on the '2+22' schedule ate more food within 2 hrs of feeding period than ad libitum rats. The 12.5% protein diet was digested almost completely by 16:30, while most of the 60% protein diet and more than half of the 30% protein diet were digested by 20:30. Although ad libitum rats also began to eat from the beginning of the dark period, Individual rats did not eat continuously (see 6). The mean value for stomach weight declined at 14:30 after which mean values increased again by the beginning of the light period (Fig. 5).

**FIG. 5**

Dried weight of rat stomach plus contents as a function of time of day. The animals had been fully adapted to various diets and feeding schedules. Each point represents the mean of four rats. Open triangle, 60% protein diet ad libitum; solid circle, 60% protein diet on '2+22'; open square, 30% protein diet on '2+22'; open circle, 12.5% protein diet on '2+22'.

Vol. 17, No. 9  Diurnal Variations in Pyridoxal Enzymes  1417
According to Fig. 6, the group fed 60% protein diet on the "2+22" schedule showed the same TAT change as shown earlier in Figure 1. Diurnal variations were also observed in both 30% and 12.5% protein groups, but the maximal activities were very low compared with that of 60% group. Moreover, the high levels of the enzyme activity were not maintained until 14:30 in both 30 and 12.5% protein groups. Thus in these groups the maximal activities were observed at 12:30. Especially in the group on 12.5% protein the decrease was so fast that the activity reached a minimum by 14:30, when the highest level was observed in the 60% protein group. In the ad libitum group the activity at 8:30 was as high as the highest level for the group on 30% protein, then it increased rapidly as observed in the group on 60% protein on the "2+22" schedule. However, after the activity reached its maximum, it decreased so slowly that a contrast was found between ad libitum and "2+22" groups by the end of the dark period.

This contrast was observed even more clearly in the case of ODC activity (Figure 7). The activity in "2+22" groups increased quickly from the beginning of the feeding. A sharp maximal value was observed at 12:30 in 30% and 60% protein groups. Although the activity increased also in the ad libitum group on 60% protein, the maximal value was not clear and was less than in the 12.5% protein group on the "2+22" schedule, and the activity did not decrease up to 20:30. The activities of both OAT and SDH depended on the protein content of the diet (Figures 8 and 9). The diurnal variation in both enzymes were not striking in any group although OAT activity in the 60% protein - "2+22" group showed a decrease from the beginning of the dark period and began to increase before the end of the dark. These changes would not be significant on a per liver basis (10).

**DISCUSSION**

A series of our studies (6,8-11) has described controlled lighting and feeding schedules, in which rats are entrained to a 24-hr cycle of 12-hr dark and 12-hr light and food is available for a limited period in the dark. The usefulness of these experimental conditions has been shown in the present report as well as in our previous work, especially in the study of enzymes with short half-lives such as TAT and ODC (Figures 1, 2, 6 and 7). The response of TAT, SDH and some other enzymes to a shift from low to high protein diet has also been studied under the "8+16" feeding schedule and in adrenalectomized rats (9,11). In the present study four pyridoxal enzymes, TAT, ODC, OAT and SDH were chosen as markers. The first two enzymes respond quickly to the nutritional changes and the other two do not respond as soon. ODC and OAT share ornithine as a substrate. The response of SDH to 60% protein is enhanced by cortisone, while the response of OAT is diminished by cortisone (12). Thus the four enzymes studied in the present report display unique responses to diet and hormone manipulation.

ODC activity increased to the maximal level on the first day of the shift from low protein to high protein diet. Although TAT responded to the shift, the highest activity of the first day did not reach the maximal level, which was attained after the 2nd day as reported previously (11). Slight increases in the activities of both OAT and SDH were observed on the 2nd day; however, it took more than four days before the full activities were induced. The clear diurnal variations in both ODC and TAT were observed in Figures 1 and 2 as reported previously (9-11). Morris and Peraleno have observed a diurnal rhythm in OAT protein synthesis which was at a maximum near the beginning of the light period, reached a minimum at the time the light went out and began to increase after approximately 6 hrs (22). However, this rhythm in the enzyme protein synthesis did not correlate
Tyrosine aminotransferase activity of liver from rats that had been fully adapted to various feeding conditions, as a function of time of day. Symbols as in Fig. 5.

Ornithine decarboxylase activity of liver from rats that had been fully adapted to various feeding conditions, as a function of time of day. Symbols as in Fig. 5.

Ornithine aminotransferase activity of liver from rats that had been fully adapted to various feeding conditions, as a function of time of day. Symbols as in Fig. 5.

Serine dehydratase activity of liver from rats that had been fully adapted to various feeding conditions, as a function of time of day. Symbols as in Fig. 5.
directly with the enzyme activity, since a clear circadian rhythm was not observed in the activity. In Figure 3 the OAT activity decreased immediately from the beginning of the dark period than it increased again during the dark period, though the variation was not so clear as observed in TAT and ODC. This change seems apparently to correlate inversely to the change in ODC activity with which OAT shares ornithine as a substrate. However, regulation of ODC activity by OAT (23) seems unlikely. Although OAT activity at saturation levels of substrate is higher than ODC activity, the latter has a much lower K and this would be highly relevant under physiological conditions. Although SDH activity seemed to increase mainly during the light period, it was not as clear as in the case of OAT activity and not always observed.

TAT activity of the ad libitum group did not decrease as rapidly as in the "2+22" groups (Figure 5). TAT synthesis may be continuously stimulated by blood amino acids in the former group, since the stomachs of the ad libitum rats were full at the end of dark period and since the enzyme activity is induced by tyrosine and other amino acids (24). The decrease in the activity of 30% and 12.5% protein groups at 14:30, when the 60% protein group showed the highest activity, may also be explained if amino acid influx in the liver was not enough to maintain TAT activity for a longer time.

Circadian rhythm of ODC activity in ad libitum fed rats, which had been reported by Hayashi et al. (25) was not served in the present study. However, the important point to be mentioned is that a feeding pattern which was observed in ad libitum rats in one laboratory is not always observed in another laboratory (6). For this reason, among others, the controlled feeding and lighting schedule is helpful for studies of enzyme regulation.

The main rationale for the controlled feeding schedule is related to the problem of episodic hormone secretion (2) which might occur along with what might be called episodic meal feeding in animals fed ad libitum (6). As emphasized earlier (2), one of the objectives in the controlled feeding schedules, and especially in the "2+22" schedule with food at the onset of the dark period (10), is to eliminate as much as possible the occurrence of episodic hormone secretion by putting the major flux within a narrow time frame. The data in the present report show that with an enzyme having a short half-life as in the case of ODC, the period of peak activity is extremely brief (Fig. 2). We had previously shown that feeding late in the dark period was less effective in achieving peak activity than feeding at the onset of the dark period (10). Now it has been shown by Nelson, Scheving and Halberg (26) that the corticosterone flux in mice fed on a "4+20" schedule shows a much sharper and higher steroid peak (40 μg per 100 ml blood) when the diet is presented at the onset of darkness than when the food is presented at the onset of the light period (26 μg per 100 ml blood). Mice fed ad libitum had a much smaller amplitude of oscillation, with a maximum of 16 μg of cortisone per 100 ml blood each day. Whether mice and rats exhibit episodic hormone secretion comparable to that seen in man (2) will be difficult to establish. We have previously published data on plasma corticosterone in rats on the "8+16" feeding schedule and the data show peak values at the light-dark transition comparable to that seen in mice (26). Mice have been successfully adapted to the "2+22" schedule after a week on the "8+16" schedule, and they show a sharp transitory rise in liver ODC slightly earlier than that in the rat (Fig. 2), while they have nearly undetectable levels of ODC at the corresponding times when food is withheld (Dr. Michael Pariza, unpublished).
Controlled feeding and lighting schedules seem to offer many advantages for experiments that attempt to assess the contributions of food intake and hormone flux on the processes that increase or decrease the activity of various enzymes, especially those with short half-lives. Applications to hepatoma and regenerating liver metabolism will be presented separately.

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