Diurnal Rhythms in Mammalian Protein Metabolism

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I. Introduction

A. Regulation of the Extracellular Fluid

Perhaps the most important advantage that accrues to mammalian cells by virtue of their communal relationship is the constancy of their immediate environment, the extracellular fluid. The cells of mammals have the good fortune to be bathed in a medium whose temperature and whose concentrations of perhaps forty important compounds vary over a surprisingly narrow range; hence these cells are never faced with the problem of surviving in the absence of glucose or water or amino acids, or of adapting to temperatures which differ from their normal mean by more than a degree or two.

There is good evidence that the constancy of several of these biochemical and physical functions (e.g., those which keep the temperature, osmolality, and calcium concentration of the extracellular fluid from

rising too high) results from the operation of a particular type of regulatory mechanism, the closed feedback loop. Moreover, the systems which control these functions appear to utilize parallel structural components, termed sensors, set-points, comparators, and effectors. The biological sensor is thought to be a group of cells, in the brain or elsewhere, which continuously monitors the level of the regulated function in the plasma; the set-point is another group (or the same cells, as in the case of the parathyroid gland) which stores information as to the highest (or lowest) absolute value that the regulated function is allowed to attain. The cells which function as comparators presumably receive inputs from both the sensor and the set-point; they subtract the former value from the latter, and if the difference is greater than zero they issue an "error signal." This signal is transmitted via neural or hormonal channels to distant effector organs, which then act to dissipate the heat, conserve the water, or remove the excess calcium. It cannot be assumed *a priori* that all compounds whose concentrations in the internal milieu remain fairly constant do so because of the operation of closed feedback loops; processes not susceptible to internal control (such as the spillage of glucose into the urine of hyperglycemic subjects) can also contribute to the stabilization of blood levels. However, more often than not, the search for a regulatory system as the basis of the constancy of a particular compound in the extracellular fluid has proved rewarding.

B. Biological Rhythms

For the greater portion of the past century, the recognition that the levels of certain compounds in the extracellular fluid remain surprisingly constant tended to obscure the fact that most of these levels could also be shown to undergo characteristic changes, albeit within a narrow range. Perhaps the most general type of variation which is exhibited by regulated functions is diurnal rhythmicity. It now appears likely that the rates of most physiological processes and the concentrations of most biochemical compounds within the cells of mammals show at least some tendency to vary as a function of time of day. These variations are reflected in changes in the composition and temperature of the extracellular fluid. For example, body temperature in humans is regulated at all times; however, the set-point around which temperature is held constant varies predictably during each 24-hour period. Early in the morning the "normal" temperature for most males is a full degree lower than it is 12 hours later. It can be shown that if the temperature is artificially elevated from 97.5°F to 98.0°F at 7:00 AM the subject will perspire; however, if the same subject's temperature is *lowered* from 98.6°F to 98.0°F at 4:00 PM, he will shiver.

This type of demonstration provides compelling evidence that the increase in body temperature observed during the hours of daylight is not simply the result of the higher ambient temperature, or of the greater heat load generated by muscular work during these hours. Similar studies, described in greater detail below, have shown that the daily rhythm in plasma amino acid levels is not simply the consequence of man's habit of presenting a load of dietary amino acids to his gut at mealtimes. The concentrations of most amino acids in human plasma have recently been shown to undergo characteristic diurnal fluctuations. Among subjects maintained on a normal activity schedule (i.e., in bed between 11:00 PM and 7:00 AM), the levels of most of the individual amino acids are lowest between 2:00 and 4:00 AM, and rise by as much as 100% during the next 8 hours (Wurtman *et al.*, 1968a). These rhythms persist when subjects are deprived of dietary protein, and must therefore reflect endogenous changes in the metabolism of the amino acids. The concentration of any amino acid in plasma depends upon two sets of rates, the rates at which the compound enters the blood from its various sources (e.g., dietary protein, the breakdown of tissue protein), and the rates at which it is removed from the blood by entering one of its "sinks" (e.g., uptake into cellular amino acid pools, transamination). Some of these rates may be regulated in the sense described above; that is, they may be controlled by feedback loops which monitor the existing level of the amino acid, and operate to maintain this level within a narrow range. In addition, almost all these rates can be modified by hormones, food intake, and other factors which also display diurnal rhythmicity. This chapter will describe diurnal rhythms in the metabolism and plasma concentrations of individual amino acids, and will consider the mechanisms which might generate such rhythms.

C. Mechanisms of Biological Rhythmicity

The mechanism of a rhythm will be defined as (1) the site of origin of the oscillating signals which instruct the cells of the "target organ" [for example, the hepatic cells which display rhythmic changes in tyrosine transaminase activity (Wurtman and Axelrod, 1967)] to vary the rate of a physiological process rhythmically, and (2) the intracellular biochemical events which mediate the rhythmic behavior. Theoretically, a daily rhythm in, for example, the activity of a hepatic enzyme could be caused by signals originating within any of three possible loci:

(1) *Within the cell itself.* Evidence has been presented that diurnal rhythms which are independent of the environment do exist among certain single-celled organisms. For example, *Euglena* incorporates more phenylalanine-^{14}C into protein during the daily light period than in dark-

ness; this rhythm persists under conditions of continuous darkness for at least 2 days (Feldman, 1968). Mammalian cells maintained in synchronous tissue cultures show distinct biochemical rhythms which are thought to reflect the time-constants of the various steps necessary for protein synthesis. The periods of these rhythms are usually considerably shorter than 24 hours (Klevecz and Ruddle, 1968). If cell-free extracts of yeasts are incubated in a medium which is continuously injected with glycolytic substrates (e.g., glucose or fructose), the activity of phosphofructokinase in the medium oscillates with periodicities of 3.5–8.6 minutes (Hess *et al.*, 1969). Oscillations in hemoglobin biosynthesis with a frequency similar to the time necessary for the synthesis of one molecule have been described in rabbit reticulocytes (Tepper *et al.*, 1969). The interaction of several such high-frequency cycles could theoretically generate a rhythm whose period approximated 24 hours.

(2) *Elsewhere in the body (e.g., in the brain)*. It is generally believed that the daily rhythms in body temperature and plasma cortisol level result from parallel changes in the set-points around which these functions are regulated (Yamamoto and Brobeck, 1965). These set-points are presumably a property of neurons localized within the brain; their oscillations are thought to be endogenous and not to depend upon the presence of environmental cycles. There is at the present time no direct evidence that any biochemical rhythm in a mammalian tissue is endogenous; there is only indirect evidence that various rhythms persist in animals deprived of one or more environmental cycles, and thus *could* be of endogenous origin. This type of proof is less than persuasive, since it is never possible to place experimental subjects in an environment which is truly devoid of cyclic inputs. Natural diurnal cycles in light, ambient temperature, food intake, and humidity can be damped without too much difficulty; however, cycles in electromagnetic field strength, gamma irradiation, and the gravitational pull of the moon persist in all terrestrial experiments, and can probably induce certain rhythms (Brown, 1965).

(3) *Outside the body*. The environment of the particular planet on which mammals happened to evolve is characterized by 24-hour cycles in a variety of physical functions. One of the cycles, the presence and absence of light, has been shown to generate daily rhythms in the biochemical activity of the pineal gland (Wurtman and Axelrod, 1965; Wurtman, 1967b). The pineal is the unique locus in mammals of an enzyme, hydroxyindole-*O*-methyl transferase (HIOMT), which synthesizes the hormone melatonin (Axelrod *et al.*, 1961). Under normal lighting conditions (i.e., 12 hours of light per day), the activity of this enzyme shows marked time-dependence; it rises several fold during the hours

of darkness and falls during the light period (Axelrod *et al.,* 1965). Rats blinded or placed in an environment of continuous darkness immediately lose the pineal enzyme rhythm; on the other hand, these treatments do not extinguish the presumably endogenous rhythms in body temperature or plasma corticosterone content (Haus *et al.,* 1967). The concentration of norepinephrine within pineal sympathetic nerve endings also varies diurnally with a rhythm which is generated by the light-dark cycle. The neural pathway through which the retinal input reaches the pineal and generates these biochemical rhythms is distinct from the visual system; it utilizes a special nerve bundle, the inferior accessory optic tract (Wurtman *et al.,* 1967a).

Even though few rhythms appear to be generated by the light-dark cycle, essentially all rhythms in mammals are influenced to some extent by this cyclic environmental input. In general, light acts as the dominant synchronizer for 24-hourly rhythms, determining the times of their maxima and minima. The light-dark cycle is thought to "entrain" endogenous rhythms, causing their period lengths to become exactly 24 hours (Aschoff, 1960; Halberg *et al.,* 1959; Haus *et al.,* 1967). The advantages to the animal of utilizing this stable environmental cycle to entrain its internal rhythms are obvious; within an individual animal, unrelated rhythmic events can be made to occur in a regular sequence, and metabolic events which generally occur at a specific time of day can be anticipated; within a species the rhythms of many individuals can be synchronized. Rhythms which are caused by the light-dark cycle and rhythms which are simply entrained by this cycle tend to show similar responses to shifts in the phase of the daily light period. If the time that subjects are exposed to light is changed from 6:00 AM—6:00 PM to noon–midnight, the times that the maxima and minima of both types of rhythms occur change until they reestablish a normal interval (termed a "phase angle"), with reference to the onset of light or darkness (Halberg *et al.,* 1959; Haus *et al.,* 1967). Rhythms generated by the light cycle can, however, be distinguished from endogenous rhythms by their response to continuous darkness: The former are immediately extinguished in the absence of light cycles (Wurtman *et al.,* 1967a), while the latter persist.

Among a small group of rhythmic functions it has been possible to demonstrate that, when animals are maintained under constant lighting conditions (i.e., continuous darkness), not only does the rhythm persist, but also the length of its period changes from exactly 24 hours to something slightly greater or less (i.e., it becomes *circadian*) (Halberg, 1959; Halberg *et al.,* 1959; Aschoff, 1960). The demonstration that a daily rhythm "free-runs" in the absence of cyclic lighting inputs is usually

offered as further evidence that the rhythm is, in fact, the product of an endogenous mechanism. It is argued that a free-running period of, for example, 23 hours and 41 minutes is not isomorphic with any known environmental cycle; thus it cannot result from a one-for-one response to an oscillating environmental input. However, even the demonstration that the period of a rhythm is not exactly 24 hours does not prove that the oscillation is of endogenous origin. Environmental cycles do exist whose periods are slightly longer or shorter than 24 hours (e.g., the lunar-tidal cycle, 24.8 hours); the resultant of the lunar and solar frequencies might appear as a circadian rhythm.

It is feasible to demonstrate circadian periodicity only when the experimental design allows the rhythmic function to be sampled repeatedly and frequently in the same subjects. In general, data of this sort cannot be obtained in studies on rhythms in the activity of tissue enzymes (Wurtman, 1967a). The necessity of killing the experimental animal to obtain the tissue specimen allows each animal to be sampled only once; hence the enzyme rhythm must be studied by analyzing tissues from groups of 6–8 animals taken at multiple intervals during a period of at least 24 hours. The difficulty in maintaining perfect synchrony among such large groups of animals, and the logistics problems involved in collecting their tissues every few hours during a full 24-hour period have, to date, made it impossible for any investigator to demonstrate that the period length of any tissue enzyme rhythm becomes circadian in mammals maintained under constant environmental conditions. In the remainder of this chapter, the term "circadian" will be applied only to rhythms (e.g., in body temperature and in the concentrations of certain substances in the plasma) which have been demonstrated to free-run under controlled environmental conditions with a period significantly different from 24 hours.

In addition to the oscillating physical inputs described above, most mammals also receive cyclic inputs of chemicals (i.e., foodstuffs) because of their tendency to confine their eating behavior to a portion of the 24-hour period. Among rats exposed to light between 6:00 AM and 6:00 PM, the portal vein and the liver receive large amounts of amino acids, glucose, and other components of the diet between 2:00 PM and 2:00 AM, when the animal consumes most of its daily food intake, and little during the rest of the day. This cyclic input of dietary amino acids generates the daily rhythm in hepatic tyrosine transaminase activity (Wurtman *et al.*, 1968b); it seems likely that additional biochemical rhythms, in the liver and perhaps elsewhere, will be shown to result from the cyclic intake of foodstuffs.

II. Twenty-four-Hour Rhythms in Hepatic Enzymes

A. Tyrosine Transaminase

1. Characteristics

If Sprague-Dawley rats are maintained under light and darkness for alternating 12-hour periods and given access *ad libitum* to a diet containing protein (e.g., Purina Chow), the activity of the enzyme tyrosine transaminase shows a marked dependence upon the hour of the day that their livers are sampled (Wurtman and Axelrod, 1967; Civen *et al.*, 1967; Shambaugh *et al.*, 1967). Tissues taken from animals killed 5 hours after the onset of darkness can catalyze the *in vitro* transamination of tyrosine about four times as fast as livers taken early in the light period (Fig. 1).

2. Mechanism

Since the concentrations of corticosterone in the adrenals and blood of the rat also vary diurnally (Fig. 1), and since the administration of this steroid is known to produce an increase in tyrosine transaminase activity (Lin and Knox, 1957; Nichol and Rosen, 1964), it initially seemed likely that the nocturnal increase in transaminase activity was a consequence of the preceding rise in plasma glucocorticoid concentration. However, this hypothesis was not supported by studies on adrenalectomized or hypophysectomized rats; in both experimental preparations, the transaminase rhythm persisted with unchanged amplitude (Fig. 2). It could thus be concluded that the cyclic signal which instructs the hepatocytes to increase their tyrosine transaminase activity is not mediated by pituitary hormones (e.g., growth hormone, ACTH, TSH), by compounds secreted in response to these hormones (e.g., corticosterone, thyroxine), or by epinephrine [which is secreted in mammals only from the adrenal medulla (Wurtman, 1966)].

Studies on intact rats had shown that injections of large amounts of tyrosine, the physiological substrate for tyrosine transaminase, or of smaller quantities of tryptophan could also produce a rise in enzyme activity (Lin and Knox, 1957). In general, these studies did not include adequate controls to rule out the possibility that the enzyme changes observed after administering the amino acid were not simply a manifestation of the same transaminase rhythm as that observed in the untreated animal. However they at least suggested that the oscillating signal responsible for the tyrosine transaminase rhythm might be an amino acid, whose availability to liver cells depended upon the time of day. To

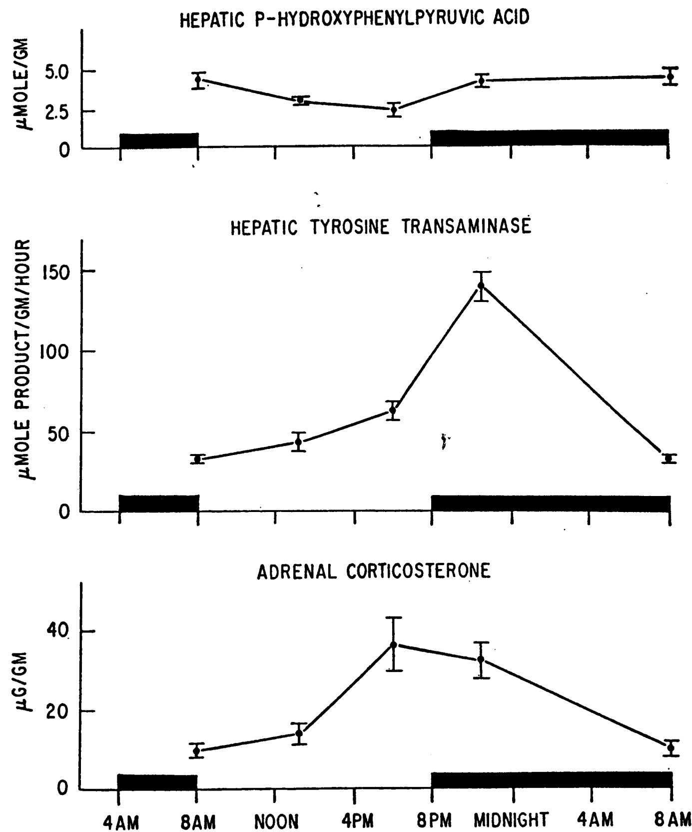

FIG. 1. Daily rhythms in hepatic tyrosine transaminase activity, hepatic content of *p*-hydroxyphenylpyruvic acid, and adrenal content of corticosterone. Rats were exposed to light from 8:00 AM to 8:00 PM for 1 week prior to assay and given access *ad libitum* to Purina Chow and water. Vertical lines in all figures refer to standard errors of the mean. (Reprinted from Wurtman and Axelrod, 1967.)

examine this possibility, livers taken at intervals throughout the day from untreated or hypophysectomized rats were assayed for tyrosine transaminase activity and for free tyrosine or tryptophan concentration; plasma samples were also assayed for free tyrosine level. It was hypothesized that, if changes in the availability of free tyrosine or tryptophan were responsible for the enzyme rhythm, it might be possible to demonstrate that the concentration of the amino acid in plasma or liver increased several hours before the daily rise in enzyme activity. No such relationship was observed between tyrosine levels and the enzyme rhythm (Fig. 3). Instead of rising several hours before the daily enzyme peak, plasma and hepatic tyrosine levels showed no change during this

period but fell during the hours following the daily rise in transaminase activity (Wurtman *et al.,* 1968b). This observation was considered compatible with the catalytic function of the enzyme; i.e., as tyrosine transaminase activity increased, more of its substrate was removed in the liver, and the concentration of its product, *p*-hydroxyphenylpyruvic acid, increased (Fig. 1). In contrast to tyrosine, hepatic tryptophan levels did vary in a manner which suggested that this amino acid might be involved in producing the transaminase rhythm: Hepatic free tryptophan content increased by about 50% several hours before the daily rise in enzyme activity (Fig. 4) (Wurtman *et al.,* 1968b).

The liver differs from other organs in that the amounts of amino

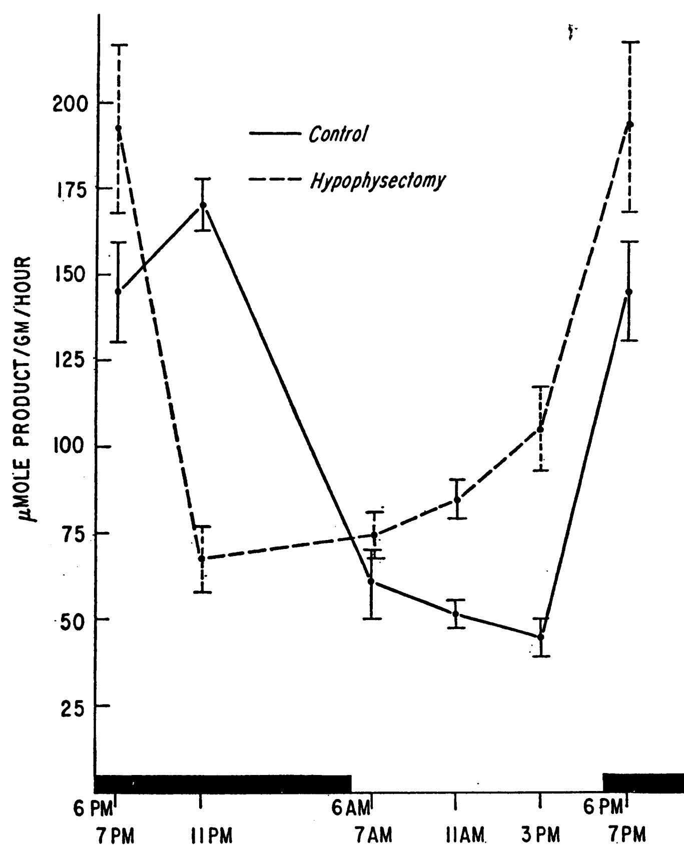

FIG. 2. Effect of hypophysectomy on the daily rhythm in hepatic tyrosine transaminase activity. Rats were hypophysectomized and kept under light from 6:00 AM to 6:00 PM for 10 days prior to assay; they had access *ad libitum* to Purina Chow and water. (Reprinted from Wurtman and Axelrod, 1967.)

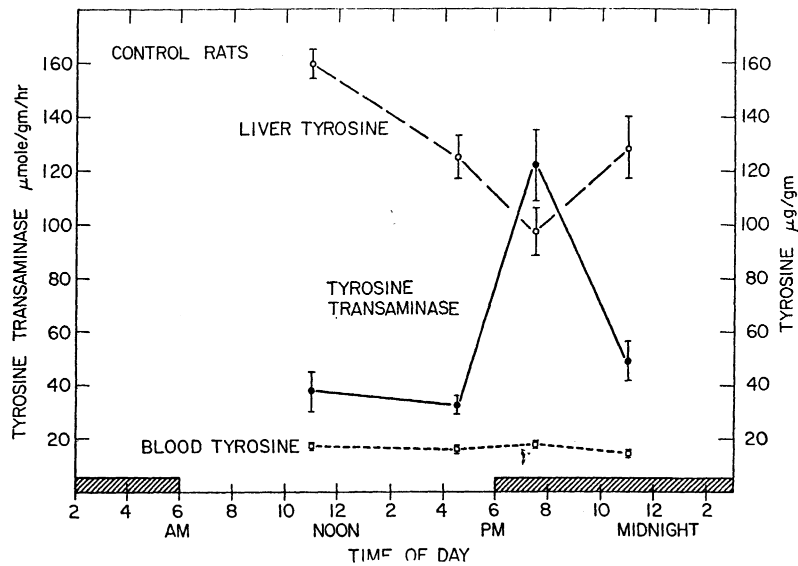

FIG. 3. Relation between blood and liver tyrosine concentrations and hepatic tyrosine transaminase activity in adult female rats given Purina Chow and water *ad libitum*. Lights were on from 6:00 AM to 6:00 PM daily. (Reprinted from Wurtman *et al.,* 1968b.)

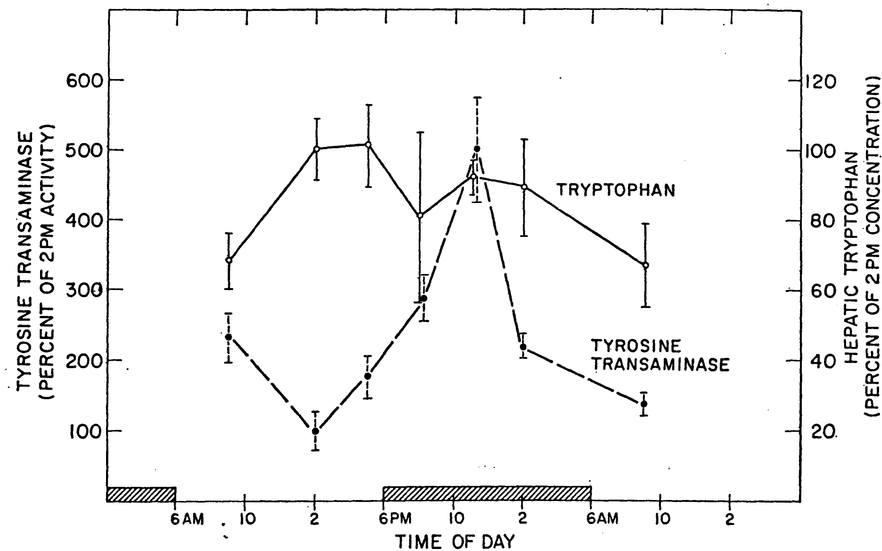

FIG. 4. Relation between hepatic tryptophan concentration and tyrosine transaminase activity in adult female rats given access *ad libitum* to Purina Chow and water. Lights were on from 6:00 AM to 6:00 PM daily. (Reprinted from Wurtman *et al.,* 1968b.)

acids with which it is perfused fluctuate markedly during the course of the day (see Chapter 38). After the animal has consumed protein, the liver receives concentrations of amino acids from the portal vascular system which are much greater than those delivered between meals from the general circulation (i.e., from the hepatic artery). Since the rat consumes its food cyclically, the quantity of tryptophan available to its liver soon after the onset of darkness, when the rate of food consumption is maximal (Wurtman *et al.,* 1968b), is far greater than the amount presented to it early in daylight period. On the basis of such reasoning it seemed likely that the oscillations in both the tryptophan content and the tyrosine transaminase activity of the liver might result from the tendency of the rat to consume dietary protein cyclically. To test this hypothesis, groups of rats were given access *ad libitum* to diets containing 18% protein (casein) or 0% protein starting soon after the onset of a daily light period; other animals were starved for 2 days. The animals were killed at intervals, and their livers were assayed for tyrosine transaminase activity. Rats having no access to protein (i.e., the 0% protein and starved groups) showed low transaminase activity throughout the first day of the experimental period (Fig. 5). On the second day, the transaminase activity in animals without protein but

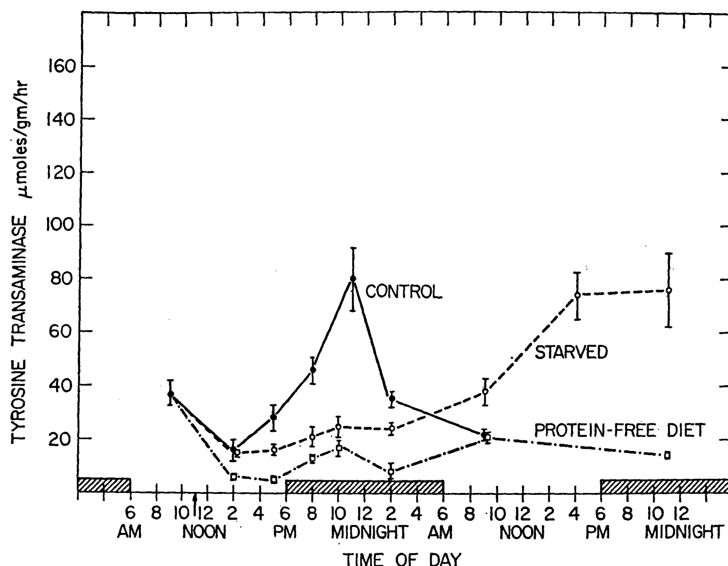

FIG. 5. Hepatic tyrosine transaminase activity in adult female rats given access to a control diet (18% protein), a 0% protein diet, or no food at all, starting at 11:00 AM. Lights were on from 6:00 AM to 6:00 PM daily. (Reprinted from Wurtman *et al.,* 1968b.)

with free access to calories (i.e., the 0% protein group) remained low, and showed little tendency to vary with time of day (Wurtman *et al.*, 1968b). At this point, enzyme activity in the starved rats rose to attain the peak evening levels observed in rats given protein (Wurtman *et al.*, 1968b). The response of the starved group presumably resulted from the increased adrenocortical secretion caused by the stress of starvation.

If rats were given access to a synthetic diet containing a complete mixture of the essential amino acids instead of protein, hepatic tyrosine transaminase activity continued to display 24-hour rhythmicity (Wurtman *et al.*, 1968b). The time of the daily peak in enzyme activity shifted slightly, possibly because amino acids from this food source did not enter the portal circulation at the same rate as amino acids derived from dietary protein. The omission of tryptophan from the amino acid mixture caused the transaminase rhythm to be extinguished (Fig. 6). It is possible that the omission of other essential amino acids might also have extinguished the enzyme rhythm; however dietary tryptophan may serve a primary function in the physiological regulation of tyrosine transaminase activity, by virtue of its special role in the aggregation of hepatic polysomes. This hypothesis is outlined below.

These studies suggested that an exogenous input, the cyclic delivery

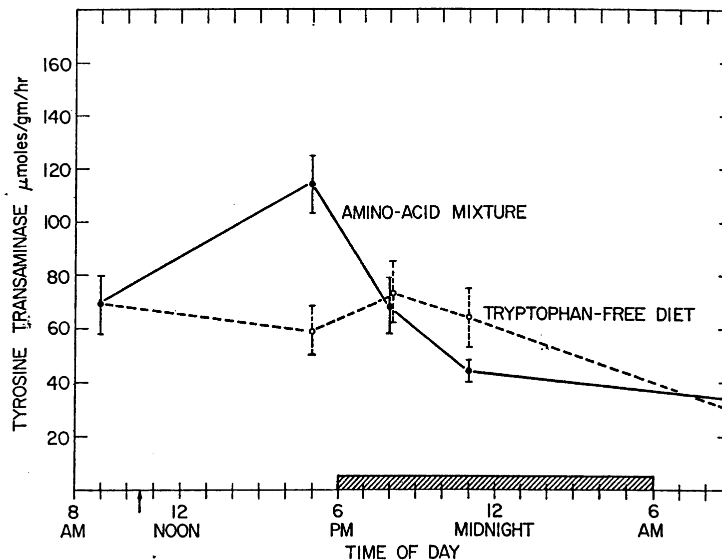

FIG. 6. Hepatic tyrosine transaminase activity in adult female rats given access to special diets starting at 10:30 AM on the day of the experiment. Lights were on from 6:00 AM to 6:00 PM daily. (Reprinted from Wurtman *et al.*, 1968b.)

to the liver of dietary amino acids, was the major factor in generating the tyrosine transaminase rhythm. This cyclic input depends upon both the composition of the diet and the tendency of the animal to confine its eating behavior to certain hours of the day. The importance of dietary composition in generating the transaminase rhythm was illustrated by the observation (Fig. 5) that the rhythm was extinguished in animals given a diet lacking in protein. Potter and his colleagues (1966) have shown that the opposite is also true; the rate at which tyrosine transaminase activity rises after an animal is given access to dietary protein is roughly proportional to the protein content of its diet. The importance of the feeding rhythm in generating the transaminase rhythm has been demonstrated by a variety of experiments: (1) The phasing of the feeding rhythm has been changed by shifting the daily onset of the light period by 12 hours. About 6 days are required for the feeding cycle to resume its normal relationship to the light cycle. During this period, the phasing of the transaminase rhythm changes at approximately the same rate as the phasing of the feeding rhythm (Zigmond *et al.*, 1969; Black and Axelrod, 1968). (2) Animals have been given access to food for only 4 hours per day (8:00 AM to noon). This constraint has shifted the phase of the tyrosine transaminase rhythm, such that the daily peak occurs several hours after the onset of eating, but almost 12 hours earlier than in animals given free access to food (Fuller and Snoddy, 1968). (3) The time of food intake has been dissociated from the time of protein ingestion by placing rats on a 0% protein diet early in the light period, and substituting an 18% protein mixture after 3, 9, or 15 hours. Under these conditions, rats continued to display a normal feeding cycle regardless of the composition of their food; however tyrosine transaminase activity did not start to rise until several hours after the animal first had access to protein (Zigmond *et al.*, 1969; Black and Axelrod, 1968). (4) Fuller has shown (1969) that mice made hyperphagic by treatment with gold thioglucose lose the tyrosine transaminase rhythm. Such animals also fail to display a normal feeding rhythm (Anliker and Mayer, 1955). If the mice are forced to eat cyclically (i.e., by being allowed access to food for only 4 hours per day), the activity of the transaminase again exhibits daily fluctuations. (5) The feeding rhythm has been extinguished by training animals to consume food in 24 equal portions during each of the 24 hours of the day. "Wheel-fed" animals prepared in this manner and exposed to light for 12 hours daily maintained a normal rhythm in corticosterone secretion; however, the transaminase rhythm was largely, but not totally, extinguished. The magnitude of the daily rise in enzyme activity was reduced to 10–15% of the increment observed among rats allowed to consume their food cyclically (Cohn *et al.*, 1970).

The persistence of a small daily rhythm in tyrosine transaminase activity among "wheel-fed" rats suggests that, in addition to the postprandial delivery of amino acids, other cyclic inputs to the liver can also generate oscillations in the activity of the enzyme. One such input might be plasma glucocorticoid level; the concentration of corticosterone in the blood of rats is known to undergo a daily rise several hours before the daily increment in enzyme activity (Guillemin *et al.,* 1959). Accordingly, the following hypothesis for the mechanism of the enzyme rhythm attributes significance to both nutritional and hormonal inputs. In the second half of the daily light period, the adrenals of the rat secrete increased amounts of glucocorticoids (Guillemin *et al.,* 1959). These agents enhance the synthesis of ribonucleic acid (RNA) within hepatic cells (Halberg *et al.,* 1958) (Fig. 7). Most of the new RNA is probably ribosomal, but some may also represent messenger coded for tyrosine transaminase (Hager and Kenney, 1968). Around this time, the animal begins to increase its rate of food consumption (Wurtman *et al.,* 1968b); this causes high concentrations of amino acids to be delivered to the liver via the portal circulation, and further stimulates RNA synthesis (Blobel and Potter, 1967; Whittle and Potter, 1968). Within the hepatic cells, dietary tryptophan (Wunner *et al.,* 1966; Sidransky *et al.,* 1967; Baliga *et al.,* 1968) and perhaps other amino acids (Mandel *et al.,* 1966) cause ribosomal and messenger RNA to

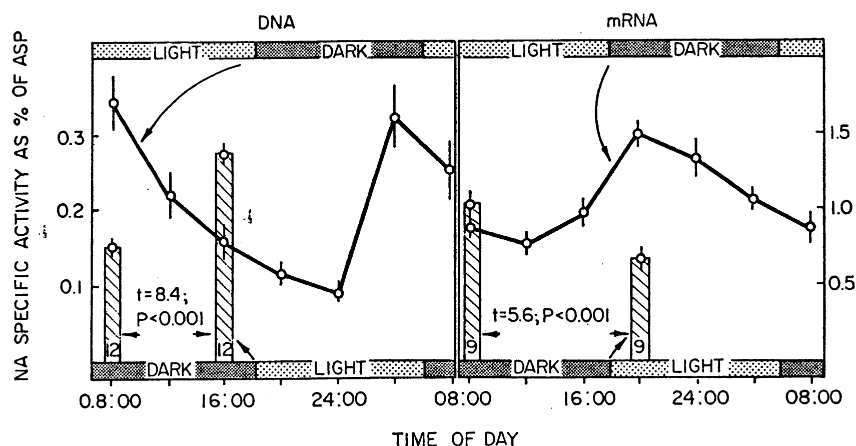

FIG. 7. Twenty-four-hour rhythms in incorporation of ^{32}P into DNA and "microsomal" RNA of mouse liver. Upper curves describe data obtained from animals kept under light from 6:00 AM to 6:00 PM; lower bars describe data obtained from animals kept under reversed lighting (i.e.. lights on from 6:00 PM to 6:00 AM) for 8–14 days. (Reprinted from Halberg *et al.,* 1959. Copyright 1959 by the American Association for the Advancement of Science.)

aggregate, forming polysomes, the units of protein synthesis. These structures then begin to synthesize tyrosine transaminase more rapidly than the protein is destroyed, hence net enzyme activity rises.

The above hypothesis rests on the assumption that the diurnal rise in transaminase activity represents an increase in the amount of enzyme protein, not simply a change in the activity of preexisting enzyme. This assumption is supported by recent evidence (D. Granner and M. Civen, unpublished observations) that the content of immunologically distinct tyrosine transaminase protein in rat liver also varies with a 24-hourly rhythm which is in phase with the rhythm in enzyme activity. Comparing the minor changes in the enzyme rhythm which follow adrenalectomy (Wurtman and Axelrod, 1967) with the near-extinction of the rhythm caused by depriving rats of dietary protein (Wurtman *et al.,* 1968b), it is apparent that the contribution of the adrenocortical cycle to the enzyme rhythm is minor. This suggests that the rate-limiting factor in the physiological regulation of tyrosine transaminase biosynthesis (i.e., in the unstressed, untreated animal) is not the amount of messenger RNA coded for this protein, but the proportion of the messenger which is bound with ribosomes in polysomal units. This proportion depends upon a nutritional input, dietary tryptophan and other amino acids, which produces its effect by acting within the cytoplasm. This hypothesis is compatible with the finding that the messenger RNA for tyrosine transaminase produced by hepatoma cells in synchronous tissue cultures is relatively long-lived (Martin *et al.,* 1968). It is also consistent with the recent observation that the proportion of hepatic RNA bound to polysomes varies diurnally in the rat (Fishman *et al.,* 1969). This proportion increases from about 50 to 73% soon after darkness among animals given free access to a diet which contains protein (Fig. 8). The rhythm is absent among animals placed on a no-protein diet.

Tyrosine transaminase activity attains a peak about 6–8 hours after the onset of darkness; it subsequently declines rapidly, even though the animal continues to consume food during the next few hours (Fig. 1). The mechanism of the daily fall in enzyme activity is entirely unknown; like the rise, it also appears to be independent of the adrenals and pituitary (Wurtman and Axelrod, 1967). The fall in tyrosine transaminase activity observed several hours after starved, adrenalectomized rats are given glucocorticoids can be blocked by inhibitors of protein synthesis (Kenney, 1967); these compounds may produce their effect by inhibiting the breakdown of the enzyme protein (see Chapter 31). The fall can also be blocked by the oral administration of casein hydrolyzates (Rosen, 1965).

The ontogenesis of the transaminase rhythm has been related to patterns of food ingestion by Honova and her colleagues (1968). An enzyme

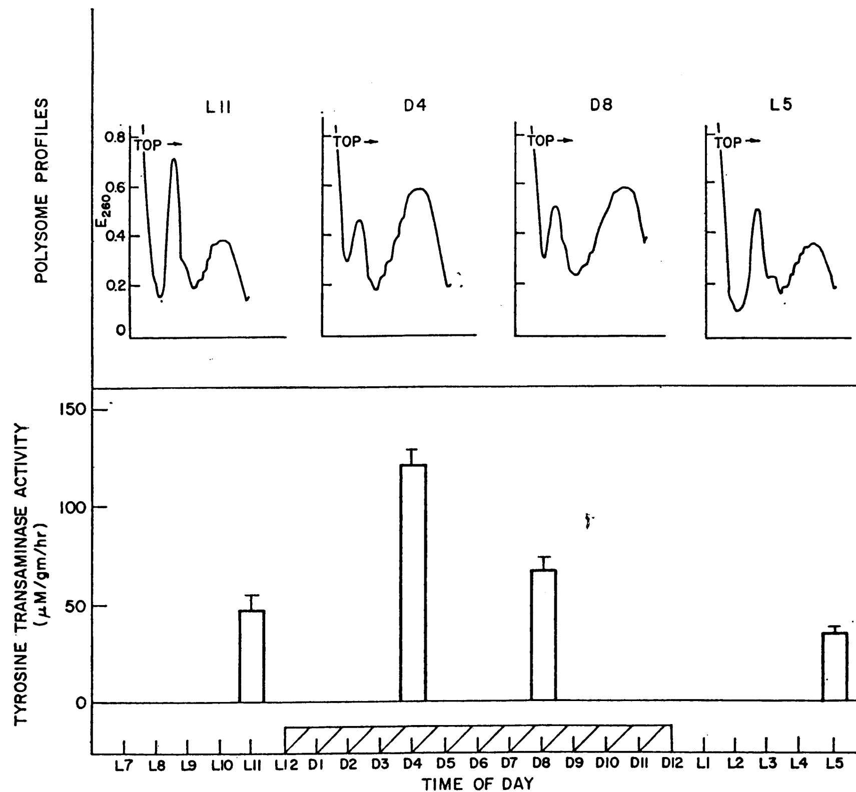

FIG. 8. Hepatic polysome profiles and tyrosine transaminase activity among groups of six rats fed Purina Chow and killed at the times indicated. Hatching along the abscissa indicates the daily dark period. (Reprinted from Fishman *et al.,* 1969.)

rhythm is present immediately after birth; however, its phasing is quite different from that seen in the adult: The daily peak in enzyme activity occurs in the morning instead of in the evening. On day 21 of life (i.e., at the time of weaning), the time of the daily peak in transaminase activity abruptly shifts to that observed in adults. The authors attribute this sudden change in phase to the fact that prior to weaning, the newborn rats ingest milk at the convenience of the mother, i.e., at times of day that she is not eating. With weaning, they suddenly assume the nocturnal feeding pattern which is characteristic of their species, and shift the time of day that they deliver dietary amino acids to their liver.

Axelrod and Black (1968) have suggested that the tyrosine transaminase rhythm is somehow related to tissue norepinephrine levels, inasmuch as rats treated with inhibitors of monoamine oxidase, an enzyme which

metabolizes norepinephrine, or with L-dihydroxyphenylalanine, a precursor of the catecholamine, exhibit both an increase in brain norepinephrine levels and a partial suppression of the hepatic transaminase rhythm. These data are difficult to interpret since they include no measurements of the rates at which the treated animals consumed food; it is possible that both compounds affected the enzyme rhythm simply by suppressing the feeding cycle; both compounds are used psychologically to modify affect and behavior. Moreover, L-dihydroxyphenylalanine, an amino acid not normally present in the circulation, might have influenced hepatic protein synthesis. Studies in other laboratories have shown that a profound pharmacological depletion of brain norepinephrine has no effect on the transaminase rhythm so long as the experimental animal continues to eat rhythmically (Wurtman *et al.*, 1968c).

Baril and Potter (1968) have observed that, following the subcutaneous injection of cycloleucine-^{14}C (a nonutilizable synthetic amino acid), the steady-state ratio of its concentrations in liver and blood varies with a daily rhythm. The ratio is highest at the time of day that transaminase activity is rising. On the basis of this observation, they have suggested that the enzyme rhythm results from a rhythm in the rate at which liver cells take up amino acids from the general circulation. This hypothesis is not compatible with the observation that adrenalectomy obliterates the rhythm in cycloleucine-^{14}C uptake (Baril and Potter, 1968) but does not alter the tyrosine transaminase rhythm (Wurtman and Axelrod, 1967). Moreover, animals given 0% protein diets retain their rhythms in plasma amino acid concentrations (Wurtman *et al.*, 1968a), but fail to display rhythms in tyrosine transaminase activity (Wurtman *et al.*, 1968b). It is well established that corticoids affect the capacity of the liver to concentrate amino acids (Chapter 4, Volume I).

The administration of glucagon (Csany *et al.*, 1967) or of insulin (Nichol and Rosen, 1964) to rats elevates tyrosine transaminase activity (see Chapter 31); hence it seemed possible that enhanced secretion of the former pancreatic hormone toward the end of the daily "starvation period" (i.e, late in the light period), or of the latter hormone soon after the animal started to eat, might be responsible for the enzyme rhythm. That this is not the case has recently been demonstrated by studies on pancreatectomized animals by Fuller (1969). Removal of this organ did not extinguish the enzyme rhythm (Fig. 9). The loss of the rhythm in intact animals given a high carbohydrate, 0% protein diet (Wurtman *et al.*, 1968b) also provides evidence that neither glucagon nor insulin participates in its genesis.

The demonstration that untreated animals, left to their own devices,

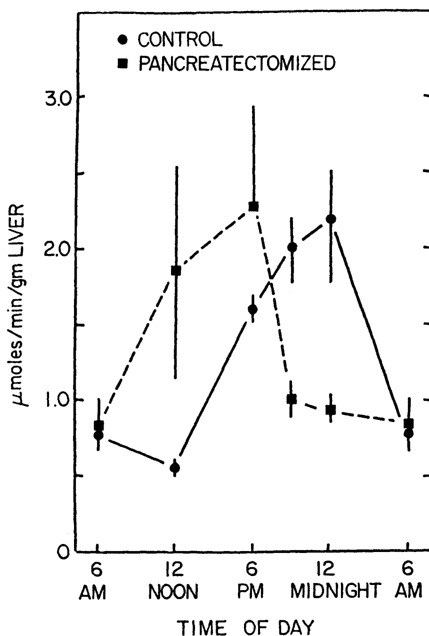

FIG. 9. Persistence of daily rhythm in hepatic tyrosine transaminase activity among pancreatectomized animals. Male rats were kept under cool-white fluorescent light from 7:30 AM to 4:30 PM and given access *ad libitum* to Purina Chow. (Figure kindly supplied by Dr. Ray Fuller, Eli Lilly Research Laboratories, Indianapolis, Indiana.)

display fourfold daily changes in tyrosine transaminase activity, and that these changes are the result of the cyclic ingestion of dietary protein, makes it mandatory that future studies on the physiological regulation of tyrosine transaminase provide considerably more information about the experimental animals than has usually been the case in the past. The environmental lighting conditions, the hour-to-hour pattern of food intake, and the composition of the diet should all be described in detail. The apparent effect of a particular experimental manipulation on transaminase activity cannot be interpreted if the reader is not told whether or not the treatment also altered the composition of the food or the time of its ingestion.

3. *Physiological Significance*

The nocturnal rise in tyrosine transaminase activity is associated with a decrease in the content of tyrosine in the liver (Fig. 3), and with an increase in the concentration of *p*-hydroxyphenylpyruvic acid (Fig. 1). If the enzyme rhythm is extinguished (by placing rats on

a 0% protein diet), hepatic tyrosine content no longer declines with the onset of darkness. The rate at which intraperitoneally injected tyrosine-^{3}H disappears from the whole mouse varies diurnally with a rhythm in phase with the hepatic tyrosine transaminase cycle (Rose *et al.*, 1969). These observations indicate that the daily rhythmic changes in tyrosine transaminase activity, measured *in vitro*, are, in fact, associated with parallel changes in the rate that tyrosine is transaminated *in vivo*. This implies that the proportion of the tyrosine in a given amount of food which remains available to the body for utilization in the synthesis of endogenous proteins depends upon the hour of its ingestion. It may be possible to increase the efficiency with which protein foodstuffs are utilized in areas in which they are in short supply by arranging for them to be eaten at metabolically favorable times of day.

During the entire part of the 24-hour day when rats eat, they tend to consume their food in multiple small meals: they nibble at a rate sufficient to keep their stomachs from becoming empty. The animals can, however, be forced to adopt human eating habits. By withholding food, the investigator can train them to eat one or two individual meals each day. Under these conditions they lose protein and accumulate fat in their carcasses (Cohn, 1966); their tissues synthesize excessive amounts of lipids from isotopically labeled precursors (Cohn and Joseph, 1967). The metabolic basis of these effects of meal-eating may be related to the mechanism of the tyrosine transaminase rhythm. The ingestion of more than threshhold amounts of protein during a short interval may cause enzyme adaptations (i.e., a rise in the activity of tyrosine transaminase, and perhaps in other transaminases) which alter the fate of any protein consumed subsequently. As a consequence of meal-eating, a larger fraction of the daily protein intake is consumed after tyrosine transaminase activity has already been increased by the prior ingestion of protein. Hence more protein is converted to precursors of lipids.

It is generally held that the major function of tyrosine transaminase relates to its role in gluconeogenesis; i.e., it deaminates an amino acid to form compounds which are utilized for the production of energy. If this hypothesis were correct, one might expect to observe in normal animals a direct relationship between the number of hours since the animal had last eaten and the level of hepatic transaminase activity; enzyme activity should rise when dietary glucose is unavailable. In contrast, it is found that the daily elevation in tyrosine transaminase activity occurs not in response to the absence of dietary carbohydrates, but to the presence of dietary proteins. It thus appears that the function of tyrosine transaminase in the unstressed, unstarved animal has little to

do with gluconeogenesis. More likely, the primary action of the enzyme is not to make its products (glycogenic and ketogenic energy sources) available, but to destroy its potentially toxic (Alam, 1965) substrate, tyrosine. When animals are starved for longer than 18–24 hours or are otherwise stressed, the secretion of glucocorticoids from the adrenal gland is stimulated, and these hormones act on the hepatic cells to induce the formation of tyrosine transaminase and other transaminases (see Chapter 31; see also, Nichol and Rosen, 1964). In this circumstance, these enzymes very likely do act to increase the rate of gluconeogenesis. Hence tyrosine transaminase activity can be regulated by two distinct inputs, depending upon the physiological state of the animal. It is enhanced by dietary protein in normal animals, and probably serves to destroy excessive amounts of its substrate; it is enhanced by adrenocortical hormones in stressed or starved animals, and serves in these circumstances to generate substrates for energy metabolism.

B. Other Enzymes

1. Demonstration of Enzyme Rhythms

Daily rhythmic changes have been demonstrated in the activities of several hepatic enzymes besides tyrosine transaminase; these include cytoplasmic enzymes which metabolize amino acids, and membrane-bound enzymes which metabolize drugs. Relatively few enzymes have been examined at intervals to determine whether they exhibit diurnal variations. Moreover, many of the publications which purport to provide evidence for the existence of enzyme rhythms lack critical experimental details without which their findings cannot be evaluated. In general, all reports on enzyme rhythmicity should include the following information: (1) the age, sex, and strain of the experimental animals; (2) the number of animals housed in each cage; (3) the lighting conditions at the times that the animals were sampled (i.e., nature of the light source; light intensity at the level of the animals; the hours each day that animals were exposed to darkness and light); (4) the number of days that the animals were maintained in their special lighting environment prior to sampling; (5) the composition of the food offered to the animals, and the hours that food and water were made available; (6) the actual hour-to-hour food intake of the animals: (7) the times of day at which enzyme activity was sampled, and the nature of the statistical method chosen to evaluate the data.

Since naturally occurring rhythms in plasma steroid concentrations have already been shown to be capable of generating some enzyme rhythms, the concentration of corticosterone in the plasma or the adre-

nals should be ascertained in samples taken at the same time as the specimens of liver. This information is especially important in justifying the conclusion that a particular enzyme does *not* show daily oscillations; i.e., if an enzyme rhythm is generated by the steroid secretory rhythm, environmental manipulations which obliterate the steroid rhythm will also extinguish the enzyme rhythm, leading the investigator to the erroneous conclusion that enzyme activity does not oscillate. The availability of data showing that the adrenal cycle is also absent will help the investigator to recognize that his particular experiment does not allow conclusions to be drawn about the existence of an enzyme rhythm.

2. *Amino Acid-Metabolizing Enzymes*

The activity of tryptophan pyrrolase in livers of male CD-1 mice exposed to light from 6:00 AM to 6:00 PM and given *ad libitum* access to mouse chow varies about twofold during each day: it is lowest at the start of the daily dark period and peaks about 8 hours later (Rapoport *et al.,* 1966). Bilateral adrenalectomy lowers the basal enzyme level; however, its activity continues to show some periodicity, now rising by about 50% between 8:00 PM and 2:00 AM. The rise in tryptophan pyrrolase activity is observed in intact animals 15–21 hours after plasma tryptophan levels reach their peak; hence the enzyme rhythm is probably not related to the delivery of tryptophan to the liver by the general circulation. The role of *dietary* tryptophan in producing the enzyme rhythm cannot be evaluated, inasmuch as no information was provided in the above study on the eating behavior of the experimental animals, nor are data available on changes in the rhythm among animals on a 0% protein diet.

The rhythm in hepatic tryptophan pyrrolase activity may be responsible for the observed relationship between the metabolic fate of a dietary tryptophan load and the hour of its ingestion. Rapoport and Beisel (1968) administered 3 gm of tryptophan per kilogram body weight to normal males, aged 20–42, at different times of the day; the subjects' urines were collected for the next 6 hours and assayed for kynurenine derivatives (formed through the action of tryptophan pyrrolase) and for indicans (synthesized by bacteria in the gut). The excretion of kynurenine derivatives was greatest if the amino acid was administered between 6:00 AM and 9:00 AM, or several hours after the daily peak in plasma cortisol levels; it was least if the tryptophan was administered between 6:00 PM and 9:00 PM.

Civen *et al.* (1967) assayed livers from adult male rats exposed to light for 12 hours daily and given Purina Chow *ad libitum* for both tyrosine transaminase and phenylalanine-pyruvate transaminase (PPT).

In contrast to the tyrosine-metabolizing enzyme, PPT activity showed no tendency to vary with time of day. Potter and his colleagues (1966) examined the activities of various enzymes in the livers of Buffalo rats which bore a transplantable tumor (Morris hepatoma No. 7793) for 149 days; the animals were exposed to light from 6:00 AM to 6:00 PM daily and given access to a diet containing 0–90% protein during the hours of darkness. Tyrosine transaminase activity exhibited a significant rhythm in host livers only among animals receiving a diet containing at least 30% protein; it is possible that the enzyme rhythm was not observed in rats eating customary amounts of protein (15–20%) because no animals were sampled between 6:00 PM and midnight. Serine dehydrase activity did not exhibit diurnal rhythmicity; ornithine transaminase, a mitochondrial enzyme, showed daily oscillations among rats whose diets contained 60% or 90% protein. No data were provided in this study on the actual amounts of food consumed by the experimental animals; such information is especially important in view of the observation (Szepesi and Freedland, 1968) that rats tend to decrease their consumption of diets which are extremely high in protein after about 4 days.

The activity of arginine transaminase in mouse kidney also varies diurnally. Among Bagg albino or C strain animals kept in light from 6:00 AM to 6:00 PM, enzyme activity was highest during the light period and attained its nadir at midnight (Van Pilsum and Halberg, 1964). The phase of the rhythm could be shifted by reversing the lighting conditions.

3. *Drug-Metabolizing Enzymes*

Radzialowski and Bosquet (1968) observed daily rhythms in the activities of several drug-metabolizing enzymes in livers of intact male Holtzman rats exposed to light from 6:30 AM to 8:00 PM daily. The oxidative enzymes which transform *p*-nitrosoanisole, aminopyrine, and hexobarbital were all 50–100% more active in livers sampled at 2:00 AM than in those removed at 2:00 PM. Bilateral adrenalectomy appeared to extinguish these rhythms; however, it had no effect on the rhythm in 4-dimethylaminoazobenzene reductase activity. No data were provided on the feeding rhythms or the protein intakes of the experimental animals. Starvation for 24 hours failed to affect the three adrenal-dependent rhythms, an observation that is difficult to reconcile with the marked effect of this stress on adrenocortical secretion.

C. Characteristics of Enzymes Displaying 24-Hour Rhythmicity

On the basis of information cited above, it can be postulated that two requirements must be met in order for a particular hepatic enzyme to display rhythmicity:

(1) The turnover time of the enzyme must be sufficiently short so that a change in the rate of enzyme production or degradation during part of a 24-hour period would generate measurable differences in enzyme activity. Thus, it is *a priori* more likely that tyrosine transaminase should display rhythmicity than arginase, an enzyme which turns over relatively slowly. All the enzymes for which rhythmicity has thus far been described have half-lives of less than 3 days (Schimke, Chapter 32).

(2) The enzyme should be induced (or activated) by a physiological input to the liver whose amplitude displays 24-hour rhythmicity. Hence enzymes which are activated or induced by components of food (e.g., protein, glucose) might be expected to vary diurnally, as might enzymes whose activities are enhanced by hormones whose plasma concentrations show similar variations: e.g., corticosterone (Guillemin *et al.*, 1959) and insulin (Freinkel *et al.*, 1968). Enzymes regulated primarily by plasma thyroxine levels would not be expected to display 24-hour rhythmicity inasmuch as the plasma concentration of this hormone shows little tendency to vary diurnally (Schatz and Volpe, 1959). The pineal organ receives a cyclic input of nerve impulses generated by the retinal response to environmental lighting (Wurtman *et al.*, 1964); these impulses drive a 24-hour rhythm in a pineal enzyme, hydroxyindole-*O*-methyl transferase (Axelrod *et al.*, 1965).

III. Twenty-four-Hour Rhythms in Plasma Concentrations of Amino Acids

A. Tyrosine

The concentration of tyrosine in human plasma is not constant but varies predictably as a function of time of day (Wurtman *et al.*, 1967b). Among healthy young men who consumed a "formula diet" (0.71 gm protein per kilogram body weight) or a "house diet" (1.50 gm protein per kilogram body weight) in four equal meals at 8:00 AM, 12:30 PM, 5:30 PM, and 10:00 PM, and who remained in bed from 11:00 PM to 7:00 AM, tyrosine levels were lowest at 2:00 AM and rose to a peak by or after 10:30 AM (Figs. 10 and 11). The magnitude of the daily rise was about 60% above the basal (2:00 AM) levels.

Subjects fed a "low-protein formula diet" (containing only 0.04 gm of protein per kilogram body weight) for 2 weeks continued to display 24-hour oscillations in plasma tyrosine levels; a sharp peak was attained at 8:00 AM (Fig. 12) (Wurtman *et al.*, 1967b, 1968a). This observation indicated that the plasma amino acid rhythm was not simply the consequence of the overflow of dietary amino acids from the portal circulation

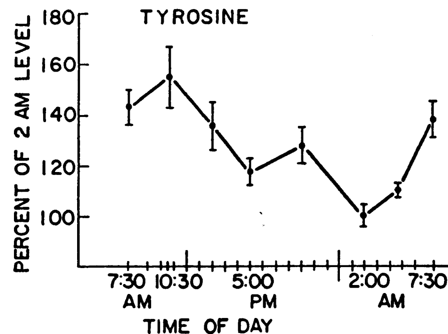

Fig. 10. Variation in plasma tyrosine concentrations in nine healthy male subjects given the "Formula Diet" (0.71 gm of egg protein per kilogram body weight). The 2:00 AM mean concentration of the amino acid was 10.04 ± 0.39 μg/ml. (Reprinted from Wurtman *et al.*, 1968a.)

to the vena cava. It also demonstrated an important difference between the rhythms in plasma tyrosine concentration and hepatic tyrosine transaminase activity. The former rhythm, but not the latter, persisted in subjects deprived of a cyclic input of dietary protein. This indicated that, although the hepatic enzyme might influence the plasma tyrosine rhythm, the primary mechanism responsible for the amino acid cycle was unrelated to alterations in the rate at which tyrosine was metabolized within the liver. Feigin and his colleagues (1968) have provided further evidence that plasma amino acid rhythms are independent of dietary protein. Subjects consumed 100 gm of protein at 9:00 AM or at 9:00 PM; this dietary load accelerated the morning rise in amino acid concentration but did not alter the expected evening decline.

To examine the possibility that the diurnal changes in plasma tyrosine level were related to man's activity rhythm, plasma samples were assayed for the amino acid before and immediately after 45 minutes of vigorous exercise. The effect of physical activity was to increase tyrosine levels only slightly (Wurtman *et al.*, 1967b); hence the daily rise in plasma tyrosine (which began at 2:00 AM, while the subjects were in bed) could not have been the result of cycles of muscular work. Changes in the sleep–wakefulness cycle can, however, entrain plasma amino acid rhythms; subjects made to sleep from 10:00 AM to 6:00 PM (instead of from 10:00 PM to 6:00 AM) showed a rapid shift in the time of peak amino acid levels. Within 48 hours, the hour of peak plasma concentrations stabilized at 4:00 AM (Feigin *et al.*, 1968). The daily rhythm in body temperature required considerably more time to resynchronize to the new activity schedule. It could not be determined whether the shift in the phase of the amino acid rhythm resulted from shifts in

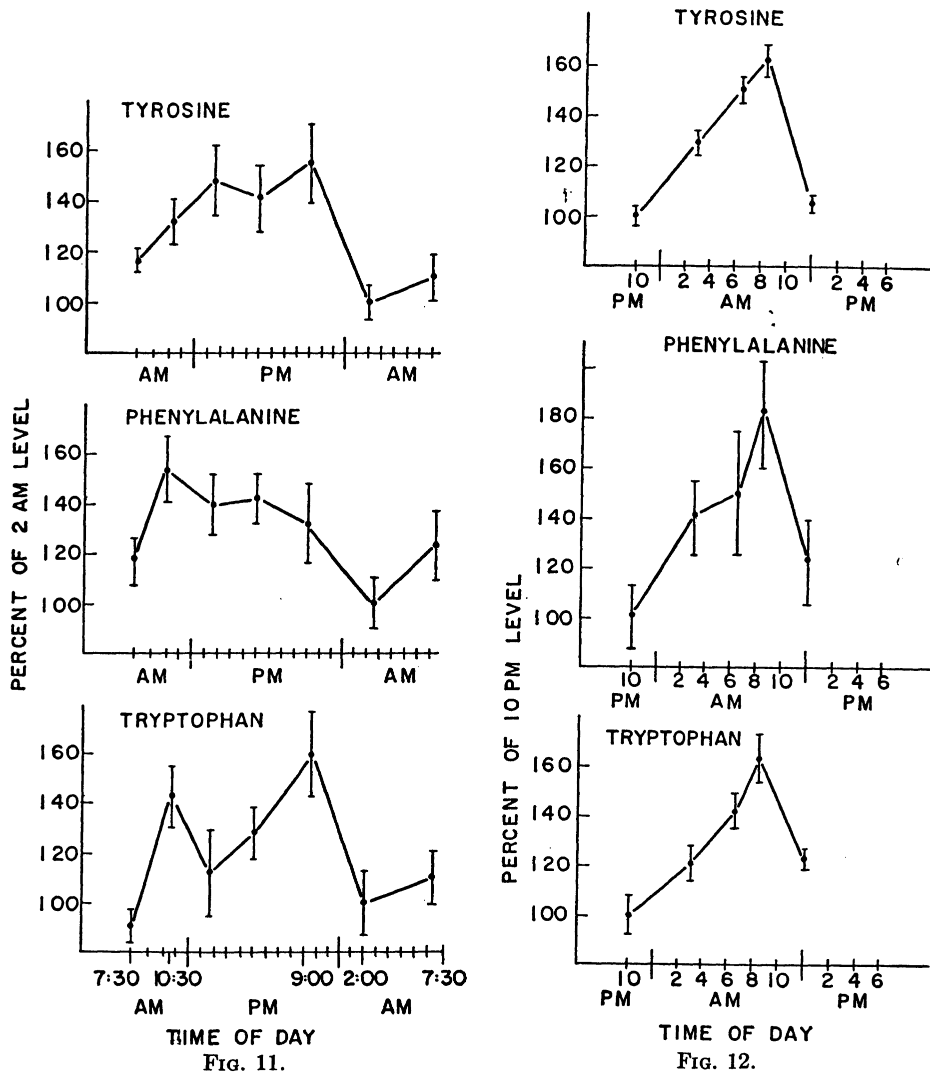

FIG. 11.

FIG. 12.

FIG. 11. Variations in plasma concentrations of tyrosine, phenylalanine, and tryptophan with time of day among six healthy male subjects given the "House Diet" (approximately 1.5 gm of protein per kilogram body weight). The 2:00 AM concentrations of the amino acids were: tyrosine: 12.20 ± 0.94 μg/ml; phenylalanine: 8.14 ± 0.85 μg/ml; tryptophan: 9.13 ± 1.22 μg/ml. (Reprinted from Wurtman *et al.*, 1968a.)

FIG. 12. Variations in plasma concentrations of tyrosine, phenylalanine, and tryptophan with time of day among eight healthy male subjects given the "Low Protein Formula Diet" (0.04 gm of protein per kilogram body weight). The 10:00 PM mean concentrations of the amino acids were: tyrosine: 7.80 ± 0.31 μg/ml; phenylalanine: 6.46 ± 0.86 μg/ml; tryptophan: 6.30 ± 0.49 μg/ml. (Reprinted from Wurtman *et al.*, 1968a.)

the sleep cycle, the times of physical activity, the hours of food ingestion, the light-dark cycle, or another cyclic behavioral or environmental input.

B. Other Amino Acids

Daily oscillations parallel to those described for tyrosine have now been observed among most other amino acids. Feigin *et al.* (1967a) measured the total concentration of 18 amino acids in plasmas of healthy young males, using a densitometric assay of spots on paper chromatograms. The total concentration was lowest (25 mg/100 gm) at 4:00 AM, and rose by 15–35% in the afternoon. Blood samples were collected at intervals of 4 hours for 6 days; there was relatively little day-to-day variability among individual subjects. Both Wurtman *et al.* (1968a) and Feigin *et al.* (1968) examined diurnal rhythms in individual amino acids. The former group observed rhythms of greatest amplitude among tyrosine, tryptophan, phenylalanine, methionine, cysteine, and isoleucine; amino acids whose plasma concentrations were highest showed the smallest percentage of variation (Table I). Feigin *et al.* (1968) found the greatest percentage differences among leucine, isoleucine, and

TABLE I

PLASMA AMINO ACID CONCENTRATIONS AT TIMES OF DAY WHEN TYROSINE LEVEL WAS HIGHEST OR LOWEST[a,b]

	"House Diet" (1.5 gm protein/kg) Amino acid concentration (μmole/ml)			"Low-Protein Formula Diet" (0.04 gm protein/kg) Amino acid concentration (μmole/ml)		
Amino acid	2:00 PM	2:00 AM	Ratio	8:30 AM	10:00 PM	Ratio
Threonine	0.17 ± 0.02	0.14 ± 0.05	1.45 ± 0.27	0.21 ± 0.10	0.15 ± 0.07	1.39 ± 0.03
Serine	0.22 ± 0.07	0.17 ± 0.05	1.39 ± 0.39	0.21 ± 0.09	0.15 ± 0.03	1.43 ± 0.19
Glutamic acid	0.28 ± 0.12	0.24 ± 0.15	1.29 ± 0.54	0.32 ± 0.10	0.26 ± 0.12	1.26 ± 0.23
Glycine	0.27 ± 0.05	0.21 ± 0.02	1.25 ± 0.17	0.41 ± 0.09	0.32 ± 0.05	1.29 ± 0.05
Alanine	0.46 ± 0.10	0.30 ± 0.07	1.59 ± 0.37	0.58 ± 0.29	0.52 ± 0.21	1.12 ± 0.33
Valine	0.36 ± 0.07	0.28 ± 0.05	1.31 ± 0.32	0.17 ± 0.03	0.11 ± 0.02	1.50 ± 0.16
Cysteine	0.08 ± 0.12	0.04 ± 0.02	1.83 ± 0.61	0.05 ± 0.02	0.03 ± 0.02	1.89 ± 0.55
Isoleucine	0.13 ± 0.02	0.09 ± 0.02	1.51 ± 0.42	0.07 ± 0.02	0.03 ± 0.01	2.16 ± 0.02
Leucine	0.23 ± 0.05	0.15 ± 0.02	1.63 ± 0.54	0.13 ± 0.03	0.06 ± 0.01	2.02 ± 0.36
Tyrosine	0.09 ± 0.02	0.05 ± 0.02	2.01 ± 1.00	0.05 ± 0.01	0.02 ± 0.02	2.13 ± 0.21
Phenylalanine	0.08 ± 0.02	0.05 ± 0.02	1.60 ± 0.44	0.06 ± 0.02	0.03 ± 0.02	1.74 ± 0.30
Lysine	0.27 ± 0.05	0.18 ± 0.05	1.58 ± 0.34	0.16 ± 0.03	0.12 ± 0.03	1.40 ± 0.09
Histidine	0.14 ± 0.02	0.11 ± 0.02	1.39 ± 0.34	0.14 ± 0.02	0.09 ± 0.02	1.60 ± 0.47
Arginine	0.13 ± 0.05	0.11 ± 0.02	1.46 ± 0.24	0.12 ± 0.02	0.08 ± 0.03	1.43 ± 0.30

[a] Reprinted from Wurtman *et al.* (1968a).

[b] Data obtained from 6 subjects on "House Diet" (1.5 gm protein/kg) and 3 subjects on "Low-Protein Formula Diet" (0.04 gm protein/kg) is presented as mean ± standard deviation.

methionine (tryptophan was not assayed); citrulline concentrations tended not to show significant diurnal oscillations.

The concentrations of tyrosine in rat plasma varies with a rhythm similar to that observed in humans, except that times of the peak and the nadir are shifted by 6–8 hours (Coburn *et al.,* 1969). Plasma phenylalanine does not display significant diurnal oscillations in this species. If rats are forced to consume all of their daily food intake between 8:00 AM and noon, the phase of the tyrosine rhythm shifts, and parallel rhythms appear in the plasma concentrations of both tyrosine and phenylalanine. Daily rhythms in free amino acid concentrations have been reported in the plasma of White Leghorn chicks (Squibb, 1966) and within the hearts of rats (Lyons *et al.,* 1967).

C. Mechanisms of Plasma Amino Acid Rhythms

A variety of factors can influence the steady-state levels of an individual amino acid in the plasma; any of these could impart rhythmicity to its net plasma concentration. Plasma tyrosine originates from a variety of "sources" and disappears by entering any of several "sinks." Its sources include the following: (1) that fraction of the dietary tyrosine which is not utilized or transformed in its passage through the portal circulation and liver; (2) tyrosine synthesized in the liver by the oxidation of phenylalanine (or, less likely, through the transamination of *p*-hydroxyphenylpyruvic acid) and secreted into the general circulation; (3) tyrosine produced in the liver and other organs from the degradation of proteins and peptides; (4) tyrosine released from intracellular depots in liver, brain, muscle, and other tissues. Its sinks include: (1) delivery to the liver via the hepatic artery, followed by deamination or incorporation into proteins or peptides; (2) uptake into tyrosine pools in peripheral tissues, followed by incorporation into proteins and such special products as catecholamines, thyroxine, or melanin; (3) excretion into the urine or feces, or decarboxylation to form tyramine (little if any tyrosine is lost via these latter pathways).

Each of the above processes depends upon many individual biochemical operations, which, if rhythmic, could impart rhythmicity to plasma tyrosine concentration. For example, the uptake of plasma tyrosine into the brain might be stimulated by an increase in the utilization of tyrosine to form proteins or catecholamines; it might also change as a result of an alteration in the steady-state ratio of intraneuronal to extracellular tyrosine, perhaps resulting from the action of a hormone.

It should be obvious that a full understanding of the mechanisms responsible for the plasma tyrosine rhythm will not be attained until considerably more information is available about the control of plasma

amino acid levels in general. Indeed, the experimental analysis of the tyrosine rhythm should provide an excellent model system for studying the physiological regulation of amino acid metabolism in the whole animal. Unlike changes in plasma amino acid levels produced by experimental manipulations, the diurnal variations in tyrosine concentration observed in untreated animals are, *prima facie,* physiologically significant.

The plasma tyrosine rhythm cannot result solely from the cyclic ingestion of dietary protein or of rhythms in the intrahepatic transamination of the amino acid, inasmuch as it persists in subjects deprived of all but trace amounts of dietary protein (Wurtman *et al.,* 1968a). Although no data are available as to the time-dependence of phenylalanine hydroxylase activity, it is not likely that the plasma tyrosine rhythm results from diurnal variations in tyrosine synthesis, inasmuch as it persists indefinitely in human subjects suffering from phenylketonuria (Coburn *et al.,* 1969). Moreover the tyrosine rhythm is in phase with a plasma phenylalanine rhythm in humans (Wurtman *et al.,* 1968a), but not in rats (Coburn *et al.,* 1969). It has not yet been determined whether the rates at which endogenous tyrosine is liberated following the degradation of proteins in the liver or elsewhere vary diurnally; however, it might be expected that more protein would be catabolized toward the middle of the daily light period (i.e., when the rate of food intake is least) than soon after the onset of darkness. Similarly, no data are available on the relation between time of day and the tendency of tyrosine pools in peripheral tissues to reenter the blood stream.

The extent to which tissues can concentrate amino acids from the plasma has been shown to vary diurnally and may provide a basis for rhythmicity in plasma tyrosine levels. Baril and Potter (1968) have shown that the steady-state ratio of the concentrations of a nonutilizable amino acid, cycloleucine-^{14}C, in liver and blood varies in relation to the number of hours since the animal last ate. The ratio is depressed by feeding, and rises during fasting. These investigators initially suggested that this rhythm was responsible for the tyrosine transaminase rhythm, i.e., higher concentrations of amino acids were presented to the liver for incorporation into protein at certain times of day. The disappearance of the rhythm in intracellular amino acid-concentrating ability among adrenalectomized animals makes this hypothesis untenable. However, it does appear likely that a daily rhythm in the ability of tissues to concentrate amino acids could contribute significantly to a plasma amino acid rhythm. Baril and Potter suggested that the rhythm in the intracellular concentration of cycloleucine-^{14}C was generated by the diurnal oscillations in plasma glucocorticoid concentrations. This

hypothesis is supported by the observation that human subjects who receive synthetic glucocorticoids chronically according to a schedule which disrupts their endogenous plasma cortisol cycle show parallel shifts in the phasing of the tyrosine rhythm (Wurtman *et al.*, 1968d). Plasma insulin levels vary diurnally (Freinkel *et al.*, 1968), and insulin can also modify the rates at which amino acids are transferred across cellular membranes (Roberts and Simonson, 1962); hence it is also possible that plasma amino acid rhythms are related to insulin secretion. This likelihood is especially great in subjects eating a high-carbohydrate, low-protein diet, as discussed below.

Preliminary data suggest that the rates at which individual tissues convert tyrosine-^{14}C to labeled protein-^{14}C depend upon the hour that the amino acid is administered. Adult male Sprague-Dawley rats exposed to light from 6:00 AM to 6:00 PM and given access to Purina Chow and water *ad libitum* received intraperitoneal injections of tyrosine-^{14}C at 6:00 AM, noon, 6:00 PM, and midnight. They were killed after 30 minutes, and the specific activities of tyrosine-^{14}C and ^{14}C-labeled protein were determined in liver and brain. The rates at which the labeled amino acid was incorporated into protein were highest in the middle of the light period and lowest during the dark period. The differences in the amounts of ^{14}C-labeled protein formed reflect rhythms in both the destruction of tyrosine-^{14}C and the rates of total protein synthesis (Rose, *et al.*, 1969). Marked changes in the rate at which intracellular tyrosine is utilized for protein synthesis could cause sufficient alterations in the uptake of the amino acid from the blood (or in its secretion from the tissues into the plasma) to produce rhythms in plasma tyrosine concentration.

In the case of the plasma amino acid rhythm observed in subjects receiving the low-protein diet (Fig. 12), it seems likely that this rhythm results from cyclic intake of dietary carbohydrate. In both man and other species, it has been shown that ingestion of carbohydrate causes a rapid fall in plasma free amino acid levels (see Chapter 10, Section III,B and Chapter 34, Section III,A for a complete review of the literature). Munro and Thomson (1953) examined this phenomenon in rats and in adult men, and showed that the reduction is maximal within 2 hours of feeding a dose of glucose. Feeding fat to fasting subjects or animals did not produce significant changes in free amino acid levels. The lowering of free amino acid concentrations by carbohydrate administration is not uniform for different amino acids; on the contrary, amino acids are removed from the plasma in the proportions in which they occur in tissue proteins generally.

Zinneman *et al.* (1966) have recorded the reduction in the levels of

22 circulating amino acids 2 hours after feeding glucose; with the single exception of glutamic acid, the percentage fall in individual amino acid levels correlates closely with the changes between maximal and minimal values on the "low-protein formula diet" shown in Table I. On the other hand, the relative differences in amino acid concentrations at two points on the cycle observed with the "house diet" (Table I) do not correlate with the pattern of change caused by glucose administration, thus suggesting that other factors are at work. Figure 13 shows these correlations.

The cause of the change in plasma amino acid concentrations after carbohydrate administration has been examined. Since the pattern of removal suggests deposition as tissue protein, a search was made by Munro and his colleagues for the site or sites of such deposition. Munro *et al.* (1959) showed that administration of glucose to fasting rats leads to actual *loss* of protein from the liver, rather than deposition. This was confirmed by injecting rats with ^{35}S-labeled methionine or ^{14}C-labeled glycine at the time of glucose administration. As compared with controls fed fat or water, glucose-fed animals showed no deposition of extra methionine or glycine in the tissue proteins of the liver or intestinal mucosa, but there was a significant accumulation in both the diaphram and leg muscles. It had previously been demonstrated that an intact insulin-secreting mechanism is required for the action of dietary carbo-

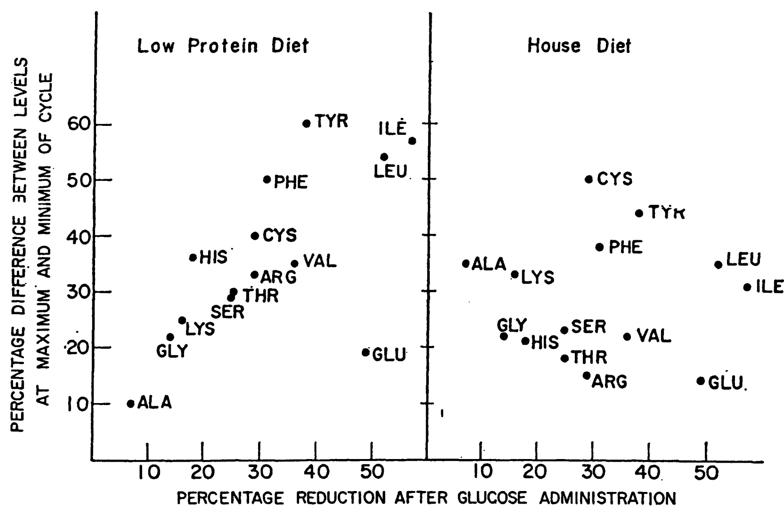

FIG. 13. Change in levels of individual amino acids after feeding glucose to fasting subjects and sampling plasma 2 hours later (Zinneman *et al.*, 1966) plotted against changes in same amino acids between daily extremes on low protein diet (left) or house diet (right).

hydrate on plasma amino acid levels (Munro, 1956). Thus it was concluded that the carbohydrate acted by stimulating the secretion of insulin; this hormone, in turn accelerates the transport of amino acids into muscle, and possibly also muscle protein synthesis. The reduction in plasma amino acid levels after giving insulin has been extensively documented (see Chapter 10, Section III,B for a review), and has been shown to occur in the eviscerated rat within 15 minutes of injecting the hormone (Ingle *et al.*, 1956). This time interval is comparable to the speed of action of insulin on carbohydrate metabolism in muscle, whereas carbohydrate metabolism in the liver responds much more sluggishly on administration of insulin to the intact animal (Renold *et al.*, 1955).

The influence of dietary carbohydrate on protein metabolism also extends to the fate of protein consumed in the same meal. As demonstrated in man by Cuthbertson and Munro (1939) and on the rat by Munro (1949), protein in the diet is better retained in the tissues if fed in a meal containing carbohydrate than if these two nutrients are fed at separate times of day. This presumably occurs because a proportion of the incoming amino acids after mixed meals is deposited in muscle as a result of the stimulus provided by the carbohydrate absorbed from the same meal. Indeed, Rabinowitz *et al.* (1966) have shown that insulin secretion is much more enhanced by a meal containing carbohydrate and protein than by carbohydrate or protein given singly. The disposal of amino acids in peripheral tissues after carbohydrate–protein meals can now be explored directly, using the arteriovenous difference technique on the arms of human subjects, as described by Foley *et al.* (1966).

As a first approximation to an explanation of the diurnal rhythms in protein metabolism disclosed in this survey, we may conclude that the main factor which causes tyrosine transaminase and perhaps other liver enzymes to undergo cyclic change in activity is variations in the levels of amino acids present in the portal vein. This hypothesis is compatible with the known sensitivity of liver protein metabolism to amino acid supply, as discussed in Chapters 10, 34, and 38. On the other hand, there is good reason to conclude that the carbohydrate absorbed from the diet exerts its major effect on the entry into, and perhaps the utilization of, amino acids within skeletal muscle. Consequently, plasma amino acid concentrations mainly reflect the ebb and flow of amino acids into and out of this large tissue mass and continue to display rhythmic change even when subjects are given diets lacking protein. When mixed meals of protein and carbohydrate are consumed, not all of the amino acids are trapped by the liver; thus a small postprandial wave of amino acids passes into the peripheral circulation, and influences the levels of the plasma amino acids. As discussed in Chapter 34, changes in plasma

free amino acid levels following a mixed meal are dependent on the mixture of amino acids provided by the dietary protein in relation to the requirements of the tissues, on the interaction with dietary carbohydrate described above, and on the later postabsorptive breakdown of labile tissue proteins (notably in liver) which returns amino acids to circulation. It is thus not surprising that cyclical changes in plasma free amino acids in subjects receiving the "house diet" show complex patterns that cannot be correlated with the disappearance of amino acids from plasma after carbohydrate is given alone (Fig. 13).

D. Plasma Amino Acid Rhythms and Disease States

The tendency of plasma amino acid concentrations to vary diurnally by as much as twofold in healthy, untreated subjects makes it necessary that studies on the effects of clinical or experimental situations on "amino acid levels" be designed so that blood can be sampled at several different times of day. If blood can be taken from a given subject at only one hour, note should at least be made of when it was obtained, when and what the subject ate, and when he slept. Rivlin and Melmon (1965) reported that plasma tyrosine levels were elevated in hyperthyroid subjects; blood was taken only once from each subject, early in the morning. In the absence of data on tyrosine levels at other times of day, these data are compatible with any of the following hypothesis: (1) hyperthyroidism increases the "set-point" around which tyrosine levels vary; (2) hyperthyroidism extinguishes the tyrosine rhythm, and plasma levels remain at the 10:30 AM level at all hours of the day; (3) hyperthyroidism causes a shift in the phase of the tyrosine rhythm, so that levels present in normal subjects at 10:30 AM now occur at 7:00 AM.

Feigin has examined the effects of experimental infections with *Pasteurella tularensis* (Feigin and Dangerfield, 1967) or a live, attenuated Venezuelan equine encephalomyelitis virus vaccine (Feigin *et al.*, 1967b) on plasma amino acid rhythms. The administration of the bacteria by inhalation was followed after 4 days by a 43% fall in total plasma amino acid concentration; subsequently amino acid rhythmicity was perturbed until the subjects had recovered. Inoculation with the viral agent also damped the amino acid rhythm, but without causing the marked decline in amino acid levels. These studies included no data on the time or quantity of food ingestion.

E. Physiological Significance of Plasma Amino Acid Rhythms

There is at present little direct evidence relating changes in the plasma concentration of an amino acid to changes in its availability to the tissues. Since all tissues except the liver are dependent upon the general

circulation for their supply of amino acids, it seems reasonable to speculate that a threshold plasma concentration must exist for each amino acid below which the tissues cannot extract sufficient material to meet their requirements; the rates of synthesis of protein and other products of amino acids (e.g., catecholamines, hormones) would then be slowed. If this hypothetical threshhold is higher than the plasma concentration of the amino acid at the daily nadir (i.e., at 2:00 AM), then the amino acid rhythm *will* be physiologically significant, and will limit the amount of the substance that is available to the tissues at certain times of day. On the other hand, if tissues are able even at 2:00 AM to extract from the blood as much of each amino acid as they need, then rhythmic changes in amino acid concentration will not exert a significant influence on the metabolism of the substances. The determination of the significance of amino acid rhythms awaits further studies.

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