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Morton I. Rapoport, William R. Beisel

J Clin Invest. 1968;47(4):934-939. <https://doi.org/10.1172/JCI105785>.

Research Article

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Circadian Periodicity of Tryptophan Metabolism

MORTON I. RAPOPORT and WILLIAM R. BEISEL with the technical assistance of
RICHARD E. DINTERMAN

*From the Division of Infectious Diseases, University of Maryland School of
Medicine, Baltimore, and the U. S. Army Medical Unit, Walter Reed
Army Medical Center, Frederick, Maryland 21701*

ABSTRACT Rhythmicity of tryptophan metabolism via the kynurenine pathway has been demonstrated in man. Normal subjects given 3 g of tryptophan at 0900 hours excreted almost three times the quantity of kynurenine, kynurenic acid, and xanthurenic acid than did subjects given the same dose at 2100 hours. Other metabolites of the kynurenine pathway varied in the same fashion but with lesser magnitude. In contrast, indican, a tryptophan metabolite not in the kynurenine pathway, varied inversely with the other metabolites measured. The data suggest that the liver enzyme tryptophan pyrrolase has a circadian rhythm in man similar to that already described in mice in a previous study.

Tryptophan tolerance tests in the future should be controlled relative to time of amino acid administration.

INTRODUCTION

Defects in tryptophan metabolism have been described in a variety of diseases. However the mechanisms underlying these defects have not been clearly established. In a recent publication Altman and Greengard (1) indicated that illness characterized by altered tryptophan metabolism could be associated with induction of the liver enzyme tryptophan pyrrolase (TP). This enzyme is the initial one in the kynurenine pathway and appears to regulate the rate of tryptophan metabolism through this pathway.

In an earlier communication (2) we reported that hepatic TP activity in mice varied relative

Received for publication 31 July 1967 and in revised form 30 October 1967.

to time of day. If a similar rhythmicity exists for man and if this enzyme is the major regulatory factor for tryptophan entering the kynurenine pathway, then one might expect that the quantity of tryptophan metabolized via this pathway would vary relative to the time the amino acid was administered.

It has long been recognized that the diazo reaction of human urine is quantitatively related to the excretion of tryptophan metabolites (3). Indeed the commonly used analytical methods for quantitation of tryptophan metabolites still employ the diazotization procedure.

In studies reported herein we have examined the urine of human subjects given tryptophan loads at various times of day and night. We have shown by an automated diazotization technique and confirmed by column chromatographic means that the metabolism of this amino acid via the kynurenine pathway is, in major part, dependent on the time of amino acid feeding, an observation compatible with circadian variation of TP in the human.

METHODS

Subjects. A total of 51 normal, ambulatory, male subjects aged 20-42 yr were studied. L-Tryptophan (Calbiochem, Los Angeles, Calif.) was given to all patients by the oral route, and no dietary restrictions were imposed. No vitamins or drugs were given before or during the period of investigation. A state of hydration was encouraged by drinking 240 ml of fluid hourly during the period of urine collections.

Urine was collected from all subjects for a period of 6 hr after ingestion of the amino acid. In most studies the 6 hr collection was obtained as a pool; however in certain selected cases, aliquot urines were obtained at

1-2-hr intervals for a 24 hr period after administration of the amino acid.

Experimental design. A dose-response relationship of limited range was established in one group of male volunteers relating quantity of tryptophan administered and μ moles of diazotizable metabolites excreted.

Studies designed to evaluate the diurnal rhythmicity of tryptophan tolerance were performed in another group of subjects. A standard dose of 3 g of tryptophan was given at 0300, 0600, 0900, 1200, 1500, 1800, 2100, or 2400 hr. Normal activity appropriate to time of day was encouraged. A number of subjects were studied more than once, with at least a 48 hr period between tolerance tests. Diurnal rhythmicity of glomerular filtration was excluded by calculating data in terms of endogenous creatinine (i.e. picric acid chromogen) excretion.

Analytical methods. Total urinary "diazo reactant" was determined by the automated method of Harvey and Brothers (4). The method is a modification of that described by Bratton and Marshall (5) and consists of diazotization with Na nitrate followed by coupling with *N*-(1-naphthyl) ethylenediamine hydrochloride. Kynurenine SO_4 (all metabolite standards were obtained from Calbiochem) was utilized for the standard calibration curve although anthranilic acid and *O*-aminohippuric acid produced similar calibration curves. Indican standards produced a color intensity which was less than one-fifth that observed using equimolar concentrations of kynurenine. In contrast xanthurenic acid, kynurenic acid, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and tryptophan were tested and found to produce no color by this method.

The elution column chromatographic method of Price, Brown, and Yess (6) was utilized in urine pools obtained from tolerance tests performed at 900 and 2100 hr. Columns were prepared with Dowex 50W-X12, and elution was accomplished with increasing concentrations of HCl. By these methods indican, kynurenine, acetylkynurenine, 3-hydroxykynurenine, anthranilic acid, anthranilic glucuronide, *O*-aminohippuric acid, kynurenic acid, and xanthurenic acid could be quantitated individually. Recovery curves ranging from 75 to 100% were obtained consistently for all metabolites.

Creatinine determinations were performed on all urine samples by an automated picric acid method.

RESULTS

In the narrow dose range selected for study, a definite linear relationship existed between ingested 0900-hr dose of L-tryptophan and the quantity of diazotizable material excreted. Most reported studies of tryptophan tolerance have utilized doses ranging from 1 to 4 g. A dose-response study was used to establish the sensitivity of the automated method for urinary diazotization. The quantity of "diazo reactant" as measured by the AutoAnalyzer method (4) had a linear relationship to the size of tryptophan load (Fig. 1). As a result of these

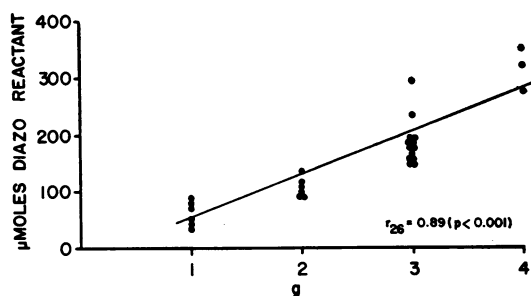


FIGURE 1 Excretion of diazotizable metabolites observed as a function of increasing doses of oral L-tryptophan. A linear relationship existed over the range tested, 1 to 4 g. Each point represents a 6 hr urine collection begun at 0900 hr, the time of tryptophan administration.

studies a standard dose of 3 g of powdered L-tryptophan was selected for all subsequent tests.

It should be noted that the various tryptophan metabolites differ in the time required for optimum color development in the diazotization and coupling reaction. Color development in the automated method is recorded after a relatively brief but consistent reaction time in contrast to the manual method of Price, Brown, and Yess (6) in which color development is allowed to become maximal. For this reason the total "diazo reactant" measured by the automated technique is not mathematically comparable to the sum of the individual metabolites measured by the manual method.

Urine was collected for 24 hr in three individuals after administration of 3 g of tryptophan given at 0900 hr. A similar study was performed on four individuals given tryptophan at 2100 hr. The mean excretion of "diazo reactant" substances during the 24 hr period (shown in Fig. 2) revealed that the peak excretion occurred 3-4 hr after oral administration of the amino acid. Values thereafter tended to fall toward basal levels. Time of administration appeared to have little influence on the length of time in which metabolites from the oral loading dose were excreted. The major quantity of "diazo reactant" excreted as a result of tryptophan administration occurred during the initial 6 hr. Based on these investigations it was elected to obtain a standard 6 hr urine collection from all individuals undergoing these tolerance studies.

The results of investigations of tryptophan tolerance performed at 3-hr intervals during the 24 hr are shown in Fig. 3. The 6 hr excretion of

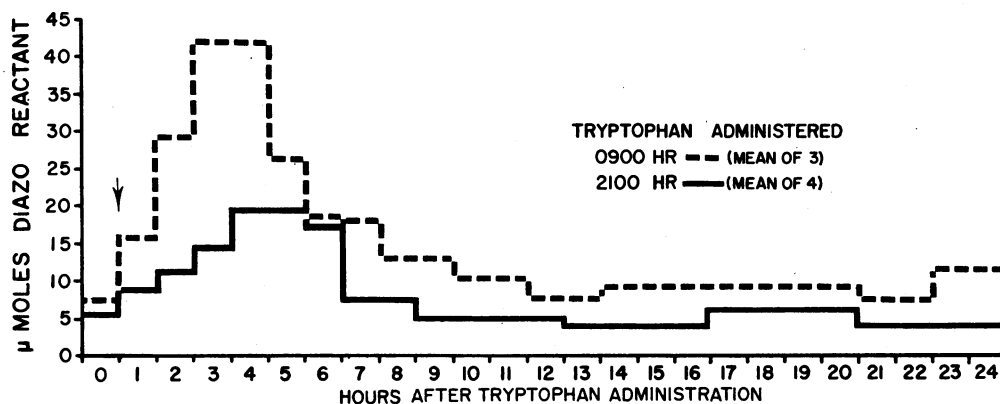


FIGURE 2 Excretion of diazotizable metabolites plotted for 24 hr after a 3 g load of L-tryptophan. The major quantity of material was excreted within 6 hr regardless of time of administration.

“diazo reactant” was lowest when tryptophan was given at 1800 and 2100 hr. In contrast, peak excretion of “diazo reactant” occurred with 0600- and 0900-hr tolerance tests. A comparison of the 2100-hr tolerance studies with the 0900- or 0600-hr studies by *t* test revealed a highly significant difference ($P < 0.001$). Therefore, circadian rhythmicity for tryptophan tolerance was evident from these studies. The magnitude of circadian change was such that the mean excretion of “diazo reactant” by volunteers given tryptophan at 0600 and 0900 hr was more than threefold greater than that of those volunteers given an identical dose of amino acid at 1800 or 2100 hr.

A relatively small group of women was studied with tryptophan loads at two times of the day. Eight tolerance tests were performed on six women at 0900 hr, and four tests were performed on four women at 2100 hr. The mean excretion of diazotizable metabolites in the 0900 hr group was 919.03

± 149.18 (SE) and for the 2100 hr group 292.58 ± 21.41 (SE). Both of these values were significantly higher than those observed in the males studied at similar times. Despite the greater excretion of metabolites, rhythmicity of tryptophan tolerance was maintained in women as in men. A difference in excretion of kynurenine pathway metabolites by men and women has previously been reported (7, 8). Rose (8) suggested that estrogens may increase the conversion of tryptophan to nicotinic acid although no definite enzymatic change could be demonstrated. The postulated estrogenic effect on tryptophan metabolism does not appear to influence its rhythmicity.

Mean excretion values for the individual tryptophan metabolites are shown in Table I. In all cases the quantity of metabolites of the kynurenine pathway excreted after the 0900 hr test was in excess of that observed after the 2100 hr test. However the ratios were dissimilar in that excretion

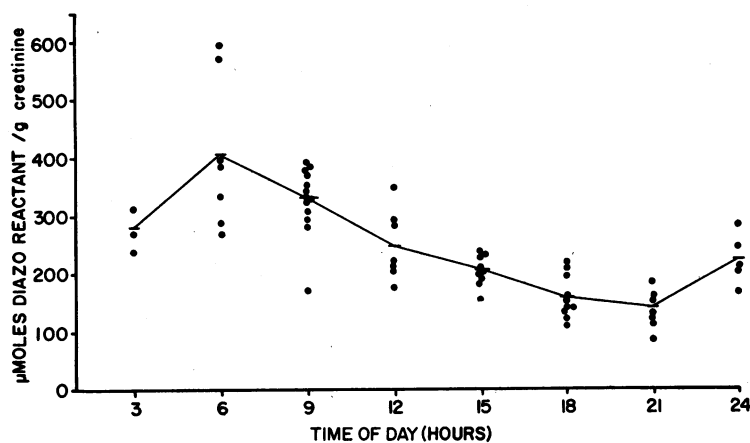


FIGURE 3 Excretion of diazotizable reactant after L-tryptophan loads at 3-hr intervals during the 24 hr clock. Values are expressed per gram of creatinine. Each point represents a 6 hr urine collection obtained after loading with 3 g of L-tryptophan at the clock-hour indicated.

TABLE I
*Mean Urinary Excretion of Tryptophan Metabolites
 after Oral Tryptophan Load of 3 g
 at 0900 and 2100 Hr*

Metabolites	Creatinine ± 1 SE	
	0900 Test	2100 Test
Indican	135 ± 26	209 ± 34
Kynurenine (includes actylkynurenine)	79 ± 15	28 ± 5
O-Aminohippuric acid	16 ± 2	10 ± 2
3-Hydroxykynurenine	28 ± 4	13 ± 3
Anthranilic acid (measured as glucuronide)	19 ± 3	12 ± 2
Kynurenic acid	96 ± 12	25 ± 6
Xanthurenic acid	42 ± 6	15 ± 4

of kynurenic acid after the 0900 hr test was almost four times greater than the 2100 hr test whereas the ratio was approximately one and one-half times greater for anthranilic acid and O-aminohippuric acid. Indican excretion, in contrast, did not conform to the rhythmicity of the other metabolites since the quantity excreted after the morning test was significantly less than that observed after the evening test.

The failure of indican excretion to adhere to the rhythmicity of other tryptophan metabolites was not unexpected. Although indican is a diazotizable metabolite of tryptophan, it is not a product of the kynurenine pathway. In fact it is a metabolite of intestinal bacteria which is absorbed from the gut and excreted in the urine.

DISCUSSION

The observed diurnal rhythmicity in tryptophan metabolism via the kynurenine pathway has a number of implications. Tryptophan tolerance tests have not heretofore been controlled relative to time of amino acid administration. Conclusions based on urinary excretion of tryptophan metabolites must be reevaluated in terms of time of administration. There is a distinct possibility that the metabolism of other amino acids may also undergo diurnal change. Plasma amino acids themselves have been shown to undergo diurnal variation in man and animals (2, 9); in addition, a diurnal rhythm for tyrosine transaminase recently has been observed by a number of investigators (10, 11). Based on these findings, it is conceivable that

feeding of protein or amino acids to patients in whom dietary nitrogen must be critically regulated, i.e. those with hepatic coma or chronic renal disease, might ultimately be adjusted by the clinician to coincide with some optimum time for their utilization.

We suggest that rhythmicity of tryptophan metabolism via the kynurenine pathway in man is based upon TP rhythmicity. While this postulate might be proven by direct measurements in liver biopsy specimens taken at varying times of day and night, indirect evidence appears to support our contention.

A diurnal rhythm for TP activity that appeared to be related in time to adrenal rhythmicity has been observed in experimental animals.

Recent studies in which liver biopsies were obtained showed that TP in humans could also be induced by pharmacologic quantities of adrenal glucocorticoids (1).

The increased excretion of tryptophan metabolites observed in this study occurred at a time that was predictable, if it were assumed that human TP varies as a function of adrenal rhythmicity. For example, if peak cortisol levels occur at 0600 hr in man, one might expect peak TP activity at approximately 0900–1100 hr (12), and peak excretion of tryptophan metabolites would be expected when the amino acid was administered at 0600 and 0900 hr, if we assume some latent period for absorption and transport to metabolic sites.

Alternative explanations for rhythmicity of the kynurenine pathway must be considered. It is possible that the enzymes subsequent to TP undergo a diurnal rhythmic inhibition causing the diazotizable metabolites to accumulate rather than to be carried to their ultimate nondiazotizable end products (i.e., niacin and quinaldic acid, etc.). However this explanation would imply the extremely unlikely possibility that all tryptophan degradative enzymes distal to TP had a similar or identical rhythmic pattern. In this context Altman and Greengard (1) indicated that at times of elevated TP activity the kynurenine degradative enzymes, kynureninase, kynurenine hydroxylase, and kynurenine transaminase, become rate-limiting. While it is functionally possible to exceed the capacity of these enzymes to degrade kynurenine, the products of these enzymes as measured herein varied in the same diurnal fashion as did kynure-

nine itself. The fact that the ratios of these metabolites were somewhat dissimilar may indeed suggest that enzymes subsequent to TP become rate-limiting during the period of maximal TP activity.

It is conceivable that the tryptophan absorptive or transport mechanisms in humans may have a diurnal rhythm. This possibility appeared to be excluded by the work of Michael, Drummond, Doeden, Anderson, and Good (7) who established that doses of tryptophan three to four times in excess of our own were 100% absorbed by the gastrointestinal tract.

Other considerations which cannot be proved or disproved from our studies are a functional pyridoxine deficiency and also a shunting of tryptophan to other pathways including protein biosynthesis. Both kynureninase and kynurenine transaminase are pyridoxine-dependent enzymes (8). However the length of time required to produce pyridoxine deficiency makes this vitamin an unlikely factor in the rhythmicity of tryptophan metabolism.

The matter of tryptophan shunting to other pathways cannot be so easily dismissed. Indeed it is this very point that requires further investigation in order to establish the primacy of pathways available for tryptophan utilization. Relative to this consideration, the serotonin pathway accounted for only 1% of urinary tryptophan metabolites according to Michael and coworkers (7). Further evidence to indicate that the serotonin pathway was limited in magnitude was the work of Weissbach, King, Sjoerdsma, and Uderfriend (13) who showed that the ingestion of 100 mg of tryptophan per kg was required to produce a detectable increase in urinary excretion of 5-hydroxyindoleacetic acid, a dose more than double that employed in our subjects.

Implications of a clinical nature, apart from rhythmic considerations, may be derived from our measurements of urinary diazotizable amines. If one is to accept the thesis that the quantity of diazotizable amines in the urine can in part reflect TP activity, then it is conceivable that certain pathologic states producing increased excretion of diazotizable amines do so by alterations of TP activity. As early as 1882 Ehrlich (14), Pelzl (15), and Brewing (16) reported that acute febrile diseases were associated with increased urinary excretion of diazotizable amines. Kotake and

Sakata (3) noted that advanced pulmonary tuberculosis could also produce an increase in the urinary diazotizable amines. Furthermore these workers established the fact that the measured amines were tryptophan metabolites. Altman and Greengard (1) have indicated that pathologic alteration in excretion of tryptophan metabolites occur as a result of adrenocortical hyperfunction, a state which would be expected to produce induction of TP. However at least one other regulatory factor must be considered. The activity of TP may be regulated by the concentrations of its heme coenzyme (17). Indeed studies by Feigelson and Greengard (18) show that TP is induced in rats with experimental porphyria as a consequence of an excess in heme coenzyme. Appropriately humans with porphyria have been shown to have increased excretion of tryptophan metabolites (19).

Finally, Berry and Smythe (20) have reported that bacterial endotoxin functions as an inhibitor of TP in experimental animals. These workers suggest that survival in endotoxemia may be correlated with maintenance of normal TP activity. The mechanism in which endotoxin inhibits TP is not completely understood. As yet no studies of tryptophan metabolism have been performed in patients with Gram-negative infections.

In summary it would appear that diurnal rhythmicity of TP in human liver may be the basis for a rhythmic change in tryptophan metabolism. Further investigations into the metabolism of this amino acid should consider the marked alterations which occur simply as a function of time of day. By the same token it would appear that the relative ease with which urinary diazotizable amines may be measured will serve as a useful tool in the study of tryptophan metabolism in a large number of clinical states.

ACKNOWLEDGMENTS

The assistance of Peggy Shank, Phebe Summers, and Pearl Flohr is gratefully acknowledged.

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