JOURNAL OF THE TENNESSEE ACADEMY OF SCIENCE 67(4):70-72 OCTOBER1992

EFFECT OF FOOD AVAILABILITY ON THE DAILY RHYTHM OF TOTAL IRON-BINDING CAPACITY IN *MUS MUSCULUS*

WILLIAM J. LAMOREAUX, CHARLES J. BIGGERS, KENNETH B. DAVIS, AND PRISCILLA S. RUSHTON

Department of Biology, Memphis State University, Memphis, TN 38152

ABSTRACT--A study of the daily rhythm of the total iron-binding capacity (TIBC) for the plasma protein transferrin was performed. Twenty mice were examined for TIBC at eight different times during the day using a microprocedure. A significantly higher TIBC occurred during the dark phase of the photoperiod. A second experiment, designed to determine if the rhythm was due to food availability, showed that all groups studied had a similar rhythm and that TIBC was still significantly higher in the dark. We conclude that the rhythm of TIBC exhibited was not directly influenced by food availability.

In vertebrates, the ability to bind and transport iron is important in the maintainence of cell function and in controlling microorganisms. The plasma protein that is responsible for binding the majority of the iron in the vertebrate is transferrin. Albumin and ceruloplasmin also have a minor role in binding iron (Aisen and Listowsky, 1980).

Transferrin is a plasma protein of low molecular-weight (MacGillivray et al., 1982) synthesized in the liver and transports ferric iron from iron-donating tissues to cells in need of iron. The major iron-donating tissues are reticuloendothelial cells, hepatocytes, and intestinal mucosa. The bulk of iron carried by transferrin is destined to be delivered to reticulocytes for heme synthesis, although, in pregnant women, 20 to 25% of the maternal iron stores are delivered to the fetus by transferrin during the last trimester (Van Dijk, 1988). When iron is delivered by transferrin to the iron-accepting cells, the protein is taken up by receptor-mediated endocytosis, with the receptor being internalized along with the transferrin (Enns et al., 1983). Subsequent pH changes result in the iron separating from the transferrin (Dautry-Varsat et al., 1983). Once the iron has been delivered to the cell, the receptor-transferrin conjugate is returned to the cell surface, the apotransferrin is released, and the receptor remains intact on the cell surface. The concentration of transferrin in human serum increases during iron-deficiency anemia and decreases during hemochromatosis. Tuil et al. (1985) suggest that regulation of transferrin concentration in serum in response to an altered level of iron is not at the transcriptional level.

A second role of transferrin is to serve as a bacteriostatic agent (Schade and Caroline, 1946). Iron is the cation whose concentration is the most important in competition between the establishment of a microbial disease and the successful suppression of the disease by animal hosts (Weinberg, 1978). Because transferrin is the iron-sequestering protein of vertebrates and will tightly bind free plasma iron at the sites of bacterial infection, it deprives invading microorganisms of the ferric iron needed for growth. Growth of *Klebsiella* is inhibited by three forms of human transferrin (Lawrence et al., 1977).

This study describes the daily rhythm of total iron-binding capacity (TIBC) and the influence of a change in food availability on the rhythm. This study may aid in attempting to explain the mechanism of iron procurement of reticulocytes for hemoglobin synthesis and to establish if there is a time when vertebrates may be more susceptible to bacterial infection.

MATERIALS AND METHODS

Retired male breeder mice, random-bred Dub-ICR *Mus musculus* (Dominion Laboratories), approximately 7 months old at the initiation of the study, were housed individually in either hanging stainless-steel cages or in polyurethane cages with stainless-steel tops. Twenty mice were entrained to a 12L:12D cycle for 1 month prior to the initiation of the study. The cycle was divided into a light phase (0600 to 1800 h) and a dark phase (1800 to 0600 h) in a light-tight room. Rodent Chow (Wayne Feeds, Memphis, Tennessee) and water were available ad lib. or as noted later. Using the entrainment light cycle and feeding conditions already described, total iron-binding capacity and hematocrit were measured eight times over a 24-h period at 0200, 0500, 0800, 1100, 1400, 1700, 2000, and 2300 h.

For the second part of the study, three groups of mice on the same 12L:12D cycle were used and grouped according to the availability of food. Group A ($n = 9$) had food during the light phase; group B ($n = 9$) had food available during the dark phase. During the initial entrainment period for the food-availability study, disturbance caused by changing food could induce activity that would mask the rhythm of TIBC. A third group (group C; $n = 8$) was included that was fed ad lib. to serve as a control. After mice were entrained to light or placed on the restrictive feeding regime, they were allowed to acclimate for a minimum of 2 weeks before sampling procedures began.

All mice were bled from the suborbital canthal sinus under ether narcosis. A minimum of four heparinized microhematocrit tubes were used per mouse per sampling time to guarantee a sufficient amount of plasma. The hematocrit was determined, and plasma was separated from the packed cells and stored at -20°C until analyzed. Each mouse was bled at each time period with a 1-week interval between sampling to prevent anemia and reduce stress.

The plasma samples were analyzed according to the microprocedure for TIBC of Caraway (1963). This assay determines the TIBC of all possible iron-binding sources. Because of the low iron-binding capacity of albumin and ceruloplasmin, they are considered to be negligible and are not considered as iron-binding sources (Aisen and Listowsky, 1980). The procedure was modified according to Williams and Conrad (1972) in that magnesium carbonate precipitate was centrifuged for an additional 5 min to remove any excess magnesium carbonate particles, which

could give a false spectrophotometric reading. Total iron-binding capacity was expressed as micrograms per milliliter.

Statistical analyses were performed using the Biomedical Data Package (BMDP; Dixon, 1983) and the Statistical Package for the Social Sciences (SPSS-9; Nie, 1975). The BMDP package was utilized to perform a repeated measure analysis (BMDP2V); the SPSS package was used for the Duncan's multiple range test, using a probability level of 0.05. The repeated measures program was used to determine if there was a significant difference among the means for the sampling times. In the second part of the study, this measurement compared the means of the three events at each sampling time to determine if there was a statistically significant difference among the means for the three feeding regimens as well as between sampling times. With a positive correlation from the repeated measures test, the Duncan's multiple range test was used to compare the means from each sampling time. The subsets from the multiple range test indicate groups whose highest and lowest means do not differ by more than the shortest significant range for a subset of that size.

RESULTS

The TIBC values (mean ± 1 *SE*) at each sampling time are shown in Fig. 1. The amplitude of the TIBCs ranged from 1.54 to 3.11 μg/ml. Statistical analyses on the TIBCs, using the repeated measures test, indicated that at least one time period was significantly different ($P < 0.05$). The lowest levels measured at 1700 h were significantly different from the highest levels at 2000 to 0200 h. This establishes that a daily rhythm exists, with a maximum concentration occurring in the dark phase of the cycle. Statistical analyses on a parallel study of hematocrit showed that there were no significant differences between mice or sampling times (43.8% $\pm$ 0.55; data not shown).

The means per time ranged from 2.73 to 4.29 μg/ml for the group fed during the light phase, 2.48 to 3.46 μg/ml for the group fed during the dark phase, and 2.38 to 3.93 μg/ml for the control group (Fig. 2). The BMDP analysis indicated that all the wave forms for all three groups were similar and that, for each time period, none of the three points per time was significantly different at $P < 0.05$. The repeated measures test showed that there was at least one time period that was significantly different when all groups from each time were combined. Duncan's multiple range test indicated that the mean concentrations at 2000 h were significantly higher ($P < 0.05$) from all other times, including the concentrations at the lowest level at 1700 h. This suggests that the rhythm is similar regardless of food availability.

DISCUSSION

These data demonstrated that TIBC exhibited a daily rhythm in mice with peak levels during the dark phase of the photoperiod. The rhythmicity of transferrin has not been widely studied; however, laboratory rats also exhibit a daily rhythm with a peak transferrin concentration in the dark phase (Schade et al., 1980). Our study shows that a similar rhythm exists in *M. musculus*. The peak concentration in rats was late in the dark phase, while the mice in our study had the peak concentration early in the dark phase. Normal values for TIBC in humans were reported by Ramsey (1958) to range from 2.5 to 4.0 μg/ml. No range of values of TIBC for mice have been previously reported.

No significant difference in hematocrit values with respect to time of sampling was found. Had there been a significant difference in hematocrit values, the difference in TIBC might have been due to a change in blood volume. The data demonstrate that the time between sampling was adequate to prevent anemia and reduce stress and validates that the plasma protein transferrin does exhibit a rhythm as described.

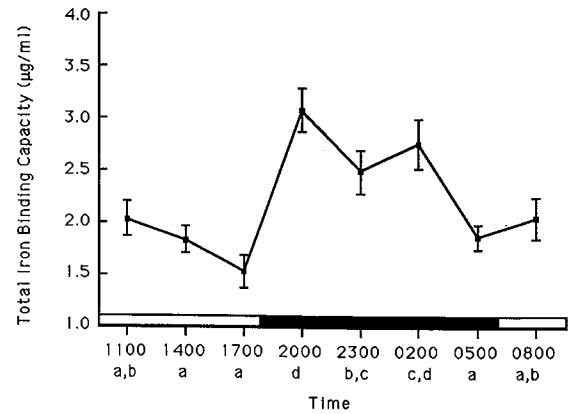

FIG. 1. Plasma total iron-binding capacity for eight times over a 24-h period (mean ± 1 *SE*). Each point represents the mean of 20 mice. The dark bar represents the dark phase of the photoperiod. The small letters under the times represent the statistical subset of the mean for that time as calsulated by Duncan's multiple range test.

The maximum concentration of TIBC coincide with feeding time. At the onset of the dark cycle, it was observed that the activity of the mice increased. The increase in activity included an increase in feeding at about 1830 to 1930 h. We believe that the peak may, therefore, be due to the demand for transport of iron from the intestine. Numerous investigators have reported the influence of feeding schedules on daily changes in tissue-wide metabolic patterns (Nelson et al., 1975; Phillipens et al., 1977; Russell et al., 1983).

The data also suggest that light was the dominant entrainment stimulus. Changing the feeding time caused no change in the daily rhythm of TIBC, indicating that the rhythm was not affected by the presence or ingestion of food. An investigation by Scheving et al. (1974) demonstrated that not all rhythms are overriden by feeding schedules. This phenomenon was also investigated by Spiteri (1982), who concluded that, when food is restricted to the light phase, rats do not become diurnal in the sense that they show a complete shift of nocturnal behavior. It is believed that those traits that do not become diurnal are controlled by endogenous factors. The increased concentration of transferrin may be brought about by some hepatic mechanism generated by cells in need of iron.

In a study comparing total plasma protein levels and γ-globulin levels in the diurnal chinchilla and nocturnal rabbit, Jakubow and Gromadzka-Ostrowska (1987) found that rabbits show a circadian rhythm in total plasma protein concentrations but not in the γ-globulin levels, The maximum total protein concentrations occurred in the dark phase of the photoperiod. In a separate study, rabbits showed similar circadian rhythms in plasma iron concentrations but no variation in plasma transferrin (Schumann and Haen, 1988). Chinchillas show a 24-h cycle for γ-globulin levels but not for plasma proteins. The highest γ-globulin levels occurred during the light phase of the photoperiod. Comparing the data on mice (present study), rats, rabbits, and chinchillas, it appears that the rhythm of immunologically important proteins is dependent on whether the animal is nocturnal or diurnal.

ACKNOWLEDGMENTS

We thank George Relyea of Statistical Services, Memphis State University, for help with the statistical analyses. This work was supported in part by a grant from the Society of Sigma Xi. It is with great

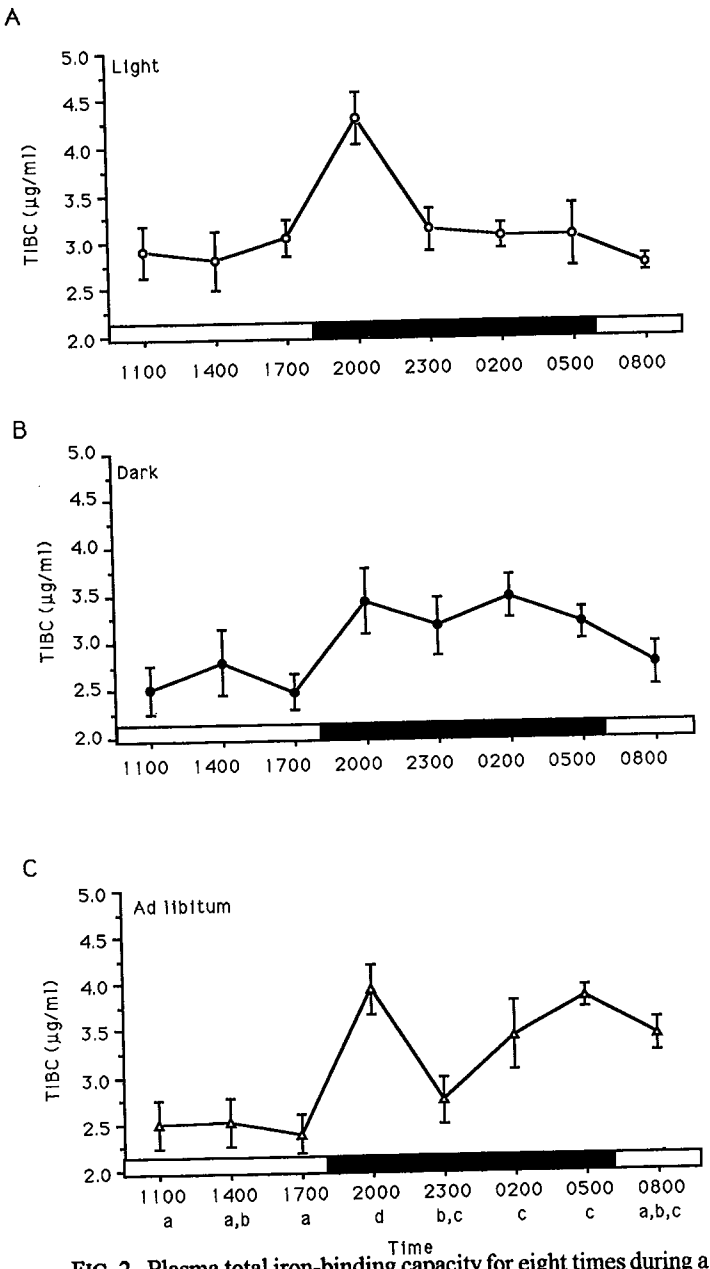

FIG. 2. Plasma total iron-binding capacity for eight times during a 24-h period for three groups of mice fed during (A) the light phase of the photoperiod (n = 9), (B) the dark phase of the photoperiod (n = 9), and (C) ad lib. (n = 8). The dark bar represents the dark phase of the photoperiod. The small letters under the time of day are the subsets from Duncan's multiple range test.

sadness that the first three authors report the death of the fourth author (P. S. Rushton) since the acceptance of the manuscript. She was an excellent teacher, researcher, and friend and will be missed by the scientific community.

LITERATURE CITED

AISEN, P., AND I. LISTOWSKY. 1980. Iron transport and storage proteins. Ann. Rev. Biochem., 49:357-393.

CARAWAY, W. T. 1963. Macro and micro methods for the determination of serum iron and iron-binding capacity. Clin. Chem., 9:188-199.

DAUTRY-VARSAT, A., A. CIECHANOVER, AND H. F. LODISH. 1983. pH and the recycling of transferrin during receptor mediated endocytosis. Proc. Natl. Acad. Sci., 80:2258-2262.

DIXON, W. J. 1983. BMDP statistical software. Univ. California Press, Berkeley.

ENNS, C. A., J. W. LARRICK, H. SUOMALAINEN, J. SCHROEDER, AND H. H. SUSSMAN. 1983. Co-migration and internalization of transferrin and its receptor on K562 cells. J. Cell. Biol., 97:579-585.

JAKUBOW, K., AND J. GROMADZKA-OSTROWKSA. 1987. Twenty-four hour changes lysozyme levels, total plasma protein concentration, γ-globulin concentration, white blood cell count and numbers of lymphocytes and granulocytes in the peripheral blood of chinchilla *Chinchilla laniger* M. and rabbits *Oryctolagus cuniculus* L. Comp. Biochem. Physiol., 86A:109-112.

LAWRENCE, III, T. H., C. J. BIGGERS, AND P. R. SIMONTON. 1977. Bacteriostatic inhibition of *Klebsiella pneumoniae* by three human transferrins. Ann. Human Biol., 4:281-284.

MACGILLVRAY, R. T. A., ET AL. 1982. The complete amino acid sequence of human serum transferrin. Proc. Natl. Acad. Sci., 79:2504-2508.

NELSON, W., L. E. SCHEVING, AND F. HALBERG. 1975. Circadian rhythms in mice fed a single daily meal at different stages of lighting regimen. J. Nutr., 105:171-184.

NIE, N. H., ED. 1975. Statistical package for the social sciences. McGraw-Hill Book Company, New York.

PHILLIPENS, K. M. H., H. VON MAYERSBACH, AND L. E. SCHEVING. 1977. Effects of the scheduling of meal-feeding at different phase of the circadian system in rats. J. Nutr., 107:176-193.

RAMSEY, W. N. M. 1958. Plasma iron. Pp. 1-39 *in* Advances in clinical chemistry. Academic Press, New York.

RUSSELL, J. E., D. J. SIMMONS, B. HUBER, AND B. A. ROOS. 1983. Meal timing as a zeitgeber for skeletal deoxyribonucleic acid and collagen synthesis rhythms. Endocrinology, 113:2035-2042.

SCHADE, A. L., AND L. CAROLINE. 1946. An iron-binding component in human plasma. Science, 104:340-341.

SCHADE, R., H. REX, D. KASTNER, A. FRIEDRICH, AND C. SPRANG. 1980. Biorhythms of four serum proteins of the laboratory rat: IgG, transferrin, α-2-acute phase protein and a VLD lipoprotein. Acta Biol. Med. Germ., 39:1183-1189.

SCHEVING, L. E., ET AL. 1974. Lighting regimen dominates interacting meal schedules and synchronizes mitotic rhythm in mouse corneal epithelium. Anat. Rec., 180:47-52.

SCHUMANN, K., AND E. HAEN. 1988. Influence of food intake on the 24-hr variations of plasma iron concentration in the rabbit. Chronobiol. Internat., 5:59-64.

SPITERI, N. J. 1982. Circadian patterning of feeding, drinking and activity during diurnal food access in rats. Physiol. Behav., 27:139-147.

TUIL, D., ET AL. 1985. Transient transcriptional inhibition of the transferrin gene by cyclic AMP. FEBS, 189:310-314.

VAN DIJK, J. P. 1988. Regulatory aspects of placental iron transfer--a comparative study. Placenta, 9:315-336.

WEINBERG, E. D. 1978. Iron and infection. Microbiol. Rev., 42:45-66.

WILLIAMS, H. L., AND M. E. CONRAD. 1972. Problems in the measurement of iron-binding capacity in serum. Clin. Chem. Acta, 37:131-140.