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Effect of Strain, Sex, and Circadian Rhythm on Rabbit Serum Bilirubin and Iron Levels[1] (37823)

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(Introduced by H. Meier)
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Serum iron (SI), total iron binding capacity (TIBC), and serum bilirubin levels have been used as diagnostic criteria in man for early clinical stages of various types of anemia. For example, Carr reported that when the ratio of SI to TIBC was less than 16%, a state of iron deficiency anemia exists whereas mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) may still be within the normal range (1). In 1971, Bloomer *et al.* (2) showed that in congenital nonhemolytic jaundice the serum bilirubin concentration is about 40 times normal. A similar level of serum bilirubin was observed by Keaster *et al.* (3) in hemolytic disease of the newborn. Jaffe and Bishop (4) reported decreased serum iron during clinical stages of Hodgkin's disease. Hargrove (5) showed a marked increase in serum bilirubin in sickle cell anemia. Caroline *et al.* (6) reported elevated serum iron without a change in total iron binding capacity in acute leukemia. These observations, along with the findings of hereditary hemolytic anemia (7, 8) and lymphosarcoma (8, 9) in the rabbit, and the single case of myeloid leukemia in the rabbit (10) pointed out the need for establishing normal baseline values for serum iron, total iron binding capacity, and bilirubin levels in the rabbit, since these parameters might be used as preclinical diagnostic criteria. Because of known variation in clinical parameters associated with circadian rhythm, strain, sex, and age in the rabbit (11–18) and in man (19, 20), we checked for such variations in these specific parameters in the rabbit prior to using them in studies of the mutant conditions. Since this baseline information was not available, we studied the variation in serum iron and serum bilirubin levels and serum total iron binding capacity for effects due to sex, strain, and time of day.

Materials and Methods. Experiment 1. Study of diurnal variation. For this experiment 24 male and 24 female genetically similar, mature rabbits were used. They were first-generation hybrids between two incipient inbred strains, III and III_c of New Zealand White origin. The experimental regimen for the investigation has been reported previously (12, 13, 16). In brief, however, blood samples were taken at 4 hr intervals, and the experiment was replicated using the same animals 3 weeks later, so that each rabbit was only bled twice, e.g., at 3-week intervals. The biochemical parameters examined were determined on aliquots of serum stored 18 months at −20° that

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TABLE I. The Effect of Circadian Rhythm on Serum Iron and Total Iron Binding Capacity.

Parameter	Sex	0400	0800	1200	1600	2000	2400
Serum iron (SI)	♀	228.02 ± 11.10[a]	218.66 ± 13.70	237.67 ± 14.42	242.46 ± 9.89	250.59 ± 9.20	212.09 ± 15.82
	♂	242.26 ± 9.98	208.45 ± 13.41	238.88 ± 14.23	246.40 ± 15.49	270.99 ± 18.27	233.93 ± 16.44
Total iron binding capacity (TIBC)	♀	211.14 ± 8.36	218.47 ± 9.83	236.00 ± 15.44	221.12 ± 10.06	228.54 ± 18.94	213.02 ± 12.18
	♂	278.92 ± 9.86	267.09 ± 8.75	251.98 ± 14.35	242.50 ± 19.22	249.27 ± 16.27	251.28 ± 22.62
SI/TIBC	♀	.9525 ± .0374	.9026 ± .0490	.9432 ± .0458	.9632 ± .0173	.9825 ± .0173	.9011 ± .0447
	♂	.8370 ± .0500	.7851 ± .0520	.8686 ± .0540	.9068 ± .0540	.9637 ± .0265	.8534 ± .0700

[a] Mean ± SE.

were part of the same blood samples used in the previous diurnal studies (12, 13, 16). Current methodology indicates that serum may be stored for 1 year at −12° and used for serum iron determinations; however, we have assayed over 200 serum samples, stored for 2 years at −20°, and found no noticeable difference when compared with a second set of 200 from the same strains and stored only 4–5 months. Because the serum samples used are from the previously reported diurnal study, direct correlations can be made between the results in this experiment and those reported in the three earlier studies.

Experiment 2. Study of strain difference. In this experiment, 10 males and 10 females from each of 10 incipient-inbred and 2 inbred strains were used. Some biochemical and physiological characteristics of the various strains used have been reported previously (14, 15, 17, 18). Water was supplied *ad lib.* Diet consisted of Purina Rabbit Chow Checkers (20% protein).

Animals were bled between approximately 9 AM and 12 noon, using a vacuum-type bleeder (21). Thirty to thirty-five cubic centimeters of blood was taken, allowed to clot at room temperature for approximately 30 min, and then, under refrigeration, centrifuged for 15 min. The serum was removed and divided into aliquots which were stored 5 months at −20° for the determination of serum levels of iron, bilirubin, and the total iron binding capacity. Additional aliquots were frozen for the determination of other parameters to be reported separately.

In order to minimize confounding serum parameters with differences due to day or time of day, the design was as follows: We bled one male and one female from each strain per day. The order of bleeding was randomized and while the animals bled were kept in outside hutches, bleeding was done during early September when the day-to-day variation in temperature was minimal and the day-to-night variation in temperature was not extreme.

Biochemical assays. Serum iron and total iron binding capacity were determined by disassociating iron from protein, reducing this to the ferrous state with hydroxylamine

hydrochloride, and subsequent formation of the typical bathophenanthroline color complex (22). Valid iron assays dictate clean acid-washed glassware (22).

The major chemical reaction for the determination of serum bilirubin involves diazotization of sulfinic acid with sodium nitrate and subsequent coupling with bilirubin to produce colored azobilirubin pigment. This method is based on the Van den Bergh reaction utilizing sucrose as a clarifier (23).

Statistical analysis. All biochemical determinations were run in duplicate on each serum sample and the mean computed to reduce technical error. Data from the diurnal study were analyzed using a factorial analysis with fixed effects of time, day, and sex. In addition, there is a nested "within rabbit between weeks" component in the "between rabbit" variation (24) as follows:

Source of variation	*df*	MS	F
Time	5	MS_1	F_1
Sex	1	MS_2	F_2
Time × sex	5	MS_3	F_3
Days	1	MS_4	F_4
Time × days	5	MS_5	F_5
Sex × days	1	MS_6	F_6
Time × sex × days	5	MS_7	F_7
Rabbits within TDS	24	EMS	
Weeks within rabbits	48		
Total	95		

Strain data were analyzed using a two-way analysis of variance with equal subclass numbers. The data were then subjected to both the Student–Newman–Keuls multiple range test and Tukeys' -*W* statistic (24). For determination of individual strain differences, Tukey's -*W* was used at the 0.01 level of significance.

Results. Experiment 1. Diurnal study. Table I shows means and standard errors for each variable separated by sex and time of day. These data are depicted graphically in Figs. 1–3. Statistical analysis reveals a highly significant effect of circadian rhythm ($P < 0.001$) on serum iron levels, but only a significant effect ($P < 0.05$) on the ratio of serum iron to total iron binding capacity (Table II). Marked sex differences were observed in total iron binding capacity ($P < 0.001$). This was reflected in the SI:TIBC ratio ($P < 0.01$). Significant interactions were observed associated with day, although as a main effect no significant pattern was noticeable.

Experiment 2. Strain study. Table III lists the data by strain and sex for each parameter given in means and standard errors, and Fig. 4 illustrates this graphically. Statistical analysis (Table IV) revealed differences associated with strain for SI, TIBC, and the SI:TIBC ratio. A significant sex difference in the SI:TIBC ratio was observed ($P < 0.01$). This was also apparent in TIBC ($P < 0.05$) but was confounded by the significant sex by strain interaction ($P < 0.05$).

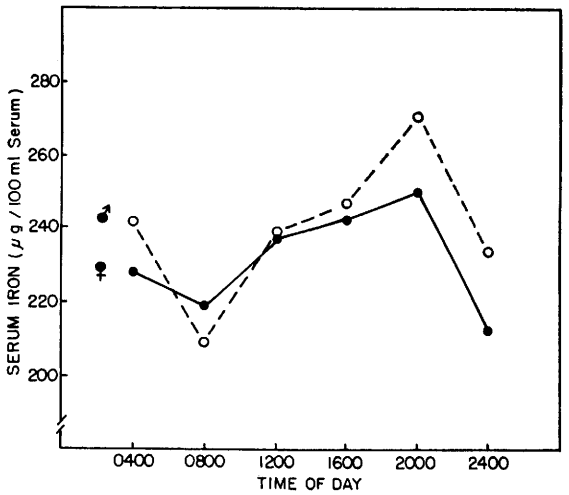

FIG. 1. The effect of time of day and sex on serum iron levels in hybrid III/III$_c$ rabbits.

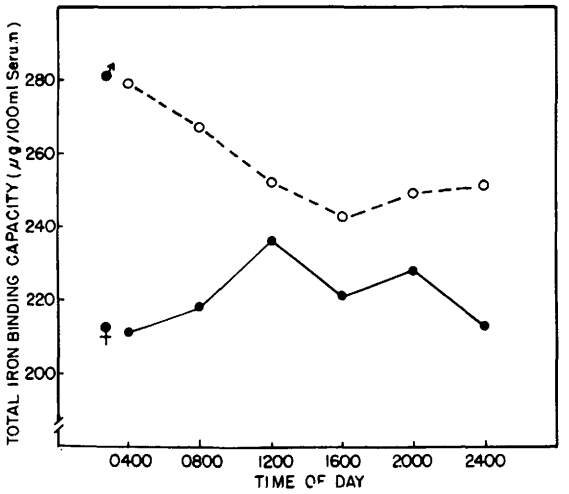

FIG. 2. The effect of time of day and sex on total iron binding capacity in hybrid III/III$_c$ rabbits.

No significant differences were observed in bilirubin levels ($P < 0.05$), due in part to the fact that rabbit sera has a very low level of bilirubin under normal conditions as seen in our data (Table III).

The differences associated with sex were consistent in both diurnal and strain studies by affecting total iron binding capacity but not serum iron levels. Serum iron levels are affected, however, by both strain and circadian rhythm. Strain differences were observed in SI, TIBC, and the ratio of these two, but bilirubin, being so low, showed no real effects.

Discussion. There is excellent agreement in the human literature on the presence of a circadian pattern to serum or plasma iron levels. Hamilton *et al.* (19), using adult human males, observed a pattern of high plasma concentration about 9:00 AM and low concentration at approximately 9:00 PM. They also summarized in tabular form five earlier studies [Hemmeler (1944); Hoyer (1944); Valquist (1941); Waldenström (1946); and Heilmeyer (1937)] (19) using both males and females which were in excellent agreement with their data. Recently, Speck (20), using males and females, also confirmed the earlier studies and showed that total iron binding capacity did not vary with time of day. Our data on the rabbit would not appear, initially, to be in accord

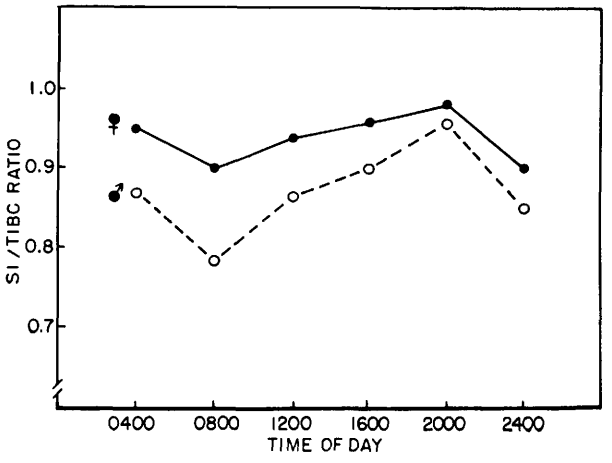

FIG. 3. The effect of time of day and sex on the SI/TIBC ratio in hybrid III/III$_c$ rabbits.

TABLE II. The *F* Values Showing the Effect of Circadian Variations on Serum Iron and Total Iron Binding Capacity.

Parameter	Time	Sex	T × S	Day	T × D	S × D	T × S × D
Serum iron (SI)	10.26***	4.14	1.46	0.19	3.34*	7.54*	8.80***
Total iron binding capacity (TIBC)	0.28	14.99***	0.80	3.14	0.63	0.06	2.13
SI/TIBC	3.23*	10.40**	0.45	0.87	1.01	4.17	2.45

* $P < .05$. ** $P < .01$. *** $P < .001$. All other *F* values not significant ($P > 0.05$).

with this. However, Hamilton *et al.* conclude that the diurnal rhythm is related to activity and sleep; they based this in part on the studies of Hoyer [Acta Med. Scand. **119**, 577 (1944)] and Waldenström [Acta Med. Scand. **170**, 252 (1946)], who showed that people working the night shift have their highest level of iron in the afternoon or early evening after waking, and that this level falls during the night while they are working to rise again during the day when they sleep (19). Hamilton *et al.* observed that 5 normal adults who were leading irregular hours of activity and sleep did not have a definite diurnal cycle. Therefore, our rabbit data on serum iron and total iron binding capacity are in complete agreement, based on activity levels and sleep, with the human literature, since the rabbit is a nocturnal animal. Based on activity levels and sleep, however, neither our data nor the human data are in accord with a study reported on the rat (25). Differences in human serum levels associated with maleness or femaleness are consistent with population studies in man. Serum iron levels are greater for males than for females,

TABLE III. Effect of Sex and Strain on Serum Iron (SI), Total Iron Binding Capacity (TIBC), and Bilirubin Levels in the Rabbit.

Strain	Sex	*n*	Serum iron (μg/100 ml)	TIBC (μg/100 ml)	SI/TIBC	Bilirubin (mg/100 ml)
III_c	♀	10	224.7 ± 8.1[a]	245.4 ± 10.5	0.90 ± 0.03	0.19 ± 0.07
	♂	10	218.8 ± 8.8	238.7 ± 7.5	0.90 ± 0.03	0.19 ± 0.06
III_{mo}	♀	10	238.4 ± 12.5	239.8 ± 11.1	0.95 ± 0.03	0.19 ± 0.08
	♂	10	246.8 ± 5.1	264.9 ± 10.4	0.91 ± 0.03	0.43 ± 0.10
AC	♀	10	191.7 ± 11.8	234.1 ± 9.7	0.82 ± 0.06	0.17 ± 0.08 (9)
	♂	10	183.7 ± 15.1	274.8 ± 9.3	0.66 ± 0.06	0.38 ± 0.15 (9)
ACEP	♀	10	236.2 ± 7.0	248.8 ± 10.3	0.92 ± 0.03	0.23 ± 0.06
	♂	10	208.1 ± 8.2	240.7 ± 11.4	0.84 ± 0.05	0.16 ± 0.06
AX	♀	10	180.8 ± 9.7	246.8 ± 16.4	0.75 ± 0.05	0.42 ± 0.12 (9)
	♂	10	210.0 ± 7.9	291.0 ± 12.8	0.73 ± 0.03	0.45 ± 0.19
Ax_{bubu}	♀	10	206.0 ± 12.7	262.8 ± 14.6	0.79 ± 0.04	0.22 ± 0.08
	♂	10	180.8 ± 7.0	270.7 ± 9.3	0.67 ± 0.02	0.20 ± 0.08
ACCR(B)	♀	10	209.6 ± 12.7	234.9 ± 6.7	0.87 ± 0.04	0.15 ± 0.10
	♂	10	196.9 ± 14.3	263.6 ± 6.8	0.74 ± 0.05	0.08 ± 0.05
C	♀	10	221.2 ± 11.1	247.7 ± 13.7	0.88 ± 0.05	0.22 ± 0.08
	♂	10	211.4 ± 7.0	247.0 ± 8.8	0.86 ± 0.03	0.24 ± 0.08 (9)
OS	♀	10	192.5 ± 11.7	239.8 ± 16.4	0.80 ± 0.04	0.08 ± 0.03
	♂	10	176.4 ± 10.6	239.5 ± 8.9	0.74 ± 0.05	0.28 ± 0.10
ACCR(Y)	♀	10	180.3 ± 11.5	296.0 ± 10.9	0.62 ± 0.06	0.08 ± 0.03 (9)
	♂	10	199.2 ± 13.2	328.2 ± 13.5	0.62 ± 0.05	0.46 ± 0.12
WH	♀	10	204.6 ± 9.2	272.6 ± 16.6	0.77 ± 0.05	0.42 ± 0.15
	♂	10	214.0 ± 16.4	276.5 ± 13.4	0.77 ± 0.07	0.25 ± 0.08
III_{vo}	♀	10	229.8 ± 13.4	309.4 ± 17.3	0.75 ± 0.06	0.18 ± 0.08
	♂	10	204.7 ± 8.3	268.2 ± 13.6	0.77 ± 0.04	0.21 ± 0.09

[a] Mean ± SE.

TABLE IV. *F* Values from Analysis of Variance of Serum Iron (SI), Total Iron Binding Capacity (TIBC), and Bilirubin Data.

Source of variance	*df*	Serum iron	TIBC	SI/ TIBC	Bili-rubin
Strain	11	5.21***	6.49***	7.26***	1.65
Sex	1	1.63	4.71*	7.14**	2.72
S × S	11	1.38	2.07*	0.84	1.34
Error	216				
Males					
Strain	11		5.66**		
Error	108				
Females					
Strain	11		3.38***		
Error	108				

* $P < .05$. ** $P < .01$. *** $P < .001$. All other *F* values not significant ($P > 0.05$).

whereas the converse is true for total iron binding capacity (20, 26). In the rabbit, however, there does not appear to be any consistent effect of sex in our population. The difference in serum iron levels associated with sex in humans and the lack thereof in rabbits may be explained in part by the failure of the rabbit to menstruate. The prevalence of iron deficiency is much greater in women than in men (26). Total iron binding capacity levels were higher for females in three strains, for males in seven strains, and about the same in two. Similarly, SI levels were higher for females in eight strains and for males in four strains. The differences in SI levels were not statistically significant. The absolute values vary depending both on the strain and on the time of day for serum iron. There does appear to be a tendency, however, for total iron binding capacity values to be greater for males than females. This was particularly noticeable in the case of the F_1 hybrids used in the diurnal experiment ($P < 0.01$), but only moderately so in the strain study ($P < 0.05$). Also, a sex by strain interaction was present. It was clearly apparent, however, in the diurnal study that the difference between the sexes is dependent upon the time of day that the serum is collected. As, for example, at 4:00 AM when the total iron binding capacity levels were maximal for the males, they were minimal for the females.

The ratio of serum iron to total iron bind-

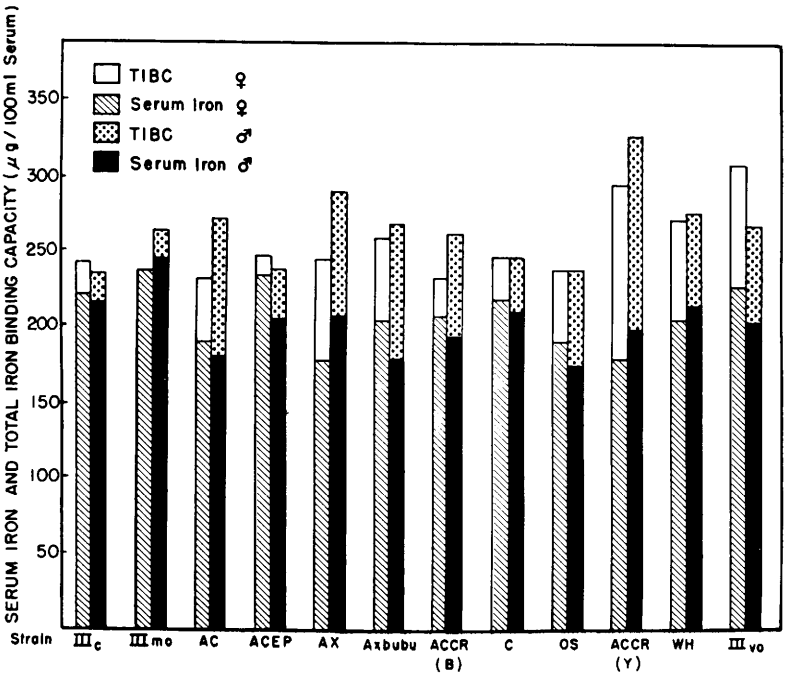

FIG. 4. The effect of strain and sex on serum iron levels and total iron binding capacity in inbred and partially inbred rabbits. Samples were taken to minimize circadian rhythm and to avoid confounding sex and strain effects with differences due to day of collection.

ing capacity, which is often used to diagnose iron deficiency anemia (1), was consistently higher for females and was affected by both strain and time of day.

Serum bilirubin values for the rabbit are so low that no real differences associated with sex or strain were observed. These values, however, of approximately 1 mg% or less are in accord with normal values published for man (2, 5, 27) and a variety of other species (28–30).

Summary. Investigation of 48 adult hybrid rabbits revealed that the time of day affects the serum iron levels and the ratio of serum iron to total iron binding capacity, and that there is a difference in the total iron binding capacity and the serum iron to total iron binding capacity ratio depending on whether males or females are used.

Analysis of the data on 240 rabbits, involving 12 inbred or partially inbred strains, showed that serum iron, total iron binding capacity, and the ratio of serum iron to total iron binding capacity are all influenced by strain, but that only total iron binding capacity and the ratio of serum iron to total iron binding capacity are influenced by the sex of the rabbit. Normal bilirubin values are so low that no real effect of sex or strain was apparent.

These data do provide baseline values for the rabbit and are consistent with man in the manner to which these parameters are affected.

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