Organ and body weights of preweanling rats treated with different doses of R2858

Treatment	Testis (mg)	Pituitary (mg)	Liver (mg)	Body weight (g)
Control	67.9±3.74	1.7±0.07	1277.2± 36.8	37.4±0.9
0.01 µg	*50.3±1.75	1.8±0.09	1412.0±115.0	39.4±3.28
0.1 µg	**40.2±2.16	1.7±0.11	1224.2± 43.92	35.1±0.52

Results are the mean± SEM of 5–12 determinations. Statistical difference compared to the control: * $p<0.05$; ** $p<0.01$.

times they were difficult to distinguish from adjacent fibroblasts (figure 2).

Discussion. Treatment with R2858 effectively retarded testicular growth in preweanling rats. Since body weights of treated rats were unchanged, testicular weight alterations were presumably not due to a pharmacologic effect of R2858. Because pituitary and liver weight were unaffected, these tissues are apparently unresponsive to estrogen at this time, unless more subtle changes have occurred. Pituitary estrogen receptors are few in young animals[9], but increase in number as the animal ages[10]. The response of the testis to estrogenic treatment in our experiments correlates well with known receptor activity in this organ[11]. Inhibition of testicular growth with the lower dose of R2858 (0.01–0.02 µg/day) indicates a remarkable sensitivity to this agent. Abetting this could be the immaturity of the steroid degrading system of the liver in preweanling animals which might allow accumulation of estrogen[12]. Our ultrastructural observations suggest that reduced testicular growth in treated rats reflects decreased Leydig cell activity. Decreased testosterone production and similar alterations of cellular morphology has been observed in older rats treated with estrogen[3,4].

The present study does not resolve whether R2858 has a direct effect upon Leydig cells or acts indirectly by decreasing pituitary gonadotrophic secretion. Diethylstilbesterol, another estrogenic substance with little affinity for α-fetoprotein, directly inhibits Leydig cell synthesis of testosterone in 28-day-old immature rats[13].

R2858 is a potent estrogenic substance in young female rats. Amounts comparable to those used in this study are uterotrophic in old animals, but similar doses of estradiol are ineffective[7]. The potency of this compound is attributed to its ability to bind with target cell receptors combined with its lack of affinity for α-fetoprotein. α-Fetoprotein is thought to protect the fetus against high levels of estrogen during pregnancy[14]. Injection of large amounts of R2858 into pregnant rats results in underdevelopment of the genital tract (seminal vesicles, prostate, genital tubercle) of the male fetuses[14]. Our study suggests that α-fetoprotein has a similar effect on preweanling animals. Although plasma estradiol is found in these animals, it is probably prevented from acting by α-fetoprotein. That estradiol is not effective in young animals is also evident from observations that a large dose of estradiol in sexually immature rats (21–28 days) had no effect on testicular growth[15].

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Effect of prolactin on human red cell sodium transport

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Summary. Incubation of red cells with higher concentrations of prolactin in vitro enhanced the cellular sodium level and produced a significant reduction in erythrocyte membrane adenosine triphosphatase activity. This effect was dose and time-dependent. It is the result of an inhibition of the active sodium pump similar to that produced by ouabain, suggesting altered red cell function and electrolyte balance in hyperprolactinemic states.

Prolactin is known to play a significant role in the regulation of the fluid and electrolyte balance, since its administration in human volunteers resulted in reduced renal excretion of sodium, potassium and water[2]. Parke and Horrobin[3] have observed a significant difference in plasma concentrations of prolactin when the same blood sample was estimated immediately on drawing and after keeping the sample for 6 h. The plasma prolactin concentrations estimated after 6 h of standing varied inversely with that of the initial concentration. This suggests the possibility that red cells may bind prolactin, exerting a sort of buffering action. Karmali et al.[4] have shown that in vitro addition of higher concentrations of prolactin (50 ng/ml) to blood samples for 6 h produced a small but very consistent fall in plasma sodium by 3% and a very significant increase in plasma osmolality with no change in potassium level. This indicates that prolactin at higher concentrations may enhance the movement of sodium into cells. Since no direct

evidence is yet available on the involvement of prolactin in red cell sodium transport, the present work was undertaken to observe the levels of red cell sodium on incubation (in vitro) with a wide range of prolactin concentrations at varied time intervals. As the movement of sodium across cells is maintained by extrusion of sodium by active pumping, ouabain, a known inhibitor of NaK dependent adenosine triphosphatase (ATPase), was used to find out whether prolactin-induced changes in red cell sodium levels could be operating through this sodium pump mechanism. Experiments were also conducted to assay erythrocyte membrane ATPase activity after incubation with prolactin and/or ouabain.

Table 1. Effect of in vitro incubation with prolactin on human red cell sodium levels (meq/l cells). Ringer phosphate buffer (pH 7.35), temperature 37 °C

Treatment	3-h incubation (mean ± SEM)	6-h incubation (mean ± SEM)
Control	9.95 ± 0.22	9.88 ± 0.32
Prolactin 5 ng/ml	10.03 ± 0.16	9.85 ± 0.30
10 ng	10.13 ± 0.19	9.91 ± 0.36
25 ng	10.15 ± 0.25	10.25 ± 0.30
50 ng	10.23 ± 0.21	11.12 ± 0.59*
100 ng	10.37 ± 0.30	11.28 ± 0.42*
500 ng	10.57 ± 0.33	11.49 ± 0.52**
5 µg	10.66 ± 0.34*	12.22 ± 0.29**

Figures represent pooled values of duplicate assays from 8 volunteers. Sodium levels of control (0 h) = 9.07 ± 0.16 meq/l. * $p < 0.05$ compared to control; ** $p < 0.01$ compared to control.

Table 2. Effect of ouabain and prolactin in vitro on red cell sodium levels (meq/l cells), temperature 37 °C

Treatment	3-h incubation (mean ± SEM)	6-h incubation (mean ± SEM)
Control	9.95 ± 0.22	9.88 ± 0.32
Ouabain 0.03 mM	10.88 ± 0.36**	10.87 ± 0.28**
Ouabain 0.03 mM + prolactin 50 ng/ml	11.15 ± 0.39**	11.90 ± 0.47** [a]
Ouabain 0.03 mM + prolactin 5 µg/ml	11.37 ± 0.34**	12.64 ± 0.43** [b]
Ouabain 3 mM	12.73 ± 0.55**	13.85 ± 0.29**
Ouabain 3 mM + prolactin 50 ng/ml	12.58 ± 0.29**	13.87 ± 0.35**
Ouabain 3 mM + prolactin 5 µg/ml	12.82 ± 0.32**	13.65 ± 0.30**

Figures represent pooled values of duplicate assays from 8 volunteers. ** $p < 0.01$ compared to control; [a] $p < 0.05$, [b] $p < 0.01$ compared to ouabain (0.03 mM) treatment value.

Table 3. ATPase activity of erythrocyte membranes

Treatment	Pi released/h/mg protein (mean ± SEM, µM) 3-h incubation	6-h incubation
Control (9)	0.40 ± 0.03	0.37 ± 0.04
Prolactin 10 ng (6)	0.39 ± 0.04	0.38 ± 0.03
50 ng (6)	0.37 ± 0.05	0.36 ± 0.03
100 ng (6)	0.34 ± 0.04	0.32 ± 0.04
500 ng (6)	0.32 ± 0.04	0.28 ± 0.03*
5 µg (7)	0.30 ± 0.04*	0.27 ± 0.05*
Ouabain 0.03 mM (6)	0.29 ± 0.05*	0.28 ± 0.03*
Ouabain 3.0 mM (6)	0.24 ± 0.05**	0.22 ± 0.03**
Ouabain 0.03 mM + prolactin 5 µg (7)	0.26 ± 0.05**	0.24 ± 0.04**
Ouabain 3.0 mM + prolactin 5 µg (7)	0.23 ± 0.05**	0.22 ± 0.05**

* $p < 0.05$ compared to control; ** $p < 0.01$ compared to control. Values in parentheses denote the number of samples subjected to analysis.

Materials and methods. Blood samples were collected by venipuncture into heparinized tubes from healthy male volunteers with hemoglobin concentrations of 13 g% and above, at 11.30 h when plasma prolactin levels were supposed to be at their lowest. Cells and plasma were separated and the cells were washed thoroughly in 0.1 M Ringer phosphate buffer (pH 7.35)[5]. 1.0 ml of washed packed cells were resuspended in Ringer phosphate buffer containing glucose (10 mM) and prolactin at various concentrations, as indicated. Controls were carried through the same procedure. Treatment with ouabain was carried out at 2 different concentrations; 0.03 mM, at which there was less inhibition of pump activity, and a higher concentration of 3 mM, at which there was maximal inhibition of NaK dependent ATPase activity[6]. The tubes were gently vortexed and left in a metabolic shaker at 37 °C. At the end of 3 or 6 h the tubes were centrifuged, the cells separated and washed twice in 5 volumes of magnesium chloride (100 mM), and the washings aspirated completely. The last traces of the medium were removed using a filter paper wick without disturbing the cells. The separated cells were hemolysed with deionised water, the volume was made up to 10 ml and sodium was estimated by flame photometry.

After incubation with prolactin and/or ouabain, the cells were separated by centrifugation at 2000 rpm and erythrocyte membrane ATPase activity was measured as described by Kaplay[7]. The phosphate liberated per h after the enzyme assay was determined as inorganic phosphorus[8] and the activity was expressed as units per mg membrane protein; protein was determined by the Lowry method[9]. The statistical significance of the results obtained was assessed using Student's t-test.

Results and discussion. Prolactin enhanced the cellular sodium levels. A dose-dependent increase in red cell sodium level was seen on incubation for 3 and 6 h, the latter producing a greater degree of increase (table 1).

Ouabain at a concentration of 0.03 mM exerted a slight degree of sodium pump inhibition resulting in a little increase in sodium levels after both 3 and 6 h of incubation. When prolactin was incubated with the same concentration of ouabain, higher levels of sodium were observed than in those samples incubated with ouabain alone (table 2). However, ouabain at a concentration of 3 mM caused a marked increase in red cell sodium. There was no further increment in cellular sodium caused by prolactin when added to the same concentration of ouabain, which is known to exert maximal inhibition of NaK ATPase. This additive effect of prolactin with only the lower and not with the maximal concentration of ouabain suggests that prolactin may affect cellular sodium levels in the same way as ouabain by affecting sodium pump activity. Prolactin is known to affect the activity of NaK ATPase[10]. Erythrocyte membrane ATPase activity was significantly less after prolactin treatment, and a further reduction in activity was evident on incubation with the lower concentration of ouabain (table 3). This suggests that prolactin inhibits NaK dependent ATPase activity, like ouabain, to elevate intracellular sodium levels. Higher levels of cellular sodium, with significant reduction in ouabain sensitive ATPase activity of erythrocyte membranes, were seen in hyperprolactinemic states (unpublished observation). A fall in plasma sodium was shown in intact rabbits by Lloyd[11] following prolactin treatment, suggesting transport into cells.

Further, Edmondson et al.[12] have also observed increased levels of leucocyte sodium in congestive cardiac failure patients in whom prolactin levels remain elevated. These reports favour the contention that higher levels of prolactin

increase intracellular sodium levels. Normal physiological concentrations of prolactin remain in the range of 5–20 ng/ml. Higher levels are encountered during lactation, stress, surgery, pituitary adenoma, galactorrhea, amenorrhea from varied causes, and also on prolonged treatment with drugs such as phenothiazines, alphamethyl DOPA, reserpine and combined contraceptive pills. The significant increase in red cell sodium observed only at concentrations of 50 ng and above, suggests that the high intracellular concentration of sodium observed in hyperprolactinemic states may be a reflection of prolactin action on cellular ATPase by selectively interacting with NaK dependent ATPase activity.

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Influence of the pineal gland on the reproductive system of the male house mouse[1]

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Summary. Procedures designed to express pineal-mediated antigonadotropic activity were performed upon male house mice. Neither blinding nor blinding plus olfactory bulbectomy of house mice resulted in testicular involution within 12 weeks. The pineal gland appears to be of little significance to reproduction in the house mouse.

The pineal gland is important for transduction of photoperiod duration into humoral factors for control of reproductive cycles in some mammalian species[4]. Photoperiods less than 12.5 h of light per day result in increased pineal activity and consequent regression of testes and sex accessory organs in the golden hamster[4,5]. In the rat, olfactory bulbectomy is required in addition to blinding for sensitization to actions of the pineal gland[6]. Sympathectomy denervates the pineal gland and renders the gland incapable of mediation of photoperiod-induced testicular regression[5].

However, the pineal gland and photoperiod may not be involved in regulation of reproduction in the house mouse, *Mus musculus*[7,8]. The studies contained herein were undertaken to determine the possible existence and nature of pineal-gonadal interactions in the house mouse.

Materials and methods. 60 mature male JC-1 house mice were utilized in this study. The animals were inbred and maintained at this institution[9], and were housed with 12 h of light daily (L/D 12/12, lights on 07.00 h). Food and water were available ad libitum. To simulate darkness, 30 adult male mice were anesthetized (methoxyflurane) and blinded via orbital enucleation. Another 30 remained intact. In each of the blinded and sighted groups, subgroups of 5 animals each were:

a) sham pinealectomized (SPX) and sham olfactory bulbectomized (SBX), b) pinealectomized (PX)[10] and SBX, c) SPX and olfactory bulbectomized (BX)[6], d) PX and BX, e) superior cervical ganglionectomized (SCGX)[11] and f) SCGX and BX. 1 group of 5 naive animals served as intact controls. In SPX groups, the superior sagittal sinus was punctured through the interval between bone disc and calvarium to stimulate bleeding, but the disc was not removed. Holes were drilled in the frontal bones for sham olfactory bulbectomy, but the bulbs were not aspirated.

12 weeks after the surgical procedures, the mice were decapitated. Body weights and weights of preputial gland, epididymides, testes and seminal vesicles (full and expressed of fluid) were measured. Testicular material was prepared for light microscopic examination (Bouin's fixative, hematoxylin and eosin stain). Data was analyzed by means of a 1-way analysis of variance performed on a Hewlett-Packard 9830-A Computer.

Results. Surgical manipulations designed to increase or decrease pineal activity did not produce significant changes in relative organ weights of the testes or accessory sex organs (table). Examination of histological material indicated testicular regression did not occur in any group. All testicular sections displayed normal spermatids and mature sperm.

Discussion. In addition to the hamster and the rat, photoperiodic influences acting through the pineal gland have also been implicated in annual reproductive rhythms in the Djungarian hamster *Phodopus sungorus*[12], and in the dark-induced sexual regression of the vole *Microtus montanus*[13]. Seasonal rhythms of reproduction in *Microtus agrestis*[14] may result from combinations of effects of the pineal gland and environmental influences.

Although reproductive capabilities of several rodent species are apparently modulated by photoperiod acting via the pineal gland, results of the present study substantiate prior work suggesting a non-involvement of the pineal gland and/or photoperiod in reproductive function in the male house mouse[7,8]. Turek et al.[8] have speculated the pineal gland is more important for the control of testicular function in species responsive to photoperiod such as the golden hamster, and may have no effect in non-photoperiodic species such as the house mouse.

Reiter[4] has suggested inbreeding of rodents for laboratory purposes may have abolished reproductive responses to changes in photoperiod. The JC-1 strain of house mouse studied herein has been inbred for about 25 years. However, the long standing commensalism between the house mouse and man may have resulted, by natural means, in a similar attenuation of photoperiodic responses noted by Reiter[4] in laboratory species.

The data presented here suggest the male house mouse