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EFFECT OF NORETHANDROLONE ON THE BILIARY EXCRETION OF BILIRUBIN IN THE MOUSE AND RAT*

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Abstract—The administration of a wide variety of dosages of norethandrolone failed to depress the excretion of exogenously administered bilirubin in normothermic rats. On the other hand, methyltestosterone produced significant depression of maximum biliary excretion (Tm) of bilirubin in two strains of rats. In mice and Simonsen rats the bilirubin Tm was significantly enhanced by norethandrolone treatment. The effect of norethandrolone on bilirubin excretion was found to be temperature-dependent. An apparent decrease in bilirubin Tm occurred only in rats which were allowed to become hypothermic, whereas an increase in Tm occurred in normothermic rats.

A NUMBER of reports can be found in the literature regarding altered liver function after the administration of anabolic steroids. Norethandrolone (17-ethyl-19-nortestosterone) is among those steroids that have been implicated with sulfobromophthalein (BSP) retention in man.[1, 2] However, conflicting reports have appeared with respect to hepatic dysfunction in animals in response to norethandrolone treatment.

Arias[3] and Hargreaves[4] have reported a decrease in the hepatic capacity to excrete bilirubin after the administration of norethandrolone to rats. The ability of norethandrolone to increase BSP retention in rabbits has been reported by Lennon.[5] Kolb *et al.*[6] measured the effects of norethandrolone on ^{131}I-labeled rose bengal in rats with bile fistulas and found a retardation in biliary elimination 1, 7 and 14 days after daily administration of norethandrolone, but none after 21 days. However, Gallagher *et al.*,[7] after studying a large number of steroids in rats, reported that norethandrolone did not impair the hepatic disposal of BSP. They suggested that species differences in the metabolism of steroids produce wide variations in response. Gass and Umberger[8] reported that daily administration of 10–100 mg norethandrolone for periods of up to 50 days had no effect on BSP retention in dogs.

In the course of exploring mechanisms involved in the potentiation of α-naphthylisothiocyanate-induced hyperbilirubinemia and cholestasis,[9] studies with norethandrolone were carried out in both rats and mice. This communication reports the results of these studies.

MATERIALS AND METHODS

Male Swiss–Webster mice (30–40 g), Simonsen Sprague–Dawley rats (300–350 g)

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and Wistar rats (110–160 g) were used in these studies. Norethandrolone* and methyltestosterone were given orally or i.p. suspended in sufficient 1% carboxymethylcellulose (CMC) to deliver the desired dose in 0·01 ml/g. Control animals received identical treatment with 1% CMC. All animals were fed (Wayne Lab-Blox stock diet) and watered *ad libitum*.

Bilirubin (Sigma Chemical Company, St. Louis, Mo.) for injection was prepared by dissolving 10 mg in 10 ml of an isotonic solution containing 0·52 g Na_2CO_3 and 0·52 g NaCl/100 ml. The solution intended for infusion in the rat was prepared by dissolving 40 mg bilirubin in 10 ml of the isotonic Na_2CO_3–NaCl solution (pH 10). For administration to the mouse, 50 mg bilirubin was dissolved in 10 ml isotonic solution and the pH adjusted to 8 with 5 N HCl.

Plasma bilirubin disappearance studies were performed by injecting mice via the tail vein with bilirubin (10 mg/kg) dissolved in the isotonic solution (0·01 ml/g). At various time intervals after administration of the bilirubin load, blood samples were obtained from separate groups of animals by cardiac puncture under ether anesthesia. Each blood sample was collected in an individual Kahn tube which had previously been treated with sodium oxalate solution and dried.

The disappearance of an exogenous bilirubin load from plasma in the absence of biliary excretion was studied by ligating the bile duct. Bile duct ligation was acomplished in mice under ether anesthesia by means of a small abdominal incision. The wound was closed with 9 mm clips and the animals were allowed to recover from the anesthesia before the bilirubin was administered. Blood samples were collected as described previously.

The plasma samples obtained were analyzed for total bilirubin content by the bilirubin oxidation method of Ferro and Ham.[10] Analysis for free and conjugated bilirubin levels was carried out by the diazotization method of Weber and Schalm.[11]

Essentially, the technique of Weinbren and Billing[12] was used in the biliary excretion studies. Rats were anesthetized with pentobarbital, and the bile duct and external jugular vein were cannulated with PE-10 tubing. A priming dose of bilirubin (20 mg/kg) was followed by constant infusion of 0·3 mg/min by means of a Harvard infusion pump (0·07 ml/min) for a 50-min period. Bile was collected continuously over the 50-min period. Bilirubin analysis and bile volume measurements were completed on the final two 10-min samples. Two 10-min samples were used to confirm the attainment of maximum excretion rates which have previously been shown to be reached 30 min after the start of the bilirubin infusion.[13] Bile volume was measured by means of a pipet graduated in 0·01 ml and the bilirubin concentrations therein were determined by the method of Weinbren and Billing.[12]

Mice weighing between 35 and 40 g were prepared in a manner identical to that used in the rat experiments. Bilirubin (0·1 mg/min) was constantly infused (0·02 ml/min) over a 60-min period. Bile was collected during two 30-min periods in tared 5-ml beakers, the bile volume being determined gravimetrically. For calculating the volume of bile collected, the specific gravity was assumed to be 1·0. The bilirubin concentration from the second 30-min sample was determined in a manner identical to that used in the rat experiments. Earlier experiments in mice demonstrated attainment of maximum excretion rates 30 min after the start of bilirubin infusion. In both the rat and mouse bilirubin infusion experiments, body temperatures were monitored

* Norethandrolone was generously supplied by Dr. V. A. Drill, G. D. Searle & Co.

and maintained at 37° by means of heat lamps. This was done to prevent the occurrence of hypothermia which would lower bilirubin excretion rates.[14]

Control and treated groups were compared statistically by the Student's *t*-test or the Mann-Whitney U test with $P < 0.05$ for rejection of the null hypothesis.[15]

RESULTS

Plasma bilirubin disappearance studies. In the initial screening of norethandrolone for effects on hepatic excretory function, plasma bilirubin disappearance studies were completed in mice (Table 1). Norethandrolone did not cause the retention of bilirubin

TABLE 1. EFFECT OF NORETHANDROLONE ON THE DISAPPEARANCE OF EXOGENOUS BILIRUBIN FROM THE PLASMA OF MICE

Groups*	Bilirubin concentration† (mg/100 ml)		
	5 min	10 min	20 min
Control	5·2 ± 0·2	4·2 ± 0·1	2·0 ± 0·1
Norethandrolone	5·1 ± 0·2	3·5 ± 0·2‡	1·7 ± 0·1

* Norethandrolone (20 mg/kg, p.o.) was given 24 and 12 hr prior to bilirubin administration (10 mg/kg, i.v.).

† Total bilirubin levels (mg/100 ml) were determined in separate groups of animals 5, 10 and 20 min after bilirubin administration. Values shown are mean values ± S.E. obtained from 10 mice.

‡ Significantly different from respective control by *t*-test ($P < 0.05$).

and may even have enhanced the disappearance of exogenous loads of bilirubin from the plasma, since the total plasma bilirubin levels were significantly less in the norethandrolone group at 10 min.

Table 2 shows the effect of norethandrolone on the disappearance pattern of exogenous bilirubin in bile duct-ligated mice. Norethandrolone did not produce bilirubin retention in the absence of biliary excretion. Conjugated bilirubin levels in

TABLE 2. EFFECT OF NORETHANDROLONE ON BILIRUBIN DISAPPEARANCE FROM THE PLASMA OF BILE DUCT-LIGATED MICE

Groups*	Bilirubin concentration† (mg/100 ml)	
	5 min	20 min
Free bilirubin		
Control BDL	5·6 ± 0·2	2·5 ± 0·1
Norethandrolone BDL	4·5 ± 0·6	2·2 ± 0·4
Conjugated bilirubin		
Control BDL	1·1 ± 0·1	1·4 ± 0·1
Norethandrolone BDL	1·9 ± 0·4‡	1·4 ± 0·3

* See Table 1 for doses. BDL = bile duct ligated.

† Free and conjugated bilirubin levels were determined by diazotization reaction in separate groups of animals 5 and 20 min after bilirubin administration. Values shown are mean values ± S.E. obtained from groups of 5 mice.

‡ Significantly different from respective control by *t*-test ($P < 0.05$).

the norethandrolone-treated animals were significantly greater than in controls at the 5-min collection period.

Biliary excretion studies. Maximum excretion rate (Tm) studies for bilirubin were conducted in the mouse and the rat. In mice norethandrolone treatment increased the biliary excretory capacity for bilirubin and the bile bilirubin concentration (Table 3). Bile flow was not significantly altered.

TABLE 3. EFFECT OF NORETHANDROLONE ON BILIRUBIN EXCRETION IN MICE

Groups*	Bilirubin Tm (μg/100 g/min)	Bile flow (μl/100 g/min)	Bilirubin concn (μg/μl)
Control	43·7 ± 7·4	6·1 ± 0·8	7·1 ± 0·6
Norethandrolone	61·9 ± 7·6†	7·7 ± 1·5	9·0 ± 1·0†

* Norethandrolone was given (20 mg/kg, p.o.) 24 and 12 hr prior to bilirubin infusion (0·1 mg/min). Bile bilirubin concentration and bile volume were determined on bile samples collected during the interval 30–60 min after the start of bilirubin infusion. Values shown are mean values ± S.E. determined from 7 control and 8 norethandrolone-treated mice.

† Significantly different from respective control by Mann-Whitney U test ($P < 0·05$).

These results in mice prompted similar studies in rats in an attempt to confirm the reported[3] inhibitory effect of norethandrolone on bilirubin excretion. In addition, methyltestosterone was included to verify its reported inhibitory effect on bilirubin excretion.[3] The results are shown in Table 4. Unexpectedly, norethandrolone significantly increased the apparent bilirubin Tm in Simonsen rats. Methyltestosterone, on the other hand, did decrease the bilirubin Tm value. Because the norethandrolone results were in disagreement with the previously reported inhibitory effect, a second

TABLE 4. EFFECT OF NORETHANDROLONE AND METHYLTESTOSTERONE ON BILIRUBIN EXCRETION IN RATS

Groups*	Bilirubin Tm (μg/100 g/min)	Bile flow (μl/100 g/min)	Bilirubin concn. (μg/μl)
Simonsen rats			
Control	63 ± 2	8·6 ± 0·7	7·6 ± 0·5
Norethandrolone	82 ± 5†	7·3 ± 0·6	11·7 ± 1·1†
Methyltestosterone	28 ± 8†	4·7 ± 1·0†	5·8 ± 1·0
Wistar Rats			
Control	95 ± 7	8·6 ± 0·4	11·0 ± 0·7
Norethandrolone	94 ± 10	9·5 ± 0·8	10·3 ± 1·4
Methyltestosterone	65 ± 13†	8·5 ± 0·9	7·3 ± 0·8†

* Norethandrolone (300 mg/kg, i.p.) and methyltestosterone (300 mg/kg, i.p.) were given once daily for 3 days. Bile bilirubin and bile volume determinations were done on bile samples collected during the interval 40–50 min after the start of bilirubin infusion. A priming dose of bilirubin (20 mg/kg) preceded the continuous infusion of 0·3 mg/min. Values shown are mean values ± S.E. from groups of 5–8 rats. The weight range for the Simonsen and Wistar rats was 330–400 g and 110–160 g respectively.

† Significantly different from respective control by *t*-test ($P < 0·05$).

series of experiments were performed with the species of rat and dosage of norethandrolone employed in experiments in which norethandrolone had exerted inhibitory effects on bilirubin excretion. Arias (personal communication) employed Wistar rats weighing 110–220 g, and a dosage range of 100–300 mg/kg daily for 3–5 days; the second experiment was performed under similar conditions. Even with these conditions, norethandrolone failed to depress the Tm values in the Wistar rats; however, methyltestosterone did depress the Tm significantly (Table 4). It is interesting to note that the Tm values in the Wistar rats were greater than those in the Simonsen rats. Whether this was due to differences in strain or whether it was due to possible differences in the proportion of liver weight to body weight in the different weight ranges employed has not been elucidated.

The possibility that a critical dose of norethandrolone was involved in eliciting an impairment of biliary excretory capacity was evaluated by employing several different doses of norethandrolone and different durations of treatment. A summary of the parameters tested is shown in Table 5. None of the various treatments with norethandrolone produced a significant depression of bilirubin Tm in either mice or rats. The Tm was either unaffected or was greater than that in the controls.

TABLE 5. SUMMARY OF DOSES OF NORETHANDROLONE EMPLOYED IN THE STUDIES ON BILIRUBIN TM

Species*	Dose† (mg/kg)		Treatment‡	No. of animals (per group)	Tm§ (% of control)
Mouse	20	p.o.	12–24 hr	8	140
	20	p.o.	3 days	5	115
	200	p.o.	3 days	6	132
	200	p.o.	7 days	2	127
	400	i.p.	1 day	4	122
	1200	i.p.	1 day	4	134
Rat	200	p.o.	1 day	3	108
	200	p.o.	3 days	4	109
	300	i.p.	3 days	7	130
	400	i.p.	2–3 hr	3	126

* All of the rats were Simonsen rats.

† The dose of norethandrolone (mg/kg) was given either orally (p.o.) or intraperitoneally (i.p.) prior to analysis of the bilirubin Tm.

‡ Norethandrolone was administered once daily for the number of days indicated. Bilirubin Tm test was determined 24 hr after the last dose. The 12 to 24-hr treatment indicates two doses given 24 and 12 hr prior to testing; the 2 to 3-hr treatment indicates one dose given 2–3 hr prior to testing.

§ The Tm values for the norethandrolone treatment group are given as a per cent of control. Under these experimental conditions, norethandrolone consistently increased or had no effect on bilirubin Tm.

Since the concentration of bilirubin in our infusate differs from that described by Arias *et al.*,[16] two additional experiments employing Wistar rats were performed by the latter method (bilirubin concentration, 1 mg/ml; infusion rate, 0·39 ml/min). In one experiment normal body temperature was maintained at 37°; in the second, no effort was made to maintain normal body temperature. The results are summarized in

Table 6. It can be seen that under these conditions norethandrolone depressed the apparent bilirubin Tm only in that experiment in which normal body temperature was not maintained. On the other hand, when the rectal temperature was maintained at 37°, norethandrolone increased the Tm.

TABLE 6. EFFECT OF TEMPERATURE ON THE BILIRUBIN TM RESPONSE TO NORETHANDROLONE*

Groups†	Final rectal temp. (°)	Bilirubin Tm (μg/100 g/min)	Bile flow (μl/100 g/min)	Bilirubin concn (μg/μl)
Experiment I				
Control	33·8 ± 0·2	96 ± 4	8·4 ± 1·0	11·9 ± 1·7
Norethandrolone	31·7 ± 0·2‡	65 ± 9‡	6·5 ± 0·3	10·0 ± 1·1
Experiment II				
Control	36·5§	104 ± 11	10·6 ± 1·1	9·8 ± 0·1
Norethandrolone	36·5	150 ± 38	11·9 ± 2·2	12·3 ± 1·0

* In contrast to Tables 4 and 5, the infusion conditions employed in these experiments were those described by Arias *et al.*[16]

† Norethandrolone (300 mg/kg, i.p.) was given once daily for 3–4 days. Bile bilirubin and bile volume determinations were done on bile samples collected during the interval 30–45 min after the start of bilirubin infusion. A priming dose of bilirubin (20 mg/kg) preceded the continuous infusion of 0·4 mg/min. Values shown are mean values ± S.E. from groups of 3 Wistar rats (150–200 g).

‡ Significantly different from respective control by *t*-test ($P < 0.05$).

§ Rectal temperature maintained between 36 and 37°.

Since norethandrolone had been given both orally and intraperitoneally, a comparison was made of its relative toxicity by these two routes of administration. In mice and rats the 24-hr LD_{50} values were about 0·5 g/kg, i.p., and 3·0 g/kg, p.o. Such a difference in lethality suggests that the route of administration of norethandrolone may be an important factor in determining the response which it elicits.

DISCUSSION

The results obtained in these studies demonstrate that norethandrolone does not impair the removal of exogenous bilirubin from the plasma of mice. There was, in fact, some suggestion that norethandrolone might enhance the plasma disappearance of bilirubin in normal mice. The early accumulation of conjugated bilirubin in mice with ligated bile ducts suggests an enhanced uptake or conjugation (or both) of the exogenously administered bilirubin. Norethandrolone has been shown to stimulate hepatic microsomal metabolism[9, 17] as well as to enhance BSP-glutathione conjugation *in vitro*.[18] However, the results obtained could also be accounted for by diminished hepatic storage of bilirubin and consequent regurgitation into the plasma.

Attempts to elicit a decrease in the apparent biliary excretory capacity for bilirubin in either mice or rats were unsuccessful even after employing a wide variety of norethandrolone dosages. In fact, in both rats and mice an actual increase in Tm was observed. The apparent conflict between these findings and the previously cited reports, where a decrease in bilirubin and BSP hepatic excretory capacity were found after norethandrolone treatment, could be due to a number of reasons. Species differences in the disposition of the norethandrolone molecule certainly could play a role. Such a possibility has been proposed by Gallagher *et al.*[7] to explain their

experimental results with various steroids. Species variation, however, does not explain the qualitative difference between our results and those of Arias, in which a decrease in biliary excretion capacity for bilirubin was found in the Wistar rat.[3]

A difference in the steroid material used or an unrecognized difference in experimental animals and technique may be involved. Three different samples of norethandrolone, obtained over a 2-year interval, were used in our studies. Infrared spectra were obtained for the last two samples and were found to be superimposable. The same biological results were obtained with all three samples. Also, two species of rats were employed in our studies, and the same qualitative response was obtained with each.

With respect to differences in experimental techniques, Arias' report[3] does not describe in detail the experimental procedure employed, but he states (personal communication) that the procedure was as described by Arias *et al.*[16] There is a difference between his method and ours. The concentration of bilirubin in the infusion solution is higher in our system. Therefore, while the amount of bilirubin infused is the same, the volume of infusate is different; Arias employs about 15 ml, whereas we employ about 4 ml/rat/hr. Otherwise, the two infusion systems seem similar since the pH of both infusates is pH 10; the pH of the bile is about 7·8 with both infusion systems; and the free bilirubin component in the bile during maximal excretion is 10–15 per cent with both systems. Our experimental system did detect the effect of methyltestosterone in the present study. It has also previously detected the depression in bilirubin Tm induced by α-naphthylisothiocyanate,[13] and has detected the increase in bilirubin Tm induced by phenobarbital.[19]

A factor which might adequately explain the differing responses to norethandrolone is body temperature. In our experimental system we find that body temperature has a marked influence on bilirubin excretion and that the hypothermia induced by pentobarbital anesthesia lowers the apparent biliary Tm values for both bilirubin and BSP.[14] A drop of 4° in rectal temperature can produce a drop in Tm of 15 μg/100 g/min. As seen in Table 6, we were able to show that body temperature made a marked difference in the effect of norethandrolone on the apparent bilirubin Tm, even when this parameter was measured with Arias' system.[16] When body temperature was allowed to drop, there was a 32 per cent *decrease* in the Tm of the norethandrolone-treated rats. However, when normal body temperature was maintained, there was a 44 per cent *increase* in the Tm. Therefore, in the hypothermic norethandrolone-treated animals we have values which are consistent with the observations of Arias[3] and Hargreaves.[4] Whereas in normothermic rats with Arias' infusion system, we have values consistent with those obtained with our own infusion system (Tables 4 and 5). It is also interesting to note that the drop in rectal temperature was greater in the norethandrolone-treated rats, thus suggesting that the apparent decrease in Tm might be due to the greater degree of hypothermia in these animals.

At this time, the reason why the effect of norethandrolone on hepatic function is apparently temperature-dependent remains to be resolved. Certainly the various factors involved warrant re-examination since they might bear on the significance of norethandrolone-impaired excretory capacity.

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