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EFFECTS OF AN ANABOLIC STEROID (NORBOLETHONE) ON THE FUNCTION OF THE ISOLATED PERFUSED RAT LIVER*

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Abstract—The effect of the anabolic steroid, Norbolethone, on the function of the isolated, perfused rat liver was evaluated by monitoring the disappearance of sulfobromophthalein and indocyanine green from the perfusate, the excretion of these dyes in the bile and the rate of bile flow. The drug was dissolved in ethanol and administered in a concentration of 20, 66 and 100 μmoles/l. The results indicate that Norbolethone impaired the ability to excrete the foreign dyes and to secrete bile. Analysis of the data suggested that the adverse effect was at the excretory level. The isolated, perfused rat liver appears a useful model for the evaluation of the effect of alkylated anabolic steroids on hepatic functions after a brief exposure of the liver to the drug.

THE ABILITY of C-17 alkyl-substituted anabolic steroids to induce hepatic dysfunction and cholestasis is well established.[1–4] The adverse effect is dose-related and may be demonstrated by morphological and biochemical studies, sulfobromophthalein (BSP) clearance and BSP bile excretion. This functional hepatic abnormality is presumed to be "canalicular" with difficulties in transport from the liver cell into the bile, although the exact site of the "cholestatic block" is unknown.

The adverse hepatic effects of the alkylated anabolic agents have been extensively studied in humans, but there have been few reports of this phenomenon in experimental animals. Impaired excretion of BSP has been observed in rats[5] and rabbits.[6] Treatment of rats with norethandrolone leads to a significant reduction of bilirubin transport.[7] Ultrastructural studies of the liver in rats and sheep that received these steroids have revealed dilated canaliculi and blunted microvilli.[5,7] Changes were also seen in the cellular membranes and in the acid phosphatase-rich lysosomes.[7] In all of these animal studies *in vivo*, the adverse hepatic effects of various anabolic steroids have been demonstrated after prolonged oral administration or intramuscular injections.

The present study was undertaken to investigate the suitability of the perfused rat liver *ex-vivo* for the study of the immediate effects of an anabolic steroid on hepatic function. Norbolethone (*dl*-13β, 17α-diethyl-17-hydroxygen-4-en-3-one) was selected as the steroid to be studied. It is an anabolic steroid, free of androgenic activity, which has been shown to produce hepatic dysfunction in humans[8] and rabbits.[6]

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In the experiments to be described, the effects of Norbolethone on the isolated, perfused rat liver were investigated by monitoring the disappearance of BSP and indocyanine green (ICG) from the perfusate, the excretion of these dyes in the bile and the rate of bile flow.

MATERIALS AND METHODS

Female, Sprague–Dawley rats (275–325 g) were used as donors of livers. The animals were maintained under standard environmental and dietary conditions and were not fasted prior to the experiments.

The method used for perfusing the liver was a modification of that described by Penhos *et al.*[9] Cannulation of the common bile duct with PE 10 tube and the portal vein with PE 205 (Intramedic, Clay–Adams) was performed under pentobarbital sodium anesthesia (50 mg/kg of body weight). This was followed by hepatectomy, and the isolated liver was then placed in a perfusion chamber (Metaloglass, Boston). Transient anoxia of the liver during the excision was minimized by passing oxygenated, heparinized buffer through the portal cannula before transfer of the liver to the chamber. The liver wet weights ranged from 9·5 to 11·0 g.

The perfusion medium was wholly synthetic and consisted of Krebs–Henseleit buffer, pH 7·4.[10] It contained, per 100 ml of total volume, 2·5 g of bovine albumin (35% solution, Pentex Biochemicals), 240 mg glucose and 3000 USP units of heparin (Liquaemin, Organon).

The thermostatic chamber was maintained at a temperature of 38 $\pm$ 0·5° during the experiment. The flow of the circulating medium was measured through a calibrated bypass and kept at a rate of 50–60 ml/min. The hydrostatic pressure of the portal vein was maintained at 17 cm water. Oxygenation of the buffer was maintained by a constant flow of O_2:CO_2 (95:5 vol. %) and continual mixing was provided with a magnetic stirrer.

Norbolethone was supplied by Wyeth Laboratories. The drug was dissolved in ethanol (absolute, USP) and added to the perfusion medium in amounts sufficient to provide concentrations of 20, 66 and 100 μmoles per l.

Ethanol was maintained in a uniform concentration of 25 m-moles per l. The need not to exceed this concentration was based on previous studies.[11] This and the relatively limited solubility of the steroid precluded administration of higher doses of Norbolethone. In some perfusions, the medium contained ethanol (25 m-moles/l.) but no steroid, in order to ascertain the effect of the vehicle in the system.

The test dyes, sulfobromophthalein (BSP)* or indocyanine green (ICG)* were added 30 min after perfusion was started, and the perfusion continued for an additional 45 min. BSP was added in a concentration of 10 mg/100 ml and ICG in a concentration of 2 mg/100 ml of perfusate.

The rate of removal of BSP by the liver was measured by obtaining 11 samples of perfusate (0·2 ml) drawn at intervals for the determination of the concentration of dye. The change in concentration of ICG in the perfusion medium was continuously recorded, using an MD-40 densitometer adapted to an "earpiece" with a flow-thru cuvette in the circuit.†

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† The Waters Co., Rochester, Minn.

Bile was collected into 20-μl pipettes, and the intervals of collection were carefully timed. A total of 42 perfusions are reported here.

The BSP concentration in perfusate and in bile was determined by a colorimetric method and read at 575 nm on a Bausch Spectronic 20 colorimeter. Readings were taken after adequate dilution with normal saline. Each sample, prior to alkalinization, served as its blank.

The ICG concentration in bile was read at 805 nm on the Spectronic 20, after proper dilution with saline of samples (20 μl/5 ml) and adequate standards. The concentration of ICG in the perfusate was directly taken from the continuous monitoring and calculated in actual recorder O.D. units.

Analysis of variance was carried out according to Snedecor.[12]

RESULTS

BSP clearance

Norbolethone, at concentrations of 66 and 100 μmoles/l., led to a significant decrease in the rate of removal of BSP from the perfusate. This is demonstrated by comparison of the observed rates of disappearance of BSP in Norbolethone-treated (100 μM) preparations compared with the controls (Fig. 1). Based on the two terms exponential model of BSP uptake by the liver,[13] the BSP clearance was also calculated as partial clearances at 15 and 45 min after addition of the dye to the perfusate and was expressed as "clearance constants" (k_1 and k_2 respectively). The k_1 values of controls, ethanol and 20 μM Norbolethone-treated livers were 10–13 μg/ml/min, while the higher

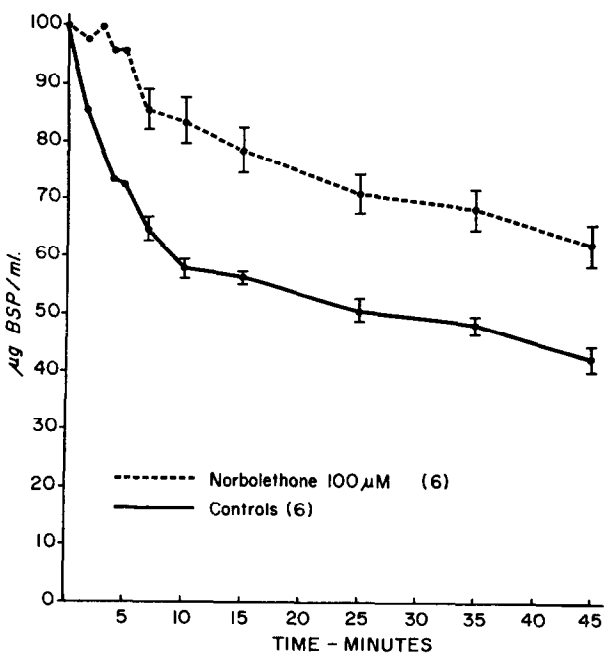

FIG. 1. BSP disappearance from the perfusate in control and Norbolethone-treated (μmoles/l.) preparations. Bars shown are S.E.M. at each point of the curves. Points of treated preparations were significantly ($P < 0{\cdot}01$) lower than controls.

doses of Norbolethone showed a significantly decreased k_1 value for the clearance (6–7 μg/ml/min). Forty-five min after BSP administration (k_2), a similar decrease in dye clearance was seen at the higher concentrations of Norbolethone. The values were 1–2 μg/ml/min compared with those of 4–6 μg/ml/min in the controls (Table 1).

TABLE 1. EFFECT OF NORBOLETHONE ON THE PARTIAL CLEARANCE* OF BSP FROM THE PERFUSATE

Treatment	Concn	N	k_1	k_2
Controls		6	13 ± 2	4 ± 0·6
Ethanol	25 mM	8	10 ± 0·1†	6 ± 0·9
Norbolethone	20 μM	6	10 ± 3	3 ± 0·9
Norbolethone	66 μM	6	6 ± 1†	2 ± 0·7†
Norbolethone	100 μM	6	7 ± 1†	1 ± 0·1†

* k_1 and k_2 were calculated as partial clearances at 15 and 45 min, respectively, by the formula $k = (\log c_a - \log c_b)/(t_b - t_a)$ where c is the perfusate concentration of BSP at intervals a and b (μg/ml) and t is the time (min). k_1 and k_2 are expressed in μg/ml/min ± S.E.M.

† Significantly different from control ($P < 0·01$).

Excretion of BSP in bile

The excretion rate of BSP in the bile was decreased in the Norbolethone-treated preparations (Table 2). The concentration of BSP in the bile of untreated livers increased during the 45 min of collection from 0·3 mg/ml at 5 min to 5·8 mg/ml at 35

TABLE 2. EFFECT OF NORBOLETHONE ON THE EXCRETION OF BSP IN BILE (mg/ml)

Treatment	Concn	N	Time after BSP addition (min)			
			5	15	25	35
Controls		6	0·3 ± 0·1	2·4 ± 0·2	4·3 ± 0·5	5·8 ± 0·7
Ethanol	25 mM	8	0·2 ± 0·05	2·0 ± 0·2	4·0 ± 0·3	5·4 ± 0·9
Norbolethone	20 μM	6	0·4 ± 0·1	2·8 ± 0·2	3·8 ± 0·2	4·0 ± 0·3*
Norbolethone	66 μM	6	0·2 ± 0·1	1·4 ± 0·3*	2·2 ± 0·2*	2·8 ± 0·3*
Norbolethone	100 μM	6	0·2 ± 0·05	1·9 ± 0·2	2·7 ± 0·3*	3·0 ± 0·2*

* S.E.M., significantly different from controls ($P < 0·01$).

min. Similar concentrations of BSP were found in the bile of ethanol-treated livers. Livers which were perfused with a 20 μM concentration of Norbolethone showed no difference in the excretion pattern of BSP; but at 35 min the dye concentration was 4·0 mg/ml, a value significantly different from that of controls ($P < 0·01$). The higher doses of Norbolethone, i.e. 66 and 100 μM, induced lower concentrations of BSP in bile. At 25 and at 35 min of collection, the dye concentrations were significantly different with respect to those of controls ($P < 0·01$).

Bile excretion

The rate of bile flow was decreased by the two larger doses of the steroid. In control livers it was 9 μl/min, and remained constant during the first 30 min of perfusion (Fig. 2). Perfusion with ethanol or the lowest dose of Norbolethone (20 μM) led to decreases

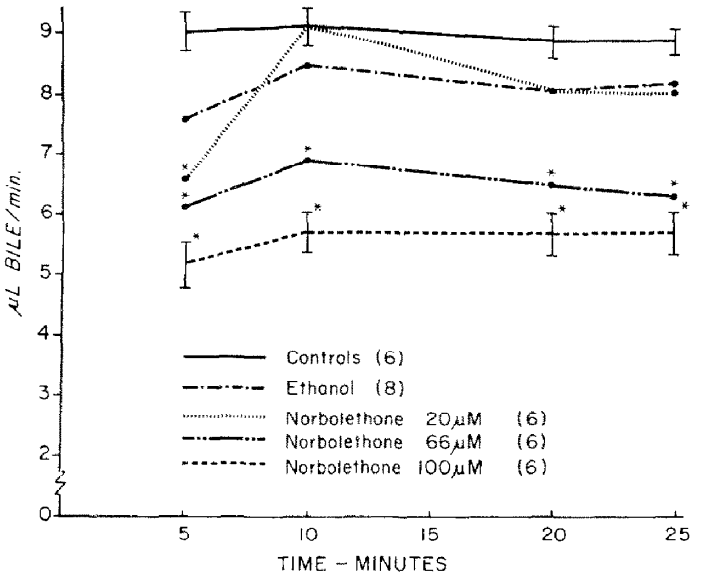

FIG. 2. Effect of Norbolethone on the rate of bile flow excreted by perfused livers (µl ± S.E.M.); number of experiments is given in parentheses. The asterisk indicates values significantly different from controls ($P < 0.01$).

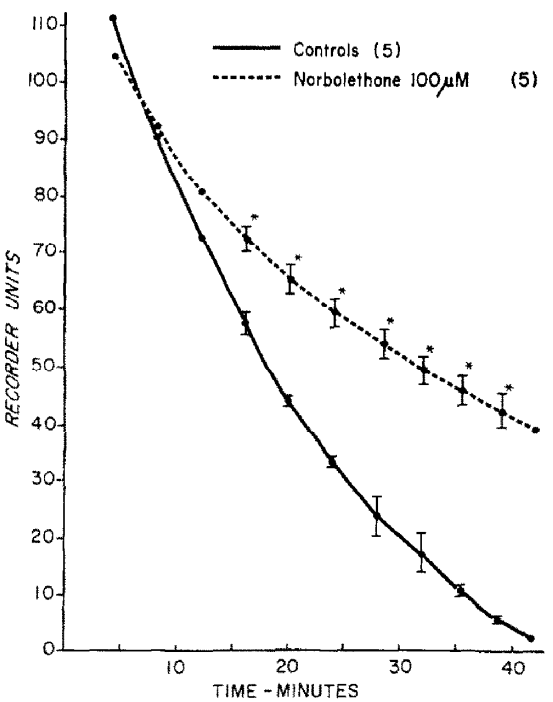

FIG. 3. Disappearance of ICG from the perfusate, expressed in recorder units; S.E.M. and statistical significance are shown. The asterisk indicates that $P < 0.01$.

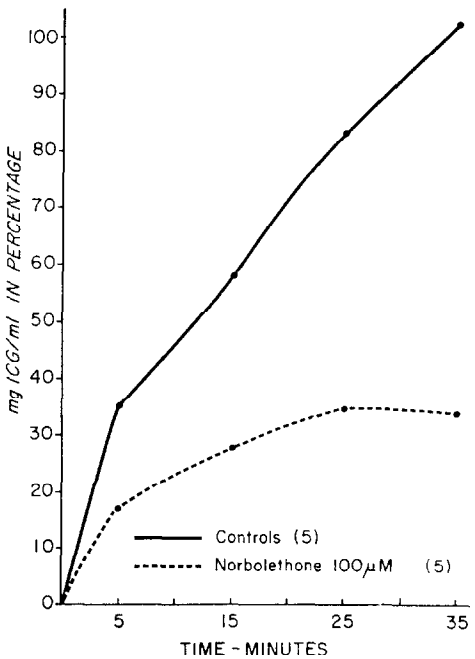

FIG. 4. Effect of Norbolethone on the excretion of ICG in the bile. Number of experiments is shown in parentheses.

in the rates of excretion (7·6–8·5 μl/min) that were not, however, statistically significant. The higher doses of Norbolethone decreased the rate of bile excretion significantly throughout the perfusion time as shown by the values of 5·2–6·9 μl bile/min.

ICG clearance and excretion in bile

The clearance of ICG from the perfusate (Fig. 3) was significantly decreased by perfusing with a 100 μM concentration of Norbolethone. The individual points on the disappearance curves became significantly ($P < 0{\cdot}01$) separated from those of the control after 15 min. Appearance of ICG in the bile was significantly lower in the 100 μM Norbolethone-treated livers than in controls (Fig. 4).

DISCUSSION

The results of the present study have demonstrated that Norbolethone interferes with removal of BSP and ICG from the perfusion medium and with the excretion of these dyes in the bile. It also decreases the rate of bile flow. These effects on hepatic function were, at least partially, dose dependent. A concentration of the steroid of 2×10^{-5} M (20 μM) produced little or no impairment of dye excretion, while concentrations of 6×10^{-5} M (66 μM) or of 10^{-4} M (100 μM) led to significant interference with clearance and excretion of the dyes and with depression of bile flow.

The demonstration that excretion of both BSP and ICG is impaired by Norbolethone suggests that defective conjugation is not responsible for the subnormal BSP excretion, since ICG does not require conjugation prior to excretion. These observations are

consistent with others that indicate the adverse effect of anabolic steroids to be at the excretory level and not to depend on inhibition of conjugation.[2,3,5,7] The decreased rate of excretion of dye in the bile induced by Norbolethone seemed greater than the decrease in rate of clearance from the perfusate. Furthermore, a concentration of Norbolethone (20 μmoles/l.) too low to decrease the observed rate of clearance led to a significant decrease in BSP excretion (Table 2).

The impaired excretion of foreign dyes and the decreased rate of bile secretion induced by this C-17 alkylated anabolic steroid in this model *in vitro* resembles the observations *in vivo* in humans and experimental animals.[6,8] These observations suggest that brief exposure of the liver to the steroid leads to changes in hepatic function similar to those produced by more prolonged treatment of the intact organism.

The perfusion medium used in this study was a synthetic salt solution with addition of bovine albumin and glucose. This medium was similar to that used by other workers.[14,15] The presence of red cells appears to be unnecessary for satisfactory oxygenation of a perfused organ *ex-vivo* where there is high flow rate and adequate oxygen mixing.[16] Livers perfused with such synthetic media appear to perform metabolic functions relatively normally.[17] In the present study, use of these synthetic media resulted in efficient clearance of foreign dyes and excretion of bile, similar to that of liver perfused with diluted rat blood.[11,18] Furthermore, the model permitted distinction of the effects of the drug on hepatic function.

The effects of Norbolethone on the perfused liver may be presumed to apply to other C-17 alkyl-substituted steroids, but this remains to be demonstrated. Indeed, the isolated perfused liver appears to be a useful experimental model for the study of the effects of other steroids, as it has been for the study of the effects of other drugs on the liver.[11,18,19] It may permit evaluation of the relationship between concentration of a drug and its adverse effects, not obscured by systemic and circulatory phenomena. Specifically, it would seem applicable to the study of the relationship between structural features of an anabolic agent and its effects on hepatic function and may be useful in studying the mechanisms by which drugs interfere with liver function.

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