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Short Communications

The Effect of 17α-Ethinyl-Substituted Steroids on Adenosine Triphosphatases of Rat Liver Plasma Membrane

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In a previous paper (Heikel & Lathe, 1970) it was shown that 17α-ethinyl-substituted oestrogens greatly decreased the bile flow of rats, whereas the parent compound oestradiol-17β had little effect. 19-Nortestosterone had no effect on bile flow and its 17α-ethinyl derivative was inhibitory, but only at much greater doses than oestrogens.

One possible explanation for the effect on bile flow was that 17α-ethinyl-substituted steroids acted by decreasing cation transport. We have now examined the effect of the addition *in vitro* of steroids, and some other substances decreasing bile flow, on the ATPases* of liver plasma membranes, as $Na^+ + K^+$-stimulated ATPase has been implicated in ion transport.

The preparation of liver plasma membranes from rats was modified from the procedure of Emmelot, Bos, Benedetti & Rümke (1964). After homogenization of liver in bicarbonate medium (approx. 1 mM, pH 7.5), fractionation and ultracentrifugation (77000$g_{av.}$ for 60 min) the membranes were suspended in bicarbonate medium, and a 5 ml portion was layered on top of a discontinuous gradient of 5 ml each of sucrose of specific gravity 1.016, 1.018 and 1.020. After a second ultracentrifugation (77000$g_{av.}$ for 60 min) the membranes were taken from the interface between the zones of specific gravity 1.016 and 1.018. The membranes were spun down (3000g) after dilution in about 4 vol. of water (4°C) and suspended in ice-cold water.

Electron micrographs showed that the preparation was rich in plasma membranes, with many tight junctions and desmosomes. Vesicular components were present, but the preparation was free from mitochondria, rough-endoplasmic reticulum, lysosomes and nuclei.

ATPase activities were determined at 37°C in the medium of Quigley & Gotterer (1969). Two sets of tubes (final vol. 1.5 ml) were set up in duplicate. One contained tris ATP (5 mmol/l), Na^+ (120 mmol/l), K^+ (20 mmol/l), Mg^{2+} (7.5 mmol/l) and tris (30 mmol/l) buffer (adjusted with HCl to pH 7.1 at 37°C). In the second set Na^+ and K^+ were omitted, for assay of Mg^{2+}-stimulated ATPase.

* Abbreviation: ATPase, adenosine triphosphatase.

Substances to be tested were added in 0.01 ml of ethanol and the controls contained an appropriate amount of ethanol. After the reaction had been started by addition of plasma-membrane suspensions, tubes were incubated for 15 min, after which they were plunged into ice, and ice-cold trichloroacetic acid was added to 8% (w/v). After centrifugation (3000g for 10 min at 4°C) the supernatant was analysed for liberated P_i (Fiske & Subbarow, 1925). Allowance was made for P_i present in membranes and that derived from non-enzymic hydrolysis of ATP. The protein in a sample of membranes was determined (Lowry, Rosebrough, Farr & Randall, 1951) with the stable copper solution of Hall & Cooking (1965), with crystalline bovine serum albumin as a standard. The amounts of protein are given in Table 1.

The 17α-substituted steroids that had inhibited bile flow were compared with the parent compounds, oestradiol-17β and 19-nortestosterone, for their effect on ATPases. We also studied some substances that have been shown to decrease bile flow, especially stilboestrol (T. A. J. Heikel, unpublished work), 17α-ethyl-19-nortestosterone (Heikel, 1967) and icterogenin (Heikel, Knight, Rimington, Ritchie & Williams, 1960). Table 1 gives the result of three typical experiments.

The introduction of a 17α-ethinyl group into oestradiol-17β was associated with a remarkable decrease in ΔP_i (the increment in ATPase activity on adding $Na^+ + K^+$). The effect was also reflected in a lowered activity ratio, i.e. the percentage decrease of the activity of the enzyme dependent on $Na^+ + K^+ + Mg^{2+}$ was higher than the percentage decrease with Mg^{2+}. The decrease in ΔP_i and activity ratio was reversed by blocking the 3-hydroxyl group, as shown with the 3-methyl ether of 17α-ethinyloestradiol-17-β.

Stilboestrol abolished the ΔP_i and greatly lowered the Mg^{2+}-stimulated ATPase activity. The activity ratio was the lowest found.

Taken as a group the nortestosterone series showed a different behaviour. All of them had some inhibitory effect on the Mg^{2+}-stimulated activity. The inhibition of ΔP_i, on the other hand,

Table. 1. *Effect of steroids, stilboestrol and icterogenin on the ATPase activity of plasma membrane from rat liver*

The compounds tested were added at a concentration of 0.1 mmol/l to three membrane preparations (containing 0.083, 0.087 and 0.104 mg of protein in the final volume of 1.5 ml), except for icterogenin, which was at 0.05 mmol/l. Each value is the mean of duplicate determinations on membranes from each of the three preparations. The range of the three mean values is given in parenthesis.

Addition	ATPase activity (μmol of P_i formed/h per mg of protein)		ΔP_i (μmol/h per mg of protein	Activity ratio
	$Na^+ + K^+ + Mg^{2+}$ (*A*)	Mg^{2+} (*B*)	(*A* − *B*)	(*A*/*B*)
None	75.3 (74.2–76.4)	63.9 (60.7–66.0)	11.4 (9.3–14.6)	1.18
Ethanol	70.7 (70.0–71.2)	62.1 (59.8–63.5)	8.6 (7.3–10.2)	1.14
Oestradiol-17β	62.6 (58.0–67.2)	55.7 (45.8–62.6)	6.9 (3.7–12.2)	1.12
17α-Ethinyloestradiol-17β	50.1 (43.8–54.7)	48.2 (38.0–53.8)	1.9 (0–5.8)	1.04
17α-Ethinyloestradiol-17β 3-*O*-methyl ether	64.8 (51.4–73.4)	55.0 (41.7–63.4)	9.8 (9.7–10.0)	1.18
Stilboestrol	20.7 (20.0–21.6)	22.4 (20.9–24.9)	0 (0–0.2)	0.92
Icterogenin	14.1 (13.4–15.1)	5.6 (3.1–9.9)	8.5 (3.8–11.2)	2.52
19-Nortestosterone	64.9 (59.8–71.4)	53.3 (52.0–55.1)	11.6 (7.8–16.3)	1.22
17α-Ethinyl-19-nortestosterone	61.9 (56.8–67.3)	51.7 (46.9–55.1)	10.2 (6.4–14.3)	1.20
17α-Ethinyl-19-nortestosterone 17β-acetate	48.3 (47.3–48.9)	48.3 (45.3–52.0)	0 (0–3.2)	1.00
17α-Ethyl-19-nortestosterone	52.1 (47.6–55.1)	42.4 (41.0–43.2)	9.7 (4.4–12.7)	1.23
Lynoestrenol	46.4 (44.6–49.7)	34.9 (32.4–36.7)	11.5 (9.3–13.0)	1.33

was marked only in one steroid, in which the 17β-hydroxyl group was acetylated and the 17α-position ethinylated.

Icterogenin (which was tested at half the concentration of other substances) showed no effect on ΔP_i, and the activity ratio was remarkably increased, indicating a very strong effect on the Mg^{2+}-stimulated ATPase. This may be due to its detergent-like action (Heikel, 1968).

With the limited number of steroids tested it is not possible to specify the structural requirements for an inhibition of $Na^+ + K^+$-stimulated activity. However, the following may be considered as a working hypothesis. High activity depends on a hydrophobic group at the 17-position and a free hydroxyl group situated at the 3-position. The increased activity, as a result of introducing a hydrophobic group at the 17-position, may depend on the interaction between enzyme and steroid occurring in a hydrophobic region of the plasma membrane. The loss of activity when the 3-hydroxyl group is blocked (by methyl) or altered to a ketone (as in the nortestosterone series) suggests that hydrogen-bonding may be involved in the steroid–enzyme interaction.

The results do not show a simple parallel between inhibition of bile flow *in vivo* and capacity to inhibit $Na^+ + K^+$-stimulated ATPase *in vitro*, but some of the exceptions may be explained. An oestrogen in which the 3-hydroxyl group was methylated was inactive *in vitro*. The activity *in vivo* may result from a demethylated product. The progestogens that were effective *in vivo* had to be given in much higher doses than oestrogens, and some of them, as well as 17α-ethyl-19-nortestosterone, have been shown to be converted into oestrogens *in vivo* (Brown & Blair, 1960; Kamyab, Fotherby & Klopper, 1968; Krüskemper, 1963). Clearly the most potent inhibitors (icterogenin excepted) have a high capacity to lower ΔP_i.

The inhibition of ATPases, especially the $Na^+ + K^+$-stimulated ATPase, by steroids that decreased bile secretion suggests that cation transport is part of the mechanism of bile production. This has

recently been emphasized by studies in which ouabain, an inhibitor of $Na^+ + K^+$-stimulated ATPase, decreased bile flow *in vivo* (Erlinger, Dumont & Benhamou, 1969).

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