Studies with Glycyrrhetic Acid: Inhibition of Metabolism of Steroids *in vitro*

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Glycyrrhizinic acid is the diglucopyranosiduronic acid of glycyrrhetic acid (Lythgoe & Trippett, 1950) which has a triterpenoid structure (Ruzicka, Jeger & Ingold, 1943) as shown in (I) (Beaton & Spring, 1955). As far as the junctions of rings A, B, C and D are concerned the stereochemistry is the same as in *allopregnane*.

Molhuysen *et al.* (1950) showed that liquorice and glycyrrhizinic acid had a deoxycorticosterone-like action in that they promoted retention of sodium and water and loss of potassium, in normal humans, but in one severe case of Addison's disease did not have these effects. On the contrary, Groen, Pelsler, Willebrands & Kamminga (1951) described two Addisonian patients who could be maintained in correct electrolyte balance with liquorice or glycyrrhizinic acid alone.

Pelsler, Willebrands, Frenkel, van der Heide & Groen (1953) showed that glycyrrhetic acid, when administered orally to Addisonian patients, was more effective than glycyrrhizinic acid. Pelsler, Willebrands & Groen (1953) found that during prolonged treatment with liquorice or glycyrrhetic acid, if the maintenance dose were not reduced after some time oedema set in. Borst, ten Holt, Molhuysen & Vries (1953) suggested that in those patients who did not respond to treatment with liquorice derivatives there was a complete lack of any remnants of functioning adrenal tissue.

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Groen, Pelsler, Willebrands, Veer & Van der Kerk (1953) investigated the effect of derivatives and analogues of glycyrrhetic acid on the electrolyte balance of Addisonian patients. They concluded that the keto group, or the carboxyl group (either as such, or as a ketol-acetate group), or both, were essential for activity.

Since glycyrrhetic acid contains an $\alpha\beta$ -unsaturated ketonic group it was thought that the effect of glycyrrhetic acid might be due to its inhibition of metabolism of adrenocortical steroid *in vivo*. It was decided therefore to investigate the action of glycyrrhetic acid on the metabolism *in vitro* of 11-deoxycorticosterone and progesterone, since these are steroids having $\alpha\beta$ -unsaturated ketonic groups known to undergo enzymic transformation *in vitro*.

EXPERIMENTAL

Materials. Glycyrrhetic acid (Stafford Allen and Sons Ltd.) had m.p. 278–282° (all melting points are uncorrected); progesterone (Organon Laboratories Ltd.), recrystallized from ethanol as prisms (α -progesterone), had m.p. 127.5–128°; 11-deoxycorticosterone was prepared from 11-deoxycorticosterone acetate (Organon Laboratories Ltd.) and had m.p. 138–142°; 11-deoxoglycyrrhetic acid had m.p. 320–323°. Methyl vinyl ketone (L. Light and Co. Ltd., Colnbrook, Bucks) was not purified further; *p*-benzoquinone was purified by sublimation and had m.p. 113–114°; acetophenone had b.p. 198–199°.

Methods. Female rats (Wistar strain) were killed by dislocation of neck vertebrae. The livers were removed and dropped into the ice-cold medium which, except where otherwise stated, was 0.25M-sucrose containing 0.12M-nicotinamide (Roche Products Ltd.). Homogenates (10%) were prepared in the same solution by grinding first in an all-glass homogenizer (Potter & Elvehjem, 1936) and subsequently in a similar apparatus with a Nylon pestle (Brownie & Grant, 1954). For some experiments, the homogenate, prepared in 0.25M-sucrose, was centrifuged for 1 hr. in an MSE refrigerated centrifuge at 0°, the super-speed unit with four-place angle head being used, at 25 000 *g* ('25 000 *g* supernatant'). The incubation medium, unless otherwise stated, contained 0.05M-KCl; 0.005M-MgSO₄; 0.002M-sodium citrate (pH 7.4) and 0.01M-potassium phosphate (pH 7.4). Where required, nicotinamide was added to incubation vessels to give a final concentration of 0.04M. Where pyridine nucleotides were added, they were dissolved in phosphate buffer and reduced with Na₂S₂O₄ (Hogeboom & Schneider, 1950). Steroids and glycyrrhetic acid were added as solutions in

propane-1:2-diol and this solvent was added to all vessels in such amounts that the final volume was the same in all cases. 11-Deoxycorticosterone is only sparingly soluble in propane-1:2-diol and was added as a solution in 0.1 ml. of ethanol. A volume (1 ml.) of enzyme preparation was added and the total volume in each vessel was 3 ml. Progesterone was determined by the method of Taylor (1954) and 11-deoxycorticosterone was determined by the method of Brownie & Grant (1954). Every incubation experiment included 'recovery controls' and 'blanks' as described by Taylor (1954).

RESULTS

Recovery of steroids in the presence of glycyrrhetic acid. It was necessary to ascertain whether or not glycyrrhetic acid was carried through the analytical procedure for steroids and appeared in the same final fraction as progesterone or 11-deoxycorticosterone, particularly since a solution of glycyrrhetic acid in ethanol gives a peak at 248 m μ (van Katwijk & Huis in't Veld, 1955). Duplicate 'blank' incubations, duplicate progesterone 'control' incubations and single 11-deoxycorticosterone 'control' incubations were carried out in the absence and in the presence of 0.001M-glycyrrhetic acid, a 10% homogenate being used. Table 1 shows that blank values and

Table 1. *Recovery of steroids added to reaction mixtures which had been incubated with glycyrrhetic acid and rat-liver homogenate*

A volume (1 ml.) of a 10% homogenate prepared in 0.25M-sucrose containing 0.12M-nicotinamide was incubated in air for 1 hr. with a medium containing 0.05M-KCl; 0.005M-MgSO₄; 0.002M-sodium citrate (pH 7.4) and 0.01M-potassium phosphate (pH 7.4) in a final volume of 3 ml. Recovery values have been corrected by 'apparent' steroid recovered in 'blank' experiments.

Progesterone added (μ g.)	0.001M-Glycyrrhetic acid	Apparent progesterone recovered (μ g.)
0 ('blank')	-	15 23
0 ('blank')	+	20 23
Progesterone recovered		
		(μ g.) (%)
395	-	364 92 368 93
395	+	368 93 384 97
11-Deoxycorticosterone added		
(μ g.)		(μ g.) (%)
610	-	573 94
610	+	591 97

Table 2. *Inhibition by glycyrrhetic acid of the metabolism of progesterone and 11-deoxycorticosterone by rat-liver homogenate*

Experimental conditions were as described for Table 1. Steroid (500 μ g.) was added in 0.05 ml. of propane-1:2-diol. 'Controls' were carried out by adding steroids after incubation. Values have been corrected by subtraction of 'apparent' steroid recovered in 'blank' experiments in which no steroids were added.

Concn. of glycyrrhetic acid (M)	0.001M-DPNH	Progesterone recovered (%)	11-Deoxycorticosterone recovered (%)
0	-	64 67	.
0	+	11 14	17 19
0.0002	-	84 87	.
0.0002	+	58 59	59 63
0.0005	-	97 98	.
0.0005	+	79 84	75 76
0.0010	-	99 99	.
0.0010	+	87 89	.
'Control'	-	100	.
'Controls'	+	98 100	94 97

recoveries of progesterone and 11-deoxycorticosterone are not significantly affected by the presence of 0.001M-glycyrrhetic acid.

Inhibition by glycyrrhetic acid of steroid metabolism by rat-liver homogenate. Schneider (1952) showed that rat-liver slices metabolize 11-deoxycorticosterone, and Wiswell & Samuels (1953) showed that progesterone is metabolized by rat-liver homogenate. Table 2 shows that glycyrrhetic acid is a powerful inhibitor of the metabolism of both 11-deoxycorticosterone and progesterone by rat-liver homogenate, both in the absence and in the presence of added reduced diphosphopyridine nucleotide (DPNH).

Inhibition by glycyrrhetic acid of progesterone metabolism by rat-liver supernatant. Tomkins & Isselbacher (1954) found that cortisone reduction was mediated by a particle-free supernatant of rat-liver homogenate in the presence of reduced triphosphopyridine nucleotide (TPNH). Glycyrrhetic acid could inhibit progesterone metabolism either indirectly by preventing the regeneration of TPNH or by inhibiting directly the reduction of progesterone. To investigate this, a 25 000 g supernatant was prepared; this still contains some

microsomes, but a higher centrifugal force was not available. This supernatant was dialysed against 1.15% (w/v) KCl solution for 4 hr. at 0°, fresh KCl solution being provided after 2 hr. This procedure removes citrate, *cis*-aconitate and *isocitrate* almost completely (Atherden, 1956). Four different progesterone concentrations were used, and recovery controls were included for each concentration. Table 3 shows that progesterone metabolism by the 25 000 g supernatant in the presence of TPNH and in the absence of *isocitrate* is inhibited by 0.0001 M-glycyrrhetic acid.

Inhibition by glycyrrhetic acid and other substances of progesterone metabolism by rat-liver homogenate. The fact that glycyrrhetic acid contains an $\alpha\beta$ -unsaturated ketone group, in common with progesterone and 11-deoxycorticosterone, was suggestive that this might be the active moiety of the inhibitor. To investigate this possibility, the effect on progesterone metabolism of various substances was tried. From Fig. 1 it may be seen that progesterone recovery is unaffected by the presence of 0.0005 M-11-deoxoglycyrrhetic acid (b), methyl vinyl ketone (c) or *p*-benzoquinone (d). It may also be seen that glycyrrhetic acid (j), methyl vinyl ketone (k) and *p*-benzoquinone (l) effect complete inhibition of progesterone metabolism, whereas 11-deoxoglycyrrhetic acid (i), acetophenone (h), ethanol (g) and acetone (f) cause much less inhibition.

DISCUSSION

Glycyrrhetic acid has been found to inhibit the destruction of both progesterone and 11-deoxycorticosterone by rat-liver homogenate (Table 2).

Table 3. *Inhibition by glycyrrhetic acid of the metabolism of progesterone by a dialysed rat-liver supernatant*

A 25 000 g supernatant was prepared from a rat-liver homogenate and dialysed against 1.15% (w/v) KCl soln. for 4 hr., the KCl soln. being changed after 2 hr. A volume (1 ml.) of the supernatant was used with an incubation medium which contained 0.05 M-KCl, 0.005 M-MgSO₄, 0.01 M-potassium phosphate (pH 7.4), 0.04 M-nicotinamide and 0.0003 M-TPNH.

Concn. of progesterone (M)	Recovery of progesterone (%)	
	No glycyrrhetic acid	0.0001 M-Glycyrrhetic acid
0.0002	54	93
'Control' 0.0002	94	—
0.0003	70	98
'Control' 0.0003	97	—
0.0004	84	96
'Control' 0.0004	101	—
0.0005	86	101
'Control' 0.0005	94	—

This may mean that the reduction of ring A of $\alpha\beta$ -unsaturated ketonic steroids is inhibited in general by glycyrrhetic acid.

To test whether glycyrrhetic acid inhibited directly progesterone reduction, a 25 000 g supernatant was dialysed (to remove citrate, *cis*-aconitate and *isocitrate*) and TPNH was added; thus the only reaction that should have occurred, as far as progesterone was concerned, was the reduction of progesterone by enzyme and pre-formed TPNH. Glycyrrhetic acid was added to this system and was found to cause complete inhibition (Table 3). Thus it may be concluded that inhibition by glycyrrhetic acid is a direct effect on the steroid enzyme system. It will be seen that to effect complete inhibition of progesterone metabolism a concentration of glycyrrhetic acid of at least 0.0005 M was required with homogenate (Table 2), whereas with supernatant (Table 3) 0.0001 M-glycyrrhetic acid was sufficient. This is

Fig. 1. Inhibition by glycyrrhetic acid and other substances of progesterone metabolism by rat-liver homogenate incubated in air for 1 hr. at 37°. The reaction mixture contained 0.05 M-KCl, 0.005 M-MgSO₄, 0.002 M-sodium citrate (pH 7.4), 0.01 M-potassium phosphate (pH 7.4) and the substances shown, in a final volume of 3 ml. Ethanolic solutions (0.1 ml.) of 11-deoxoglycyrrhetic acid, methyl vinyl ketone, *p*-benzoquinone and acetophenone were used; final concentrations of these substances and of glycyrrhetic acid and acetone were 0.0005 M. About 500 μ g. of progesterone was added in 0.05 ml. of propane-1:2-diol. Double lines at the ends of bars indicate duplicate values.

possibly due to glycyrrhetic acid itself being metabolized to a greater extent with the homogenate than with the supernatant.

The fact that 11-deoxoglycyrrhetic acid inhibits to a small extent only (Fig. 1) demonstrates that the 11-oxo group is required for inhibition. Glycyrrhetic acid, administered to Addisonian patients maintained on 11-deoxycorticosterone, was found to have a 11-deoxycorticosterone-sparing effect in that it assisted in the maintenance of a positive sodium balance (Groen *et al.* 1951); Groen, Willebrands & Pelser (1956) found that analogues of glycyrrhetic acid without the 11-oxo group, when administered to patients, did not maintain a positive sodium balance characteristic of glycyrrhetic acid. This agreement between *in vivo* and *in vitro* results supports the postulate that glycyrrhetic acid acts *in vivo* by inhibiting steroid reduction.

The requirement for a keto group and the fact that methyl vinyl ketone and *p*-benzoquinone, which both contain $\alpha\beta$ -unsaturated ketonic groups, inhibited progesterone reduction (Fig. 1), though not proof, is suggestive that the $\alpha\beta$ -unsaturated ketonic group of glycyrrhetic acid is the active part of the molecule. Ketones such as acetophenone and acetone inhibited to a small extent only (Fig. 1).

SUMMARY

1. Glycyrrhetic acid has been found to inhibit the metabolism of progesterone and 11-deoxycorticosterone by rat-liver homogenate.

2. Methyl vinyl ketone and *p*-benzoquinone have also been found to inhibit the metabolism of progesterone by rat-liver homogenate.

3. Glycyrrhetic acid inhibited progesterone metabolism by reduced triphosphopyridine nucleotide with a dialysed supernatant, free from citrate, *cis*-aconitate or *iso*-citrate.

4. These results, and clinical results with glycyrrhetic acid and its analogues, are discussed.

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