[1] R. C. WARNER, in H. NEURATH AND K. BAILEY, *The Proteins*, Vol. II, Part A, Academic Press Inc., New York, 1954, p. 435.
[2] D. K. MECHAM AND H. S. OLCOTT, *J. Am. Chem. Soc.*, 71 (1949) 3670.
[3] G. ALDERTON AND H. L. FEVOLD, *Arch. Biochem.*, 8 (1945) 415.
[4] H. SUGANO, *J. Biochem. (Tokyo)*, 45 (1958) 393.
[5] F. J. JOUBERT AND W. H. COOK, *Can. J. Biochem. and Physiol.*, 36 (1958) 389.
[6] R. K. MORTON, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. I, Academic Press, Inc., New York, 1955, p. 40.
[7] C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
[8] F. C. KOCH AND T. L. MCMEEKIN, *J. Am. Chem. Soc.*, 46 (1924) 2066.
[9] A. L. LEVY AND D. CHUNG, *Anal. Chem.*, 25 (1953) 396.
[10] C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.

Received July 20th, 1959

Biochim. Biophys. Acta, 38 (1960) 360–362

Differences between oxosteroids in their behaviour towards the Girard reagent

The formation of water-soluble derivatives of oxosteroids by treatment with Girard's reagent T is frequently utilized to effect the separation of these steroids from non-ketonic material in lipid extracts of urine[1,2] and tissues[3]. The free oxosteroids can be regenerated from such derivatives by acid hydrolysis. Recently, this procedure has been adapted to the purification of certain highly labile carbonyl compounds by the introduction of two modifications[4]: the use of a carboxylic resin instead of glacial acetic acid as catalyst for the condensation reaction, and treatment with formaldehyde in neutral solution, replacing acid hydrolysis, as a means of splitting the Girard complexes. It has been suggested[5] that the modified procedure may be applied with advantage to oxosteroids, since it eliminates the risks of causing acetylation of hydroxysteroids and partial destruction of labile compounds.

A number of steroids were subjected to the new method of separation in this laboratory. Crystalline steroids (20–200 μg) were dissolved in 1 ml ethanol containing 20 mg Girard reagent T and 5 mg of the resin Amberlite IRC-50 [H] (Rohm & Haas), and either heated to boiling under reflux for 1 h (Series I), or allowed to stand at room temperature for 12 h (Series II). After filtration and dilution with 10 ml water, the "non-ketonic fraction" was separated by extracting 3 times with an equal volume of ethyl acetate. 2 ml 36 % (w/v) formaldehyde solution were then added to the aqueous phase, which was allowed to stand for 7 h at room temperature before re-extracting with ethyl acetate to obtain the "ketonic fraction A". The aqueous residue was then acidified by the addition of 0.4 ml 10 *N* HCl or of 1.5 ml pyruvic acid, and 5–7 h later extracted with ethyl acetate to yield the "ketonic fraction B". The fractions thus obtained were washed to remove residual acid and formaldehyde and taken to dryness. The steroids were then separated by paper chromatography[6] and determined individually.

The experiments, results of which are summarized in Tables I and II, showed that the steroids examined differed markedly both in the rate of formation of a Girard derivative and in the ease with which these complexes could be split. The 3-oxo group of α,β-unsaturated 3-oxosteroids combined readily with the Girard reagent

Abbreviations: Girard's reagent T, trimethylaminoacetohydrazide hydrochloride; trivial names of steroids, as defined in Tables I and II.

TABLE I

BEHAVIOUR OF STEROIDS AFTER TREATMENT WITH GIRARD'S REAGENT AND RESIN IRC-50[H] IN BOILING ETHANOL (SERIES I)

Data give the range of values obtained for individual compounds in each group.

Compound	*Recovery (%)*		
	Non-ketonic fraction	*Ketonic fraction A*	*Ketonic fraction B*
Progesterone (pregn-4-ene-3,20-dione) Testosterone (17β-hydroxy-androst-4-ene-3-one)	< 1	88–94	0–5
Oestrone(3-hydroxyoestra-1,3,5(10)-trien-17-one) Androsterone (3α-hydroxy-5α-androstan-17-one)	< 2	8–13	79–86
Androst-4-ene-3,17-dione 11β-Hydroxyandrost-4-ene-3,17-dione	< 1	8–13	79–86

TABLE II

BEHAVIOUR OF STEROIDS AFTER TREATMENT WITH GIRARD'S REAGENT AND RESIN IRC-50(H) IN ETHANOL AT ROOM TEMPERATURE (SERIES II)

Compound	*Recovery (%)*		
	Non-ketonic fraction	*Ketonic fraction A*	*Ketonic fraction B*
Progesterone Testosterone Corticosterone (11β,21-dihydroxypregn-4-ene-3,20-dione Cortisone (17α-21-dihydroxypregn-4-ene-3,11,20-trione) Cortisol (11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione)	< 1	86–95	0–7
Oestrone Androsterone	17–25	6–12	61–70
Androst-4-ene-3,17-dione 11β-Hydroxyandrost-4-ene-3,17-dione	< 1	21–28	67–74
17α-Hydroxypregn-4-ene-3,20-dione	< 1	60–68	28–38
Tetrahydrocortisol (3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one)	90–95	None detected	0–2

at room temperature, and quantitative recovery of free progresterone and testosterone was obtained by treatment with excess of formaldehyde, in accordance with TAYLOR's observations[5]. On the other hand, in the case of 17-oxosteroids the recoveries were small: After treatment with Girard's reagent at high temperature, amounts of oestrone, androsterone, androst-4-ene-3,17-dione and 11β-hydroxyandrost-4-ene-3,17-dione released from the Girard complexes by formalin did not exceed 13 %; the remainder could only be recovered by acid hydrolysis. A larger proportion (21–28 %) of the Girard derivatives of the 3,17-diones was amenable to decomposition by formalin treatment if the condensation had been carried out at room temperature; this increase in the formalin-labile fraction probably reflects incomplete coupling of the 17-oxo group of these steroids with Girard's reagent under the milder conditions, as suggested by the presence of 17–25 % of androsterone and oestrone in the "non-ketonic fraction" after similar treatment.

The percentage of cortisone, cortisol or tetrahydrocortisol recovered after treatment with the Girard reagent at high temperature was small; of the total amount recovered, the larger portion was released only by acid hydrolysis. In contrast, the Girard complexes of cortisone or cortisol prepared by the milder procedure (Series II) were readily split by formalin. The difference must be due to inability of the dihydroxyacetone side-chain to combine with Girard's reagent at room temperature, since the bulk of tetrahydrocortisol was recovered from the "non-ketonic fraction" under these conditions. At the same time, the difference observed between the rates of hydrolysis of the Girard derivatives of progesterone and of 17α-hydroxypregn-4-ene-3,20-dione on addition of formalin (Table II) suggests that 21-deoxysteroids, unlike the preceding group, combine at room temperature with Girard's reagent at C-20, and that the complex so formed is more resistant to hydrolysis if a hydroxyl group is present in position 17.

It was noticed that the treatment of corticosterone, cortisol, and 11β-hydroxyandrostenedione with aq. formaldehyde gives rise to small amounts (2 %) of artefacts, appearing on Bush-type paper chromatograms[6] as u.v.-absorbing components more polar than the corresponding parent compounds. The derivatives resemble the respective parent compounds as regards the presence of an α-ketolic side-chain or of a 17-oxo function. The 11β-hydroxy group appears to be implicated in the production of these artefacts, since closely related compounds lacking such a group, *e.g.* cortisone and androstenedione, fail to produce them.

Early work[1,3] has demonstrated that the Girard complexes of $\alpha\beta$-unsaturated 3-oxosteroids are more resistant to acid hydrolysis than those formed by the saturated 3-oxo-5α steroids. The present study indicates that further differences exist between oxosteroids in their reactions with the Girard reagent, depending on the location of carbonyl groups and the presence of substituents in neighbouring positions; these differences largely determine which procedure is best suited for the purification of any particular class of steroids by means of this agent. While it can be confirmed that a number of oxosteroids, such as progesterone and testosterone, can be conveniently separated from non-ketonic material by use of the mild and selective method adopted by TAYLOR[5], our experiments show that it is not applicable to oxosteroids in general; in particular, it cannot be used for the purification of urinary 17-oxosteroids.

I am much indebted to the M.R.C. Steroid Reference Collection (Dr. W. KLYNE) for steroid reference substances and to Dr. T. MANN, F.R.S. for the interest taken in this study. The work was carried out during the tenure of a Studentship of the Commonwealth Scientific and Industrial Research Organisation of Australia.

Agricultural Research Council Unit for Reproductive Physiology and Biochemistry, Department of Veterinary Clinical Studies, University of Cambridge (Great Britain) H. R. LINDNER

[1] A. GIRARD AND G. SANDULESCU, *Helv. Chim. Acta*, 19 (1936) 1095.
[2] G. PINCUS AND W. H. PEARLMAN, *Endocrinology*, 29 (1941) 413.
[3] T. REICHSTEIN, *Helv. Chim. Acta*, 19 (1936) 1107.
[4] C. L. TEITELBAUM, *J. Org. Chem.*, 23 (1958) 646.
[5] W. TAYLOR, *Nature*, 182 (1958) 1735.
[6] L. E. BUSH, *Biochem. J.*, 50 (1952) 370.

Received July 25th, 1959