CHARACTERIZATION OF Δ^4-3-OXO-C_{21}-STEROIDS ON THIN-LAYER CHROMATOGRAMS BY "*IN SITU*" COLOUR REACTIONS

B. P. LISBOA

Hormone Laboratory, Department of Women's Diseases, Karolinska Sjukhuset, Stockholm (Sweden)

(Received January 7th, 1964)

A previous communication described the application of thin-layer chromatography with a binder, using Silica Gel G as adsorbent, to the separation of Δ^4-3-ketosteroids of the pregnane series[31]. At that time only a small number of reactions was employed for the detection of the spots following chromatography, *e.g.* the formation of isonicotinic acid hydrazones, the reduction of phosphotungstomolybdic acid and the anisaldehyde–sulphuric acid reaction.

The application to thin-layer chromatography of reactions for α,β-unsaturated ketosteroids, reducing steroids, ketonic steroids, as well as structural reactions for the side-chain of pregnane steroids and unspecific general reactions for steroids, is reported in this paper.

EXPERIMENTAL

Materials

Reagents. In addition to the substances indicated in previous papers[31,32,34], the following reagents were used.

Phosphomolybdic acid (No. 532), triphenyltetrazolium chloride (No. 8380), potassium permanganate (No. 5083), ammonium acetate (No. 1115), 2,4-pentanedione (No. 9600), sodium nitroprusside (No. 6540), mercury(II) iodide (No. 4420), ferric ammonium sulphate (No. 3792), ammonium molybdate (No. 1182) and phenylhydrazine hydrochloride (No. 7253) were obtained from Merck A.G., Darmstadt, Germany.

Sodium arsenate, silver nitrate and potassium periodate were purchased from May & Baker, England.

Chloroform was from AB Kebo; it was purified by fractionation.

Steroids. The systematic names, trivial names, abbreviations and sources of the thirty-seven Δ^4-3-keto-C_{21}-steroids used throughout this investigation have been previously indicated[31]. One or more *hydroxy-*, *keto-* or *aldehyde*-groups are indicated in the abbreviation by *-ol*, *-one* or *-al* respectively and P denotes *pregnane*. Each systematic name can easily be derived from its abbreviation, for instance: pregna-4,6-diene-3,20-dione from $P^{4,6}$ 3,20 one (6-dehydro-progesterone). For the formation of trivial names the steroids were regarded as derivatives of the following compounds: *progesterone* (pregn-4-ene-3,20-dione), *cortexone* (21-hydroxypregn-4-ene-3,20-dione), *Reichstein's compound "S"* (17α,21-dihydroxypregn-4-ene-3,20-dione), *corticosterone* (11β,21-dihydroxypregn-4-ene-3,20-dione), *cortisol* (11β,17α,21-trihydroxypregn-4-ene-3,20-dione) and *cortisone* (17α,21-dihydroxypregn-4-ene-3,11,20-trione).

The source and systematic names of other steroids—oestrogens and androgens—cited in this work, have been indicated in previous papers[32,33].

Ultraviolet lamp. The lamp used (λ = 254 mμ) was model PL 360 NN/15/44 from Quartzlampen GmbH, Hanau, Main, Germany.

Colours. The colours developed were recorded as previously described[34].

METHODS AND RESULTS

A. Detection of α,β-unsaturated ketosteroids

(*1*) *Absorption in ultraviolet light.* The α,β-unsaturated ketosteroids were detected by their absorption at 240 mμ after spraying with a dilute ethanolic solution of fluorescein for contrast[11]. This method of detection is not specific since many other steroids absorb in this region (see *e.g.* refs. 16, 17).

Direct observation at 240 mμ without fluorescein spraying is impossible because of the very strong absorption of Silica Gel G at this wavelength. Steroids of the pregnane series may be recovered from the plate by elution with chloroform since fluorescein does not react with these steroids.

(*2*) *Isonicotinic acid hydrazones.* The isonicotinic acid hydrazones of α,β-unsaturated ketosteroids were obtained by spraying with isonicotinic acid hydrazine as described in a previous paper[31]. This reaction permits differentiation between Δ^4-3-keto-, $\Delta^{4,6}$-3-keto-, $\Delta^{1,4}$- or Δ^1-3-keto-steroids. The presence of a $\Delta^{4,6}$-dien-3-one configuration, such as that of 6-dehydroprogesterone or 20-hydroxypregna-4,6-dien-3-one is shown by an immediate greenish yellow colour (sensitivity 1 μg), which can be differentiated from the less sensitive (4–5 μg) pale yellow colour produced by Δ^4-3-ketosteroids.

(*3*) *Bodansky-Kollonitsch's reaction*[7]. This reaction with *p*-phenylenediamine and phthalic acid, which was carried out as previously described[31], also allows differentiation between a Δ^4-3-keto- and a $\Delta^{4,6}$-3-keto-configuration.

All the Δ^4-3-ketosteroids tested revealed a yellow-olive to yellow-brown colour while 6-dehydroprogesterone and 20β-hydroxypregna-4,6-dien-3-one gave a yellow-orange to orange-brown colour. The sensitivity of the reaction is 2 to 3 μg. This reaction seems to be specific for α,β-unsaturated ketones.

Especially noteworthy is the fact that aldosterone gives an initial grey-brown colour which changes to purple as heating is continued. None of the other pregnane steroids tested gave these colours.

(*4*) *Formation of π-complexes with tetranitromethane*[63,46]. The reaction was carried out as described by LISBOA AND DICZFALUSY[34]. The Δ^4-3-keto- and $\Delta^{4,6}$-3-keto-steroids of the pregnane series give a pale lemon colour of low sensitivity (10–15 μg). The reaction is, however, more sensitive for steroids with a benzenoid configuration, such as phenolic oestrogens, an olive-yellow colour being obtained.

(*5*) *Osmate esters.* These esters were obtained by exposure of the steroid to osmium tetroxide vapours[4,35] in sealed tanks. A positive reaction is indicated by the formation of a black colour. Isolated double bonds such as occur in pregna-4,17-dien-3-one, 16-dehydroprogesterone, 16-dehydropregnenolone* and androst-2-ene-7,17-dione give a positive reaction after 5 to 10 min exposure to the vapours. The other Δ^4-3-keto-

* For a generous supply of 16-dehydropregnenolone (3β-hydroxypregna-5,16-dien-20-one) the author is indebted to Dr. O. A. DE BRUIN, N.V. PHILIPS-Duphar, Weesp, The Netherlands.

and $\Delta^{4,6}$-3-keto-steroids tested, which contained no isolated double bonds, gave a positive reaction only after 30 to 150 min exposure.

(6) *Sodium hydroxide*[10] *or tert.-butanolic sodium butoxide*[2]. It was impossible to employ these reactions on plates since no fluorescence developed.

B. Reduction reactions

(1) *Phosphomolybdic acid reagent*[29]. The plates were sprayed with a 10 % ethanolic solution of phosphomolybdic acid and heated to 90° for 15 min. The molybdenum blue colour obtained with this reagent is observed with 2 μg of steroid. Reichstein's compounds "E" and "*epi*-E" and the 20-dehydro substance "S" gave a blue-violet colour, possibly because of a side-reaction with the phosphoric acid released by the heating of phosphomolybdic acid.

(2) *Phosphotungstomolybdic acid reagent*[24]. This reagent was used as previously described by LISBOA[31]. It is less sensitive than that described above, requiring 5 μg of steroids.

(3) *Arsenomolybdic acid reagent.* This reagent, which was used for corticosteroids by SCHWARZ[54], was prepared according to NELSON[44]. The sprayed plates were heated for 5 to 10 min at 100°. The steroid is visualized as a blue spot on a yellow-green background. This is the most sensitive of the three molybdic acid reagents and as little as 0.5 μg of α,β-unsaturated steroids could be detected.

(4) *Triphenyltetrazolium reaction.* This reaction, whereby a red formazan is formed, was carried out as described by SHULL *et al.*[55]. Triphenyltetrazolium allows differentiation between α,β-unsaturated ketosteroids and α,β-ketolic steroids; the latter form a red formazan within 5 min at room teperature, with a sensitivity of 2 μg.

(5) *Alkaline potassium permanganate reagent.* The reaction was carried out as described for paper chromatography by BURTON *et al.*[9]. After spraying with a 0.2 % solution of potassium permanganate in 5 % sodium carbonate, all the Δ^4-3-ketosteroids tested immediately developed an olive-yellow colour on a violet background. When left overnight at room temperature following spraying, the steroids appeared as olive spots on a pale yellow background. This reaction is sensitive enough to detect 2 μg of steroid.

(6) *Reduction of p-amino-diethylaniline sulphur dioxide.* Plates were sprayed with a 0.5 % *p*-amino-diethylaniline sulphur dioxide in 5 % sodium bicarbonate solution and allowed to stand at room temperature overnight.

17α,21-Dihydroxy-20-ketosteroids without a hydroxyl group at C_{16}, such as compound "S", cortisol and *epi*-cortisol, give a red-orange colour with a sensitivity between 2 and 5 μg. 16α-Hydroxycortisol, 16α-hydroxy-compound "S", 16α-hydroxycortisone and the ketolic steroids, however, give an orange or yellow-orange colour. 17,20,21-Trihydroxy and 21-desoxy-Δ^4-3-keto-steroids do not reveal any colour even at 15–20 μg levels. Some oestrogenic steroids give a positive reaction: 16-keto-oestrone gives an orange colour almost immediately, while 16-ketolic oestrogens show the same colour after standing overnight.

(7) *Reduction of ferricyanide*[59]. The plates were sprayed with a solution of 0.1 % potassium ferricyanide in 0.25 % sodium carbonate and heated for 30 min at 80°. After cooling, the plates were sprayed with a 0.2 % solution of ferric ammonium sulphate, to each 100 ml of which 5 ml of concentrated (85 %) phosphoric acid had been added. The Prussian blue colour of ferric ferrocyanide appears immediately

or after a few minutes. The sensitivity of this reaction is between 2 and 5 μg.

This reaction is stronger with the 17,21-dihydroxy-20-keto- and 20,21-ketolic steroids; 17-hydroxy-C_{21}-steroids reduce more strongly than 17-deoxy-C_{21}-steroids. All other Δ^4-3-ketosteroids require at least 5 μg for detection.

(*8*) *Dragendorf reaction*[49]. The plates were sprayed with the reagent as modified by LISBOA[32]. Using this modified reagent, less than 1 μg of Δ^4-3-ketosteroid gives an orange colour on a yellow background. The latter turns to grey-lilac giving an even greater contrast. This reaction, however, is not specific.

(*9*) *Reduction of Tollens reagent.* The reaction was carried out as described for paper chromatography by ZAFFARONI *et al.*[65]. The reduction of ammoniacal silver nitrate requires more than 10 μg of an α,β-ketolic steroid. The reduction of *Nessler's reagent*[40] requires even larger quantities than the above reduction and thus is not considered suitable for use on chromatoplates.

C. Ketonic reactions

(*1*) *Reaction of Gornall and MacDonald*[21]. The plates were sprayed with a 10 % (v/v) ethanolic solution of hydrochloric acid containing 0.1 % 2,4-dinitrophenylhydrazine. The colour develops immediately upon spraying and its intensity may be enhanced by heating at 60° for a few minutes.

Steroids with only a Δ^4-3-keto group react to give an orange-red colour, *e.g.* pregn-4-en-3-one, pregna-4,17(20)-dien-3-one, 20β-hydroxypregna-4,6-dien-3-one, 20β- and 20α-hydroxypregn-4-en-3-one. If a 20-keto group is present, an orange yellow colour appears, *e.g.* progesterone, 17α-hydroxyprogesterone, 11-ketoprogesterone, 6- and 16-dehydroprogesterone. A 17α,21-dihydroxy-20-ketosteroid reacts more strongly than a 20,21-ketol; cortisol, cortisone, compound "S", 16α-hydroxy-compound "S", 16α-hydroxy-cortisone and 16α-hydroxy-cortisol give an orange colour, while corticosterone, *epi*-corticosterone, cortexone and aldosterone give a yellow colour. This reaction allows detection of 2 μg of the steroid.

The sensitivity can be increased, as suggested by STUPNICKI AND STUPNICKA[60] by using successively a solution of 0.1 % potassium permanganate in 1 % aqueous carbonate and an 0.2 % aqueous solution of ascorbic acid. If this method is applied the sensitivity of the reaction is increased, but the differentiation noted above is no longer possible.

(*2*) *The Zimmermann reaction.* This reaction for ketonic steroids with an *ortho* unsubstituted methylene group, was carried out as previously described[32]. Plates were sprayed with a solution containing equal parts of a 2 % alcoholic *m*-dinitrobenzene and a 1.25 *N* alcoholic potassium hydroxide solution (carbonate free) and dried unter a hot air stream or in a ventilated oven at 40°.

Five non-polar steroids with a Δ^4-3-keto-group gave a blue-violet colour with this reagent. Progesterone and other 20-keto-21-deoxysteroids, *e.g.* 16-dehydroprogesterone, 11-ketoprogesterone and 17α-hydroxyprogesterone react with a blue-grey colour, while ketolic and dihydroxy-ketolic steroids give a pink colour. By means of this reaction 2 to 3 μg of cortexone could be detected.

The GORNALL AND ZIMMERMANN reactions[21,66] were found to take place in much the same manner on chromatoplates as on paper chromatograms[10]. The GORNALL reaction shows greater sensitivity with polar steroids, while the ZIMMERMANN reaction is found to be more sensitive with the less polar 3-ketosteroids.

D. Side-chain reactions

(*1*) *17,21-Dihydroxy-20-ketosteroids.* These steroids can be detected by the PORTER–SILBER reaction[50], using phenylhydrazine–sulphuric acid reagent as described by SILBER AND PORTER[57]. After spraying, the plates may be observed after one hour if left at room temperature, or in a few minutes if heated to 60°. 17,21-Dihydroxy-20-ketosteroids give a dihydrazone as the end product with an E_{max} at about 400 mμ. However, 21-aldo-20-ketosteroids[58], 21-hydroxy-20-keto-16-dehydropregnene- and 16,21-dihydroxy-20-ketopregnane-steroids[37] also give a positive reaction.

The entire group of 17α,21-dihydroxy-20-ketosteroids tested give a positive PORTER–SILBER reaction with a sensitivity of 1 μg, with the exception of 16α-hydroxycortisol, 16α-hydroxycortisone and 16α-hydroxy-compound "S". These exceptions are in agreement with the observation of BERNSTEIN AND SILBER[5] on the reactivity of triamcinolone and may perhaps be explained by the fact that these compounds are unable to form the 16-dehydro-derivative which is an intermediate in the formation of the PORTER-SILBER chromogen[62].

Steroids such as corticosterone, *epi*-corticosterone, cortexone, aldosterone, 19-hydroxycortexone and others that do not possess the dihydroxyketone structure, and the above 16-hydroxysteroids give a very weak positive reaction following prolonged heating. This reaction, which was described for corticosterone and 11-dehydrocorticosterone on paper by NEHER AND WETTSTEIN[43] is probably due to the formation of the 3-phenylhydrazone of the Δ^4-3-keto-steroids, which shows an E_{max} between 340 and 360 mμ[50].

16α-Hydroxycortexone gave a negative reaction when the procedure was carried out as described above, but when observed again after several hours, the reaction had become positive.

Steroids with a 20-hydroxy group may give a positive reaction if the plates are heated or observed after prolonged exposure at room temperature. The colour produced differs, however, from the typical PORTER–SILBER reactions as follows: 20α- and 20β-hydroxy-pregn-4-en-3-ones: yellow-orange; 20β-hydroxypregna-4,6-dien-3-one: brown-orange; 20-dihydro-compound "S": olive; Reichstein's compound "E": dark blue-grey; Reichstein's compound *epi*-"E": pale grey-violet; and Reichstein's compound *epi*-"U": pale rose. The colours may result from a reaction between the steroid and the sulphuric acid of the reagent.

(*2*) *17-Deoxy-α-ketolic steroids.* These steroids could be detected after oxidation to their corresponding glyoxals by means of cupric acetate[30].

The plates were sprayed with a 0.01 *M* methanolic cupric acetate solution and left at room temperature overnight. Subsequently they were sprayed in the same manner as described for the PORTER–SILBER reaction above.

Of the six 17-deoxy-α-ketolic steroids tested in the course of this work, cortexone, corticosterone and *epi*-corticosterone give positive reactions which are as strong as those of 17α,21-dihydroxy-20-ketosteroids. 19-Hydroxycortexone, 16α-hydroxycortexone and aldosterone, however, give a very weak positive reaction. The colour appears almost immediately after spraying the plates.

The reaction of LEWBART AND MATTOX[30] appears to be a good method for the identification of corticosterone, *epi*-corticosterone and cortexone on silica gel plates. Since the glyoxal derivatives of 17,21-dihydroxy-20-ketosteroids give an almost instantaneous PORTER–SILBER reaction (E_{max} 425–450 mμ), this oxidation method

may also be employed for the identification of these steroids as suggested by BIRMINGHAM[6]. This is especially useful when 17,21-dihydroxy-20-keto- and 17-deoxy-20,21-ketolic steroids are present together.

(3) *Formaldehydogenic steroids.* These were detected by the method of SCHWARTZ[53] as described for paper chromatography by PAN[48]. The plates were sprayed with a 1 % solution of potassium periodate in 70 % (v/v) ethanol, and after 10 min at room temperature, were sprayed again before they were dry with a methanolic solution containing 15 % ammonium acetate, 1 % acetic acid and 1 % 2,4-pentanedione. After 15 to 20 min formaldehydogenic steroids show a yellow spot in daylight, yellow-green in U.V. light (252 mμ lamp). The sensitivity is to 1–2 μg. Thirteen formaldehydogenic steroids with a 20,21-ketolic, 17,20,21-trihydroxy- and 17,21-dihydroxy-20-keto-structure have been shown to be positive in the SCHWARTZ reaction with a sensitivity of 2 μg: corticosterone, *epi*-corticosterone, cortisol, *epi*-cortisol, 16α-hydroxycortisone, 16α-hydroxycortisol, 16α-hydroxy-compound "S", cortisone, 6α-hydroxycorticosterone, 11-dehydrocorticosterone, Reichstein's compound "S", cortexone and 16α-hydroxycortexone. No 20,21-glycol-steroid was, however, tested. In U.V. light the yellow-green colour permits the detection of less than 1 μg.

Various non-formaldehydogenic steroids with 20-hydroxy-21-deoxy- and 20-keto-21-deoxy-structure were negative in this test.

(4) *21-Deoxy-20-ketosteroids.* These were revealed *in situ* by the nitroprusside reaction of FEIGL[18] for methyl ketones as applied to steroid methyl ketones by PAN[47].

This method is suitable for thin-layer chromatography when the plates are protected by a filter-paper (Whatman No. 2) prior to the application of the methanolic nitroprusside paste reagent.

Amounts of 5 to 6 μg of 21-deoxy-20-ketosteroids, such as progesterone, 6-dehydro-, 17α-hydroxy-, 6β-hydroxy-, 11α-hydroxy- and 11β-hydroxyprogesterone give a bright violet spot after 60 min at room temperature. This can be observed on the uncovered surface of the plate. 16-Dehydroprogesterone and the 21-hydroxy-steroids proved to be negative in this reaction.

(5) *Vanillin–phosphoric acid reagent*[13]. This reagent was used for the identification of *17-hydroxy-20-keto-21-deoxysteroids* as suggested by MCALEER AND KOZLOWSKI[38]. The plates were sprayed with a 2 % solution of vanillin in phosphoric acid (85 %) and then heated for 20 min at 90–95°. A bright orange colour indicates the presence of the above steroids.

The results of the application of the CHABROL reaction to Δ^4-3-keto-C_{21}-steroids on thin-layer chromatograms are summarized in Table I. Some steroids develop a colour after 15 min heating at 95–100°.

Using 1 μg amounts, pregna-4,17(20)-dien-3-one, 20β-hydroxypregna-4,6-dien-3-one, 20α- and 20β-hydroxypregn-4-en-3-one give a lilac-grey colour, 20-dihydro-compound "S" and Reichstein's compound *epi*-"E" a violet-grey, Reichstein's compound "E" a pale violet-blue and Reichstein's compound "U" a carmine-brown colour.

In 1 μg amounts 17α-hydroxy- and 11β,17α-dihydroxyprogesterone develop the characteristic orange colour described for 17α-hydroxy-20-keto-21-deoxysteroids, while 17α-hydroxyprogesterone could be detected in 0.5 μg amounts.

Reichstein's compound "U", 11β-hydroxyprogesterone, corticosterone and 6β-

TABLE I

APPLICATION OF CONCENTRATED PHOSPHORIC ACID AND VANILLIN–PHOSPHORIC ACID REAGENTS TO THE DETECTION OF THIRTY-SIX Δ^4-3-KETOPREGNANE-STEROIDS

Amount of steroid: 8–10 μg

Steroid	Conc. phosphoric acid (at least 85%)		Vanillin–phosphoric acid reagents	
	Daylight	U.V. light	15 min	30 min
P^4 3 one	—	—	—	lc.gy.
$P^{4,17(20)}$ 3 one	ce.	y.gr.	gy.lc.	bh.lc.*
20α ol P^4 3 one	ce.	—	gy.lc.	bh.lc.*
20β ol P^4 3 one	ce.	—	gy.lc.	bh.lc.*
20β ol $P^{4,6}$ 3 one	ce.	—	gy.lc.	bh.lc.*
17α,20β,21 ol P^4 3 one	p.gy.br.	y.or.	v.gy.	bkh.ol.*
11β,17α,20β,21 ol P^4 3 one	gr.gy.	—	bl.v.	pu.v.*
11α,17α,20β,21 ol P^4 3 one	p.pu.	—	bl.v.	pu.v.*
17α,20β,21 ol P^4 3,11 one	—	—	p.sm.	br.
17α,20α,21 ol P^4 3,11 one	or.r.-or.br.	y.or.	c.bh.	c.br.*
P^4 3,20 one	—	—	—	bl.gy.
$P^{4,6}$ 3,20 one	—	—	—	p.or.r.
$P^{4,16}$ 3,20 one	—	—	—	p.ol.gy.
6β ol P^4 3,20 one	—	—	—	p.y.br.
11β ol P^4 3,20 one	ce.	—	p.or.r.	br.ol.
11α ol P^4 3,20 one	—	—	—	p.gy.br.
16α ol P^4 3,20 one	—	—	—	gy.
17α ol P^4 3,20 one	p.br.	r.	or.	m.*
21 ol P^4 3,20 one	ol.br.	y.or.	—	bl.gy.
11β,17α ol P^4 3,20 one	gh.y	y.	or.	or.bh.
11β,21 ol P^4 3,20 one	gy.br.	p.bu.gr.	p.or.r.	gy.br.
11α,21 ol P^4 3,20 one	—	p.bu.gr.	—	y.br.
16α,21 ol P^4 3,20 one	lc.re.	pk.	—	gy.
17α,21 ol P^4 3,20 one	lc.re.	pk.	—	lc.gy.
19,21 ol P^4 3,20 one	—	gh.bl.	p.pu.	br.lc.
11β,21 ol 18 al P^4 3,20 one	—	—	—	p.gy.br.
6β,11β,21 ol P^4 3,20 one	—	—	p.pu.	p.v.
11β,17α,21 ol P^4 3,20 one	—	—	—	bf.
11α,17α,21 ol P^4 3,20 one	—	p.bu.gr.	—	gy.br.
16α,17α,21 ol P^4 3,20 one	—	—	—	gy.
11β,16α,17α,21 ol P^4 3,20 one	p.br.	gh.y.	p.ol.y.	bh.ol.
P^4 3,11,20 one	p.gy.br.	p.or.	—	gy.br.
21 ol P^4 3,11,20 one	—	—	—	bf.
17α,21 ol P^4 3,11,20 one	ol.	gh.y.	—	br.ol.
6β,17α,21 ol P^4 3,11,20 one	—	—	p.sm.	lc.re.
16α,17α,21 ol P^4 3,11,20 one	—	—	—	y.

* Colour developed for 1 μg of steroid.

Abbreviations:

bf.	= buff	c.	= carmine	m.	= minium	r.	= red
bh.	= brownish	ce.	= cream	ol.	= olive	re.	= rose
bl.	= blue	gh.	= greenish	or.	= orange	sm.	= salmon
br.	= brown	gr.	= green	p.	= pale	v.	= violet
bu.	= bluish	gy.	= grey	pk.	= pink	y.	= yellow
bkh.	= blackish	lc.	= lilac	pu.	= purple		

hydroxycortisone also show this orange colour, but with a much lower sensitivity. Pregna-4,17(20)-dien-3-one and the three steroids with a 20-hydroxy-21-deoxy-structure tested, developed a yellow fluorescence under U.V. light early on in this reaction. This disappeared on heating.

The CHABROL reaction, described for steroids of the cholane series, is regarded[1,27]

as a specific reaction for cholic acid depending upon the concentration of phosphoric acid in the reagent. The specificity of this reaction for 7α-hydroxycholane-steroids was established by CHABROL *et al.*[13], CHARONNAT AND GAUTHIER[14] and HÄUSSLER[22,23] and later confirmed by CERRI AND SPIALTINI[12]; apocholic acid—a bile acid with an 8(14)-unsaturated bond—reacts as cholic acid[51]. Later MCALEER AND KOZLOWSKI[38] described the reaction as specific for 17-hydroxy-20-keto-21-deoxysteroids.

In our experience, however, other C_{21}-deoxysteroids react with a sensitivity similar to the extremely sensitive orange colour noticed by MCALEER AND KOZLOWSKI. They have, however, different absorption maxima. Structurally different steroids react just as strongly, for example: 7α-hydroxy-oestradiol (celadon-green), 20-dihydro-compound "S" (black-olive) and 17β-hydroxy-5α-androstane-3,7-dione (yellow-orange). Since some steroids react with concentrated phosphoric acid, the colour reaction of a steroid with the vanillin–phosphoric acid reaction must be compared with that produced with phosphoric acid prior to evaluation.

E. Non-specific reactions for steroids

(1) *Phosphoric acid reaction.* The chromatoplates were sprayed with concentrated phosphoric acid (85 %) and heated at 90–95° for 15 min. Colours were observed in daylight and U.V. light. With many of the steroids studied here, concentrated phosphoric acid develops colours in daylight or in U.V. light, some of the reactions being quite specific. Reichstein's compound "S" and 16α-hydroxycortexone, for instance, appear as lilac-rose spots in daylight, which turn to pink under U.V. light. The fluorescence in U.V. light of 20 steroids tested are indicated in Table I.

Dilute phosphoric acid solution[43], concentrated phosphoric acid[20,15] or so-called 100 % phosphoric acid[45] are employed to detect steroids of the pregnane series. However, for Δ^4-3-keto-C_{21}-steroids this reaction is not as sensitive as for others, such as alcoholic pregnane-steroids, oestrogens, Δ^5-3β- or Δ^5-7-hydroxysteroids.

(2) *Sulphuric acid reaction.* This reaction was carried out as previously described[33]. The colours developed are not specific, but in U.V. light some fluorescence can be noted: red for cortexone and Reichstein's compound "S", orange for 19-hydroxy- and 17α,19-dihydroxycortexone, green for corticosterone, *epi*-corticosterone, *epi*-cortisol, pregna-4,17(20)-dien-3-one, 20α- and 20β-hydroxypregn-4-en-3-one; and yellow for 20β-hydroxypregna-4,6-dien-3-one and compounds "E" and *epi*-"E" of Reichstein.

(3) *Liebermann-Burchard reaction.* This was carried out as described for paper chromatograms by NEHER AND WETTSTEIN[43]. The grey-brown or brown colours developed after 15 min heating at 85–90° are not specific. Under U.V. light, the spots (15–20 μg) develop the following fluorescence: *bluish-green* for cortisone, 16α-hydroxycortisone, cortisol, 16α-hydroxycortisol, *epi*-corticosterone, 11-dehydrocorticosterone, aldosterone, 16α-hydroxy-compound "S", progesterone, 6-dehydro-, 6α-hydroxy-, 11α-hydroxy- and 16α-hydroxy-progesterone; *yellow-green or greenish-yellow* for compound "U" (Reichstein), *epi*-cortisol, 11-keto-progesterone, 16-dehydroprogesterone, 19-hydroxy- and 17α,19-dihydroxy-cortexone; *yellow* for Reichstein's compound *epi*-"E", cortexone 16α-hydroxycortexone, pregna-4,17(20)-dien-3-one, 20β-hydroxypregna-4,6-dien-3-one and epimeric 20-hydroxypregn-4-en-3-one; *yellow-brown* or *brown* for corticosterone and 17α-hydroxyprogesterone, and *orange* for Reichstein's compounds "E" and "S".

TABLE II

COLOUR REACTIONS OF THIRTY-SEVEN Δ^4-3-KETO-C_{21}-STEROIDS ON THIN-LAYER CHROMATOGRAMS AFTER TREATMENT WITH ANISALDEHYDE–SULPHURIC ACID AND VANILLIN–SULPHURIC ACID REAGENTS

Amount of steroid: 4–5 μg. The colours developed at 95–100°. The time required for the colour to be observed for anisaldehyde–sulphuric acid reagent is indicated in parentheses; whenever no time is noted for vanillin–sulphuric acid reagent this signifies 12–15 min.

Steroid	*Reagent*	
	Anisaldehyde–sulphuric acid	*Vanillin–sulphuric acid*
P^4 3 one	y.or.(10) – or.br.(12–15) – cr.(16–18)	or.
$P^{4,17(20)}$ 3 one	gy.lc.(12) – bh.v.(15)	bl.gy.
20β ol P^4 3 one	bf.(5–6) – dk.v.bl.(10–12)	bl.gy.(8) – dk.bl.gy.(15)
20α ol P^4 3 one	dk.v.bl.(10–12)	bl.gy.
20β ol $P^{4,6}$ 3 one	pk.(10–12) – or.br.(16) (U.V. = or.)	p.v.(8–10) – v.gy.(15)
17α,20β,21 P^4 3 one	p.v.(10–12) – gy.v.(16)*	gy.v.(5–6) – pu.v.
11β,17α,20β,21 ol P^4 3 one	or.br.(5–6) – gy.v.(10–12)	dk.rh.lc.(5–6) – bh.v.
11α,17α,20β,21 ol P^4 3 one	or.br.(5–6) – gy.v.(10–12)	dk.rh.lc.(5–6) – bh.v.
17α,20α,21 ol P^4 3,11 one	p.sm.(5–6) – br.r.(10–12)*	pk.(5–6) – br.lc.
17α,20β,21 ol P^4 3,11 one	sm.(5–6) – br.lc.(10–12)	pk.(5–6) – r.lc.
P^4 3,20 one	y.(5–6) – y.br.(10–12) – c.br.(18)	or.y.(10–12) – br.(15)
$P^{4,6}$ 3,20 one	sm.(5–6) – r.or.(12–15) (U.V. = or.)	or.br.(15)
$P^{4,16}$ 3,20 one	y.or.(5–6) – y.bh.(12) – c.r. (16)	or.(10–12) – r.br.(15)
6β ol P^4 3,20 one	or.(6) – r.br.(10–12) (U.V. = y.)	or.y.(5–6) – c.br.
11β ol P^4 3,20 one	y.or.(10–12) – or.br.(16) (U.V. = y.)	or.y.(5–6) – c.br.
11α ol P^4 3,20 one	y.or.(10–12) – c.br.(16) (U.V. = y.)	or.br.
16α ol P^4 3,20 one	or.(5–6) – y.br.(12) – r.br.(16) (U.V. = y.)	y.br.
17α ol P^4 3,20 one	y.or.(10–12) – br.(12) – br.v.(16) (U.V. = y.)	p.r.(5–6) – br.r.(12–15)
21 ol P^4 3,20 one	gr.gy.(10–12) – br.(16)	br.r.
11β,17α ol P^4 3,20 one	y.br.(12)	r.
11β,21 ol P^4 3,20 one	bl.gy.(12) – r.br.(16)	br.r.
11α,21 ol P^4 3,20 one	bl.gy.(12) – bh.r.(16)	br.r.
16α,21 ol P^4 3,20 one	y.br.(10–12) – r.br.(16)	y.br.
17α,21 ol P^4 3,20 one	lc.br.(12)	r.br.
19,21 ol P^4 3,20 one	ol.(5–6) – s.bl.(10–12)*	pk(5–6) – br.lc.
11β,21 ol 18 al P^4 3,20 one	sm.(5–6) – c.r.(10–12)*	or.(5–6) – r.br.
6β,11β,21 ol P^4 3,20 one	c.(5–6) – pu.(10–12)*	or.y.(5–6) – bg.v.*
11β,17α,21 ol P^4 3,20 one	r.or.(10–12) – c.br.(16)	br.r.
11α,17α,21 ol P^4 3,20 one	r.or.(10–12) – br.lc.(16)	br.r.
16α,17α,21 ol P^4 3,20 one	dl.y.(5–6) – ol.(10–12) – p.or.r.(16) (U.V. = y.)	r.br.
17α,19,21 ol P^4 3,20 one	p.sm.(5–6) – br.r.(10–12)*	pk.(5–6) – br.lc.
16α,11β,17α,21 ol P^4 3,20 one	y.(5–6) – ol.y.(10–12) – or.br.(16) (U.V. = y.)	r.br.
P^4 3,11,20 one	y.or.(10–12) – r.br.(16)	or.
21α ol P^4 3,11,20 one	c.br.(12)	br.r.
17α,21 ol P^4 3,11,20 one	or.(5–6) – c.r.(12)	br.r.
6β,17α,21 ol P^4 3,11,20 one	or.(5–6) dk.re.lc.(10–12)	sm.(5–6) – br.lc.
16α,17α,21 ol P^4 3,11,20 one	r.or.(5–6) – or.br.(10–12) – br.r.(16) (U.V. = or.)	bh.r.

* Colour developed with 1.5–2.0 μg of steroid

Abbreviations:

bf.	= buff	dk.	= dark	ol.	= olive	sm.	= salmon
bg.	= bright	dl.	= dull	or.	= orange	sy.	= straw-yellow
bh.	= brownish	gr.	= greenish	p.	= pale	s.gr.	= sea-green
bl.	= blue	gy.	= green	pk.	= pink	s.bl.	= sea-blue
br.	= brown	lc.	= grey	pu.	= purple	v.	= violet
bu.	= bluish	lt.	= lilac	r.	= red	y.	= yellow
c.	= carmine	lt.	= light	re.	= rose		
ce.	= cream	mhre	= maroon	rh.	= reddish		

(*4*) *Antimony trichloride reaction.* This reaction was carried out as described in a previous paper[34]. It is less sensitive for Δ^4-3-keto-C_{21}-steroids than for Δ^5-3β-hydroxy-steroids or oestrogens, and for a large number of the steroids tested, 20 μg developed no colour whatsoever or only a non-specific grey or brownish one.

There are, however, some exceptions: grey-lilac or pale violet spots (U.V. pale orange) are obtained with pregna-4,17(20)-dien-3-one, 20β-hydroxypregna-4,6-dien-3-one and epimeric 20-hydroxypregn-4-en-3-one; and brownish red (U.V. red-orange) spots are noted with 20-dihydro-compound "S" and Reichstein's compound "E".

(*5*) *Sulphuric acid and aromatic aldehydes.* The sulphuric acid–aromatic aldehyde reaction was carried out as previously described[34]. The results of the application of this reagent to thirty steroids utilising anisaldehyde, vanillin, benzaldehyde, salicylaldehyde and *p*-dimethylaminobenzaldehyde as the aromatic aldehyde are shown in Tables II and III.

In this reaction, the colours developed are dependent upon temperature, time of heating and intensity of spraying. The described colours can be obtained only when the stated experimental conditions are observed. The change of colours during and after heating make the use of standards for simultaneous reactions advisable. A freshly prepared 1 % solution of the aromatic aldehyde gives the best results. If higher concentrations are used, a deep background coloration occurs, as described for paper chromatography[26].

Even though a direct relationship between the structure of the steroid and the colour obtained cannot be determined, many steroids with similar structural groups show similar colours. 16-Hydroxysteroids and many hydroxy-derivatives of progesterone react with all the aldehydes tested, a yellow or yellow-brown colour being obtained.

The 11-ketosteroids tested, compound A, cortisone, Reichstein's compounds "E" and *epi*-"E" and 11-ketoprogesterone, appear as red or carmine spots with anisaldehyde.

The colours of 20α-hydroxy- and 20β-hydroxy-pregn-4-en-3-one and pregna-4,17(20)-dien-3-one are similar with the five aldehydes: blue-grey (vanillin), violet (anisaldehyde) or carmine-brown (benzaldehyde, salicylaldehyde or *p*-dimethylaminobenzaldehyde). The colours developed with steroids containing a side-chain with the 17,20,21-trihydroxy-structure, such as Reichstein's 20-dihydro-substance "S" and Reichstein's compounds "E" and *epi*-"E" are also lar in each of the five reactions.

The red colour developed with vanillin for 17α-hydroxy- and 11β,17α-dihydroxy-progesterone is typical, as is the pink colour, changing to brown-lilac, obtained for 19-hydroxy- and 17α,19-dihydroxy-deoxycorticosterone, with the same aldehyde. Just as typical is the green-grey colour which appears when deoxycorticosterone is treated with the anisaldehyde–sulphuric acid reagent.

The three 6β-hydroxysteroids tested, 6β-hydroxyprogesterone, 6β-hydroxycorticosterone and 6β-hydroxycortisone, develop similar colours with each of the aldehydes employed.

When *p*-dimethylaminobenzaldehyde is used as the aromatic aldehyde, the majority of the progesterone and deoxycorticosterone hydroxy-derivatives develop a non-specific or a yellow-brown colour with a yellow or orange U.V. fluorescence. With *p*-dimethylaminobenzaldehyde 16α-, 17α- and 19-hydroxy-deoxycorticosterones,

TABLE III

COLOUR REACTIONS OF THIRTY-SEVEN Δ^4-3-KETO-C_{21}-STEROIDS ON THIN-LAYER CHROMATOGRAMS AFTER TREATMENT WITH SULPHURIC ACID–AROMATIC ALDEHYDE REAGENTS

Amount of steroid: 4–5 μg. The colours are recorded in daylight and U.V. light after 12–15 min heating at 95–100°. For abbreviations see Table II.

Steroid	*Aldehyde*					
	Benzaldehyde		*Salicylaldehyde*		*p-Dimethylaminobenzaldehyde*	
	Daylight	*U.V. light*	*Daylight*	*U.V. light*	*Daylight*	*U.V. light*
P^4 3 one	dl.y.	—	or.y.	p.or.r.	y.or.	y.
$P^{4,17(20)}$ 3 one	p.pk.(5–6)* – c.br.	y.	c.br.	ol.gr.	r.br.(5–6) – c.br.	or.
20β ol P^4 3 one	p.br.lc.(5–6) – c.br.	y.	c.br.	ol.gr.	y.br.(5–6) – c.br.	or.
20α ol P^4 3 one	p.br.lc.(5–6) – c.br.	y.	c.br.	ol.gr.	y.br.(5–6) – c.br.	or.
20β ol $P^{4,6}$ 3 one	v.	r.br.	r.br.	y.br.	ol.br.	y.
17α,20β,21α P^4 3 one	p.pu.(8) – p.v.	bf.	lc.(3) – p.v.	or.	dl.pu.(5–6) – or.br.	or.
11β,17α,20β,21 ol P^4 3 one	pk.(5–6) – gy.lc.	r.or.	lc.pu.(5–6) – d.gy.bl.	y.gr.	lg.sm.(5–6) – y.br.	or.
11α,17α,20β,21 ol P^4 3 one	pk.(5–6) – gy.lc.	or.	lc.pu.(5–6) – d.gy.bl.	y.gr.	bf.(5–6) – y.br.	or.
17α,20α,21 ol P^4 3,11 one	sy.(8) – gy.br.	—	y.gr.(5–6) – s.gr.	gr.	y.gr.(5–6) – y.ol.	bf.
17α,20β,21 ol P^4 3,11 one	sy.	or.	ol.	y.gr.	y.	y.
P^4 3,20 one	dl.y.	dl.y.	dl.y.	dl.y.	y.or.	y.
$P^{4,6}$ 3,20 one	dl.y.	y.	gh.y.	s.gr.	gh.y.(8) – ol.y.	gh.y.
$P^{4,16}$ 3,20 one	dl.y.	dl.y.	or.y.	dl.y.	y.or.	y.
6β ol P^4 3,20 one	y.or.	y.or.	y.gr.(5–6) – y.ol.	lg.bl.	y.(5–6) – or.br.	or.
11β ol P^4 3,20 one	y.	y.gr.	y.	ce.	y.or.	y.or.

11α ol P[4] 3,20 one	y.or.	y.	y.	y.gr.	y.or.	y.or.
16α ol P[4] 3,20 one	p.br.	p.or.	y.	lg.bl.	p.y.(5–6) – ol.y.	y.
17α ol P[4] 3,20 one	gh.y.	y.or.	y.gr.	gr.	or.y.(5–6) – or.br.	or.
21 ol P[4] 3,20 one	br.ol.	y.	lc.gy.	gy.bu.	y.br.	or.
11β,17α ol P[4] 3,20 one	br.ol.	or.	y.ol.	or.	y.or.	sm.
11β,21 ol P[4] 3,20 one	br.ol.	y.	br.ol.	y.or.	y.br.	or.
11α,21 ol P[4] 3,20 one	br.ol.	y.	br.ol.	or.	y.br.	or.
16α,21 ol P[4] 3,20 one	br.ol.	y.	y.ol.	y.gr.	ol.	y.or.
17α,21 ol P[4] 3,20 one	dk.gr.gy.	or.br.	ol.	dl.y.	ol.	y.or.
19,21 ol P[4] 3,20 one	sg.(8) – gy.br.	—	ol.(5–6)	lg.bl.	y.gr.(5–6) – y.ol.	bf.
11β,21 ol 18 al P[4] 3,20 one	sy.	or.	y.(8) – oc.	p.gh.bl.	y.gr.	y.
6β,11β,21 ol P[4] 3,20 one	p.br.(8) – y.br.	—	y.gr.(5–6)** – s.gr.	p.gh.bl.	y.(5–6) – y.or.**	or.
11β,17α,21 ol P[4] 3,20 one	br.ol.	y.gr.	ol.	y.or.	y.br.	or.
11α,17α,21 ol P[4] 3,20 one	br.ol.	y.gr.	ol.	y.or.	y.br.	or.
16α,17α,21 ol P[4] 3,20 one	p.br.	p.or.	sy.	ce.	y.gr.	y.
17α,19,21 ol P[4] 3,20 one	sy.(8) – gy.br.	—	y.gr.(5–6)	gr.	y.gr.(5–6) – y. ol.	bf.
11β,16α,17α,21 ol P[4] 3,20 one	p.br.	p.or.	y.br.	or.	y.gr.	y.or.
P[4] 3,11,20 one	y.	dl.y.	or.y.	dl.y.	y.or.	y.
21 ol P[4] 3,11,20 one	ol.y.	y.bh.	ol.y.	dl.y.	y.or.	y.or.
17α,21 ol P[4] 3,11,20 one	ol.y.	y.bh.	ol.y.	dl.y.	y.or.	y.or.
6β,17α,21 ol P[4] 3,11,20 one	b[illegible]	r.or.	ol.y.	p.gh.bl.	y.(8) – p.sm.	y.or.
16α,17α,21 ol P[4] 3,11,20 one	p.[illegible]	p.or.	bf.	y.	y.gr.	y.

* Time required (in minutes) for colour development, whenever this differs from that given above.
** Colour developed with 1.5–2.0 μg of steroid.

however, appear as yellow spots, and 16α-hydroxy-derivatives of Reichstein's compound "S", cortisone and cortisol as yellow-green spots.

The sulphuric acid–aromatic acid reaction can be used to characterize progesterone and 6-dehydroprogesterone, when anisaldehyde or benzaldehyde is employed. In contradistinction to progesterone and 16-dehydroprogesterone, which give a feeble reaction with benzaldehyde, 6-dehydroprogesterone appears as a greenish yellow spot, sea-green under U.V. light. When anisaldehyde is used, the red-orange spot with orange fluorescence from 6-dehydroprogesterone can also be differentiated from the yellow or yellow-orange spot of progesterone and 16-dehydroprogesterone.

With the anisaldehyde–sulphuric acid reaction it was found best to observe the spots in daylight; on the other hand, very good fluorescent spots occurred when salicylaldehyde was employed as the aromatic aldehyde.

(6) *Mylius reaction*[41]. The reaction with iodine–potassium iodide (Lugol solution) was carried out according to BURTON *et al.*[9]: the plates were sprayed with a 0.3 % iodine solution in a 0.5 % aqueous solution of potassium iodide. After the original colour was observed the plates were resprayed with ether[39], which modifies the reaction of many steroids.

TABLE IV

RESULTS OF THE APPLICATION OF THE MYLIUS REACTION (IODINE–POTASSIUM IODIDE) TO THIRTY-SEVEN Δ^4-3-KETO-C_{21}-STEROIDS BEFORE (I) AND AFTER (II) ETHER TREATMENT

Amount of steroid: 8–10 μg

Steroid	*I*	*II*	*Steroid*	*I*	*II*
P^4 3 one	y.	y.	11β,17α ol P^4 3,20 one	—	—
$P^{4,17(20)}$ 3 one	y.	y.	11β,21 ol P^4 3,20 one	y.	y.
20β ol P^4 3 one	y.	y.	11α,21 ol P^4 3,20 one	bl.[d]	bl.
20α ol P^4 3 one	br.oc.	br.oc.	16α,21 ol P^4 3,20 one	lg.v.gy.	bl.
20β ol $P^{4,6}$ 3 one	or.	or.	17α,21 ol P^4 3,20 one	y.	or.br.
17α,20β,21 ol P^4 3 one	y.	y.	19,21 ol P^4 3,20 one	—	p.y.br.
11β,17α,20β 21 ol P^4 3 one	—	y.	11β,21 ol 18 al P^4 3,20 one	y.	y.
11α,17α,20β 21 ol P^4 3 one	—	y.	6β,11β,21 ol P^4 3,20 one	y.	y.
17α,20β,21 ol P^4 3,11 one	y.	y.[b]	11β,17α,21 ol P^4 3,20 one	y.[e]	bl.
17α,20α,21 ol P^4 3,11 one	y.	y.[b]	11α,17α,21 ol P^4 3,20 one	y.	y.
P^4 3,20 one	y.	bl.	16α,17α,21 ol P^4 3,20 one	—	—
$P^{4,6}$ 3,20 one	y.	y.	17α,19,21 ol P^4 3,20 one	—	y.
$P^{4,16}$ 3,20 one	y.or.	r.br.	11β,16α,17α,21 ol P^4 3,20 one	—	—
6β ol P^4 3,20 one	y.	y.	P^4 3,11,20 one	y.	r.br.
11β ol P^4 3,20 one	y.	y.	21 ol P^4 3,11,20 one	y.	y.
11α ol P^4 3,20 one	bl.	bl.	17α,21 ol P^4 3,11,20 one	bl.	bl.
16α ol P^4 3,20 one	lg.v.bl.	br.oc.	6β,17α,21 ol P^4 3,11,20 one	—	—
17α ol P^4 3,20 one	or.br.[a]	or.br.[a]	16α,17α,21 ol P^4 3,11,20 one	—[f]	—
21 ol P^4 3,20 one	y.	br.oc.[c]			

[a] With 15 μg steroid: blue.
[b] Increased sensitivity.
[c] With 50 μg steroid: red-orange; 7[illegible] μg: blue.
[d] After 5 min the blue colour disapp[illegible]rs.
[e] After longer spraying: yellow char[illegible]s to blue.
[f] With 20 μg steroid: feeble blue; 30 [illegible]g: blue.

Abbreviations:

bl.	= blue	br.oc.	= brown ochre	lg.v.bl.	= light violet blue
or.	= orange	or.br.	= orange brown	p.y.br.	= pale yellow brown
r.br.	= red brown	y.	= yellow	y.or.	= yellow orange.

The results of the application of this reaction to thirty-seven steroids are listed in Table IV. Some colours are not stable and the sensitivity varies from one steroid to another. The blue colour of *epi*-corticosterone disappears in 5 min and the yellow colour obtained with cortisol may change into blue after prolonged spraying with Lugol's solution without ether treatment.

According to SZENT-GYÖRGYI[61] the blue colour obtained with some steroids is due to a charge transfer, in which the complex (I_3^-), formed by the interaction of I_2 and KI, is an electron donor, and the steroids act as acceptors.

In this work a reaction is stated to be positive whenever a colour develops owing to the presence of a steroid. This is the same interpretation as used previously by KRITCHEVSKY AND CALVIN[28] and HOHENSEE AND HÜTTENRAUCH[25], who did not restrict the positive reaction to the classical blue colour obtained by MYLIUS[41] with cholic acid.

The sensitivity of this method is in our hands not in complete agreement with results previously reported using paper chromatography. For instance 17α-hydroxyprogesterone was detectable on plates at the level of 15 μg, while ZAFFARONI AND BURTON[64] and FUJISAKI *et al.*[19] could detect a much smaller quantity on paper. On the other hand 8 μg of 16α-hydroxyprogesterone could easily be spotted, although MCALEER AND KOZLOWSKI[39] could not detect less than 15 μg of this substance on chromatograms. In general the use of ether does not increase the sensitivity of the colour reactions on plates, even for those steroids for which ether had been shown to be an important enhancing factor on paper. On a thin-layer, for example, 75 μg of cortexone are necessary to obtain a blue colour, following spraying with ether.

The detection of cortisone on chromatoplates at the level of 15 μg confirms previous reports by ZAFFARONI *et al.*[65] and BASSIL AND BOSCOTT[3]. However, the use of iodine vapours[29] is not recommended for plate chromatograms, in agreement with the results previously reported by MATHEWS *et al.*[36].

DISCUSSION

For the development of spots on paper chromatograms of Δ^4-3-keto-C_{21}-steroids a large number of reactions has been available for a long time (see *e.g.* refs. 52, 56 and 42).

Some of the reactions presented here were especially developed for thin-layer chromatography, in order to increase the sensitivity or to make their application on chromatoplates possible.

Many of these reactions have been previously applied for a small number of Δ^4-3-ketopregnane-steroids only. The extension of these reactions to a large number of steroids under similar conditions permits an adequate study of their specificity as well as their easy application for identification of steroids.

Impurities sometimes make the application of a colour reaction on chromatoplates for the identification of steroids derived from biological extracts difficult.

A suitable method for purification of the steroids before their identification by means of the colour reactions here proposed consists of elution of the area which includes the substance, followed by the microsublimation of the steroid according to the technique of BREUER AND KASSAU[8]. After sublimation and prior to identification, the steroid must be rechromatographed to prove that it has not changed during the operation.

The R_F values in the eight solvent systems developed for Δ^4-3-keto-C_{21}-steroids, the application of the functional and individual colour reactions here described and the formation of derivatives, such as hydrazones, acetates, 17-keto-C_{19}-steroids and etiocholanic acids, also separable by thin-layer chromatography (unpublished results), constitute a good approach to steroid identification.

ACKNOWLEDGEMENTS

The author wishes to thank Doc. Dr. E. DICZFALUSY, head of the Hormone Laboratory, Karolinska Sjukhuset, Stockholm, for stimulating discussions, helpful advice and generous support in this work. For valuable discussions the author is also indebted to Dr. R. WILSON, Toronto, Canada.

The author gratefully acknowledges gifts of steroids from Dr. S. BERNSTEIN (American Cyanamid Co., Pearl River, U.S.A.), Prof. H. BREUER (Chirurgische Universitätsklinik Bonn-Venusberg, Germany), Dr. O. A. DE BRUIN (N.V. Philips-Duphar, Weesp, The Netherlands), Dr. J. JOSKA (Academy of Sciences, Prague, Czechoslovakia), Prof. K. JUNKMANN (Schering A.G., Berlin, Germany), Prof. W. KLYNE (Medical Research Council, London, Great Britain), Dipl. Eng. I. KÖNYVES (A.B. Leo, Hälsingborg, Sweden), Dr. R. NEHER, (Ciba Ltd. Basel, Switzerland), Prof. J. SCHMIDT-THOMÉ (Farbwerke Hoechst A.G., Frankfurt a.M.-Hoechst, Germany) and Prof. J. ZANDER (Köln University, Germany).

SUMMARY

The application of thirty-four reactions for the characterization of thirty-seven Δ^4-3-keto-C_{21}-steroids *in situ*, on thin-layer chromatograms, is described.

The methods involve the detection of (a) α,β-unsaturated ketosteroids; (b) reducing corticosteroids; (c) ketonic steroids; (d) 17α,21-dihydroxy-20-ketosteroids; (e) 17-deoxy-α-ketolic steroids; (f) formaldehydogenic steroids; (g) 21-deoxy-20-ketosteroids; (h) 17-hydroxy-20-keto-21-deoxysteroids and (i) individual steroids (no specific general reactions for steroids).

The sensitivity, specificity, optimal conditions and as far as possible, the reaction mechanisms are discussed. In some cases a comparison is made between the results of paper and thin-layer chromatography.

REFERENCES

1 Y. ABE, *J. Biochem. (Tokyo)*, 25 (1937) 181.
2 D. ABELSON AND P. K. BONDY, *Anal. Chem.*, 28 (1956) 1922.
3 G. T. BASSIL AND R. J. BOSCOTT, *Biochem. J.*, 48 (1951) xlviii.
4 H. S. BENNET, *Proc. Soc. Exptl. Biol. Med.*, 42 (1939) 768.
5 S. BERNSTEIN AND R. H. SILBER, in discussion to S. BERNSTEIN, *Recent Progr. Hormone Res.*, 14 (1958) 1.
6 M. K. BIRMINGHAM, *Nature*, 184 (1959) B.A. 67.
7 A. BODANSKY AND J. KOLLONITSCH, *Nature*, 175 (1955) 729.
8 H. BREUER AND E. KASSAU, *Acta Endocrinol., Suppl.*, 51 (1960) 1113.
9 R. B. BURTON, A. ZAFFARONI AND E. H. KEUTMANN, *J. Biol. Chem.*, 188 (1951) 763.
10 I. E. BUSH, *The Chromatography of Steroids*, Pergamon Press, Oxford, 1961.
11 V. ČERNY, *3rd. Conf. Inst. Chem., Prague, Dec. 21st, 1951*, quoted by Z. PROCHÁZKA, *Chem. Listy*, 47 (1953) 718.
12 O. CERRI AND A. SPIALTINI, *Boll. Chim. Farm.*, 96 (1957) 193.

13 E. CHABROL, R. CHARONNAT, J. COTTET AND P. BLONDE, *Compt. Rend. Soc. Biol.*, 115 (1934) 834.
14 R. CHARONNAT AND B. GAUTHIER, *Compt. Rend.*, 223 (1946) 1009.
15 R. I. COX, *Nature*, 181 (1958) 638.
16 H. DANNENBERG, *Abhandl. Preuss. Akad. Wiss., Math.-Naturw. Kl.*, 21 (1939) 3.
17 L. DORFMAN, *Chem. Rev.*, 53 (1953) 47.
18 F. FEIGL, *Spot Tests in Organic Analysis*, 5th Ed., Elsevier, Amsterdam, 1956.
19 M. FUJISAKI, Y. ARAI, T. KON AND M. ITOH, *Folia Endocrinol. Japon.*, 29 (1953) 148.
20 S. GOLDBERG AND M. FINKELSTEIN, *Bull. Res. Council Israel*, A5 (1956) 310.
21 A. G. GORNALL AND M. P. MACDONALD, *J. Biol. Chem.*, 201 (1953) 279.
22 E. P. HÄUSSLER, *Z. Physiol. Chem.*, 290 (1952) 155.
23 E. P. HÄUSSLER, *Z. Physiol. Chem.*, 291 (1952) 168.
24 R. D. H. HEARD AND H. SOBEL, *J. Biol. Chem.*, 165 (1946) 687.
25 F. HOHENSEE AND R. HÜTTENRAUCH, *Z. Physiol. Chem.*, 310 (1958) 19.
26 S. KATZ, *Arch. Biochem. Biophys.*, 91 (1960) 54.
27 S. KAWAGUCHI, *J. Biochem. (Tokyo)*, 28 (1938) 445.
28 D. KRITCHEVSKY AND M. CALVIN, *J. Am. Chem. Soc.*, 72 (1950) 4330.
29 D. KRITCHEVSKY AND M. R. KIRK, *Arch. Biochem. Biophys.*, 35 (1952) 346.
30 M. L. LEWBART AND V. R. MATTOX, *Anal. Chem.*, 33 (1961) 559.
31 B. P. LISBOA, *Acta Endocrinol.*, 43 (1963) 47.
32 B. P. LISBOA, *J. Chromatog.*, 13 (1964) 391.
33 B. P. LISBOA AND E. DICZFALUSY, *Acta Endocrinol.*, 40 (1962) 60.
34 B. P. LISBOA AND E. DICZFALUSY, *Acta Endocrinol.*, 43 (1963) 545.
35 J. M. MANARO AND A. ZYGMUNTOWICZ, *Endocrinology*, 48 (1951) 114.
36 J. S. MATTHEWS, V. A. L. PEREDA AND P. A. AGUILERA, *J. Chromatog.*, 9 (1962) 331.
37 V. R. MATTOX, H. L. MASON AND A. ALBERT, *Proc. Mayo Clinic*, 28 (1953) 569.
38 W. J. MCALEER AND M. A. KOZLOWSKI, *Arch. Biochem. Biophys.*, 62 (1956) 196.
39 W. J. MCALEER AND M. A. KOZLOWSKI, *Arch. Biochem. Biophys.*, 66 (1957) 125.
40 C. MONDER AND A. WHITE, *Endocrinology*, 68 (1961) 159.
41 F. MYLIUS, *Z. Physiol. Chem.*, 11 (1887) 306.
42 R. NEHER, *J. Chromatog.*, 1 (1958) 205.
43 R. NEHER AND A. WETTSTEIN, *Helv. Chim. Acta*, 34 (1951) 2278.
44 N. NELSON, *J. Biol. Chem.*, 153 (1944) 375.
45 W. J. NOWACZINSKI AND P. R. STEYERMARK, *Can. J. Biochem. Physiol.*, 34 (1956) 592.
46 I. OSTROMISSLENSKY, *J. Russ. Phys. Chem. Soc.*, 12 (1909) 731.
47 S. C. PAN, *J. Chromatog.*, 8 (1962) 449.
48 S. C. PAN, *J. Chromatog.*, 9 (1962) 81.
49 V. PELCOVÁ, unpublished results, quoted by O. SIBLÍKOVÁ, M. ČERNÁ AND I. M. HAIS, *Cesk. Farm.*, 11 (1962) 187.
50 C. C. PORTER AND R. H. SILBER, *J. Biol. Chem.*, 185 (1950) 201.
51 E. L. PRATT AND H. B. CORBITT, *Anal. Chem.*, 24 (1952) 1665.
52 L. M. REINEKE, *Anal. Chem.*, 28 (1956) 1853.
53 D. P. SCHWARTZ, *Anal. Chem.*, 30 (1958) 1855.
54 V. SCHWARZ, *Nature*, 169 (1952) 506.
55 G. M. SHULL, J. L. SARDINAS AND R. C. NUBEL, *Arch. Biochem. Biophys.*, 37 (1952) 186.
56 O. SIBLÍKOVÁ AND I. M. HAIS, *Cesk. Farm.*, 7 (1958) 1.
57 R. H. SILBER AND C. C. PORTER, *J. Biol. Chem.*, 210 (1954) 923.
58 R. H. SILBER AND C. C. PORTER, *Methods Biochem. Anal.*, 4 (1957) 139.
59 N. R. STEPHENSON, *Can. J. Biochem. Physiol.*, 37 (1959) 391.
60 R. STUPNICKI AND E. STUPNICKA, *J. Chromatog.*, 9 (1962) 235.
61 A. SZENT-GYÖRGYI, *Life Sciences*, (1963) 112.
62 A. WALSER AND H. P. SCHLUNKE, *Experientia*, 15 (1959) 71.
63 A. WERNER, *Ber.*, 42 (1909) 4324.
64 A. ZAFFARONI AND R. B. BURTON, *J. Biol. Chem.*, 193 (1951) 749.
65 A. ZAFFARONI, R. B. BURTON AND E. H. KEUTMANN, *Science*, 111 (1950) 6.
66 W. Z. ZIMMERMANN, *Z. Physiol. Chem.*, 233 (1935) 257.