DEMONSTRATION OF STEROIDAL FUNCTIONAL GROUPS ON PAPER CHROMATOGRAMS

I. KETONES AND ALCOHOLS

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Numerous methods are known for the detection of steroid spots on paper chromatograms[1-4]. Most of them involve the formation of colored products through reactions between the spray reagents and certain functional groups of the steroids. These methods could obviously serve not only the purpose of detecting the steroid spots, but also of demonstrating the presence of those functional groups. During our studies on the microbial conversions of steroids, it has been considered highly desirable that reagents for demonstrating those functional groups for which detection methods have so far been lacking can also be made available. Quite a few new reagents have since been found suitable for such purposes and are reported here as well as in subsequent papers.

As will be discussed more in detail below, the use of 2,4-dinitrophenylhydrazine for the detection of steroid ketones has not produced entirely uniform results among different workers. A study on the effectiveness of these known methods was therefore undertaken, leading to the development of two procedures which were shown to be applicable to all types of steroid ketones. Description of these two procedures will constitute the first part of the present report.

STEROID KETONES

While the detection of Δ^4-3-ketones by U.V. scanning[4,5] and of 17-ketones by the Zimmerman reaction[4] have been widely adopted, the use of 2,4-dinitrophenylhydrazine (DNPH*) for detecting steroid ketones in general produced rather variable results. AXELROD[6,7], using a reagent in aqueous HCl, recognized it as specific for 3-ketones, while SAVARD[8] used virtually the same reagent for the detection of 3- and 20-ketones. REINEKE[9] and KOCHAKIAN AND STIDWORTHY[10] used a reagent in an acidified alcoholic solution and concluded that a 3-ketone is the most reactive while the reactivity of a 20-ketone is affected by neighboring groups. Both REINEKE[9] and NEHER[4] recognized an order of sensitivity among different steroid ketones toward DNPH with 17-ketones, α-ketols and $\Delta^{1,4}$-3-ketones as the least reactive.

While testing these reported procedures, it was recognized that the low sensitivity of a 17-ketone or an α-ketol is not due to the difficulty with which these ketones form

* 2,4-Dinitrophenylhydrazine is abbreviated as DNPH throughout this paper.

hydrazones*, but rather to the fact that their light yellow colored hydrazones are not well distinguished from the yellow background (DNPH color). As will be described below, when the background color is efficiently washed off, practically all steroid hydrazones become readily discernible spots, 11-ketones being an exception.

Neher's washing procedure

NEHER's "unpublished procedure"[4] was found very effective for washing off the background color. Since he did not describe the details, the procedure used in the present study is given below:

After the steroids are chromatographed with proper solvent systems (*cf.* Table I), the dried papergram** is drawn through a solution prepared by dissolving 3 g DNPH in 900 ml methanol containing 3 ml conc. HCl. After being heated at 90° for 5 min, the strip is drawn back and forth four times through 2 *N* aqueous NaOH and then through water until most of the dark purplish color is washed away. The washing is repeated with 2 *N* aqueous HCl and water.

The data from this experiment are presented in Table I. The Δ^4-3-ketones appear as orange and $\Delta^{1,4}$-3-ketones appear as deep yellow spots immediately after heating. The background becomes light yellow after washing and all steroid ketones, including 3-, 17- and 20-ketones become readily discernible yellow spots. Substitutions at 17- and 21-positions with hydroxyl or acetoxyl groups do not affect the "sensitivity" of the 20-ketones.

Since in this procedure the background is not free from the yellow DNPH color, steroid ketones forming very light yellow hydrazones will remain undetectable. The hydrazones of the "hindered" 3-ketones of the triterpenoid series, such as the chromic acid oxidation products of eburicoic acid and lanosterol (*cf.* ref. 11) were found to be of this type. Of course, other explanations of their undetectability might also be possible. For these ketones, the following procedure was found effective.

Washing procedure for use with aqueous DNPH reagent

The dried papergram is placed in a shallow dish and flooded with a reagent prepared by dissolving 1 g of DNPH in 100 ml conc. HCl with warming and diluting to 1 l with water. One hour later, the strip is washed first with 6 *N* HCl once and then four times with water. The background becomes almost colorless.

This procedure was developed for the following reason: The reaction between DNPH and the hindered 3-ketones is slow, while it is hard to wash off the DNPH from the paper after complete or partial drying. Immersion in the aqueous DNPH reagent for one hour permits the completion of the reaction, yet keeps the paper wet. As will be expected, most steroid ketones can also be detected by this procedure. However, it was shown that a 20-ketone flanked by hydroxyl or acetoxyl groups on both sides failed to give a positive test. It is not known whether this is due to the interference by the substituent groups or to the fact that those hydrazones are easily washed off the paper. A $\Delta^{1,4}$-3-ketone also fails to respond to this test.

The data from this experiment are also presented in Table I. It can be seen that as a general detection method for steroid ketones, NEHER's procedure proves effective. When a hindered 3-ketone is suspected, the washing procedure for aqueous DNPH

* 2,4-Dinitrophenylhydrazone is abbreviated as "hydrazone" throughout this paper.

** ZAFFARONI type papergrams are dried in a 100° oven equipped with a forced air draft system.

TABLE I

DEMONSTRATION OF STEROID KETONES WITH 2,4-DINITROPHENYLHYDRAZINE REAGENTS

Compounds	*Structure tested*	*Solvent system*	R_F	*Neher's method*		*DNPH in aq. HCl plus washing*
				Before washing	*After washing*	
3-Keto-Δ^4-pregnen-20β-ol	Δ^4-3-ketone	TPG	0.75	OR	OR	OR
5α-Pregnane-3,20-dione[b]	3,20-dione	MCHC	0.52	—	Y	Y
5α-Cholestan-3-one[b]	3-ketone	KP	0.45	—	Y	Y
Pregnenolone[b]	20-ketone	TPG	0.75	—	Y	Y
Androsterone[b]	17-ketone	MCHC	0.14	—	Y	Y
Dehydroepiandrosterone acetate[b]	17-ketone	MCHC	0.65	—	Y	Y
3β,21-Dihydroxy-Δ^5-pregnen-20-one[a]	20-one-21-ol	TPG	0.32	—	Y	Y
21-Acetoxy-5α-pregnan-20-one[a]	21-acetoxy-20-one	MCHC	0.83	—	Y	Y
3β,17α-Dihydroxy-5α-pregnan-20-one[a]	17α-ol-20-one	TPG	0.16	—	Y	—
3β,17α,21-Trihydroxy-Δ^5-pregnen-20-one[a]	17α,21-diol-20-one	HTMW	0.23	—	Y	—
3β,17α-Dihydroxy-21-acetoxy-5α-pregnane-11,20-dione[a]	21-acetoxy-17α-ol-20-one	HTMW	0.27	—	Y	—
3α,11β,17α,21-Tetra-hydroxy-5β-pregnan-20-one[a]	17α,21-diol-20-one	CMW	0.63	—	Y	—
Δ^1-Testololactone[b]	$\Delta^{1,4}$-3-ketone	TPG	0.46	Y-OR	Y-OR	—
Δ^1-Cortexolone[b]	$\Delta^{1,4}$-3-ketone	HTMW	0.24	Y-OR	Y-OR	—
3-Keto-17α-hydroxy-$\Delta^{1,4}$-etiadienic acid[d]	$\Delta^{1,4}$-3-ketone	CMA	0.20	Y-OR	Y-OR	—
$\Delta^{8,24}$-Lanostadien-3-one[c]	4α,4β-dimethyl-3-one	KP	0.83	—	—	Y
$\Delta^{8,24(28)}$-Ergostadien-3-one-21-oic acid[c]	4α,4β-dimethyl-3-one	BEPEG	0.42	—	—	Y
Pregnan-11-one[a]	(tested by direct spotting)			—	—	—

TPG = toluene/propylene glycol system[4]; MCHC = methyl cyclohexane/carbitol system[4]; KP = kerosene/2-phenoxyethanol system[4]; CMW = chloroform–methanol–water (2:1:2) by TUZSON's technique[12]; CMA = chloroform–methanol–1 *N* NH_4OH (1:1:1)[11]; BEPEG = *n*-butyl ether–piperidine/ethylene glycol–piperidine system[11]; HTMW = BUSH B-1, system at 37°, *n*-hexane–toluene–methanol–water (5:5:7:3)[4]. Y = yellow; OR = orange; Y-OR = deep yellow.

[a] From U.S.P. reference standards.

[b] From Squibb steroid collection or commercial sources.

[c] Chromic acid oxidation products of lanosterol and eburicoic acid by microchemical techniques (see refs. 11, 13). These compounds are not isolated and the major spot is regarded as representing the compounds listed.

[d] Periodate oxidation product of Δ^1-cortexolone by the same microchemical technique[11,13].

reagent must be resorted to. Although no attempt has been made to correlate the hydrazone color with the structure of the steroid ketones, it is to be noted that a Δ^4-3-ketone forms the well known characteristic orange hydrazone by either procedure. The sharp contrast between the results obtained by applying these two procedures to a $\Delta^{1,4}$-3-ketone spot is, however, remarkable (*cf.* Table I). A yellow-orange hydrazone is produced with NEHER's procedure while a virtually negative result is obtained with aqueous DNPH. Such a treatment could very well be used as a characteristic test for a $\Delta^{1,4}$-3-ketone in the absence of other ketone groups. An 11-ketone is known to be inert and does not respond to the DNPH reaction by either procedure.

It should also be mentioned in this connection that for applying the washing techniques, it is more convenient to use the small scale paper chromatogram as described by UNDERWOOD AND ROCKLAND[14] as well as in another publication from this laboratory[11].

Deepening of the hydrazone color

After either washing procedure, the light yellow hydrazone spot can be made more conspicuous by either of the following two procedures:

(a) Dip the dried paper strip in 1 *N* alcoholic NaOH[15]. The spots become dark reddish-brown on a light violet background.

(b) Dip the paper strip in 1 % $SnCl_2$ solution in 1 *N* HCl. Allow the strip to air dry for one hour. Spray with a reagent containing 500 mg of *p*-dimethylamino-benzaldehyde and 1 ml of conc. HCl in 100 ml of 95 % ethanol[16,17]. The spots become deep orange-brown on a light pink to brown background.

STEROID ALCOHOLS

By means of the methods developed for detecting steroid ketones, a steroid alcohol (secondary) which contains no ketone groups can naturally be detected by oxidation to a ketone with chromic acid directly on a papergram (*cf.* ref. 18). AXELROD described such a method[7]. However, the filter paper after treatment with chromic acid generates some ketonic compounds which form yellow hydrazones with DNPH. If the steroid ketone produced by oxidation forms orange hydrazone, AXELROD's method will offer a ready means for its detection. For those steroid alcohols from which the ketones form only yellow hydrazones, the spot cannot be differentiated from the background. The procedure described below can solve such a problem.

A part of the papergram bearing the spot of a steroid alcohol is sprayed with a reagent containing 5 mg of CrO_3 and 8 mg of conc. H_2SO_4 per ml of 95 % acetic acid, while the other part of the paper strip is covered with a piece of cardboard as shown by the diagram of Fig. 1. One hour later, when the chromic acid is completely consumed

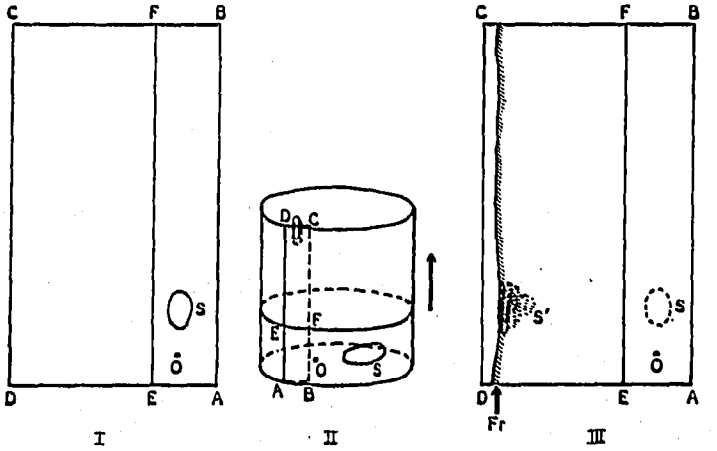

Fig. 1. Detection of steroid alcohols by direct chromic acid oxidation. O = origin; S = spot of a steroid alcohol; S′ = ketone spot detected; Fr = front of 1:1 MeOH–$CHCl_3$ irrigation. (I) Area ABFE is sprayed with a CrO_3 reagent while area EFCD is covered with a cardboard. (II) The paper strip is rolled to form a cylinder and irrigated with 1:1 MeOH–$CHCl_3$ in the direction of the arrow-head. (III) Treat the area EFCD with DNPH reagent, with area ABFE cut off.

by the paper, the strip is rolled to form a hollow cylinder and placed in a jar containing a shallow layer of a 1:1 mixture of methanol–chloroform in the same manner as the development of an ascending chromatogram, as described by WILLIAMS AND KIRBY[19]. The ketone spots now move with the solvent front. The dried strip is then treated with DNPH as described above. A yellow hydrazone spot in the solvent front would indicate the presence of an alcohol spot on the original papergram (see Fig. 1).

For papergrams developed with ZAFFARONI type systems, it was found necessary to wash the hot air dried paper chromatogram with water to completely remove any residual non-volatile solvent*. Washing by descending solvent flow as in chromatography was found more efficient than washing by rinsing. By the use of this technique, positive results were obtained with cholestanol and lanosterol on papergrams developed with a kerosene/2-phenoxyethanol system**[4,20–22], desoxycholic acid on a chloroform–methanol–1 *N* NH_4OH system (1:1:1)[11] and eburicoic acid on a *n*-butyl ether–piperidine/ethylene glycol–piperidine system[11]. Although all these compounds tested are 3-ols, the method could be applicable to other steroid alcohols provided interfering groups are absent.

It should be noted that even after washing, there is always a narrow band of DNPH positive material present at the front of methanol–chloroform irrigation. This does not interfere with the test as the steroid ketones appear as outstanding spots easily differentiated from the band (see Fig. 1).

METHYL KETONES

Steroids with an acetyl side chain undergo reactions characteristic for a methyl ketone. Although the well-known iodoform test[23] works well in test tubes, it is not easily adapted to the detection of a steroid spot on a papergram. Another characteristic reaction, involving the use of sodium nitroprusside and Na_2CO_3, was originally developed for water soluble methyl ketones[24]. STANLEY AND TAFT recently modified the reagent so that it becomes applicable to water insoluble methyl ketones[27]. It was found that this modified method not only works well with steroid methyl ketones in test tubes but can be adapted to their detection directly on a papergram. The detailed procedure is as follows:

Mix intimately in a shallow dish: 6 parts (by wt.) of powdered sodium nitroprusside, 100 parts (by wt.) of anhydrous Na_2CO_3 and 100 parts (by wt.) of ammonium acetate. Add enough methanol to make a thin paste and spread the paste uniformly over the bottom of the dish. Lay the dried papergram upon the paste. There should be no visible excess liquid when the paper is thoroughly wetted. For a 7 in. × 12 in. dish, use approximately 2.4 g of sodium nitroprusside, 40 g of Na_2CO_3, 40 g of ammonium acetate and 30–35 ml of methanol. Cover the dish with a metal or glass plate to reduce evaporation and set it aside for 1 h. Methyl ketones appear as deep violet spots on an almost colorless background. A 5 γ progesterone spot occupying an area of 1 cm^2 can be readily detected.

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* This washing technique is applicable only to highly non-polar steroids, *e.g.* cholesterol or eburicoic acid. More polar steroids are washed off the paper by water and the present test is applicable only after they are chromatographed on the BUSH type systems.

** This is essentially the *n*-heptane/2-phenoxyethanol system. More satisfactory results were obtained by substituting kerosene for *n*-heptane.

Different steroidal methyl ketones were tested with this method and the results are summarized in Table II. It can be seen that steroids which are not methyl ketones all fail to show the characteristic color, while all methyl ketones were positive, including progesterones variously substituted with hydroxyl- or oxo-groups. The only exceptions are 16α, 17α-dihydroxyprogesterone and its acetonide, both giving very weakly positive results, and 16-oxo- and Δ^{16}-progesterone which completely fail. These exceptions are understandable on the ground that the methyl ketone structure is severely influenced by the neighboring substituents.

TABLE II

METHYL KETONE TEST

Compounds[a]	*Substituent groups*	*Results*
5(α or β)-Pregnane-3,20-dione		+
Progesterone	none	+
Progesterone[b]	−OH at 6β,19,7α,11α,11β,12α, 15α,16α,17α	+
Progesterone[b]	16α, 17α-di-OH	±
Progesterone[b]	16α, 17α-di-OH, acetonide	±
Progesterone[b]	oxo- at 6, 11, 12, 15, 19	+
Pregnenolone		+
A-Norprogesterone[c]		+
Progesterone	21-OH	—[e]
Androstenedione		—[e]
Testololactone[b]		—
Testosterone		—
Progesterone	Δ^{16}-, 16-oxo	—[e]
Pregnenolone	Δ^{16}-	—
Progesterone	16α, 17α-oxido	+ (deep green-blue)
5α-Pregnane-3α,20α-diol[d]		—

[a] The compounds used in these tests, besides those specified below, were obtained commercially.
[b] These steroids are from the Squibb steroid collection, mostly conversion products by microorganisms.
[c] Synthesized by WEISENBORN AND APPLEGATE[25].
[d] U.S.P. reference standards.
[e] These compounds appear as tan colored spots. The test is considered negative.

It should be noted that 16α, 17α-oxidoprogesterone gives a strongly positive test although the color is different—deep green-blue instead of blue-violet. Since Δ^{16}-progesterone can be readily converted to 16α, 17α-oxidoprogesterone by alkaline hydrogen peroxide[26], the following procedure has been developed for showing the Δ^{16}-20-one structure. The dried papergram is sprayed with a reagent containing 1.2 ml 30% H_2O_2, 0.2 ml 6 *N* KOH in 20 ml methanol. The sprayed strip is immediately suspended in a closed jar containing some of the reagent on the bottom for 4 h at room temperature. It is then air dried and tested with the method given above. Both Δ^{16}-progesterone and Δ^{16}-pregnenolone appear as deep green-blue spots. It is obvious that a spot becomes diffuse on the alkaline H_2O_2 treatment. For spots which are already quite diffuse, it was found necessary that they be eluted and respotted on a piece of filter paper before applying the alkaline H_2O_2 reagent.

SECONDARY METHYL CARBINOLS

From the methods described above, it is clear that a secondary methyl carbinol can also be demonstrated by the procedure described above for steroid alcohols. The methyl ketone test instead of the DNPH treatment after the irrigation with 1:1 methanol–chloroform is applied. Such a procedure has been shown to work satisfactorily for a spot of 5α (or β)-pregnane-3β,20 α-diol or 20β-hydroxy-Δ^4-pregnen-3-one on a papergram of toluene/propylene glycol system. No washing of the papergram is necessary as methyl ketones are not easily generated from common solvents or impurities in the filter paper.

DISCUSSION

It is clear from the description above that these tests definitely can serve the purpose of detecting spots on papergrams as well as demonstrating the presence of certain functional groups. When spots are detected by other more convenient means, such as U.V.-scanning for a Δ^4-3-ketone or phosphomolybdic acid[11] for most steroids, the tests described here can be used just to reveal these functional groups. For this purpose, often a sliver of the spot is enough for a test. Of course, it should be cautioned that the use of a sliver is not recommended for testing a steroid alcohol, including the secondary methyl carbinol, because the solvent front always shows a positive test.

It is obvious that the methods for ketones are generally applicable regardless of the presence of hydroxyl-groups. The method for an alcohol is applicable only when the compound contains no pre-existing ketone groups and the chromic acid oxidation product is a ketone. For naturally occurring steroids, only the 3-hydroxyl-group of sterols and related compounds meets these requirements.

It is always advisable to test a known compound which will give a positive result and another which will give a negative result as controls when an unknown is being tested with the methods reported here.

SUMMARY

Simple and effective methods are developed for detecting or demonstrating:

1. A steroid ketone spot on a paper chromatogram by applying a 2,4-dinitrophenylhydrazine reagent with subsequent washing.

2. A steroid alcohol by direct chromic acid oxidation on the paper, permitting the ketone produced to migrate out of the area sprayed with chromic acid and detecting the ketone formed with 2,4-dinitrophenylhydrazine.

3. A methyl ketone with a sodium nitroprusside reagent.

4. A secondary methyl carbinol by direct chromic acid oxidation and detection of the methyl ketone formed.

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