DEMONSTRATION OF STEROIDAL FUNCTIONAL GROUPS ON PAPER CHROMATOGRAMS

II. α-KETOLS AND GLYCOLS

S. C. PAN

Squibb Institute for Medical Research, New Brunswick, N.J. (U.S.A.)

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As a continuation of a previous communication[1], reporting the search for spot test methods to demonstrate certain functional groups of steroids on paper chromatograms, the present paper will deal with methods for detecting an α-ketol or a *vic*-glycol grouping.

Periodate has been widely used for the detection of polyols and non-reducing sugars[2–5], deoxysugars[6–8] and hydroxy-amino acids[9] on paper chromatograms. These methods are developed either on the basis that periodate is consumed by the polyols or that characteristic products—formaldehyde, acetaldehyde or malonaldehyde—are formed on oxidation. Periodate oxidation of steroidal α-ketols and 1,2-diols yields similar products. In fact, the identification of steroidal α-ketol and 1,2-diol structures by virtue of the characteristic oxidation products has been thoroughly studied and widely applied[10,11]. However, besides the demonstration of the formation of a 17-ketone through periodate oxidation of a 17,20-diol[12,13], no methods have been reported to identify other oxidation products directly on a paper chromatogram. In the present paper, four tests, namely, those for formaldehydogenic, acetaldehydogenic, aldehydogenic (non-volatile) and acidogenic steroids, which can be directly applied to papergrams will be described.

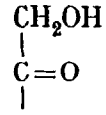
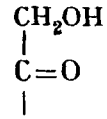
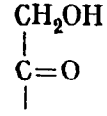
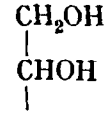
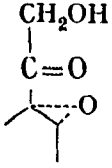
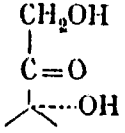
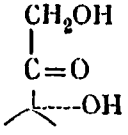
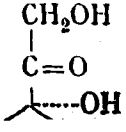
TEST FOR FORMALDEHYDOGENIC STEROIDS

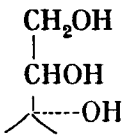
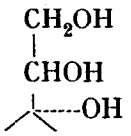
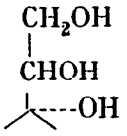
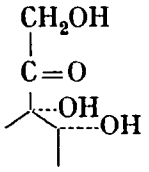
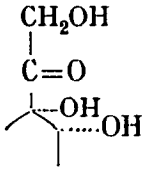
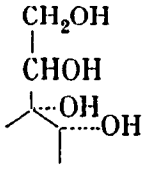
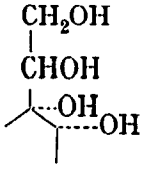
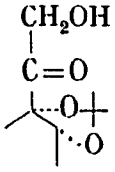
Steroids (C_{21}) with a 21-ol-20-one or a 20,21-diol structure produce formaldehyde on periodate oxidation[10,11]. The procedure described below is adapted from the method of SCHWARTZ for the detection of serine on papergrams[9].

The dried papergram is sprayed with a reagent consisting of 1 vol. of a saturated aqueous potassium periodate solution plus 3 vol. of 95 % ethanol. Four to eight minutes later, the partially dried paper is sprayed with a freshly prepared reagent containing 15 g of ammonium acetate, 0.3 ml of glacial acetic acid and 1 ml of 2,4-pentanedione in 100 ml of methanol. Ten minutes after the second spray, a formaldehydogenic steroid appears as a yellow-green fluorescent spot on a dark background under U.V. ("Mineral Light"). The spot becomes yellow under ordinary light on a colorless background in 30–60 min. The background gradually turns yellow on longer standing. The development of the yellow color at the end of 30 min is regarded as a more reliable

TABLE I

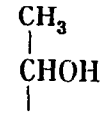
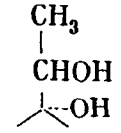
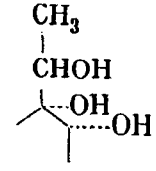
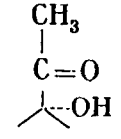
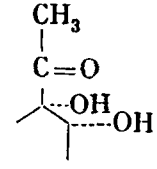
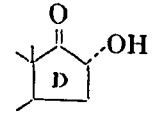
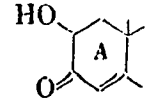
RESULTS OF TESTS FOR STEROIDAL α-KETOLS AND GLYCOLS

Compound	*Structure under test*	*Solvent system*	R_F	*Test for formaldehydogenic steroids*	*Test for acetaldehydogenic steroids*	*Test for aldehydogenic steroids*	*Test for acidogenic steroids*	*Phenol red borate test*	*Tetrazolium reduction test*
Cortexone[a]		HTMW	0.81	+	—	—	+	—	+
3β,21-Dihydroxy-5α-pregnan-20-one[b]		HTMW	0.74	+		—	+	—	+
Corticosterone[a]		HTMW	0.34	+			+	—	+
20β-Dihydrocortexone[c]		HTMW	0.39	+		+	—	—	—
16α,17α-Oxidocortexone[a]		HTMW	0.76	+			+		—
Cortexolone[a]		BMW	0.70	+	—	—	+	—	+
3β,17α,21-Trihydroxy-Δ[5]-pregnen-20-one[b]		BMW	0.59	+			+	—	+
Cortisol[a]		BMW	0.12	+		—	+	—	+

20β-Dihydrocortexolone[c]		BMW	0.35	+	±	—	—	—
20β-Dihydrocortisol[c]		CMW	0.52	+	+	—	—	—
3α,17α,20α,21-Tetrahydroxy-5α-pregnan-11-one[b]		CMW	0.56	+	±	—	—	—
16α-Hydroxy-cortexolone[a]		CMW	0.88	+	—	+	+	+
Triamcinolone[a]		CMW	0.22	+	—	+	+	+
16α,17α,20β,21-Tetrahydroxy-Δ^4-pregnen-3-one[c]		CMW	0.66	+	+	—	+	—
9α-Fluoro-11β,16α,17α,20β,21-pentahydroxy-Δ^4-pregnen-3-one[c]		CMW	0.15	+	+	—	+	—
Triamcinolone-16α,17α-acetonide[a]		HTMW	0.21	—		—	—	+

(continued on p. 84)

TABLE I (*continued*)

Compound	*Structure under test*	*Solvent system*	R_F	*Test for formaldehydogenic steroids*	*Test for acetaldehydogenic steroids*	*Test for aldehydogenic steroids*	*Test for acidogenic steroids*	*Phenol red borate test*	*Tetrazolium reduction test*
3-Keto-Δ^4-pregnen-20β-ol[a]		HTMW	0.88	—	—			—	—
3-Keto-Δ^4-pregnene-17α,20β-diol[c]		HTMW	0.53	—	+			—	—
3-Keto-Δ^4-pregnene-16α,17α,20β-triol[c]		HTMW	0.22	—	+			+	—
17αHydroxy-progesterone[a]		HTMW	0.72	—	—	—		—	—
16α,17α-Dihydroxyprogesterone[a]		HTMW	0.59	—	—	+	—	+	—
16α-Hydroxyandrostenedione[a]		HTMW	0.50	—	—	+	+	—	+
2β-Hydroxytestosterone[a]		HTMW	0.45	—		+	—	—	+

direct spotting)			—	—	—	—	—	—
Androstenedione[a] (tested by direct spotting)			—	—	—	—	—	—
Testosterone[a]	HTMW	0.70	—	—	—	—	—	—
Δ^1-Testotolactone[a]	HTMW	0.40	—	—	—	—	—	—

HTMW = Bush B-1 system, *n*-hexane being used in place of petroleum ether, run at 37°; BMW = Benzene–methanol–water (1:1:1), by Tuzson's technique[14]; CMW = Chloroform–methanol–water (2:1:2) by Tuzson's technique[14].

[a] From Squibb steroid collection including those obtained commercially and those produced by microbial conversions.
[b] From USP reference standards.
[c] Prepared by sodium borohydride reduction of the corresponding 20-ones. See text under the section of acetaldehydogenic steroids.

criterion than the yellow fluorescence produced in 10 min after the second spray. Certain non-formaldehydogenic steroids, *e.g.* 16α-hydroxyandrostenedione and 16α, 17α-dihydroxyprogesterone, produce a doubtful trace of fluorescence but no characteristic bright yellow color.

The compounds used in studying this test are listed in Table I. All steroids with a 21-ol-20-one or 20,21-diol structure respond well to this test while those without either of these two structures give negative results. Steroids in which an α-ketol side-chain is joined to an isopropylidene-dioxy-group (a 16,17-acetonide) form exceptions to this test. A separate experiment, carried out in test tubes, showed that such a "hindered" α-ketol resists oxidation in a dilute potassium periodate solution; more drastic conditions, *e.g.* treatment with a 2 % periodic acid solution at 37° overnight, is required for oxidation. It is not known whether a hindered 20,21-diol behaves similarly.

A 10 γ cortexone spot on an area of 1 sq. cm can be readily demonstrated on a papergram. The method is not applicable to ZAFFARONI type papergrams because traces of non-volatile solvent or impurities contained therein are often also formaldehydogenic.

TEST FOR ACETALDEHYDOGENIC STEROIDS

Only the 21-unsubstituted C_{21} steroids with a 17,20-diol structure yield acetaldehyde on periodate oxidation. The procedure developed, which is a modification of SCHWARTZ's[9] method for threonine is as follows.

The dried papergram is sprayed with a reagent prepared by dissolving 2.5 g of periodic acid in 10 ml of water and diluting the resulting solution with 90 ml of *tert.*-butanol. Three to seven minutes later, the partially dried paper is sprayed with a freshly prepared reagent containing 2 g of sodium nitroprusside, 15 ml of piperidine in 100 ml of methanol. Ten to fifteen minutes after the second spray, an acetaldehydogenic steroid appears as a blue spot on a straw colored background. The whole background turns blue after 20 minutes. Exposing the sprayed paper strip momentarily to ammonia vapor intensifies the blue color.

As shown by the data given in Table I, only 3-keto-Δ^4-pregnene-17α,20β-diol and 3-keto-Δ^4-pregnene-16α,17α,20β-triol, among all the compounds tested showed a positive reaction. These two compounds were prepared on a microscale[15-17] by reducing 17α-hydroxyprogesterone and 16α,17α-dihydroxyprogesterone respectively with sodium borohydride using the method of NORYMBERSKI AND WOODS[18] as described below. The fact that no steroids tested besides the two mentioned showed a positive reaction conclusively demonstrated that the method is specific for acetaldehydogenic steroids.

To a solution containing 500 γ of 17α-hydroxyprogesterone in 0.1 ml of methanol which had been cooled to 4° in a cold room, 0.1 ml of a freshly prepared 0.1 % solution of $NaBH_4$ in methanol which had also been cooled to 4° was added. The test tube was closed with a polyethylene stopper and kept at 4° for one hour. The reaction was stopped by the addition of 0.1 ml of 3 *M* acetic acid in water and the steroids were extracted with 2.5 ml of chloroform after the addition of 0.7 ml of a saturated solution of ammonium sulfate. The chloroform extract was evaporated to dryness with the aid of a gentle current of air (*cf.* ref. 19). The residue was taken up in a small volume of

1:1 methanol–chloroform and an aliquot was chromatographed. The major spot which was detectable on a HAINES' U.V.-scanner[20] and moved with an R_F value considerably lower than that of 17α-hydroxyprogesterone was taken to represent the expected product—3-keto-Δ^4-pregnene-17α,20β-diol. The compound itself was not isolated in pure form. Other steroidal 20β-ols, as listed in Table I, were prepared by exactly the same procedure.

The nitroprusside reaction for acetaldehyde and secondary amines is well known[21]. It has been widely used for the determination of acetaldehydogenic glycols[22], in-including steroids[23]. As a spray reagent for paper chromatography, HULME AND ARTHINGTON used it for the detection of proline[24]; WALDRON and EDWARD used it for 6-deoxysugars[6,7] and SCHWARTZ used it for threonine[9]. The method reported here is just another example for its adaptation as a spray reagent. The use of *tert.*-butanol as the solvent for periodic acid is probably the only unique feature of the present technique. In fact, other lower alkanols can also be used; *tert.*-butanol seems to give the most satisfactory result.

A 10γ papergram spot of 3-keto-Δ^4-pregnene-17α,20β-diol occupying an area of 1 sq. cm. is readily detectable. The method is again not applicable to ZAFFARONI type papergrams.

TEST FOR ALDEHYDOGENIC STEROIDS

A steroid containing a secondary α-ketol or a *vic*-glycol structure, except a 17,20-diol, generates a non-volatile aldehyde on periodate oxidation. It is obvious that such an aldehyde cannot be detected by double spraying technique as used in the tests described above for formaldehydo- or acetaldehydogenic steroids because the cellulose of the paper also produces aldehydes on oxidation. This problem can obviously be solved by using the same technique developed for demonstrating a steroid alcohol by direct chromic acid oxidation on a papergram[1].

The part of the papergram bearing the spot to be tested is sprayed with a reagent prepared by dissolving 2.5 g of periodic acid in 10 ml of water and diluting the resulting solution with 90 ml 95 % ethanol. The other part of the papergram is covered with a piece of cardboard at the time of spraying. (See the drawings in a previous paper[1].) Immediately after spraying, the paper strip is rolled on the form of a hollow cylinder and kept for four hours in an atmosphere saturated with 95 % ethanol. Upon removal, it is air dried and then transferred, still in the form of a cylinder, to another jar which contains a shallow layer of 1:1 methanol–chloroform mixture on the bottom. An ascending type chromatogram is developed until the solvent front moves at least 2 in. beyond the area which has been wetted by the periodic acid solution. The strip is again air dried, the part which has been wetted by the periodic acid solution is cut off and the rest is sprayed with Schiff's reagent[25]. One hour later the air dried strip is placed in a closed container to prevent the evaporation of sulfur dioxide and left at room temperature overnight. The aldehyde spot now situated in the front becomes deep blue on an almost colorless background. Traces of red or purple areas along the front are not considered as a positive result.

This test is applicable to papergrams developed with both ZAFFARONI* and BUSH systems. The compounds used in this study are also listed in Table I. Both side-chain or ring *vic*-glycols and secondary α-ketols respond well to this test. The 16α,17α-diol

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

structure of triamcinolone and related compounds constitutes an exception. A separate experiment carried out in test tubes showed that the 16α,17α-diol structure of triamcinolone is destroyed after the treatment with periodic acid but the product gives a negative Schiff's test. Probably rearrangement similar to that reported by SMITH *et al.*[26] could have taken place during the oxidation, yielding a non-aldehydic product.

Repeated tests with steroids with a 17α,20,21-triol structure showed that the results were variable (see Table I). Apparently the factors influencing the rate of oxidation which in turn determines whether a 17-ketone or a 17-aldehydo structure would be formed as the end product are not easily controlled. In view of this variation together with the failure of the 16α,17α-diol structure of triamcinolone to give a positive test, it is to be emphasized that in using this test, although a positive result indicates unequivocally the presence of a 1,2-diol or a *sec.*-α-ketol structure, a negative result does not necessarily mean the absence of such groupings.

Although Schiff's reagent has been used for detecting certain steroidal aldehydes[27], is was found in the present study that the reaction between Schiff's reagent and a water-insoluble steroidal aldehyde is very slow. It is therefore essential to keep the sprayed and dried paper strip inside a closed container to keep the background from turning deep red due to the loss of sulfur dioxide. Many new reagents are known for detecting aldehydes[28–30]. Schiff's reagent has been regarded as a rather standard reagent for aldehydes, and has been used in the present study; it is entirely possible that the newer reagents might be more advantageous.

TEST FOR ACIDOGENIC STEROIDS

Periodate oxidation of a steroidal α-ketol generates a non-volatile acid as one of the products. It is considered feasible to detect the acid formation from readily oxidizable neutral steroids directly on a papergram. The following procedure proves satisfactory for such a test.

The dried papergram is sprayed with a reagent containing 1 vol. of a saturated aqueous KIO_4 solution and 3 vol. of 95 % ethanol. One hour later, the paper strip is sprayed with the phenol red–Tris buffer reagent developed for steroid acids[31]. The acidogenic steroids appear immediately as yellow spots on a light pink background. The spots become more conspicuous on drying.

From the results given in Table I, it can be seen that all primary steroid α-ketols respond well to this test. Secondary α-ketols, *e.g.* 2β-hydroxytestosterone, give negative results. Separate microchemical experiments carried out in test tubes showed that these α-ketols are not readily oxidized by a brief treatment with a dilute KIO_4 solution. It is also to be noted that a ring D α-ketol, namely 16α-hydroxyandrostenedione, gives a strong positive test and as discussed under the test for formaldehydogenic steroids, a side chain α-ketol joined to a 16,17-acetonide, namely triamcinolone-16,17-acetonide, is not expected to respond to this test.

This test is also not applicable to ZAFFARONI type paper chromatographs.

PHENOL RED BORATE BUFFER SPRAY FOR CERTAIN STEROID GLYCOLS

This test is a direct adaptation of the methods reported by BRADFIELD AND FLOOD[32] and HOCKENHULL[33] for polyols. The reagent is prepared by mixing 2 ml of a 0.1 %

solution of phenol red in 95% ethanol, 1 ml of an aqueous 0.15 *M* H_3BO_3 and 20 ml of methanol. To this mixture, 0.1 *N* NaOH is added dropwise until the solution becomes pink and does not produce any more perceptible change in color on further addition of 1 to 2 drops. Immediately after spraying, steroidal ring *cis*-1,2-diols appear as yellow spots on a light pink background. The spots become more conspicuous on drying. To exclude the possibility that the spot could be an acid, another identical strip should be sprayed with phenol red–Tris buffer reagent[31] where a glycol would not change the color of the indicator.

As shown by data listed in Table I, all steroid 16α,17α-diols responded to this test without exception, while steroids with a 20β,21-diol, a 17α,20α,21-triol, or a 17α,21-diol-20-one structure gave negative results. These results indicate that a ring *cis*-1,2-diol structure is required for a positive test, agreeing with the information known for carbohydrates[34]. A 1,2- or a 1,3-diol in the side chain apparently does not lower the pH far enough to turn the color of phenol red, although it is known that such glycols also form "chelated orthoborates"[35]. No steroidal diaxial 1,3-diols were tested. It is therefore not known if such a structure would respond to the borate test. In view of the fact that a steroidal diaxial 1,3-diol forms an acetonide[36] readily, such a possibility must be taken into consideration in interpreting a positive result obtained with this test.

This test is applicable to papergrams of both BUSH and ZAFFARONI* types. A 10 γ papergram spot of 16α,17α-dihydroxyprogesterone on an area of 1 sq. cm can be readily detected.

TETRAZOLIUM REDUCTION TEST FOR α-KETOLS

To complete the picture concerning tests characteristic for α-ketols and glycols, the results obtained with tetrazolium reduction tests are also included in Table I. The reagent used was a solution of 1 mg of tetrazolium chloride per ml of 1 *N* KOH in 90% aqueous methanol (v/v), which was essentially the same as that reported by NOWACZYNSKI *et al.*[37]. While primary α-ketols, *e.g.* cortexone, develop a highly intense color within 5 minutes after the reagent is applied by spraying or dipping, the secondary α-ketols, *e.g.* 2β-hydroxytestosterone, produce only a weak color, indistinguishable from that produced by many non-reducing Δ^4-3-ketones, *e.g.* androstenedione or 7α-hydroxyprogesterone (*cf.* ref. 38). However, when the sprayed paper strip is allowed to air dry thoroughly (40 min or longer) and then heated at 80°–90° for 5 min, the color produced by 2β-hydroxytestosterone becomes as intense as that of cortexone, while that produced by non-reducing Δ^4-3-ketones undergoes only slight changes. On the basis of these results, it is recommended that to determine if an unknown is a secondary α-ketol by using the tetrazolium reduction test, known compounds, *e.g.* those mentioned above, should be used as positive and negative controls and the increase in the color intensity on heating should be used as the criterion. Commercial samples of both 2,3,5-triphenyltetrazolium chloride or blue tetrazolium—3,3-dianisole-bis-4,4-(3,5-diphenyl)-tetrazolium chloride—were found to give virtually identical results, the latter giving more intense coloration. Since only the qualitative aspect of the reduction was emphasized in the present study, the impure

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

nature of the commercial blue tetrazolium samples as pointed out by BUSH AND GALE[39] was not seriously considered.

DISCUSSION

Since all the tests reported here are based on well established reactions, the results should mean an indication of the presence or absence of the structural elements tested. However, in view of the exceptions observed, namely, the negative result obtained with triamcinolone acetonide in the test for formaldehydogenic steroids and non-aldehydogenic nature of the structure of 16α,17α,21-triol-20-one, a negative result should be interpreted with caution. The principal theme of the present study is really a development of shortcut methods whereby a well established reaction can be carried out directly on a paper chromatogram.

Although the different tests reported here can be used for the detection of spots on a papergram, they are not as sensitive as many other methods, such as U.V.-scanning or phosphomolybdic acid[31]. They are rather recommended for the demonstration of certain structural elements after it is ascertained that a paper gram spot contains a sufficient quantity of the compound to be tested. Furthermore, it is always advisable that in testing an unknown, a compound known to give a positive test and another known to give a negative test should be used at the same time as controls.

SUMMARY

Simple and convenient methods are described whereby a steroid which forms formaldehyde, acetaldehyde, a non-volatile aldehyde or a non-volatile acid on periodate oxidation can be demonstrated directly on a paper chromatogram. A method for demonstrating certain steroidal glycols by using a borate–phenol red spray and a modified procedure for applying the tetrazolium reduction test on a paper chromatogram are also presented.

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