THE USE OF *tert.*-BUTYL CHROMATE FOR THE LOCATION OF STEROIDS ON PAPER CHROMATOGRAMS

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MENINI AND NORYMBERSKI[1] described the systematic use of *tert.*-butyl chromate in the oxidation of steroids, particularly demonstrating the value of the smooth conversion of the 3β-hydroxy-steroid-5-enes to 6β-hydroxy-steroid-4-ene-3-ketones and to steroid-4-ene-3,6-diketones. This reagent has now been modified and applied for the location of reactive steroids on paper chromatograms.

These observations have been communicated in preliminary form[2] and are part of a general study of the chromatography of the steroids[3].

PROCEDURE

Reagents

Tert.-butyl chromate stock solution (MENINI AND NORYMBERSKI[1]). *tert.*-Butanol (10 ml) was diluted with CCl_4 (10 ml) and cooled in an ice bath. Chromium trioxide (5 g) was added in small portions whilst stirring. After standing 10 min and diluting with CCl_4 (65 ml) the solution was percolated through Na_2SO_4 (anhydrous 5 g) supported on a sintered filter. A further portion of CCl_4 (10 ml) was used to wash the apparatus and filter. Na_2SO_4 (anhydrous, 1 g) was added before storage at 4°.

This reagent has shown no signs of deterioration after 6 months storage at this temperature and in the dark.

The location reagent. Immediately before use the stock solution (1 ml) was added to a mixture of xylene (80 ml) and pyridine (20 ml) and shaken well.

Reference steroids were largely purchased from Messrs. Steraloids, of Croydon, London, and were checked for correctness of melting point, chromatographic properties and chemical reaction. Others were kindly provided by Prof. W. KLYNE from the M.R.C. Reference steroid collection.

The location procedures

The oxidation. The papers were sprayed evenly with location reagent and heated at 100 $\pm$ 5° for 5 min. One of the following procedures was then applied.

Alkaline fluorescence. The paper was dipped through *N* NaOH and dried at 100°. Fluorescence emission was sought by examining under a 360 mμ U.V. lamp.

Zimmermann reaction. The alkali treated paper from the fluorescence developement was dipped through ethanolic *m*-dinitrobenzene (2 %, w/v). On warming with a hair drier the purple or blue spots locating ketones appeared.

Alternatively, with better sensitivity and colour specificity, the procedure detailed elsewhere[3] was used.

The Zimmermann reagent also detects ketonic steroids present without the intervention of the oxidation. These were detected on unoxidized duplicate chromatograms.

The sensitivity of each of the location procedures was investigated by spotting serial dilutions of several steroids on paper and noting the least concentration/cm^2 which could be clearly discerned.

RESULTS

The yellow fluorescence after oxidation and alkali treatment

3β-Hydroxy-steroid-5-enes. Twenty-seven examples gave the yellow fluorescence after the alkali dip and heating. These included androstane, pregnane, cholane, cholestane and spirostane derivatives. Substituents, singly or multiply, included 12α-, 17α-, 17β-, 20α-, 20β-, or 21-hydroxyls; 21-acetoxy; 17-, 20-, 22- and 25-carbonyls; 16-unsaturation; 17-alkyl, and 17β-, 22- or 24-carboxyl or carboxylic methyl esters. Sensitivity for pregnenolone, diosgenin and cholesterol, 1 μg/cm^2.

Seven derivatives did not give the yellow fluorescence and these bore a 6-methyl, 7-keto- or 7-unsaturation. Both the 7-keto and 7-unsaturated derivatives of cholesterol gave a brownish red fluorescence. The blue fluorescence of 7-keto-dehydro*epi*androsterone is discussed below.

Esterification of the 3β-hydroxyl of steroids which were capable of producing the yellow fluorescence blocked the fluorescence production. Acetylation (5 examples) and formylation (3 examples) were carefully investigated. 3β-Acetoxy-chol-5-enic acid and 3β-acetoxy-bisnor-chol-5-enic acid, however, gave areas of very strong absorption of 360 mμ U.V. light, but no fluorescence.

Steroid-4-ene-3-ketones. Thirty-two examples gave the yellow fluorescence including androstane, pregnane, cholane and cholestane derivatives. Substituents, singly or multiply, included 2α-, 6β-, 11α-, 11β-, 17α-, 17β-, 18-, 20α-, 20β-, 21-, and 22-hydroxyls; 17α- and 21 acetates; 6-, 11-, 17-, 20-, 22-, and 25-ketones; 16-unsaturation; 9α-fluoro; 17-alkyl- and 19-nor-derivatives.

Three derivatives did not give the fluorescence and each possessed an additional unsaturation at the 1-position.

The limit of sensitivity observed for progesterone, testosterone and cholestenone was 5 μg/cm^2.

Other steroids giving the yellow fluorescence. (i) These were steroids with structures closely related to the 3β-hydroxy-steroid-5-enes and the steroid-4-ene-3-ketones, namely, pregn-4-ene-3β, 20β-diol, pregn-5-ene-3, 20-dione, androst-4-ene-3β, 17β-diol and cholest-5-en-3-one. (ii) Derivatives with a 6-oxygenation including hyodeoxycholic acid.

Blue fluorescence after oxidation and alkali treatment

Oestrogens. Oestrone, oestradiol-17α, oestradiol-17β and oestriol gave blue fluorescences with a sensitivity limit of 2 μg/cm^2. All but oestradiol-17α gave a red colour on oxidation with the *tert.*-butyl chromate, sensitivity 0.5 μg/cm^2.

Others. 3β-Hydroxy-androst-5-ene-7,17-dione.

Zimmermann reaction

Ketonic functional groups react with this reagent without intervention of the oxidation. Extra reacting centres detected only after oxidation were: (i) Blue colours from the oxidation of 3α- or 3β-hydroxyls to 3-ketones. The colour was particularly strong when 6-hydroxyls were produced or already present. (ii) 17α- and 17β-hydroxy-androstane derivatives were not oxidised to 17-ketones but 17α-hydroxy-pregnane-20-ketones and 20-alcohols were and gave the familiar 17-ketone purple colour.

The yellow reaction turning black was only observed when the 6-oxygen was present in the molecule before oxidation.

The sensitivity limit was 1 μg/cm^2 when purple reacting 17-ketones or 3,6-diketones were produced, the same for blue reacting 6-hydroxylated-3-ketones but 5 μg/cm^2 for simple 3-ketones.

DISCUSSION

MENINI AND NORYMBERSKI[1] showed by careful preparative and isolation procedures that 6-oxygenated derivatives were obtained when 3β-hydroxy-steroid-5-enes were oxidized with *tert.*-butyl chromate. Other steroids were not 6-oxygenated, most notably the 4-ene-3-ketones. Steroids already possessing the 6-hydroxyl group were oxidized to 6-ketones and likewise 3-, 11-, 17-, and 20-hydroxy derivatives to the corresponding ketones. The 17-hydroxy-20-ketones were oxidized to 17-ketones.

These reactions have now been successfully reproduced on paper chromatograms providing structurally specific location reactions. To do this it was necessary to dilute the reagent of MENINI AND NORYMBERSKI with xylene–pyridine and to heat at 100° to obtain a satisfactory reaction condition in which the 6-oxygenated products were obtained and not further destroyed. Solvents other than xylene–pyridine mixture were unsatisfactory as were temperatures other than 100°. The paper background was pale yellow and did not interfere with the subsequent procedures.

Application of the reagent on paper evidently did not provide such powerful oxidation as MENINI AND NORYMBERSKI obtained in solution since 17α- and β-androstane derivatives were not oxidized to 17-ketones and 6β-hydroxy derivatives were not oxidized to 6-ketones. Similarly 20α- and β-hydroxy derivatives were only poorly oxidized to 20-ketones (detected by the nitroprusside reagent of PAN[4]).

The reagent performed the following oxidations:

(1) 3β-Hydroxy-steroid-5-enes were converted to 6-hydroxy-steroid-4-ene-3-ketones which gave the yellow fluorescence on alkali treatment and strong blue Zimmermann colour. 6-Ketones were not formed since the yellow colour changing to black with the Zimmermann reagent was not observed.

(2) Steroid-4-ene-3-ketones were 6-hydroxylated. This contrasts with the observations of MENINI AND NORYMBERSKI and was first considered to be due to the "soda fluorescence" reaction described by BUSH[5] and due to the effect of the heating with alkali alone. This is not so, however, as the sensitivity of detection of progesterone is greatly increased by the *tert.*-butyl chromate oxidation.

(3) A similar reaction was also observed for the 3-hydroxy-4-unsaturated steroids and the 3-keto-5-unsaturated steroids.

(4) Steroids already 6-oxygenated also gave the yellow fluorescence distinguishing, for example, hyodeoxycholic acid from the non-6-oxygenated bile acids.

(5) 17-Hydroxy-20-oxygenated pregnane derivatives were converted to 17-ketones which gave the characteristic Zimmermann reaction.

These observations support two generalizations.

Firstly, oxidation and treatment with hot alkali produces a yellow fluorescence from 3-oxygenated, 4- or 5-unsaturated steroids, or from 6-oxygenated steroids. Secondly, a purple Zimmermann reaction is obtained from the 17-hydroxy-pregnane derivatives.

On investigating the effect of the common steroid substituents on the production of the yellow fluorescence most have been shown to have no effect. Only A and B ring substituents changed or abolished the reaction. Thus 7-substitution resulted in fluorescence emissions of colours other than yellow and the 6-methyl-derivatives and the 1,4-diunsaturated 3-ketones gave no reaction.

The colours produced with the Zimmermann reaction are not readily interpreted because 3-ketones produce a blue colour whether 6-hydroxylated or not, although 6-hydroxylation results in a five-fold increase in sensitivity. The purple reaction from the 17-hydroxy-pregnane derivatives may be rather difficult to distinguish in the presence of reactive A and B ring structures and the positive reaction would be best confirmed by use of the more specific lead tetraacetate oxidation as described by BUSH[6].

The observation that oestrogens produce a red colour on oxidation with *tert.*-butyl chromate, giving a blue fluorescence on the alkali treatment, may prove useful in oestrogen work. It might be profitable to extend the investigation of the utility of the reagent to a wider range of phenolic steroids than are available to the present writers.

It is hoped that the value of the *tert.*-butyl chromate reagent in application may soon be illustrated by an account of its application in the characterization of the steroids excreted by patients with congenital adrenal hyperplasia due to the 3β-hydroxy-steroid dehydrogenase defect.

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SUMMARY

The use of the *tert.*-butyl chromate reagent on paper chromatograms has been shown (i) to detect 3-hydroxy- or 3-keto-, 4- or 5-unsaturated steroids in the androstane, pregnane, cholane, cholestane, and spirostane series if followed by heating with alkali and examination for fluorescence emission under a 360 mμ U.V. light, and (ii) to detect 17-hydroxy-20-hydroxy- or 17-hydroxy-20-ketopregnane derivatives if followed by Zimmermann's reagent for the 17-ketosteroids produced.

Oestrogens gave a red colour on *tert.*-butyl chromate oxidation and blue fluorescence on alkali treatment.

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