The experiments showed that microgram quantities of other ketosteroids might be similarly assayed.

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Specific Colorimetric Reaction for Quantitative Determination of 3-Hydroxy-$\Delta^{1,5}$-Diene Grouping in Steroids

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Abstract □ A quantitative colorimetric procedure, which appears to be specific for the 3-hydroxy-$\Delta^{1,5}$-diene grouping in steroids, was developed. The reaction results in the formation of a stable red color when the steroid reacts with aluminum chloride in a benzene–nitrobenzene system. The anabolic hormone, 17α-methyl-1,5-androstadiene-3β,17β-diol, was used to develop the procedure.

Keyphrases □ 3-Hydroxy-$\Delta^{1,5}$-diene steroids—reaction with aluminum chloride in benzene–nitrobenzene, colorimetric analysis □ Steroids—reaction with aluminum chloride in benzene–nitrobenzene, colorimetric analysis for 3-hydroxy-$\Delta^{1,5}$-diene group □ Colorimetry—analysis, 3-hydroxy-$\Delta^{1,5}$-diene steroids

An analytical procedure based on the color developed by heating an anabolic hormone with aluminum chloride, followed by condensation with anisaldehyde, was reported by Kato (1, 2). The functionality responsible for the reaction was found to be a double bond, which originally existed in the molecule or which was produced by the elimination of a hydroxyl group during the reaction. In all cases, a necessary condition was that these double bonds must not be conjugated with any carbonyl group present in the molecule. Cholestane, cholestanol, cholesterol, cyclohexene, and cyclohexanol were some of the many compounds reported by Kato that gave a positive reaction

The analysis of 17α-methyl-1,5-androstadiene-3β,-17β-diol (I) by Kato's procedure (1, 2) was tried but without success; the mixture turned an olive-green color and had a very poorly defined absorption maximum.

Tauber (3) and Lange *et al.* (4) reported color formation for many steroids in chloroform when reacted with 70% perchloric acid. Lange *et al.* (4) reported that colorless, well-forme dcrystals of sterol–perchloric acid were precipitated when sterols containing the 3-hydroxy-5-ene grouping were dissolved in chloroform and then treated with a stoichiometric amount of perchloric acid. When an excess of perchloric acid was used, the crystals redissolved and characteristic colors were produced. Alteration of either of the two essential groups in the sterol resulted in failure to give a precipitate, but color formation still took place.

Early work on the color formation of cholesterol when treated with sulfuric acid–acetic anhydride (Liebermann–Burchard reaction) (5, 6) and zinc chloride–acetyl chloride (Tschugaeff reaction) (7) has formed the basis for more recent work. The latter papers deal with colors formed with unsaturated steroids when treated with an acid–iron reagent (8–10).

When the steroid (I) was tested in these systems, it gave a positive color reaction with all of them. Since such a large variety of steroids give positive color reactions with these systems, it was desirable to try to obtain a reaction that would be specific for the reactive grouping of I.

In the course of the Kato reaction, it was noted that I produced a satisfactory red color by merely allowing aluminum chloride in nitrobenzene to react with the steroid at room temperature. The utilization of this color as an analytical procedure for I is reported here.

EXPERIMENTAL

Apparatus—The absorption curves were obtained using a spectrophotometer[1] with 1-cm. cells.

Reagents and Solvents—The following were used: aluminum chloride, anhydrous[2]; benzene, analytical reagent, thiophene free[2]; and nitrobenzene[3], m.p. 5–6°. The aluminum chloride and benzene were used without further purification. Solutions of aluminum chloride in nitrobenzene became increasingly dark (in the absence of added steroid) as the nitrobenzene aged. To minimize this effect conveniently, the nitrobenzene was washed twice with 0.5 N HCl and then three times with 0.5 N NaOH, dried with anhydrous

[1] Carey model 15.
[2] Mallinckrodt.
[3] Matheson, Coleman, and Bell.

Table I—Effect of Nitrobenzene–Benzene on Absorbance

Nitrobenzene–Benzene[a]	5:1	5:3	5:5	5:10	5:15
Absorbance[b] (495 nm.)	0.748	0.710	0.676	0.588	0.565

[a] Milliliters of benzene added to 5 ml. nitrobenzene. Dilution to 25 ml. with benzene was made 15 min. after mixing two solvents. [b] Steroid (I) concentration 0.696 mg./25 ml. final dilution.

sodium sulfate, and filtered. Blanks prepared using this nitrobenzene remained satisfactorily light colored during the analysis.

Aluminum Chloride Solution—Dissolve 7 g. aluminum chloride in 100 ml. nitrobenzene.

Steroid Solution—Dissolve 20–150 mg. 17α-methyl-1,5-androstadiene-3β,17β-diol in 100 ml. benzene.

Procedure—In a 25-ml. volumetric flask, add 2 ml. steroid solution and 3 ml. benzene. Add 5 ml. aluminum chloride solution, mix by swirling, and allow to stand at room temperature for 15 min. Add benzene to the mark and mix well.

A blank is prepared in the same manner, with the substitution of 2 ml. benzene for the steroid solution.

Measure the absorbance, at 495 nm., of the sample *versus* the blank. All samples should be read within 30 min. after the final dilution is made.

DISCUSSION

The rate of color formation was found to be greatest when the reaction was run in neat nitrobenzene. However, the analytical absorption maximum at 495 nm. decreased in intensity somewhat rapidly with time and was partially obscured by a second maximum at 450 nm.

Other solvents were tried, but the red color failed to form. In benzene or toluene, a red precipitate was obtained instead of a red solution. A combination of nitrobenzene and benzene, however, proved satisfactory; the undesirable peak at 450 nm. was strongly suppressed and the color stability substantially improved. The color was rapidly discharged when alcohol, chloroform, or carbon tetrachloride was added to the system.

The initial ratio of nitrobenzene to benzene used (see *Experimental* section) and the time allowed for color development prior to final dilution had an effect on color intensity and stability.

Effect of Ratio of Nitrobenzene to Benzene—The color intensity varied inversely with increasing amounts of benzene relative to nitrobenzene. The data in Table I were obtained by adding varying amounts of benzene to a fixed volume of a solution of aluminum chloride in nitrobenzene at a constant steroid concentration.

The 5:5 ratio was found to be convenient in the analyses performed, using pharmaceutical preparations containing the steroid.

The intensity of the color reached a maximum approximately 15 min. after the two solvents were mixed and showed good stability up to 1 hr. after final dilution.

The absorbance followed a linear relationship with concentration and had a molar absorptivity of 8300 (a = 27.0).

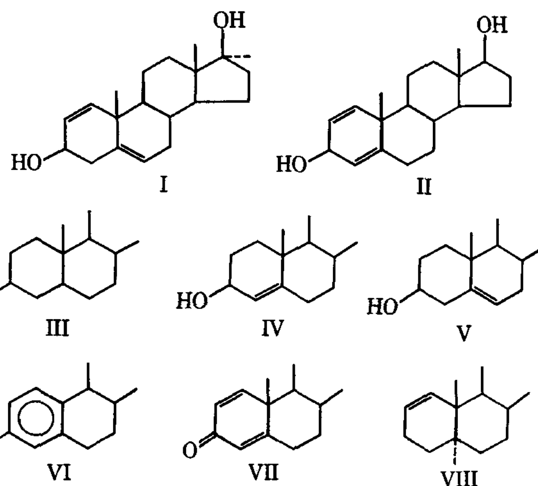
Other Reagents and Solvents—Nitrobenzene solutions of acetic acid, trichloroacetic acid, ferric chloride, tin tetrachloride, and zinc chloride were prepared. Only tin tetrachloride produced a red color with the steroid.

Tin tetrachloride was then dissolved in acetonitrile, benzene, dimethyl sulfoxide, nitrobenzene, and 1-nitropropane. Only in nitrobenzene and nitropropane was the color produced.

The intensity of the color produced in the tin tetrachloride–nitropropane–steroid system was only about one-fifth as intense as that in the aluminum chloride–nitrobenzene–steroid system. Since the stability of the color was also poorer in the former system, there appeared to be no advantage in using it over the latter one.

Other Compounds—The reaction, performed as described in this paper, appears to be specific for the 3-hydroxy-$\Delta^{1,5}$-diene grouping of I. The only other material tried that gave a positive reaction was $\Delta^{1,4}$-androstadiene-3β,17β-diol (II). However, the color would only form in neat nitrobenzene, and a much larger amount of steroid was necessary to obtain an intensity comparable to that of I.

Other compounds that gave no color by this procedure were (shown in partial structure): cholestan-3β-ol (III), Δ^4-androstene-3α,11β,17β-triol-11-acetate (IV), 25-hydroxycholesterol (V), estradiol (VI), betamethasone (VII), Δ^1-cholestene (VIII), ethyl morphine, phenol, and allyl alcohol.



Investigation of Reaction—To try to obtain some mechanistic information, the reaction was run on a larger scale by two methods.

Method 1—Compound I, 400 mg., was dissolved in nitrobenzene, and a solution of $AlCl_3$ in nitrobenzene was added to it. After 5 min. at room temperature, water was added. The nitrobenzene phase was then separated, dried with anhydrous Na_2SO_4, and filtered; the filtrate was evaporated under N_2 to a brown, oily residue. The residue was then triturated with methanol and a tan powder, slightly soluble in methanol, remained; yield = 150 mg.

Method 2—Compound I, 500 mg., was dissolved in 500 ml. benzene, and 3 g. $AlCl_3$ was added to it. The mixture was stirred for 4 hr. at room temperature and then handled as in Method 1. The methanol-insoluble residue was also obtained; yield = 200 mg.

The residual powders were subjected to various spectrometric analyses and to elemental analysis. The elemental analysis gave essentially identical results for the two procedures: C (87.6%), H (9.50%), Cl (0.9%), N (0.0%), O (3.5%). The starting material, $C_{20}H_{30}O_2$, has: C (79.5%), H (9.90%), O (10.6%). Since identical results were obtained by both methods, the incorporation of a solvent molecule into the methanol-insoluble material may be disregarded.

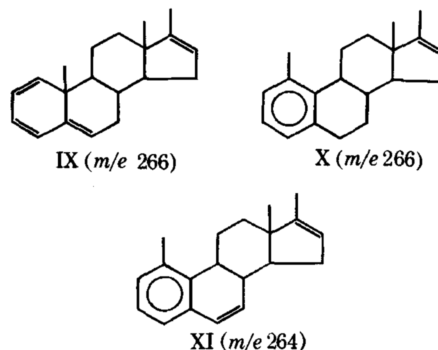
Replicate runs were made, and the elemental analysis was obtained for each run. The C and H results were essentially constant, but the results for oxygen varied from a low of 1.1% to a high of 4.3%. This variability made the selection of a possible empirical formula impossible.

The IR spectrum, run as a mineral oil mull and as a carbon tetrachloride solution, showed the material to be essentially a hydrocarbon; there was no carbonyl and very little OH absorption. Bands assignable to conjugated double bonds and possibly to an aromatic system were clearly present.

The NMR spectrum gave very poor resolution and was useless in structure elucidation.

The visible and UV spectra in isooctane were obtained. A complex curve resulted, which showed a maximum at 480 nm. and shoulders at 450 and 420 nm. in the visible region. The UV gave shoulders at 290 and 260 nm. and a maximum at 234 nm. (a = 45.0 at 234 nm.). The starting material, I, has no absorption above 210 nm. The UV data, therefore, support the IR spectrum in that a conjugated system was generated by the reaction.

Both GLC, using 3% OV-1, and TLC, on silica gel, revealed that the powder is a multicomponent material.



In the mass spectrum, the highest mass ion was found at 615 and the base peak at 532 m/e. Although the material fragmented into a great many ions, the highest concentration of them clustered into two groups: one ranged from 530 to 536 and the other from 262 to 270 m/e. Since the starting material has a molecular weight of 302, the possibility that the ion at 615 m/e is the parent ion of a dimer of the starting material may be ruled out. The ion could possibly arise, however, from the fragmentation of a trimer formed from an intermediate generated during the reaction. The ions in the groups at m/e 530–536 and 262–270 could then be due to dimers and monomers, respectively, fragmenting from the trimers or present in the mixture obtained from the reaction.

Of the many structures possible, three monomeric ones that can be written for the ions and that appear to be consistent with the various spectral data and with the starting material are shown in IX, X, and XI.

Structures such as X and XI can arise from a rearrangement similar to that given for the dienol–benzene rearrangement (11).

It seems, therefore, that the initial reaction is a Friedel–Crafts reaction on the allylic alcohol group of I with the formation of an allyl carbonium ion. This carbonium ion can be stabilized not only by the excess aluminum chloride but also by the nitrobenzene (12, 13). The stabilized carbonium-ion complex, conjugated with one or more double bonds, results in the red color observed in the analytical reaction. Red oils were reported previously in reactions of aluminum chloride, or bromide, with aromatic hydrocarbons (14). When the complex is destroyed by the addition of water, polymeric material is formed by attack of the carbonium ions on the various double bonds initially present in the molecule or generated during the reaction.

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Paper Chromatographic Determination of Oxytetracycline

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Abstract □ A paper chromatographic method for the quantitative separation and determination of oxytetracycline in the presence of its degradation products is presented. The separation of the antibiotic was carried out by making use of its complexation with urea on the chromatographic paper. Oxytetracycline and its degradation products gave characteristic fluorescence under UV light, and their respective R_f values were given. The recovery of this method was satisfactory (mean deviation = −3.0%) and was successfully applied to the assay of oxytetracycline in bulk and other pharmaceutical dosage forms. Hydrocortisone acetate and polymyxin B sulfate did not interfere with the chromatographic separation.

Keyphrases □ Oxytetracycline—separation, analysis from degradation products, urea-impregnated chromatographic paper □ Paper chromatography—separation, analysis, oxytetracycline □ Urea complexes—separation, analysis of oxytetracycline □ UV spectrophotometry—identification, oxytetracycline

The microbiological methods widely used for the determination of tetracycline antibiotics are not satisfactory, since some degradation products of these antibiotics proved to have antibacterial activity (1, 2). In view of the findings that relate a reversible fanconi-type syndrome to the ingestion of degraded tetracycline capsules (3–7), interest has developed in the analytical determination of tetracycline antibiotics in the presence of their degradation products.

Novelli *et al.* (8) pointed out the need of applying experimental correction factors, which are different for each component, in the spectrophotometric determination of tetracycline and its degradation products. This is necessary to make the results coincide with the theories and with the microbiological determinations.

A review of the literature dealing with the quantitative paper chromatographic determination of oxytetracycline revealed that there are only scanty publications concerning this aspect.

Selzer and Wright (9) separated different tetracyclines and their epimers on a paper chromatogram, previously impregnated with McIlvaine's buffer, pH 3.5. Sztaricskai (10) described a circular paper chromatographic method for determining oxytetracycline in the presence of its acidic decomposition products, using Whatman No. 1 chromatographic paper previously impregnated with phosphate buffer, pH 3.0, and a butanol–acetic acid–water (4:1:5) solvent system overnight. This method was claimed to be useful for estimating oxytetracycline in intermediary products and animal nutrients, but it was not reported to be applicable for the determination of oxytetracycline in pharmaceutical preparations. On applying this method to a sample of degraded oxytetracycline, we were unable to detect the fluorescent zones under UV lamp according to the description of the author; furthermore, no sharp separation of the degradation products was observed, and the experiment was time consuming (overnight running).