OXOSTEROIDS

THE USE OF PHENOLIC HYDRAZIDES FOR DETECTION, CHARACTERISATION AND ESTIMATION

BERNARD CAMBER

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This book is dedicated
TO MY MOTHER

Contents

Part 1. Introduction

Part 2. Preliminary Investigations

Part 3. 2-Hydroxy-3-Naphthoic Acid Hydrazide

Part 4. β-Resorcylic Acid Hydrazide

OXOSTEROIDS

The Use of Phenolic Hydrazides for Detection, Characterisation and Estimation

PART 1

Introduction

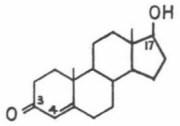
The Analysis of Steroid Mixtures from Biological Sources

The qualitative and quantitative analysis of the steroids occurring in body fluids and tissues is of value both as an aid to diagnosis in clinical medicine and also as a mode of investigation of fundamental physiological processes.

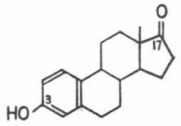
Unfortunately, the methods available for making such an analysis are not only laborious, time-consuming and complicated, but in addition they are often of insufficient sensitivity or specificity, and therefore unreliable. Indeed, concern has been expressed at the widespread application of such techniques and the uncritical deductions drawn therefrom, and the plea has been made for a "sabbatical year" devoted to attempts to improve the methodology.

The object of the work undertaken and reported here was to attempt to find improved reagents for the detection and recognition of steroids.

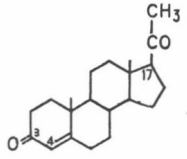
In fact, steroid compounds with hormonal activity are for the most part ketones, and recognition of and assignment of position to



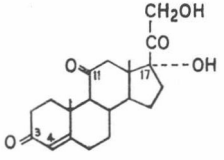
Testosterone



Oestrone



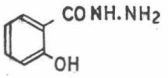
Progesterone



Cortisone

the ketone group is of primary importance in the detection, recognition and determination of steroid compounds in biological fluids and tissues. This importance of the ketone group is exemplified in testosterone, a Δ^4-3-ketone, oestrone, a 17-ketone, progesterone, a Δ^4-3 : 20-diketone, and cortisone, a Δ^4-3 : 11 : 20-triketone. The Δ^4-3-ketone group, it may be noted, is particularly associated with physiological activity.

A reagent which could allocate the position of the ketone group within the steroid molecule would therefore be of great value. A reagent with the desired properties has been discovered by the author, namely salicyloyl hydrazide (I):



salicyloyl hydrazide (I)

Present Chemical Methods for the Detection and Characterisation of Oxosteroids

Characteristically, a number of stages are employed in the study of steroids of biological origin. Firstly a crude extract is obtained from the body fluids or tissues, which may then be chemically fractionated (e.g. with the Girard reagents) to produce a material consisting largely of oxosteroids together with adventitious impurities. Finally, the steroids are separated from one another and from extraneous matter by physicochemical methods, of which the most commonly employed are adsorption and partition chromatography (see, for example, Dingemanse *et al.* 1946 and 1952 (adsorption on alumina); Keltie and Wade 1957 (gradient elution of alumina column); Dobriner, Lieberman and Rhoads 1948 (adsorption on magnesium trisilicate); Jones and Stitch 1953 (partition on silica gel); Nyc *et al.* 1951 (partition on powdered rubber); Zaffaroni and Burton 1951, Bush 1952, Heftmann 1955 (paper chromatography)).

The problem which arises in the last stage is that of making visible the colourless steroids, firstly in order that the course of the separation may be followed, and secondly for differentiating and identifying the steroids so isolated. The requirements in the two cases are rather different.

Adsorption chromatography is often followed by taking samples of the eluate and reacting them with a chemical reagent, e.g. the Zimmermann reagent (Zimmermann 1935, 1936, 1944), to give a colour. If the reaction is quantitative, colorimetric estimation of each

portion of the eluate can be carried out and peaks observed as each separated steroid moves out of the column.

In paper chromatography, the paper is dried after the chromatogram has been developed for a certain length of time, the reagent applied in the form of a spray, and the colour reaction made to take place on the paper, so that the separated steroids show up as spots of colour. To some extent the requirements for the reagent here resemble those for a staining reagent in histochemistry, where the colour reaction must also take place, *in situ*. They are insolubility of the coloured material, sensitivity, specificity, quantitative or semi-quantitative nature, and permanence of colour.

Of the fairly large number of reagents which may be used for oxosteroids, many are non-specific, i.e. they react with all steroids, and sometimes with other compounds as well, to give the same colour. For example, the Liebermann-Burchard reagent (1 ml. concentrated sulphuric acid, 20 ml. acetic anhydride, 50 ml. chloroform) reacts with all steroids of the cholesterol type, i.e. those having a double bond adjacent to a ring junction, to give a green colour with no fluorescence.

Iodine vapour, or a solution of iodine in potassium iodide, is even less specific, forming as it does a kind of solution in almost any organic compound, giving brown spots on papergrams from which the iodine slowly sublimes away.

2 : 4-Dinitrophenylhydrazine reacts with oxosteroids, as it does with all ketones, to give an orange spot. It is, therefore, a fairly useful reagent for detecting separated oxosteroids on a papergram, though it is incapable of distinguishing between them. Recently other reagents have been found which are capable of differentiation in some instances, because they give different colours with different steroids; the colours have been determined empirically, and no reasons have been advanced or unifying theory sought for their differences. Examples are phosphoric acid (15% aqueous solution), a reagent containing anisaldehyde (0·5 ml. anisaldehyde, 50 ml. glacial acetic acid, 1 ml. conc. H_2SO_4) and antimony trichloride (30% solution in chloroform) (Neher and Wettstein 1951). The different colours may display further characteristics, indicative of the identity of the original steroid, when they are subjected to further treatment, e.g. heating the paper (for the antimony trichloride reaction) or examination for fluorescence under ultraviolet light (after the phosphoric acid treatment).

Several other reagents exist, which are specific for particular chemical groupings within the molecule. The simplest reaction was discovered by Bush (1952) and consists in spraying the paper with methanolic caustic soda solution (10% w/v in 60% v/v aqueous

methanol), drying, and examining under ultraviolet light. All those steroids containing the physiologically active group Δ^4-3-ketone (e.g. testosterone, corticosterone, progesterone) fluoresce a brilliant yellow colour; to this reaction there seem to be no exceptions. What is more, a wide variety of other unsaturated oxosteroids (Δ^1-3-ketones, $\Delta^{1,4}$-3-ketones, $\Delta^{1,4,6}$-3-ketones, $\Delta^{4,6}$-3-ketones, $\Delta^{9(11)}$-12-ketones, Δ^{16}-20-ketones, $\Delta^{8(9)}$-7-ketones and Δ^5-7-ketones) do not give the fluorescence.

Reagents specific to the α-ketol group, characteristic of the corticosteroids, usually depend on the reducing power of this group, and include triphenyltetrazolium chloride (0·2% in water, mixed just before use with an equal volume of 10% NaOH) (Burton, Zaffaroni and Keutmann 1951), aqueous alkaline silver nitrate (Zaffaroni, Burton and Keutmann 1950) and the Porter-Silber reagent (65 mg. phenylhydrazine hydrochloride in 100 ml. dilute H_2SO_4) (Porter and Silber 1950).

Certain colour reactions, originally used as spot tests for particular steroids, give some indication of a type of chemical grouping in the molecule. For example, the blue colour with chloral hydrate or trichloracetic acid, which Rosenheim (1929) regarded as a specific test for ergosterol, was shown by Schoenheimer and Evans (1936) to be indicative of an actual or potential conjugated diene system in a steroid. The Tortelli-Jaffe test (1915) is supposedly specific for ditertiary "bridge-head" double bonds, but may not work when such a double bond is conjugated with a keto group. The selenium dioxide test for Δ^7-stenols (Fieser 1953) depends on the extreme rapidity with which this double bond is oxidised.

The more general reagents, used for detection, can sometimes be used for differentiating between steroids. Thus, Idler and Baumann (1953) studied the time at which the maximum intensity of colour appeared with the Liebermann-Burchard reagent under carefully controlled conditions (cf. Schoenheimer and Sperry 1934). Δ^7-Stenols gave a strong coloration, which reached its maximum in 90 seconds; $\Delta^{8(9)}$, $\Delta^{8(14)}$ and $\Delta^{14(15)}$stenols took 340 seconds, 15 minutes and 15 minutes, respectively, to reach a maximum of about the same intensity; the intensity with Δ^5-stenols continued to increase steadily with time; the reaction with Δ^6 was weak and hardly increased with time.

Another example of the use of a general reagent to identify specific groups, this time on paper chromatograms, is afforded by Bush's use of 2 : 4-dinitrobenzene (Bush 1954). The Zimmermann reagent is not very suitable as a spray reagent, forming as it does a red colour on its own on exposure to air, but Bush was able to differentiate between the brown colour given by 20-ketones, the purple given by

17-ketones, and the blue due to 3-ketones. The Zimmermann reagent can afford more detailed differentiation in the test-tube. Following the Medical Research Council method for using the reagent, in which the conditions are very carefully specified (*The Lancet* 1951), Broadbent and Klyne (1954) obtained the values shown in Table 1.

TABLE 1

Steroid type	λ max. at 5 min. (mμ)	$10^{-3}\,\varepsilon$ at 5 min.	λ max. at 60 min. (mμ)	$10^{-3}\,\varepsilon$ at 60 min.
2-ketone	540	11·0	530, broader	7·2
3-ketone, A/B trans	540	6·1–6·8	No peak, absorption falls continuously 300–600 m.	
3-ketone, A/B cis	360	ca. 6	ditto	ditto
4-ketone	—	—	~320, 420	—
15-ketone	—	—	ca. 400	2·2–2·6
17-ketone	—	—	520	15·2
20-ketone / Δ^{16}-20-ketone	—	—	490	2·4

Concentrated sulphuric acid, observed to give characteristic colour reactions with some steroid spots on paper chromatograms (e.g. red with compound S, yellow-green with green fluorescence with 11-hydroxy-steroids) (Zaffaroni and Burton 1951) was examined by Zaffaroni (1950) with a view to correlating spectral absorption peaks with the presence of functional groups in the molecule. His results may be analysed as shown in Table 2. A maximum at 280 mμ is shown to be specific for 4-en-3-ones; other correlatives are not so certain, but the analysis suggests, tentatively, that a maximum at about 340 mμ is associated with 11-oxygen, and that one at about 400 mμ is caused by a dihydroxy-acetone side-chain.

Apart from its fallibility, the Zaffaroni identification procedure has the disadvantage that it is necessary for the compounds to be examined in the test-tube for identification.

The same limitation applies to such physical methods of identifying steroids, as examination of ultraviolet absorption spectra (where these exist), measurement of optical rotation, melting point, and infra red spectra. The paper chromatographer, and even more so the histologist, requires a reagent which will mark the oxosteroid *in situ*, giving an indication of its position. If he could establish at the

same time the steroid's identity, or at least the chemical group which it contains, then a real advance would be made.

TABLE 2. SULPHURIC ACID CHROMOGENS

Cpd.	3-one	4-one	280 mμ	11-OH	11-one	340 mμ	20-one	21-OH	17-OH	400 mμ	Others
1	+	+	+	+	−	−	+	+	+	+	475
2	+	+	+	−	+	+	+	+	+	+	—
3	+	+	+	+	−	+	+	+	−	−	373, 455
4	+	+	+	−	+	+	+	+	−	+	—
5	+	+	+	−	−	−	+	+	+	−	535
6	+	+	+	−	−	−	+	+	−	−	370, 440
7				+	−	+	+	+	+	+	510
8				−	+	+	+	+	−	+	—
9				+	−	−	+	+	−	+	315
10	+	−	270	−	+	+	+	+	+	+	
13	+	+	+	−	+	−	−	−	one	−	
14	+	−	−	−	−	−	−	−	one	−	

+ = present — = absent

(Compounds numbered as in Zaffaroni's list: 1, 17-hydroxy-corticosterone; 2, 17-hydroxy-11-dehydrocorticosterone; 3, corticosterone; 4, 11-dehydrocorticosterone; 5, 17-hydroxy-11-desoxy-corticosterone; 6, 11-desoxycorticosterone.

7, *allo*-Pregnane-3, 11, 17, 21-tetrol-20-one; 8, *allo*-pregnane-3, 17, 21-triol-11, 20-dione; 9, *allo*-pregnane-3, 17, 21-triol-20-one.

10, Pregnane-17, 21-diol-3, 11, 20-trione.

13, Androst-4-ene-3, 11, 17-trione; 14, Androstane-3, 11, 17-trione.)

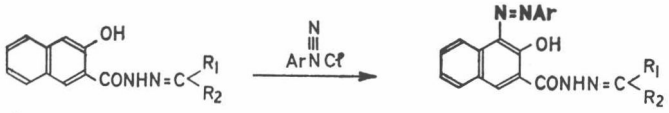
IDENTIFICATION OF OXOSTEROIDS BY MEANS OF PHENOLIC HYDRAZIDES

The search for a reagent which would detect oxosteroids and at the same time differentiate between them is described in the following pages. The three most useful compounds found were all phenolic acid hydrazides: 2-hydroxy-3-naphthoic acid hydrazide, β-resorcylic acid hydrazide, and salicylic acid hydrazide.

Their mode of action depends on the formation of coloured derivatives of the oxosteroids. Condensation of the hydrazide group with the ketone group of the oxosteroid results in colourless hydrazone derivatives:

$$HO.Ar.CO.NH.NH_2 + >C=O \longrightarrow HO.Ar.CO.NH.N=C<$$

It was found that the hydrazones (II) obtained from 2-hydroxy-3-naphthoic acid hydrazide could be converted in turn into coloured compounds. The presence of the phenolic group in these derivatives enables them to be coupled with a diazonium salt to form a dye:



2-Hydroxy-3-naphthoyl hydrazone (II)

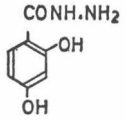
Azo dye

This was the first demonstration that oxosteroids could be converted into a new type of coloured compound, namely an azohydrazone.

The reagent has also been applied histochemically and its use was first described by the author in 1949 (Camber 1949). The method has since been used by many other workers in the histochemical field for the staining of ketonic compounds. Previously, the only reagents available were the weakly staining phenylhydrazine (Bennett 1940) and 2 : 4-dinitrophenylhydrazine (Dempsey and Wislocki 1946).

The azo-dyes which result from the above reaction are high-molecular-weight compounds and, depending on the diazonium component chosen, can be rendered highly insoluble and deeply coloured. There are, however, two major disadvantages with the reagent: firstly, a lack of specificity in that only one colour results from all ketonic compounds, and secondly the necessity for a two-stage method, with all the attendant disadvantages of two-stage procedures, namely multiplication of artefacts, geometric diminution of yield, and increased possibility of interference from unrelated substances, all of which increase the difficulties of interpretation.

The same type of two-stage reaction, this time using β-resorcyloyl hydrazide (2 : 4-dihydroxy-benzoic acid hydrazide) was found to be an improvement, in that some differentiation between oxosteroids



β-resorcyloyl hydrazide (III)

was obtained. Two activated positions (3- and 5-) are available in this molecule, and two different azo derivatives (3- and 3,5-) are possible, with different depths of colour. Pink and purple dyes (from the same diazo compound) have in fact been prepared, and the final colour seems to depend on the position of the keto-group in the

original steroid. Similar shades, namely pink and purple, have been obtained in tissue sections with the same reagents.

The β-resorcyloyl hydrazones are themselves coloured, if their characteristic fluorescence is excited by ultraviolet light screened by Wood's glass. The following colours were observed for five oxosteroid β-resorcyloyl hydrazones:

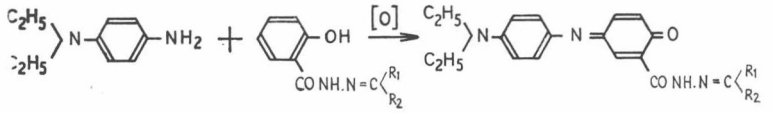
Dehydro*epi*androsterone	Intense blue
Oestrone	Intense blue
Testosterone	Weak dull purplish
Progesterone	Weak dull purplish
Desoxycorticosterone	Weak dull green

This was the first indication that a one-stage procedure could give derivatives in which the inherent differences between steroid molecules could be made manifest by colour differences. However, the specificity was still limited, and the reagent had the disadvantage of instability, with a known tendency to decarboxylate.

Salicyloyl Hydrazide

With the use of salicyloyl hydrazide, many of the difficulties and disadvantages of the earlier reagents were overcome. The hydrazide itself is a stable, crystalline, easily purified substance. Its reaction with oxosteroids gives crystalline hydrazones, which fluoresce under Wood's light in colours which enabled the oxosteroids examined to be divided into seven distinct groups, each related to a position and type of ketone grouping in the molecule. The reaction is quantitative, and takes place under mild conditions; it is extremely sensitive, and amounts as small as 0·5 μg. in a filter paper spot can be successfully detected and identified.

The salicyloyl hydrazones are very difficult to couple with diazonium salts, but a derivative that is coloured in ordinary light can be obtained by an *oxidative* coupling with p-alkylamino-anilines. When the reaction was carried out in the presence of potassium ferricyanide as oxidising agent, it proceeded smoothly with the formation of blue-green indoanilines:



This reaction, which is positive with all oxosteroid salicyloyl hydrazones, makes visible those few which show little or no fluor-

escence colour, and confirms the position on papergrams of those which do fluoresce. Specificity is lost at this stage, the derivatives of all oxosteroids being uniform in colour.

Present Methods for the Estimation of Oxosteroids

Chemical and physical methods of estimating hormones and their metabolites are usually based on the properties of ketone groups as such, or when influenced by neighbouring groups or bonds. For example, the height of the absorption peak at 240 mμ to which the 4-en-3-one group gives rise can be used for the estimation of the hormones in which it occurs. More recently, a fluorimetric method for this group using its reaction with *tert*-butoxide has been introduced (Abelson and Bondy 1955).

17-Oxosteroids give an intense colour with the Zimmermann reagent which is suitable for assay purposes. Other oxosteroids give weak colours (cf. Callow, Callow and Emmens (1932) and also Broadbent and Klyne (1954)). Unfortunately, the intensity and absorption maximum change with time, and the reagents are very liable to decomposition, so that a considerable degree of skill is necessary for reproducible results. Discrepancies of 15% have been observed by the author between the results from different laboratories, and even from a single laboratory the reproducibility attained is seldom more than $\pm 5\%$.

The 17-oxosteroid content can also be estimated by the Pincus method (Pincus 1943), which is a colorimetric method depending on the antimony trichloride reaction. The handling of this intensely poisonous compound presents a problem, and the reaction is not reliable. One of the most important of urinary 17-oxosteroids, dehydro*epi*androsterone, is not even chromogenic with antimony trichloride (Pincus 1945). By contrast, another method employing sulphuric acid under certain conditions (Patterson 1947, Patterson and Swale 1953) is believed to be specific for DHA alone. In view of the widespread action of sulphuric acid on steroids to produce colours, it would seem to be unwise to rely on this being true.

Phenolic steroids (e.g. oestrone, equilenin) can be estimated by a fluorimetric method involving the measurement of the fluorescence produced when they are heated with either concentrated sulphuric acid (Bates and Cohen 1947, Jailer 1947) or phosphoric acid (Finkelstein, Hestric and Koch 1947), or by a colorimetric method involving the reaction with β-naphthol and sulphuric acid (Kober 1938). These reactions destroy the molecule they are designed to estimate, and in consequence are very dependent on the conditions, and the time taken for the determination.

The reducing power of α-ketols, characteristic grouping of the corticosteroids, enables them to be estimated either by sugar reagents —alkaline copper (Talbot, Saltzmann, Wixom and Wolfe 1945) or phosphomolybdate (Heard and Sobel 1946; Heard, Sobel and Venning 1946)—or by liberating formaldehyde by oxidation with periodate and estimating this with chromotropic acid (Lowenstein, Corcoran and Page 1946; Daughaday, Jaffe and Williams 1948). Such methods are completely non-specific, since they measure only the reducing power of the substance or mixture. In any case, the formaldehyde method as usually employed was tested by the addition of pure cortisone and thereby shown to be inaccurate (Hollander, DiMauro and Pearson (1951)). Paterson and Marrian (1953) have investigated the reaction and come to the same conclusion.

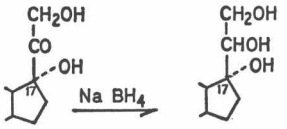
The Clark (1955) modification of the Dische diphenylamine reaction (Dische 1930) provides a colorimetric method for corticosteroids, and some differentiation between them. Although a formal relationship can be discerned between a particular group of atoms in corticosteroids and the laevulinic aldehyde molecule, which reacts with diphenylamine to give a colour, the basis of the reaction is not understood.

Two recently introduced methods are Brown's method for oestrogens, which represents a significant advance in this difficult field, and Norymberski's selective determination of different types of corticosteroids.

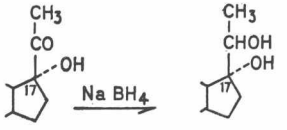
Brown's method is a modification of the Kober technique, using quinol both as reducing agent and as a diluent of the sulphuric acid. The difficulties and causes of interference with the reaction have been studied by Bauld in the same laboratory, and the procedure modified and standardised so as to overcome them (Bauld 1954).

Norymberski's methods (Appleby, Gibson, Norymberski and Stubbs 1954a) give separate estimations of two types of corticosteroids, those with a dihydroxyacetone side-chain (17α,21-dihydroxy-20-ones) (IV) and those with a monohydroxyacetone side-chain (17α-hydroxy-20-ones) (IVa).

The first stage of the standard determination employs sodium borohydride reduction. Both types of compound IV and IVa form vicinal glycols:



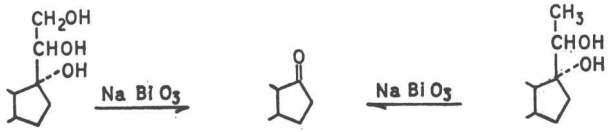
(IV)



(IVa)

and all other ketone groups in the molecule are eliminated by

reduction to alcohols (so that they cannot affect the later Zimmermann estimation). In the second stage, treatment with sodium bismuthate selectively oxidises the side-chain glycols to 17-ketones:



which are finally estimated by the Zimmermann method.

An extension of the method employs a bismuthate oxidation before the first stage, in order to eliminate the dihydroxyacetone side-chain (IV) while leaving the monohydroxyacetone side-chain (IVa) intact. The latter is therefore selectively estimated by the subsequent procedure, and the amount of the former is calculated by difference.

The method has been applied to urinary extracts (Appleby, Gibson, Norymberski and Stubbs 1954b). It is unfortunate that so elegant a method depends for the last stage of the estimation on the Zimmermann reaction.

An addition to the existing methods for estimating oxosteroids is provided by the use of salicyloyl hydrazide.

Estimation of Oxosteroids by Means of Salicyloyl Hydrazide

A technique has been evolved whereby the fluorescence of the salicyloyl hydrazones of oxosteroids has been enhanced and the interference from excess reagent diminished; a simple, direct, one-stage fluorimetric method for certain oxosteroids results.

One of the limitations in the fluorescence method is that certain types of oxosteroids are not fluorigenic under standard conditions. This has been overcome by converting the non-fluorescent salicyloyl hydrazones into compounds of the indoaniline type, which are estimated colorimetrically. These techniques, arising out of the investigations herein reported, provide two new methods for oxosteroid assay.