THE USE OF PHENOLIC HYDRAZIDES FOR THE DETECTION, CHARACTERISATION AND ESTIMATION OF OXOSTEROIDS

A Thesis submitted for the degree of

Doctor of Medicine

by

Bernard Camber

27, Wimpole Street,
London, W.1.

1957

CONTENTS

Part 1. Introduction

Part 2. Preliminary Investigations

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

PART I

INTRODUCTION

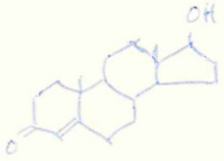
The Analysis of Steroid Mixtures from Biological Sources

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.

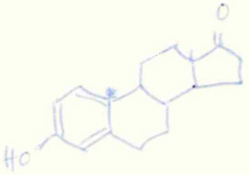
Unfortunately, the methods available for making such an analysis are not only laborious, time-consuming and complicated, but in addition they are often of insufficient sensitivity or specificity, and therefore unreliable. Indeed, concern has been expressed at the wide-spread application of such techniques 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.

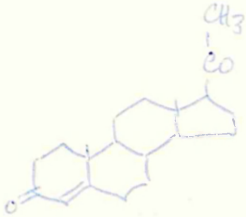
In fact, steroid compounds with hormonal activity are for the most part ketones, and recognition of and assignment of position to the ketone group is of primary importance in the detection, recognition and determination of steroid compounds in biological fluids and tissues. This importance of the ketone 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.



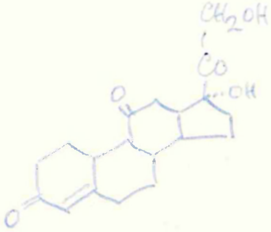
Testosterone



Oestrone

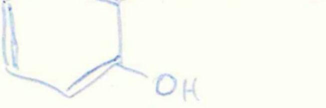


Progesterone



Cortisone

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



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 + 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, <u>in situ</u>. 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 oxo-steroids (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	λmax at 5 min. (mμ)	$10^{-3}\varepsilon$ at 5 min.	λmax at 60 min. (mμ).	$10^{-3}\varepsilon$ at 60 min.
2-ketone	540	11.0	530, broader	7.2
3-ketone A/B	540	6.1-6.8	No peak, absorption falls continuously 300-600 mμ	
3-ketone, A/B cis	360	ca. 6	" " "	
4-ketone	-	-	320, 420	-
15-ketone	-	-	ca.400	2.2-2.6
17-ketone	-	-	520	15.2
20-ketone				
Δ^{16}-20-ketone	-	-	390	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 dihydroxyacetone side-chain.

Table 2. Sulphuric acid chromogens

Cpd.	3-one	4-ene	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 in the molecule under consideration.

(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-desoxycorticosterone; 6,11-desoxycorticosterone.

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

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

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

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 infrared 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.

Identification of Oxosteroids by Means of Phenolic Hydrazides

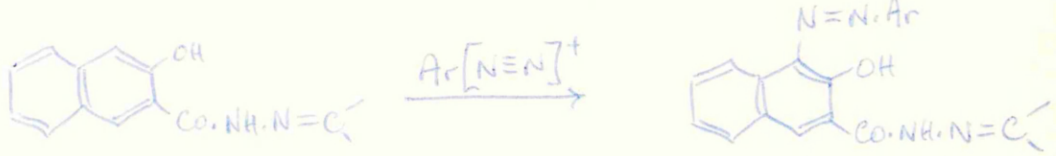
The search for a reagent which would detect oxosteroids and at the same time differentiate between them is described in the following pages. The three most useful compounds found were all phenolic acid hydrazides: 2-hydroxy-3-naphthoic acid hydrazide, β-resorcylic acid hydrazide, and salicylic acid hydrazide.

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:-

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

which are insoluble, crystalline compounds.

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)

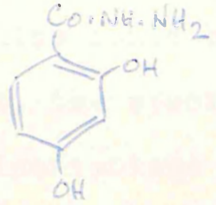
Azo-dye

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

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-resorcylcoylhydrazide (2:4-dihydroxy-benzoic acid hydrazide) was found to be an improvement, in that some differentiation between oxosteroids 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 (Camber 1957c).



β-resorcyloyl hydrazide

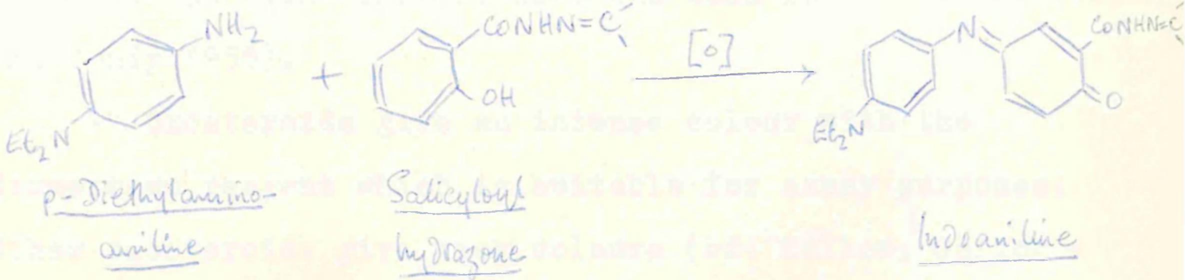
The β-resorcyloylhydrazones are themselves coloured, if their characteristic fluorescence is excited by ultraviolet light screened by Wood's glass. The following colours were observed for five oxosteroid β-resorcyloylhydrazones:-

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.

With the use of salicyloylhydrazide, 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 0.5μg. 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 fluorescence 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-butozide 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 & Emmens (1932) and also Broadbent & 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 ±5%.

The total 17-oxosteroid content can 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, dehydroepiandrosterone, 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. oesterone, 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 a-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.

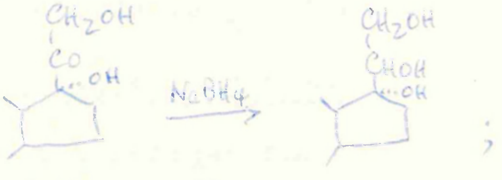
The Clark (1955) modification of the Dische diphenylamine reaction (Dische 1930) provides a colorimetric method for corticosteroids, and some differentiation between them. Although a formal relationship can be discerned between a particular group of atoms in corticosteroids and the laevulinic aldehyde 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 (17_{α},21-dihydroxy-20-ones) (IV) and those with a monohydroxyacetone side-chain (17_{α}-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:-

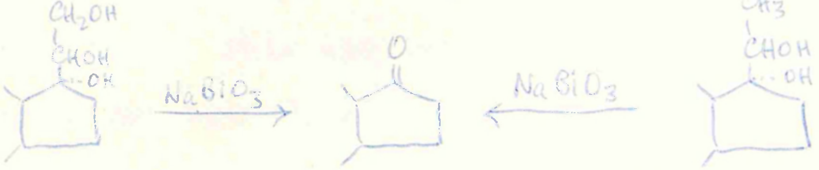


(IV)

;

(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 method.

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.

PART 2

PRELIMINARY INVESTIGATIONS

Introduction

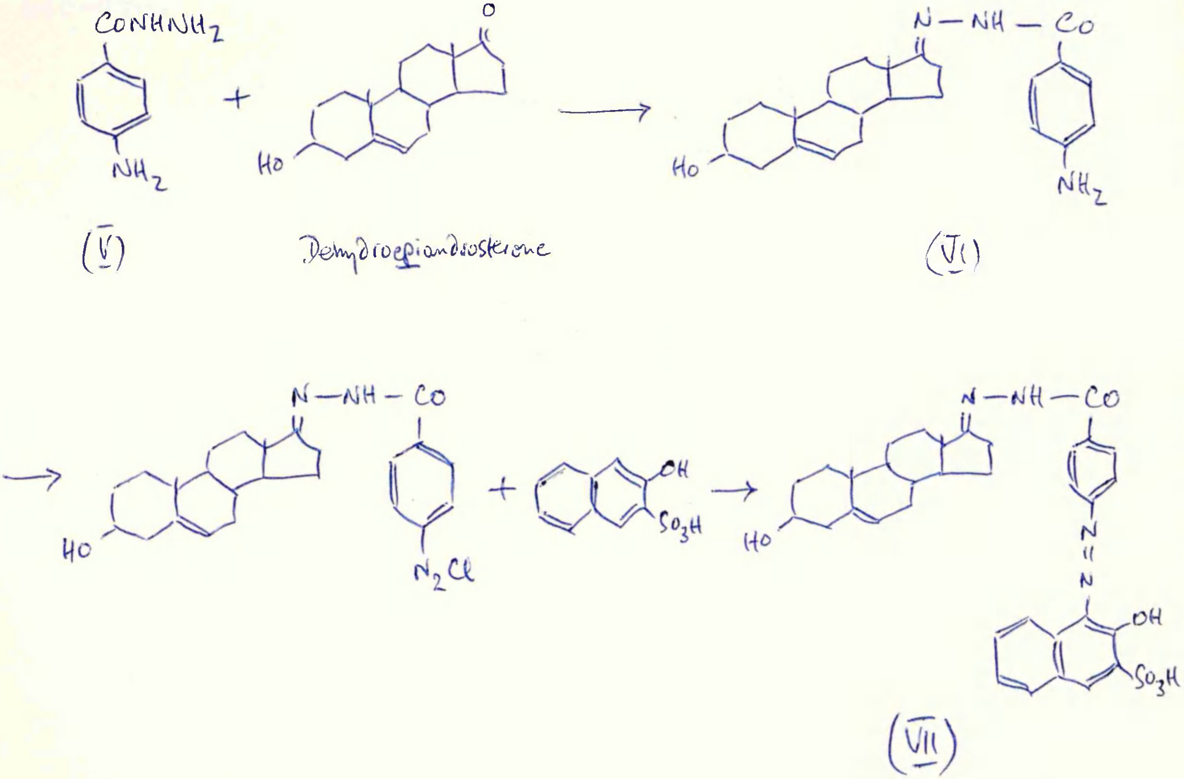
Whilst engaged on the chromatography of urinary extracts by the methods of Dingemanse _et al_. (1946, 1952), the author was impressed by the vividly and diversely coloured, sharply demarcated bands which the urinary pigments form on an alumina column. This incidental observation led to considerations of possible means whereby the steroids themselves might be rendered similarly highly coloured.

The basic idea was to react the urinary oxosteroids with an aryl hydrazine to form hydrazones. The aryl hydrazine was to have either a primary amino or a hydroxyl group, so that azo dyes could be formed from the hydrazone and estimated colorimetrically. The dyes so formed it was hoped might lend themselves to adsorption or partition chromatography more readily than the original steroids, because their physicochemical properties (in particular water-solubility) could be varied at will by appropriate choice of the arylhydrazine or diazonium compound. They might also be useful histologically.

It was realised that by attaching further large groups to the already large steroid molecule, differences between individual steroid monoketones might be diminished. On the other hand, if steroid diketones were to form dihydrazones, and these in turn reacted with two molecules of diazonium salt, the differences would be accentuated; and it is amongst the diketones that the corticosteroids are to be found.

p-Aminobenzoic Acid Hydrazide

The first substance to be investigated was p-aminobenzoic acid hydrazide (V) prepared according to the method of Curtius (1917). The preparation proceeds easily and results in good yields from the readily available ethyl ester of p-aminobenzoic acid (Benzocaine, B.P.). It was proposed to form the hydrazone (VI), diazotise it and couple with a phenol to give an azo-dye (VII):



(V)

Dehydroepiandrosterone

(VI)

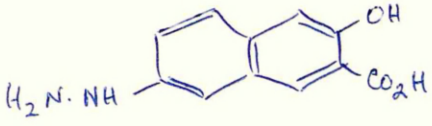
(VII)

The reagent refused to form hydrazones with simple model carbonyl compounds under any of the conditions investigated. Formation was attempted in both organic and aqueous solvents, in acid and alkaline media and under different temperature conditions. Catalysts such as sulphur dioxide and aluminium trichloride were used without effect. No reaction occurred with benzaldehyde, acetophenone, cyclohexanone, benzophenone, benzil or benzoin. The author is unable to adduce any explanation for the unreactivity of this compound.

The idea was then reversed, so that a phenolic instead of an amino hydrazine was employed as the reagent. In virtue of its phenolic character, it was thought that the hydrazone would couple with a diazonium salt to form the azo-dye.

6-Hydrazino-2-hydroxy-3-naphthoic acid

The formation of 6-hydrazino-2-hydroxy-3-naphthoic acid (VIII),



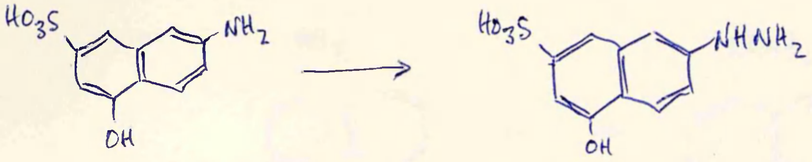
(VIII)

a substance related to well-known azo-dye substrates, involved a long and difficult synthesis. 2-Hydroxy-3-naphthoic acid was methylated with methanol and concentrated sulphuric acid, and the methyl ester converted to 2-methoxy-3-naphthoic methyl ester with methyl iodide in sodium methoxide. The ester group was hydrolysed off, and the resultant 2-methoxy-3-naphthoic acid nitrated to give a mixture of nitro derivatives, with 6-nitro-2-methoxy-3-naphthoic acid predominating. This was reduced to the 6-amino derivative and the methyl ether group removed with hydriodic acid. The 6-amino-2-hydroxy-3-naphthoic acid was diazotised and reduced to give the hydrochloride of 6-hydrazino-2-hydroxy-3-naphthoic acid, the desired reagent.

The substance decomposes readily: the solid oxidises visibly in air and dissolves in water to give highly coloured solutions. It was found to condense only sluggishly with carbonyl compounds (e.g. acetone, benzaldyhyde, dehydro<u>epi</u>androsterone, in 5% acetic acid containing HCl), and the product formed amorphous precipitates. For all these reasons, 6-hydrazino-2-hydroxy-3-naphthoic acid was considered unsuitable as a reagent.

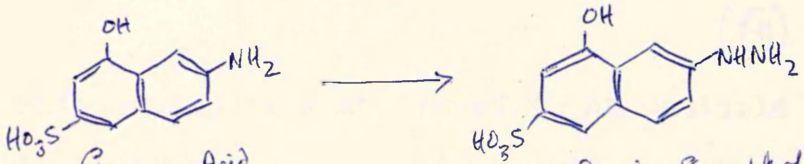
Hydrazinonaphtholsulphonic acids

The compounds IX and X were synthesised from the readily available corresponding amino compounds by diazotisation followed by reduction with stannous chloride :-



J-Acid

2-hydrazino-5-naphthol-7-sulphonic acid (IX)



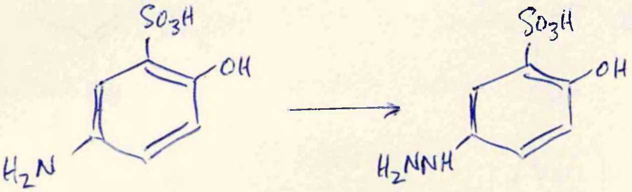
Gamma Acid

2-hydrazino-8-naphthol-6-sulphonic acid (X)

They suffered the same disadvantage as the related carboxylic acid described above, and were also only very slightly soluble in alcohol and water.

2-Hydroxy-5-hydrazinobenzenesulphonic acid

A mononuclear relative (XI) of the above sulphonyl compounds was synthesised as follows:-

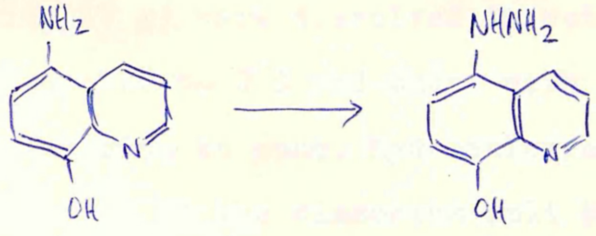


(XI)

The substance proved to be unstable and unreactive in condensation.

5-Hydrazino-8-hydroxyquinoline

This quinoline equivalent (XII) of a hydrazinonaphthol was synthesised from its parent amine:-

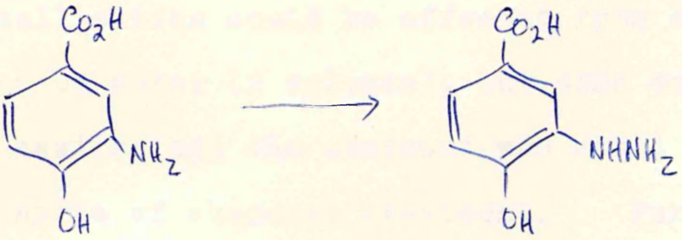


(XII)

This substance also proved to be unstable and unreactive in condensation.

3-Hydrazino-4-hydroxybenzoic acid hydrochloride

This new compound (XIII) was synthesised, like the hydrazines above, from the corresponding amine:-



(XIII)

The compound proved to be extremely reactive, forming immediate precipitates of hydrazones at room temperature, even with ketones such as α-ionone, reported unreactive with phenylhydrazine and 2:4-dinitrophenylhydrazine.

-o-

EXPERIMENTAL

Synthesis

Sodium 3-amino-4-hydroxybenzoate (17.6 g) and sodium nitrite (7 g) were dissolved in water (150 ml), the solution cooled to 0°C and added with efficient cooling and stirring to conc. hydrochloric acid (200 ml). Yellowish crystals of the diazonium salt began to separate after the addition of two-thirds of the solution. Without further attempt at isolation, the reaction mixture was added with stirring to a solution of stannous chloride (60 g) in conc. HCl (200 ml), the temperature being kept below 5°C. A profuse, flocculent white precipitate of 3-hydrazino-4-hydroxybenzoic acid hydrochloride (XIII) separated immediately. The reaction mixture was stirred for a further 15 minutes and filtered with suction.

Recrystallisation could be effected from conc.HCl (8 volumes) plus water (2 volumes), but some exidation occurred on heating and the compound was still slightly coloured in spite of charcoal treatment. Pure white crystalline needles were obtained by dissolving the reagent in the minimum amount of water at room temperature, cooling to 0°C and passing gaseous hydrogen chloride into the solution with slow stirring between 0° and 5°C. The needles which separated in good yield were isolated by rapid filtration and dried over flakes of sodium hydroxide.

Found: C 38.5, H 4.8, N 12.3, Cl 16.1. Reqd. for $C_7H_9O_3N_2Cl.H_2O$: C 37.8, H 5.0, N 12.6, Cl 16.0

Formation of hydrazones.

Acetone. A precipitate formed immediately when a drop of acetone was added to an aqueous solution of the reagent containing one equivalent of sodium acetate.

Found: C 58.65, H 5.70, N 12.0. Reqd. for $C_{10}H_{12}O_3N_2$: C 57.7, H 5.8, N 13.4.

Benzaldehyde. A dilute solution of benzaldehyde in ethanol was added to the aqueous solution of the reagent containing one equivalent of sodium acetate.

Found: C 65.6, H 4.7, N 10.3. Reqd. for $C_{14}H_{11}O_3N_2$: C 65.6, H 4.7, N 10.9

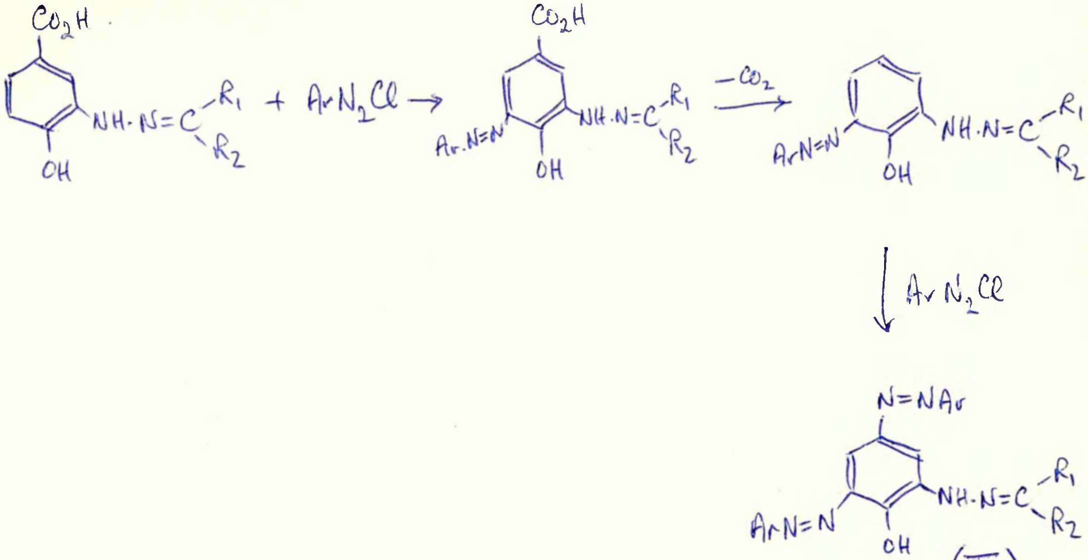
p-Nitrobenzaldehyde. As for benzaldehyde.

Found: C 53.6, H 4.2, N 13.9. Reqd. for $C_{14}H_{11}O_5N_3$: C 55.8, H 3.65, N 13.95.

-o-

Formation of Blue Pigment

It was anticipated that reaction of the hydrazones with diazonium salts would result first in diazo-coupling *ortho*- to the phenol group; then, since p-hydroxybenzoic acid readily decarboxylates, loss of the carboxyl group would follow and a second coupling would produce a highly coloured, insoluble compound (XIV):-



(XIV)

However, when the hydrazone derivatives of acetone, benzaldehyde, cyclopentanone, cyclohexanone, and pyruvic acid were tested under various conditions with the high-molecular-weight stabilised diazonium salts Black K, Blue RR, Blue BB and dianisidine, only deep red to purplish colorations were obtained.

The first successful production of an insoluble coloured compound was obtained by mixing a solution of stabilised Blue RR with a solution of the benzaldehyde hydrazone in alcohol, and adding dilute sodium carbonate dropwise to neutralise the acetic acid. At the neutral point, an intensely blue precipitate separated, leaving a water-clear supernatant liquid. Precipitation occurred at a concentration of 30-50 micrograms in 1 ml. ethanol, and a blue colour could be developed at a concentration of only 0.5 micrograms per ml. of ethanol.

The blue compound proved to be insoluble in all solvents except boiling acetic acid, and this insolubility suggested that it might be useful in histochemical applications. However, the compound did not behave like an azo-dye. It could be reduced to a leuco derivative by warming with zinc dust and acetic acid or with sodium hydrosulphite and alkali, and the colour could be restored by the addition of quinone.

The suspicion that it was not an azo-dye was confirmed when it was shown that the presence of a diazonium salt was not necessary to the formation of the blue pigment. The essential ingredient was the zinc chloride in the stabilising solution of the diazonium compound, and pigment formation was actually due to auto-oxidation of the hydrazone (a reaction which hydrazones undergo fairly readily, cf. Pansacker 1950). Aqueous copper sulphate (1%) was even more effective as a catalyst. Unknown impurities also assist the reaction, since freshly prepared and carefully recrystallised simple ketonic hydrazones do not react, but will do so after varying times in alcoholic solution. This "ageing" process in alcohol could not be replaced by oxidative treatments such as the following:-

(a) bubbling air or oxygen through the solution,

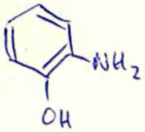
(b) addition of benzoyl peroxide or lead tetraacetate, or

(c) exposure to ultraviolet light.

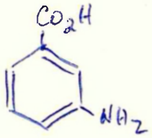
Glass wool was an effective catalyst, producing immediate reaction with freshly recrystallised benzaldehyde hydrazone, but requiring several hours with ketonic derivatives. The reaction also took place at the surface of several powdered salts, e.g. sodium carbonate, calcium carbonate, and barium hydroxide. Sodium and potassium bicarbonates and bismuth carbonate produced a red-brown colour. A far better reaction medium than alcohol was acetic acid, diluted with water after the hydrazone had been dissolved in it.

In order to determine what groups of the reagent were required for the blue reaction, the following compounds were prepared and tested:-

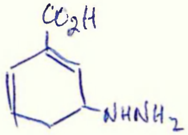
1.



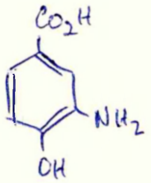
2.



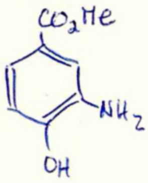
3.



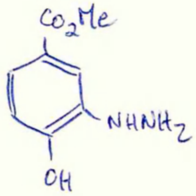
4.



5.



6.



A positive reaction was obtained with compounds 4, 5 and 6 and it was concluded that the following groups are essential:-

(a) carboxy (or carbomethoxy),

(b) 3-amino or hydrazino,

(c) 4-hydroxy.

-o-

EXPERIMENTAL

To 0.1 ml of solution containing the reagent (10-50μg) in N-acetic acid was added industrial spirit (0.3 ml), 1% aqueous copper sulphate (o.1 ml) and saturated sodium acetate solution (o.1 ml). For reagents which gave the reaction, there appeared to be a ranging of colours in the region 10-40μg. Precipitation occurred at the 50μg level.

-o-

The reagent 3-hydrazino-4-hydroxybenzoic acid, and the formation of blue pigment by its derivatives, was not investigated further, although it gave some evidence of specificity in histochemical applications (the adrenal cortex, but not the medulla, gave a blue zoning appearance). The significance of such observations was regarded as altogether too conjectural, in view of the obscure nature of the pigment and the dependence of its colour on changing conditions. Above all, with no steroid derivative of the reagent could a blue pigment be obtained. The search was continued for a simple reagent, not overburdened with reactive groups, from which a definite steroidal derivative could be obtained by a standard procedure.

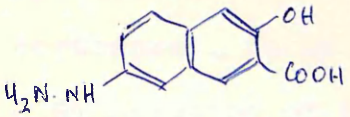
PART 3

2-HYDROXY-3-NAPHTHOIC ACID HYDRAZIDE

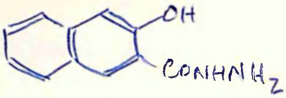
Introduction

Fundamental objections can be raised to the use of several of the compounds investigated in the preliminary experiments described in Part 2. Even if it had been possible to condense p-aminobenzoic acid hydrazide with ketones, there is evidence (Wieland and Horner 1937) that the nitrous acid used in the subsequent diazotisation might split the hydrazone linkage. The formation of the blue pigment from 3-hydrazino-4-hydroxy-benzoic acid showed that it is a molecule highly subject to the free-radical type of auto-oxidation. This might be expected from a nucleus so heavily loaded with active and activating groups.

There is no such fundamental objection to 6-hydrazino-2-hydroxy 3-naphthoic acid; here the objections were purely practical, in that the reagent reacted so sluggishly with ketones. If the reactive groups are rearranged slightly, we obtain the compound 2-hydroxy-3-naphthoic acid hydrazide:-



6-hydrazino-2-hydroxy 3-naphthoic acid



2-hydroxy-3-naphthoic acid hydrazide

and this was the next compound investigated. The parent compound, 2-hydroxy-3-naphthoic acid, is known to be very reactive in azo-coupling (it is a classical parent substance of the German dye industry), and to be stable to oxidation. There are, of course, electronic reasons why an acid hydrazide should be more reactive in condensation than a hydrazine, which augured well for the ketone reactions; while the neutralisation of the carboxyl function reduces the tendency to autoxidation. Also, the hydrazide is fluorescent, and it was anticipated that the fluorescence of derivatives might be used for following the course of chromatographic separations.

The reagent is easily synthesised (Franzen and Eichler 1908) by reacting hydrazine with ethyl 2-hydroxy-3-naphthoate. It crystallises in silvery plates, m.p. 204-205° from aqueous ethanolic acetic acid.

Formation of 2-hydroxy-3-naphthoyl hydrazones

The reagent proved to be very reactive in condensation with ketones, in accordance with prediction. For the hydrophobic oxosteroids (e.g. desoxycorticosterone, cortisone), it is necessary merely to dissolve them in a 0.8% solution of 2-hydroxy-3-naphthoyl hydrazide in glacial acetic acid, and dilute with 20 volumes of 5% methanol in water, when the hydrazones precipitate out.

Hydrazones can also be formed in hot alcoholic solution, from which they precipitate out on diluting with water and cooling. In general, however, the presence of acetic acid seems to favour condensation.

-o-

EXPERIMENTAL

Cholestanone-hydrazone. Cholestanone (250 mg) was added to 2-hydroxy-3-naphthoyl hydrazide (160 mg) in glacial acetic acid (20 ml). Some cloudiness was apparent before all the ketone had dissolved. Left overnight: ppt. 291 mg, m.p. 236-239°. Recrys. ex methanol-chloroform.

Corticosteroid hydrazones. Similar to cholestanone, except that dilution with 5% methanol in water was necessary to precipitate the derivative.

Dehydroepiandrosterone-hydrazone. A mixture of DHA (5 mg) and hydrazide (10 mg) in ethanol (20 ml) was refluxed 10 min., then concentrated to 5 ml on the steam bath. One drop of water was added, and the mixture cooled. White ppt. recrys. from ethanol, m.p. 266-268° (turning red).

Precipitation of steroids from urine as 2-hydroxy-3-naphthoyl hydrazones

The most interesting feature of 2-hydroxy-3-naphthoyl hydrazide was that it appeared to precipitate the water-soluble oxosteroid conjugates of urine as their insoluble hydrazones, when one-fortieth volume of a 0.8% solution of the reagent in glacial acetic acid was simply added to the urine at room temperature and the mixture allowed to stand. Some typical results are shown in Table 3.

Table 3. Precipitates of hydrazone from unhydrolysed urine

Fresh urine	Reagent	Acetic Acid	Dried ppt.	Ppt. per litre of urine
10,950 ml.	1 g.	125 ml.	2.879 g.	263 mg.
14,360 ml.	2 g.	250 ml.	3.974 g.	278 mg.
9,040 ml.	1 g.	125 ml.	1.714 g.	190 mg.

Apart from the fact that acetic acid is a good solvent for the reagent, it is obviously important to keep the pH low to achieve maximum precipitation, for the hydrazones are soluble in alkali by reason of their phenolic group. Overnight cooling of the solution in the refrigerator. assists precipitation.

The direct precipitation of oxosteroid conjugates at low temperatures would represent an advance on previous methods for oxosteroid extraction, because the initial hydrolysis in other methods, designed to split off the water-solubilising portion of the conjugates and enable the steroids to be separated from hydrophilic urinary constituents, demonstrably causes degradation and loss of steroid material. Recent methods attempt to overcome the disadvantages of hot acid hydrolysis by ether/ethanol extraction after saturation with ammonium chloride (Edwards, Keltie and Wade 1953; Keltie and Wade 1957).

Chromatography of the precipitated 2-hydroxy-3-naphthoyl-hydrazones

Two standard methods were adopted for the analysis of the mixture of precipitated hydrazones: partition chromatography and adsorption chromatography.

The first system tried was that of triethylamine, pyridine and water on paper, and was not very successful, because of the inadequate solubility of the hydrazones in water. It was found that the best results were obtained when pure triethylamine was used, when the paper had been left in contact with the vapour of the solvents until equilibrium was reached, and when the strips were run at a somewhat elevated temperature (37°C). These desirable conditions were shortly afterwards described by Bush (1952) in a well-known paper.

The water-washed precipitate from normal young men's urine was successfully resolved by paper chromatography in a triethylamine-tert-butanol-water system into at least 10 components which fluoresced under blue light (Camber 1950). The spots were observed fluorimetrically, and a permanent record made by soaking the dried paper in a solution of Blue 2B, and exposing to ammonia fumes to produce a blue spot.

Further resolution was obtained by the formation of sulphanilic azo dyes (by coupling _ortho_- to the phenolic group) and carrying out a second dimensional partition of these coloured derivatives in a water-saturated sec-butanol system. The formation of these dyes has a solubilising effect, with consequent alteration in the chromatographic behaviour.

The addition of a known oxosteroid conjugate (e.g. sodium androsterone sulphate) to the urine resulted in the enlargement of one of the spots. The addition to urine of hexoses, pentoses, ascorbic acid, caffeine, urea, and uric acid failed to alter the chromatrographic pattern of the precipitated substances. An artificial mixture of the hydrazones of steroids known to occur in the β-ketonic fraction from acid-hydrolysed urine (_epi_androsterone, dehydro_epi_androsterone, _allo_pregnan-3 -ol-20-one) was resolved in an _iso_propylether-triethylamine-water system at 0°C and also in an _iso_propylether-trimethylamine-methanol-water system at room temperature. Results were not easily reproducible on any of these chromatrograms, and it was not possible to identify any of the individual components.

Resolution of the urinary precipitate mixture into five components was achieved on a silica gel column, using chloroform and then increasingly concentrated solutions of methanol in chloroform as eluant. Peaks were detected by observing the intensity of colour developed when the eluted fractions were evaporated to dryness, dissolved in ethanolic ammonia and treated with a saturated solution of Blue 2B. Peaks occurred only when the concentration of methanol had just been increased, and a considerable amount of material was left on the column, so this method of separation was deemed a failure.

Rather similar results were obtained in the first experiments with adsorption chromatography, on florisil (synthetic magnesium silicate) columns. The hydrazones were placed on the column in 95% ethanol and eluted with this solvent containing increasing quantities of water. The five fractions all resulted only at the time of increasing the water content. An improvement was effected by using an acid medium, 60% methyl cellosolve and 40% 0.1N hydrochloric acid, which have sharp bands both on florisil and on alumina, although the hydrazones were not fully soluble in this system.

The solvent system in which the hydrazones were most freely soluble was 60-80% aqueous dioxan, and some evidence of separation on alumina using this solvent was obtained. However, the method was not considered as suitable as paper chromatography for the separation, and was not investigated further.

Attempted acid hydrolysis of steroidal 2-hydroxy-3-naphthoylhydrazones

When the oxosteroids or their conjugates have been separated as their hydrazones, it may be desirable to regenerate the carbonyl compound by hydrolytic fission of the hydrazone linkage. The conventional method of doing this, by acid hydrolysis, was unsuccessful, presumably because of the low solubility of the hydrazones in the hydrolysing medium.

-o-

EXPERIMENTAL

Urinary hydrazone precipitate (1 g) was added to 95% ethanol (200 ml) and refluxed for 1 hour. Only about half the solid dissolved. Conc. HCl (25 ml) and water (50 ml) were added, and refluxing continued for 1½ hours. The contents of the reaction flask were poured into ice-water, extracted with ether, and the ether solution extracted with alkali. Only 50 mg.

of neutral ether extract resulted, of which only 25 mg. reacted with the Girard T reagent. The remaining material was all alkali-soluble, and therefore still contained phenolic groups.

-o-

Hydrolysis by means of hot mineral acids would, of course, destroy the conjugates and would also tend to decompose and degrade the steroids in the way that has been avoided by the cold precipitation of the oxo-steroids in the first place. A new approach was investigated, namely, the reversing of the hydrazone formation by the use of oxidised cellulose.

Effects Observed with Oxidised Cellulose Columns

If cellulose is partially oxidised with periodic acid, free aldehyde groups are formed. If a solution of hydrazones is then passed through a column of such oxidised cellulose, the hydrazide moiety leaves the hydrazone and combines with the aldehydic groups of the column, so liberating the free ketones which are recovered from the eluate. This effect, which does not appear to have been described previously, was designated by the author (Camber 1950) "Group Exchange". Although time did not permit of complete investigation of the phenomenon and its usefulness, the novelty of the effect and its potential value as a technique for controlled organic reactions are held to warrant some detailed discussion here.

-o-

EXPERIMENTAL

(a) Oxidised cellulose. Laboratory oxidation of starch with periodic acid was found to be tedious (oxidation requires 1 month) and unsatisfactory -- the starch dried into hard lumps of low activity, and if the oxidised mass was kept wet, it tended to turn yellow and form a gel. Of the synthetic carbonyl resins investigated, one showed no activity even with the hydrazide itself, while the other was water-soluble. Commercial oxycellulose was therefore used; its activity depended on the degree of drying (diminished activity) and on the particle size (the smaller the more active). The raw material (from Hercules Powder Co., N.J.) was shredded in a Waring blendor for 10-15 min. and packed into a column (6 in. x 0.5 in.) above a cotton-wool plug. It was washed with water and the milky filtrate recycled until as many of the very fine particles as possible were trapped in the column.

(b) Eluant. Application of the hydrazones in 5% acetic acid in 50% aqueous ethanol was unsuccessful. Acetate and phosphate buffers, as well as mercuric chloride, silica gel. ammonium chloride and tetramethylammonium chloride (all said to have a catalytic effect on dissociation were added to this solution without effecting improvement. The essential process was found to be to wash the column first with 4 to 5 volumes of 1-5% aqueous acetic acid, then add the hydrazone (0.25 mg) in 95% ethanol (0.25 ml), continuing the elution with 95% ethanol.

(c) Following the reaction. The column was observed by Wood's light, in which the hydrazones showed up as a bright yellow band against the bright blue of the column, the bright blue fading to a dull white appearance as transfer was effected. The observations were confirmed by coupling the column with a coloured diazonium salt.

-o-

On all columns, the hydrazone derivative (e.g. that from cyclohexanone) formed a narrow band at the top of the column immediately on application, and an intense yellow fluorescence moved down the column from this band, thinning and disappearing as cleavage became complete. This was interpreted as meaning that the top band represented the immediate coupling to the column of the hydrazide moiety which was already free when the solution was applied to the column, so that the width and intensity of the top band is proportional to the degree of dissociation, or equilibrium constant $(\frac{k_1}{k_2})$ of the dissociation:-

$$\text{Oxosteroid hydrazone} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Oxosteroid + Hydrazide}$$

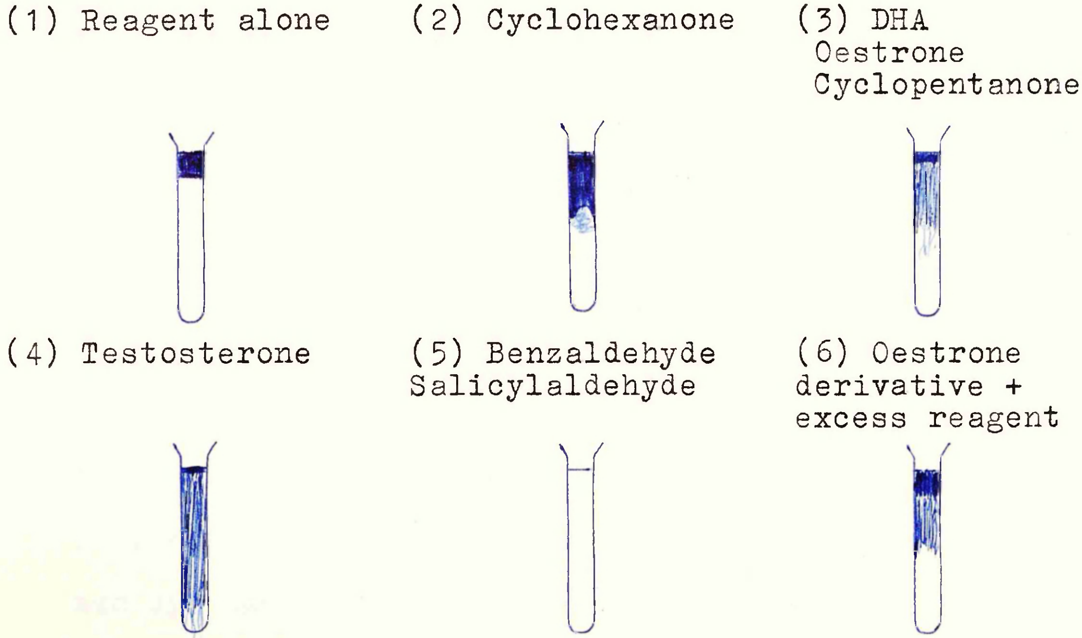
The width of the less intense fluorescent band, in which dissociation and coupling are taking place as the solution moves down the column, is a measure of the velocity constant k_1 of the dissociation.

Immediate coupling of the column to 2-hydroxy-3-naphthoyl hydrazide was established in a separate experiment. The degree of dissociation in the solution to be applied depends not only on the dissociation constant of the particular hydrazine, but also on the pH, the temperature, the nature of the organic solvent and the amount of water it contains. The rate of dissociation depends on all these factors as well as on the rate constant of the particular hydrazone. The particular effect of pH was investigated using aqueous acetic acid and phosphate buffers in the pH range 4 to 8. As regards water, aqueous conditions were found to be essential to cleavage; this was reconciled with the hydrophobic nature of the steroid hydrazones by washing the column with the aqueous acid or buffer solution, then applying the ethanol solution. As the rate of dissociation is usually rather slow, negative pressure was usually applied to the column to retard the flow of solution.

From the literature (see, for instance, Reichstein 1936) on the ease of hydrolysis of steroid hydrazones, it appears that they fall into four classes, viz. (in increasing order of ease of hydrolysis): (1) saturated ketones in 6-rings, of which cyclohexanone is a model, (2) 20-ketones, (3) 17-ketones, of which cyclopentanone is a model, and (4) $\alpha\beta$-unsaturated ketones, e.g. testosterone. In accordance with this, it was observed that cyclohexanone splits much more easily on the cellulose column than cyclopentanone; Zimmermann-positive material was demonstrated in the aqueous eluate. It therefore appears that, with a mixture of steroid hydrazones, cleavage and separation might be achieved in one operation; and that an individual oxosteroid might be allocated to one of the four groups by visual observation of the dissociation constants on a standard column.

Some confirmation of this was obtained, using cyclohexanone (as model for 3-oxosteroids); dehydro*epi*androsterone, oestrone, and cyclopen-tanone (17-ones and model); and testosterone and desoxycorticosterone (4-en-3-ones). Aldehyde hydrazone derivatives failed to cleave on the column, and no fluorescence was visible. A sample of oestrone 2-hydroxy-3-naphthoylhydrazone, prepared for analysis, was examined in a standard column and shown by this means to contain a considerable quantity of excess reagent(Fig.1.).

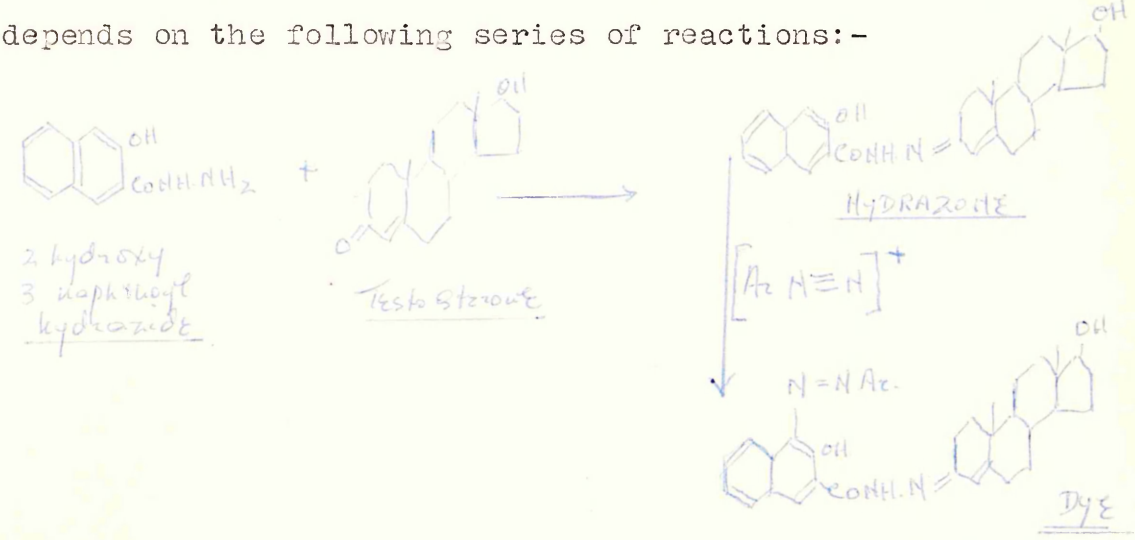
Fig.1. Differentiation of 2-hydroxy-3-naphthoylhydrazones



Histochemical Applications

The formation of azo-dyes by coupling 2-hydroxy-3-naphthoylhydrazones with diazotised sulphanilic acid was mentioned briefly in the discussion of the separation of the hydrazones by paper chromatography. The reaction was found to be quite general, and the reactivity of the hydrazones to be high, in accordance with expectation, so that immediate coupling with diazotised amines takes place at room temperature.

The method was applied to the staining of tissue sections. Previous use of hydrazone formation for staining (Bennett 1940, Dempsey and Wislocki 1946) had depended on the colour of the hydrazones themselves, and because of their low staining intensity thick sections had to be used. Thin sections (5 μ) may be used with the new reagent. Another advantage of the method, which depends on the following series of reactions:-



is that the colour and degree of solubility of the final azo dye can be varied by selecting the aromatic group of the diazonium salt. The technique involved permits the staining of blocks of fixed tissues, the use of paraffin embedding, counterstaining of the sections and clearing and mounting by the usual techniques. The most successful method of application found was that published by the author in 1949 (Camber 1949).

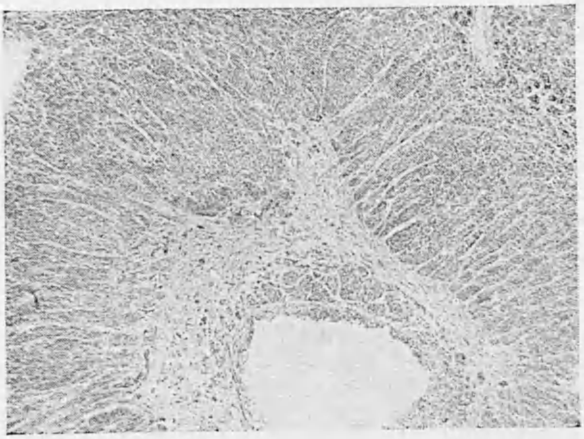
-o-

EXPERIMENTAL

Formalin-fixed tissue, thoroughly washed in running water, is sectioned on the freezing microtone. The

The distribution of coloured material in otherwise unstained adrenal tissue is illustrated below (from the original paper, Camber, 1949).

Whilst the appearances could be construed as evidence indicating the presence of oxosteroids in the adrenal cortex, it was considered that no claims were warranted at this stage of the investigation.



Section of human adrenal gland, stained with 2-hydroxy-3-naphthoic acid hydrazide coupled to tetrazotized benzidine. (× 40)

(Facing page 48).

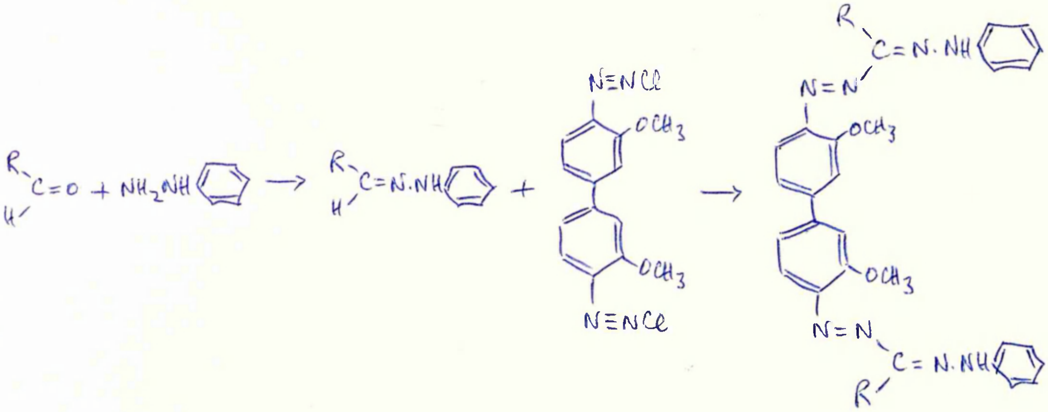
sections (5_μ) are allowed to remain overnight in a saturated aqueous solution of 2-hydroxy-3-naphthoyl hydrazide and washed in N/1000 hydrochloric acid and then in distilled water to remove excess reagent, immersed in a solution of 0.4% sodium hydroxide and transferred to a dilute solution of the diazonium salt. This may be prepared from a stabilised diazonium salt, or a fresh diazotisation mixture of milli-equivalent parts of primary aromatic amine, hydrochloric acid and sodium nitrite may be made up. Excess nitrous acid would lead to rupture of the hydrazone linkage (Wieland and Horner 1937) and is disposed of by means of sulphamic acid. Approximately 0.5 ml. of the diazonium salt solution added to 250 ml. of distilled water provides a suitable concentration for coupling. If the diazonium salt solution is too strong, staining tends to be patchy. The sections may then be counterstained, cleared and mounted in the usual way. (For appearance see illustration facing this page).

-o-

It is to be noted that the method employs a slow reaction, in accordance with a general histochemical principle that differentiation demands a technique of gradualness in staining. For this reason it is to be regretted that Ashbel and Seligman, who investigated the reaction subsequently (1949) modified the technique in order to obtain greater speed and intensity, with corresponding loss of detail. Their use of 50% ethanol for the hydrazone formation is also questionable, since the steroids are liable to dissolve and be displaced in this solvent.

The question which immediately arises is whether the reagent is specific for oxosteroids, for it will presumably react with all ketones, and also with aldehydes, which invariably accompany oxosteroids in tissue. Seligman and Ashbel (1951) strove to show that the staining (which occurs only after formalin-fixing) was not due to reaction with aldehydes; and the point has been extensively discussed by Gomori (1952). A summary of the discussion follows.

Several standard reactions for aldehydes were applied to formalin-fixed nervous tissue, with which a positive reaction had been obtained with the hydrazide. All these failed to indicate the presence of aldehydes. For example, the sections showed a negative formazan reaction. Aldehyde phenylhydrazones react with diazonium salts to form purplish formazans (Rutenburg _et_ _al_. 1950), but ketone phenylhydrazones cannot do so, because they have no replaceable hydrogen:-



Again, the Angeli-Rimini test for aldehydes (Angeli and Angelico 1904, Rimini 1908) was negative. Finally the staining reaction was not blocked by prior treatment with sulphanilic acid or aniline, which are known to block aldehydes selectively, (Oster and Mulinos 1944, Boscott and Mandl 1949). The absence of any visible reaction with 2:4-dinitrophenylhydrazine (Boscott and Mandl) can be attributed to the weak staining properties of this reagent as contrasted with those of 2-hydroxy-3-naphthoyl hydrazide.

Gomori is not convinced by these arguments. He points out that formazans are not given by all aldehydes: for instance, periodate-treated glycogen is completely negative. Similarly, the Angeli-Rimini test is known to be negative with a number of aldehydes even in test-tube experiments (Angeli and Angelico 1903; Angeli and Marchetti 1908), and is invariably negative histochemically with unquestionably aldehydic substances.

He maintains that the results of blocking experiments permit no conclusions whatever as to the aldehydic or ketonic nature of carbonyl groups, a somewhat sweeping generalisation. He states that the carbonyl reactions in the tissues are so intense that they must be due to a substance which constitutes a substantial percentage of the lipid material, a dubiously valid statement since the author has shown that the azo-compounds formed are in fact very intensely coloured.

All in all, the balance of evidence seems to lie on the side that maintains that the staining reaction is not due to aldehydes (since the attempted aldehyde reactions are of several different types) but to ketones. On the other hand, the weakness lies in the fact that prior fixation with formaldehyde (or with a saturated solution of picric acid in 50% alcohol) is necessary for the reaction to take place, which makes one suspect that oxidation of unsaturated linkages is taking place to form carbonyl compounds. Even if it is accepted that ketones, not aldehydes, are responsible, no evidence has been brought forward that the ketones are steroidal in nature.

It is evident, then, that although the use of 2-hydroxy-3-naphthoic acid hydrazide followed by azo-coupling is a useful technique in histochemistry, and has already found considerable application in the field (see, for example, the detection of the aldehyde reaction of the sugar moiety of nucleic acids, Pearse 1951), its specificity for oxosteroids is at least questionable. In the author's experience, results with the reagent were much too unsatisfactory and vague to support the weight of argument that was being erected upon them. What was more, it did not seem likely that mere assiduous application of the reaction would lead to clarification of the issues involved. It was decided, therefore, to eschew experiments designed merely to support one side of the argument or the other, to admit the plain fact of the inadequacies of the reagent, and to continue the search for a more satisfactory reagent which might provide answers to the problems that had been raised rather than solved by the introduction and use of 2-hydroxy-3-naphthoyl hydrazide.

PART 4

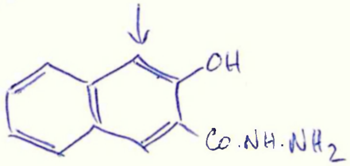
β-RESORCYLIC ACID HYDRAZIDE

Introduction

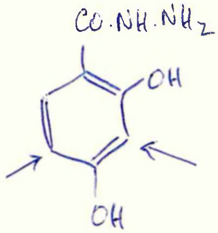
One of the major difficulties in demonstrating the presence of oxosteroids histochemically is the minute quantity of the compounds to be expected in a tissue section. Olson et al. (1944) estimated the oxosteroid content of extracts from tissue, and the amount of oxosteroid to be expected per section, based on these extraction values, is of the order of 0.02μg. ?

For this reason, after the first suggestion (Bennett 1940) of the use of phenylhydrazine for the staining of oxosteroids, the more deeply coloured 2:4-dinitrophenyl-hydrazine came into use (Dempsey and Wislocki 1946). Even the derivatives of this reagent were not deeply coloured enough, for Boscott and Mandl (1949) were unable to obtain a visible reaction of oxosteroids after the elimination of tissue aldehydes. A new ketonic reagent giving intensely coloured and insoluble derivatives was at this stage highly desirable.

The reaction with 2-hydroxy-3-naphthoic acid hydrazide, followed by azo-coupling, met this need to some extent, and gave greater intensity of staining. A related phenolic acid hydrazide in the benzene series, 2:4-dihydroxybenzoyl hydrazide (β-resorcyloyl hydrazide), would be expected to give even more intense staining, since it contains two activated nuclear positions at which azo-coupling can take place, instead of one:-



2-hydroxy-3-naphthoic acid hydrazide



2:4-dihydroxybenzoyl hydrazide

β-Resorcylic acid hydrazide had not been described in the literature; it was therefore synthesised and examined.

Synthesis

The synthesis, carried out in conjunction with D.D. Dziewiatkowski and described in 1951 (Camber and Dziewiatkowski 1951), involved the condensation of the commercially available ethyl ester of β-resorcylic acid with hydrazine hydrate under reflux in absolute ethanol.

-o-

EXPERIMENTAL

To ethyl β-resorcylate (18.2 g, 0.1 mole), dissolved in absolute ethanol (10 ml) by warming, 100% hydrazine hydrate (8 ml) was added. Most of the ethanol was removed at this stage by distillation, and the reaction mixture refluxed for two hours. At the end of one hour, a further addition of hydrazine hydrate (4 ml) was made.

On cooling to room temperature, the reaction mixture solidified. The flask with the reaction mixture was placed in a vacuum desiccator over calcium chloride at room temperature and left there for three days to drive the reaction to completion by removing ethanol. The solid material in the flask was dissolved in the minimum quantity, about 200 ml., of boiling 95% ethanol. A light tan crystalline precipitate was deposited on cooling to room temperature, its bulk being increased by further cooling at 0°C for 3 hours. The filtered precipitate was redissolved in boiling 95% ethanol (130 ml), the solution decolorised with activated carbon (Darco, Grade G-60) and left at 0°C overnight. The nearly white crystalline precipitate was dried briefly in air and then for 24 hours in vacuo over calcium chloride at roon temperature. The yield was 5.7 g. (34%), m.p. 240-241° (uncorr.). The synthesis was repeated with the same results.

Two more recrystallisations from 95% ethanol gave colourless lath-like needles with a tendency to form rosettes (Fig.2), m.p. 245-246° (uncorr.). This material was analysed. Found: C, 50.0; H, 4.8; N, 16.7. Calc. for $C_7H_8O_3N_2$: C, 50.0; H, 4.8; N, 16.7.

β-Resorcyloylhydrazones of simple aldehydes and ketones

The formation of derivatives of the reagent with carbonyl compounds was investigated (in part, with Dziewiatkowski) *in vitro*, in order to prove the identity of the reagent as much as to assess its condensing power.

In each instance the aldehyde or ketone (50 or 100 mg) was added to a saturated ethanolic solution of the hydrazide (10 ml). One drop of glacial acetic acid was added and the mixture kept hot, short of boiling, for 20-30 minutes. On cooling, whether a precipitate was evident or not, an equal volume of water was added and the flask set aside at 0°C for at least 24 hours. The precipitates were filtered on a sintered glass plate, washed repeatedly with water and then repeatedly with ether. They were recrystallised from aqueous alcohol, washed with water and ether, and then dried in vacuo over calcium chloride for at least 24 hours.

A summary of the physical characteristics of the hydrazones is given in Table 3.

Table 3.

Aldehyde or Ketone	β-Resorcyloylhydrazone M.p. °C.	Crystalline form and colour
Formaldehyde	295 (dec.)	Amorphous, white
Acetaldehyde	118	Plates, white
Benzaldehyde	239	Rosettes, light yellow
Salicylaldehyde	268-9	Needles forming rosettes, yellow
Acetone	250	Rectangular plates, white
Methyl propyl ketone	199-200	Micro-needles forming rosettes, white
Pyruvic acid	255 (dec.)	Amorphous, white
Cyclopentanone	265 (dec.)	Micro-needles forming rosettes, white

The hydrazones of benzaldehyde and cyclopentanone were further dried in vacuo over phosphorus pentoxide for 3 days at room temperature and then for 1 hour at 78°C, and submitted for analysis.

Hydrazone of benzaldehyde: C, 65.6; H, 4.9; N, 11.1. Calc. for $C_{14}H_{12}O_3N_2$: C, 65.6; H,4.7; N, 10.9.

Hydrazone of cyclopentanone: C, 61.6; H, 6.0; N, 12.4. Calc. for $C_{12}H_{14}O_3N_2$: C, 61.5; H, 6.0; N,12.0.

β-Resorcyloylhydrazones of steroidal ketones

The hydrazone derivatives of five oxosteroids were prepared under rather more aqueous conditions than above. For example, hot solutions of dehydroepiandrosterone (50 mg) in methanol (1.5 ml) and of β-resorcyloyl hydrazide (30 mh., approximately 1 equivalent) in water (1.5 ml) were mixed, and 2 drops of glacial acetic acid were added. No precipitation occurred until the mixture had been refluxed for 1 minute. Refluxing was continued for 10 minutes and the crystals were recovered from the cooled colution.

Oestrone required 4.5 ml. methanol for solution, and some of the methanol had to be evaporated off before precipitation occurred. For the diketones progesterone and desoxycorticosterone, 2 equivalents of reagent were used.

-o-

The physical characteristics of the steroidal hydrazone derivatives are shown in Table 4.

Table 4.

Oxosteroid	β-Resorcyloylhydrazone	
	M.p. °C	Crystalline form and colour
Dehydro*epi*androsterone	245-251	Squarish micro-plates, yellow
Oestrone	215	Needles forming rosettes, white
Testosterone	269-70	Amorphous, white
Progesterone	243-5	Amorphous, white
Desoxycorticosterone	230-5	Elongated hexagons forming rosettes, yellow

Coupling of the steroidal hydrazones with azo-dyes: specificity of the reagent

The steroidal hydrazone derivatives were coupled with Fast Black Salt K (p-nitrobenzene-azo-5-dimethoxy-aniline, Heftmann 1950). The interesting result was obtained that two different colours of azo-derivatives, depending on the original steroid, resulted. These were further differentiated on treatment with acid or alkali (Table 5).

-o-

EXPERIMENTAL

The hydrazones were dissolved in dilute ammoniacal methanol (0.5 mg per 100 ml), and to the solution was added one drop of a freshly filtered colution of diazotised Fast Black Salt K. The same results were obtained if coupling took place in 95% ethanol solution to which one drop of 0.2M phosphate buffer, pH 7.1, had been added. Changes of colour were noted when the solution was acidified with one drop of N sulphuric acid, or made alkaline with 2 drops of N sodium hydroxide.

-o-

Table 5

β-Recorcyloyl-hydrazone of	Colour on coupling	On acidification	On alkalisation
Dehydroepiandrosterone (17-one)	Pink	Pink	Pink
Oestrone (17-one)	Purple	Pink	Pink
Testosterone (4-en-3-one)	Purple	Purple	Purple
Progesterone (4-en-3,20-dione)	Purple	Purple	Purple-blue
Desoxycorticosterone (4-en-3,20-dione)	Purple	Purple	Blue
-	-	-	-
(Reagent	Pink	Pink	Pink)

The results in Table 5 provide the first example of the formation of a dye whose colour depends to some extent on the nature of the original steroid material, and offer what was lacking in 2-hydroxy-3-naphthoylhydrazide, namely some degree of specificity. If the same range of colour could be observed histochemically, it might be possible to identify particular substances in tissues

THE PHOTOMICROGRAPH MAY BE REMOVED
FROM ENVELOPE FOR INSPECTION.

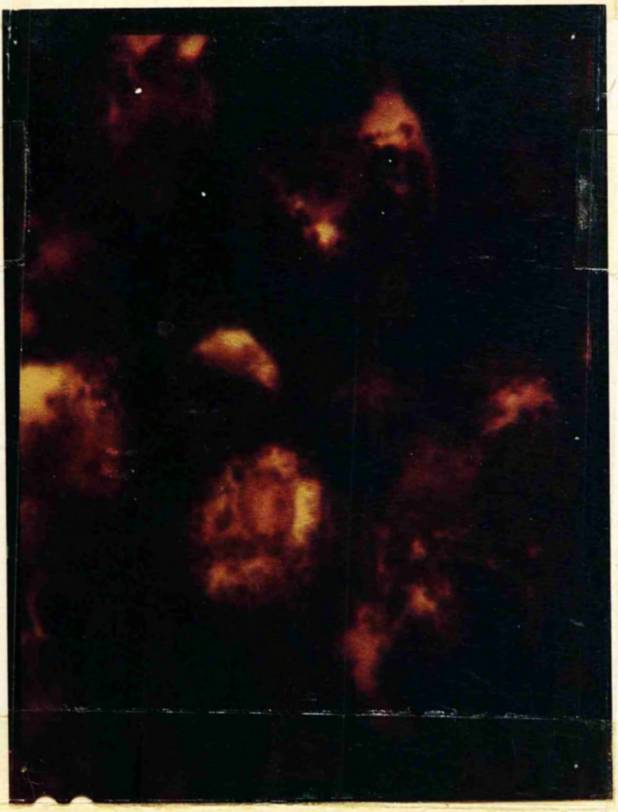


Fig. 2. Section of rat adrenal gland stained with β-resorcyloyl hydrazide followed by azo-coupling with Fast Black Salt K. Note pink and purple staining.

by colour alone.

Formalin-fixed frozen sections (5μ) of rat adrenal, thoroughly washed in running distilled water for 10 minutes, were transferred to a saturated solution of β-resorcylic acid hydrazide in aqueous acetic acid (0.25%) and left at room temperature overnight, or warmed at 55-60^{o} for 1 hour. The sections were then washed in running distilled water to remove excess reagent, transferred to aqueous sodium carbonate solution (1%) for 1 minute, and then to a freshly filtered aqueous solution of Fast Black Salt K. Coupling commenced immediately, but was allowed to continue for 1 hour, by which time the section appeared blue-black to the naked eye. After washing in running distilled water for 5 minutes, the sections were mounted in Farrant's medium, and examined under oil-immersion.

The cells of the fascicular zone of the adrenal cortex appeared to be of two types. One was a diffusely red-stained cell, the other a clear cell showing granules of two colours, purple and pink. These colours were similar to those of the azo derivatives noted in Table 5. The differentiation is clearly shown in the coloured photomicrograph, Fig. 2.

In rats treated with ACTH, the purple granules were more numerous and more intense in colour, and the number and intensity as judged roughly by inspection appeared to be proportional to the dose of ACTH employed.

The range of colours observed in the test-tube seems, therefore, to be observed in the cell also, but in interpreting these results caution must be exercised. That the reagent reacts with tissue aldehydes was illustrated by two observations. If formalin-fixed rat adrenal sections were hydrolysed with hydrochloric acid before hydrazide treatment, the cell <u>nuclei</u> showed granules similar to those obtained in the Feulgen reaction, but red-brown in colour. In cartilage and glycogen-containing tissues, otherwise unreactive, prior oxidation with periodic acid brought about a positive reaction with the hydrazide, again red-brown in colour. The pink and purple colours mentioned above might also be due to reactions with aldehydes, and not with oxosteroids at all.

However, there is room for optimism, since these colours are in fact quite different in appearance. A variety of colours is possible with the reagent: when it was applied to the adrenal cortex, various

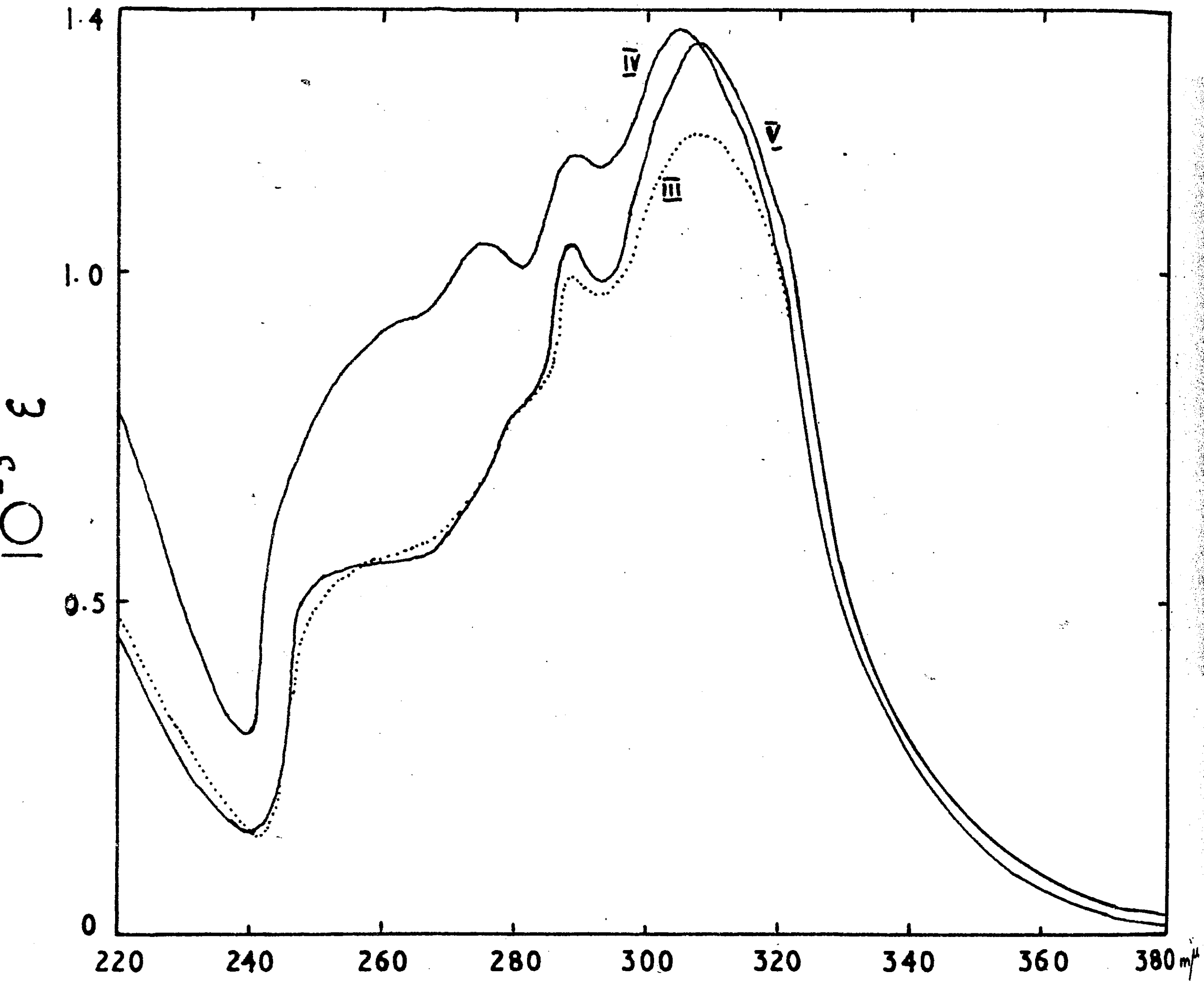


Fig. 6. Ultraviolet absorption spectra of β-resorcyloyl hydrazones after coupling with Fast Black Salt K. III, testosterone. IV, progesterone. V, desoxycorticosterone.

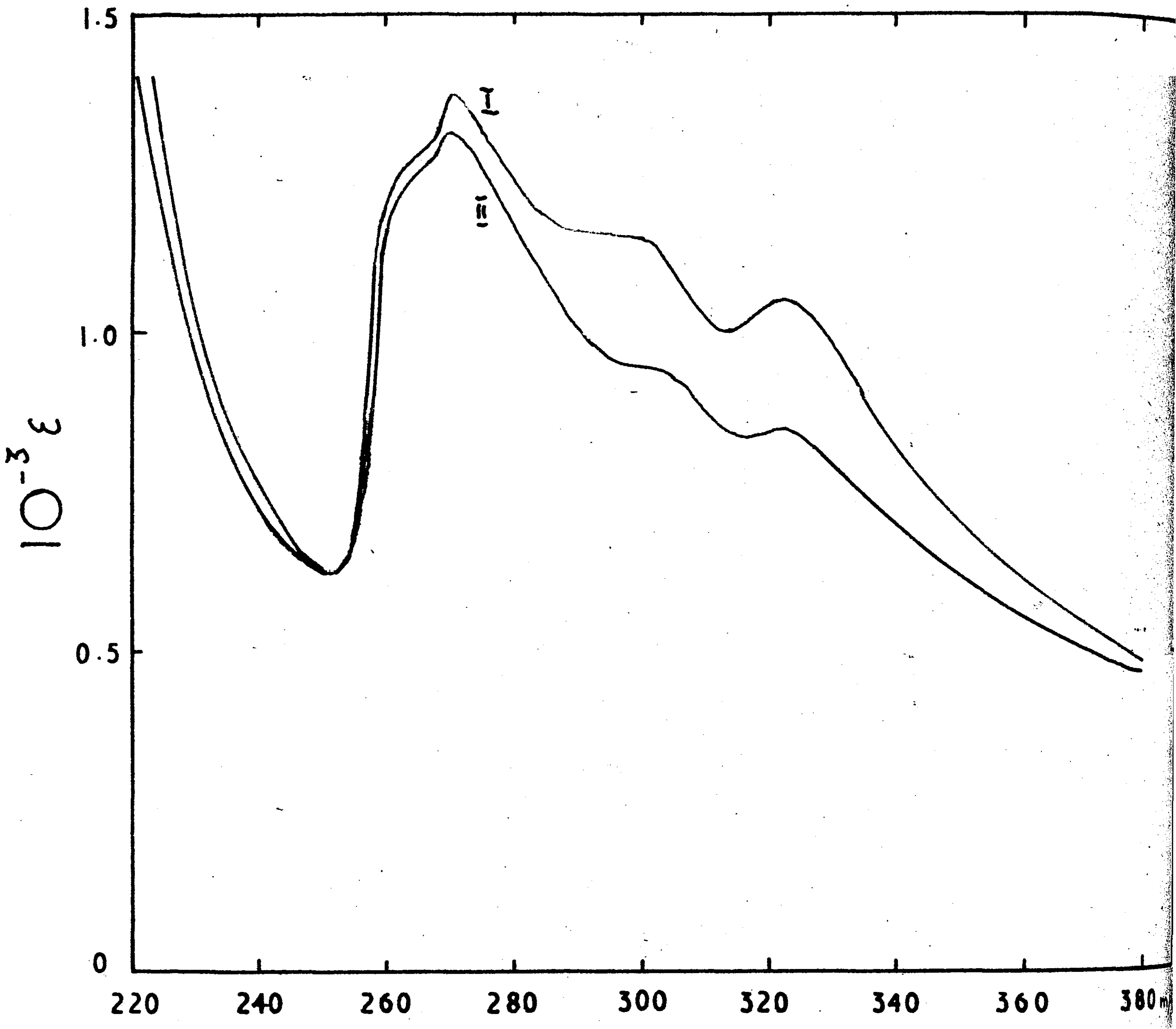


Fig. 5. Ultraviolet absorption spectra of β-resorcyloyl hydrazones after coupling with Fast Black Salt K. I, dehydro*epi*androsterone. II, oestrone.

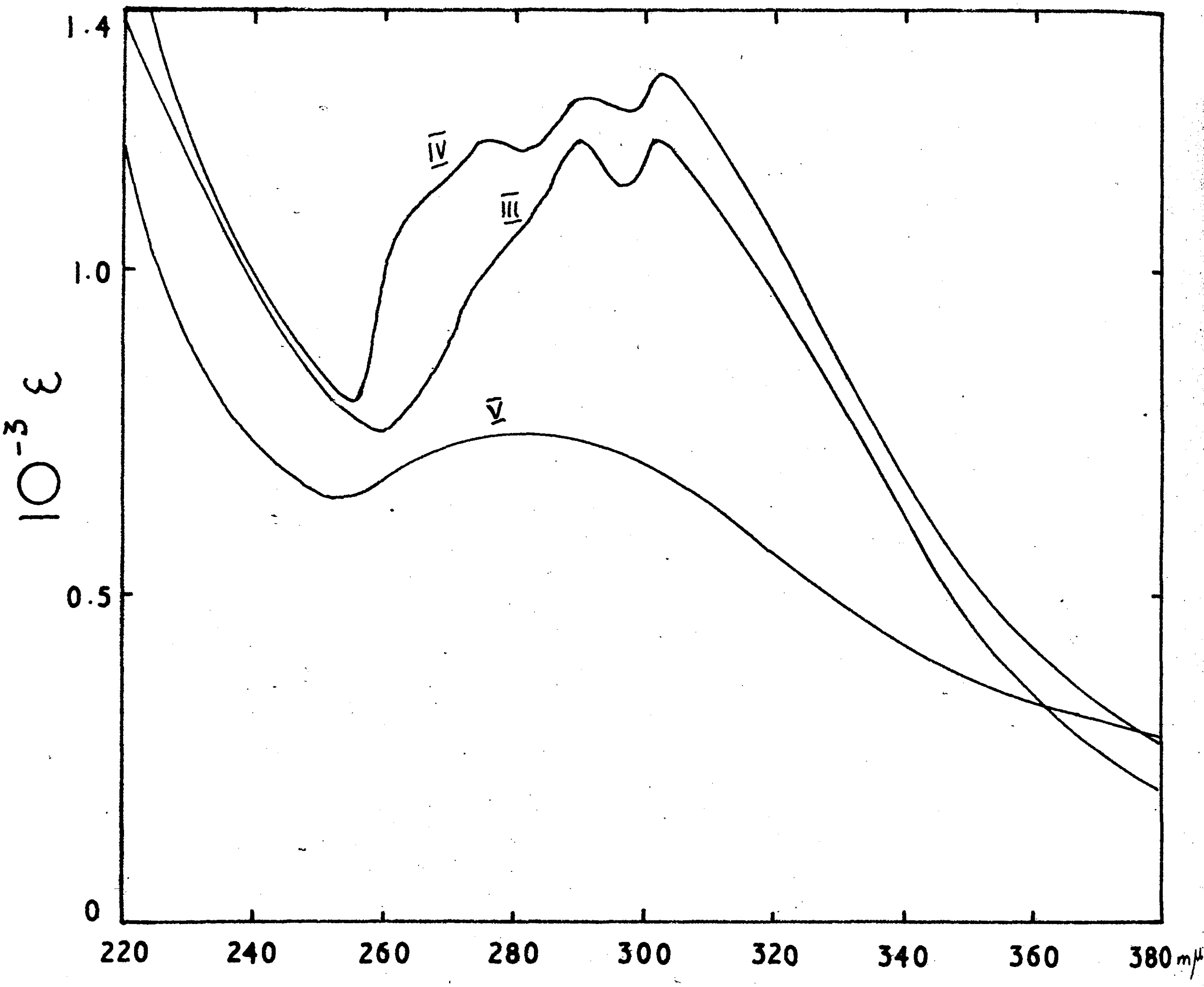


Fig. 4. Ultraviolet absorption spectra of oxosteroid derivatives of β-resorcyloyl hydrazide. III, testosterone. IV, progesterone. V, desoxycorticosterone.

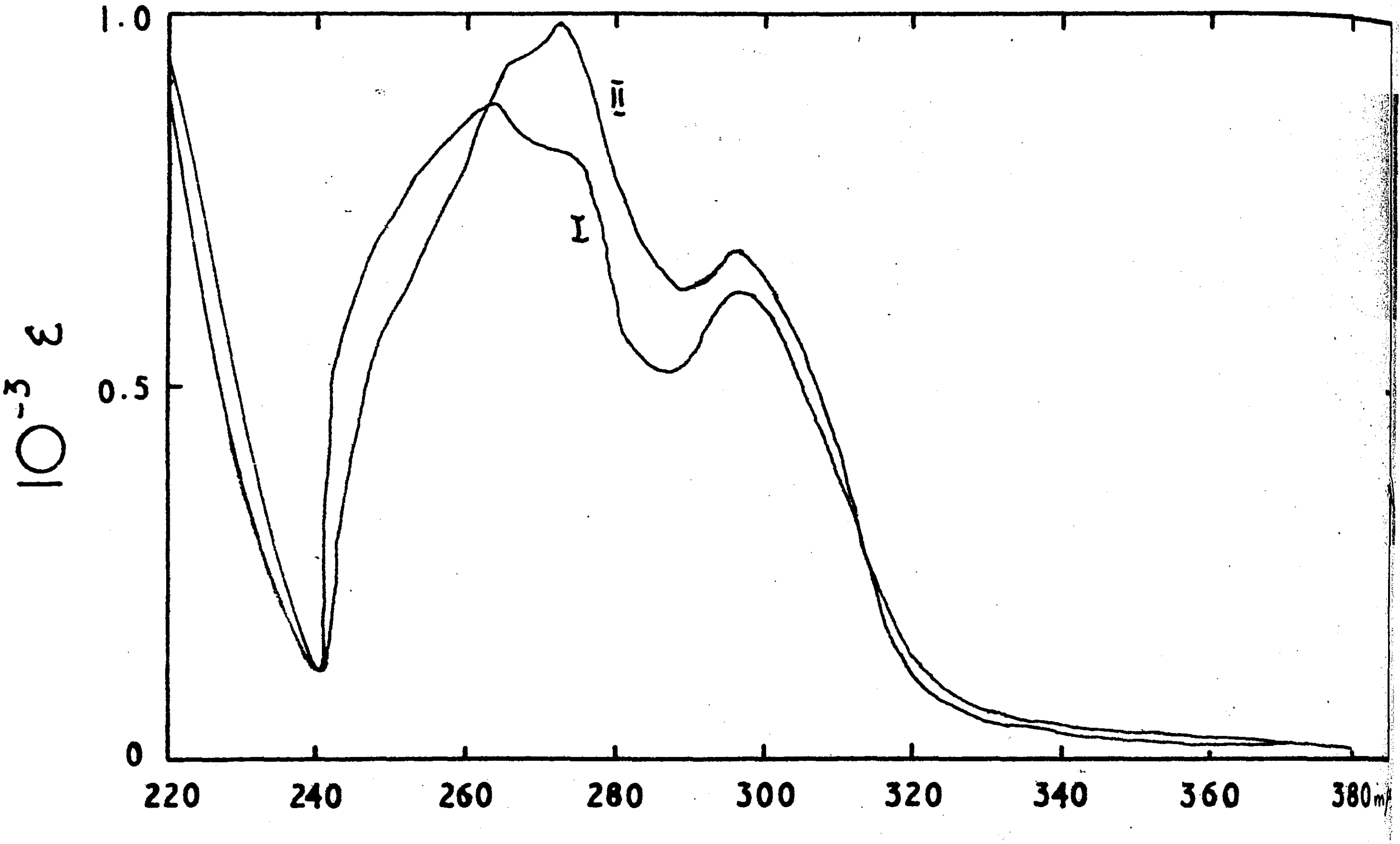


Fig. 3. Ultraviolet absorption spectra of oxosteroid derivatives of β-resorcyloyl hydrazide. I, dehydro*epi*androsterone. II, oestrone.

zones were clearly demarcated in shades of red, blue, and yellow. Myelinated tissue, which with 2-hydroxy-3-naphthoyl hydrazide gives the same colour as adrenal cortical tissue, stained orange with the new reagent.

Further differentiation of steroidal β-resorcycloylhydrazones

By measuring the ultraviolet absorption spectra of the steroidal hydrazones, greater differences than the colour differences between the azo derivatives could be observed. The ultraviolet spectra are shown in Figs. 3 and 4: Figs. 5 and 6 show the spectra of the azo-derivatives. (The absorption spectra of the latter in *visible* light merely separates the azo-dyes into two classes as before: the purple dyes have an absorption maximum at 560 mμ ; the pink have one at 475 mμ .) The ultraviolet absorption maxima are shown in Table 7.

Table 7

λmax of hydrazone derivative mμ		λmax of coupled hydrazone derivative mμ
Dehydro*epi*androsterone (17-one)	264*, 275 (shoulder), 295	265, 270*, 298 (shelf) 321
Oestrone (17-one)	265 (shoulder), 272*, 295	265, 270*, 300 (shelf) 321
Testosterone (4-en-3-one)	280 (inflexion), 288, 308*	280 (infl.) 290, 302
Progesterone (4-en-3,20-dione)	265, 275, 289, 305*	265, 275, 290, 303
Desoxycorticosterone (4-en-3,20-dione)	280 (inflexion), 288, 308*	283 (broad)
-	-	-
(Reagent	260, 295	257*, 294)

(* indicates the most important peak)

The general shapes of the curves for dehydro*epi*androsterone and for oestrone are very similar. The curves for the β-resorcyloylhydrazones of the other three sterones, while differing in detail, are very similar to one another; the same observation applies to the curves for the azo derivatives from testosterone and progesterone, while the azo derivatives from desoxycorticosterone differs from all the others.

Further differentiation between β-resorcyloylhydrazones could be achieved by observing their fluorescence in that portion of ultraviolet light which passes through a Wood's glass filter. The colours which were observed in solution in 95% ethanol are shown in Table 8.

Table 8

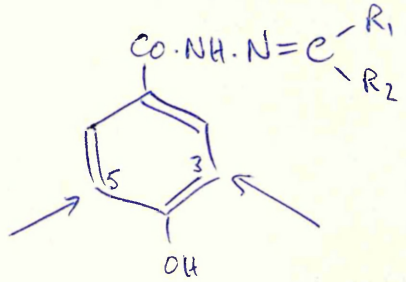
β-Resorcyloylhydrazone of	Fluorescent colour under Wood's light
Dehydroepiandrosterone	Intense blue
Oestrone	Intense blue
Testosterone	Weak dull purplish
Progesterone	Weak dull purplish
Desoxycorticosterone	Weak dull green
-	-
(Reagent	Nil)

It is evident that the five sterones examined fall into two groups: dehydroepiandrosterone and oestrone on the one hand, testosterone, progesterone and (with reservations) desoxycorticosterone on the other. Certain conclusions can be drawn about the chemical constitution of the derivatives from the data given.

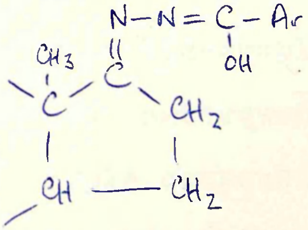
The ultraviolet spectra of the β-resorcyloylhydrazones indicate that the peaks 265, 275, 295mμ are typical of derivatives of the saturated 17-ones, while 280, 289 and 308 mμ are the corresponding peaks from 4-en-3-ones, in which the absorbing group has been extended by a conjugated double bond. The hydrazone of progesterone shows, besides the second group of three, 265 and 275mμ. These peaks could be explained as the contribution of a 20-hydrazone, were it not that testosterone (as well as desoxycorticosterone) also shows some indication of the peaks at 265 and 275mμ. Nevertheless it seems likely that 3,20-dihydrazones are in fact formed by both progesterone and desoxycorticosterone (see later).

The features of these spectra are retained after azo-coupling in all cases (including that of the reagent) except that of desoxycorticosterone resorcyloylhydrazone. Here, all characteristic peaks disappear after coupling, leaving a single broad peak at 283mμ. This observation suggests a decomposition of the hydrazone with loss of the steroid moiety, see later, which might account for the uncharacteristic behaviour of the "azo derivative" in other respects (alkalisation).

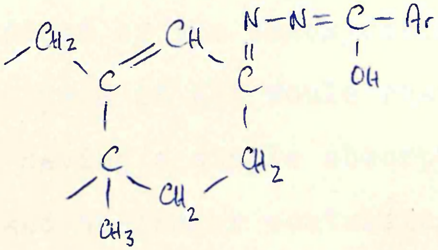
The production of pink and purple dyes by different β-resorcyloylhydrazones may be understood by considering the molecule of the reagent, in which two nuclear positions are <u>ortho</u>- to a phenolic group and therefore activated for diazo coupling. Coupling normally takes



place at position 5, giving a pink dye with β-resorcyloyl hydrazide itself. Increased activation of position 3 will result in the formation of the bis-azo compounds having the deeper purple colour. Precisely why 4-en-3-one residues should produce this extra activation whereas 17-ones do not is rather obscure, since the electronic environments of the connecting carbon atoms are not very different in the two cases:-



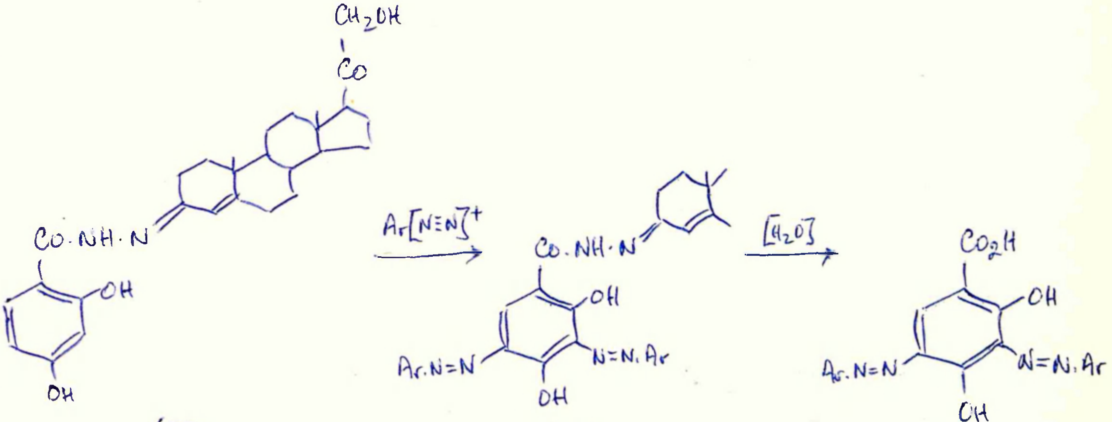
17-ones



4-en-3-ones

At any rate, the difference is sufficiently great to give the useful differentiation.

Raising the pH of the diazotising solution with sodium hydrozide has no visible effect on the pink dyes, and merely deepens the colour in the case of the 4-en-3-one testosterone; but with progesterone a blue colour appears, presumably from the 20-one. For desoxycorticosterone, it is suggested that the blue colour is produced by the compound XV, whose formation is indicated by the change in the ultraviolet spectrum noted above. Treatment of XV with the diazonium salt will result in bis-coupling induced by the desoxycorticosterone residue; subsequent hydrolysis of XVI would result in the purple compound XVII, having a single absorption peak in the ultraviolet, and no longer containing a steroid residue.



(XV) (XVI) (XVII)

Finally, the fluorescence differences between the two groups of hydrazones can be explained on the basis of the extra conjugated double bond introduced in the

4-en-3-ones. This changes the energy levels available to the system, and would account for the difference in intensity and colour of fluorescence. Blue is apparently associated with 17-one derivatives, purple with the 4-en-3-one group, and green with 20-ketones. For desoxycorticosterone, the green colour at the 20-position apparently swamps the purple colour from position 3 of the steroid molecule.

The existence of these differing properties is certainly of interest. Nevertheless, there were certain deficiencies in β-resorcyloyl hydrazide which could not be glossed over, notably the failure to obtain satisfactory elementary analysis of oxosteroid hydrazones. It was clear that the disadvantages of the reagent were of a fundamental nature: its tendency to decarboxylate and to undergo side-reactions rendered interpretation difficult, and the continued necessity for a two-stage process was especially disappointing. It was felt that β-resorcyloyl hydrazide was not the answer that was being sought, and another reagent in this series was therefore investigated.

PART 5

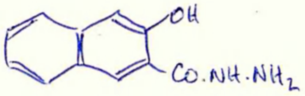
SALICYLIC ACID HYDRAZIDE

Introduction

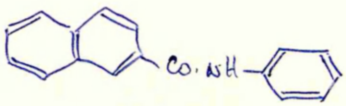
Although both 2-hydroxy-3-naphthoyl hydrazide and β-resorcyloylhydrazide had proved promising as chemical and histochemical reagents for oxosteroids, both were open to criticism on both practical and theoretical grounds.

For example, 2-hydroxy-3-naphthoyl hydrazide itself is difficult to crystallise, and oxidation occurs readily enough for the final product of recrystallisation to be more highly coloured than the starting material, despite charcoaling at each stage. The reagent is relatively insoluble in water and most other solvents; its low solubility may explain why fairly high concentrations of oxosteroids are necessary to produce a hydrazone precipitate. The hydrazone precipitates formed were gummy or colloidal, and frequently had to be separated by extraction (benzene-ethyl acetate vs. 5% aqueous sodium bicarbonate). The inability to carry out in vitro precipitation using quantities of oxosteroids even remotely approximating to those expected to occur in body cells renders such a reagent of doubtful value in histochemistry.

Further, formalin fixation proved to be a necessary prerequisite to obtaining a positive reaction in tissues. The use of a carbonyl compound as a precondition of demonstrating the presence of carbonyl compounds raises doubts as to the significance of a positive reaction, especially since the reagent itself reacts readily with formalin. Again, the reagent is very closely related in structure to the "Anilid-Säure" of the dye industry:-



2-Hydroxy-3-naphthoyl hydrazide

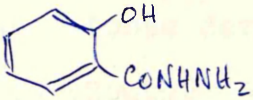


Naphthol-AS

The value of Anilid-Saure to the industry lay in its special capacity for adsorption onto textiles in the presence of formaldehyde. This was considered to be by virtue of the CONH-function--which it shares with 2-hydroxy-3-naphthoyl hydrazide. The possibility therefore arises that the union of the latter with tissues might be adsorptive rather than chemical. The microscopic appearance of the section after azo dye formation reveals much blurring and background staining, which is not inconsistent with some degree of adsorption of the hydrazide. This still further complicates an obscure and unsatisfactory situation.

β-Resorcyloylhydrazide was also somewhat unreactive in condensation. The parent acid of the reagent readily decarboxylates under a variety of conditions, to yield resorcinol or its derivatives: this happens, for example, on azo-coupling, the product being a mixture of mono- and bis-azo derivatives of resorcinol. Although the hydrazide is more stable, and the hydrazone derivatives even more so, similar reactions, with loss of the steroid moiety, were observed. In general, the presence of the two phenol groups rendered the molecule too reactive, and raised doubts as to the identity and purity of the products of any reaction.

In contrast, salicylic acid hydrazide (XVIII) is a simple substance, easily prepared from methyl salicylate (Curtius,



(XVIII)

Struve and Radenhausen (1895), crystalline and stable. It is readily soluble in aqueous and glacial acetic acid and reactive in condensation with carbonyl compounds, forming crystalline precipitates. Although the hydrazino group is active in condensation, the benzene nucleus is stable, obviating any tendency to side-reactions; this was a disadvantage when the attempt was made to form a coloured derivative by azo-coupling. However, another method of forming coloured derivatives was discovered, which involved oxidative coupling with an aromatic amine to produce an indoaniline compound.

Fluorescence Properties of Salicyloylhydrazones derived from Simple Aldehydes and Ketones

The most useful property of derivatives of salicyloylhydrazide from aldehydes and ketones was their ability to fluoresce when illuminated by ultraviolet light using a Wood's glass filter (though not in direct ultraviolet light). Simple aldehydes are easily differentiated from simple ketones, because the former give derivatives which fluoresce brilliantly in diverse colours, whereas ketones develop only a dim uniform blue fluorescence (Camber 1954). The ketonic fluorescence can be intensified by the action of zinc acetate, which also introduces marked colour differences ranging from sky blue (cyclohexanone) to lilac (cyclopentanone). Further charactertistic changes occur on raising the pH of the solution with NaOH, either before or after the zinc acetate treatment (Table 9). Aluminium, cadmium and silver salts have a fluorigenic action similar to zinc acetate, but the luminescence produced is much less intense. For certain carbonyl compounds (e.g. salicylaldehyde), the relative effects of cadmium and zinc salts are reversed.

Neither the reagent nor the carbonyl compound separately shows any luminescence in acid solution. The procedure can be adapted as a spot test on filter paper, although in this case there is some bluish luminescence from excess reagent which has dried on the paper.

-o-

EXPERIMENTAL (Camber, 1957)

Routine fluorescence observations were carried out with an Osram High Pressure Mercury Vapour Lamp (125 W/Class MBW), made of Wood's glass.

Water. Traces of copper in distilled water affected the reproducibility of results, and distilled water from an all-glass still was used.

Glassware. Unsatisfactory results were obtained with glassware cleaned with chromic acid. It was cleaned mechanically with magnesium carbonate, kept overnight with a solution of dilute hydrochloric acid and the detergent Santomerse (Monsanto Chemicals), and finally washed with distilled water.

Salicyloylhydrazide. The reagent should be pure and free from disalicyloylhydrazide. The following procedure gave a suitable material.

Hydrazine hydrate (90-100%; 1.5 moles) was boiled under reflux and methyl salicylate (1.0 mole) added dropwise through the condenser. The mixture was boiled under reflux for 2 hours, cooled, and the colourless solid hydrazide filtered and recrystallised from distilled water (copper-free), giving a product m.p. 149° (Curtius, Struve and Radenhausen 1895 give m.p. 146°). The hydrazide, which is stable in the solid state, should not come into contact with traces of metal at any stage, as it readily forms chelates.

Preparation of hydrazones. Acetic acid was found to be the best solvent for the preparation of derivatives.

Reagents: Solution A: Saturated solution of salicyloylhydrazide in 5% aqueous acetic acid (ca.1% solution).

Solution B: Saturated solution of salicyloylhydrazide in glacial acetic acid (ca. 15% solution).

A few drops of the saturated solution of the carbonyl compound in 5% acetic acid were added to solution A. The crystalline hydrazone separated immediately. Alternatively, if solution B was used, dilution with water effected precipitation. The reagent is not precipitated by dilution. The product was crystallised from dilute acetic acid.

(Table 10 ..to face p.76)

Table 10

Carbonyl compound	Fluorescence of salicyloylhydrazone	Effect of saturated aqueous solution of zinc acetate	Effect of N NaOH on	
			Salicyloylhydrazone pre-treated with zinc acetate soln.	Direct on salicyloylhydrazone.
Benzaldehyde	brilliant electric blue	colour persists	colour persists	complete quenching
2:4-Dihydroxybenzaldehyde	pale blue	intensified	colour persists	gradual quenching
Salicylaldehyde	brilliant apple green	changes to electric blue	gradual quenching	gradual quenching
p-Dimethylaminobenzaldehyde	electric blue	intensified	colour persists	changes to purple
p-Nitrobenzaldehyde	no fluorescence	nil	nil	nil
Hexaldehyde	dull purplish blue	intensified	colour persists	intensified
n-Heptaldehyde	bright blue	greatly intensified	colour persists	gradual quenching
Cyclohexanone	trace purple	brilliant sky blue	colour persists	greatly intensified
Cyclopentanone	trace mauve	brilliant lilac	colour persists	greatly intensified
Acetone	trace mauve	brilliant mauve	colour persists	greatly intensified
Pyruvic acid	nil	nil	nil	nil
Laevulinic acid	dull blue	intense purple	colour persists	intensified

Spot Tests.

Reagents: Solution C: a freshly made mixture of 2% salicyloylhydrazide in glacial acetic acid (1 vol) and absolute ethanol (5 vol).

Zinc acetate solution: a saturated aqueous solution of zinc acetate (ca. 25%).

A drop of a saturated solution of the carbonyl compound in 5% acetic acid was put on filter paper and a concentric drop of reagent solution A was superimposed. The range of fluorescent colours exhibited by some simple hydrazones when treated with (a) zinc acetate, (b) zinc acetate followed by alkali treatment, and (c) alkali prior to zinc acetate treatment, are indicated in Table 10.

-o-

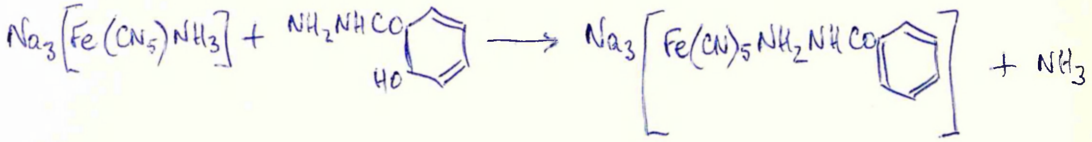
Preliminary studies were made on the wavelength of the light needed to activate aldehydes and ketones respectively, by the use of a monochromatic source. Aldehyde derivatives are activated by ultraviolet light of long wavelength, and can be demonstrated by the standard illuminating sources. Ketone derivatives show scarcely any fluorescence under the microscope under these conditions; examination of paper spot tests with high-angle monochromatic light (Hilger's instrument) showed that the fluorescence began at about 3466 Å and 3504 Å. The strong fluorescence observed with a mercury vapour lamp is due to the fairly high output of weak radiations below 3650 Å. These radiations could not be used in fluorescence microscopy, because it is essential there to have a high intrinsic output of the activating wavelength. The only satisfactory way of examining the fluorescence microscopically would be to use a high sensitivity spark between cadmium or magnesium electrodes and obtain the activating wavelengths by means of a double dispersion quartz monochromator.

Fluorescence properties of salicyloylhydrazones derived from steroidal ketones

Application of the above reaction to oxosteroids gives striking results. Hydrazones are formed which exhibit the dim blue fluorescence charactertistic of ketones; zinc acetate enhances the fluorescence and introduces new colours. Treatment with sodium hydroxide also gives rise to characteristic changes in the fluorescence, and altogether seven distinct types of fluorigenic behaviour can be distinguished.

Aluminium oxide was found to be the only other transition-metal salt which had an enhancing effect, though not all the possible combinations of metal salt and steroid were tried. It is possible that further differentiation could be achieved by these means. Moreover, as a similar effect is found with resorcyloylhydrazones of oxosteroids, further investigation of this reaction may be of value.

The compounds are formed immediately on applying the reagent to oxosteroid spots on filter paper. The spray reagent solution is stable and colourless, and salicyloylhydrazide itself is not fluorescent in solution in aqueous or alcoholic acetic acid. However, it does display a bluish luminescence after drying on the paper, which interferes with the detection of micro-amounts of salicyloylhydrazones, the background effect being increased after treatment with zinc acetate. Under alkaline conditions the hydrazide fluoresces considerably both in the solid state and in solution. It had, therefore, to be removed or diluted and a variety of methods, including oxidation with potassium periodate or treatment with p-nitrobenzaldehyde or with boric acid, were examined. The most successful, however, involved the treatment of the paper with sodium pentacyanoammine ferroate (abridged to PAF) (XIX, Feigl, Anger and Frehden 1934), whereby the salicyloylhydrazide reacted with PAF



XIX XX

to form a deeply coloured purple water-soluble compound, though to have the structure XX. Immersion of the paper in a solution of PAF followed by washing with water thus eliminated unreacted hydrazide. An important limitation to this procedure is that zinc acetate interferes with the PAF reaction and should, therefore, not be used before it. The same limitation applies to the use of zinc acetate in the indoaniline reaction (see later).

-o-

EXPERIMENTAL

EXPERIMENTAL

Preparation of oxosteroidal salicyloylhydrazones.

The oxosteroid (200 mg) in glacial acetic acid (5-10 ml) at 55-65° was treated with solution B (1.5 moles) and kept at that temperature for 3-5 minutes. Crystallisation was, if necessary, induced by the addition of a few drops of water and was complete on cooling. The hydrazone was crystallised from dilute acetic acid.

Spot Tests.

Reagents: Acidified zinc acetate solution: a saturated aqueous solution of zinc acetate (AnalaR) in 5% acetic acid (AnalaR).

Pentacyanoammine ferroate (PAF) solution: a freshly prepared mixture of a 2% aqueous solution of sodium pentacyanoammine ferroate (1 vol) and a 5% aqueous sodium bicarbonate solution (1 vol).

The oxosteroid (1-50 mg) on filter paper was treated with solution C either by immersion or spraying. (oxygen-free nitrogen being used for spraying). The paper was dried in a hot-air oven and immersed in the PAF solution. The intense purple water-soluble complex was washed out of the paper by agitation in a water-bath, and excess water removed by blotting. The paper was then ready for treatment with N NaOH, or with the acidified zinc acetate solution.

The fluorigenic behaviour of the oxosteroids separated them into seven well-defined groups, and some degree of correlation with the position of the ketone group in the molecule was achieved (Table 11).

Table II. Differentiation of steroid salicyloylhydrazones by fluorescent spot-testing under Wood's light

Examples	Characteristic grouping
Group 1. Blue fluorescence, enhanced by zinc acetate solution or by alkali.	
Androstan-17β-ol-3-one	3-one
Dehydroepiandrosterone Dehydroepiandrosterone acetate Epiandrosterone Androsterone Aetiocholan-3α-ol-17-one 11β-Hydroxyaetiocholan-3α-ol-17-one Oestrone	17-ones
Δ^5-Pregnen-3β-ol-20-one Allopregnan-3β-ol20-one Pregnan-3α-ol-20-one Pregnan-$3\alpha,17\alpha$-diol-20-one	20-ones
5α-Androstane-3,17-dione 5β-Androstane-3,17-dione	3,17-diones
5α-Pregnane-3,20-dione 5β-Pregnane-3,20-dione	3,20-diones
Group II. Faint blue fluorescence, quenched by zinc acetate and by alkali.	
Tetrahydro-compound E (pregnan-3α, 17α,21-triol-11,20-dione Tetrahydro-compound F (pregnan-3α, 11β,17α,21-tetrol-20-one)	20-on-21-ols possessing no other ketone group

Examples	Characteristic grouping
Group III. Faint blue fluorescence, quenched by zinc acetate, only slightly diminished by alkali.	
Tetrahydro-compound E 21-acetate Allopregnan-3α,21-diol-20-one 21-acetate	20-one-21-acetates possessing no other ketone group
Group IV. Yellow fluorescence, enhanced by zinc acetate, quenched by alkali.	
Testosterone Epitestosterone	4-en-3-ones
Cortisone Cortisol	4-en-3,20-dion-21-ols
Cortisone acetate Cortisol acetate	4-en-3,20-dione-21-acetates
Group V. Yellow fluorescence with zinc acetate, blue fluorescence with alkali.	
Progesterone 17α-Hydroxyprogesterone	4-en-3,20-diones
Androst-4-ene-3,17-dione	4-en-3,17-dione
Group VI. Faint blue fluorescence, changed to vivid green by zinc acetate, quenched by alkali.	
Prednisol acetate 17β-Hydroxyandrosta-1,4-dien-3-one	1,4-dien-3-ones

(The above groupings differ slightly from those reported in Clin. Chim. Acta (Camber 1957) because of further experimental work since the publication of that paper.

Group 1 contains 3-, 17- and 20-oxosteroids with no conjugated double bond. The sensitivity of the reaction with different oxosteroids is given below, in Table 12, from which it can be deduced that in general, 3-ones are much more powerful fluorigens than 17- or 20-ones (although oestrone is an exceptionally powerful 17-one). When more than one of these saturated keto groups is present in a molecule, each appears to exert its effect without interference, enhacing the overall sensitivity. On the other hand, the presence of a 21-hydroxyl group does interfere with the fluorescence of a 20-one (although the chemical reactivity of a 20-one is normally enhanced by 21-hydroxylation) and 20-one-21-ols are therefore in a different category. Acetylation of the 21-hydroxyl (Group III) tends to remove its quenching effect, so that alkali now brings up a rather weak blue fluorescence, although this is still not visible with zinc acetate.

The yellow fluorescence (Group IV) due to 4-en-3-ones in zinc acetate is quenched by alkali. When a saturated 17-one or 20-one is added to the molecule (Group V), this quenching allows the blue fluorescence due to these added groups to show up (cf. Group I). 21-Hydroxylation prevents this effect in cortisone and cortisol, but in these cases acetylation of the

21-hydroxyl does not, apparently, remove the quenching effect. Consequently, cortisone acetate, cortisol acetate, cortisone and cortisol are all in Group IV, not V.

The brilliant green fluorescence characteristic of 1,4-dien-3-ones provides a useful spot test for distinguishing between prednisol and cortisone.

Table 12. Sensitivity of oxosteroid spot tests with salicyloylhydrazide

		Colour and sensitivity (μg)*		
Gp.	Oxosteroid	Reagent + ZnAc	Reagent + NaOH	Reagent + both
I	Androstan-17β-ol-3-one	Blue, 4	Blue, 5-6	
	Dehydroepiandrosterone	" 5-10	" 2	Blue, 5-10
	Epiandrosterone	" 15	" 3	" 25
	Androsterone	" 15	" 2	" 25
	Aetiocholan-3α-ol-17-one	" 10	" 4	" 15
	11β-Hydroxyaetiocholan-3α-ol-17-one	" 15	" 3-4	" 15
	11-Oxoaetiocholanolone+	" 15	" 5-10	" 15
	Oestrone	" 2	" 2	
	Δ^5-Pregnen-3β-ol20-one	" 10	" 2	" 15
	Allopregnan-3β-ol-20-one	" 10	" 2-4	
	Pregnan-3β-ol-20-one	" 10	" 3	
	Pregnan-3α,17 -diol-20-one	" 8-10	" 2-3	

Gp.	Oxosteroid	Colour and sensitivity (μg)*		
		Reagent + ZnAc	Reagent + NaOH	Reagent + both
I	5β-Androstane-3,17-dione	Blue, 3	Blue, 1-2	
	5α-Pregnane-3,20-dione	" 4	" 1-2	
II	Tetrahydro-compound E	Nil	Nil	
III	Tetrahydro-compound E acetate	Nil	Blue, 15	
	Allopregnane-3α,21-diol-20-one 21-acetate	Nil	" 10-12	
IV	Cortisone	Yellow, 3	Nil	
	Cortisol	" 1	"	
	Cortisone acetate	" 4	"	
	Cortisol acetate	" 1	"	
V	Progesterone	" 2-3	Blue, 3 (15)	
	17α-Hydroxyprogesterone	" 2	" 5-10	
	Androst-4-ene-3,17-dione	" 3	" 15	
VI	Prednisol acetate	Green, 25		

*The term "sensitivity" here refers to the minimum amount of oxosteroid (up to 50μg) that will give a reaction in a 1.5-cm. diameter spot on filter paper.

+11-Ketones do not react under any conditions, so that this compound is effectively a 17-monoketone. Reproducibility is poor with this compound.

Aldosterone may be considered to fall into a seventh Group of oxosteroids. It reacts like a simple aldehyde, in that it gives a strong yellow fluorescence in the absence of zinc acetate, addition of which does not affect its colour or intensity.

Paper chromatograms have been successfully followed using salicyloylhydrazide as a spray. The advantage over sprays already in use, apart from its stability and colourlessness, lies in the possibility of detailed allocation of chemical groups within the molecule. If standard ketones are available for comparison, identification of individual steroids may be made in favourable cases, since colours in Group I, for example, vary from sky blue to lilac. The record on the papergram is permanent, unlike those sprays which depend on transient colour reactions.

A particularly useful and neat technique for observing the fluorescent colours has been developed. It consists in forming the derivative in a cellophane membrane instead of on filter paper (Camber 1957b). The method is of general application to all spot tests, provided they are carried out in a solvent which permeates cellophane. The details of the technique depend on whether the substance to be tested is volatile or not.

For the oxosteroid reaction, a 5% aqueous acetic acid solution of the compound is placed in a test-tube covered with a square of cellophane previously moistened with the solvent, the cellophane being firmly secured with a rubber band. The test-tube is inverted so that the lower surface of the cellophane is wetted, and a drop of a saturated solution of salicyloylhydrazide in 5% acetic acid is put on top of the cellophane, through which it permeates to meet the steroid solution in the membrane. Colours can be seen much more clearly, and colour comparison is greatly facilitated.

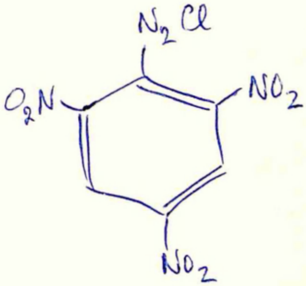
A volatile substance is simply placed in the test-tube and allowed to volatilise through the cellophane until it meets the reagent solution permeating from the top. Interference from non-volatile substances is avoided and, since the volatile substance is virtually sealed in, contamination of and by neighbouring tests is prevented. Excess reagent can be removed by simply wiping the outer surface of the cellophane with a damp cloth.

Formation of coloured derivatives of salicyloylhydrazones

Some salicyloylhydrazones, e.g. those from Group II oxosteroids, do not fluoresce in Wood's light. An attempt was made to prepare a coloured derivative, for cross-checking purposes, by azo-coupling in an analogous fashion to 2-hydroxy-3-naphthoylhydrazones and β-resorcyl-oylhydrazones. However, a variety of azo-dyes was tried 1-

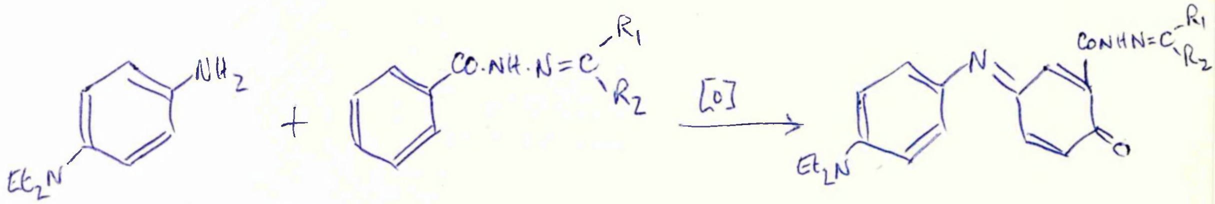
oylhydrazones. However, a variety of azo-dyes was tried
under many different conditions, and salicyloylhydrazones could not be induced to couple with them.

This result was not altogether unexpected: in comparative studies on the reactivities of different phenols in azo-coupling, salicylic acid is shown as the least reactive (Saunders 1936). Even with the extremely reactive diazotised picramide (XXI), prepared according to the method of Missline et al. (1920), no reaction with salicyloylhydrazones could be observed, so that no further attempt to couple them with azo-dyes was made.



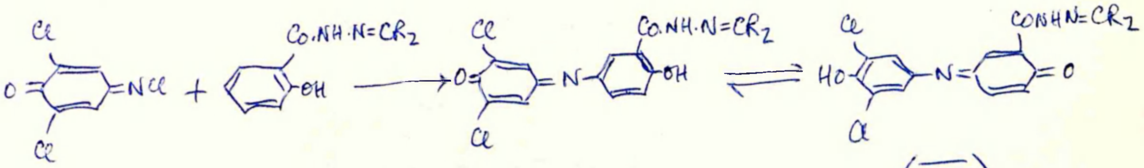
(XXI)

However, a different type of dyestuff resulted from an oxidative coupling with p-amino-diethylaniline (Fischer 1914), the product being a blue-green indoaniline:



Spots on a papergram can be made visible in this way (the excess reagent being removed first by the PAF procedure), the sensitivity for oxosteroids being 0.1μg. on filter paper. The reaction appears to be quite general for phenolic substances, some colour variation being obtained with different phenols.

A somewhat similar reaction of dehydroepiandrosterone salicyloylhydrazone with chloroimide in ethanol at pH (buffered) of 8.32 gave an intensely coloured blue derivative (XXII), solutions of which obeyed Beer's Law;



(XXII)

but the reaction with p-aminodiethylaniline was found to be more generally satisfactory, both for qualitative and (see below) quantitative purposes.

Any differences between salicyloylhydrazones of different steroids disappear when the oxidative coupling is carried out. As mentioned above, zinc acetate interferes with the reaction and must not be applied before it.

-o-

EXPERIMENTAL

p-Aminodiethylaniline mixture. Immediately before use, the following solutions are mixed: (i) a 0.2% aqueous solution of stabilised p-aminodiethylaniline ("Genochrome", May and Baker, Ltd.) (1.vol) and (ii) a 5% aqueous sodium bicarbonate solution (1 vol).

Indoaniline spot test for oxosteroids. Immersion of the paper in saturated aqueous potassium periodate solution (ca. 0.33% w/v) develops the intense blue-green colour of the indoaniline. Besides potassium periodate, potassium ferricyanide (Camber 1955), silver nitrate and mercuric chloride can be used as oxidising agent.

-o-

Quantitative estimation of oxosteroids using salicyloylhydrazide

The qualitative methods described above have been adapted to provide fluorimetric and absorptiometric methods of quantitative analysis of oxosteroids. As

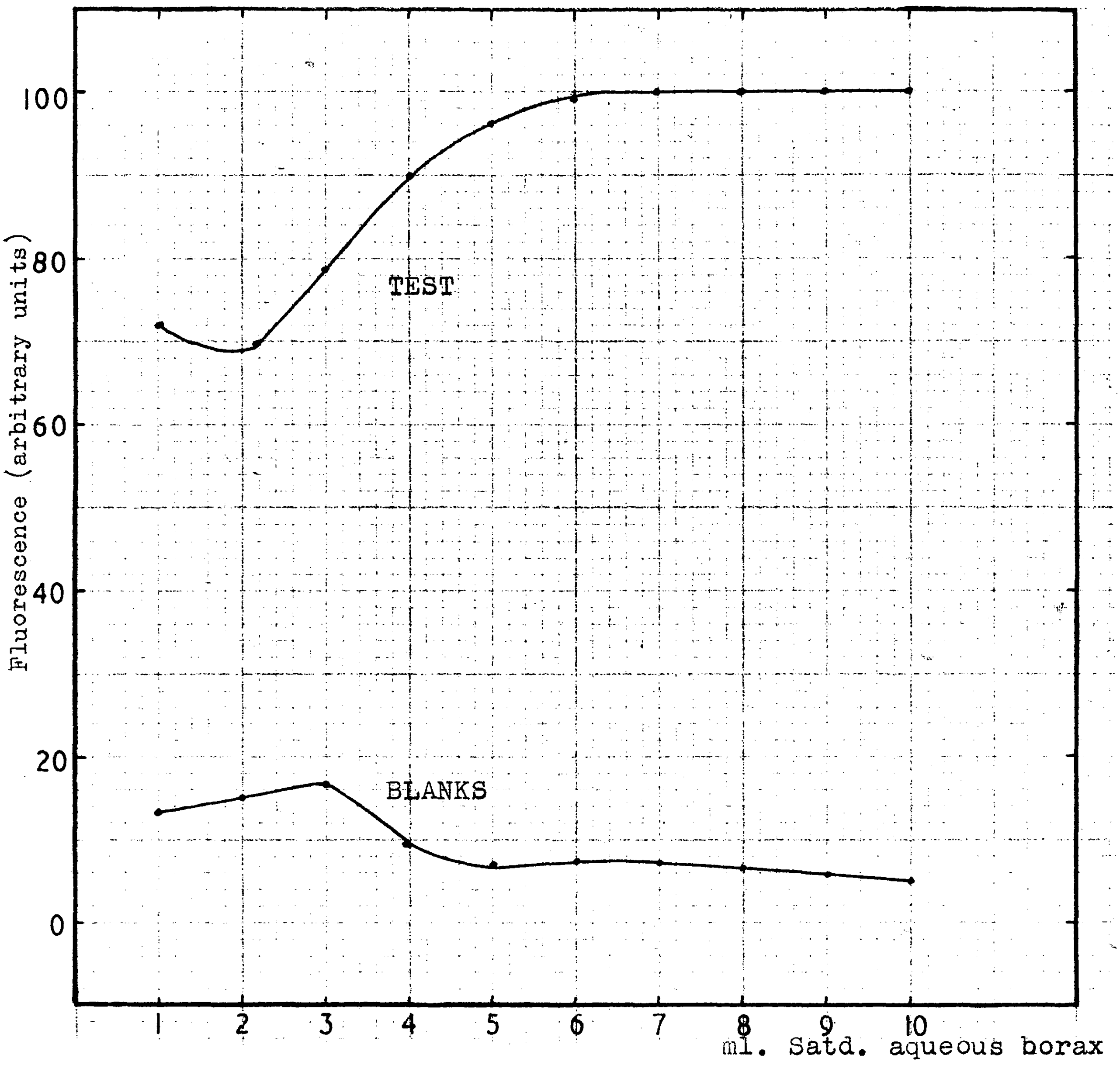


Fig. 8. Selective quenching of reagent fluorescence with concomitant intensification of hydrazone fluorescence by addition of sodium borate.

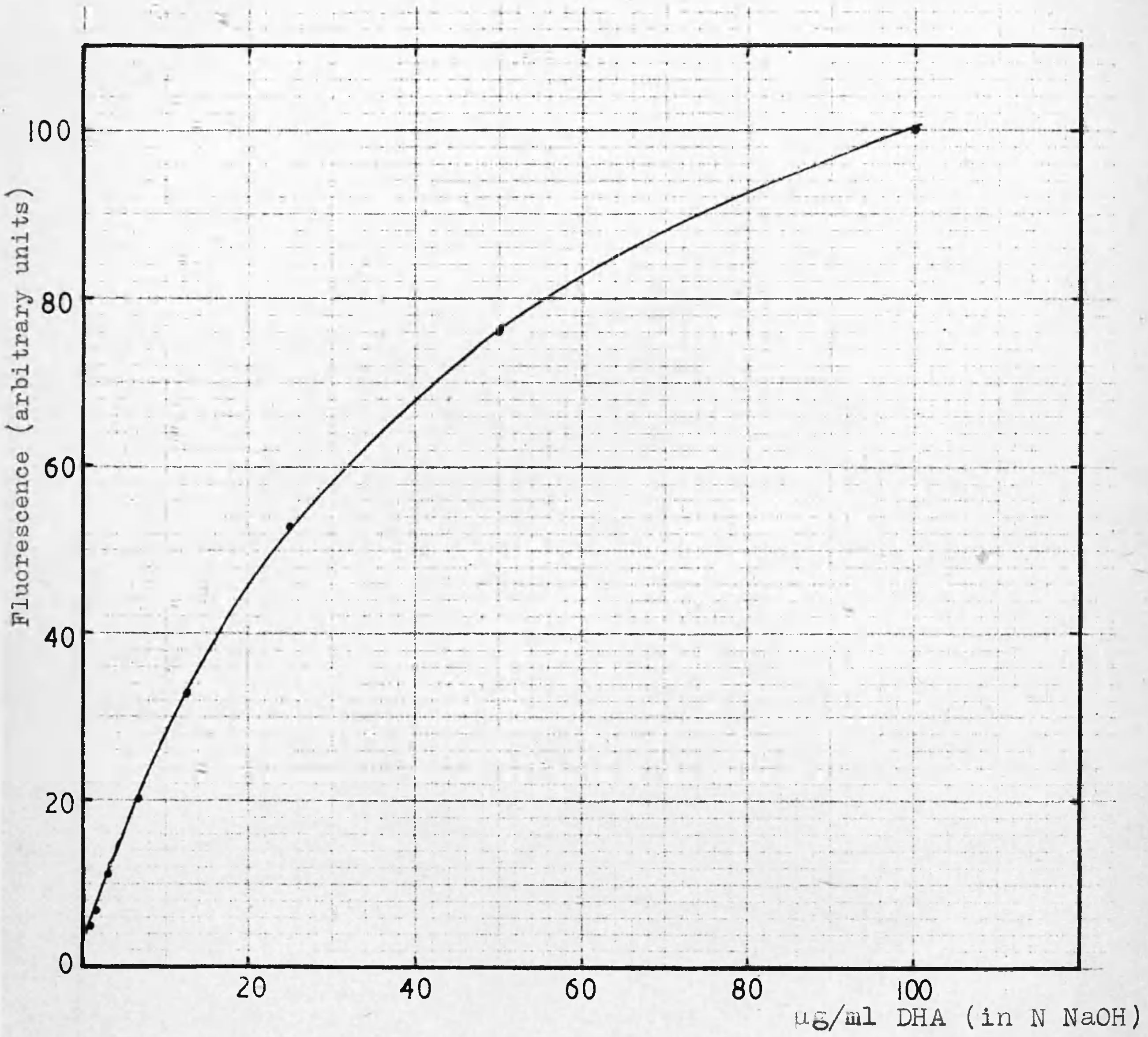


Fig. 7. Concentration-fluorescence curve of dehydro*epi*androsterone salicyloylhydrazone.

Fig. 7 shows, the concentration-fluorescence curve for salicyloylhydrazones in N NaOH solution is not linear. However, a simple one-stage fluorimetric method for Group I oxosteroids was finally developed as indicated below. Under specified conditions a linear relationship between concentration (1 to 50 μg) and fluorescence was obtained.

Before this could be achieved, supplementary experiments were performed to discover the optimum conditions for suppression of fluorescence due to unchanged reagent. It was found that alkaline sodium borate not only did this, but also intensified the fluorescence due to the salicyloylhydrazone. (Fig.8.) As can be seen from Fig.8, the reagent blank fluorescence progressively falls, as the proportion of borate increases, to a maintained level of 5%, whereas the fluorescence of the salicyloylhydrazine increases to a maximum. The flattening out of the curves indicates that over a wide range the absolute concentration of borate is not highly critical.

Subsequent experiments showed that :-

(a) the curve of salicyloylhydrazone concentration continued at a maximum level if the relative concentration of borate was further increased, but no relative diminution of the blank resulted;

(b) no better results were obtained when potassium pyroborate was substituted for sodium borate; and

(c) sodium bicarbonate was less effective than sodium borate in that the relative fluorescence of the blank could not be diminished below 12% as against 5% for sodium borate.

-o-

EXPERIMENTAL

Dehydroepiandrosterone (2.5 mg) was deposited in a test tube, a second test tube being used for blank determination. To each tube was added glacial acetic acid (0.5 ml) containing salicyloylhydrazide (10 mg). The tubes were warmed at 60° for ten minutes and N NaOH (15 ml) was added to each tube. Duplicate portions of the reaction mixture (1.5 ml) were pipetted into each of eleven test tubes and duplicate portions of the reagent blank (1.5 ml) into each of another series of eleven test tubes. To the first tube and to its reagent blank was added 10 ml. water, to the second tube and to its reagent blank 1 ml. saturated aqueous solution of sodium borate and 9 ml. water. Graded volumes of the borate solution were substituted for water up to the eleventh

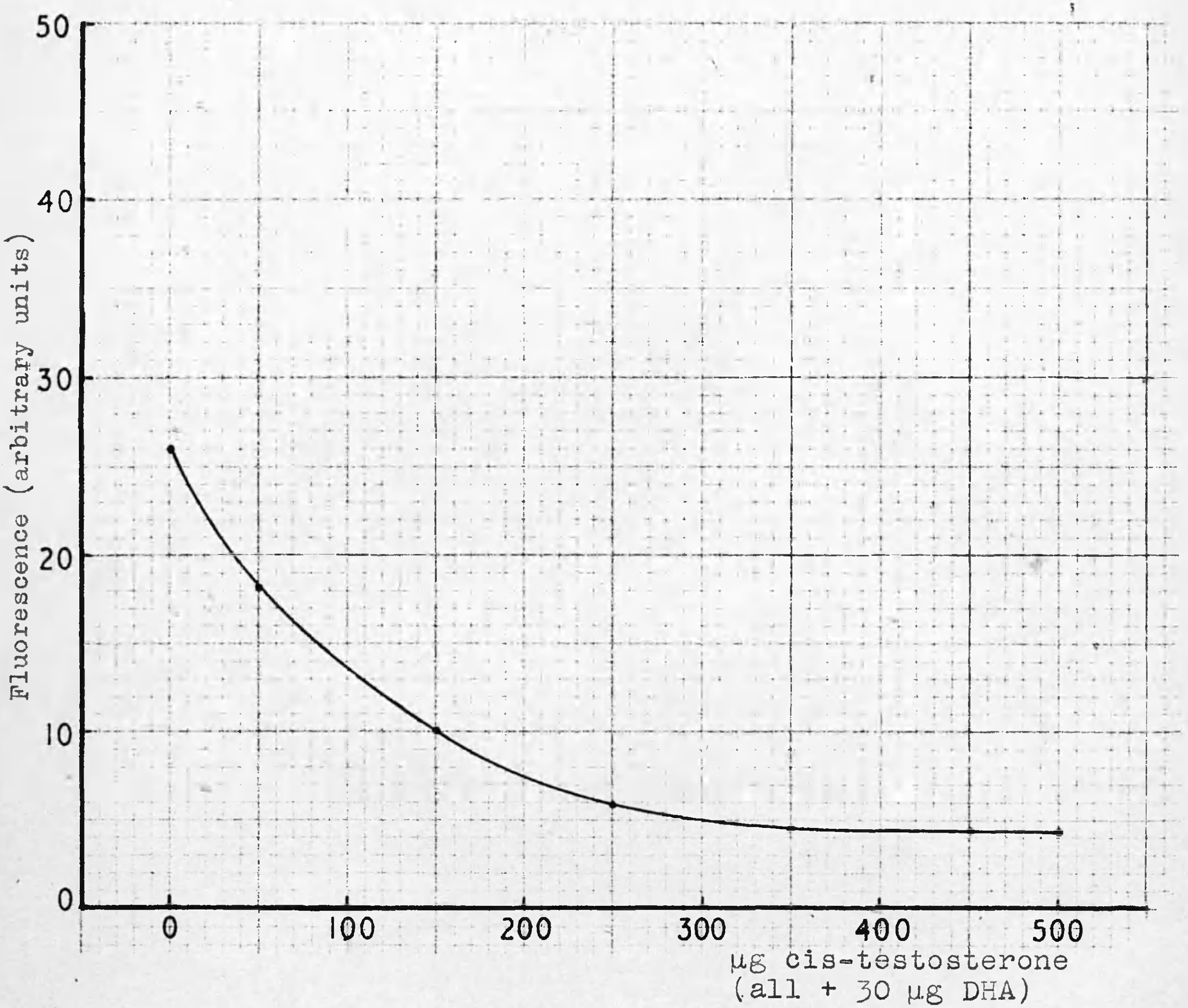


Fig. 9. Quenching of fluorescence of dehydroepiandrosterone salicyloylhydrazone by cis-testosterone.

pair of tubes, to each of which were added 10 ml. borate solution.

The relative fluorescence of the tubes was read in the fluorimeter against the eleventh reaction tube as standard. (Fig.8).

-o-

The fluorimetric estimation of Group I steroids which utilises this reaction is described below. Unfortunately, steroids other than Group I interfere by quenching the fluorescence at concentrations above 50 µg. (Fig. 9). On the other hand, under these conditions it was found that the fluorescence due to individual members of Group I are additive, i.e., the method is capable of providing a measure of total Group I steroid in the sample. The validity of the method was supported by experiments which showed that the condensation of the steroids with salicyloylhydrazide is quantitative and, under the conditions of the determination, immediate.

Preliminary work also indicated that this method may be adapted to estimate oxosteroids present in urinary extracts.

Fluorimetric method for Group I steroids. Reagents:-

1. Solution D: freshly prepared 2% w/v salicyloylhydrazide in glacial acetic acid.

2. Saturated aqueous sodium borate.

Known quantities (1-50μg) of the steroid were placed in different tubes. Solution D (0.1 ml) was added to each and the mixtures were kept at 60° for 10 minutes. Distilled water (1 ml) at 60° was added dropwise with cooling, in order to drive the reaction to completion by precipitation. When cool, N sodium hydroxide (3 ml) and sodium borate solution (10 ml) were added, and the resulting solutions transferred to cuvettes for analysis. All readings were made against the tube containing the highest concentration of steroid -- this tube therefore acting as an arbitrary standard. A "blank" determination was also carried out.

For dehydro*epi*androsterone salicyloylhydrazone, for example, a linear relationship between concentration and the drum reading (which indicates the relative intensity of fluorescence of an unknown solution compared with an arbitrary standard) was obtained. Subtraction of the fluorescence due to the reagent "blank" gave a calibration curve, passing through the origin, which could be used for the analysis of solution of unknown concentration.

To show that the condensation was quantitative. A series of graduated amounts of dehydroepiandrosterone in the range 1-10 μg. was estimated fluorimetrically by the standard method described. A parallel set of tubes was set up in which were deposited amounts of dehydroepiandrosterone salicyloylhydrazone in the range equivalent to 1-10 g. oxosteroid. To each tube was added N NaOH (3 ml) followed by sodium borate solution (10 ml), and finally 0.1 ml. of the reagent solution. These were estimated fluorimetrically. The curves obtained for the two sets of tubes were virtually superimposable, indicating that condensation is quantitative during the course of ah estimation by the standard method down to 1 μg. level of oxosteroid.

Additivity of Group I steroids. Mixtures of androsterone and dehydroepiandrosterone (200 μg + 50 μg; 150 μg + 100 μg; 100 μg + 150 μg) gave the same fluorescence as 250 μg of either compound alone.

Provisional fluorimetric method for Group I steroids present in urinary extracts. A method was worked out for the application of the reaction to the oxosteroids in urine. It seemed satisfactory, giving values slightly higher than those by the Callow-Zimmermann method, but was not extensively proved over a large number of samples.

Hydrolysis and extraction of urine. The method used was based on a modification of that recommended by The Lancet (1951). Hydrochloric acid (20 ml; d. 1.18; AnalaR) was added to urine (200 ml) and the mixture boiled under reflux. After 10 minutes the mixture was allowed to cool, benzene (60 ml) was added, and the mixture again boiled for 10 minutes. The benzene layer was separated and the process was repeated. The combined benzene extracts were washed with water (40 ml), 2N sodium hydroxide (2x40 ml), 2N hydrochloric acid (40 ml), and water (40 ml). The benzene layer was then evaporated to dryness to leave the extract.

Method of analysis. The following tubes were set up:

1. Reagent blank: Solution D (0.1 ml).

2. Unknown: Solution D (0.1 ml). Urine extract or unknown, in the range 1.50 µg Zimmermann equivalent of oxosteroid.

3. Standard: Solution D (0.1 ml), dehydro*epi*androsterone (50 µg). Condensation with salicyloylhydrazide was carried out as described for the standard fluorimetric procedure, benzene (5 ml) was added and the tubes were shaken. The hydrazones were extracted with N sodium hydroxide (3 ml) and a portion (2 ml) of the alkaline

extract was treated with the borate solution (10 ml). Fluorimetry was then carried out as previously described.

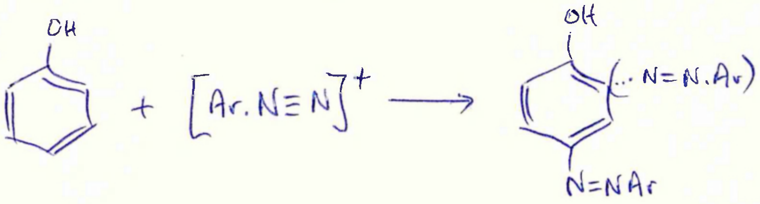
Steroids of Groups other than Group I can be estimated by converting the corresponding salicyl~~l~~oyl-hydrazones into the indoaniline derivatives, which are then estimated absorptiometrically, unchanged reagent having been removed as its derivative with pyruvic acid, which is soluble in alkali (cf. Camber 1954).

This method was developed as an improvement on existing methods for the estimation of phenols, which are reviewed in the next section.

The Use of the Indoaniline Reaction in the Estimation of Phenols

Methods of the estimation of phenols currently in use are (a) the Fox and Gauge (1920) diazonium-coupling method; (b) the Folin and Ciocalteu (1927) reduction of phospho-tungsto-molybdic acid; (c) the Gibbs (1927) indophenol method using 2:6-dibrome-quinonimide; and (d) the recently introduced method depending on the oxidative coupling with amino-antipyrine (Powell and Smith 1954, Kind and King 1954).

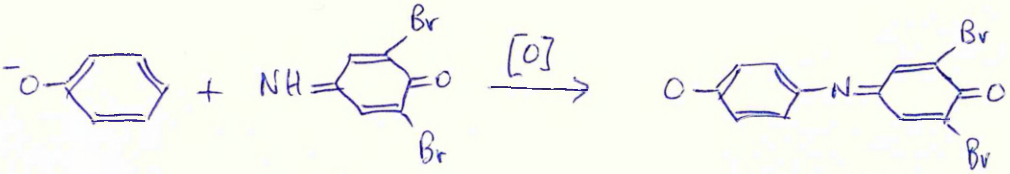
The Fox and Gauge method (a) depends on the ability of phenols to couple with diazonium salts to give azo-dyes, which are estimated colorimetrically. Coupling takes place preferentially in the p-position, but in the o-position if this is blocked.



Diazotised sulphanilic acid or diazotised p-nitraniline are commonly used as the diazonium salt. The method is satisfactory, but aromatic amines and imidazoles can be expected to give some interference.

The complex mixture produced when phosphoric, molybdic and tungstic acids are boiled together is very unstable, and is readily reduced in alkaline solution to a blue colloid. A large range of substances will effect this reduction, but with certain previous precautions, the method may be used for the estimation of phenols; consequently the mixture of acids has come to be known as the Folin-Ciocalteu phenol reagent. The non-specificity of the reagent makes method (b) quite unsuitable for estimation of biological samples in which appropriate pre-treatment cannot be applied.

The Gibbs indophenol method (c) depends on the coupling of 2:6-dibromo-quinonimide with the phenol in a buffered alkaline solution, with the formation of an indophenol:-

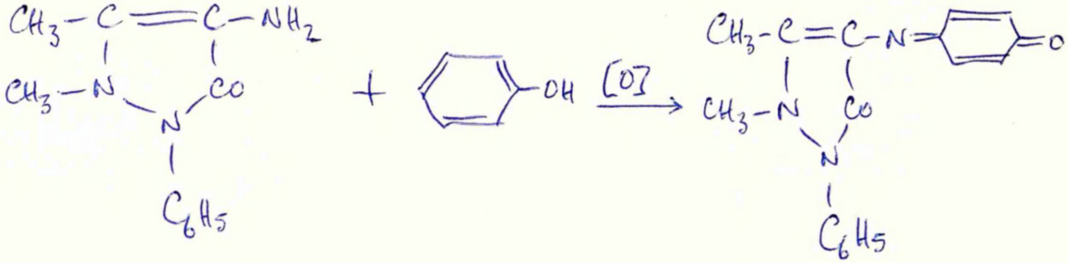


Coupling takes place in the p-position, and is an <u>oxidative</u> coupling, a second molecule of the quinonimide acting as the necessary oxidising agent. The blue dye is estimated colorimetrically; as it is an indicator, the colour composition must be made in a highly buffered solution at a given pH; as it is also an oxidation-reduction indicator, reducing substances must be absent. The sensitivity is high (1 in 10^8), but if the colour is to develop in a reasonable time (30 minutes) the phenol must be concentrated to about 1 mg. per 100 ml. Otherwise, the test solutions must be left overnight.

In an attempt to overcome this disadvantage, Houghton and Pelly (1936) employed an oxidative (sodium hypochlorite) coupling of p-aminodimethylaniline with the phenol. They called the product an indophenol, a name hallowed by usage, but not as accurate as the term indoaniline (see Vittum and Weissberger 1954), as consideration of the formulae will show:

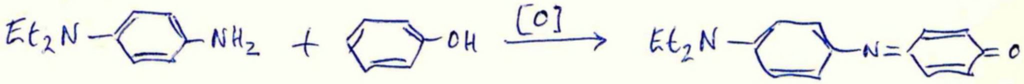
The method has the advantage that although coupling also takes place with amines, the colours of the indamines formed are very much less intense and in a different part of the spectrum. However, Houghton and Pelly's procedure was not adopted, because the fundamental principles of the colour formation were unsound: even a slight excess of hypochlorite weakened and then bleached the colour produced, so that the point of maximum colour intensity was a matter of judgment. A further disadvantage was that the amine had to be prepared fresh from p-introsodimethylaniline for each estimation.

The use of 4-aminoantipyrine in method (d) for phenols (in particular, those from plasma phosphatases) is based on the oxidative coupling of the amine with the p-position of the phenol to give a red or purple quinone (Emerson 1943).



Gottlieb and March (1946), using alkaline ferricyamide as the oxidant, devised a method, based on this reaction, for the estimation of certain phenolic fungicides. The procedures of Powell and Smith (1954) and of Kind and King (1954) are based on the proposed modifications of Grifols-Lucas (1951); the method of Kind and King was compared with the King and Armstrong (1934) procedure, which employed the Folin-Ciocalteu reaction, and was found to be simpler and quicker (because the pre-treatment was no longer necessary), and comparable in accuracy.

The indoaniline reaction described in this Part is essentially an oxidative coupling of the phenol group of the arylhydrazones with an amine, to produce an indoaniline similar to that of Houghton and Pelly.



However, the use of potassium ferricyanide as oxidising agent in place of hypochlorite was found to be very much more satisfactory, since an excess of it did not affect the colour. Again, the colour maximum was obtained in a few minutes instead of the three-quarters of an hour required for indophenols. The amine used was p-aminodiethylaniline, because this is available in a very convenient stabilised form, the sulphur-dioxide complex commercially known as "Genochrome" (May and Baker Ltd.), supplied for use in colour photography.

Some investigation of the colorimetric estimation of model phenols was undertaken. A preliminary qualitative examination of phenols by the method described previously yielded the observations summarised in Table 13.

Table 13

Phenol	Indoaniline colour on paper
α-Naphthol	Purple
Phenol	Purple
2,4,6-Tribromophenol	Green
o-Hydroxy-diphenyl	No reaction
β-Naphthol	Green
p-Chlorophenol	No reaction
3,5-Xylen-1-ol	Blue
m-Cresol	Purplish blue
o-Chlorophenol	Blue-green

-o-

EXPERIMENTAL

A solution of α-naphthol in 70% ethyl acetate (AR) was diluted to 30 μg/ml. Portions of this solution (multiples of 0.1 ml) were made up to 1 ml. with 70% ethyl acetate, and 0.1 ml of stabilised p-aminodiethylaniline ("Genochrome", 0.1% w/v aqueous solution) added, followed by 2 ml of potassium ferricyanide solution (0.33% w/v) and 2 ml of sodium bicarbonate solution (8% w/v). The mixture was vigorously shaken, and 3 ml brine added to separate the indoaniline, which was extracted with ethyl acetate (10 ml). Solid sodium chloride was added to give an optically clear solution, which was estimated in the Spekker absorptiometer against a blank, using Filter No. 6. A linear relationship between reading and concentration from 3 to 15 μg/ml was obtained.

-o-

Estimations of cyclopentanone and of dehydro<u>epi</u>androsterone by preliminary formation of their salicyloylhydrazones as described for the fluorimetric estimation, followed by indoaniline formation and colorimetric estimation, established that the reaction was quantitative and gave accurate results from 1 to 50 μg. of ketone. The reaction was extended to the examination of urinary extracts, and the reaction is described for both estimations.

-o-

EXPERIMENTAL

Reagents:

1. Aqueous (0.1% w/v) stabilised p-aminodiethylaniline.
2. Aqueous (0.33% w/v) potassium ferricyanide.
3. Aqueous (ca. 1% w/v) solution of a higher alkylbenzenesulphonic acid detergent (Santomerse).
4. Aqueous (5% w/v) potassium pyroborate.
5. Aqueous (1% w/v) pyruvic acid.
6. Aqueous (8% w/v) sodium bicarbonate.

The extract of the urinary hydrolysate was prepared by the standard methods. Graded quantities, in the range 10-50 μ g Zimmermann equivalent oxosteroid, of the extract were placed in test-tubes. An additional tube was used for the "blank". Alternatively, known quantities of the ketone (cyclopentanone or dehydroepiandosterone) were placed in different tubes. Condensation with salicyloylhydrazide was carried out as described in the fluorimetric method for Group I steroids. Pyruvic acid solution (1 ml) was then added to each tube and the crystalline condensation product allowed to separate (10 minutes). After the addition of sodium bicarbonate (5 ml), in which this product is soluble, the oxosteroid salicyloylhydrazone was removed by extraction with ethyl acetate (3 ml). A portion (1 ml) of the extract was then treated with the aqueous solutions of p-aminodiethylaniline (0.8 ml), potassium ferricyanide (1 ml), diacetin (2 ml), detergent (2 ml), and pyroborate (1 ml). After being vigorously shaken the mixture was extracted with ethyl acetate (7 ml). The supernatant layer was transferred to a cuvette and read in a Spekker absorptiometer against the reagent blank, using filter 608. A linear relation between concentration and optical density in the range 10-50 µg Zimmermann equivalent oxosteroid was obtained.

Histochemical Applications of Salicyloylhydrazide

The application of salicyloylhydrazide to histochemica problems has hardly been investigated. Partly in view of the current controversy on the validity of the conclusions to be drawn from histochemical observations, it was felt more worthwhile to obtain fundamental quantitative data rather than precipitate controversy by premature attempts at histochemical applications.

However, some indication has been obtained that the distinction between aldehydes and ketones that the reagent makes in vitro can be observed cytologically. The fluid from ground fresh beef adrenals was treated with salicyloylhydrazide in 5% acetic acid. Different types of crystals were microscopically observed to be precipitated. One type was fluorescent under Wood's light, and another type "lit up" with fluorescence when a drop of zinc acetate solution was allowed to spread over the slide.

PART 6

SUMMARY

Part 1.

Introduction

The need for improved reagents for the detection and recognition of steroids is stressed.

A description is given of the methods for detection and characterisation at present available. Beginning with non-specific reagents which can be used with oxosteroids, the description goes on to the use of reagents in which some degree of specificity and of differentiating power between individual steroids is empirically known but not theoretically understood. Certain reagents which are completely specific to particular groupings in biologically important steroid molecules are then described, together with others which were at first thought to be completely specific to a single compound, but which investigation has revealed to be actually indicative of a particular grouping, which that steroid contains.

Efforts to extend the usefulness of the general reagents, by enabling them to differentiate between steroids, are dealt with in some detail. The methods employed usually involve the employment of carefully standardised conditions for the reaction, and examination of the absorption spectrum of the reaction mixture (in either visible or ultraviolet light).

Reference is made to methods currently available for the <u>estimation</u> of oxosteroids, by spectrophotometric, colorimetric, fluorimetric and chemical means.

An outline is given at this stage of the progress made in these fields by means of the new acid hydrazide reagents described in this work.

Part 2.

Preliminary Investigations

The general principle which was the starting point for the present investigations is described in this Part. The aim was to condense the carbonyl group of the oxosteroids with an aryl hydrazine or hydrazide which also contained either an amino or a phenol group, so that azo-coupling could take place with another large molecule to form an insoluble, highly coloured dye. This would be useful in detecting the steroids on chromatograms or histochemically, and in estimating them colorimetrically; some differentiation might also be possible under the right circumstances.

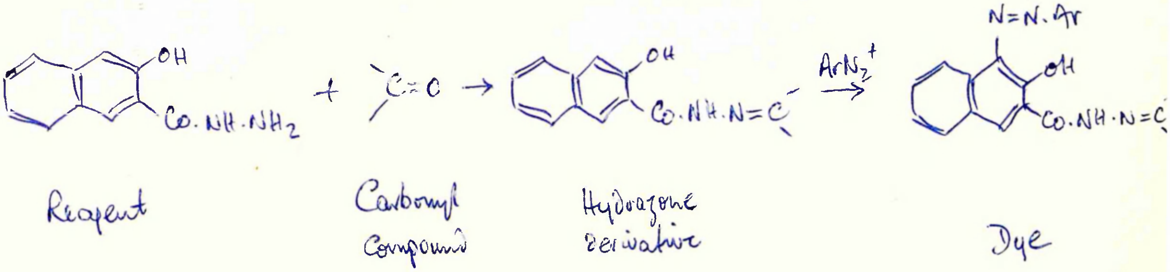
The investigation of several possible reagents is described. The first compounds tested were unreactive and sluggish in the attempted condensation with the ketone group, or very susceptible to oxidation in solution, or both. The most promising was 3-hydrazino-4-hydroxybenzoic acid, whose hydrochloride was soluble in water and reacted with alcoholic solutions of simple ketones to give the desired hydrazone derivatives. These reacted with stabilised solutions of diazonium salts to give an insoluble blue pigment, but it was shown that this was not an azo-dye, but the product of some form of autoxidation.

The reaction could not be induced to give a pigment with oxosteroid derivatives *in vitro*. The last observation confirmed the feeling, already engendered by the difficulty of handling the reagent, that it was not really suitable for the purpose in mind, and it was not investigated further.

Part 3.

2-Hydroxy-3-naphthoyl hydrazide

This compound was very reactive in condensation with carbonyl compounds, to such an extent that it would precipitate oxosteroid conjugates, as their hydrazones, from unhydrolysed urine. The mixture of compounds thus obtained was separated by paper chromatography into at least ten components; and coloured derivatives with azo compounds could also be made:-



This reaction was applied histochemically, strong localised staining being obtained in tissues where the oxosteroid content is expected to be high. However, the attribution of the staining reaction to the presence of oxosteroids has been questioned by some workers, and in the author's opinion with complete justification.

The extrapolation from *in vitro* chemistry to histochemistry must take place over a very wide gap, the conditions in tissue sections being so vastly more complicated than in the test tube. If to the complexity of the cell is added a complexity of reagent, as in the two-stage azo-coupling technique, the significance of any appearance obtained must be highly questionable.

The author believed that there were too many indeterminate factors in the demonstration of oxosteroids by these means, and endeavoured, as throughout this work, to obtain facts rather than generate opinions. The reagent has, however, found enthusiastic supporters particularly in U.S.A. as a histochemical detector of carbonyl groups in general.

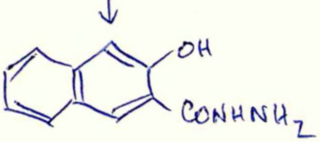
Some attention was given to a procedure for regenerating the oxosteroids from their hydrazone derivatives by fission on an oxidised cellulose column; the observations were in accordance with the idea of "group exchange", in which the hydrazide moiety of the compound is preferentially attached to the aldehyde groups of the oxidised cellulose, with regeneration of the ketone.

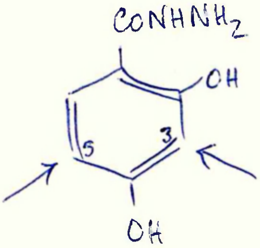
The conclusion of a Fellowship at the Rockefeller Institute brought this investigation prematurely to an end, but in any case many difficulties in the separation of mixtures of steroid hydrazones had already been encountered, and the method did not seem promising as a simple technique for this purpose. The author is aware that many subjects in the course of this work have been only incompletely investigated. It was decided to adopt the strategy of closing lines of investigation, however interesting in themselves when they seemed unlikely to lead to the ultimate goal, which was to discover a simpler, more reliable reagent for oxosteroids than those hitherto available.

Part 4

β-Resorcylic acid hydrazide

The search for a reagent giving an even more intense staining reaction was pursued by investigating the properties of β-resorcylic acid hydrazide (2:4-dihydroxybenzoic acid hydrazide), which had one more phenolic group than 2-hydroxy-3-naphthoyl hydrazide and one more nuclear position activated for possible azo-coupling.





The compound was synthesised by the author in collaboration with D.D. Dziewiatkowski, derivatives were successfully prepared from simple aldehydes and ketones and from oxosteroids. The oxosteroid derivatives were coupled with Fast Black Salt K to give derivatives coloured either pink or purple. The difference was attributed to coupling either at the 3-position or at both the 3- and the 5-positions. The reagent could also be used histochemically, and staining in discrete spots of pink and purple, using Fast Black Salt K as the azo-dye, was achieved in rat adrenal sections.

Although this observation makes it tempting to extrapolate easily and confidently from test-tube to tissue section and claim that the presence of particular steroids has been demonstrated histochemically, the more important result of this work was the first indication that oxosteroids might be separated into distinct groups on the basis of the properties of their hydrazone derivatives. The colours obtained on coupling five different oxosteroid hydrazones with Fast Black Salt K, the ultraviolet absorption spectra of the resultant dyes and of the hydrazones themselves, and the fluorescence colours given by the hydrazones under Wood's light, were compared, and significant differences between the five oxosteroids obtained. A tentative division of the five compounds into two groups could be made, a division which was confirmed when the compounds were included in the more extended examination of derivatives of salicyloylhydrazide.

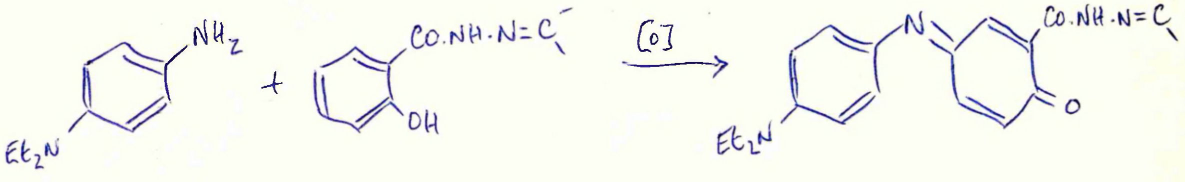
It is to be noted that the conclusions to be drawn from results with one steroid (desoxycorticosterone) were made uncertain because of the known tendency of the reagent to decarboxylate. The possibility of this complication is one of the reasons for caution in interpreting the histochemical results.

Part 5

Salicylic acid hydrazide

The properties and use of this reagent in connection with oxosteroids have constituted the most important discovery in this study. Differentiation of oxosteroids into seven distinct groups has been achieved by a one-stage reaction of an essentially simple nature. The relation between the distinctive behaviour of the seven groups and the position of the ketone group in the molecule has been elucidated, and the reaction therefore has potential usefulness in allocating structural features in unknown compounds as well as in identifying known components after separation from a mixture.

The reaction with oxosteroids having no $\alpha\beta$-unsaturated group has been made the basis of a fluorimetric estimation. Quantitative estimation of salicyloylhydrazones of other types of oxosteroids has been effected, using an oxidative coupling with an amine to produce an indoaniline:-



The indoaniline is blue-green and is estimated colorimetrically.

The indoaniline reaction was brought from the field of colour photography and provided a clear-cut reaction very suitable for colorimetric estimation. Some basic work was done on its application to the estimation of phenols in general, and should prove useful for work with phenolic derivatives of many different kinds of compounds.

By-products of the salicyloyl hydrazide investigation were the discovery of a new way to distinguish between aldehydes (vivid fluorescence of salicyloylhydrazones) and ketones (dim blue fluorescence only), and the general use of the indoaniline reaction to estimate aromatic amines and phenols.

The usefulness of salicyloyl hydrazide in histochemistry is left to other workers. Although there is a great difference between test-tube and cell conditions, progress must consist in going forward from the known to the unknown, and it has been the author's endeavour to make this possible by establishing on a firm basis the qualitative and quantitative nature of the salicyloylhydrazide reaction. The reaction with oxosteroids, which leads to compounds having fluorescence properties characteristic of those steroids, is a simple one and is in accordance with established chemical principles; the reagent is stable, not subject to decarboxylation or autoxidation, non-colloidal and easy to handle. Whilst it is hoped that some of the incidental observations made in the course of this study may prove capable of further development, the results with salicyloyl hydrazide and the indoaniline reaction should prove a solid foundation on which further studies may be securely based.

Acknowledgments

The work herein reported has been carried out during the tenure of the following appointments:

Great Britain

1 Aug. 46 - 30 Sep. 47	Honorary Clinical Assistant to Lord Cohen of Birkenhead, Liverpool Royal Infirmary.
1 Oct. 47 - 25 Mar. 49	Honorary Research Assistant to Professor John Beattie, Royal College of Surgeons.

USA

1 Apr. 49 - 30 Mar. 50	Advanced Medical Fellow of the Commonwealth Fund.
1 Apr. 49 - 30 Mar. 50	Visiting Investigator to the Hospital of the Rockefeller Institute : with Dr. R.M. Archibald.
1 Apr. 50 - 30 Mar. 51	Fellow of the Rockefeller Institute : with Dr. R.M. Archibald.
1 Apr. 51 - 30 Jun. 51	Research Fellow of College of Physicians and Surgeons, Columbia University : with Dr. Seymour Liebermann.

Great Britain

1 Sep. 51 - 31 Aug. 52	Fellow of Medical Research Council at the Chester Beatty Research Institute, London, S.W.3. with Professor Alexander Haddow.
1 Sep. 52 - 31 Aug. 56	Research Associate in Endocrinology in departments of Mr L.R. Broster and Professor J. Patterson, Charing Cross Hospital.

I am deeply indebted to Dr. R.K. Callow for having read and commented on the manuscript.

References

Abelson, D. & Bondy, P.K. (1955). Arch. Biochem. Biophys. 57, 208.

Albert, S. & Leblond, C.P. (1946). Endocrinology, 39, 386.

Angeli, A. & Angelico, F. (1903). Gazz.chim.ital. 33-11, 245.

Angeli, A. & Angelico, F. (1904). Gazz.chim.ital. 34-1, 50.

Angeli, A. & Marchetti, G. (1908). Atti reale Accad. Lincei, ser. 5,cl.di sc.fis., mat e nat., 17-11, 360.

Appleby, J.I., Gibson, G., Norymberski, J.K. & Stubbs, R.D. (1954a). Biochem.J. 57, xiv.

Appleby, J.I., Gibson, G., Norymberski, J.K. & Stubbs, R.D., (1954b). Biochem.J. 58, xxix.

Ashbel, R. & Seligman, A.M. (1949). Endocrinology, 44, 565.

Bates, R.W. & Cohen, H. (1947). Fed.Proc. 6, 236.

Bauld, W.S. (1954). Biochem J. 56, 426.

Bennett, H.S. (1940). Amer.J.Anat. 67, 151.

Boscott, R.J. & Mandl, A.M. (1949). J.Endocrinol. 6, 132.

Broadbent, I.E. & Klyne, W. (1954). Biochem.J. 56, xxx.

Brown, J.B. (1954). J.Endocrinol. 8, 196.

Burton, R.B., Zaffaroni, A. & Keutmann, E.H. (1951). J.biol.chem. 188, 763.

Bush., I.E. (1952). Biochem.J. 50, 370.

Bush., I.E. (1954). Rec.Prog.Horm.Res. 9, 321.

Camber, B. (1949). Nature, 163, 285.

Camber, B. (1950). Fed.Proc. 9.

Camber, B. (1954). Nature, 174, 1107.

Camber, B. (1955). Nature, 175, 1085.

Camber, B. (1957). Clin.Chim.Acta, 2, 188.

Camber, B. (1957b). Nature, 180, in the press.

Camber, B. (1957c). J.Endocrinol., in the press.

Camber, B. & Dziewiatkowski, D.D. (1951). J.Amer,chem.soc. 73, 4021.

Claesson, L. & Hillarp, D.A. (1947) Acta anat. 3, 109.

Clark, I. (1955). Nature, 175, 123.

Curtius, Th. (1917). J.prakt.Chem. 95, 336.

Curtius, Th., Struve, A., & Radenhausen, R. (1895). J.prakt.Chem. 52(2), 227.

Danielli, J.F. (1949). Quart.J.Micro.Sci. 90, 67.

Daughaday, W.H., Jaffe, H., & Williams, R.H. (1948). J.Clin.Endocrinol. 8, 166.

Dingemanse, E., Huis in 't Veld, J.G., & De laat, B.M.(1946) J.Clin.Endocrinol. 6, 555.

Dingemanse, E., Huis in 't Veld, L.G., & Hartogh-Katz, S.L. (1952) J.Clin.Endocrinol. 12, 66.

Dempsey, E.W. & Wislocki, G.B. (1946). Physiol.Rev. 26, 1.

Dische, Z.(1930). Mikrochemie, 8, 4.

Dobriner, K., Lieberman, S., & Rhoads, C.P. (1948). J.biol.chem. 172, 241.

Edwards, R.W.H., Kellie, A.E. & Wade, A.P. (1953). Memoirs Soc.Endocrinol. 2, 53.

Emerson, E. (1943). J.org.Chem.8, 417.

Feigl, F., Anger, V., & Frehden, O. (1934). Mikrochemie, 15, 184.

Fieser, L.F. (1953). J.Amer,chem,soc. 75, 4400-4401.

Finkelstein, M., Hestrin, S., & Koch, W. (1947). Proc.Soc. Exptl.Biol.Med. 64, 64.

Fischer, R.(1914). German Pat. 253,335; Fischer, R. & Siegrist, H. Phot.Korr. 51,18; Fischer, R. U.S.Pat. 1,102,028.

Folin, O. & Ciocalteu, V. (1927). J.biol.chem. 73, 627.

Fox, J.J. & Gauge, A.H. (1920) J.Soc.Chem.Ind. 39, 260T.

Franzen & Eichler (1908). J.prakt.Chem. 78, 164.

Gibbs, H. (1927). J.biol.Chem. 72, 649.

Gomori, G. (1942). Proc.Soc.Exp.Biol., N.Y. 51, 133.

Gomori, G. (1952). J.Lab. & Clin.Med. 39,649.

Gottlieb, S. & Marsh, P.B. (1946). Ind.Eng.Chem.(Anal.Ed.) 18, 16.

Grifols-Lucas, J.A. (1951). Brit.med.J. 2, 295.

Heard, R.D.H., & Sobel, H. (1946). J.biol.chem. 165, 687.

Heard, R.D.H., Sobel, H., and Venning, E.H. (1946) J.biol.chem. 165, 699.

Heftmann, E.(1950). Science, 111, 571.

Heftmann, E.(1955). Chem.Revs. 55, 679.

Hollander, V.P., Di Mauro, S., & Pearson, O.H. (1951) Endocrinol. 49, 617.

Houghton, G.U. & Pelly, R.G. (1937) Analyst, 62, 117.

Idler, D.R. & Baumann, C.A. (1953). *J.biol.chem.* 203, 389.

Jailer, J.W. (1947). *Endocrinol.* 41, 198.

Jones, J.K.N. & Stitch, S.R. (1953). *Biochem.J.* 53, 679.

Kellie, A.E. & Wade, A.P. (1957). *Biochem.J.* 66, 196.

Kind, P.R.N. & King, E.J. (1954). *J.clin.Pathol.* 7, 322.

King, E.J. & Armstrong,AR. (1934). *Canad.med.Ass.J.* 31, 376.

Kober, S. (1938). *Biochem.J.* 32, 357.

Lancet, The (1951). 261, 585.

Lowenstein, B.E. Corcoran, A.C., & Page, I.H. (1946). *Endocrinol.* 39, 82.

Missline, (1920). *Helv.chim.Acta*, 3, 626.

Neher, R. & Wettstein, A.(1951). *Helv.chim.Acta*, 34, 2278.

Nyc, J.F. Maron, D.M., Gavst, J.B. & Friedgood, H.B. (1951) *Proc.Soc.Exp.Biol. N.Y.*, 77, 466.

Norymberski, J.K. Stubbs, R.D, & West, H.F. (1953) *Lancet*, 1, 1276.

Olson, R.E., Jacobs, F.A., Richert, D., Thayer, S.A., Kopp,L.J & Wade, N.J. (1944). *Endocrinol.* 35, 430.

Oster, K.A. & Mulinos, M.G. (1944). *J.Pharmacol & Exper.Therap* 80, 132.

Paterson, J.Y.F., & Marrian, G.F. (1953). *Memoirs Soc. Endocrinol.* 2, 1.

Patterson, J. (1947). *Lancet*, 2, 580.

Patterson, J. & Swale, J.(1953). *J.Endocrinol.Memoirs No 2*.41.

Pausacker, K.H.(1950) *J.Amer.chem.soc.* 3478.

Pearse, A.G.E. (1951). J.Clin.Path. 4, 1.

Pincus, G. (1945). J.Clin.Endocrinol. 5, 291.

Pincus, G. & Pearlman, W.H. (1943). Vits.& Hormones, 1, 285.

Porter, C.C., & Silber, R.H. (1950). J.biol.chem. 185, 201.

Powell, M.E.A. & Smith, M.J.H. (1954). J.clin.Pathol. 7, 245.

Reichstein, T. (1936). Helv.chim.Acta, 19, 29, 1107.

Rimini, E. (1908). Atti reale Accad.Lincei, ser.5, 17-11.360.

Rosenheim, O. (1929). Biochem.J. 23, 47.

Rutenburg. A.M. Gofstein, R. & Seligman, A.M. (1950). Cancer Research, 10, 113.

Saunders (1936). "The Aromatic Diazo-Compounds and their Technical Application", p.108.

Seligman, A.M. & Ashbel,R. (1951). Cancer. 4, 579.

Talbot, N.B., Saltzmann, A.H., Wixom, R.L., & Wolfe, I.K. (1945). J.biol.chem. 160, 535.

Tortelli, & Jaffe, (1915). Chem.Ztg. 39, 14; see also Heilbron & Spring (1930). Biochem.J. 24, 133.

Vittum, P.W. & Weissberger, A. (1954). J.phot.sci. 2, 81.

Wieland & Horner (1937). Ann. 528, 95.

Zaffaroni, A. (1950). J.Amer.chem.soc. 72, 3828.

Zaffaroni, A. & Burton, R.B. (1951). J.biol.chem.193, 749.

Zaffaroni, A. Burton, R.B. & Keutmann, E.H. (1949). J.biol.chem. 177, 109.

Zaffaroni, A. Burton, R.B., & Keutmann, E.H. (1950) Science,111, 6.

Zimmermann, W. (1935). Hoppe-Seyl.Z. 233, 257.

Zimmermann, W. (1936). Hoppe-Seyl.Z. 245, 47.

Zimmermann, W. (1944). Vitam.u.Horm. 5, 1.