Chromogenic and Fluorogenic Reactions of Adrenocortical and Other Steroids in Concentrated Acids

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It has been known for many years that various steroids, when treated with certain concentrated or anhydrous acids, fluoresce brightly under suitable illumination. Natural oestrogenic hormones were found to develop a yellowish orange colour and a bright-green fluorescence when dissolved in a mixture of concentrated sulphuric acid and acetic anhydride (Wieland, Straub & Dorfmüller, 1929; Marrian, 1930), or in concentrated sulphuric acid alone (Schwenk & Hildebrandt, 1932). Cortisone, hydrocortisone (17-hydroxycorticosterone) and certain other adrenocortical steroids behaved similarly, although cortisone was said not to do so after chromatographic purification (Wintersteiner & Pfiffner, 1938; Reichstein & Shoppee, 1943). Similar colour or fluorescence phenomena have been reported from time to time in a variety of steroids dissolved in different reagents such as concentrated phosphoric acid (Finkelstein, Hestrin & Koch, 1947; Boscott, 1948; Finkelstein, 1952), phthalic anhydride and zinc chloride (Garst, Nyc, Maron & Friedgood, 1950), perchloric acid (Tauber, 1952; Pontius, Beckmann & Voigt, 1955), anthrone in sulphuric acid (Graff, 1952), thiophen in sulphuric and acetic acids (Levine & Tao, 1953), diphenylamine in sulphuric and acetic acids (Clark, 1955), phosphorus pentoxide in sulphuric acid (Steyermark & Nowaczynski, 1955) and vanillin in phosphoric acid (McAleer & Kozlowski, 1956).

The fluorescence phenomena have been utilized in the development of qualitative tests for the detection of various steroids, and of fluorophotometric procedures for their measurement in extracts of biological materials. Colorimetric procedures involving reactions in similar acid media (e.g. the Liebermann–Burchard reaction, the Kober reaction) have found even wider application. Such tests have of necessity been devised empirically, because the nature of the changes wrought by the various acids employed remains unknown. Bierry & Gouzon (1936) were the first to perform a spectrographic analysis of the fluorescence of natural oestrogens in mixed sulphuric and acetic acids. Partial studies of the absorption and fluorescence spectra of acid solutions of numerous other steroids have been carried out, chiefly in relation to the development of the fluorophotometric and colorimetric procedures mentioned above (e.g. Umberger & Curtis, 1949; Bates & Cohen, 1950a, b; Bompani, 1951; Braunsberg, 1952; Goldzieher, Bodenchuk & Nolan, 1952, 1954; Diczfalusy, 1953; Sweat, 1954). Many of these studies have been made for the purpose of selecting the most suitable primary illumination and secondary filters for use in the excitation and measurement of fluorescence in the various steroid–acid reaction mixtures under consideration. Implicit in this application of the results is the assumption that for a given reaction mixture the absorption and fluorescence maxima are those of a single molecular species.

There is no evidence available, however, to support this assumption. Indeed, almost nothing is known about the number and identity of the products resulting from the action of acids upon steroids. Zaffaroni (1953) demonstrated the rapid formation of a Δ¹¹ dehydration product when corticosterone was dissolved in concentrated sulphuric acid. However, Neher & Wettstein (1951) showed that fluorogenic products could be obtained by the action of phosphoric or sulphuric acid on other steroids which could not possibly undergo the same internal dehydration.

Zaffaroni (1950) had shown previously that the absorption spectrum of any steroid dissolved in concentrated sulphuric acid underwent progressive change with the passage of time. Umberger & Curtis (1949) had demonstrated other changes in the absorption spectra, dependent upon the concentration of acid used. Linford and his associates (Linford, 1952; Linford & Paulson, 1952; Linford & Fleming, 1953), and Dirscherl & Breuer (1954) observed other changes in the absorption spectra, on dilution of the reaction mixtures with alcohol or with water. Linford and his co-workers carried out very extensive and detailed studies of the effects of time, temperature and dilution, in an effort to correlate the wavelengths of absorption maxima with molecular structure and configuration of the steroids.

Neher & Wettstein (1951) had found phosphoric acid, sulphuric acid and acetic anhydride all capable of producing the same fluorescence.
phenomena with adrenocortical steroids on paper chromatograms. This seemed difficult to reconcile with the findings of several groups of investigators (Bompiani, 1951; Braunsberg, 1952; Goldzieher et al. 1952) to the effect that concentrated sulphuric acid and concentrated phosphoric acid gave different absorption spectra with the same steroid. In these comparative studies, however, 85% phosphoric acid was used. Axelrod (1953) had shown a considerable difference between the spectra obtained with concentrated sulphuric and with fuming sulphuric acid. Therefore it seemed possible that the use of 100% phosphoric acid might yield spectra more closely comparable with those obtained with concentrated sulphuric acid. Nowaczyński & Steyermark (1955) have published detailed observations on the absorption spectra of steroids in anhydrous phosphoric acid (100%). At the time the present work was done, however, their publication had not appeared.

In summary, the observations of the many workers cited are not in full agreement with respect to the structural correlation of the absorption or fluorescence maxima, to the similarity or dissimilarity of action of sulphuric, phosphoric and other acids tested, or to the optimum conditions for demonstration of the fluorescence properties of the various reaction products. On the contrary, they demonstrate a remarkably complex pattern of chemical behaviour for each steroid tested, such that serious doubts arise concerning the specificity of the acid-steroid interactions under any one set of reaction conditions.

Because of the potential advantages inherent in the great sensitivity of fluorometric methods of measurement of steroids, it seemed worthwhile to explore further the problem of the nature of action of concentrated acids on steroids, and the absorption and fluorescence properties of the products. It was hoped that if the reaction mechanisms could be explained, much greater sensitivity and specificity of fluorometric measurement might be achieved by purposeful direction of the reaction. The results presented here permit a unified interpretation of some of the apparently discordant observations mentioned above. In addition, they have led to a partial fulfilment of the hope for improved quantitative fluorophotometry.

**EXPERIMENTAL**

**Apparatus**

Absorption spectrophotometry of the various steroid-acid reaction mixtures and isolated fractions was carried out with a Beckman DU spectrophotometer and with a Warren Spectrorecord automatic-recording spectrophotometer (kindly lent by the Fisher Scientific Co., Toronto, Canada) incorporating the Beckman quartz optical system and an RCA 1P28 electron-multiplier phototube.

Fluorescence-emission spectra were studied by means of a spectrofluorometric adaptation of the Beckman DU spectrophotometer (Gornall & Kalant, 1955). The RCA 1P28 phototube used in this adaptation is very insensitive to the red end of the spectrum. In order to study fluorescence in the red region therefore use was made of a Hilger Medium Quartz spectrograph. The spectra were recorded on Ilford Rapid Process panchromatic plates. A General Electric H-85-C-3 quartz mercury arc source was employed. Light from the arc was directed via a hole in an asbestos mask, through an appropriate primary filter, into the quartz lens of a standard Beckman fluorescence accessory. In this accessory, the incident light is reflected upward through the optically polished bottom of a Corex fluorescence cuvette. This cuvette shows 50% transmission at 300 μm, and virtually complete and uniform transmission above 340 μm. Fluorescence emitted at 90° to the axis of primary illumination passed through the polished side of the cuvette directly into the slit of the spectrograph. Since no collecting lens was employed, exposure times of 30–60 min. were used at a slit width of 0.3 mm. The spectrographic plates were scanned in a Leeds and Northrop microphotometer with continuous recording attachment, in order to obtain density readings at each wavelength.

**Reagents**

*Phosphoric acid.* Concentrated orthophosphoric acid (sp.gr. 1.75) of AnaALR grade was chilled in ice-water, and AnaALR P₂O₅ was added gradually, with slow swirling, until sp.gr. 1.96 was attained. This corresponds to a concentration of more than 99% H₃PO₄; 100% H₃PO₄ was not used because it crystallizes at room temperature. Although the application of heat speeds the solution of the added P₂O₅, it tends to drive off water and forms pyrophosphoric acid. The latter was found to give no fluorescence with steroids under the conditions of this study. Metaphosphoric acid is probably also inactive in this respect, since mixtures of meta- and ortho-phosphoric acids, prepared from ice and P₂O₅, were considerably less effective than was pure H₃PO₄.

For convenience, the H₃PO₄ of sp.gr. 1.96, prepared as described above, will be referred to as 100%, and dilutions will be designated by reference to it. Thus 1 part (by vol.) of this acid mixed with 3 parts of water will be considered as 25% H₃PO₄.

*Sulphuric acid.* Reagent-grade H₂SO₄, at least 96% H₂SO₄, obtained from Canadian Industries, Ltd., was employed without redistillation or other purification. For convenience, this will also be referred to hereafter as 100% H₂SO₄, and dilutions prepared from it will be designated in the same manner as the dilutions of H₃PO₄.

*Alcohols.* Absolute methanol and ethanol were twice-distilled before use, the first distillation being done over 2:4-dinitrophenylhydrazine.

*Dichloromethane.* Technical-grade dichloromethane (Dow Chemical Co.) was purified by fractional distillation. The fraction of b.p. 40–1°C was used.

**Absorption spectra of steroids treated with 100% phosphoric and sulphuric acid**

Samples of various steroids in methanolic solution, ranging from 50 to 100 μg. in amount, were measured into individual Pyrex reaction tubes with ground-glass stoppers. They were evaporated to dryness in an oven at 100°C. Each dry residue was redissolved in 0.1 ml. of absolute ethanol.
For $\text{H}_2\text{SO}_4$—chromogen curves (Zaffaroni, 1950), 4 ml. of 100% sulphuric acid was added to each sample and mixed thoroughly. The stoppered tube was left in the dark for 2 hr., after which the contents were transferred to a Beckman quartz cuvette of 1 cm. light path for absorption spectrophotometry.

For $\text{H}_3\text{PO}_4$—chromogen curves, 4 ml. of the specially prepared 100% $\text{H}_3\text{PO}_4$ was added to each tube and mixed thoroughly. The stoppered tubes, including an acid—ethanol blank, were placed in a boiling-water bath, in the dark, for exactly 5 min. The contents of each tube were mixed a second time during the first minute of heating, when the viscosity of the acid had decreased greatly. After heating, the tubes were transferred at once to a cold-water bath until their contents were at approximately room temperature. Heat was used for the $\text{H}_3\text{PO}_4$—chromogen curves because no reaction was found to occur in several hours at room temperature. Five minutes was selected as the optimum time of heating, on the basis of preliminary trials with periods of from 2 to 30 min. The absorption spectra obtained in $\text{H}_3\text{PO}_4$ in this way were found to be stable for at least 24 hr. (Kalant, 1954).

RESULTS

In Fig. 1 are shown typical absorption spectra obtained by the use of sulphuric and phosphoric acid with a number of different steroids. For

![Fig. 1. Comparison of absorption spectra of steroids dissolved in concentrated $\text{H}_2\text{SO}_4$ (●) and in 100% $\text{H}_3\text{PO}_4$ (■).](image)

(a) Corticosterone; (b) hydrocortisone; (c) cortisone; (d) 11-deoxycorticosterone; (e) aldosterone; (f) 17-hydroxy-11-deoxycorticosterone.

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cortisosterone and hydrocortisone the curves in the two acids are almost identical in form. With cortisone there is partial resemblance of the two curves; with aldosterone and 17-hydroxy-11-deoxycorticosterone almost none.

These results do not clearly show the essential similarity or dissimilarity of action of sulphuric and phosphoric acids on steroids. The fact that the two acids yielded practically identical absorption spectra in some cases, made it unlikely that their actions were basically different. The instances in which corresponding absorption spectra in the two acids were partially or entirely dissimilar might conceivably be explained on the basis of different degrees of reactivity of the two acids towards some of the steroids. Therefore some effects of time and temperature on the steroid–chromogen curves were studied, as described in the next section.

**Effects of time and temperature on absorption spectra of steroid–acid reaction mixtures**

Samples of various steroids were treated with 100 % sulphuric acid as described above. Absorption spectra were studied at 2 and 4 hr. The samples were returned to their respective reaction vessels and left in the dark at room temperature for varying lengths of time, after which the absorption spectra were again measured. The hydrocortisone curve showed no appreciable change from 2 to 24 hr. In the same interval the cortisone curve showed marked change, coming to resemble much more closely the curve obtained in phosphoric acid (compare Figs. 1c and 2a). Between 2 and 96 hr. the curve for corticosterone showed only some minor changes in the relative heights of the various absorption peaks, with some further resolution of the shoulder at 320 mµ and a shift of the 460 mµ peak to 445 mµ. During the same interval the 3:17α-dihydroxyallo pregnane-11:20-dione spectrum showed considerable change (Fig. 2b).

Duplicate samples of 17-hydroxy-11-deoxycorticosterone were treated with sulphuric acid at different temperatures. One was left at 25° throughout. The other was placed in a boiling-water bath for 10 min., then cooled rapidly to 25°. The absorption spectra were compared after 2 hr. The sample which had been heated was left in the dark at 25° for a further 120 hr., after which its absorption spectrum was again measured. All three curves are shown in Fig. 3. It can be seen that heating at 100° produced marked changes in both the heights and the locations of all the principal absorption maxima. These changes progressed only slightly in 5 days more at room temperature.

Samples of cortisone, hydrocortisone, aldosterone and corticosterone were also treated with 100 % sulphuric acid at 100° for 10 min., as described above. The absorption curves of the heated reaction mixtures are shown in Fig. 4. With cortisone, a completely new and quite marked absorption maximum appeared at 375 mµ. A new maximum appeared at 320–325 mµ in the hydrocortisone

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**Fig. 2.** Effect of time on absorption spectra of steroids dissolved in concentrated H₂SO₄. (a) Cortisone, observations made after 2 hr. (•) and 24 hr. (○); (b) 3:17α-dihydroxyallo pregnane-11:20-dione, observations made after 2 hr. (□) and 96 hr. (●) at room temperature.

**Fig. 3.** Effects of heat and time on the absorption spectrum of 17-hydroxy-11-deoxycorticosterone in concentrated H₂SO₄: 2 hr. at 25° (●); 10 min. at 100° followed by 110 min. at 25° (□); 10 min. at 100° followed by 120 hr. at 25° (▲).
curve and the previously existing maxima decreased in height, merging into a higher base-line of non-specific absorption. The only change in the corticosterone curve was the disappearance of the shoulder at 320 mμ so that the curve came to resemble that obtained in phosphoric acid (cf. Fig. 1e). With aldosterone, suggestions of two new maxima appeared at 245 mμ and at 370 mμ, so that this curve also bore more resemblance to that obtained in phosphoric acid. This effect of heat on the aldosterone–chromogen spectrum is similar to that reported by Simpson et al. (1954), who heated the aldosterone-reaction mixture for a longer period and obtained an absorption spectrum resembling even more closely that obtained in phosphoric acid (Fig. 1e).

Samples of progesterone and of allopregnane-3:20-dione were dissolved in concentrated sulphuric acid at room temperature, and the absorption spectra were measured after 2 hr. The solutions were then heated to 100° for 10 min., and the spectra were measured again. Heating produced a marked effect in both cases (Fig. 5), especially upon the progesterone spectrum, in which the single peak at 290 mμ was resolved into two widely separated and lower maxima.

**Effects of dilution on steroid–chromogen spectra**

Phosphoric acid reaction mixtures were prepared with cortisone, hydrocortisone, corticosterone, 17-hydroxy-11-deoxycorticosterone, aldosterone, oestriol and testosterone in the following manner. For each steroid, a set of five equal samples were measured into separate tubes, dried and redissolved in ethanol as described previously. To one tube of each set was added 0·5 ml. of acid and, to each of the remaining tubes, 1 ml. instead of the usual 4 ml. After the heating period, the volume in each tube was made to 4 ml. with suitable amounts of phosphoric acid and water, such that the final acid concentrations in each series of five tubes were 100, 75, 50, 25 and 12·5%. The absorption spectra were studied by comparison with acid–water blanks of corresponding acid concentration. Some of the results are shown graphically in Fig. 6.

With all the corticosteroids and testosterone, reduction of the final acid concentration led to a progressive shift of the principal absorption maximum from 290 to 300 mμ towards the position at 240–245 mμ, which is characteristic of compounds containing the Δ4-C-3 ketone grouping. Dilution of the oestriol solution led similarly to a suppression of the absorption maximum at 360 mμ and corresponding augmentation of that at about 265 mμ.

The behaviour of the maxima at higher wavelengths was quite varied and unpredictable. Thus the maxima at 360–370 mμ shown by cortisone and aldosterone in 100% phosphoric acid simply disappeared on dilution. With corticosterone and hydrocortisone, the maxima in the blue and near ultraviolet disappeared on dilution, but a new plateau appeared in the region of 300–360 mμ. The peaks at 495 and 380 mμ in the testosterone–chromogen spectrum shifted to slightly shorter wavelengths on dilution to 50% acid concentration, and a high new peak appeared at 595 mμ. On further dilution these all disappeared, and a new low peak at 350 mμ was detected (Fig. 6d). Again, the peaks at 460 and 500 mμ in the oestriol spectrum showed sharp increases on moderate dilution, and then decreases on further dilution.

The effects of dilution with water upon the absorption spectra of steroids dissolved in concentrated sulphuric acid were observed in the same manner. As illustrated in Fig. 7, progressive
dilution produced sequences of change in the absorption spectra, quite comparable with those observed for the same steroids in phosphoric acid solution. Similar observations were made by Linford & Paulson (1952) and by Linford & Fleming (1953) on dilution with ethanol.

Similar changes occurred on dilution of heated sulphuric acid-reaction mixtures. Aldosterone, for example, after being heated with 100 % sulphuric acid for 15 min. at 100°, was diluted with an equal volume of absolute ethanol. The resulting changes in absorption spectrum included a decrease in height of the original maximum at 290 mµ, and the resolution of a new maximum at 350 mµ.

Effects of steroid concentration on steroid–chromogen spectra

The effect of relatively large variations in the concentration of steroid upon the absorption spectra of undiluted and diluted reaction mixtures was studied in the following manner. Into two reaction tubes were placed 2 and 0.2 mg. of corticosterone. The dried steroid samples were each treated with 4 ml. of concentrated sulphuric acid, so that the concentrations of steroid in the reaction mixture were 500 µg./ml. (solution I) and 50 µg./ml. (solution II) respectively. After 2 hr. in the dark at room temperature, solution I was diluted to 40 ml. with concentrated sulphuric acid, so that the concentration of steroid or steroid derivatives was also equivalent to an initial value of 50 µg./ml. Portions (1 ml.) of solutions I and II were then diluted to 4 ml. with appropriate amounts of water and acid to bring the final acid concentrations to 25, 50, 75 and 100 % (v/v). The absorption spectra were determined by comparison with corresponding acid–water blanks.

The experiment was repeated with hydrocortisone and with cortisone. In all cases, the absorption spectra of corresponding dilutions made from solutions I and II were observed to differ somewhat, although the extents of observed differences varied from one steroid to another, and from one final dilution to another for each steroid. Some of these slight differences are illustrated in Fig. 8. These results, indicative of a small but definite effect of the steroid concentration upon the reaction pattern, are discussed later.

Fig. 6. Effects of dilution with water on absorption spectra of steroids dissolved in 100 % H₃PO₄. (a) Corticosterone; (b) cortisone; (c) oestriol; (d) testosterone. Final acid concentrations indicated are 100 % (○), 75 % (●), 50 % (■) and 25 % (▲).
sections, it seemed possible that the differences in fluorescence behaviour of steroids encountered by the various authors cited, might be due to these same factors.

Phosphoric acid–chromogen solutions were prepared as already described from samples of various steroids. Each was viewed under these different types of illumination: (1) Mercury arc lamp (General Electric H-100-SP-4), and Corning 5874 filter (chiefly 365 mμ). (2) Mercury arc lamp and Corning 9863 filter (broad band including the 302, 313 and 365 mμ mercury lines). (3) Mercury arc lamp and Corning 5113–3389 filter combination (chiefly 436 mμ). (4) Tungsten lamp and Evelyn 490 mμ narrow-band pass filter. (5) Tungsten lamp and Evelyn 570 mμ narrow-band pass filter.

**Fluorescence properties of steroid–acid reaction mixtures**

In the qualitative fluorescence test described by Neher & Wettstein (1951), all the physiologically active adrenocortical steroids then known were found to give distinctive colours of fluorescence, whether the acid employed was phosphoric or sulphuric acids or acetic anhydride. Linford & Paulson (1952) demonstrated complex fluorescence spectra for a number of different steroids, including 11-deoxycorticosterone in concentrated sulphuric acid solution. However, Finkelstein (1952) and Sweat (1954) were able to measure appreciable fluorescence in solution, by their respective methods, only with corticosterone and hydrocortisone.

In view of the marked influence on absorption spectra exerted by such factors as temperature, time and dilution, as shown in the preceding

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**Fig. 7.** Effects of dilution with water on absorption spectra of steroids dissolved in concentrated H₂SO₄. (a) Corticosterone; (b) cortisone; (c) hydrocortisone. Final acid concentrations indicated are 100 % (○), 75 % (●), 50 % (■) and 25 % (▲).

**Fig. 8.** Effects of steroid concentration on absorption spectra in H₂SO₄. Initial concentration of steroid in concentrated H₂SO₄ was either 50 μg./ml. (○) or 500 μg./ml. (●). For spectrophotometric study, the 500 μg./ml. solutions were diluted tenfold with acid, then both solutions were diluted with water to the final acid concentrations indicated. (a) Corticosterone in 75% H₂SO₄; (b) cortisone in 100% acid; (c) hydrocortisone in 25% acid.
After completion of these observations, the steroid–chromogen solutions were diluted by addition of sufficient water to lower the final acid concentration to 90%, and viewed again under the various types of illumination. The solutions were diluted again repeatedly, lowering the acid concentration by similar successive stages, with re-examination under the various illuminations at each stage. Some of the results are summarized in Table 1.

Similar sequences of fluorescence change were observed on dilution with absolute ethanol instead of water, although the intensity of fluorescence in general appeared weaker than when water was used. Also the degree of dilution at which each change occurred was not the same in the ethanol series as in the water series. Sulphuric acid–chromogen solutions showed comparable behaviour when treated in the same manner.

The effect of extreme dilution was tested with a solution of 1 mg. of corticosterone in 1 ml. of concentrated sulphuric acid. After 3-5 hr. at room temperature the solution had an intense orange-red colour and showed strong green fluorescence under 436 m\(\mu\) illumination. Under the 9863 filter it showed a relatively weak greenish white fluorescence. The solution was chilled in ice–water and diluted with ice-cold water. At 25% acid concentration, the solution assumed a purple colour, with intense mustard-yellow fluorescence under the 9863 filter, and none under the 436 m\(\mu\) light. At 10% acid concentration the solution became pale orange–yellow in colour, and showed a brilliant green fluorescence under the 9863 filter. This visible colour and green fluorescence persisted despite further dilution down to 0.5% acid concentration.

This solution in very dilute acid was then neutralized by gradual addition of sodium hydroxide. No change occurred in either the visible colour or the fluorescence properties.

A more precise and quantitatively valid examination of the steroid–chromogen solutions was undertaken by means of spectrophotometric and spectrographic analysis of their fluorescence. For this purpose, replicate samples of each steroid were treated with 100% phosphoric acid and subsequently diluted to the same final volume with varying proportions of acid and water as described

Table 1. Effects of dilution on visible fluorescence of steroid–phosphoric acid reaction mixtures

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Primary illumination</th>
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<tr>
<td></td>
<td>302, 313 and 365 m(\mu)</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>Faint blue–white in 100% acid, turning green below 50% acid, and grows progressively more intense</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>Weak yellow–white in 100% acid, turning yellow–green and becoming brilliant with high dilution</td>
</tr>
<tr>
<td>Cortisone</td>
<td>Moderate blue in 100% acid, fading on dilution below 70% acid</td>
</tr>
<tr>
<td>17-Hydroxy-11-deoxycorticosterone</td>
<td>Weak mauve in 100% acid, turning yellow and more intense on dilution below 50%</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>Mauve in 100% acid, turning yellow at 50% and green at 25%</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>None</td>
</tr>
<tr>
<td>Oestriol</td>
<td>Pale yellow, turning green and much more intense on dilution</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Faint whitish green, turning to strong green on dilution below 50%</td>
</tr>
<tr>
<td>Progesterone</td>
<td>None</td>
</tr>
</tbody>
</table>
previously, so that fluorescence intensity measurements could be compared. The results, some of which are shown in Figs. 9 and 10, indicated that the colours of fluorescence seen visually were in most cases composite colours resulting from the blending of two or more fluorescence bands, and that the changes resulting from dilution were produced in part by alterations in the relative heights of these bands.

A mixed sample, of approximately 100 \( \mu \)g, each of cortisone and of hydrocortisone, was used to prepare a sulphuric acid–chromogen solution as described. Its fluorescence was examined spectrographically, under primary illumination with the 365 m\( \mu \) mercury line. As seen in Fig. 11, a composite spectrum was obtained, which appears to be a qualitative fusion of the separate spectra of the two steroids. This will be discussed below.

Isolation of products from reaction mixtures

Cortisone. Cortisone (2 mg.) was dissolved in 1 ml. of concentrated sulphuric acid at room temperature, and left in the dark for 2 hr. It was then placed in a refrigerator at 4\( ^\circ \) and diluted to 200 ml. by dropwise addition of cold water over a period of 12 hr. The absorption spectrum, determined by comparison with a sulphuric acid blank treated in the same manner, showed complete reversion to the typical pattern of a free corticosteroid, i.e. a single peak at 245 m\( \mu \). From the height of this peak the solution was calculated to contain 1.98 mg. of steroid, accounting for practically all the cortisone originally used.

The weakly acid solution was extracted with dichloromethane and the extract chromatographed on paper by the method of Bush (1953). Four separate zones on the chromatograms were located by ultraviolet contact photography (Fig. 12). A zone in the running position of cortisone appeared to contain about 100–150 \( \mu \)g of material. Another, about equal in amount, remained at the starting line, suggesting that the material was of a very polar nature. A third zone, appearing to contain approximately 25 \( \mu \)g of material, had migrated about three-quarters as fast as the cortisone area. Zone X, the fourth and much the largest, was rechromatographed by the method of Zaffaroni, Burton & Keutmann (1950). In addition to zones X-1 and X-2 (Fig. 12), it yielded a third zone which did not absorb ultraviolet illumination of the wavelength used for photography, but gave a mauve fluorescence under 365 m\( \mu \) illumination (Fig. 13).

Each of the zones was eluted and redissolved in concentrated sulphuric acid. All the solutions showed immediate development of a yellow or orange–yellow colour, the speed of appearance being in striking contrast with the slow and weak colour development in the original cortisone–chromogen solution. The absorption and fluorescence properties were examined, both in the concentrated solutions and after subsequent dilution, as already described. The absorption spectra of some of these fractions dissolved in concentrated sulphuric acid are shown in Fig. 13. None of the absorption curves was the same as that of the original cortisone–chromogen solution, but all exhibited return of the main absorption peak to the 245 m\( \mu \) region on redilution of the solutions with water. In previous experiments it had been found that eluates from blank segments of the chromatograms, when treated in the same manner, showed only a non-specific absorption falling off rapidly from 220 to 250 m\( \mu \). Therefore such blanks were not used in the present work.

Hydrocortisone. A similar set of experiments was carried out with a solution of 2.4 mg. of hydrocortisone in concentrated sulphuric acid, left at room temperature for 4 hr. Dilution of the reaction mixture caused disappearance of the typical absorption spectrum, but the resulting pattern was still different from that of a free corticosteroid.

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**Fig. 9.** Effects of dilution on fluorescence spectra of steroids dissolved in 100% \( \text{H}_2\text{PO}_4 \). (a) Corticosterone; (b) cortisone. Measured in spectrofluorometric adaptation of Beckman DU spectrophotometer. Reaction mixtures were diluted with water to final acid concentrations shown: 100% (○); 75% (□); 50% (▲). Excitation with 365 m\( \mu \) source.
Fig. 10. Fluorescence spectra of steroids dissolved in H₂SO₄. Primary radiation from high-pressure mercury arc with filters indicated below. Microphotometric tracings of spectrographic plates. (a) Hydrocortisone in concentrated H₂SO₄ with Corning 9863 primary filter (—) and Corning 5113 + 3389 primary filters (---). (b) 3:17α-Dihydroxy-allopregnane-11:20-dione (—) and allopregnane-3:20-dione (---) in concentrated H₂SO₄, with Corning 5113 + 3389 primary filter. (c) 11-Deoxycorticosterone: in concentrated H₂SO₄ with Corning 9863 primary filter (—), in 50% H₂SO₄ with Corning 9863 primary filter (---) and in 50% H₂SO₄ with Corning 5113 + 3389 primary filter (···). (d) Cortisone, with Corning 9863 primary filter: in concentrated H₂SO₄ (——) and in 50% H₂SO₄ (···). Concentrated H₂SO₄ blank with Corning 9863 primary filter (···).
STEROID FLUORESCENCE IN CONCENTRATED ACIDS

There was a sharp maximum at 240 m\(\mu\), and a low rounded one at 360 m\(\mu\). If the peak at 240 m\(\mu\) is considered indicative of a \(\Delta^4\)-C-3 ketone grouping, its height suggested a total of 2-25 mg. of material, or about 94% of the original amount. Chromatography of the dichloromethane extract revealed five zones: minute amounts in the positions of hydrocortisone and cortisone, a moderate amount of highly polar material remaining close to the starting line, and two very dense bands (Y and Fl) running close behind the solvent front. The more rapid of these two zones (Fl) showed a brilliant yellow-green fluorescence under ultraviolet illumination. Rechromatography of the Y band in the Zaffaroni system yielded two fractions Y-1 and Y-2 (Fig. 12).

The various fractions were eluted separately with methanol and their absorption spectra determined. These all showed only a single main peak at 235-245 m\(\mu\), except for that of Fl, which showed also a low plateau adjacent to the base of the main peak, in the region of 300-360 m\(\mu\), similar to that noted on dilution of hydrocortisone-chromogen solutions. The methanolic solution of Fl showed bright yellow-green fluorescence under 365 m\(\mu\) illumination. When the fractions were dried and redissolved in concentrated sulphuric acid, they showed immediate development of a yellow colour. The acid-chromogen absorption spectra of most of the fractions showed only a main peak at 285-295 m\(\mu\), but the spectra obtained from the Fl and Y-1 fractions were exceptions, and are shown in Fig. 13.

The solution of Y-1 in concentrated acid showed a moderately intense blue fluorescence under 365 m\(\mu\) illumination. The solution of Fl showed a dull yellowish white fluorescence under 365 m\(\mu\).

Fig. 12. Products isolated from diluted steroid-H\(\text{SO}_4\) reaction mixtures. Ultraviolet contact photographs of paper chromatograms. (a) Dichloromethane extract of cortisone-reaction mixture, developed in benzene-methanol-water system (Bush, 1952); E, cortisone; X, unknown. (b) Zone X eluted from (a) and developed in toluene-propylene glycol system (Zaffaroni et al. 1950); X-1 and X-2 obtained. (c) Dichloromethane extract of diluted hydrocortisone-reaction mixture, developed in benzene-methanol-water system; F, hydrocortisone; E, cortisone; Y, unknown; Fl, zone with intense yellow-green fluorescence under 365 m\(\mu\) illumination. (d) Zone Y eluted from (c) and developed in toluene-propylene glycol system: Y-1 and Y-2 obtained.

Fig. 11. Fluorescence spectrum of mixed solution of cortisone and hydrocortisone in concentrated H\(\text{SO}_4\). Corning 9863 primary filter. Microphotometric tracing of spectrographic plate.
illumination, and a strong green under 436 m.μ. When this solution was diluted with water, 365 m.μ excitation evoked a brilliant yellow-green fluorescence whereas 436 m.μ light caused almost no fluorescence at all. This was exactly the same sequence of changes as that noted above on dilution of the original hydrocortisone–sulphuric acid reaction mixture.

**DISCUSSION**

The findings obtained in the first three groups of experiments described above appear to support the concept of a common mechanism of action of sulphuric and phosphoric acids on steroids. Although the acid–chromogen spectra of some steroids in 100% phosphoric and sulphuric acid differ rather widely, some of the other steroids tested show close agreement between their corresponding spectra in the two acids. The changes in the sulphuric acid–chromogen spectra which occur with time and with heating tend to decrease considerably the differences from the corresponding phosphoric acid–chromogen spectra. Finally, the same sequences of change were observed in both sets of spectra on dilution of the reaction mixtures with water. Phosphoric acid appears to react less readily with steroids than does sulphuric acid, as shown by the necessity of heating the phosphoric acid–reaction mixtures, in order to produce their characteristic absorption and fluorescence properties. The tendency for differences between corresponding sulphuric acid and phosphoric acid–chromogen spectra to diminish with time, or on heating of the sulphuric acid–reaction mixtures, suggests that various intermediate reactions of differing activation energies occur with sulphuric acid, whereas the reactions with phosphoric acid require higher energy of activation but go at once to completion, in so far as this term may be applied here.

The effects which both acids produce in the steroid–reaction mixtures are of two types. From the observations described above, as well as those of Zaffaroni (1953), Linford & Paulson (1952), Linford & Fleming (1953), Bernstein & Lenhard (1953) and many others, it is clear that steroids containing a Δ^4-C-3 ketone grouping give rise in acid solution to an absorption maximum in the region of 290 m.μ. This is based on a reversible reaction, because on progressive dilution of the reaction mixtures with water, this maximum gradually reverts to the position at 240–245 m.μ, characteristic of the free steroids. The fact that the same maximum at 290 m.μ in acid solution, and reversion to 240–245 m.μ on redilution, was seen with the products isolated chromatographically from steroid–reaction mixtures, indicates that these products retained the Δ^4-C-3 ketone grouping.

A similar reversible reaction may be postulated for the phenolic C-3 hydroxyl of oestriol, which shows an absorption maximum at 360 m.μ in concentrated phosphoric acid, reverting to 270 m.μ on dilution.

In addition to these reversible changes, various irreversible ones occur spontaneously, and on heating or dilution. The multiplicity of products obtained chromatographically from extracts of the diluted reaction mixtures is evidence of such change. It might be argued that a reversible dehydronation reaction could give rise to several different products on dilution of the reaction mixture. Thus Δ^11-11-deoxy corticosterone, obtained by Zaffaroni (1953) from a corticosterone–sulphuric acid reaction mixture, would give rise to 11α-, 11β- and 9-hydroxy isomers if random rehydration were possible. All of these, however, should give rise to the original acid–chromogen spectrum of corticosterone on being dissolved a second time in concentrated acid. In fact, the various fractions obtained after dilution did not give rise to the original chromogen spectra when placed in concentrated acid, indicating that dilution of the reaction mixtures had not merely reversed a previous reaction but had induced new irreversible changes.

The recovery of small amounts of the original steroids from their diluted reaction mixtures is therefore probably indicative of the relative slowness of the acid–steroid reaction. Hydrocortisone developed its typical acid–chromogen absorption spectrum very rapidly, and only minute amounts of hydrocortisone were recovered after dilution; cortisone developed its chromogen spectrum much more slowly, and appreciable amounts of cortisone

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**Fig. 13.** Absorption spectra of H₂SO₄-chromogen solutions prepared from fractions isolated from steroid–acid reaction mixtures: ●, F₁; ○, Y-1; ▲, X-1; △, X-2 (see Fig. 12 for explanation). ●, Mauve fluorescent material obtained from cortisone reaction mixture. It does not appear in Fig. 12 (a) because it does not absorb ultraviolet radiation of the wavelength employed in the contact photography.
were recovered after dilution. In both cases, however, the other products obtained after dilution reacted instantaneously on solution in concentrated acid, indicating that they were indeed quite different from their parent steroids.

Proof of the occurrence of irreversible change even before dilution is provided by the demonstration that the green fluorescence of the hydrocortisone-acid solution was due to a stable substance, no longer dependent upon an acid medium, running chromatographically in a single zone and showing its same fluorescence in the dry state or in methanolic solution. The exact similarity of fluorescence behaviour of the acid solution of this material, and of the original hydrocortisone-chromogen solution, suggest very strongly that this material was present in the latter solution and was responsible for its fluorescence properties.

Any hypothesis concerning the reaction mechanism must take into account both the reversible and irreversible changes. The $\Delta^4$-C-3 ketone grouping (or the phenolic C-3 hydroxyl of oestriol) probably acts as a proton acceptor in strong-acid media. The resulting ion could not be capable of internal stabilization by association with reactive groups such as hydroxyl or keto groups at C-11 and C-17, because of the rigidity of the steroid nucleus. However, intermolecular association at such reactive positions could occur. In this case it might be expected that the concentration of steroid in the reaction mixture would affect the pattern of reaction, and some evidence is available to bear this out. As described in the Results section, the concentration of steroid during the time of reaction with concentrated acid had a demonstrable effect on the absorption spectra obtained. Even at the highest steroid concentration employed, however, the acid was present in great excess, and can probably be regarded as a constant factor. Therefore the differences observed appear to support the concept of intermolecular reaction between the protonated steroid molecules. These postulated intermolecular association complexes would presumably give rise to stable products by spontaneous degradation, which could be hastened by heat or dilution with water. The end products must be degradation products rather than polymers or other addition products, as shown by their chromatographic behaviour. Moreover, the ultraviolet-absorption patterns of the fractions obtained chromatographically indicate that the protonated $\Delta^4$-C-3 ketone group must be left intact during the degradation reactions.

Zaffaroni (1953) has suggested that dehydration at C-9-C-11 gives rise to a carbonium ion by protonation at the unsaturation, analogous to that seen with unsaturated hydrocarbons in acid solution (Gold & Tye, 1952). The absorption spectrum of the acid-chromogen solution, he suggests, would really be that of the carbonium ion. Such a simplified generalization fails to account for the behaviour of steroids such as 11-dehydrocorticosterone and 11-deoxycorticosterone, which cannot undergo preliminary dehydration. The hypothesis advanced above would cover these cases, and seems more probable. A study of the reaction of steroids in sulphuric acid, by the method of Newman & Deno (1951), might help to choose between these hypotheses, by indicating the number of stages in the reaction.

It is clear from the fluorescence behaviour of the reaction mixtures examined here that fluorescence in acid solution is not a specific attribute of a small number of steroids. The specificity described by Sweat (1954), Goldzieher et al. (1954) and others, related to various substituent groups within the steroid molecule, is valid only with respect to the reaction conditions described by these authors. From the present work it seems probable that under suitable conditions a great many steroids are capable of yielding fluorescent products in acid.

It is also evident that the fluorescence of each reaction mixture is not a simple monochromatic emission, but a complex of two or more fluorescence bands which probably derive from different constituents of the mixture. Since both the absorption and the fluorescence spectra are composite, it is impossible to correlate the peaks of the one with those of the other, as can be done with the spectra of a pure substance. Such correlations, as attempted by Bates (1954), Linford (1952) and Aitken & Preedy (1953), are evidently fortuitous, and cannot be generally valid in the selection of optimum wavelengths for quantitative fluorometry. This is well illustrated by the green fluorescence at 530 m$\mu$ shown by corticosterone solutions in 100% phosphoric acid. This band seems reasonably enough to correspond to the re-emission of energy absorbed at the 480 m$\mu$ maximum. However, the latter absorption peak is abolished by dilution of the reaction mixture with water, whereas the fluorescence is greatly increased, but is now dependent upon primary illumination of 365 m$\mu$ or shorter. This suggests that some of the maxima in the absorption spectra are due to products which do not fluoresce within the visible range, whereas some of the fluorescence bands must correspond to structures which do not show specific absorption maxima within the range examined, but which are nevertheless capable of excitation by non-specific absorption of sufficient energy.

A detailed study has been published recently by Linford (1957), in which he has attempted to explain all the colour reactions of steroids in strong acids on the basis of a common mechanism. He has sought to correlate the spectral-absorption bands of
such reaction mixtures with the presence of specific chemical groups in the steroid molecule, and concludes that colour and fluorescence are due to the formation of charged structures in the acid media. The effects of dilution, or of the addition of furfural, electrophilic salts and other reagents, would be to extend the charged structures.

The evidence which Linford has amassed from many sources is indeed impressive, and his hypothesis offers a plausible explanation of the light-modifying properties of a given reaction mixture at any instant. It fails, however, to offer any explanation of the changes which occur in each reaction mixture with the passage of time. Further, the proffered explanation of the effects of dilution is inadequate to account for the findings described above, e.g. the formation of multiple products on dilution of the corticosterone-reaction mixture, one of which exhibited the fluorescence properties of the original mixture. Finally, the alterations in absorption spectra which occur on dilution, as reported above, do not support Linford's interpretation. Thus he states that dilution shifts each absorption band in the near ultraviolet and visible regions to a secondary position of longer wavelength. Fig. 6 (d) seems to demonstrate exactly the opposite trend, all the comparable peaks appearing to move to positions of shorter wavelengths on progressive dilution.

It seems probable therefore that an explanation of all the observed optical properties and chemical behaviour of solutions of steroids in strong acids requires both Linford's concept and that advanced in the present discussion.

The mixed emission spectrum obtained with cortisone and hydrocortisone in sulphuric acid under 365 m\(\mu\) illumination (Fig. 11) may possibly be explained on the assumption that part of the blue fluorescence of the cortisone derivative acts as the appropriate primary illumination for the hydrocortisone derivative. However, the fact that components of both emission spectra were present, together with the demonstration by Zaffaroni (1953) that the absorption spectra are additive, indicates that the presence of the two steroids in the same reaction mixture does not interfere with the characteristic reaction pattern of each. Since different acid concentrations and different excitation wavelengths are required for optimum demonstration of the individual fluorescences, it is possible that selective suppression of one or other fluorescent species may be achieved, thereby avoiding the risk of mutual quenching or sensitization. If this proves true, it may be possible to select suitable conditions for fluorimetry of each corticosteroid in a mixture without the need of previous separation.

**SUMMARY**

1. Spectrophotometric and spectrofluorometric studies have been made of the reaction mixtures obtained by dissolving various steroids in concentrated sulphuric and concentrated phosphoric acids. The effects of time, heat and subsequent dilution of the reaction mixtures were also studied, with respect to the absorption and fluorescence properties. An attempt was made to isolate the reaction products of cortisone and of hydrocortisone in concentrated sulphuric acid, and to correlate their light-modifying properties with those of the parent steroids.

2. The mechanism of reaction of a given steroid is essentially the same in either acid, differing in rate rather than in nature.

3. Appropriate variations of the time and temperature of reaction, and dilution of the reaction mixture with water or with ethanol, tend to diminish or remove the differences observed in the behaviour of any steroid in these two acids.

4. The absorption and fluorescence spectra of each reaction mixture are complex spectra resulting from the contributions of two or more reaction products in each case. With hydrocortisone, the most characteristic fluorescence band is associated with a stable product which resists change on dilution and can be isolated chromatographically. The characteristic blue fluorescence of the cortisone reaction mixture is destroyed by dilution.

5. The observed changes are tentatively explained on the basis of a three-stage-reaction pattern. Reversible protonation of the \(\Delta^1\)-C-3 ketone of corticosteroids or testosterone, or of the phenolic C-3 hydroxyl of oestradiol, is the first step. The protonated steroid is assumed to form intermolecular association complexes at reactive substituent sites of other steroid molecules. These postulated complexes are thought to break down irreversibly to yield a series of products, via reactions which are markedly affected by dilution.

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Fluorimetric Measurement of Adrenocortical Steroids in Concentrated Acid Solution

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A number of steroids of various types, when dissolved in concentrated sulphuric, phosphoric or other acids, exhibit visible fluorescence under suitable ultraviolet or visible illumination. This phenomenon has been adapted quantitatively for fluorophotometric measurement of various oestrogenic steroids, and numerous modifications of technique have been devised (see review by Bates, 1954). Very much less attention seems to have been directed to the quantitative possibilities of the fluorescence shown by adrenocortical steroids in acid solution.

Interest in this phenomenon was revived by the finding of Neher & Wettstein (1951) that all the physiologically active corticosteroids, when isolated on paper chromatograms, fluoresce with distinctive colours under ultraviolet illumination, after the chromatogram has been heated with phosphoric or sulphuric acid or acetic anhydride. Finkelstein (1952) developed a quantitative fluorophotometric assay for certain corticosteroids dissolved in 85% phosphoric acid with the aid of heat. Sweat (1954) reported a more sensitive method, in which the steroids were dissolved in concentrated acid.