CV. STUDIES ON COLOUR TESTS FOR STEROLS AND VITAMIN A.

I. STEROL TESTS.

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Numerous colour tests for sterols have been put forward during the last fifty years, the reagents employed including concentrated sulphuric acid [Salkowski, 1872], concentrated sulphuric acid with acetic anhydride [Liebermann, 1885], acetyl chloride with zinc chloride [Tschugaieff, 1900], arsenic and antimony trichlorides [Kahlenberg, 1922], and antimony pentachloride [Steinle and Kahlenberg, 1926]. In some cases the production of colour is activated by addition of benzoyl peroxide [Lifschutz, 1898], nitric acid [Whitby, 1923] or formaldehyde [Whitby, 1923]. The initial colours obtained are either blue or red, or a blend of these.

The first colour test for vitamin A, put forward by Drummond and Watson [1922], was based on the old Salkowski [1872] reaction which had long been used to estimate the "activity" of cod-liver oil. Many years before vitamins had been discovered, this characteristic reaction with concentrated sulphuric acid had been shown to be due to a constituent residing in the unsaponifiable fraction of the oil, and destroyed by oxidation when the oil became rancid. Other "vitamin" reagents (*e.g.* arsenic trichloride [Rosenheim and Drummond, 1925], antimony trichloride [Carr and Price, 1926]) would also appear to be closely related to sterol tests, and give similar, though more transient, colours.

In view of these facts, and of the gradually accumulating evidence in favour of vitamin A being a sterol derivative, it was thought of interest to make a comparative study of some of these colour tests put forward for sterols and for vitamin A.

Experimental.

Sources of sterols employed.

The cholesterol derivatives were prepared by Mr W. A. B. Sexton, working at Liverpool University, from pure cholesterol obtained from cod-liver oil. Feeding tests showed this cholesterol to be free from vitamins A and D. The purity of these derivatives, and the absence of ergosterol, was spectroscopically controlled. The ergosterol was obtained from ergot, and the sitosterol from wheat germ oil. Analytical data obtained on these sterols and derivatives are given in Table I.

Reagents.

These were usually prepared and applied according to the directions of their respective authors. In certain cases modifications were introduced on account of factors such as time, temperature, nature of solvent, concentration, presence of catalysts, which have been found to influence the results obtained.

Table I. *Sterols and sterol derivatives examined.*

Sterol or derivative	Formula	M. Pt.	No. of double bonds	$[\alpha]_D^{20°}$
Cholesterol[1]	$C_{27}H_{45}OH$	148·5°	1	− 39·2°
Cholesteryl acetate	$C_{27}H_{45}COOCH_3$	114·5°	1	—
Cholesteryl chloride	$C_{27}H_{45}Cl$	96°	1	—
α-Cholesterylene	$C_{27}H_{44}$	78–79°	2	−102·1°
Cholestene	$C_{27}H_{46}$	92°	1	− 53·05°
ψ-Cholestene	$C_{27}H_{46}$	80°	1	+ 60·13°
Cholesterylmethylxanthogenic ester	$C_{27}H_{45}OCSSCH_3$	127°	—	—
Cholestenone	$C_{27}H_{44}O$	80°	2	—
Dicholesteryl ether	$(C_{27}H_{45})_2O$	194–195°	2	—
Hydroxycholesterylene	$C_{27}H_{42}O$	110°	3	—
β-Hydroxycholestenol acetate	$C_{27}H_{43}O_2COCH_3$	156°	—	—
Ergosterol	$C_{27}H_{42}O$	162°	3	−127°
Sitosterol	$C_{27}H_{46}O$	132°	—	− 34·4°

[1] Freed from ergosterol by boiling in alcoholic solution with norite for several hours, and fractional crystallisation.

Results.

These may be summarised as follows.

1. Pure cholesterol, freed from ergosterol, gives with the "vitamin" reagents (concentrated sulphuric acid, arsenic and antimony trichlorides) red colours persisting for many hours. Similar results are obtained with cholesteryl acetate and chloride, α-cholesterylene, cholestene and ψ-cholestene, but with the last two more time may be required for the colour to develop.

2. Cholesterol, cholesteryl acetate or chloride, cholestene and ψ-cholestene in chloroform solution, left in contact with concentrated sulphuric acid for some hours, and then diluted with more chloroform, give a purple or violet colour. Similar colours can be obtained by removal of the chloroform solution from the acid after less than a minute's contact, and addition to the former of a drop of formalin.

3. No colours are produced by formaldehyde alone with chloroform solutions of cholesterol or its derivatives which have not been in contact with concentrated sulphuric acid.

4. Irradiation of sterol derivatives generally has the effect of rendering the colours more transient. In the case of cholesterol, however, irradiation under certain conditions may develop the property of producing with the "vitamin" reagents blue colours, changing to red on standing for some hours.

5. Activation with other agents, such as acetic anhydride, benzoyl peroxide or formaldehyde, may lead to blue or purple colours being obtained on addition of the "vitamin" reagents.

6. Cholestenone, which has been suggested [Heilbron, Morton and Sexton, 1928] as being a typical product of irradiation, gives transient red colours with the "vitamin" reagents, and negative results (including red instead of blue with Liebermann's and Lifschutz's tests) with all other tests.

7. The other oxidation products of cholesterol which have been tested give negative results in all cases (dicholesteryl ether can be made to give red colours with modified Lifschutz's, Liebermann's, Tschugaieff's and "vitamin" tests).

8. Antimony pentachloride gives the colour sequence red → blue → red with all cholesterol derivatives examined except the oxidation products. Of these dicholesteryl ether, containing one proportion of oxygen, gives both red and blue, cholestenone and hydroxycholesterylene, containing two proportions of oxygen, give red only, and β-hydroxycholestenol acetate, containing four proportions of oxygen, gives negative results.

9. Introduction of sulphur into the side chain, as in cholesterylmethylxanthogenic ester, retards the development of the colours, but does not necessarily prevent it after liberation of sulphur compounds has taken place.

10. Ergosterol differs from cholesterol only in its results with the "vitamin" reagents. These give with ordinary concentrations of ergosterol the usual red, but if a higher initial concentration be employed (about 0·05 g. to 1 cc. of reagent) the red colour given by arsenic or antimony trichloride changes to purple or blue, on diluting with more reagent after a few moments.

11. Sitosterol gives similar results to cholesterol, but more slowly.

Details of the individual tests are given in Table II.

Table II. *Colour tests on sterols and derivatives.*

	"Vitamin" reagents									
Sterol or derivative	Salkowski, Drummond and Watson	Rosenheim and Drummond	Carr and Price	Liebermann	Lifschutz[1]	Rosenheim (methylal and $AsCl_3$)	Whitby *A*	Whitby *B*	Tschugaieff	Steinle and Kahlenberg
Cholesterol[2]	R	R	R	R→B	B→R	B→R	B	R[3]	R	R→B
Cholesteryl acetate	R	R.	R	R→B	B→R	B→R	B	R	R	R→B
Cholesteryl chloride	R	R	R	R→B	B→R	B→R	B	R	R	R→B
α-Cholesterylene	R	R	R	R→B	P→R	B	B	R	R	R→P
Cholesterol, irradiated[4]	B→R*	B→R*	B→R*	R→B*	B→R*	B*	P	R	R	R→B
α-Cholesterylene, irradiated[5]	R	R	R	R→B	B→R	B	P	R	R	R→P
Cholestene	→R	→R	→R	R→P	R	B	B	R	R	R→P
ψ-Cholestene	→R	→R	→R	R→P	R	B	B	R	R	R→P
Cholesterylmethylxanthogenic ester	→R	→R	→R	R→P	→R	→R	P	→R	R	R→P
Cholestenone	→R	→R	→R	R	R*	—	—	—	—	R
Dicholesteryl ether	→R	→R	→R	→R	R*	—	—	—	R*	R→B
Hydroxycholesterylene	—	—	—	—	—	—	—	—	—	R
β-Hydroxycholestenol acetate	—	—	—	—	—	—	—	—	—	—
Ergosterol	R	R[3] B→R	R[3] B→R	R→B	B	B	B	R	R	R→B
Sitosterol	R	R	R	R→B	B	B	P	R	R	R→B

[1] Heated in chloroform solution for a few hours at 37°, then cooled, and arsenic or antimony trichloride added.
[2] Freed from ergosterol as previously described.
[3] In high concentration the initial red colour changes rapidly to a blue which may persist for several hours.
[4] Irradiated half an hour at melting point.
[5] Irradiated until characteristic absorption bands disappeared.

"B" signifies a blue colour, "P" purple and "R" red.
→ Indicates development of colour on standing. * Denotes that the colour is more transient.

Discussion.

In the case of colour tests put forward for vitamin A, it was first suggested by Rosenheim and Drummond [1925], and Takahashi *et al.* [1925], that the initial blue colour obtained, rather than the red colour which develops on standing, is indicative of vitamin content. This initial blue colour has since been employed by a number of investigators to detect or estimate the vitamin in cod-liver oil and other sterol-containing natural products [Drummond, Channon and Coward, 1925; Carr and Price, 1926; Peacock, 1926; Rosenheim and Webster, 1926; 1927, 1, 2; Willimott, Moore and Wokes, 1926; Willimott and Wokes, 1927, 1, 2; Wokes and Willimott, 1927, 1, 2, 3]. Some interest, therefore, attaches to any case in which a sterol derivative produces with "vitamin" reagents blue colours similar to those attributed to the vitamin.

The results given above show that blue colours may be obtained from sterols under the following conditions:

(1) with antimony pentachloride;

(2) with acetic anhydride and concentrated sulphuric acid;

(3) with "vitamin" reagents on ergosterol in high initial concentration;

(4) with "vitamin" reagents on sterols treated with "oxidising" agents, formaldehyde or acetic anhydride.

It is true that these sterol colours are fairly stable, and may take a day or more to change to red or red-brown, whereas the "vitamin" colours are more transient, the blue colour in general having disappeared within a few minutes. But the above results show that irradiation of the sterols may cause the colours to be more transient, while it has been found possible, in the case of antimony trichloride on a physiologically tested cod-liver oil, to make the "vitamin" blue persist in measurable quantity for nearly an hour [Wokes and Willimott, 1927, 2].

Another characteristic property of the "vitamin" colours is their sequence, blue → red. In the case of antimony pentachloride, of acetic anhydride and concentrated sulphuric acid, and of arsenic or antimony trichloride on ergosterol in high concentration, the initial colour obtained is red. The blue → red sequence has, however, been obtained with cholesterol either irradiated at its melting point or oxidised under given conditions.

The case of ergosterol is exceptional in requiring a high initial concentration of sterol. Usually in sterol tests the most satisfactory concentration of sterol appears to be between 0·05 and 0·2 %. It has been shown by Wokes and Willimott [1927, 3] that the best results with antimony trichloride as a quantitative reagent for vitamin A in cod-liver oil are given by a concentration of oil of 1 to 5 %. Taking the unsaponifiable fraction of cod-liver oil as about 1 %, the optimum concentration of this fraction for "vitamin" tests would be from 0·01 to 0·05 %, or not very different from the optimum concentration for sterol tests.

Thus, of the four classes of blue colours given by sterols and their derivatives, it is those which are obtained by addition of "vitamin" reagents to cholesterol or certain of its derivatives or to ergosterol, after "oxidation" with benzoyl peroxide or nascent formaldehyde, which resemble most closely the "vitamin" colours. In regard to cholesterol itself (not purified from ergosterol) it was shown by Lifschutz many years ago [1908] that it gave with concentrated sulphuric acid, after treatment with benzoyl peroxide, a definite blue colour. Marston [1924] found that "oxycholesterol" prepared according to Lifschutz's method also gave a blue colour with arsenic trichloride, a reagent which had previously been shown by Kahlenberg [1922] to give a red colour with untreated cholesterol. Robertson [1925] came to the conclusion that when cholesterol is oxidised by Lifschutz's method the composition of the product varies, and found it more satisfactory to aerate in aqueous colloidal suspension at 100° in presence of an acetone extract of brain tissue. Rosenheim [1927] pointed out that cholesterol or ergosterol, after treatment with either benzoyl peroxide or nascent formaldehyde (obtained from methylal) will give blue colours with arsenic or antimony trichloride. Moore and Willimott [1927] showed that cholesterol, after heating in air to about 150° for half to two hours will give ultramarine colours, persisting some hours, with antimony trichloride. The same colours were obtained from cholesterol which had been heated in colloidal aqueous suspension at 90° for an hour or more, the presence of brain tissue extract not being found essential.

Turning to cholesterol derivatives, the new results here recorded may, perhaps, permit certain deductions to be drawn. Whitby [1923] suggested that in a typical sterol colour test the first stage consisted in the production of a colourless hydrocarbon, such as cholesterylene, by the action on cholesterol of the condensing agent (*e.g.* concentrated sulphuric acid), and that this product then coupled with a second substance (*e.g.* formaldehyde) to give the coloured product. But the writer has failed to obtain any colours with cholesterylene or cholestene when treated with formaldehyde only (either as formalin solution or produced in the nascent condition by warming a methylal solution of the sterol with dilute acid). This would seem to be evidence against Whitby's theory. The only noticeable result of the loss of the hydroxyl group was a distinct retardation in the rate of development of the colour in the case of cholestene and ψ-cholestene, but not in the case of cholesterylene, which contains an additional double bond. Variation in the position of the double bond, or replacement of the hydroxyl group by an acid radicle (acetate or chloride) made no appreciable difference to the colours obtained.

The most interesting results have been obtained with "vitamin" reagents on cholesterol which has either been irradiated or treated with oxidising or other agents, such as benzoyl peroxide, formaldehyde and acetic anhydride. From the recent work of Heilbron, Morton and Sexton [1928] the inference

might be drawn that when cholesterol is irradiated, dehydrogenation takes place, with formation of oxidation products similar to cholestenone. Tests applied by the writer to the latter compound have shown it to give with the "vitamin" reagents rather unstable red colours only, in contradistinction to initial blue colours obtained from irradiated cholesterol. A failure to obtain blue colours was also experienced after irradiation of cholesterylene and certain other cholesterol derivatives. These results do not necessarily imply that cholestenone is not a product of the irradiation of cholesterol, but there seems to be a strong possibility that, under suitable conditions, there is formed some other chromogen which is responsible for the initial blue colour. Further oxidation may lead to formation of chromogens giving red colours only, and finally to completely negative results. Oxidation of cholesterol can be made to give mixed products which when treated with "vitamin" reagents yield initial blue colours, changing gradually to red, which very closely resemble the "vitamin" colours given by the same reagents on cod-liver oil and other natural sources of vitamin A. But it has not yet been possible to isolate from these oxidation mixtures pure substances giving initial blue colours with the vitamin reagents, and only red chromogens have so far been obtained. With sterols as with the "vitamin" colours, the blue chromogen is unstable, and is destroyed by attempts to isolate it.

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