# VIII. A SPECIFIC COLOUR REACTION FOR ERGOSTEROL.

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The unique function of ergosterol as the parent substance of vitamin D made it desirable to find a colour reaction for it, by means of which it could be detected in the presence of other sterols. In searching for such a reaction, the property of formaldehyde of shifting the colour from the red into the blue part of the spectrum in the usual colour reactions of sterols [Whitby, 1923; Rosenheim, 1927] suggested an investigation of the behaviour towards sterols of the aldehyde corresponding to trichloroacetic acid. This acid is known to give rise to colour reactions with cholesterol [Tschugajeff, 1900].

Although anhydrous chloral proved to be non-reactive, it was found that both chloral hydrate and trichloroacetic acid give a characteristic blue colour reaction with ergosterol, whilst all the other naturally occurring sterols investigated, when purified from ergosterol, remain colourless under the same conditions. The specimens of ergosterol used in these experiments had been fractionally recrystallised at 36° and possessed the highest optical activity, $[\alpha]_D$ − 132°, so far recorded [Tanret, 1908; see also Bills and Honeywell, 1928]. The reactions to be described are therefore unlikely to be due to impurities or degradation products of ergosterol.

In contradistinction to naturally occurring sterols it was found, when studying the reaction of sterol derivatives, that the production of an immediate red colour with either of these reagents is specific for the $\Delta^{1,2}$ (or $\Delta^{1,13}$) linkage of the sterol ring system. These observations suggest an explanation of the mechanism of sterol colour reactions, which is applicable to all of them and will be discussed later.

## I. *Chloral hydrate reaction.*

When a few crystals, 1 mg. or less, of ergosterol are added to about 0·5 g. chloral hydrate, liquefied by warming in a water-bath, they dissolve and immediately give rise to a carmine red solution, showing a broad absorption band at 500 $\mu\mu$. The red colour changes within a minute into a green and finally into a deep blue, which persists for a considerable time. The esters of ergosterol react in the same way. The colour is discharged rapidly

by water or alcohol, more slowly by chloroform, benzene, toluene or other anhydrous solvents not possessing a hydroxyl group.

Although the blue colour is discharged by dilution with water, a saturated aqueous solution of chloral hydrate (80 %) reacts in the typical manner when a drop of concentrated HCl is added. It would therefore appear that traces of an acid are essential for the reaction.

Freshly distilled anhydrous chloral dissolves ergosterol. The colourless solution undergoes the above-described changes on the addition of one drop of water. On keeping under laboratory conditions, chloral, or its chloroform solution, attracts moisture and gradually becomes "activated," so as to give the reaction with ergosterol without the addition of water.

Colourless solutions are given under these conditions by all the other naturally occurring sterols, their esters and their reduction products. The following carefully purified sterols were examined: cholesterol, sitosterol, $\gamma$-sitosterol (1 double bond); stigmasterol (2 double bonds); zymosterol, fungisterol (3 double bonds); *iso*cholesterol (saturated?), amyrol, coprosterol and dihydrositosterol (saturated). In the preparation of zymosterol from yeast and fungisterol from ergot [Rosenheim and Webster, 1928], it was found that the crude specimens gave the reaction strongly. The intensity of the reaction decreased, however, proportionately to their progressive purification and the removal of ergosterol. The agreement of the spectroscopic and biological examination with the colorimetric results justifies the conclusion that the slight positive reaction of the purest specimens of zymosterol and fungisterol obtained so far is due to admixture of ergosterol.

### II. *Trichloroacetic acid reaction.*

An aqueous solution, prepared by dissolving nine parts of the pure crystallised acid in one part water, was found to be the most suitable reagent. When this is added to ergosterol dissolved in a few drops of chloroform, an immediate red solution (band at 500 $\mu\mu$) is produced, which changes gradually into a clear blue (bands at 570–580 and at 650–680 $\mu\mu$), without showing the intermediate green phase of the chloral hydrate reaction. In distinction from the latter, the reaction takes place at ordinary temperature and has the further advantage of yielding a final blue solution, which may be diluted for colorimetric purposes with the reagent itself or with chloroform. Excessive dilution with chloroform or other solvents tends to change the colour into a bluish-green of the same shade as that given by ergosterol with a saturated chloroform solution of trichloroacetic acid. The latter solution undergoes decomposition on keeping, liberating phosgene and hydrochloric acid, and is not so suitable a reagent as the aqueous solution.

*The sensitiveness of the reaction* was determined by adding three drops of the reagent to 0·1 cc. of a chloroform solution containing known amounts of ergosterol. It was found that 0·01 mg. ergosterol still gave a marked reaction

within 5 minutes, and that the colour is just recognisable with 0·005 mg. when compared with the colourless control. The sensitiveness is therefore of approximately the same order as that of the usual sterol reactions.

Employing mixtures of cholesterol and ergosterol, prepared by mixing their solutions in chloroform, a strong reaction is obtained in the presence of 0·5 % ergosterol in 0·1 g. cholesterol, and even smaller quantities down to 0·1 % can be detected by comparison with the colourless cholesterol control. The amount of ergosterol in ordinary cholesterol, as estimated by the spectroscopic test, may vary from 0–0·1 % according to the method of purification used and depending on the source of the sterol. Cholesterol prepared from brain or cod-liver oil, having undergone charcoal treatment, may occasionally be free from ergosterol [Rosenheim and Webster, 1927], whilst as much as 0·12 % may be present in preparations from spinal cord [Bills, Honeywell and MacNair, 1928]. Examination of a large number of "pure" specimens, M.P. 147–148°, prepared from brain, gallstones, liver, spleen, skin, blood, ovaries (pig), eggs (frog) and cod-liver oil gave negative tests in some cases (brain, gallstones) and positive reactions in most. The intensity of the reaction in descending order was approximately: eggs (frog), ovaries, liver, skin (pig), brain, spleen, blood. Specimens of sitosterol from wheat and maize also gave positive reactions before purification by the bromine method. Whilst the colour reaction for ergosterol in sterols probably does not equal that of the biological or spectroscopic test in sensitiveness, it affords chemical evidence for the assumption that the impurity in ordinary cholesterol, which gives rise to vitamin D on irradiation, is identical with ergosterol.

The other naturally occurring sterols examined (see above) do not react with the freshly prepared reagent and remain colourless for more than 30 hours when kept at room temperature in the dark. On warming, however, cholesterol [Tschugajeff, 1900; Hirschsohn, 1902] and the other unsaturated sterols rapidly give a red solution, showing an absorption band at 500 $\mu\mu$.

*A specific reaction for the* $\Delta^{1,2}$ *(or* $\Delta^{1,13}$*) linkage in sterols.*

A study of the behaviour of the two reagents towards cholesterol derivatives, in which known chemical changes had been produced, showed that esterification of the hydroxyl group, its replacement by chlorine, or its complete removal, has no effect, for cholesterol acetate or chloride and cholestene gave colourless solutions. Reduction or bromination, etc., of the double bond are equally without influence, since dihydrocholesterol, cholestane, cholesterol dibromide and cholesterol oxide are also non-reactive. The doubly unsaturated ketone, "oxycholesterilene" and dicholesteryl ether, as well as digitaligenin, also gave negative results.

On applying the chloral hydrate and the trichloroacetic acid reaction to *allo*cholesterol and *allo*sitosterol, however, an immediate yellow → orange colour was produced, which rapidly deepened into a permanent carmine red. In both cases the red solutions showed an absorption band at 500 $\mu\mu$. In

the $AsCl_3$ and $SbCl_3$ reactions, the behaviour of the *allo*-compounds is identical with that which they show with the above reagents.

The conversion of cholesterol into the isomeride *allo*cholesterol was effected by means of hydrochloric acid by Windaus [1927], who also showed conclusively that a shifting of the double bond from $C_{6,7}$ to $C_{1,2}$ (or $C_{1,13}$) takes place under these conditions (see formulae I and II, p. 52).

The above results indicate that an immediate red colour reaction is directly dependent on the presence of the $\Delta^{1,2}$ linkage[1], and it seemed therefore of interest to test the behaviour of other sterol derivatives, in which the existence of the same linkage had been postulated. Heilbron and Sexton [1928] arrived at the conclusion that "one of the ethenoid linkages in cholesterilene must occupy the same position as in $\psi$-cholestene," which latter substance possesses only one double linkage, presumably in the same position as in *allo*cholesterol. Specimens of cholesterilene, $\psi$-cholestene and $\psi$-cholestane, prepared by these authors, were kindly put at my disposal by Prof. Heilbron, and it was found that both cholesterilene and $\psi$-cholestene gave an intense red reaction, the latter somewhat more slowly than the former. On the other hand, $\psi$-cholestane remained colourless, as was to be expected in the case of this saturated hydrocarbon.

The positive results confirm Heilbron and Sexton's conclusions, and may be taken as supplementary evidence for the specificity of the reaction. They lead further to the suggestion that the primary red phase of the ergosterol reaction (see above) is due to the presence of the $\Delta^{1,2}$ linkage. This conclusion is in agreement with that reached by Heilbron, Morton and Sexton [1928] from their studies of the ultra-violet absorption spectrum of cholesterilene, which led them to infer "that of the three double bonds in ergosterol, two occupy the same position as in cholesterilene." The final blue stage of the ergosterol reaction may therefore justifiably be ascribed to the influence of the third double linkage, the position of which is at present unknown.

It is of interest to note further that an immediate red reaction is also given by $\beta$-cholesterol, an isomeride of cholesterol obtained by Diels and Abderhalden [1908], by heating cholesterol to 310°. The position of the double bond in this isomeride remains unknown. It seems permissible to infer from the positive result of the above colour reaction that $\beta$-cholesterol also contains the $\Delta^{1,2}$ linkage, its isomerism with *allo*cholesterol being due to the attachment of the hydrogen atom to the carbon atom $C_2$ (or $C_{13}$) in either the *cis*- or *trans*-position.

*The bathychromic effect of formaldehyde.* In two colour reactions of cholesterol, namely with concentrated $H_2SO_4$ and with $AsCl_3$, formaldehyde has a bathychromic effect, *i.e.* it deepens the colour from red to violet [Whitby, 1923; Rosenheim, 1927]. The same effect is observed in the trichloroacetic acid reaction with *allo*cholesterol and *allo*sitosterol: if a few drops of formalin

[1] For convenience of discussion, the symbol $\Delta^{1,2}$ only is used in the following, implying also the possibility of the $\Delta^{1,13}$ linkage.

are added when the red phase has been reached, the colour changes gradually into a purple and violet. The reaction is conveniently carried out with a saturated solution of the acid in formalin as a reagent, which produces rapidly a violet reaction with the *allo*-compounds, showing a band at 590–610 $\mu\mu$. Cholesterol behaves in the same way with trichloroacetic acid and formalin, but heating in this case is necessary [Golodetz, 1908]. In the reaction with ergosterol, on the other hand, formaldehyde exerts a hypsochromic effect, and prevents the formation of the blue phase. When added to the reaction mixture at the blue stage, it gradually weakens and discharges the colour.

It has been shown previously [Rosenheim, 1927] that, on heating cholesterol in chloroform solution with benzoyl peroxide, the white product obtained gives a blue colour with the reagents which usually give rise to a red colour. When similarly treated, *allo*cholesterol also gives a blue colour with trichloroacetic acid. The gentian-blue colour of this reaction is, however, easily distinguished by the naked eye from the colour of the ergosterol reaction (starch-iodine-blue), and spectroscopic examination shows in the former solution the characteristic band of "oxycholesterol" only. The blue reaction of "oxycholesterol" may simulate the presence of ergosterol in specimens of cholesterol which have been exposed to light or ultra-violet irradiation in presence of air, but is easily differentiated by its absorption band from the ergosterol reaction[1].

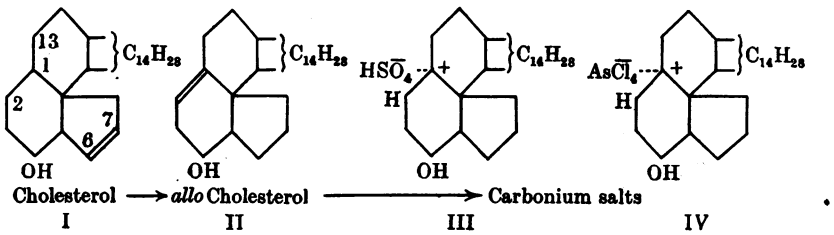
*The mechanism of sterol colour reactions.*

Various attempts have been made to find an explanation for the numerous sterol colour reactions, which are obviously due to one common factor. Mauthner [1909] considered the presence of double linkages as essential, since the reactions are negative with completely reduced derivatives of cholesterol. In explanation of Liebermann's reaction (acetic anhydride and concentrated $H_2SO_4$) Wieland and Weil [1913] suggest that the coloured substances are halochromic sulphates of ketones, the latter being produced by the action of acetic anhydride on a reactive double linkage. On the other hand, Whitby [1923] assumes that the sterol colours are produced by the condensation of an aldehydic coupling substance (*e.g.* formaldehyde) with a hydrocarbon (*e.g.* cholesterilene), both of which are presumably formed from the sterol by the condensing agent used (*e.g.* sulphuric acid). There appears to be, however, no evidence for the formation of the postulated aldehydic coupling substance under the conditions of the test, and on other grounds also Whitby's explanation has been criticised [Wokes, 1928].

The reactions described above, which show that an immediate red colour reaction is given only by those sterols or their derivatives which contain the $\Delta^{1,2}$ linkage, suggest a general explanation for the chromogenic properties of sterols. According to this, the primary reaction consists in the isomerisation

[1] The oxidising action of charcoal also gives rise to "oxycholesterol" formation. [Blix and Löwenhielm, 1928.]

of the sterol, under the influence of a strong acid, giving rise to an isomeride containing the $\Delta^{1,2}$ linkage. In these compounds (II) the carbon atom $C_1$ has become doubly linked. Owing to its position at the junction of two rings, $C_1$ is assumed to have acquired a property similar to that of the tervalent carbon atom in triphenylmethyl, and to be able, like the latter [Baeyer, 1905, *int. al.*] to form carbonium salts, which are coloured (III and IV).




This assumption is supported by the fact that the sterol colours are discharged by water or alcohol, and that in their formation strong acids are used (*e.g.* concentrated $H_2SO_4$ in Salkowski's, Liebermann's, Whitby's, etc. reactions), or generated from the reagent (HCl from $AsCl_3$ in Kahlenberg's reaction). In the latter case, as well as in the trichloroacetic acid reaction, it is significant in this connection that a rapid colour change with sterols other than *allo*cholesterol, etc., requires heat, which also favours isomerisation: a solution of cholesterol in $AsCl_3$ or trichloroacetic acid remains colourless when kept at 0°. The identity of the absorption band in all these reactions with that of *allo*cholesterol is a further indication of the similarity in constitution of the coloured substances formed.

## Summary.

1. Ergosterol gives a blue colour reaction with chloral hydrate and with trichloroacetic acid, by means of which it may be detected in the presence of other naturally occurring sterols.

2. An immediate red colour reaction with the above reagents is specific for those sterol derivatives which possess the $\Delta^{1,2}$ (or $\Delta^{1,13}$) linkage.

3. It is suggested that the primary reaction in all sterol colour reactions consists in the shifting of the double linkage into the $C_{1,2}$ (or $C_{1,13}$) position and the subsequent formation of coloured carbonium salts.

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