XI. COLOUR REACTIONS OF STEROLS WITH NITRIC ACID.

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IN the course of experiments on the dehydrogenation of zymosterol by means of mercuric acetate, it was noticed that nitric acid, used for cleaning the reaction vessel, gave an intense blue colour reaction with fragments of the reaction mixture left in the flask. This chance observation led to a systematic study of the reaction of nitric acid with sterols. The characteristic colours produced with certain sterols were found to be somewhat transient when pure colourless nitric acid was employed. The oxidising action of the acid was retarded and the colour reactions were stabilised to a certain extent by using a mixture of glacial acetic acid with nitric acid (1:4). Finally it was found that nitric acid containing the acetates of metals (magnesium, cerium, cadmium, mercury, uranium, etc.) gave fairly stable colour reactions. They differed to some extent with different metals, and in order to simplify description the following notes are restricted to the reactions of the "mercury reagent." The reagent is prepared by dissolving 25 g. of mercuric acetate in 100 cc. of nitric acid (sp. gr. 1.42). Nitrous acid interferes with the colour reactions, and it is necessary to decolorise the solution with a few crystals of urea. When this precaution is used the reagent apparently keeps indefinitely.

The reaction is carried out by adding to a chloroform solution of the sterol an equal volume of the reagent, and shaking immediately. On account of the high specific gravity of the mercury reagent, the mixture separates rapidly into an upper coloured chloroform layer and a lower, usually colourless, layer of the reagent.

1. *Red colours* are given by *allo*-cholesterol, *allo*-sitosterol, cholesterolene, ψ -cholestene and β -cholesterol. The tint of the red colours varies somewhat with different sterols and is orange, carmine red or magenta according to concentration, but all the solutions show selective absorption in the region of $500\mu\mu$. A red colour is given only by those sterols which are believed to possess a $\Delta^{1,2}$ (or $\Delta^{1,13}$) ethenoid linkage in the molecule and which have been found previously [Rosenheim, 1929] to give a red colour reaction with trichloroacetic acid. It may be assumed therefore that the mechanism of the reaction consists, as in the latter case, in the formation of coloured carbonium salts and that a red colour reaction with the mercury reagent is specific for

and dependent on the presence of a $\Delta^{1,2}$ (or $\Delta^{1,13}$) linkage in the sterol ring system.

Considerations of the relationship of colour reactions and absorption spectra of sterols to their structure have led Heilbron and Spring [1930] to the conclusion that "selective absorption of sterols is conditioned by the presence of two ethenoid linkages in the molecule, one of which must apparently be in the $\Delta^{1,13}$ (or $\Delta^{1,2}$) position." The fact that oxycholesterilene possesses two ethenoid linkages and a highly selective absorption at $280\mu\mu$ [Rosenheim and Adam, 1929], but gives a negative reaction with both the mercury reagent and trichloroacetic acid (see later) weakens the evidence adduced by Heilbron and Spring for such a generalisation.

2. *A blue colour*, following a transient pink, is given by ergosterol and its esters when the reaction is carried out with dilute solutions (5 mg. or less in 1 cc. chloroform), a marked reaction still showing with 0.01 mg. The tint is a characteristic peacock-blue, and the solution does not show any selective absorption. On prolonged standing the blue colour changes through magenta into a dull shade of red. The lower acid layer rapidly loses its initial rose-red colour and becomes colourless.

An entirely different reaction results when relatively concentrated solutions of ergosterol (10 mg. or more in 1 cc. chloroform) are used. The same initial pink colour appears on mixing with the reagent, but it rapidly changes into orange and a clear yellow, instead of into blue. The acid layer remains colourless throughout.

The latter reaction has been found useful as an index of the purity of ergosterol, since oxidised or otherwise changed specimens give finally an intense green reaction under the above conditions [Callow, 1931]. The sensitiveness with which the reaction indicates such changes is demonstrated by allowing a 1% chloroform solution of ergosterol to stand in a stoppered cylinder exposed to diffused daylight. Within a few hours the still colourless solution gives with the mercury reagent a purple and finally an intense green colour, showing a green fluorescence. The resemblance of this reaction to that of *isoergosterol* (see later), and the observation that this change does not occur in solutions protected from light, suggests that it is due to the production of *isoergosterol*. Under the influence of light, chloroform decomposes into phosgene and hydrochloric acid, and the latter is known to isomerise ergosterol into *isoergosterol* at ordinary temperatures. The liberation of hydrochloric acid from chloroform has been shown to be the cause also of the change from red to green when a chloroform solution of bilirubin is exposed to sunlight [Thudichum, 1883].

Ergosterol peroxide [Windaus and Brunken, 1928] and dehydroergosterol peroxide [Windaus and Linsert, 1928] yield greenish-blue reactions similar to that of dilute ergosterol solutions.

The reaction of *isoergosterol* [Reindel, Walter and Rauch, 1927] differs essentially from that of ergosterol. The colour changes end in olive green after

passing rapidly through red to purple and blue. In the blue stage a well defined absorption band at $550\mu\mu$ can be observed, and the final green stage shows a green fluorescence. A further distinction from ergosterol is seen in that the colour changes are identical in concentrated or dilute solutions. The influence of the complex Hg ion is evident in this reaction: when cadmium acetate replaces mercuric acetate, the *isoergosterol* reaction stops at the red stage. The various *isoergosterols* of Bills and Cox [1930] have not been investigated.

3. *Colourless solutions* are obtained in the reactions with (1) cholesterol and its derivatives (cholesteryl chloride, α -cholesterol oxide, α -cholestanetriol, cholestenone, oxycholestenone, oxycholesterilene, dihydrocholesterol), (2) coprosterol and coprostanone, (3) the phytosterols: stigmasterol, sitosterol, γ -sitosterol, dihydrositosterol, (4) zymosterol (impure preparations give an intense blue-green reaction, due to ergosterol), lanosterol (from lanolin) and β -amyrol, (5) dihydroergosterol and tetrahydroergosterol.

From these facts it is apparent that only those sterols can give colour reactions with nitric acid or with the mercury reagent which possess either the $\Delta^{1,2}$ (or $\Delta^{1,13}$) linkage or the unknown ethenoid linkages of the ergosterol molecule. The non-reactivity of the two partially reduced ergosterols is not surprising, since a shifting of the double linkages may have taken place during reduction. Both still contain an "inert" ethenoid linkage, which Heilbron and Spring [1930] connect with a positive bromine reaction (Tortelli-Jaffé). It may be suggested however that the latter reaction is a secondary one and connected with the destructive action of bromine on the ergosterol molecule. In favour of this view is the fact, found by us, that iodine replacing bromine in the Tortelli-Jaffé reaction, gives a green colour with ergosterol, but does not react with either dihydroergosterol or tetrahydroergosterol.

Other reactions. (1) *Vitamin A and carotene.* Most cod-liver oils give with the mercury reagent a series of indefinite colour reactions ending in red. Other liver oils, which are many times as rich in vitamin A as cod-liver oil [Rosenheim and Webster, 1927], such as certain mammalian liver oils, salmon-liver oil, etc., give a brilliant transient blue colour with the reagent. The unsaponifiable fraction of these oils, and also of cod-liver oil, yields an intense and rather more stable blue colour, which is apparently identical with the colour produced with the unsaponifiable fraction by AsCl_3 , SbCl_3 and trichloroacetic acid [Rosenheim and Webster, 1926]. The relationship of these colour reactions to vitamin A remains still to be defined.

In view of the resemblance of the colour reactions of cod-liver oil to those of carotene, which originally suggested a relationship of the latter to vitamin A [Rosenheim and Drummond, 1925] it was not surprising to find that carotene also gives a blue colour reaction with the mercury reagent. In distinction from the trichloroacetic acid reaction of carotene, the reaction is a transient one.

(2) *Vitamin D.* Irradiated ergosterol, freed from unchanged ergosterol

by digitonin, gives a transient red colour, followed by a permanent olive green with both the mercury reagent and trichloroacetic acid. Since the same reaction is given by biologically inactive, over-irradiated or oxidised specimens, it is probably due to one or more of the inactive substances produced from ergosterol by irradiation.

(3) *Bile acids and their derivatives.* Desoxycholic acid and cholic acid possess the same ring system as the saturated sterols and were accordingly found to be non-reactive. On distillation they form, by loss of water, the unsaturated choladienic and cholatrienic acids [Wieland and Weil, 1912], specimens of which we owe to the kindness of Professor Wieland. Whilst choladienic acid is non-reactive, cholatrienic acid in dilute chloroform solution gives with the mercury reagent a gentian-blue colour reaction, the lower acid layer turning deep red. An intense blue colour slowly develops with trichloroacetic acid on standing for a few hours, the blue solutions in both reactions showing a band in the red region of the spectrum with a maximum at $650\mu\mu$. It seems therefore that the ethenoid linkages connected with C_3 and C_7 , which are common to both acids, are unable to react, and that the colour reaction of cholatrienic acid depends on the presence of the $\Delta^{11,12}$ or $\Delta^{12,13}$ linkage.

(4) "*Oxycholesterol*," the mixture of substances obtained by debromination of cholesterol dibromide with sodium acetate [Lifschütz, 1919] or by treatment of cholesterol with benzoyl peroxide, gives with the mercury reagent an immediate gentian-blue colour, showing the characteristic "oxycholesterol" absorption band at $650\text{--}680\mu\mu$. Traces of this product, sufficient to be detected by the reagent, are formed from cholesterol under all conditions where oxidation may take place. A positive reaction is given by cholesterol after recrystallisation in the presence of charcoal, after irradiation in air or after heating to the melting point in air, etc. Since "oxycholesterol" gives an identical reaction also with trichloroacetic acid, AsCl_3 or SbCl_3 [Rosenheim, 1927, 1929], and thus behaves like an unsaturated sterol, it may be suggested that the substance contains an additional ethenoid linkage, and is formed by dehydrogenation of cholesterol. Such a suggestion is strengthened by the ease with which oxidising agents like perbenzoic acid dehydrogenate sterols [Windaus and Lüttringhaus, 1930].

SUMMARY.

1. Nitric acid gives characteristic colour reactions with certain sterols. A solution of mercuric acetate in nitric acid ("mercury reagent") is recommended as a general reagent.

2. A red colour with the mercury reagent indicates the presence of the $\Delta^{1,2}$ (or $\Delta^{1,13}$) linkage in sterols.

3. A greenish-blue colour is given by ergosterol in dilute solutions, whilst a yellow colour results when concentrated solutions of ergosterol are employed. The latter reaction serves as a useful index for the purity of ergosterol.

4. A gentian-blue colour is given by products containing vitamin A (certain liver oils and the "unsaponifiable" of cod-liver oil).

REFERENCES.

- Bills and Cox (1930). *J. Biol. Chem.* **84**, 455.
Callow (1931). *Biochem. J.* **25**, 87.
Heilbron and Spring (1930). *Biochem. J.* **24**, 133.
Lifschütz (1919). *Z. physiol. Chem.* **106**, 279.
Reindel, Walter and Rauch (1927). *Liebig's Ann.* **452**, 34.
Rosenheim (1927). *Biochem. J.* **21**, 386.
—— (1929). *Biochem. J.* **23**, 49.
—— and Adam (1929). *Proc. Roy. Soc. Lond. B* **105**, 422.
—— and Drummond (1925). *Biochem. J.* **19**, 753.
—— and Webster (1926). *Biochem. J.* **20**, 1342.
—— — (1927). *Nature* **120**, 440.
Thudichum (1883). *Moleschott's Beitr.* **13**, 327.
Wieland and Weil (1912). *Z. physiol. Chem.* **80**, 287.
Windaus and Brunken (1928). *Liebig's Ann.* **460**, 225.
—— and Linsert (1928). *Liebig's Ann.* **465**, 148.
—— and Lüttringhaus (1930). *Liebig's Ann.* **481**, 119.