I. SOME NEW REACTIONS FOR THE DETECTION OF STEROLS.

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Observations made in relation to the colour reactions in vogue for the detection of sterols have indicated that in their general character all or most of these reactions are in essence similar, and have led to the discovery of several new reactions which present certain interesting features including a greater degree of delicacy than that possessed by reactions now employed. In what follows, first three new reactions for sterols and a reaction for sterolins are described, and then some considerations are brought forward concerning the mechanism of the colour reactions shown by sterols.

Reaction A. To 2 cc. of a chloroform solution of the sterol (containing preferably 1–2 mg. of the sterol) are added 2 cc. of a reagent prepared by mixing conc. sulphuric acid and formalin in the proportions 50 vols. : 1 vol., and the contents of the test-tube are then shaken. When the layers have separated, the upper, chloroform layer is seen to be cherry-red in colour, and the lower, sulphuric acid layer to be brownish-red in colour and to show an intense green fluorescence. The upper layer is then poured off into a dry test-tube and treated with two or three drops of acetic anhydride; as a result it assumes a bright blue colour, which lasts for a considerable time, but passes into a green within, say, an hour.

The reaction is much more sensitive and striking than the Salkowski [1872] reaction, of which it may be regarded as an elaboration. In the Salkowski reaction, a chloroform solution of a sterol is shaken with an equal volume of conc. sulphuric acid; when the liquids have separated, the chloroform layer is seen to be brownish-yellow, and the sulphuric acid layer to be yellow-brown with a green fluorescence; on allowing the test-tube to stand for several hours, the sulphuric acid layer becomes deeper and redder in colour and more strongly fluorescent, while the chloroform layer assumes, if the sterol was present in sufficient amount, a cherry-red or purple colour. Reaction A described above has several advantages over the just-described Salkowski reaction, namely: (*a*) the cherry-red colour is obtained immediately in the former, whereas in the latter it is necessary to wait several hours for its appearance; (*b*) the former reaction is given by a much smaller quantity of the sterol than the latter (*vide infra*); (*c*) the coloured chloroform solution obtained in the

former is converted by acetic anhydride into a comparatively lasting blue, whereas when the cherry-red solution obtained in the latter is treated with the same reagent a blue stage, if recognisable, is very transient, and does not add a well-defined feature to the reaction.

A comparison of the degree of delicacy of Reaction A and the Salkowski reaction showed that the former is more than ten times as sensitive as the latter.

2 cc. of cholesterol solution were used in each of the tests made in this connection. When 0·01 mg., *i.e.* 1 part in 100,000 parts, of cholesterol was present, in Reaction A a faint fluorescence could be seen in the sulphuric acid layer on allowing the contents of the test-tube to stand or on warming them, athough no colour could be seen in the chloroform layer. This amount of sterol represents approximately the limit at which Reaction A gives any result. The Salkowski reaction gave no result with this amount. When 0·1 mg. sterol was present, Reaction A gave a comparatively deep reddish-brown colour and a very marked fluorescence in the sulphuric acid layer, and, if the contents of the test-tube were heated, a barely discernible purple colour in the chloroform. When the same amount of sterol was present, the Salkowski reaction gave no colour in the chloroform and no colour or fluorescence in the sulphuric, although a faint colour and fluorescence developed in the latter if the contents of the tube were heated and then allowed to stand for about ten minutes. Even when 1 mg. of sterol was present, a cherry-red colour in the chloroform was not usually noticeable in the Salkowski reaction; any faint colour which might possibly have developed during the night having disappeared by the morning. With 1 mg. of sterol, Reaction A gave an immediate cherry-red colour in the chloroform.

Reaction A was applied with positive and essentially identical results to three samples of cholesterol and five samples of phytosterol[1]. In this reaction, amyrin gives in the chloroform no colour but in the sulphuric acid an orange-red colour (which soon becomes blood-red) and a deep green fluorescence; abietic acid gives nothing very characteristic: the chloroform becomes merely pale brown and the sulphuric acid dark red-brown and slightly fluorescent.

A modification of the Salkowski reaction, made by Hesse [1881], and consisting in using sulphuric acid of specific gravity 1·76 instead of 1·84, has the advantage over the Salkowski reaction of giving a characteristic colour (rose) in the chloroform immediately, but it has the disadvantage of not giving fluorescence in the acid and of being less sensitive than the ordinary Salkowski reaction. It must be considered as less generally useful than either the Salkowski reaction or Reaction A.

[1] These samples were as follows: (*a*) a sterol from the resin of *Hevea* rubber [Whitby and Dolid, 1921]; (*b*) phytosterols from *Adonis vernalis*; (*c*) sitosterol from *Echinacea* root; (*d*) and (*e*) preparations of sitosterol from wheat. The author is much indebted to Dr F. W. Heyl, the Upjohn Co., Kalamazoo, Mich. for samples (*b*) and (*c*), and to Prof. L. Kahlenberg, University of Wisconsin, for samples (*d*) and (*e*).

Reaction B. To 2 cc. of a solution of sterol in glacial acetic acid, containing conveniently 0·2–0·5 mg., are added with shaking 25 drops of a reagent prepared by mixing conc. sulphuric acid and formalin in the proportion 50 vols. : 1 vol. The result is a rose-coloured and fluorescent solution.

The rose colour, which has a tinge of purple, is not permanent, but on standing changes to yellow-brown. When only 0·01 mg. of sterol is present, the rose colour changes quickly, but more usually (*i.e.* with an amount of sterol of the order first mentioned) no change in the full, initial brightness of the colour is apparent for about two minutes, and the change to brown is not complete until an hour or more has elapsed.

This reaction is more sensitive than any colour reaction hitherto proposed for the detection of sterols. It is approximately twice as sensitive as the Liebermann-Burchard reaction [Liebermann, 1885; Burchard, 1889], although, unlike the latter, it is not suited to the quantitative determination of sterols. In Reaction B colour is just recognisable with 0·005 mg. of sterol per cc., *i.e.* the limit of sensitiveness of the reaction is 1 in 200,000. With 1 mg. per cc. the colour developed is too deep to allow one to see through the solution in a test-tube. A suitable concentration at which to apply the reaction is 0·1–0·25 mg. sterol per cc.

The reaction may be applied to an ethereal instead of an acetic acid solution of a sterol; but in this connection it should be noted that on standing for several hours a blank test will show a brown colour.

In Reaction B amyrin gives a cherry-red, strongly fluorescent solution, which becomes brown on long standing; abietic acid, a not very characteristic green-brown colour[1].

Reaction C. A few milligrams of a sterol are added to one drop of acetic anhydride on a piece of porcelain and gently heated until it has melted and excess of anhydride has been driven off. The melt is allowed to cool completely, and is then moistened with conc. nitric acid. Within a few seconds the material assumes a blue or blue-green colour.

This reaction is chiefly of value for cholesterol; it is of less value for phytosterols. Five samples of phytosterol[2] to which the reaction was applied behaved somewhat differently from cholesterol: even when the reaction was carried out with great care (the melt being distributed in as thin a layer as possible), they gave a much less intense colour than did cholesterol. The difference in behaviour of cholesterol and phytosterols in this reaction is not, however, sufficiently sharp to allow of the reactions being used as a means of distinguishing animal and vegetable sterols.

[1] The behaviour of amyrin and abietic acid in certain other sterol colour reactions may be put on record here. In Tschugajeff's reaction (see later) amyrin gives a red, which is somewhat browner than that given by a sterol and is unaccompanied by fluorescence; abietic acid gives a still browner red which is, however, accompanied by fluorescence. In Lifschütz's reaction (see later) neither substance gives a characteristic colour but merely a pale brown colour.

[2] Described in footnote, p. 6.

The author could not confirm the statement of Kahlenberg [1922] that arsenic chloride provides a means of distinguishing cholesterol from phytosterols by yielding a cherry-red solution with the former and a colourless solution with the latter. Each of the five samples of phytosterol referred to above gave a cherry-red solution in arsenic chloride.

A fusion test for the detection of cholesterol has been described by Obermüller [1891], but does not appear to be very useful, as it would seem to demand exceptionally dry samples of cholesterol for success. The present author obtained negative results on applying Obermüller's reaction to samples of cholesterol taken directly from stock bottles.

Sterolin reaction. Sterolins[1] give most of the colour reactions of sterols (*vide infra*). The following reaction, which depends in part on the sterol portion and in part on the glucose portion of the molecule, has been designed in conjunction with Mr J. Dolid, for recognising sterolins and at the same time distinguishing between sterolins and sterols.

1–2 cc. of conc. sulphuric acid is poured on to a few particles of a sterolin contained in a test-tube, and the mixture is warmed gently until the solid has gone into solution; the liquid is then cooled, and a cold, saturated, aqueous solution of thymol is poured on top of it. The result is an orange colour and a strong green fluorescence in the lower layer and a violet ring at the junction of the upper and lower layers. On allowing the test-tube to stand, the violet colour tends to spread through the upper layer.

When the reaction is applied to a sterol, a lower layer similar to that described above is obtained, but a violet ring is missing.

The sample of sterolin to which the above reaction was applied was isolated from the resin of *Hevea* rubber [Whitby and Dolid, 1921]. Its behaviour in the various sterol colour reactions was as follows. It gave Reaction B, the Liebermann-Burchard, the Tschugajeff, and the Lifschütz reactions. In Reaction A its behaviour was slightly different from that of a sterol, due doubtless to the circumstance that it is only very slightly soluble in chloroform: fluorescence occurred in the sulphuric acid layer, as in the case of a sterol; the colour in the chloroform layer was not, however, as in the case of a sterol, cherry-red, but a brownish yellow, which soon deepened to a red-brown; and, when the chloroform layer was poured off and treated with acetic anhydride, it became blue, but the blue remained for a shorter time than in the case of a sterol.

A consideration of the colour reactions applicable to the detection of sterols in solution indicates the existence of a general similarity between these superficially different reactions. In all or nearly all the reactions in question three points in common are discernible, namely, (*a*) the dehydration of the sterol

[1] The name phytosterolins has been given by Power and Salway [1913] to glucosides of phytosterols, such as have been recognised during recent years in a considerable number of plants.

molecule with the production of a colourless substance, (*b*) the appearance of a coloured product by the interaction of this first substance with a second (coupling) substance derived from the sterol or introduced in carrying out the reaction, (*c*) the use of an agent for rendering the medium anhydrous. As is pointed out below, the first substance is probably a hydrocarbon—*e.g.* a cholesterylene or cholesterylin—formed by the withdrawal of the elements of water from the sterol. The dehydrating agent responsible for its production may or may not also be the agent used to render the medium anhydrous. The coloured products obtained in the sterol colour reactions are very sensitive to traces of moisture. The actual colour obtained depends upon both the thoroughness with which the medium is dehydrated and the nature of the second (coupling) substance. These points can be illustrated by considering some of the individual reactions.

The Salkowski reaction. If the cherry-red chloroform layer is poured into another test-tube, it is usually seen to lose its colour as a result of the transference. That the destruction of the colour is due simply to the small amount of moisture present in a nominally dry test-tube is shown by, among other observations, the fact that the colour can be restored, not only by conc. sulphuric acid (as was noticed by Salkowski), but also by phosphorus pentoxide.

In the Salkowski reaction the sulphuric acid acts both to produce the first, colourless substance by its action on the sterol and to render the chloroform anhydrous. The presence, immediately after the introduction of the sulphuric acid, of the first, colourless substance, capable, by reacting with a second substance, of giving a coloured product, is shown by the observation that, if the chloroform layer is poured off at once, before a cherry-red colour has developed, and is then treated with formaldehyde[1], which serves as the coupling substance (cf. Reaction A, *infra*), a cherry-red or purple colour is obtained at once. In the ordinary Salkowski reaction a second (coupling) substance apparently arises only slowly. The rate at which the second substance necessary for the appearance of colour arises seems to be different in different solvents, and is, for example, noticeably slower when chlorobenzene is used as a solvent for the sterol than when chloroform is used.

Reaction A. In the reaction the first substance couples at once with formaldehyde to give a cherry-red product. Unlike the paler cherry-red colour produced in the ordinary Salkowski reaction, the colour produced in Reaction A is not destroyed by pouring the chloroform layer into another test-tube. Acetic anhydride changes the colour through purple and then through blue to green. The characteristic feature here is the comparative persistence of the blue stage, which has hitherto been discernible in sterol colour reactions (*e.g.* in the Liebermann-Burchard reaction) only as a very fleeting stage. By choosing a suitable solvent, in which the blue colouring matter is insoluble, the latter can be actually isolated as a solid.

[1] In, say, chloroform or ethereal solution.

Two drops of conc. sulphuric acid were added to 3 cc. of a solution of cholesterol in ethyl bromide. The solution immediately took on an orange colour (which became blood-red within a few minutes) and appeared fluorescent. A small amount of a brown solid separated in flocks. The blood-red solution was treated with a few drops of acetic anhydride, and then allowed to stand for half-an-hour. While standing, the solution passed through a purple stage, then became blue, and gradually deposited a blue solid. That this solid represents the blue product seen in solution in various of the sterol reactions, is indicated by the fact that it was found to be soluble in chloroform, and, when dissolved in the latter, to suffer a change of colour to green on treatment with acetic anhydride.

Reaction B. Here, as in the previous reaction, sulphuric acid both dehydrates the medium and acts on the sterol to produce the first, colourless substance, and the latter then reacts with formaldehyde to give a coloured product. This reaction is more sensitive than Reaction A because of the miscibility of the sulphuric acid with the solvent. In the case of the Salkowski reaction and Reaction A some of the colour-producing substance goes into the sulphuric acid layer. In this connection the following observations may be noted.

(1) That, alike in the Salkowski reaction and in Reaction A, the same substance is in question in the two layers, is indicated by the observation that formaldehyde, which, as already mentioned, intensifies the colour in the chloroform, also intensifies the depth of the colour and the fluorescence in the sulphuric acid.

(2) If in Reaction A the two layers are shaken together vigorously, the upper layer can be rendered almost colourless, owing to the transference of colour to the sulphuric acid layer.

(3) If conc. sulphuric acid is dropped into a solution of cholesterol in carbon tetrachloride, the latter assumes at once, as the acid drops through it, a deep yellow colour, and the sulphuric acid forms a colourless layer at the bottom; if now the two layers are shaken together well, the colour goes almost completely into the sulphuric acid layer, the upper layer becoming nearly colourless.

In connection with carbon tetrachloride, it is interesting to note that the purple-coloured product obtained in various of the sterol colour reactions is insoluble in this solvent and can be isolated by its use.

(*a*) The above-mentioned yellow, carbon tetrachloride layer was poured off and treated with acetic anhydride. A dark-purple solid gradually separated, the liquid itself finally becoming green. The solid was removed, and was found to be soluble in warm chloroform. The purple chloroform solution of the solid, when treated with acetic anhydride, became converted through blue to green.

(*b*) The yellow carbon tetrachloride solution was treated with formaldehyde. There separated a purple-coloured solid with properties similar to those of the purple solid just mentioned.

It thus appears that the purple solid, mentioned under (*a*), and obtained by treating the first, colourless substance in carbon tetrachloride with acetic anhydride, is essentially identical with the purple or cherry-red substance obtained by treating the first colourless substance with formaldehyde.

Liebermann-Burchard reaction. In this reaction [Liebermann, 1885; Burchard, 1889] a green colour is produced by the addition of a drop of conc. sulphuric acid to a solution of a sterol in acetic acid containing some acetic anhydride. The sulphuric acid produces the first, colourless substance from the sterol, and the acetic anhydride then reacts with this substance to give a purple-coloured product, which it then changes to a blue and finally to a green product. The acetic anhydride also serves to dehydrate the medium. The power of acetic anhydride to change the colour of the earlier coloured stages and at the same time to dehydrate the medium, is shown in the following observations.

(*a*) The cherry-red chloroform layer obtained in Reaction A was poured off and diluted with chloroform from a stock bottle until it became colourless. (The amount of moisture in an ordinary sample of chloroform is sufficient to discharge the colour.) Acetic anhydride was then added. The result was to produce a blue colour (which later turned green), just as it would have been had the colour not first been discharged.

(*b*) To a blue chloroform solution obtained in Reaction A drops of water were added until the colour was discharged. (The colour could be restored by adding phosphorus pentoxide.) A little acetic anhydride was then added. The result was a green colour.

Tschugajeff reaction. In this reaction [Tschugajeff, 1900] an eösin-red colour is obtained by boiling an acetic acid solution of a sterol with zinc chloride and acetyl chloride[1]. The zinc chloride acts both to withdraw water from the sterol molecule and to dehydrate the medium. In the latter function it is doubtless assisted by the acid chloride.

It was found that if an acetic acid solution of a sterol is boiled with powdered, dry zinc chloride, without the addition of an acid chloride, a purple colour develops. Hence it would appear that zinc chloride alone is capable of producing from a sterol both a first and a second substance, which together give rise to what has previously been referred to as the first coloured stage (cherry-red, purple, or rose). As in the case of the Salkowski reaction, the amount of the second substance formed is apparently insufficient to lead to the full development of colour which the amount of the first colourless substance produced is capable of yielding; for, if a little formaldehyde is introduced (as vapour) into the solution, the purple colour obtained on boiling is deepened.

Lifschütz reaction. In this reaction [Lifschütz, 1898] benzoyl peroxide serves to dehydrate the medium and to react with the first, colourless substance, formed from the sterol by the action of sulphuric acid.

It would appear that the hydrocarbons—*e.g.* cholesterylenes and cholesterylins—which can be obtained from sterols by the action of sulphuric acid

[1] It was found that the acetyl chloride may, with identical results, be replaced by benzoyl chloride.

or zinc chloride, and have been studied by Zwenger [1848, 1849] and by Mauthner and Suida [1896], are concerned in the reactions discussed above.

A mixture of hydrocarbons, prepared from cholesterol according to the procedure of these authors, was dissolved in chloroform. The solution thus obtained was colourless, but when warmed with phosphorus pentoxide became rose- or cherry-red. If a little formaldehyde vapour was passed into the solution before the addition of the pentoxide, a deeper purple colour was obtained. If the purple solution was poured off and treated with acetic anhydride, its colour changed through blue to green. These results are strictly parallel with those observed in connection with the sterol colour reactions.

It appears that in the mixture of hydrocarbons (? with possibly other substances) which sulphuric acid produces from cholesterol there are present both the substances previously referred to as the first, colourless substance and the second (coupling) substance; as it is sufficient to dissolve the mixture in a thoroughly dehydrated medium for a cherry-red colour to make its appearance. It appears, further, that, as was seen to be the case in the Salkowski and Tschugajeff reactions, here too an insufficient amount of the second (coupling) substance is present; as the introduction of formaldehyde deepens the colour obtained.

Summary.

Three new colour reactions (A, B and C) for the detection of sterols are described. Reaction A is more striking and sensitive than the usual chloroform-sulphuric acid reaction. Reaction B is more sensitive than any sterol colour reaction hitherto described.

A reaction is described for detecting sterolins and distinguishing them from sterols.

In a general discussion of the reactions applicable to the detection of sterols in solution it is concluded that all or almost all such reactions are, in their essential features, similar.

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