XVIII. A NEW COLOUR REACTION FOR "OXYCHOLESTEROL."

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In a previous communication dealing with the presence of "Oxycholesterol" in brain [1914], I have mentioned that this substance, when moistened with dimethyl sulphate (techn.) gives immediately a characteristic purple colour reaction, while under the same conditions cholesterol remains unaffected¹. The latter assumes gradually, especially on warming, a bright raspberry-red colour. Under identical conditions pure dimethyl sulphate fails to react with either substance.

When the colouring matters were examined in chloroform solution, they were found to possess characteristic spectra.

An interesting reaction occurs in the presence of oxidising agents, of which ferric chloride is the most convenient for practical purposes. On addition of a drop of ferric chloride in glacial acetic acid solution, the bright raspberry-red colour of the cholesterol reaction changes into purple, while the purple of the "oxycholesterol" reaction changes into a brilliant emerald green. The latter shows a well-defined and characteristic absorption band in the red between λ 650– λ 630, which is identical with that obtained in the Lifschütz reaction [1908]. I found further that *pure* dimethyl sulphate in the presence of ferric chloride also gave the same emerald green colour with "oxycholesterol," while cholesterol under the same conditions gave no colour reaction even on prolonged warming. These reactions can be used to detect "oxycholesterol" in presence of cholesterol, and also to estimate its quantity spectrometrically.

The striking difference between the behaviour of technical and of pure

¹ This reaction has since been successfully applied by Lamb [1914] in a study of the influence of atmospheric oxygen on the oxidation of cholesterol under histological conditions.

dimethyl sulphate was next investigated. The active substance in technical dimethyl sulphate which reacts with "oxycholesterol" and with cholesterol in the absence of ferric chloride is evidently an impurity and my experiments tend to show that it is monomethyl sulphate. This probably owes its origin to partial hydrolysis of dimethyl sulphate in a moist atmosphere, and its formation explains the fact that even inactive *pure* dimethyl sulphate gradually becomes active on keeping under ordinary laboratory conditions.

The dimethyl sulphate reaction cannot serve for the distinction of animal and vegetable cholesterol, as I have found that sitosterol as well as "oxysitosterol" give colour reactions identical with those of cholesterol and "oxycholesterol" respectively.

EXPERIMENTAL.

The cholesterol used was prepared from human brain by the method of O. Rosenheim [1906]. "Oxycholesterol" was prepared from it by treatment with benzoyl peroxide in glacial acetic acid solution, according to Lifschütz [1913]. The spectroscopic observations were made with a Zeiss comparison spectroscope. The depths of the fluids examined were measured in a special absorption vessel, which allows measurements to 0.05 mm. A small electric arc lamp, at 20 cm. distance from the apparatus and screened by a plate of matted glass, served throughout the experiments as the source of light. The width of the spectroscope slit was 0.2 mm. in all observations.

I. *Reaction with technical dimethyl sulphate.*

(a) *Cholesterol.* On adding a few drops of technical dimethyl sulphate (Kahlbaum) to a solution of cholesterol in chloroform, the solution remains colourless for some minutes, but on slightly warming in a water-bath it gradually assumes a bright raspberry-red colour¹. In sunlight, or in the light of the electric arc, it shows a blue fluorescence. Solutions of cholesterol in carbon tetrachloride, benzene, toluene or xylene give similar colour reactions, the colours tending to be more blue, less transparent and more fluorescent. Solutions in ether, alcohol, acetone, amyl acetate and amyl alcohol failed to give any reactions. The colour of the chloroform solution is discharged by the addition of water or alcohol. This fact distinguishes the reaction from that of Neuberg and Rauchwerger [1904] with methylfurfural, which is carried out in alcoholic solution. This also gives a raspberry-red solution, but shows a different spectrum.

¹ This reaction was discovered accidentally by Dr O. Rosenheim when carrying out experiments on the methylation of cholesterol.

On adding a drop of ferric chloride solution (5 % ferric chloride in glacial acetic acid), the colour is intensified and changes into a deep purple. The reaction is distinguished by great permanency and was observed to persist for more than a week.

For the spectroscopic examination 0.2 cc. of Kahlbaum's dimethyl sulphate (techn.) was added to 2 cc. of a 5 % cholesterol solution in dry chloroform. A deep raspberry-red colour developed on slight warming which showed two characteristic bands, one between *D* and *E* and the other near *F*. The following observations in the absorption vessel were made:

Depth of fluid

5.0 mm. only red light transmitted.

3.0 „ one dense band in yellow and green from λ 595– λ 460.

1.5 „ one dense band in yellow and green from λ 585– λ 485.

1.0 „ two bands from λ 575– λ 525 and from λ 505– λ 488.

0.7 „ „ from λ 565– λ 535 and from λ 505– λ 485.

0.5 „ „ from λ 560– λ 550 and from λ 500– λ 490.

0.4 „ one band at λ 500.

The appearance of the two characteristic bands is shown in Fig. 1 (Plate III).

When one drop of the ferric chloride reagent was added, the solution became so intensely coloured that accurate observations were impossible without dilution. The experiment was therefore repeated using a 1 % solution of cholesterol. On addition of ferric chloride the solution became purple and transmitted only red light when examined in 5 mm. thickness.

The following observations were made:

Depth of fluid

5.0 mm. only red light transmitted.

1.3 „ blue light also is transmitted.

1.0 „ two bands from λ 540– λ 530 and from λ 510– λ 420.

0.5 „ one band only from λ 505– λ 480.

The reaction is of approximately the same sensitiveness as Liebermann's cholesterol reaction, the limit being reached when 2 cc. of the solution contained 0.02 mg. of cholesterol.

(b) "*Oxycholesterol*." A solution of this substance in chloroform gives on the addition of a few drops of technical dimethyl sulphate an immediate purple colour without warming (stage 1). A drop of the ferric chloride reagent added changes this at once into a greenish blue which gradually turns into an emerald green (stage 2) showing a green fluorescence. The

Fig. 1. Spectrum of Cholesterol Reaction, 0.02 g. in 2 cc. chloroform, thickness of layer: 1 mm.
Fig. 2. Spectrum of "Oxycholesterol" Reaction, 1st stage 0.02 g. in 2 cc. chloroform, thickness of layer: 0.5 mm.
Fig. 3. Spectrum of "Oxycholesterol" Reaction, 2nd stage, 0.02 g. in 2 cc. chloroform, thickness of layer: 0.5 mm.

green colour is discharged by water, acetone, alcohol or amyl alcohol, but not by glacial acetic acid, chloroform or benzene. Glacial acetic acid appears to be the most suitable diluent, the solution remaining transparent, whilst chloroform or benzene gives rise to some turbidity.

For spectroscopic examination 0.2 cc. of dimethyl sulphate (techn.) was added to 2 cc. of a 1 % "oxycholesterol" solution. Observations could only be made in thicknesses from 1.8 mm. downwards. All the spectral colours pass through the solution and there is only one band near the *D* line which shows best in a thickness of from 0.5 to 0.3 mm.

Depth of fluid

- 1.8 mm. one deep broad band from λ 620 to λ 520.
- 0.5 ,, one broad band; blue, green and red light transmitted brilliantly.
- 0.3 ,, one distinct band in the yellow, from λ 600— λ 580.
- 0.15 ,, one faint band at *D* line—at λ 590.

The characteristic band of the first stage is shown in Fig. 2 (Plate III). On addition of two drops of glacial acetic acid and one drop of the ferric chloride reagent, the solution becomes greenish blue at once and rapidly reaches the second stage of emerald green. This also shows one band which is however in the red. The solution had to be diluted with an equal volume of acetic acid before satisfactory measurements could be made. The following observations were made with a freshly prepared solution containing only 0.5 % of "oxycholesterol."

Depth of fluid

- 10.0 mm. absorption of red end of spectrum as far as λ 560.
- 6.5 ,, red light transmitted; blue and green come through undiminished.
- 5.0 ,, one band from λ 670— λ 630.
- 4.0 ,, one sharply defined band from λ 660— λ 630.
- 3.0 ,, " " " " "
- 1.2 ,, one band still shows faintly at λ 650.

Fig. 3 (Plate III) shows the typical band of the second stage in a 1.0 % solution of 0.5 mm. thickness.

The sensitiveness of the "oxycholesterol" reaction is somewhat less than that of the cholesterol reaction under the same conditions. The limit is reached when 0.2 mg. of "oxycholesterol" is contained in 2 cc. of chloroform; but on the other hand, the colour once produced in a stronger solution stands an amount of dilution which is much greater than the above limit.

The formation of "oxycholesterol" from cholesterol may be very well shown by means of the above reaction, when a small quantity of cholesterol is boiled in acetic acid solution with benzoyl peroxide, according to Lifschütz [1908]. On the addition of a few drops of dimethyl sulphate (techn.) a brilliant blue colour develops at once, which changes into green on standing, or immediately after the addition of a drop of ferric chloride solution. The green solution shows the single band in the red of the "oxycholesterol" reaction in the typical manner.

II. *Reaction with pure dimethyl sulphate.*

(a) *Cholesterol.* Pure dimethyl sulphate does not give rise to any colour reaction with either dry cholesterol or its solutions in chloroform or other solvents. The solutions remain colourless also on warming in a water-bath or after the addition of a drop of the ferric chloride reagent.

(b) "*Oxycholesterol.*" Strong "oxycholesterol" solutions in chloroform show, after the addition of pure dimethyl sulphate and warming in the water-bath, a slight purple colour which changes after the addition of ferric chloride into the typical green colour, showing the characteristic single band in the red. Even dilute "oxycholesterol" solutions which remain colourless with pure dimethyl sulphate, still turn green after the addition of ferric chloride, and show the "oxycholesterol" band in the red.

III. *The nature of the active substance in technical dimethyl sulphate.*

The fact that pure dimethyl sulphate fails to react with cholesterol makes it evident that the active substance in the technical product is of the nature of an impurity. An attempt was made to isolate this impurity by fractional distillation, but it was found that all the fractions as well as the residue in the distilling flask gave equally strong reactions. It was next observed that inactive pure dimethyl sulphate became active when it was simply heated to boiling in a test tube. At the same time it became strongly acid to Congo paper, while it was previously neutral. It was further noticed that a sample of inactive pure dimethyl sulphate, which had been kept for a considerable time in a bottle under ordinary laboratory conditions, had become active and had at the same time acquired an acid reaction. A control sample, kept in a sealed tube for the same length of time, had remained inactive. Further, it was found that all the samples of active technical dimethyl sulphate examined possessed an acid reaction to Congo paper.

It is well known that dimethyl sulphate is partially decomposed into methyl alcohol and monomethyl sulphate on heating to the boiling point, as well as by partial hydrolysis, and monomethyl sulphate may be further hydrolysed into methyl alcohol and sulphuric acid. The latter cannot be considered as the active substance since the addition of small quantities of concentrated sulphuric acid to pure dimethyl sulphate failed to activate it. Further, sulphuric acid gives rise to the well-known Salkowski's reaction, from which the dimethyl sulphate reaction is characteristically different, both in its appearance and in its behaviour towards ferric chloride.

It seemed therefore most likely that the acid monomethyl sulphate was responsible for the reaction. Since this substance forms a barium salt which is insoluble in chloroform, an indirect proof for this assumption may be obtained. A sample of technical dimethyl sulphate, dissolved in chloroform, was treated with solid barium carbonate until the effervescence had ceased. The filtered solution was found to be inactive and left, on careful evaporation of the chloroform, an oil which reacted neutral towards Congo paper. The inactive dimethyl sulphate thus prepared was easily activated again by boiling it for a short time in a test tube. Since the preparation of pure monomethyl sulphate from its barium salt is a matter of some difficulty, I have refrained from further testing this question.

There remained still the possibility that the activity of technical dimethyl sulphate might be due to traces of formaldehyde or formic acid, formed by gradual oxidation of methyl alcohol which is set free by hydrolysis. Formaldehyde and formic acid were indeed found to give rise to colour reactions with cholesterol under certain conditions, which need not be described here. But pure dimethyl sulphate does not produce any colour reaction in the presence of a trace of formaldehyde or formic acid, and there is therefore no reason to ascribe to either of them any share in the reaction of technical dimethyl sulphate with cholesterol.

The reaction of cholesterol with monomethyl sulphate may be used conversely for testing the purity of dimethyl sulphate preparations. Since technical dimethyl sulphate is probably manufactured by distillation under ordinary pressure, most of the samples contain monomethyl sulphate in varying amounts, whilst a distillation *in vacuo* furnishes a pure product which does not react with cholesterol.

It may be mentioned that diethyl sulphate behaves in exactly the same way. The pure substance does not react with cholesterol, but with "oxy-cholesterol" gives rise to a typical green reaction with ferric chloride, showing

the characteristic absorption band between λ 650 and λ 630. By heating the pure product to boiling in a test tube it also became active towards cholesterol and then behaved just like technical dimethyl sulphate.

Cholesterol esters give the dimethyl sulphate reaction in a less degree but vegetable cholesterols (phytosterols) behave exactly like cholesterol. A phytosterol prepared from hemp-seed and sitosterol from wheat embryos (prepared according to Ritter [1902]) were tested. The corresponding "oxysitosterol" reaction also was spectroscopically indistinguishable from that of "oxycholesterol."

The relationship of cholesterol to the terpenes suggested an examination of the behaviour of some of these substances towards dimethyl sulphate. It was found that borneol, camphor and abietinic acid (prepared from colophony), as well as cholic acid, all give rise to colour reactions with technical dimethyl sulphate, the description of which is outside the scope of this communication. But none of these substances, when subjected to oxidation with benzoyl peroxide, as in the preparation of "oxycholesterol," gave rise to the typical "oxycholesterol" reaction with dimethyl sulphate.

SUMMARY.

1. A colour reaction of cholesterol with technical dimethyl sulphate is described, the spectrum showing two bands, from λ 560– λ 550 and from λ 500– λ 490.

2. This reaction is not given by *pure* dimethyl sulphate and appears to be due to the presence of monomethyl sulphate.

3. "Oxycholesterol" gives a purple colour reaction with technical dimethyl sulphate showing an absorption band in the yellow (λ 600– λ 580).

4. Pure or technical dimethyl sulphate, after the addition of ferric chloride, gives a typical green colour reaction with "oxycholesterol," showing a well-defined absorption band in the red between λ 650 and λ 630.

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