LXXXII. ON THE DEVELOPMENT OF CHROMOGENIC PROPERTIES IN CHOLESTEROL BY THE ACTION OF HEAT.

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"OXYCHOLESTEROL," a brown resinous substance first obtained by Lifschütz [1908] from cholesterol by the action of benzoyl peroxide in acetic acid solution, came into prominence through certain similarities shown by its colour reactions to those associated with vitamin A [Takahashi *et al.*, 1924, 1925]. More recently Rosenheim [1927], working on the arsenious chloride reaction, has also obtained a chromogenic substance from cholesterol by the use of the same reagent, but in chloroform solution, which is found to simulate the "vitamin" colour reactions closely in many respects, though proving unstable in the presence of oils. It is intimated that further experiments are being undertaken using ergosterol, the precursor of vitamin D, in place of cholesterol.

In the meantime certain experiments have come to our notice in which "oxycholesterol" has been formed from cholesterol by other means than the use of benzoyl peroxide or a similar oxidising agent.

(1) It will be recalled that Rosenheim and Webster [1926] suggested that "oxycholesterol" might be formed on the irradiation of cholesterol in air, thus increasing the non-precipitable fraction without raising the antirachitic value. Shear and Kramer [1926] have now confirmed the appearance of this substance under such conditions, having obtained by the prolonged irradiation of cholesterol in air a yellow amorphous product, "U.V. Oil," which gives the typical colour reaction of "oxycholesterol" as described by Lifschütz.

(2) In the course of experiments on the multiplication of infusoria, Robertson [1925], by the aeration of an aqueous colloidal solution of cholesterol at boiling-point in the presence of traces of an acetone extract of brain, has obtained on evaporation a "light brown pasty mass," resembling "oxycholesterol." This material is found to give the typical colour reaction, and is supposed to be produced from the sterol by the action of a catalyst present in the brain extract, in the absence of which no "oxidation" takes place.

In the course of experiments designed to confirm such observations we were led to examine the colour reactions of cholesterol which had been gently heated, both in aqueous colloidal solution and in the solid state. The results stated below would indicate that caution is necessary in accepting the production of "oxycholesterol" as a specific effect of some particular mechanism in cases where heat is also involved.

Experimental.

The experiments were first carried out on a specimen of cholesterol obtained by five recrystallisations from a crude cod-liver oil product supplied by Messrs Joseph Nathan, Ltd. They were repeated on an "almost pure" sample, M.P. 148°, from the same source, obtained by Prof. Heilbron in the course of his recent spectroscopic work, and almost free from ergosterol. Both specimens gave negative results when tested with (1) a 1-5 sulphuric-acetic acid mixture, followed by a drop of 2 % ferric chloride in acetic acid (Lifschütz), or (2) a chloroform solution of antimony trichloride [Carr and Price, 1926], which were the reagents employed to test for "oxycholesterol."

The production of "oxycholesterol" in molten cholesterol.

Samples of cholesterol were heated to temperatures slightly above the melting-point in an open dish on a carefully regulated sand-bath. After some time a slight yellow tint was observed, and on cooling the cholesterol was now found to give positive reactions with the reagents mentioned above, the nature of such reactions depending upon the duration of heating and the temperature attained.

(a) If the cholesterol were warmed for a few minutes at a temperature barely above the melting-point a very faint yellow tint was acquired. With the antimony trichloride reagent a blue coloration was given, which faded in a few minutes to a dull brown. With the Lifschütz reagents a preliminary violet was given, which became ultramarine and then greenish blue, but not emerald green, on the addition of ferric chloride. (b) If the cholesterol were kept at the same temperature for a longer period (30-120 mins.) the yellow tint developed was more pronounced. A bright ultramarine, permanent for hours, was now given with the antimony trichloride reagent, while in the Lifschütz test a definite green was obtained on the addition of ferric chloride. (c) Prolonged heating at more elevated temperatures converted the cholesterol entirely into brown vitreous resins. Some resins gave the complete "oxycholesterol" colour sequence with the Lifschütz reagents, and with antimony trichloride an indigo blue coloration; other resins, in the production of which still higher temperatures were attained, gave no colorations other than brown.

It would seem, therefore, that the production of "oxycholesterol," effected by Shear and Kramer through the agency of ultra-violet radiations, may equally well be effected by thermal means. These workers have also found that irradiated cholesterol gives on boiling with a mixture of 15 parts aniline and 1 part hydrochloric acid a red or reddish brown coloration, which, in spite of strong criticism by Rosenheim and Webster, is still attributed to vitamin D. It may be stated, in support of the view of Rosenheim and Webster, that samples of melted cholesterol were found to react positively to this test, though the original cholesterol gave no coloration. Moreover, samples of a cholesterol,

freed from ergosterol by bromination and reduction, after melting gave positive reactions in this test and in those for "oxycholesterol." The aniline hydrochloride test cannot be considered specific to vitamin D.

The "oxidation" of cholesterol in colloidal solution.

In confirmation of Robertson's work it was found that stable colloidal solutions of cholesterol could be obtained by pouring an alcoholic solution of the latter into hot water, with constant agitation. In our experiments 5 cc. of a 0.5 % solution were poured into 100 cc. of hot distilled water, 105 cc. of solution therefore contained 25 mg. of cholesterol. The solutions so obtained displayed a slight orange opalescence in transmitted light and remained stable for days. As the solutions were not to be used in physiological experiments it was thought unnecessary to follow Robertson's procedure for the removal of alcohol and the solutions were used without further treatment.

After aeration of the colloidal solutions under a reflux condenser at 90–100° for 6 hours with traces of acetone extracts of brain, liver, or kidney, the residues after evaporation gave erratic results when tested with the Lifschütz "oxycholesterol" reagents, the initial colours obtained varying from bright purple to dull brown, which was at first mistaken for a negative reaction. It soon became apparent, however, that the colour produced was entirely dependent on the care taken during evaporation. Cautious evaporation resulted in the production of the normal purple of "oxycholesterol," even in the absence of tissue extracts; overheating in the final stages resulted in a brown colour reaction. Moreover, plain solutions which had not been aerated for 6 hours but merely evaporated carefully to dryness gave positive tests for "oxycholesterol." For a further investigation of the phenomenon it was necessary to evaporate the solutions at a lower temperature. Accordingly they were poured after aeration into large Petri dishes, and left in a hot room at 37° until dry.

Immediate colorations with the Lifschütz and antimony trichloride reagents were given by residues so obtained from solutions heated, without tissue extracts, to 90° for periods down to 1 hour, with or without aeration. No colorations were produced by residues from (a) a solution made up and aerated for 48 hours at 37°, (b) a solution made up hot and immediately cooled, and (c) an alcoholic solution of cholesterol evaporated directly.

A further phenomenon is perhaps worthy of mention. The colloidal cholesterol solutions immediately after preparation were very sensitive to the precipitating action of electrolytes; after heating for some hours they became much less sensitive. Such increased stability would seem to coincide with the formation of the resinous "oxidation" products, but the question has not been investigated in detail.

DISCUSSION.

Cholesterol has long been known to undergo changes on heating. The above experiments show that chromogenic properties appear on such treatment, and consequently in all experiments where the presence or production

of "oxycholesterol" is observed care must be taken to rule out such thermal effects as might arise in the course of the experiment or during the processes of isolation. Thus Lifschütz [1914] has described experiments in which "oxycholesterol" is produced by heating defatted blood with cholesterol in acetic acid solution at 60–65° for several days. While it is possible that the blood acts as a catalyst under these conditions, such evidence does not, in our opinion, justify the assumption of a similar catalytic action in normal physiological processes.

In regard to the experiments of Robertson, as stated above, we have been unable to confirm the reported stability of cholesterol towards "oxidation" when aerated in colloidal solution in the absence of brain extract. Since it is stated that the cholesterol to resist oxidation must be "perfectly pure," we took pains to repeat our experiments on as pure a specimen of cholesterol as was available to us, but still obtained the "oxycholesterol" colour reactions on evaporation. In the absence of a more detailed description of Robertson's experiments we are at a loss to explain this discrepancy.

Cholesterol, of course, may be boiled in most organic solvents without the formation of "oxycholesterol," and the processes of crystallisation and evaporation from such solvents only present danger when excessive temperatures are attained. Assuming that due precautions were taken against this in the experiments of Shear and Kramer all that can be said is that the changes produced in cholesterol by heating and by irradiation in air are superficially very similar.

In conclusion, it should be noted that in our own experiments the cholesterol was usually only in part converted into resinous products, and in some cases only a fraction of such resins must have possessed chromogenic properties. Heat was usually much less efficient in the production of chromogenic substances than the benzoyl peroxide procedure described by Lifschütz.

SUMMARY.

Brown resinous substances are produced in cholesterol on melting or on heating in aqueous colloidal solution. At the same time chromogenic properties similar to those of "oxycholesterol" are acquired.

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