CLXV. ON THE OXIDATION OF CHOLESTEROL BY MOLECULAR OXYGEN.

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I.

Whilst the ability of the animal organism to synthesise cholesterol seems to be clearly proven, the question if, or to what extent, the cholesterol is broken down in oxidative or other ways in the body is still very obscure. This question is, indeed, not only of physiological interest, but in view of the diseases which are known to be accompanied by an abnormal accumulation of cholesterol in blood or tissues it is also of a definite pathological-clinical interest.

What is known about this question we owe to a great extent to Lifschütz [1927]. According to this author cholesterol is transformed by the action of permanganate or, better, benzoyl peroxide in acetic acid solution into a resinous product called "oxycholesterol." This substance can be recognised and estimated with the aid of a colour reaction with glacial acetic acid and sulphuric acid not given by the cholesterol itself. Since oxycholesterol has not been obtained crystalline and has no definite melting-point, the purity of the material is not yet assured. Lifschütz ascribes to it the formula $C_{26}H_{47}O_2$.

Oxycholesterol has been found by Lifschütz (and others) to be a normal constituent of the blood. It also seems to be present in certain other tissues. Lifschütz regards it as the first step in the break-down of cholesterol in the body. In spite of its indefinite chemical nature it merits, nevertheless, due attention, especially as a possible normal catabolite of cholesterol.

When protected from light pure cholesterol kept at room temperature seems to be very stable against the oxygen of the air. In the dry state it remains under such circumstances unchanged at least for weeks. Suspended in water it can be aerated for hours at 90–100° without perceptible changes (see below). Aeration of an alcoholic solution at 60–70° for a few hours at any rate (as we have found) does not affect it.

The mechanism by which the oxycholesterol is produced in the organism is not known. Lifschütz [1914] has found that oxycholesterol is formed when pure cholesterol in glacial acetic acid is heated for some days together with small amounts of dried, fat-free blood. He therefore is of the opinion that the primary oxidative attack on the cholesterol takes place in the blood. More recently Robertson [1925], in the course of experiments on the multiplication

of infusoria, found that cholesterol suspended in water in the presence of mere traces of substances from the acetone extract of brain tissue, when aerated at 100°, was oxidised to oxycholesterol. Perfectly pure cholesterol did not undergo oxidation under these conditions.

These observations of Robertson seemed to us to form a possible starting-point in the study of the mechanism by which the cholesterol is physiologically broken down. When we were already engaged in our work a paper was published by Moore and Willimott [1927] bearing on this subject. These authors found that when pure cholesterol in open dishes was heated to a temperature slightly above the melting-point it acquired after a few minutes a faintly yellow tint and afterwards gave a positive oxycholesterol reaction; they were not able to confirm Robertson's results that pure cholesterol suspended in water was stable when aerated at 90–100°. In such instances they also obtained invariably a positive oxycholesterol reaction. Moreover, pure suspensions without aeration directly evaporated at 100° gave similar positive tests. On the other hand the residues obtained after aeration for 48 hours at 37° with subsequent evaporation at the same temperature gave no coloration with the Lifschütz reagent. On account of these findings Moore and Willimott seem to be of the opinion that in the cases mentioned formation of resinous products with chromogenic properties similar to those of oxycholesterol is not due to an oxidation by oxygen but is an effect merely of heat. They recommend caution "in accepting the production of oxycholesterol as a specific effect of some particular mechanism in cases where heat is also involved."

That in the first of the above-mentioned observations the formation of oxycholesterol is due not merely to heat but also to the action of oxygen is, however, easily shown.

Two portions of pure cholesterol weighing each about 0·04 g. were heated at 150–155° for 20 minutes. Portion 1 was lying on an open watch-glass, portion 2 was placed in a small flask through which was led a very slow stream of pure nitrogen. After solidifying, portion 1 had a faintly yellow colour, was semi-transparent, and gave an intensively positive reaction for oxycholesterol. Portion 2 remained perfectly white and opaque and gave no trace of a positive Lifschütz reaction. When heated to about 155° in oxygen instead of in air the cholesterol acquired a yellow tint in a few minutes and showed a strongly positive oxycholesterol reaction. It was also found that if cholesterol suspensions, which, either "pure" or with an activating agent added, on aeration at 100° gave oxycholesterol, were treated with nitrogen at the same temperature, no oxycholesterol was formed.

The indispensable rôle of oxygen for the formation of oxycholesterol in these cases seems to be thus quite clear. The question next arises regarding the physical or chemical conditions necessary for the oxidation of cholesterol suspended in water by molecular oxygen. The following investigations are to be considered mainly as an inquiry into this question. Our experiments have been restricted to simple chemical systems.

II.

At first our experience of the formation of oxycholesterol in "pure" suspensions was similar to that of Moore and Willimott. We had at our disposal a sample of cholesterol prepared in this institute from human gall stones. The preparation was perfectly white and gave no reaction for oxycholesterol. The melting-point was not determined. 0·5 and 1 % suspensions of this cholesterol were prepared by dissolving the substance in a little alcohol and pouring the solution with stirring into water heated to 60–70°. The suspensions were aerated at 90–100° with and without addition of acetone extract from brain tissue for 30 minutes up to 5 hours. No consistent results were obtained. In most cases the residues gave a strongly positive reaction for oxycholesterol. Occasionally, however, negative reactions were obtained, also in cases where brain extract had been added. In order to get reproducible results we tried to standardise as far as possible the experimental conditions, and to avoid impurities from different sources. Accordingly we submitted the cholesterol to purification by recrystallising it seven times from alcohol. Thereafter it was no longer possible to get suspensions of the same strength as before, since most of the cholesterol flocculated immediately. In order to obtain as concentrated suspensions as possible we followed the directions given by Svedberg [1924], who emphasises the importance of boiling off the solvent completely to obtain stable colloids. Accordingly, the alcoholic solution of the cholesterol was ejected in small portions into boiling water through the end of a glass tube of fine bore. Most of the alcohol thereby immediately volatilised. The rest was driven off by distilling the fluid to half the bulk, but we did not succeed in obtaining more concentrated suspensions than could be obtained by simply pouring the alcoholic solution with stirring into hot water. The highest concentrations attained were 0·07–0·08 %.

Even after the measures mentioned oxycholesterol was formed on aeration of "pure" cholesterol suspensions at 90–100°. The results, although less irregular than in the first series of experiments, were, however, quantitatively not reproducible. The cholesterol was then further purified in different ways: (1) by bromination and subsequent reduction with zinc dust, (2) by boiling for one hour with 0·5 N alcoholic potassium hydroxide, diluting with water, extracting with ether and taking up the residue after evaporation of the ether in light petroleum, (3) by boiling an alcoholic solution of cholesterol twice with blood charcoal (Merck's pure) for half an hour. After these purifications the cholesterol was once recrystallised from alcohol and then aerated at 90° for one hour. In all instances the Lifschütz reaction of the residue was positive. Considering the possibility that traces of oxycholesterol (or decomposition products of it) might favour its production by autocatalysis the residues obtained by evaporating the mother-liquors from the above-mentioned recrystallisations were tested for oxycholesterol. They all gave a positive reaction. The cholesterol purified with charcoal was then further recrystallised from

alcohol (4 times) until the residue from the mother-liquor no longer gave any reaction for oxycholesterol. The suspensions made with cholesterol thus prepared could be aerated for several hours (up to 7) without production of oxycholesterol in demonstrable amounts or, sometimes, with the formation of oxycholesterol in amounts giving only a faintly positive reaction. The method of purification at last adopted was the following. The crude cholesterol from gall stones was, after four recrystallisations from alcohol, boiled twice with blood charcoal in alcoholic solution for half an hour. The cholesterol was then further recrystallised from alcohol (3 or 4 times) until the residue from the mother-liquor no longer gave any positive test for oxycholesterol. The preparation thus obtained melted at 148° (corr.). Whether oxycholesterol actually acts autocatalytically was not further investigated. All that can be said is that the mode of purification above described proved to be reliable in repeated experiments for our purpose[1]. The highest concentrations of the suspensions prepared from this cholesterol were from 0·05 to 0·06 %.

III.

In the course of this investigation we made an observation which seemed to us to be worth closer study. If merely a trace of an alkali soap was added to the cholesterol suspension we found regularly an abundance of oxycholesterol on aeration at 90°.

Experimental.

(1) The suspensions were prepared either according to Svedberg or simply by dissolving 0·05 g. cholesterol in 10 cc. alcohol and pouring the solution with stirring into 100 cc. water at 60–70°. In the earlier experiments we distilled off all the alcohol, but later found that this was not necessary. In some instances the long boiling necessary to drive off the alcohol seemed to make the suspension more prone to the formation of oxycholesterol, but with suspensions prepared in this way we obtained residues giving no reaction for oxycholesterol after aeration at 90° up to 7 hours.

(2) For the aeration 20 cc. of the suspensions were placed in a wide test-tube closed with a rubber stopper provided with three holes, respectively for inlet and outlet of air and for a thermometer. The glass tube for the inlet of air was in its lower submerged end enlarged to a bulb perforated with small holes. The outlet tube was connected with a reflux condenser in its turn connected with a suction pump. The air was in most experiments purified by filtering through cotton wool and by passing through three wash-bottles containing strong alkali, concentrated sulphuric acid and water respectively. The test-tube was immersed in a water-bath heated to the desired temperature. When the air was sucked through the suspensions in the boiling water-bath

[1] It was found that if the cholesterol thus purified was again boiled with charcoal the residue from the mother-liquor in the first recrystallisation from alcohol gave a positive reaction. Obviously small amounts of oxycholesterol are formed by the boiling with charcoal.

the temperature of the former sank, of course, below 100°, the more rapid the air stream, the lower the temperature. The temperature of the suspension therefore served as a relative measure of the rate of the air stream. By regulating the suction pump so that the temperature of the suspension was constantly at 90° we had a simple means of getting approximately the same rate of air stream in the different experiments at this temperature.

(3) The evaporation of the suspensions was performed in porcelain dishes placed in an air stream at room temperature. As the soaps interfere with the Lifschütz reaction the residues were extracted with light petroleum (B.P. 35–55°) and the latter was thereafter driven off. All vessels used were carefully cleansed with hot sulphuric acid-dichromate mixture.

(4) In some experiments estimation of the oxycholesterol was made roughly by direct inspection of the intensity of the colour reaction, in others by spectrophotometrical determination. A sample of oxycholesterol, prepared according to Lifschütz by oxidation of cholesterol by benzoyl peroxide, served as a standard. To 1 cc. of a chloroform solution of oxycholesterol was added 2 cc. of the reagent. Instead of adding, as Lifschütz did, the ferric chloride afterwards, we dissolved this substance in a concentration of 1/1000 directly in the reagent (9 vol. glacial acetic acid and 1 vol. conc. sulphuric acid). The spectrophotometrical reading was made after the mixture had been left for 15 minutes at room temperature. The spectral range used was that between 625 and 645$\mu\mu$. The relation between the concentration of the oxycholesterol and the extinction coefficient was determined in a series of oxycholesterol solutions of varying concentrations. The spectrophotometer used was one of Hüfner's type.

The sodium stearate and sodium oleate used were pure preparations from Merck, the rest of the soaps tried were prepared by adding the calculated amount of alkali to the free fatty acids (Kahlbaum's). Table I shows the amount of oxycholesterol formed on addition of sodium stearate in varying concentrations. The suspensions were aerated for one hour at 90°. Repeated control experiments (without soap) at 90° and lower temperatures gave negative results. The amount of oxycholesterol formed is given as a percentage of the amount of cholesterol taken (the difference in the molecular weights referable to the oxygen uptake not being taken into consideration).

Table I.

Stearate concentration %	Oxycholesterol formed %
0·02	71
0·02	52
0·004	45
0·004	65
0·001	49
0·001	42
0·0002	5
0·0002	0
0·0002	0

Table II shows the production of oxycholesterol on aeration at different temperatures.

Table II.

A. Stearate concentration 0·004 %		
Aeration (hours)	Temperature	Oxycholesterol formed %
1	90°	65
1	85°	65
1	80°	40
1	75°	25
1	70°	10
1	65°	0
1	65°	0

B. Stearate concentration 0·02 %		
Aeration (hours)	Temperature	Lifschütz reaction
1	90°	Strongly positive
1	70°	Negative
2	70°	Positive
4	70°	Strongly positive
1	60°	Negative
3	60°	Faintly positive
6	60°	Strongly positive
3	55°	Negative
6	55°	,,
12	55°	Positive
12	45°	Negative

Tables III and IV show series of similar experiments with addition of sodium oleate. The controls were negative.

Table III.

Aeration for 1 hour at 90°.

Oleate concentration %	Oxycholesterol formed %
0·02	57
0·02	57
0·02	98
0·004	25
0·004	14
0·004	25
0·001	0
0·001	13
0·001	10
0·0002	0
0·0002	0

Table IV.

Oleate concentration 0·02 %.

Aeration (hours)	Temperature	Lifschütz reaction
1	90°	Strongly positive
1	80°	Positive
3	80°	Strongly positive
1	70°	Negative
3	70°	,,
6	70°	,,
9	70°	Positive

No close reproducibility as regards the quantitative data could be obtained. Qualitatively the results are unequivocal. On aeration at 90° the addition of 0·001–0·02 % sodium stearate or 0·004–0·02 % sodium oleate regularly brings about a rapid formation of oxycholesterol. The residues obtained in these cases were quite transparent and only very faintly yellow. Higher soap concentrations were not tried on account of excessive foaming. If the temperature is lowered the rate of oxycholesterol formation is also lowered. There

seems to be a steep fall of the rate of oxycholesterol formation in the stearate experiments at 60–55° and in the oleate experiments at 80–70°, which is probably not compatible with a normal temperature coefficient of the rate of the reaction.

In a few experiments with sodium palmitate, potassium palmitate and potassium stearate no oxycholesterol was at first formed on aeration at 90°. As other experiments had shown that the addition of alkali inhibited the effect of sodium stearate on oxycholesterol formation, we added small amounts of sulphuric acid to the soap solutions used (the amount of acid being so small that the fatty acids were not precipitated and the solutions were still faintly red to phenolphthalein). After the addition of acid the soap solutions brought about a liberal formation of oxycholesterol, while the addition of alkali in excess also, in the case of these soaps, inhibited the oxycholesterol production.

IV.

The observation of the marked effect of the soaps led us to investigate the effect of adding other hydrophile colloids. Thus a series of proteins (glutin, edestin, ovomucoid, peptone) was tried, further two carbohydrates, dextrin and gum arabic and finally unsaturated phosphatides from egg-yolk. Of these substances the ovomucoid and the phosphatides were prepared by ourselves, the others were commercial specimens. The concentrations used were the same as in the soap experiments. The aerations were carried out at 90° and also at lower temperatures. In some experiments the hydrogen ion concentration was varied by addition of small amounts of acid or alkali. In no instance was any formation of oxycholesterol promoted. The addition of a protein to a cholesterol suspension was, indeed, found to inhibit the effect of soaps.

The easy oxidation of the cholesterol at temperatures above its melting-point led us to ascertain whether, in suspensions containing in the colloid state substances in which cholesterol is soluble, the latter was possibly prone to oxidation by oxygen. Accordingly mixed suspensions of different triglycerides and of higher fatty acids and cholesterol were prepared. In no instance was oxycholesterol formed on aeration at 90°. (As the fatty acids and the fats interfere with the Lifschütz reaction, the residues were in these cases dissolved in 5–10 cc. alcoholic sodium hydroxide, the alcohol was evaporated to dryness on the water-bath with exclusion of air and the residues were extracted with light petroleum to separate the soaps.) No oxycholesterol was formed on aeration of a mixed cholesteryl oleate-cholesterol suspension.

Considering the well-known rôle of many heavy metal salts as catalysts in oxidation reactions we tried the effect of the addition of small amounts of ferric chloride, ferrous sulphate and cupric chloride. As the cholesterol suspensions are very easily precipitated by addition of such salts, they must be used in very low concentrations. In a strength of 1/100,000 they had no effect on the formation of oxycholesterol in pure suspensions. On the addition of ferric chloride in 0·005 % and 0·001 % concentrations to cholesterol suspensions

containing 0·02–0·04 % phosphatides we observed a formation of oxycholesterol at room temperature and without aeration. The amounts of oxycholesterol formed were relatively small (5–25 %) and irregular, so that we do not regard these results as very definite. Aeration and higher temperature did not increase the oxycholesterol formation in these cases.

V.

The experimental results direct the attention to a certain physical factor which to all appearance is of the greatest importance for the attack of oxygen. It is very improbable that the soaps actually partake in the oxidation reaction. On the other hand the alkali soaps doubtless profoundly influence the colloidal state of the cholesterol. Simple inspection gives clear evidence of this fact. On adding (*e.g.*) 0·02 % sodium stearate to a 0·05 % cholesterol suspension, the mixture, when slowly heated, gradually becomes clearer. There is a distinct diminution of the turbidity at 40°, between 50–60° there is a further very marked clearing of the suspension, and at 60° it is nearly water-clear. Its turbidity equals then approximately that of a pure 0·05 % cholesterol suspension diluted with 7–8 parts of water. The clearness of the suspension contrasts strikingly with the milky appearance of a pure cholesterol suspension at the same temperature. When a cholesterol suspension containing 0·004 % sodium oleate is slowly heated, the turbidity remains practically unchanged up to between 70° and 80°, when it is rapidly reduced. (A 0·02 % sodium oleate solution itself is still rather milky at 90°.)

It should be noted that at the same temperatures at which the clearing of the suspensions takes place, there is—especially in the oleate experiments—a marked increase in the rate of the oxycholesterol formation, an increase which can hardly be an ordinary temperature effect on the rate of the reaction. It may also be mentioned that none of the other colloids tried brought about any visible clarification of the cholesterol suspensions.

There can be no doubt that this marked clearing of the suspensions is referable in the main to an increased degree of dispersion of the cholesterol. This is in itself no remarkable occurrence, the powerful peptising action of the alkali soaps being a well-known phenomenon. The point lies in the observation that parallel with the increased degree of dispersion brought about by the soaps the oxidisability of cholesterol by oxygen is essentially increased[1]. This observation suggests that, provided that the cholesterol exists in a sufficiently high degree of dispersion in water, the substance may require no particular catalyst for a relatively rapid oxidation by oxygen even at body temperature. As the cholesterol in the blood normally exists in a high degree of dispersion, the results obtained support to a limited degree the opinion that cholesterol

[1] In heterogeneous systems the rate of the reaction is a function of the surface area between the phases containing the reacting substances. The degree of dispersion, therefore, strongly influences the rate of the reaction. It can, however, not be excluded that the soaps act catalytically also in some other way than by changing the degree of dispersion of the cholesterol.

may undergo oxidative changes in the blood. The observed inhibition of the soap effect by alkali and the resistance of cholesterol in alcoholic solution against oxygen suggest, *inter alia*, that other factors than the degree of dispersion are of decisive importance.

With regard to the question of the catalysing substances present in impure cholesterol, there are certain evidences of their acting in the same manner as the soaps. The fact that with impure cholesterol it was possible to prepare more concentrated suspensions than with the quite pure substance points to the presence of peptising agents in the impure preparations. The non-formation of oxycholesterol on aeration at 37° for 48 hours (in the experiment of Moore and Willimott) as compared with the liberal amounts of oxycholesterol formed on direct evaporation of the suspensions on the boiling water-bath indicates an abnormal temperature coefficient of the rate of the reaction in the same way as in the soap experiments. Furthermore, in our first series of experiments (p. 1315) with impure cholesterol we often noted a very marked clearing of the suspensions in cases where oxycholesterol was formed. As soaps are claimed to be normal constituents of the bile there is the possibility that the active impurities (in our preparations) were actually soaps. The active principle of the brain extract was not investigated.

As to the failure to get quantitatively reproducible results we think that it is most likely due to the difficulty to reproduce exactly the same colloidal state (or series of states) in the different experiments.

The observations made call for caution against the action of oxygen in the determination of oxycholesterol in blood or tissues. Finally the relative character of the qualitative results obtained with the Lifschütz reaction may be emphasised. It is clear that if greater quantities of the suspensions had been used in the aeration experiments than those employed by us, positive Lifschütz reactions might have been encountered in cases where we obtained negative ones, and conversely if smaller quantities of suspensions had been taken negative reactions might have been found where we got positive ones.

Summary.

The formation of oxycholesterol on heating cholesterol in the air at temperatures slightly above the melting-point or on aeration of cholesterol suspensions is due to the action of the atmospheric oxygen. The observation by Robertson that suspensions of pure cholesterol are, even at boiling temperature, very stable against the oxygen of the air is confirmed. Impurities very stubbornly adhering to the cholesterol catalyse the formation of oxycholesterol on aeration. Alkali soaps have a marked catalysing effect on the oxidation of the cholesterol by molecular oxygen. A series of other hydrophile colloids tried had no catalytic action. The same was the case with a series of higher fatty acids, triglycerides and heavy metal salts. The action of the soaps in all probability is mainly due to their peptising action on the cholesterol. The catalysing impurities probably act in the same way. The observations

made call attention to the great importance of the colloidal state of the cholesterol—especially its degree of dispersion—for the oxidisability of the substance by molecular oxygen.

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