CCXVII. STUDIES IN THE CHOLESTEROL CONTENT OF HUMAN PLASMA.

V. THE NATURE OF THE STEROLS IN BLOOD-PLASMA.

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It is now generally recognised that sterol is excreted in human faeces mainly in the form of coprosterol, and that this is accompanied by smaller quantities of dihydrocholesterol (β -cholestanol) and cholesterol. It was shown long ago by Böhm [1911] and in recent years confirmed by Schönheimer *et al.* [1930], in a series of elegant experiments, that the dihydrocholesterol is excreted by the wall of the large intestine. Schönheimer *et al.* [1930] have further demonstrated the presence of dihydrocholesterol to the extent of as much as 10 % in gall-stone cholesterol; and they also claimed that they had found it in smaller proportion in the cholesterol isolated from various tissues. It is generally assumed that coprosterol is produced by bacterial reduction of cholesterol in the gut, though the two substances are not in the same stereochemical series. Schönheimer *et al.* [1930] have further convincingly shown that both dihydrocholesterol and coprosterol, when administered in food, are not absorbed from the gut into the chyle.

Another sterol, allocholesterol, an isomeride of cholesterol, has been considered as associated with sterol metabolism, firstly because its catalytic reduction *in vitro* gives rise to coprosterol, secondly because Enderlen *et al.* [1928] have shown that it is the only sterol, other than cholesterol, which when fed to an animal leads to any noteworthy quantitative increase in bile acid production.

From these observations it seemed to us desirable to investigate more closely the blood-sterol for evidence of the presence of any of these substances.

In our routine analytical work we had preserved the cholesterol digitonides obtained and these were kept in separate batches according to their origin, whether from free or ester cholesterol, or from plasma, red blood corpuscles or whole blood, from normal or diseased individuals or from pregnant women. The cholesterol from each of these digitonides was recovered by the xylene method of Windaus, and each was submitted to an elaborate process of fractional crystallisation from alcohol. The melting-points of the initial fractions from different sources were 148–149°, subsequent fractions melting at 147–148°, with no appreciable progressive lowering of the melting-point during the fractionation. The residual material in the mother-liquor was small in quantity and dried up to a transparent glassy residue, which still gave the Burchardt and Liebermann colour test, and from which some further very small quantity of cholesterol could be obtained by precipitation with digitonin. The bulk of this residual

material was found to be a resin contained in the commercial rectified xylene which we had used. This was shown by a blank experiment in which the digitonide was omitted—the resin thus obtained yielded an atypical colour change with the Burchardt-Liebermann test. In subsequent experiments, using pure sulphur-free xylene, the amount of the resinous material was almost negligible.

Micro-polarimetric estimations were made of the specific rotation of the various fractions and the results varied between -37.5 and -41.5° . These however being micro-estimations we are not prepared to say how far these variations were due to errors of experiment. The constancy of the melting-point in the successive fractions during crystallisation, and the high negative specific rotatory power make it very unlikely that any appreciable quantity of positively rotatory dihydrocholesterol could be present. The unfortunate contamination of our tail fractions with the xylene resin made it impossible to say that no other sterol was present. We therefore collected large quantities of human blood, mainly from hyperpietic patients. The plasma-cholesterol, free and ester separately, was isolated from these by Fex's method combined with the use of digitonin; cholesterol from the erythrocytes was also obtained in a similar manner. The digitonides thus obtained were decomposed with pure sulphur-free xylene, and the sterols obtained were fractionally crystallised. The tail portions were glassy in character and contained very little xylene resin. The final fractions of sterol were obtained as before by reprecipitation from the glassy mass with digitonin. In this way practically the whole of the sterol present was recovered, and no other sterol than cholesterol could be isolated.

About this time Schönheimer *et al.* [1930] reported on a method of estimating β -cholestanol in the presence of cholesterol based on the fact that cholesterol dibromide, left in solution after bromination of an alcoholic solution of cholesterol, is not precipitated by digitonin, whereas β -cholestanol forms an insoluble digitonide. In this process the alcoholic solution of cholesterol was brominated quantitatively by addition of an alcoholic solution of bromine; the crystalline dibromide which separated after standing in the cold was filtered off and the filtrate treated with an alcoholic solution of digitonin in the usual way; any saturated sterol precipitable by digitonin separated out in 48 hours. This method of bromination seemed at first sight to present considerable advantages over bromination by the method of Windaus, *viz.* precipitation of an ethereal solution of cholesterol by a solution of bromine in glacial acetic acid, because the latter involved the extraction of any dihydrocholesterol that was present, before the precipitation with digitonin—a method used by one of us [Gardner, 1921] in the separation of faecal sterols. In Schönheimer's method, after separation of the cholesterol dibromide, the filtrate is treated with digitonin and allowed to stand in the dark, when the precipitation of β -cholestanol digitonide takes place within 48 hours, and if it is present in quantity it begins to precipitate very quickly.

We made a series of experiments using cholesterol from human blood obtained by venesection, performed mainly for hyperpiesia and right heart failure. The cholesterol used was from total cholesterol of plasma, red blood cells and from whole blood, and in each experiment 2–4 g. were used. In none of these experiments did we obtain the slightest precipitation of a digitonide in 48 hours, and in some cases even in 3 to 4 days. If therefore this method is strictly reliable β -cholestanol was not present in the sterols we had isolated from blood. This agrees with the fractional crystallisation experiments and polarimetric determinations quoted above. On the other hand when the liquid was allowed to

concentrate spontaneously in the dark precipitates gradually appeared. These were filtered off and the filtrates allowed to concentrate further. Precipitation continued during the course of weeks and so further crops were obtained. These precipitates were separated, washed with a little alcohol and much ether, and in some cases were warmed for a few minutes with hot xylene to take out any cholesterol dibromide which might have crystallised out along with the precipitate during concentration. The precipitates were then decomposed with xylene vapour in the usual manner and the product, which still contained some bromo-derivative, was purified by crystallisation, pure cholesterol being obtained, recognised by crystalline form, melting-point and properties of the acetate. The presence of this cholesterol must have been due either to the formation of the dibromide being incomplete in alcoholic solution, even though some excess of bromine had been added, or to the cholesterol dibromide remaining in the alcoholic solution being debrominated by the action of the alcohol during the spontaneous evaporation. This latter explanation of the formation of cholesterol from its dibromide is difficult to account for chemically but it recalls the finding of Windaus and Lüders [1920] as regards the debromination of cholesterol dibromide by an alcoholic solution of sodium acetate. Nevertheless some such action seemed probable because the solution, as it became more concentrated, became more acid and contained increasing quantities of ionic bromine. In order to test this we did the following experiments:

A sample of cholesterol dibromide, m.p. 112°, prepared by bromination in alcohol, was heated in alcoholic solution on an electric bath at about 40°. After 19 hours, aliquot portions were titrated with *N*/10 NaOH and also with silver nitrate. It was found that hydrobromic acid was present to the extent of about 75 % of the theoretical value if the total bromine had been removed as HBr. 3 hours' further heating produced no further change. To the main solution chalk was added to neutralise the HBr, the solution was then filtered, found to be neutral, and was then concentrated on a water-bath to small bulk. On standing, some crystalline matter separated mixed with a brown oil. The crystalline matter was recrystallised twice from methyl alcohol and finally from ethyl alcohol. Cholesterol was obtained in typical crystalline form, m.p. 147–148°, and to the extent of 14 % of the original cholesterol used.

Another sample of cholesterol dibromide, m.p. 124°, prepared by bromination in ether and acetic acid according to the method of Windaus was treated in portions in a similar manner, some on the electric bath, and others at laboratory temperature. The heated samples showed decomposition to the same extent as before, *viz.* to about 75 % of the theoretical maximum; in the unheated samples the action was much slower, and in these specimens the liberated cholesterol was precipitated by digitonin and amounted to 2 to 3 % of the original cholesterol dibromide used, after a period of standing of 2 to 3 days.

Following this evidence of the instability of cholesterol dibromide in alcohol solution at ordinary temperatures we were led to repeat Schönheimer's experiments on gall-stone cholesterol.

5.36 g. of a specimen of cholesterol obtained from gall-stones by extraction with benzene and subsequent recrystallisation from 95 % alcohol were treated by Schönheimer's method in every detail. The cholesterol used had m.p. 148.5°, $[\alpha]_D^{17} - 37.4^\circ$. If we take the specific rotation of cholesterol as -41° and that of β -cholestanol as $+34^\circ$ the rotation observed for this specimen corresponds to a β -cholestanol content of 5 %.

The digitonide precipitate obtained in the first 48 hours after the separation of the dibromide weighed 0.9429 g. This was decomposed by xylene, and after crystallisation 0.0188 g. of recognisable β -cholestanol was obtained. It gave no Burchardt and Liebermann reaction. Subsequent crops from the crystallisation contained no recognisable β -cholestanol. Thus the amount of this

substance obtained equalled 0.35 % of the original cholesterol taken. The original liquid after removal of the first digitonide precipitate, on long standing gradually deposited further quantities of white precipitate which we examined. By treatment with xylene these were found to consist mainly of cholesterol digitonide.

We then repeated this experiment with 24.0 g. of cholesterol obtained in similar manner from mixed gall-stones of various types. This specimen melted at 145–146° and had $[\alpha]_D^{19} - 35.6^\circ$, corresponding to a β -cholestanol content of 7.2 %. This was treated in alcohol with alcoholic solution of bromine, and the dibromide which separated weighed 21.0 g.; hence at least 12 g. of cholesterol dibromide remained in solution in the $2\frac{1}{2}$ litres of alcohol used. Digitonin was added in amount sufficient to correspond with Schönheimer's figures of up to 10 % of β -cholestanol in gall-stone cholesterol. The precipitate obtained in 48 hours weighed 1.1162 g., and the filtrate therefrom was allowed to stand in open beakers in the dark for further periods, and subsequent crops of precipitate on the 5th, 19th and 33rd days were separately filtered off and examined. The initial precipitate, supposed to be β -cholestanol digitonide, was decomposed by xylene and the residue tested to show that decomposition was complete. The sterol, after removal of the xylene, was fractionally crystallised from alcohol, and the first crop weighed 0.1029 g. This gave only a trace of colour with the Burchardt and Liebermann test and had a specific rotation of -4.7° . Evidently the material was β -cholestanol but, reckoning from the rotation, contained about 30 % of cholesterol. It was then brominated by the Windaus method, a slight precipitate which formed was filtered off, the trace of bromine left was removed by sulphur dioxide and the recovered β -cholestanol after recrystallisation from alcohol melted correctly at 142–143° and gave no B. and L. reaction even on standing. We could not isolate any more β -cholestanol from subsequent crops of the first deposit.

The later deposits from the brominated solution, on further examination, yielded white crystalline materials with specific rotations of -36° to -42° , m.p. 130° to 148°, which gave typical cholesterol dibromide on bromination.

If we reckon the whole of the first crop of sterol recovered from the first 48 hours' digitonide precipitate as β -cholestanol then only 0.4 % of β -cholestanol was isolated from the original gall-stone cholesterol.

We can therefore confirm Schönheimer's finding of β -cholestanol in gall-stone cholesterol, though in our specimens the actual identifiable quantity was less than in his specimens. Nevertheless from our experience the method is of qualitative value only, as the digitonide obtained in the first 48 hours is not entirely that of β -cholestanol but contains cholesterol digitonide and possibly some digitonin if this is added in excess. We think the method is unreliable for the examination of small quantities of material, especially as we have shown above that cholesterol dibromide in alcoholic solution is unstable at ordinary temperatures. It seems to us unfair to assume, in the examination of sterols in small quantities of tissues, that any small precipitate of digitonide obtained by Schönheimer's method in 48 hours represents β -cholestanol unless this substance is further specifically identified.

We also made a series of experiments in an endeavour to detect whether any allocholesterol is present in human blood. It seemed possible that by using large quantities of fresh blood, rapidly drying by the addition of plaster of Paris and extracting with ether alone, we might be enabled to extract any allocholesterol largely unchanged along with the other ether-soluble substances. We hoped that by taking the dry ether solution we might be able to reduce any allocholesterol in this material in the cold with platinum black, according to the method of Willstätter and Meyer [1908] to the stable coprosterol, the cholesterol present being reduced to β -cholestanol. The experiments were not successful, as we were unable to get complete reduction, probably as a result of some substance in the ether extract interfering with the activity of the catalyst. We isolated cholesterol and some β -cholestanol from the partially reduced material but could not find

any coprosterol. Unfortunately the ether extract of the dried blood was slightly acid and this probably also militated against a successful result by this method. We hope to return to this investigation shortly.

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