CCXVI. STUDIES IN THE CHOLESTEROL CONTENT OF HUMAN PLASMA.

IV. THE NON-STEROL UNSAPONIFIABLE MATTER OF HUMAN PLASMA.

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SOME years ago Wacker [1912] noted the presence of unsaponifiable matter other than sterols in tissue extracts. He called this material "Begleitsubstanz des Cholesterins" and stated that it gave sterol colour reactions in modified manner. In our analyses of plasma-cholesterol we have always recorded the amount of this non-sterol unsaponifiable matter which we refer to as N.S. Its amount, reckoned as if present in the plasma, varies between 0.15 and 1.2 %. This amount is quite unrelated to the sterol content whether free, combined as ester or total. In the course of these analyses we accumulated about 10 g. of this material and it seemed worth while to submit it to closer investigation. We found that it was easily separable by means of light petroleum into an insoluble portion A and a readily soluble portion B. In individual analyses these portions A and B bore no definite relation to each other.

The insoluble portion A was soluble in alcohol, ether or chloroform and on evaporation it dried to a resin or brittle scale. In chloroform solution with acetic anhydride and sulphuric acid it gave a brownish coloration which sometimes attained a dirty green tinge. It was not volatile in superheated steam nor could it be distilled in a high vacuum. It contained traces of nitrogen and sulphur but no phosphorus. We found that by heating sodium ethoxide in alcoholic solution with ether under a reflux, in similar manner to our usual saponification procedure, the N.S. obtained consisted of material resembling the A portion. This and other experiments suggested that this portion was produced by condensation of the impurities in our reagents with one another and possibly with constituents of plasma. We then made a series of plasma analyses using carefully purified alcohol and ether, when this constituent of the N.S. practically vanished. We therefore did not consider that it merited further investigation.

The portion B soluble in light petroleum was entirely volatile in superheated steam; it could be distilled in high vacuum without decomposition at a temperature of 150 to 220°/1 to 2 mm. After evaporation from light petroleum it was left as a vaseline-like substance and on fractional crystallisation from alcohol, methyl alcohol, ethyl acetate or acetone it yielded crystalline paraffins and also hydrocarbons of liquid consistency. Three different crystalline specimens had melting points 56–57°, 62–63°, and 63–64°. Analysis of representative specimens yielded the following results.

1	H 14.40 %	C 85.35 %	Mol. wt. (Rast)	496.5
2	H 14.66 %	C 85.37 %	"	438
3	H 14.64 %	C 85.21 %	"	340.5

These were obviously hydrocarbons. Some specimens however seemed to contain traces of oxygen suggesting the presence of some fatty acid or higher alcohol *etc.*

It seemed to be highly unlikely, in view of the generally accepted knowledge of the composition of blood-plasma, that paraffins in such quantities as we found could be real constituents of the plasma, and therefore it seemed probable that they were artefacts. We thought that the vaseline used to grease the stopcocks of the separating funnels might have been extracted in our analyses in sufficient quantity to yield these results. Though unlikely we also considered whether liquid paraffin [Channon and Collinson, 1929] frequently administered to hospital patients, could be a source of the N.S. in the blood. We therefore took the hospital liquid paraffin and the laboratory vaseline and redistilled them fractionally in high vacuum as we did the plasma N.S. The paraffinum liquidum yielded only liquid fractions which would not solidify even on freezing, and which appeared to consist of hydrocarbons of the naphthene series. They were quite unlike the material obtained from the plasma N.S. and hence could be eliminated. The vaseline however readily separated into a series of fractions which solidified on cooling and when crystallised from alcohol or acetone yielded hydrocarbons physically precisely similar and of similar composition to those described above. It therefore seemed likely that the tap vaseline was a source of the hydrocarbons in our plasma N.S. It was found that we could get such grease into solution in quantities comparable with those obtained in our blood analyses by extracting dilute caustic soda with ether in separating funnels, using quantities such as would correspond with a definite number of our usual analyses. In order to test whether the whole of the N.S. soluble in light petroleum was of this origin we made a number of plasma analyses with carefully purified alcohol and ether, using ungreased taps in the separators, and when aqueous caustic soda was required this was always carefully filtered lest traces of paraffin waxes used by the dealers for sealing the corks of the alkali bottles could have contaminated the material. The results showed a marked reduction in the amount of N.S. soluble in light petroleum but it was not eliminated completely as the following examples show.

	Amount of plasma (g.)	Part soluble in light petroleum		Part insoluble in light petroleum		Total N.S.	
		g.	% of plasma	g.	% of plasma	g.	% of plasma
1	15.49	0.0083	0.0540	0.0033	0.0210	0.0110	0.0750
2	13.76	0.0063	0.0460	0.0009	0.0060	0.0072	0.0520
3	7.69	—	—	—	—	0.0052	0.0670

A series of N.S. residues of this kind showed considerable diversity of properties. They were generally acidic in character, though not always so, but contained soaps. No. 3, for example, on ignition left an alkaline ash in minute quantity.

Thus we see that a non-sterol substance is always obtained, even after extreme precautions are used, in the analysis of plasma to the extent of 0.05–0.07 % of the plasma, and that this material consists of neutral sodium salts or acid sodium salts of fatty acids.

In order to determine if these substances were produced in our analytical procedure from the plasma-lipin material we made the following experiments, using the same precautions as regards the purity of all reagents and the complete absence of all added grease.

(a) 1.0062 g. of a commercial specimen of cholesterol oleate, which on heating softened at 40°, M.P. 49°, was saponified and yielded 0.057 g. of N.S., of which 0.0539 g. was soluble in light petroleum. 0.0536 g. of this was dissolved in standard alcoholic potash and titrated with standard acid. It was equivalent to 6.0 ml. of *N*/50 acid. The equivalent weight of the N.S. was thus 447 which corresponds closely to a formula of $(C_{18}H_{34}O_2)_2C_{18}H_{33}O_2Na$ or a mixture of free acid and soap in such proportion, the equivalent weight of which is 434.

(b) 1 ml. of ethyl oleate was treated in the same manner. The N.S. was wholly soluble in light petroleum; weight 0.0091 g. It was dissolved in alcoholic potash and corresponded in titration to 3.09 ml. of *N*/50 standard acid. The equivalent of the material was thus 147, which corresponds to a dibasic acid of similar molecular weight to oleic acid (mol. wt. 282), or to a polybasic acid of higher molecular weight, or a monobasic acid of half the size of the oleic acid molecule.

(c) 1 ml. of ethyl palmitate was similarly treated. The N.S. was dissolved in ammonia to form a colloidal frothy solution, and a silver salt was prepared. This weighed 0.0218 g. and on heating yielded 0.013 g. of silver. The mol. wt. of the acid of the silver salt (assuming it to be monobasic) was therefore 74.

(d) A specimen of oleic acid, which had been in stock for some years, was redistilled *in vacuo* at 2–3 mm. pressure, and was collected in three fractions at 160–164°, 164–185°, and 185° and upwards. The first fraction showed a correct titration value; if it were taken two portions, one of which was heated with sodium ethoxide in pure alcohol, the other with alcoholic potash made from pure reagents, and the N.S. was extracted in both experiments.

(1) 1.05 g. of oleic acid yielded 0.048 g. of N.S.

(2) 1.34 g. of oleic acid yielded 0.052 g. of N.S.

Neither of these specimens possessed any acidity whatever.

(e) 0.9998 g. of pure palmitic acid was heated with a solution of 1 g. of sodium in pure ethyl alcohol. The solution remained colourless and little or no deposit formed. The N.S. found was 0.155 g.; this was dissolved in standard alcoholic alkali and found equivalent to 1.1 ml. of *N*/5 acid. Equivalent weight of N.S. is therefore 70.4.

(f) 0.9992 g. of a specially pure commercial specimen of stearic acid was heated with alcoholic sodium ethoxide exactly as in the previous experiment. The solution behaved quite differently, as it became deep brown in colour after heating, presumably owing to impurities. It was then diluted with water and thoroughly shaken out with ether in the usual way. A bulky white precipitate separated out between the ethereal and aqueous layers. It was removed by filtration and was found to be insoluble in water or ether. After washing thoroughly with these solvents it was dried and weighed 1.015 g. It was then dissolved in alcohol giving a soapy solution of alkaline character; on spontaneous evaporation it left a dry crystalline powder. Of this 0.2995 g. was ignited and the sodium present estimated as sulphate, of which 0.0611 g. was obtained. Hence this substance contained 6.61 % of sodium. This would correspond to the formula

which requires Na 6.5 %.

The ether-soluble matter, the N.S., weighed 0.073 g. and was practically all soluble in light-petroleum. The portion thus soluble weighed 0.0714 g.; it was dissolved in alcoholic NaOH and found by titration to be equivalent to 2.21 ml. of *N*/5 acid, yielding an equivalent weight of 158.5.

Thus we see that, after all saponifications of fatty acids or esters thereof under these conditions, unsaponifiable matter is produced and can be extracted. Such material is almost entirely soluble in light petroleum, is either acid or neutral in reaction, and presumably consists of acid salts of fatty acids or soaps. It seems probable that these substances arise from the partial hydrolysis of the soaps, the products of this hydrolysis passing into the ether layer during extraction. This appears to agree with the results of Lester Smith [1928]. Some of the equivalent weights of these acid bodies which we determined are very low, but we do not put sufficient reliance on these micro-estimations to be able to discuss these figures.

CONCLUSION.

There can be little doubt that the so-called N.S. (Begleitsubstanz des Cholesterins) obtained in the analysis of blood-plasma does not contain any sterol compound. The material obtained is really an artefact produced in three ways, firstly by condensation of the impurities in the reagents during saponification, secondly from the grease used in the separating funnel stopcocks, and thirdly from the hydrolysis of the soaps with production of ether-soluble substances in the extraction process.

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