XXI. STUDIES ON THE CHOLESTEROL CONTENT OF NORMAL HUMAN PLASMA. PART II.

THE ATTRACTION OF THE PROTEINS OF PLASMA FOR STEROLS.

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It has long been recognised that the whole of the cholesterol and cholesterol esters cannot be separated from blood plasma and serum by simple shaking with ether.

That, in native serum, the ether-soluble constituents are associated in some way with the serum proteins would appear to follow from the work of Hardy [1905], Haslam [1913] and Chick [1914], and, as they are present in relatively very small amounts they acquire the solubility properties of proteins. As Hartley [1915] has pointed out, when the conditions are reversed, traces of proteins may acquire the solubility properties of the "lipoid" with which they are associated.

As far as we are aware, Bang was the first to suggest that the cholesterol of serum may be in combination with proteins, and most probably with the globulins. A verbal communication by Bang on the subject is quoted by Thaysen [1913], and Bang [1918] gave two analyses of dog's globulin and one of human in which considerable proportions of cholesterol were found, and suggested that this might form a useful subject for further study.

In a series of papers published by Handovsky, Lohmann and Bosse [1925], Bosse [1925], Bosse and Handovsky [1925], Handovsky [1925], Handovsky and Lohmann [1925], these authors determined the total cholesterol in a large number of ox-sera, and found also that on precipitating the globulins by various salts a proportion of the whole sterol was precipitated along with the globulins. They concluded that the globulin has a greater attraction for cholesterol in serum than the albumin, and attempted to gain an idea of the quantity of cholesterol in association with the globulin, and the firmness of the union, by determining the proportion of the whole sterol which can be obtained by directly shaking out the serum with ether under rigid but arbitrary conditions.

Their figures show that when the albumin fraction of the protein is high the amount that can be directly shaken out with ether is high, but when the albumin fraction is low, relatively to the globulin, the percentage of the whole cholesterol that can be shaken out with ether is low. They concluded that in normal ox-sera about 25 % of the total cholesterol is firmly bound to globulin; the rest should be bound to phosphatides. The intensity of this union, measured by the capacity of being directly shaken out with ether, is dependent on the salt and water content of the sera. They took however no account of the fact that cholesterol is present both in the free and ester conditions. Young [1922] on the basis of his polariscopic investigation assumed that the cholesterol esters are associated in a loose way with the serum-albumin. Cholesterol is precipitated with these and can, both from the serum-albumin and also from the serum, be extracted with light petroleum or with ether. Troensegaard and Koudall [1926] obtained some evidence of the existence of cholesterol as a prosthetic group in serum-globulin, from which by simple extraction no cholesterol was obtained, by the fractionation of the acetyl bases of the different blood proteins. From serum-globulin they obtained hydrocarbons $C_{16}H_{28}$ and $C_{18}H_{32}$ which they considered must have originated from a cholesterol complex present in the globulin, since they were not obtained from either globin or albumin.

Much more work will evidently have to be done on this reaction before their conclusions can be regarded as firmly established.

In the same year Theorell [1926] analysed the proteins precipitated from horse plasma by fractional saturation with ammonium sulphate. These fractions, even after dialysis and redissolution, were found to contain considerable quantities of lipins. 77 % of the total cholesterol of the plasma was thus precipitated.

The ratios of the cholesterol to ether-soluble phosphorus of fibrinogen, globulin and albumin fractions were respectively 20.5, 16.9 and 9.7, which may be taken as an indication of the distribution of cholesterol and phosphatides between these fractions. In this connection we may point out that Wacker and Hueck [1913] in very careful experiments with horse blood showed that pure fibrin contains no cholesterol, though commercial samples do contain small amounts.

For some time past we have been working on this problem, and in view of the recent papers quoted above, we think it may be of interest to give an account of our work to date.

Cholesterol is quite insoluble in water, in alkalis and in dilute mineral acids, but Porges and Neubauer [1908] found that colloidal suspensions in water may be prepared by adding a solution of cholesterol in acetone to water and dialysing. They concluded from the properties of the fluid that it was an anodic suspensoid. This work was verified by Partington [1911] who observed that a solution of cholesterol in alcohol gave a colloidal solution when added in small quantities to water with stirring. The alcohol was removed by

dialysis and the colloidal solution was left for several months without very marked deposition. It was immediately precipitated by mineral acids, fixed alkalis and by various salts. We find that similar colloidal suspensions, though more dilute, may be made by dropping an alcohol or an acetone solution into boiling physiological saline. Similar colloidal solutions of esters, such as cholesterol palmitate, may also be readily prepared. Such colloidal solutions are markedly opalescent, whereas plasmas, containing much larger concentrations of cholesterol, are often perfectly clear and transparent to the eye, so that in plasma even if the cholesterol be not in chemical or physical union with other constituents, it must be in a quite different state to that in the artificial suspensions.

Colloidal suspensions of cholesterol, even when very dilute, we find are readily precipitated by half saturation with ammonium sulphate, and the precipitation is complete. For example, 250 cc. of a colloidal suspension of cholesterol in saline, containing 0.0055 g. cholesterol, was half saturated with ammonium sulphate, the precipitate separated and dissolved in ether and estimated by means of digitonin. It gave 0.0246 g. cholesterol digitonide, equivalent to 0.00598 g. of cholesterol. Similar colloidal suspensions of cholesterol palmitate are also flocculated by half saturation with ammonium sulphate, but we are not sure that the precipitation is quite complete. For example, 200 cc. of a suspension of palmitate on evaporation to dryness and solution in alcohol yielded 0.0211 g. cholesterol digitonide, equivalent to 0.00256 %. 250 cc. of the same suspension after half saturation with ammonium sulphate yielded a precipitate which on solution in alcohol and precipitation with digitonin gave 0.0208 g. compound, equivalent to 0.00202 %. Evidently approximately 22 % of the cholesterol palmitate was not precipitated. The actual differences in weight however are not much beyond the limits of experimental error.

If the cholesterol in plasma is in a similar state of colloidal suspension we should expect the whole or almost the whole would be precipitated along with the globulin on half saturation of the plasma with ammonium sulphate.

In order to gain some idea of the proportion of the free cholesterol and cholesterol esters thrown down on simple separation of the proteins by means of ammonium sulphate, 102 g. of plasma from a case of hyperpiesia, which analysis had shown to contain 0.087 % of free cholesterol and 0.166 % in ester form, were diluted with an equal bulk of water, and precipitated by the addition of 204 cc. of saturated ammonium sulphate. The precipitated globulins were filtered and well washed with half saturated ammonium sulphate solution. The filtrate and washings were then saturated with ammonium sulphate, and the precipitated albumins filtered and washed with a saturated solution of ammonium sulphate. The globulin precipitate was then separated into euglobulin and pseudoglobulin by digestion with saturated sodium chloride solution. The proteins thus separated were not dried and weighed, but were at once dissolved in 2 % caustic soda solution and extracted with

ether according to the method of Fex [1920]. The free and ester cholesterol were then determined in the various ether extracts, with the following results.

Table I.

Protein	Wt. of digitonin comp. in g. from		Sterols as % of original plasma			Sterols as % of cholesterol of plasma		
	Free chole- sterol	Ester chole- sterol	Free chole- sterol	Ester chole- sterol	Total chole- sterol	Free chole- sterol	Ester chole- sterol	Total chole- sterol
Euglobulin	0.0826	0.0768	0.0197	0.0183	0.0380	22.6	11.0	15.0
Pseudoglobulin	0.0648	0.1136	0.0155	0.0271	0.0426	17.8	16.3	16.8
Albumin	0.1771	0.3258	0.0422	0.0777	0.1199	48.5	46.8	47.4
			0.0774	0.1231	0.2005	88.9	74.1	79.2

The protein-free filtrates were thoroughly extracted with ether, but no sterol, either free or ester, was obtained. On boiling these filtrates with acetic acid for many hours, and re-extraction, a minute portion of cholesterol was obtained, weighing in the form of digitonin compound only 1.4 mg.

From this experiment it is clear that the whole of the cholesterol and cholesterol esters is thrown down with the proteins on precipitation with ammonium sulphate. There is a loss however of about 20 % of the sterol, in the proportion of about 1 of free to 2.3 of ester. The loss must represent sterol so intimately associated with one or other of the proteins as to be difficult of extraction by shaking the alkaline solution with ether.

Further experiments with other samples of plasma showed that by repeated re-solution and re-precipitation of the protein fractions, the protein could be largely separated from the sterol thrown down with it in the initial precipitation; and also that the difficultly extractable portions were associated rather with the globulins than the albumin.

We therefore decided to obtain a large sample of blood from a normal individual, and subject the plasma to a more thorough fractionation.

Mr C. S. Coleman, a vigorous young man aged 19, in perfect health, volunteered to give us 450 cc. of blood, and we take this opportunity of expressing to him our sincere thanks.

The plasma was light brown in colour, clear, and showed no trace of haemolysis. On analysis it was found to contain 0.0445 % free, 0.1099 % ester and 0.0057 % of cholesterol isolated only by acid hydrolysis. The total was thus 0.1602 %. 200 cc. of this plasma were diluted to 1000 cc. with water and mixed with 1000 cc. of a saturated solution of ammonium sulphate. The globulins were filtered and washed with half saturated ammonium sulphate. The filtrate and washings were then saturated with ammonium sulphate and the albumin filtered and washed with saturated ammonium sulphate. The globulins were then dissolved in physiological saline and the albumin in water, carefully filtered, and made up to 1 litre each. The globulin solution was slightly opalescent and the albumin perfectly clear and transparent. The globulin solution was then again precipitated by half saturation with ammonium sulphate and the globulin washed. The filtrate and washings were

then saturated with ammonium sulphate and the precipitated albumin, after washing, dissolved in water and added to the main albumin solution. The aqueous albumin solution was then half saturated with ammonium sulphate and a small quantity of globulin which separated was added after washing to the main globulin fraction.

The albumin solution was then saturated with ammonium sulphate and the precipitated albumin filtered and washed with saturated ammonium sulphate. This process was then repeated a third time, when the albumin fraction separated from the globulin, and the globulin separated from the albumin fraction, were practically negligible.

The globulins were then re-dissolved in saline, made up to a litre and re-precipitated by half saturation with ammonium sulphate. This process was repeated four times. The albumin was then dissolved in a litre of water, carefully filtered and re-precipitated by saturation with ammonium sulphate. This process was also repeated four times. The final albumin dissolved in water, giving a perfectly clear and transparent solution.

The final globulin precipitate was then digested over night in a considerable bulk of saturated sodium chloride, and thus approximately separated into pseudoglobulin and euglobulin. This process was not repeated more than once.

The free protein fractions were then coagulated by heat in the usual way, thoroughly washed free from salts, dried *in vacuo* over sulphuric acid and weighed.

In this way 0.7129 g. of pseudoglobulin, 2.7077 g. of euglobulin and 10.133 g. of albumin were obtained.

These proteins were allowed to soak in 2 % caustic soda solution until they swelled to jelly, and were then heated on the water-bath with a suitable further quantity of the alkali, until they went into solution, which took half to one hour. After cooling, the alkaline fluids were thoroughly extracted with ether in the manner described for plasma in Part I, and the free and ester cholesterol determined by digitonin. The extracted alkaline fluids were then evaporated to dryness and heated for 5 to 6 hours with glacial acetic acid containing hydrochloric acid. The fluid was then diluted, neutralised and extracted again with ether. After distilling off the ether, the residue was hydrolysed by means of alcoholic sodium ethoxide to saponify any cholesterol acetate. After extraction the cholesterol set free by acid hydrolysis was precipitated by digitonin.

The results are summarised in Table II.

It was noticed that the surface of the filter papers used in filtering the globulin became covered with a varnish-like substance, insoluble in physiological salt solution. The papers were therefore cut up, thoroughly washed with saline, dried and extracted in an all-glass extraction apparatus with ether. On evaporating the ethereal solution, 0.495 g. of a brownish oil was obtained. This on treatment with digitonin yielded 0.2236 g. of compound

It will be noticed from these figures that the different proteins show much the same power of retention for cholesterol in the free as in the ester state. We do not know whether it would have been possible to eliminate all occluded sterol from the pseudoglobulin and the albumin by a more extended resolution and re-precipitation, but it is highly unlikely that this would have been the case with the euglobulin.

It is difficult to form a precise picture from our results, or those of other workers, as to the true relationship between the sterols and the proteins in natural plasma, but apparently there must be a close association with the euglobulin. It is not possible to conclude that the union is chemical in the ordinary sense, but probably there is (in such complex substances) really no sharp distinction between so-called physical adsorption and chemical union of associated substances.

It seems not improbable that changes in the globulin albumin ratio in plasma may have something to do with the variations in sterol content of plasma in disease, and even under normal physiological conditions. We hope however to deal with this in another communication.

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