IX. THE CHOLESTEROL OF THE BRAIN. II. THE PRESENCE OF "OXYCHOLESTEROL" AND ITS ESTERS.

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In a previous communication [1906] I was able to show, by the methods then available, that Baumstark's statement with regard to the occurrence of cholesterol esters in brain must be considered erroneous. My observations were in accordance with those of R. Bünz [1905], whose work was published a few months before mine. The methods used by Bünz and by myself were of an indirect nature, depending on variations in melting points etc., and my failure to isolate cholesterol esters from the oily mother liquors of the brain-cholesterol might be ascribed to the well-known difficulty inherent in this procedure. Since this work has been carried out, Windaus [1910] has described a quantitative method for the estimation of free and combined cholesterol, which depends on the insolubility of the digitonin-cholesterol compound. It seemed therefore of interest to reinvestigate the question of the presence of cholesterol esters in brain by the help of this new method. This was all the more desirable as indications of their presence may be taken from statements occurring in the recent literature on the subject. (Lapworth [1910], Lorrain Smith and Mair [1911], Lifschütz [1913, 1].)

Cholesterol esters are now usually estimated in an indirect way, i.e. by calculating as combined cholesterol the difference between the free and the total cholesterol content by Windaus' method, estimated in relatively small aliquot parts of suitably prepared extracts before and after saponification. As my previous work showed conclusively that cholesterol esters, if present at all in brain, would be found only in exceedingly small amounts, this indirect method of estimation does not offer much hope of success, as the limits of error of the method are greater than the possible differences to be looked for. It was therefore decided to work up the whole brain and to

remove the cholesterol completely by precipitation with digitonin, leaving the esters, if present, in the filtrate. One half of the filtrate was to be used for the actual preparation of the esters, whilst the other half was to be saponified, and the cholesterol, originally present as ester, precipitated with digitonin.

Although a whole adult human brain was examined in this way for cholesterol esters, no trace of them could be found. The same result was obtained with a child's brain. In both cases, however, digitonin produced a distinct weighable precipitate in the final solution. The examination of this precipitate brought out the fact that the substance in question certainly did not consist of cholesterol. The substance recovered from its digitonin compound, in contradistinction from cholesterol, gave with glacial acetic and sulphuric acid a violet colour, which on the addition of ferric chloride turned to a bright emerald green. This green solution, on spectroscopic examination, showed a very characteristic band in the red (between λ 630 and λ 650), which has been described by Lifschütz [1913, 1] as typical for the compound which he termed "oxycholesterol." According to him, "oxycholesterol" is present in blood and in most organs with the exception of the liver and bile¹ (see also Unna and Golodetz [1909], Lifschütz [1913, 2], Schreiber and Lénard [1913, 1 and 2]).

Lifschütz ascribes to this chemically ill-defined substance an important rôle in the metabolism of cholesterol, which seems to deserve more attention than it has hitherto received, the discussion of which, however, is outside the scope of this communication. The results of the present investigation in any case seem to justify the conclusion that in human brain a substance of this nature exists mainly in the form of esters.

EXPERIMENTAL.

For the first experiment the brain of a child of three months was used. Cause of death, pneumonia; weight of brain, 512 g. After removal of the membranes, the brain was finely minced. A water estimation was made in a sample of the mixed brain and showed 85.8 per cent. In order to obtain an idea as to the amount of digitonin necessary for the complete removal of cholesterol from the brain extracts, the percentage of cholesterol in the brain was estimated quantitatively. As the application of Windaus' method to the brain is complicated by the presence of the large amounts of lipoids, the

¹ In this connection it may be mentioned that, according to my observations, gallstones contain "oxycholesterol." If this result should be confirmed by a more extensive investigation, it might have a considerable bearing on the problem of their formation.

following method was used, which represents a combination of O. Rosenheim's method for the preparation of cholesterol [1906] with that of Windaus for its quantitative estimation. As the same method was used in the subsequent experiments it may be described here in detail.

Five grams of minced brain were mixed with 15 g. of plaster of Paris¹. After the mass had set hard, it was broken up and the drying completed in a desiccator. The brain plaster mixture was then extracted with acetone in a Soxhlet apparatus for several days, the extraction flask being changed repeatedly. The extraction was considered complete when the last extract after evaporation of the solvent left only a trace of residue, which did not give any precipitate when digitonin was added to its alcoholic solution.

The combined acetone extracts were evaporated to dryness, the residue being taken up in 70 cc. of 95 per cent. alcohol, filtered and precipitated hot with a large excess (35 cc.) of a 1 % solution of digitonin (Merck) in 90 % alcohol. The contents of the flask were kept boiling for a few minutes and the precipitate allowed to settle at room temperature over-night. The digitonin-cholesterol was filtered on a weighed Gooch crucible, washed with alcohol and ether and dried to constant weight in a toluene oven at 105°. From 5 g. of fresh brain was obtained 0.1435 g. digitonin-cholesterol (mean of two analyses). According to Windaus the amount of cholesterol contained in digitonin-cholesterol is found by multiplication with the factor 0.2431; therefore the fresh brain contained 0.70 per cent. cholesterol.

The bulk of the minced brain was put into 1 litre of acetone, allowed to stand overnight and then filtered through a Buchner funnel under pressure. Five subsequent extracts each with 500 cc. cold acetone were made, and the extraction then continued three times with 800 cc. boiling acetone. Previous experience with other organs had shown me that by this procedure cholesterol and cholesterol esters were completely removed.

The dry residue of the combined extracts was treated repeatedly with boiling 95 % alcohol and filtered hot. An alcoholic extract, amounting to 1 litre, was thus obtained, which contained the whole of the cholesterol, free and combined, present in the brain.

The total quantity of free cholesterol contained in the whole brain, as calculated from the result of the quantitative estimation (see above), amounts to 3.5 g., which would require about 10.5 g. of digitonin. In order to ensure complete precipitation, 13 g. of digitonin dissolved in 1300 cc. of 90 % alcohol were added to the hot alcoholic brain extract. A copious precipitate formed and was allowed to settle over night. The complete removal of cholesterol was tested for by the formation of a precipitate on adding a few drops of an alcoholic solution of cholesterol to a sample (a few cc.) of the clear supernatant fluid. The precipitate was well washed with 90 % alcohol and with ether. A clear filtrate was thus obtained which should contain the whole of the cholesterol esters present in brain.

¹ It is essential that the best quality of plaster of Paris should be used. The "superfine" quality as sold by makers of plaster-casts was found to be suitable.

The filtrate was heated on the water-bath until most of the alcohol had evaporated and then transferred to an automatic extraction apparatus (Maassen's), in which it was extracted for many days with a mixture of ether and petroleum ether until the extraction was complete.

The ethereal extract was evaporated, the residue taken up with boiling absolute alcohol and saponified by boiling with sodium ethylate. Water was added and the saponified extract transferred again to the extraction apparatus. The final ethereal extract, which should contain the cholesterol of the whole of the cholesterol esters, was again evaporated, taken up in 70 cc. of hot 95 % alcohol and 70 cc. of 1 % digitonin solution were added. There was no immediate precipitate, but after standing for some time at room temperature a small flocculent precipitate settled, which appeared to be quite different from the crystallised digitonin-cholesterol as usually obtained. The precipitate was treated in the usual way and when dry weighed 0.2131 g.

It will be shown in connection with the next experiment that this precipitate did not consist of digitonin-cholesterol, but it is of interest to note that on the assumption that one quarter of it represents cholesterol, the percentage of combined cholesterol in the whole brain would only amount to 0.01 per cent.

In the absence of any evidence to the contrary, it may be argued that cholesterol esters, even if absent in a child's brain, may be present in the adult human brain.

In the next experiment, therefore, the whole of an adult human brain was treated in a similar manner. The brain of a man (cause of death, heart disease) weighing 1270 g. contained 78.9 per cent. of water and 1.95 per cent. of free cholesterol (mean of two determinations) estimated as in the previous experiment.

The extraction was carried out as in the first experiment, ten cold and three hot extractions with acetone having been made. In order to economise the valuable digitonin, of which at least 80 g. would have been necessary, a large bulk of the free cholesterol was allowed to crystallise out from the combined extracts. As the first crystallisation might possibly carry down any cholesterol ester present, the product was recrystallised twice from a mixture of alcohol and acetone, the mother liquors being added to the main extract. In this way, 21 g. of pure cholesterol were separated.

To the final extract, dissolved in 95 % alcohol, 850 cc. of 1 per cent. digitonin solution were added and the precipitated digitonin cholesterol removed as previously described. The whole of the free cholesterol was thus removed. The resulting filtrate was extracted with ether in the Maassen's

extraction apparatus as above described. One half (200 cc.) of the ether extract was reserved for the isolation of the esters as such, and the other half was saponified as before, and again extracted with ether. The final residue dissolved in 50 cc. of 95 % alcohol was precipitated with 70 cc. of 1 % digitonin solution. The formation and appearance of the precipitate resembled closely that observed in the previous case. The dry precipitate weighed 0.2635 g. The whole brain would therefore yield 0.5270 g. of this substance. Assuming again that one quarter of it consists of cholesterol, the percentage of combined cholesterol in the whole brain would amount to 0.01 g. per cent., i.e. exactly the same figure as calculated for the child's brain.

Examination of the digitonin compound.

Having obtained a weighable amount of a digitonin compound in the two cases, it became necessary to investigate it further and to establish its identity or otherwise with digitonin-cholesterol. Windaus has already shown that, by means of boiling xylene, cholesterol can be recovered from its combination with digitonin. Before subjecting the substances obtained from brain to this treatment, it was thought advisable to test the reliability of this method when dealing with such small quantities as are obtained from brain. For this purpose 0.26 g. of digitonin-cholesterol, as prepared by direct precipitation of a brain extract, was treated in a suitable extraction apparatus with boiling xylene for 7.5 hours. From the xylene extract, cholesterol was isolated in the typical crystals, without any difficulty.

The digitonin precipitates, obtained from brain after the removal of free cholesterol, were then treated separately with xylene in the extraction apparatus. In both cases I was unable to detect any cholesterol crystals. The xylene extract left on evaporation an oily residue (approximately 35 % of the digitonin compound), in which under the polarising microscope some anisotropic sphero-crystals could be detected. All attempts to bring this residue to crystallisation were unsuccessful.

The behaviour of the substance suggested a similarity to the product described by Lifschütz [1913, 1] as "oxycholesterol," which according to him also occurs as ester in ox brain. With this name Lifschütz designates for convenience sake the primary neutral oxidation products of cholesterol, which are as yet insufficiently defined chemically, but which under certain conditions give rise to a colour reaction possessing a highly characteristic absorption spectrum. By means of this spectroscopic behaviour "oxycholesterol" can not only be detected in the presence of cholesterol, but its quantity may be

estimated spectrometrically by comparison with a standard "oxycholesterol" solution.

In order to compare the substances obtained from brain with "oxycholesterol," I prepared some of this substance according to Lifschütz by oxidising pure cholesterol with benzoyl peroxide in glacial acetic acid solution. Following scrupulously the detailed directions of Lifschütz, there is no difficulty in obtaining the sticky amorphous product as described by him. This was boiled with alcoholic potash, extracted with ether and the residue from the ether extract purified by means of methyl alcohol. After careful evaporation of the methyl alcohol and drying *in vacuo*, the product obtained was a yellowish brittle resin, insoluble in water, but soluble in all the usual organic solvents.

This substance gave the typical reaction as described by Lifschütz. One milligram dissolved in chloroform gave with glacial acetic acid and sulphuric acid the first phase of the Lifschütz reaction (violet), which changed almost instantaneously on the addition of ferric chloride to a bright emerald green, showing a well defined band in the red between λ 630—650. A cholesterol solution remained perfectly colourless under the same conditions¹. When these tests were carried out with the substances isolated as above described by means of their digitonin compound from brain, it was found that they gave the typical "oxycholesterol" reactions, even in minute quantities. One milligram of the substances gave a strong Lifschütz colour reaction, and the green solution showed the well marked typical absorption band in the red.

From some preliminary experiments, in which the coloured solutions were compared with a standard "oxycholesterol" solution in a Zeiss comparison spectroscope, it seems that their spectroscopic value is at least as high as that of the standard.

It is evident therefore that "oxycholesterol" exists in the brain in the form of esters and that free "oxycholesterol" is precipitated from its alcoholic solution by digitonin. This latter fact has also been demonstrated by Lifschütz [1913, 3].

¹ In conjunction with Dr O. Rosenheim I have found another characteristic reaction of "oxycholesterol" which may be carried out with the dry substance. If a minute quantity of dry "oxycholesterol" (or the residue of a solution to be tested) is put on a slide or watch-glass and a drop of methyl sulphate (techn.) is added, a brilliant purple colour is at once produced. Pure cholesterol remains unchanged under these conditions, but on warming or on prolonged standing it develops a bright cherry red colour. The reaction is not given with pure methyl sulphate. The reason for this as well as the characteristic behaviour of cholesterol, oxycholesterol and other substances towards methyl sulphate in solution and in the presence of ferric chloride etc. will be described in a subsequent communication.

With regard to the quantity of the esters present we may form an approximate estimate from the amount of the digitonin compound weighed as such. If we assume that in analogy to digitonin-cholesterol it contains a quarter of its weight of "oxycholesterol," the amount of the latter as ester would be 0.01 % in the fresh brain. Its amount, however, may be larger as we have at present no experimental data as to the completeness of its precipitation by digitonin.

An unsuccessful attempt was made to isolate the esters as such from the solution reserved for this purpose (see above, p. 78). Just as is the case with free "oxycholesterol," so also its esters are evidently not readily crystallisable [see also Lifschütz, 1913, 3]. Various solvents were tried, but the substance did not show any tendency to crystallise, although under the polarising microscope some anisotropic needles could be detected in it. It gave no reaction for free "oxycholesterol," but did so readily after saponification.

In this connection an experiment may be mentioned which I carried out in order to satisfy myself that "oxycholesterol" is not an artifact, i.e. a product formed from cholesterol esters during the lengthy process of saponification, etc. For this purpose 0.25 g. pure cholesterol palmitate was carried through the process in exactly the same way as the solution of esters described above. The product finally obtained did not give any trace of an oxycholesterol reaction.

The occurrence of free "oxycholesterol" in brain.

As the above results have clearly demonstrated the existence of "oxycholesterol" esters in brain, it seemed of interest to search for the presence of free "oxycholesterol." This substance forms, just like cholesterol, a compound with digitonin (Merck)¹, and it might therefore be expected that any free "oxycholesterol" present would be found in the primary digitonin precipitate. Its presence in this can be easily shown if a small quantity be dissolved in warm glacial acetic acid. After cooling, the solution gives

¹ This fact makes it unfortunately impossible to accept unconditionally the results of cholesterol determinations according to Windaus' method, unless the digitonin compound after weighing is tested qualitatively, and if necessary, quantitatively, for "oxycholesterol." The question becomes still more complicated since Windaus and Schneckenburger [1913] have recently discovered that "crystallised digitonin" contains, besides digitonin, in variable quantities, an amorphous glucoside called gintonin. It is possible that "oxycholesterol" is precipitated by "crystallised digitonin" only owing to the presence of gintonin. This would explain the contradictory statements of Lifschütz [1913, 3] and of Schreiber [1913], according to whom "oxycholesterol" is not precipitated by purified digitonin. In any case, the whole method of cholesterol estimation in tissues by means of digitonin seems to require further investigation.

Lifschütz's reaction. The dry digitonin-oxycholesterol compound also gives the methyl sulphate reaction mentioned in the foot-note on p. 79.

By means of these reactions I was able to show the presence of "oxycholesterol" in the adult brain referred to above, but not in the brain of the child.

This result seemed suggestive if we remember that in the brain of new-born children the cholesterol and galactoside percentage is known to be low, whilst the percentage of unsaturated phosphatides is high as compared with an adult's brain. One might assume that a similar relationship holds good for "oxycholesterol." In order to test this suggestion still further, I examined the extracts of another adult human brain, of a child's brain (5 days old) and that of a human foetus (36 weeks old). Here again the adult brain gave a strong "oxycholesterol" reaction, whilst that of the child gave a negative result. The brain of the foetus gave a positive reaction.

Before coming to a final conclusion with regard to this question, however, more experiments seem to be necessary.

CONCLUSIONS.

(1) The examination of the whole human brain by means of Windaus' method has shown the complete absence of cholesterol esters.

(2) Human brain contains "oxycholesterol" esters to the extent of at least 0.01 per cent.

(3) Free "oxycholesterol" seems to be present in adult human brain, but not in the brain of young children.

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REFERENCES.

- Bünz, R. (1905), *Zeitsch. physiol. Chem.* **46**, 47.
 Lapworth, A. (1910), *J. Path. Bact.* **15**, 254.
 Lifschütz, I. (1913, 1), *Biochem. Zeitsch.* **48**, 373.
 — (1913, 2), *Biochem. Zeitsch.* **52**, 206.
 — (1913, 3), *Biochem. Zeitsch.* **54**, 212.
 Rosenheim, M. C. (Tebb, M. C.), (1906), *J. Physiol.* **34**, 106.
 Rosenheim, O. (1906), *J. Physiol.* **34**, 104.
 Schreiber, E. (1913), *Münch. med. Woch.* **60**, 2001.
 — and Lénard (1913, 1), *Biochem. Zeitsch.* **49**, 458.
 — (1913, 2), *Biochem. Zeitsch.* **54**, 291.
 Smith, J. Lorrain, and Mair, W. (1911), *J. Path. Bact.* **16**, 131.
 Unna, P. G. and Golodetz, L. (1909), *Biochem. Zeitsch.* **20**, 469.
 Windaus, A. (1910), *Zeitsch. physiol. Chem.* **65**, 110.
 — and Schneckenburger, A. (1913), *Ber.* **46**, 2628.