ON THE LIPOIDS OF ANCIENT EGYPTIAN BRAINS.¹

By W. MAIR.

From the Pathological Laboratory, University of Manchester.

THROUGH the kindness of Professor Elliot Smith I had placed at my disposal samples of ancient brain material dating from different periods, and it was thought that the analysis of these by the newer methods might throw an interesting light on the changes which the brain lipoids undergo during the lapse of ages. The material of which the largest quantity was available was Coptic, from the Christian cemetery, Island of Biga, date 400 to 600 A.D.; of the older specimens only small samples could be obtained. For the sample of Coptic brain of which a quantitative analysis is given below I am indebted to the kindness of Dr. Armand Ruffer.

Cholesterin was first isolated from mummy tissues by W. A. Schmidt (1907¹, 1908²). Professor Schmidt informs me that he found both the Coptic and the prehistoric brain material rich in cholesterin. Abderhalden (1911³) also has isolated cholesterin from ancient brain.

In my first observations I used the method described by Lorrain Smith and Mair (1913⁴) for the analysis of brain lipoids, but as it soon appeared that the chloroform extract consisted chiefly of fatty acids, that it contained no phosphorus (or only traces where the extraction had been very prolonged), and no cerebroside, it became clear that somewhat modified methods were more applicable. In some cases it was found that complete extraction with boiling chloroform was very slow, and the method finally adopted was as follows:—

A weighed sample of the material was transferred to a Soxhlet thimble and extracted continuously in the hot extraction apparatus for twenty-four hours, first with boiling methyl alcohol and then for a second period of twenty-four hours with boiling chloroform. The residue in the thimble was dried and weighed and the weight of the extract calculated by difference. The united extracts were transferred to an evaporating dish, boiled on a water bath, and neutralised with a measured quantity of N/2 barium hydrate in 80 per cent. methyl alcohol. As the extract is of a dark brown colour, the end-

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point was determined by transferring a drop to phenol-phthalein paper. It was noted that some ammonia was given off from the hot extract even before the addition of baryta. During neutralisation ammonia came off freely. The alcohol and chloroform were boiled off; the residue was dried, transferred to a Soxhlet thimble, and extracted for twenty-four hours with boiling acetone.

The acetone solution was of a light yellow colour, while the barium soaps left in the thimble were deeply pigmented. On standing, an almost white precipitate settled out from the cold acetone; this was separated, washed with acetone, dried, and weighed. The filtrate and washings were evaporated and the residue weighed.

The nature of the substance insoluble in cold acetone will be discussed below. The cold acetone soluble substance was used for the estimation of free cholesterin and cholesterin present as esters soluble in cold acetone.

The total acetone extract subtracted from the methyl-alcohol-chloroform extract gives the weight of fatty acids, amido-acids, ammonium soaps, and pigment retained by the baryta. Since the amount of barium used is known, an approximation to the mean molecular weight of the fatty acids, etc., can also be obtained.

ISOLATION OF CHLORESTERYL ESTERS INSOLUBLE IN COLD ACETONE.

We may now consider the nature of the cold acetone insoluble substance mentioned above.

In a preliminary analysis of 10 grms. of Coptic brain only 0.72 per cent. of free cholesterin was found by the digitonin method. On the other hand, the cold acetone insoluble substance amounted to more than 7 per cent. After treatment with a mixture of hot methyl alcohol and chloroform it was obtained as a pure white waxy substance giving the cholesterin reaction with sulphuric acid, and containing neither nitrogen nor phosphorus. It showed a fluid crystalline phase which cleared at 78° C. After boiling for four hours in N/5 methyl-alcoholic baryta it was recovered unchanged. On boiling for sixteen hours in alcoholic potash it yielded over 50 per cent. of cholesterin. The acetone insoluble substance therefore consists of cholesteryl esters which are not saponifiable by methyl-alcoholic baryta.

In order to obtain these esters in larger amount 75 grms. of Coptic brain were extracted, and a yield of 6 grms. of the esters in a fairly pure condition was obtained. About 2 grms. of this were hydrolysed with alcoholic potash and the fatty acids separated. The fatty acids were distilled *in vacuo* in a bent test-tube, and by this means a partial separation was effected. The more volatile acid separated out in small amount as a fine white deposit on the side of the test-tube. This gave a melting-point of 61° C. (palmitic acid, M.P. 62°-6). The bulk of the distillate solidified in a mass which could be easily separated. After a second distillation this portion also showed a melting-point of 61° C., but its boiling-point at 100 m.m. pressure was 290° C. A sample of Kahlbaum's stearic acid tested under the same conditions gave the same boiling-point.

The remainder of the sample of insoluble esters was recrystallised from absolute alcohol, it was then boiled with methyl-alcoholic baryta and finally purified by repeated recrystallisation from ethyl acetate. A finely crystalline product was obtained with melting-point 80° C. On hydrolysis this yielded only one fatty acid with melting-point 68° C. (stearic acid, M.P. 69°-2) and boiling-point at 100 m.m. 290° C.

As I (1913⁵) have shown, the melting-point of pure cholesteryl stearate, prepared from stearyl chloride, is 85° C., and this ester is unaffected by boiling with methyl-alcoholic baryta. When a little of the natural ester (M.P. 80°) was added to a sample of the synthetic ester (M.P. 85°) the mixture melted sharply at 84° , and the crystalline appearance as seen on the stage of the microscope was not altered. There can therefore be little doubt as to the identity of the two substances, the lower melting-point of the natural ester being accounted for by the presence of traces of the palmitate.

The cold acetone insoluble substance therefore consists chiefly of a mixture of cholesteryl stearate, and palmitate, the stearate being present in much the larger amount.

Coptic brain contains from 7 to 14 per cent. of these esters, and less than 1 per cent. of free cholesterin. In the quantitative analyses given below, the cholesterin present as insoluble esters is reckoned as 50 per cent. of the total cold acetone insoluble substance. This is a sufficiently close approximation to the truth; cholesteryl stearate contains 59 per cent. of cholesterin, and some allowance is thus made for the presence of impurities.

ESTIMATION OF CHOLESTERIN FREE, AND IN CHOLESTERYL ESTERS SOLUBLE IN COLD ACETONE.

After evaporating off the acetone, the residue was dissolved in boiling 95 per cent. alcohol and precipitated with excess of digitonin. The filtrate was evaporated to dryness and treated with ether to separate the excess of digitonin; the ether soluble portion was then evaporated and boiled for several hours with methyl-alcoholic potash. It was then acidified with hydrochloric acid and brought back to the neutral-point with methyl-alcoholic baryta, dried and extracted with hot acetone in the Soxhlet. The acetone extract was filtered cold to get rid of traces of soaps and then evaporated, taken up with 95 per cent. alcohol, and precipitated with digitonin. After again separating the excess of digitonin there was left in each case a small amount of an oily unsaponifiable residue. Since cholesteryl oleate is soluble in cold acetone it is not improbable that this ester is present in the cold acetone extract, but an attempt to isolate it in a pure condition from the Coptic brain was not successful.

It may here be remarked that the amounts of cholesterin found by the two methods described above were very small in the case of the older brains, but in each case some definite evidence of the presence of cholesteryl esters was obtained. Thus in the prehistoric sample (4000 B.C.), although the cold acetone insoluble substance amounted only to a few milligrammes, it showed, when examined on the hot stage of the microscope, a fluid crystalline phase clearing at 115° C. In the sample dating from 3000 B.C. it showed a fluid crystalline phase clearing at 76° C. In the sample dating from 600 B.C. the evidence for the presence of insoluble esters was not so

good, but in this case quite an appreciable amount of esters soluble in acetone was present.

In addition to the above analyses of the lipid constituents, estimations of nitrogen and phosphorus were made by the Kjeldahl and Neumann methods in the residue and in the chloroform extract or in the original material. Finally, the ash in the residue was estimated by incineration in a silica crucible with the addition of nitric acid to complete the combustion.

Similar estimations were made of a recent brain, dried after fixation in formalin, for comparison with the ancient material.

The results of the quantitative analyses expressed in percentages of the dried brain are given in Table I.

TABLE I.—Percentages of Dried Brain Material.

	Prehistoric, 4000 B.C.	Old Kingdom, 3000 B.C.	Persian vason, 600 B.C.	Coptic, A.D. 500.	Recent.
Extract	36	36	61	78	46
Residue	64	64	39	22	54
Cholesterin { Free	0	0.18	0.30	0.40	11.3
As { Soluble in cold	0.02	0.02	0.30	1	0
Esters { acetone					
Insoluble in cold	0.02	0.48	0.23	7	0
acetone					
Total	0.04	0.68	0.83	8.4	11.3
Fatty acids, amido-acids, etc.	35	31	53	52	0
Molecular weight of do. (stearic acid = 1)	0.78	0.88	0.89	1.27	...
Nitrogen { Lipoid	...	1.13	1.90	3.39	0.67
Residual	3.35	5.06	3.31	1.78	8.96
Total	...	6.19	5.21	5.17	9.63
Phosphorus { Lipoid	0	0	0	0	0.82
Residual	0.13	1.80	0.80	0.29	0.45
Total	0.13	1.80	0.80	0.29	1.27
Ash in residue	7.25	9.53	3.31	1.59	2.01

In order to follow the changes which have occurred in the various constituents, it is desirable to have some idea of the amount of the original dried brain represented by the samples examined. The only figures which appear likely to give information on this point are those for the ash. On the assumption that the percentage of ash in each sample was originally the same as in the sample of modern brain the figures given in Table II. are obtained, representing percentages of the various constituents calculated on the original dried brain from which they were derived.

The results of these analyses are in several ways remarkable and unexpected. It appears at once that the Coptic brain takes a position by itself. In it there has been practically no disappearance of

TABLE II.—*Percentages of the Dried Brain originally present, calculated from the Ash.*

	Prehistoric, 4000 B.C.	Old Kingdom, 3000 B.C.	In- vasion, 600 B.C.	Coptic, A. D. 500.	Recent.
Extract	10	8	37	99	46
Residue	18	13	24	28	54
Cholesterin	0·012	0·14	0·51	10·7	11·3
Nitrogen {	...	0·24	1·16	4·32	0·67
	Lipoid	0·94	1·06	2·02	8·96
	Residual	...	1·30	3·18	9·63
Phosphorus	0·036	0·38	0·57	0·37	1·27
Ash	2·01	2·01	2·01	2·01	2·01
Amount of sample equivalent to 100 parts of original	28	21	61	127	100

cholesterin, but whereas in the recent brain all the cholesterin is free, in the Coptic it has undergone almost complete esterification. In the next older sample (600 B.C.) 95 per cent. of the cholesterin has disappeared. On the other hand, in the Coptic brain 70 per cent. of the phosphorus has disappeared, while in the older sample (600 B.C.) only 55 per cent. of the phosphorus has gone. These results seem to indicate a difference in the conditions under which the material has been preserved. As a matter of fact, the Coptic bodies were packed in salt before burial in the dry sand, while the older specimens were simply buried. In neither case has there been any access of water. The increase of weight which has occurred in the Coptic material is presumably due to oxidation. The reduction in the residual nitrogen may be taken as a measure of the amount of putrefactive change which has occurred. This is much the same in the Coptic and in the next older sample, and there is apparently a progressive diminution in the nitrogen at a slower rate after putrefaction has ceased. It will be observed that a large amount of the nitrogen set free from the residue has been retained in the lipid extract. This is most marked in the Coptic material, and no doubt accounts for the high percentage of extract and the high molecular weight of the fatty acid compounds. There is also a progressive disappearance of phosphorus and cholesterin in the older samples. The disappearance of phosphorus is difficult to account for, especially as it seems to have proceeded after all putrefactive processes had stopped. With regard to the disappearance of cholesterin, it is of interest to note that on heating this substance with stearic acid it is easy to decompose it with the formation of volatile products and pigment which strikingly resemble that present in the lipid extract of the ancient brains (1913⁵). In both cases the dark brown solution shows green fluorescence and no definite absorption

bands. It has been suggested that the effect of time on chemical reactions is the same as that of heating—that is to say, a reaction which takes place in a short time at a high temperature will take place at low temperature in the course of ages. The combination of cholesterin with stearic acid and the subsequent destruction of the cholesterin in the ancient brains illustrates the truth of this. It is an experiment which can readily be carried out in a short time at a high temperature, but there is an interesting difference to be noted in the nature of the cholesteryl ester formed in the two cases. As has already been shown, the naturally occurring ester corresponds to that made synthetically from the acid chloride, whereas on heating cholesterin with the acid it is the more easily saponifiable ester which is formed (1913 ⁵).

In conclusion, I wish to express my thanks to Professor Lapworth for much kind assistance and advice during the course of this investigation.

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