XVI. THE CHOLESTEROL CONTENT OF HUMAN MILK.

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THE following work on the nature and composition of the "unsaponifiable" constituents of human milk fat was undertaken originally to supply data required for some experiments on cholesterol metabolism in breast-fed infants.

It has long been known that cholesterol, free or in ester form, occurs in human milk, but the quantitative figures published in the earlier literature were little more than estimations of the crude unsaponifiable matter left on hydrolysis of the fat. This so-called unsaponifiable matter consists only in part of cholesterol. The rest consists of low melting oily substances containing oxygen, and of somewhat similar empirical composition to sterols, with perhaps traces of sterol esters. It also contains more or less resinous matter produced in the hydrolytic process [Gardner and Fox, 1921]. It is only in recent years since the development of colorimetric methods and more particularly of the digitonin method of estimating cholesterol, that comparatively reliable quantitative results have been obtained.

In 1918, Denis and Minot published [1918] a series of analyses in which the cholesterol was determined by Bloor's colorimetric procedure [1916], and the fat by a nephelometric method also devised by Bloor. Most of these results refer to late periods of lactation. In Table I we give their figures arranged in descending values of cholesterol content.

In the same year Beumer [1918] published two analyses by the Windaus digitonin method, respectively 0.0105 and 0.011 % total cholesterol.

In 1920 Gamble, using a modification of Bloor's colorimetric method gave, as an average of ten samples, 0.013 %, but without details of individual samples [1920]. The most detailed study has been made by Wacker and Beck [1921] using the digitonin procedure of Windaus. Most of their results refer to late periods of lactation. In Table II we give their results in the order of descending values of cholesterol.

A consideration of the modern literature on the composition of human milk shows that there is some variation in the proportion of the various constituents with the duration of lactation; for instance the percentage of fat appears to increase, of protein to decrease, of the ash to decrease, whilst

Table I. [*Denis and Minot, 1918.*]

Duration of lactation	Fat in 100 cc. milk	Cholesterol in 100 cc. milk	Duration of lactation	Fat in 100 cc. milk	Cholesterol in 100 cc. milk
330	5.4	0.038	14	5.2	0.018
—	6.8	0.031	28	3.6	0.018
330	8.0	0.029	30	4.2	0.018
30	7.2	0.028	330	5.4	0.018
330	8.0	0.028	21	1.8	0.017
90	3.8	0.027	28	4.5	0.016
—	5.2	0.026	60	2.9	0.016
60	6.6	0.026	120	1.2	0.016
60	5.5	0.024	120	2.1	0.016
42	8.0	0.024	49	3.2	0.016
35	7.1	0.024	24	2.4	0.015
63	5.4	0.024	29	1.4	0.015
330	2.5	0.024	25	2.0	0.014
21	—	0.023	28	2.6	0.014
275	5.5	0.023	210	2.2	0.014
30	5.6	0.022	25	1.6	0.014
14	6.8	0.022	5	1.5	0.013
150	2.7	0.021	330	1.5	0.013
240	—	0.020	56	0.8	0.012
14	4.0	0.020	35	0.7	0.011
330	2.7	0.019	—	1.2	0.010
180	2.0	0.019	—	2.3	0.010
Average (44 Exps.):			Cholesterol in 100 g. milk fat 0.490.		
113	3.88	0.0190			

Table II. [*Wacker and Beck, 1921.*]

Duration of lactation	Fat in 100 cc. milk	Cholesterol in 100 cc. milk	Duration of lactation	Fat in 100 cc. milk	Cholesterol in 100 cc. milk
—	3.3	0.036	16	3.4	0.014
14	2.3	0.019	17	3.9	0.013
46	3.4	0.016	315	3.5	0.013
44	3.7	0.016	345	2.8	0.012
210	3.7	0.016	280	3.5	0.012
37	2.6	0.015	345	4.1	0.012
39	2.7	0.015	290	4.6	0.012
14	3.3	0.015	270	3.0	0.011
45	3.5	0.015	315	3.4	0.011
15	3.7	0.015	315	3.1	0.010
290	4.3	0.015	345	1.9	0.007
345	3.7	0.014	345	2.1	0.007
280	3.5	0.014	280	3.0	0.007
Average (26 Exps.):			Cholesterol in 100 g. milk fat 0.424.		
200	3.29	0.0139			

the percentage of sugar remains more or less constant. This however is only apparent when averages of a large number of analyses are considered, since individual variations are large, and the differences of the averages of different lactation periods are within the range of the individual variations on which the averages are based. A difficulty met with in trying to arrive at, for instance, an average fat content by comparing the results of different ob-

servers, arises from the fact that when samples of milk are taken consecutively from the same breast, a more or less marked increase in fat content is observed. This is well known in the case of cow's milk, but does not appear to have been sufficiently taken into account by all writers on human milk. Further the percentages of fat from different individuals under apparently similar conditions vary much more markedly than in the case of animals such as the cow, which long generations of domestic use and systematic milking have no doubt modified.

It seemed to us of interest to find out whether any variation of cholesterol or cholesterol ester content with the duration of lactation could be detected, and how far the cholesterol content varied with progressive milking.

EXPERIMENTAL.

Methods of analysis.

All our analyses were made on the fat as separated by the well-known Gottlieb-Rose method.

The estimations of the non-ester and ester cholesterol and of the total cholesterol were made by the digitonin method fully described in previous papers by one of us, and particularly in our paper on cow's milk [1923].

Sampling.

As many of our results were required for metabolic experiments on infants, samples were drawn before and after nursing and mixed. In other cases the milk was withdrawn in the morning as completely as possible by the breast pump. A great difficulty in dealing with the human subject is that one cannot obtain the milk with the same systematic regularity and under the same controlled conditions as in dealing with an animal. The ideal method would be to work mainly on the milk from wet nurses, but these cannot easily be obtained in this country, so that one is at some disadvantage compared for instance with one's French confrères.

Results.

In Table III we give the results of our experiments in descending order of cholesterol.

DISCUSSION OF RESULTS.

It will be seen from the above tables that whereas the bulk of our own estimations were made on milk in the early days of lactation—the so-called colostrual milk—those of other observers were mainly on milk at later periods. It seemed therefore that by combining all the results quoted an answer to the questions whether the sterols vary with duration of lactation, and whether there is any relation between fat and cholesterol could be obtained.

The milk during the early days of lactation is known as colostrual milk and differs from the later milk both in colour, which is often though *not always*

Table III.

Duration of lactation	Fat in 100 g. milk. g.	Unsaponifiable matter in 100 g. milk. g.	Cholesterol in 100 g. milk. g.	Duration of lactation	Fat in 100 g. milk. g.	Unsaponifiable matter in 100 g. milk. g.	Cholesterol in 100 g. milk. g.
30	3.31	0.20	0.047	4	2.18	—	0.027
4	3.80	0.13	0.045	5	2.38	0.10	0.027
8	2.29	—	0.044	—	4.02	0.11	0.027
6	3.07	0.13	0.041	390	2.28	0.06	0.025
6	4.48	0.10	0.039	6	4.02	0.21	0.025
5	2.64	0.32	0.038	253	5.03	—	0.025
10	3.53	—	0.033	3	2.38	0.14	0.024
8	4.43	0.12	0.032	—	2.18	0.13	0.023
254	4.78	—	0.031	249	3.19	—	0.023
—	3.37	0.08	0.029	9	2.05	0.17	0.019
3	1.19	0.12	0.029	7	2.85	0.12	0.019
5	1.24	0.19	0.029	10	1.78	0.14	0.014
6	1.46	0.18	0.027	18	2.59	0.26	0.013
248	6.75	—	0.027				
Average (27 Exps.):				Cholesterol in 100 g. milk fat 0.94.			
	3.08	0.15	0.0289				

more or less yellow, and in composition, being less rich in fat, but richer in mineral matter. It is said, moreover, to have some peculiar physiological properties and is generally considered to be of "educational" importance in developing the digestive activities of the child. This colostrum milk gradually gives place to "true milk" which remains of a fairly uniform nature throughout the rest of the period of lactation.

Various writers differ somewhat in their definitions of colostrum milk and in the date at which they consider it gives place to ordinary milk.

The most thorough study of these changes in composition with regard to fat, sugar, protein and ash have been made by L. E. Holt and her collaborators [1915]. They distinguish four periods each more or less marked by differences in chemical composition, namely (1) colostrum 1-12 days, (2) transitional 12-20 days, (3) mature 30-270 days, (4) late 10-20 months. Adopting this perhaps rather arbitrary classification of Holt, we have grouped the average figures of Denis and Minot, Wacker and Beck, and ourselves in Table IV in which our own results have been adjusted from parts per 100 g. to parts per 100 cc. to make them strictly comparable.

Table IV.

Days of lactation	Denis and Minot			Wacker and Beck			Average total cholesterol per 100 g. fat
	Number of experiments on which average is based	Average fat per 100 cc. milk in grams	Average total cholesterol per 100 cc. milk in grams	Number of experiments on which average is based	Average fat per 100 cc. milk in grams	Average total cholesterol per 100 cc. milk in grams	
1-12	1	1.5	0.013	1	3.30	0.036	
12-30	15	3.78	0.017	5	3.32	0.015	
30-270	15	3.61	0.019	7	3.23	0.015	
11-20 (months)	8	4.87	0.024	13	3.35	0.0112	
	Fox and Gardner			Average results of all these observers			
	Number of experiments on which average is based	Average fat per 100 cc. milk in grams	Average total cholesterol per 100 cc. milk in grams	Number of experiments on which average is based	Average fat per 100 cc. milk in grams	Average total cholesterol per 100 cc. milk in grams	
1-12	19	2.83	0.030	21	2.79	0.0295	1.057
12-30	2	3.10	0.031	22	3.61	0.0178	0.494
30-270	4	5.09	0.027	26	3.73	0.0192	0.515
11-20 (months)	1	2.37	0.026	22	3.86	0.0165	0.427

It will be seen from this table, particularly columns 11, 12 and 13, that the early milk is richer in total cholesterol than that of the later periods. The total cholesterol of the 1-12 day milk is higher than the average of the other three periods in the ratio 1 : 0.59.

This is also borne out by the following analyses of milk obtained from three French wet nurses, from whom we obtained the whole day's supply.

Table V.

Period of lactation	Fat in g. per 100 cc.	Total cholesterol in g. per 100 cc.
6th day	3.07	0.041
Intermediate, exact date not known	4.02	0.027
13th month	2.28	0.025

On comparing the data in Tables I and II it will be noticed that the values given by Denis and Minot done by a colour method are on the whole slightly higher than those of Wacker and Beck which were done by the Windaus digitonin method. It has been shown that Bloor's method gives somewhat higher results for blood than the digitonin method and even than other colorimetric procedures, and this is probably the case for milk, since, as we show later, the unsaponifiable matter of milk fat contains, besides cholesterol, oils which give greenish colours with acetic anhydride and sulphuric acid and this must to some extent at any rate modify the cholesterol reaction with these reagents. On the other hand it is conceivable that Wacker and Beck's results may be low owing to diet. From the date of their paper it would seem probable that the samples of milk were obtained and analysed during the period covering the end of the war and the first years after the armistice, when the German people are generally admitted to have suffered from dietetic deficiencies. Generally speaking, diet, *if sufficient*, is known to have but little effect, either quantitatively or qualitatively, on the milk at any rate as far as the main constituents are concerned. As to what would be the effect of a prolonged period of more or less deficient diet, particularly if defective in accessory factors, on the fat-cholesterol content of the milk we have no data upon which to base any opinion. On the other hand the average values for cholesterol in cow's milk obtained by Denis and Minot, Wacker and Beck, and ourselves, quoted in our paper on cows' milk [1923], show close agreement.

Relation of fat to total cholesterol.

From a consideration of the figures given in Tables I, II and III we have been unable to detect any relation whatever between the percentage of fat and total cholesterol in individual cases. From column 2 of Table IV however it will be seen that in the early milk (1-12 days) the average fat value is lower than the average of the later periods in the ratio of 1 : 1.35.

The average percentage of total cholesterol in the fat of the three periods of normal human milk is 0.476. This is considerably higher than the average for cow's milk viz. 0.351 given in our former paper [1923]. The higher per-

centage of cholesterol in the ether extract of the early milk as compared with that from milk of later periods is clearly shown in the last column of the table.

The high cholesterol content of the early milk compared with later milk recalls the changes which occur in the cholesterol content of blood during pregnancy. Hermann and Neumann [1911, 1912] were the first to show that during pregnancy the cholesterol content of the serum is increased, and their observations have been abundantly confirmed by Chauffard, Laroche and Grigaut [1911], Klinkert [1913], Schlimpert and Huffmann [1912] and others. During the last few months of pregnancy the increase above normal is often very marked. As an average of 17 cases, between the seventh and ninth months Klinkert finds 0.2633 % against a normal of 0.18. Hermann and Neumann also showed that the increase or retention of cholesterol during pregnancy was followed by an elimination during the puerperium. The chief means of excretion is the milk and they noted that the state of hyper-cholesterolaemia passed off more rapidly in women who nursed their own children than in those who did not.

Though there is no reason to doubt the truth of these general conclusions, much reliance cannot be placed on the absolute figures obtained by these various workers since the recent work of Fex [1920] has conclusively shown that the methods of extraction of fat and cholesterol from blood and serum hitherto used are open to very grave errors. We hope, however, to return to this subject in a later paper, since E. Pritchard [1922, p. 94] has expressed rather definitely the opinion that "the gradual change from colostrum to milk which occurs between the first day of life and the time when true milk is excreted—generally the third or fourth day—gives the stomach and the intestines time and opportunity to settle down to their newly acquired function. It is impossible to conceive of any nutritive fluid being less irritating to the stomach and intestines than colostrum for, as already stated, it is practically maternal lymph, the same fluid that has bathed the foetal tissue during the previous nine months of intra-uterine life."

Ratio of free or non-ester cholesterol to cholesterol in the form of esters.

Wacker and Beck and also Beumer in his 1918 paper stated that the whole of the cholesterol of milk (in general) is present in the form of esters. In a later paper [1922] however, Beumer gives for a sample of human milk the following figures.

Free cholesterol 0.021 %, total 0.038 and ester 0.017, a ratio of free to ester cholesterol of 1:0.81.

We have analysed a number of samples for free and ester cholesterol and the results are given in Table V.

The average ratio of free to ester cholesterol from these results is therefore 1:0.53. In our paper on cow's milk we gave the ratio 1:0.54. As far as these few results give any information, there appears to be no connection between the ester cholesterol and the fat.

Table VI.

	I	II	III	IV	V
Period of lactation	3 days	?	?	6 days	390 days
Fat in g. per 100 g. milk	2.38	3.77	4.02	3.07	2.28
Total cholesterol, g. per 100 g. milk	0.0235	0.0288	0.0265	0.0413	0.0254
Free	0.0115	0.0211	0.0207	0.0280	0.0155
Ester	0.0120	0.0087	0.0058	0.0133	0.0099
Ratio of free to ester cholesterol	1 : 1.04	1 : 0.43	1 : 0.28	1 : 0.47	1 : 0.63

Variation of cholesterol content during milking.

It is well-known in the case of the cow that the strippings of milk are much richer in fat than the earlier fractions, though the other constituents are less variable. This appears also to be the case in the human subject, and is well illustrated by the figures due to Schlossmann [1900] given in Table VII.

Table VII.

	Vol. of milk withdrawn cc.	Fat %	N %	Sugar %
Breast				
Right	77	5.90	0.2	6.8
Left	18.5	4.40	0.14	8.04
	37.0	4.40	0.20	7.15
	26.0	8.47	0.20	7.10
	11.2	10.36	0.21	8.00
	15.6	10.58	0.26	7.98

Denis and Talbot [1919] published analyses of samples of milk taken before and after nursing in 56 cases which show the same phenomenon, though on the whole the differences are, as one might expect, less exaggerated.

It was necessary for the purpose of our metabolic experiments to ascertain whether the cholesterol content before and after nursing showed any marked variation as in the case of fat or was more or less constant as in the case of other constituents.

We found that though the percentage of cholesterol of the milk varies markedly in different individuals, and is not always the same from the two breasts of the same woman; on progressive milking the cholesterol content of the different fractions does not show the regular variation noted in the case of fat. This will be clear from Table VIII.

Table VIII.

Age of baby	Breast	Weight of milk with- drawn in g.	Fat per 100 g. milk in g.	Unsaponi- fiable matter per 100 g. milk	Total cholesterol per 100 g. milk	Total cholesterol per 100 g. of milk fat
11 days	Right	55 (whole)	1.78	0.14	0.014	0.79
	Left	26	1.14	0.09	0.016	1.40
		24	1.86	0.21	0.022	1.18
		29	2.27	0.10	lost	—
1 month	Right	112 (whole)	3.31	0.20	0.047	1.42
	Left	23	2.61	0.15	0.035	1.34
		24	2.19	0.20	0.028	1.28
		27	3.04	0.16	0.039	1.28
		22	3.99	0.20	0.042	1.05

It seemed therefore justifiable to determine the intake of cholesterol of a suckling child on the basis of analysis of samples of milk taken immediately before and after nursing, but there would be danger of very appreciable error in calculating this intake from average values applied to an individual mother.

Qualitative examination of the unsaponifiable matter of human milk.

In Table III we have given the quantities of unsaponifiable matter obtained by hydrolysis of the ether extract per 100 g. of milk. From these figures it will be seen that the sterol precipitable by digitonin, *i.e.* the cholesterol, constitutes only a fraction of the total unsaponifiable matter. The remainder consists of sticky yellow oils, very inactive and not easy to characterise. The figures for this unsaponifiable matter are very variable and too high, being vitiated from a quantitative point of view by the presence of small variable amounts of resinous matter produced during the hydrolysis of the fat by alkalis in alcoholic solution [Gardner and Fox, 1921]. This error is very dependent on conditions of reaction.

Sterols precipitable by digitonin.

The cholesterol digitonides obtained in our analyses were decomposed in the vapour of boiling xylene as recommended by Windäus. The crystalline matter obtained in this process was recrystallised from alcohol and found to be cholesterol from its crystalline form, its melting point and the properties of its benzoate. We had not sufficient material however to subject it to the elaborate fractional crystallisation which we performed on the similar material obtained from cow's milk.

Oil not precipitated by digitonin.

The golden yellow oil obtained after removal of the cholesterol digitonide was soluble in hot alcohol. On cooling traces of a white gelatinous substance separated. This was recrystallised several times from hot alcohol, and was finally obtained in white flakes which on heating softened at 59° and melted at 62–63°. It gave no sterol colour reactions. There was not sufficient for analysis or further examination.

The oil left after separating the last traces of the above substance by freezing in acetone solution was distilled in a high vacuum from a metal-bath as a mobile amber coloured oil leaving a slight tarry residue. This oil was again distilled in a high vacuum, but with the quantity at our disposal (0.4 g.) it was impossible to determine accurately its boiling point. On treatment in chloroform solution with acetic anhydride and sulphuric acid it gave a dark reddish brown colour which soon changed to a dusky green. On combustion it was found to contain 79.34 % of carbon and 11.64 % of hydrogen.

It was evidently a mixture, but as far as we could ascertain from the small quantity in hand it recalled in properties the similar oils we obtained from cow's milk.

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