XIII. THE STEROL CONTENT OF COW'S MILK.

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THE following account of the nature and composition of the so-called "unsaponifiable matter" found in milk fat, originated from analyses required for some experiments on cholesterol metabolism in adults. Ordinary cow's milk contains nearly one-tenth per cent. of a mixture of substances which can be extracted with ether or light petroleum after saponification of the fat by alkalis, and after evaporation of the ether is obtained as a soft golden yellow solid. In spite of the large number of investigations that have been made upon milk fat, this fraction has been invariably dismissed, until recently, by saying that it consisted of traces of cholesterol, wax, colouring matter etc.; this was no doubt largely due to the lack of any good method of separation or estimation of the constituents.

In 1883, Schmidt-Mülheim [1883] drew attention to a substance present in milk which he considered to be cholesterol on account of its colour reaction. Menozzi [1903] was perhaps the first to investigate the substance more closely and he showed that the sterol was identical in physical and chemical properties with the cholesterol isolated from bile. The earlier quantitative estimations of the cholesterol content of milk were made by Magnus-Levy [1910], Tolmatscheff [1867], Siegfeld [1906], Grigaut [1913], and others. Their values range from about 0.01 to 0.04 g. per 100 cc. milk.

In 1918 Denis and Minot [1918] published a series of analyses both of cow's and human milk made by Bloor's colorimetric method [1916]; this method however is known to give rather too high results for some tissues. Their figures for the fat and total cholesterol in 100 cc. of cow's milk are given in Table I.

Table I.

Fat	5.0	4.6	4.4	4.2	4.7	3.4	5.3	3.4
Cholesterol	0.0176	0.0176	0.0164	0.0162	0.0158	0.0156	0.0152	0.0152
Fat	4.0	3.9	3.3	4.5	3.7	3.4	3.2	
Cholesterol	0.0144	0.0140	0.0136	0.0128	0.012	0.0112	0.0105	
Average fat, 4.07.				Average cholesterol, 0.0145.				

The average percentage of cholesterol per 100 g. of fat is thus 0.356. Last year, while our work was in progress, Wacker and Beck [1921] published a series of determinations made by the digitonin method of Windaus, which are summarised in Table II.

Table II.

Fat in 100 cc. milk	4.02	4.00	3.75	3.72	3.69	3.46	3.31	3.31
Cholesterol in 100 cc. milk	0.014	0.014	0.016	0.012	0.011	0.012	0.011	0.011
Cholesterol in 100 g. fat	0.351	0.352	0.424	0.315	0.300	0.347	0.329	0.327

Averages: 3.65; 0.0126; 0.343.

The work to be described in the present paper may for convenience be divided into three parts: (1) quantitative estimation of cholesterol content of fresh and condensed milk; (2) qualitative examination of the fraction of the sterols precipitable by digitonin; (3) separation and quantitative examination of the residual non-precipitable oils.

METHOD OF ANALYSIS.

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

The fat was divided into two portions, one for determination of the free and the other for determination of the total free and combined cholesterol. The estimation was made by the method described by Fraser and Gardner [1910] with some slight modifications, which we detail briefly. To estimate the total free and combined cholesterol the fat was dissolved in ether and a large excess of a solution of sodium in 97 % pure alcohol, containing about 8 to 10 times the theoretical quantity of sodium required, added. This mixture was allowed to stand 24 hours, and then heated under a reflux on the water bath for 4 to 5 hours, in order to ensure the complete hydrolysis of cholesterol esters. It was then largely diluted with water and repeatedly extracted with ether until nothing more was dissolved. The ethereal solutions were united, thoroughly washed by shaking with water until quite free from traces of soap and made up to known volume. A suitable aliquot portion was then evaporated until free from ether, dissolved in alcohol and precipitated with a considerable excess of a 1 % alcoholic solution of digitonin. After standing overnight the alcohol was completely evaporated at the lowest convenient temperature. The residue was washed by decantation with ether to remove unprecipitated oily matter, then with warm water to remove excess digitonin. Finally the precipitate of cholesterol-digitonide was brought on to the Gooch, the washing completed and the substance dried and weighed.

For estimation of free cholesterol the fat was dissolved in alcohol and precipitated directly with excess of 1 % digitonin solution. In this case it was found advantageous not to evaporate the alcohol completely, but to leave

about one-fourth of the total volume, otherwise it is sometimes difficult to wash away all traces of fat and colouring matter with ether. After filtering off the alcohol and washing the precipitate with ether the rest of the procedure was as described above.

Fresh milk.

For fresh milk we used two samples of "Grade A certified milk," as defined by the milk section of the Ministry of Health, and two samples of so-called "Nursery Milk" stated to be obtained from a herd of tuberculin tested cattle which yield a milk higher than the average in butter-fat. The results are given in Table III.

Table III.

No.	Description	Fat per 100 cc. g.	Unsaponifiable matter in 100 cc. milk g.	Free cholesterol per 100 cc. g.	Ester cholesterol per 100 cc. g.	Total cholesterol per 100 cc. g.	Total cholesterol in 100 grams of fat g.
(I)	Nursery milk (1922)	4.46	—	0.001	0.015	0.016	0.358
(II)	" " "	4.14	0.0725	0.0144	0.0029	0.0173	0.418
(III)	Grade A (1922)	3.38	0.082	0.0114	nil	0.0114	0.334
(IV)	" "	3.47	0.066	0.0113	0.0044	0.0157	0.344
	Mean	3.86	0.0735	0.0095	0.0056	0.0151	0.3635

Condensed milk.

In order to cover a wider range of conditions, we also examined a number of samples of condensed milk. These were ordinary shop samples, described as consisting of whole milk, and had been prepared in England, United States, Norway, Denmark and Switzerland. The results are given in Table IV.

Table IV. *Condensed milk, bought 1920 and 1921.*

Description	Specific gravity	Total solids per 100 g. milk	Ash per 100 g. milk	Protein total N $\times 6.38$ per 100 g.	Fat	Total unsaponifiable matter per 100 g. milk	Total cholesterol free and ester per 100 g. milk	Total cholesterol per 100 g. milk fat	Unsaponifiable matter not precipitated by digitonin per 100 g. milk fat	Remarks
(1) Unsweetened	1.085	28.12	1.43	7.44	8.47	0.118	0.0336	0.401	1.000	} same makers
(2) Sweetened (a)	1.284	80.52	2.14	9.77	10.50	0.133	0.0268	0.255	1.010	
" (b)	—	—	—	—	—	0.145	0.0227	0.216	1.060	
(3) Unsweetened	1.074	25.80	1.38	7.12	7.98	0.062	0.0227	0.256	0.529	} same makers
(4) "	1.091	32.68	1.63	8.38	10.12	0.067	0.0317	0.314	0.329	
(5) Sweetened	1.294	74.45	1.60	7.70	9.32	0.082	0.0310	0.333	0.542	
(6) Unsweetened	1.072	25.77	1.32	6.61	8.34	0.125	0.0428	0.513	0.990	} same makers
(7) Sweetened	1.309	75.58	1.70	7.33	8.65	0.161	0.0486	0.562	1.298	
(8) "	1.288	73.20	1.78	7.60	9.30	0.053	0.0248	0.256	0.314	
(9) Unsweetened	1.073	26.40	1.47	7.05	8.22	0.044	0.0201	0.245	0.293	} same makers
(10) Sweetened	1.308	76.16	1.81	8.03	8.96	0.055	0.0266	0.295	0.335	
(11) "	1.305	77.23	1.94	8.78	10.89	0.085	0.0317	0.291	0.490	
					Mean	9.16	0.094	0.0303	0.328	0.691

For the sake of comparison, the average results of various observers are brought together in Table V.

Table V.

	Fat per 100 cc. milk	Cholesterol per 100 cc. milk	Cholesterol per 100 g. milk fat
Denis and Minot	4.07	0.0145	0.356
Wacker and Beck	3.65	0.0126	0.343
Fox and Gardner (fresh milk)	3.86	0.0151	0.364
„ „ (condensed milk)	—	—	0.339
Mean	3.86	0.0141	0.351

It will be seen from the above tables that the percentages of cholesterol found by the various methods quoted are in fairly close agreement.

Ratio of total cholesterol to fat.

The most obvious preliminary point to settle seems to be whether there is any definite relation between cholesterol and fat content. The average figures in Table V would at first sight indicate that there is a rough proportionality. This has already been pointed out by Denis and Minot, and their table, quoted above, which is arranged in order of descending values of cholesterol is suggestive, although there are several quite marked exceptions. Neither the figures of Wacker and Beck nor of ourselves however point to any exact ratio. It may also be mentioned that no such exact proportionality is found in tissues, blood etc. It will be noticed that the condensed milks show a much greater variation in the percentage of cholesterol in the fat than the specimens of fresh milk examined. Assuming that these variations are real and do not merely result from the removal or addition of cream or from the various processes of manufacture employed, they may perhaps be accounted for by differences of breed, feeding, climate, etc.

Breed. The percentage of fat varies very markedly with the breed of cow. The extent of the variation is well shown by the following figures, compiled by Wing [1897] from a large number of analyses.

Breed:—	Friesian- Holstein	Shorthorn	Ayrshire	Jersey	Guernsey
Average % of fat in milk	3.51	3.65	3.68	4.78	5.02

In actual practice the range may be considerably higher as it is not very uncommon for a Friesian cow to give milk with a fat percentage below 3, and for a Guernsey to reach 6 or even 7 %. In this connection however it must be remembered that the total volume of milk produced also varies and in the opposite sense, so that the actual output of fat in a given period according to Henry [1911] is nearly the same for all dairy breeds.

In order to test the effect of breed on the fat and cholesterol content of the milk we secured two samples, one from a Friesian cow known to yield a milk low in fat and a second from a good average Jersey. The analyses are given in Table VI.

Table VI.

Breed	Fat per 100 cc.	Cholesterol per 100 cc.		Total	Total cholesterol per 100 g. fat
		Free	Ester		
Friesian	2.95	0.0096	0.0023	0.0119	0.404
Jersey	5.96	0.0180	0.0047	0.0227	0.381

Here the output of cholesterol appears to follow approximately the output of fat.

Diet. Of the influence of this factor upon the amount of cholesterol in milk little can be said with certainty. Generally speaking, diet, if sufficient, is stated to have little effect on the milk, either quantitatively or qualitatively. Thus it appears to be easier to increase the yield by constant, regular and complete milking than by increased or richer feeding. What would be the effect of a prolonged period of more or less deficient diet on the fat-cholesterol content of the milk we do not know, but it is perhaps not without significance that three samples of milk, two purchased in London and the other in Cambridge for metabolic experiments in the summer of 1917 and early spring of 1918, gave the following results.

	Fat per 100 cc.	Total cholesterol per 100 cc.	Total cholesterol per 100 g. fat
(1)	3.73	0.007	0.188
(2)	3.50	0.007	0.200
(3)	2.92	0.009	0.308

It is well known that certain feeding stuffs such as cotton and linseed meal have an effect on the physical character of the fat. Further in a series of interesting experiments Palmer and Eckles [1914] were able to show that almost colourless butter can be obtained if the diet is kept sufficiently free from the lipochrome carotene. Here also the shortage is at first made good by reserves in the tissues; this is the probable explanation of "pale" winter butter sometimes met with. Apparently we have here a case of one constituent of milk being directly dependent for its supply upon the diet. Whether the same applies to the sterol there are no data to say, but experiments are in progress which we hope may throw light on this point.

Lactation period. As the lactation advances some changes in the fat content of milk occur, but we do not know whether the sterol follows suit. We have not had an opportunity of examining cow's colostrum from this point of view, but we agree with Wacker and Beck in finding that human colostrum is much richer in cholesterol than normal human milk. This however we propose to deal with in another communication.

Proportion of free cholesterol to cholesterol in form of esters.

According to Wacker and Beck cholesterol is not found in the free state in milk but in the form of esters, presumably esters of oleic and palmitic acids. This conclusion was based on the following experiment. Milk fat was shaken in a separating funnel with hot alcohol. Since free cholesterol is easily soluble

in hot alcohol, while the oleic and palmitic esters are insoluble or difficultly soluble, they expected that any free cholesterol would separate out from the alcohol solution in characteristic crystals, or could be detected by the digitonin reaction. They were unsuccessful in finding free cholesterol by this method. They assumed however without much justification that the presence of fat would not influence the solubility in alcohol. Our own conclusions are almost exactly the opposite. As will be seen from Table III, in all except the first case we found that the main proportion was in the free state. The average ratio of free to ester cholesterol was 1 : 0.54. In the case of the Friesian and Jersey cows (Table VI) the proportion of free to ester cholesterol was in the first case 1 : 0.24 and in the second 1 : 0.26.

Distribution of cholesterol in milk.

Little is known of the manner in which the cholesterol is distributed throughout the milk. Being quite insoluble in water it might be supposed that it was merely dissolved in the globules of fat, but as Wacker has already pointed out this scarcely explains the facts. He finds that the liquid which remains after the fat has been removed either by the separator or during churning is richer in cholesterol than can be accounted for on this hypothesis by the small amount of fat still remaining. According to his analyses the fat in skim milk would contain 0.80 % of cholesterol instead of 0.38. We came to the same conclusion in another way since we noticed that butter fat invariably shows a lower percentage of total cholesterol than the whole milk fat. This difference will be seen from a comparison of the average percentage cholesterol content of milk fat—0.35 in Table IV with the following analyses of samples of genuine butter given in Table VII.

Table VII.

	Total cholesterol %
(1) Reading Agricultural Dept. College Farm herd	0.278
(2) Butter made from milk of Jersey cow	0.291
(3) Good commercial sample	0.225
(4) Commercial sample	0.254
Mean	0.244

In order to confirm this we made the following analyses of samples of separated milk and butter milk kindly procured for us by Lord Rayleigh Dairy Co. (Table VIII).

Table VIII.

	Fat per 100 cc.	Cholesterol per 100 cc.		Total	Percentage of total cholesterol in fat
		Free	Ester		
Skim milk	0.187	0.0029	0.0008	0.0037	1.912
Butter milk	0.511	0.0089	0.0012	0.0101	1.977

These facts rather suggest that while no doubt most of the cholesterol—free and ester—is present dissolved in the fat globules, a certain amount may be present in some other form, probably in colloidal solution, or possibly even

combined with the protein. We have found a somewhat similar state of things when examining ascitic fluids, which, even when apparently clear and capable of being filtered through paper without any residue, often contain quite considerable quantities of cholesterol and cholesterol esters.

QUALITATIVE EXAMINATIONS OF THE STEROLS OF MILK.

The sterol which can be precipitated by digitonin, as will be seen from the tables, forms only about one-third to half of the total unsaponifiable matter of the fat, the remainder consisting of sticky yellow oils, very inactive and not easy to deal with or characterise.

In order to examine both precipitable and non-precipitable portions more closely we isolated a considerable quantity, using butter as a convenient source. The bulk of the fat was removed by saponification with caustic soda and the unsaponified portion was subjected to a final saponification by means of sodium ethoxide in alcoholic solution, the mixture being boiled for several hours, in addition to standing 24 hours, in order to ensure decomposition of difficultly hydrolysable sterol esters.

The yield of unsaponifiable matter from butter is somewhat lower than from milk since as mentioned above some sterol remains behind in the butter milk. A further minor source of error, which accentuates this difference between the percentage of unsaponifiable matter in milk fat and butter fat, is due to the small amount of resinification which takes place on hydrolysis by alkalis in alcoholic solution [Gardner and Fox, 1921]. This error is proportionally greater when small quantities of fat are analysed, so that the figures given in the tables for unsaponifiable matter are probably rather too high.

Sterols precipitated by digitonin.

The crude sterol was separated as far as possible from oily matter by crystallisation from acetone or alcohol, and the last traces removed from the oils by precipitation with digitonin. The digitonide was filtered off and decomposed as recommended by Windaus by heating in the vapours of boiling xylene. The whole crystalline matter was now subjected to a thorough fractional crystallisation, melting points taken and the benzoates of several fractions prepared. All the fractions corresponded in properties to cholesterol and cholesterol only. The final residues, too small to purify by further crystallisation, were benzoylated by the pyridine method, but again the result was a typical cholesterol benzoate.

We therefore conclude that the precipitable portion of the unsaponifiable matter of milk fat consists entirely of cholesterol.

Oils not precipitated by digitonin.

We were not successful in isolating any pure substance from these oils, but they appeared to consist in part at least of stable esters which escaped saponification by alkali. We attempted to purify the oils by distillation in

super-heated steam. This helps to remove resinous matter, but also appears to hydrolyse some of the very stable esters which occur in these mixtures. The distillation proceeded very slowly and there was not quite the same tendency for the distillate to assume the characteristic candle-like solid emulsion noted in the case of similar faecal oils [Gardner, 1921]. The distillate was dissolved in ether and the ethereal solution shaken out with alkali. From the alkaline solution a small quantity of a crystalline acid was obtained, which was purified by distillation *in vacuo*. It had no definite melting point and on titration with alkali proved to have an average molecular weight of about 288. The neutral portion after evaporating the ether was distilled in a high vacuum. It passed over between 170–180° for the most part. An attempt was made to fractionate this and eventually two main fractions were obtained: (1) distilling up to 175° (2 mm.) and (2) above 175°. Fraction (1) was a mobile golden yellow liquid at ordinary temperature, but solidified in a freezing mixture. It gave on treatment in chloroform solution with acetic anhydride and a drop of sulphuric acid a dark brown colour which more or less rapidly changed to a dusky olive green very similar to the colour obtained with faecal oils [Gardner, 1921]. On combustion 0.1885 gave 0.2049 H₂O and 0.5629 CO₂. C = 81.44 %, H = 12.08 %.

The fraction 2 was again hydrolysed, this time by heating in a sealed tube at 110° with alcoholic potash. The liquid was diluted and extracted with ether and the ethereal solution shaken out with alkali as before. A very small fraction of acid, apparently a mixture of a liquid and a solid acid was obtained. This was distilled *in vacuo* and by titration with alkali an average molecular weight of about 274 was indicated. The acids were too small in amount for identification, but the liquid portion appeared to be unsaturated.

The neutral portion after removal of the ether distilled in a high vacuum at about 200°. It came over as a clear golden yellow amorphous glass which just melted on a warm day. On freezing out from acetone a trace of a white wax-like substance was obtained too small however for further purification. On combustion of the amorphous glass 0.1543 gave 0.1579 H₂O and 0.4582 CO₂. C = 81.00 %, H = 11.37 %.

On testing an alcoholic solution of this oil with digitonin a mere trace of a digitonide was precipitated, far too small in quantity however to do anything with.

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REFERENCES.

- Bloor, W. R. (1916). *J. Biol. Chem.* **24**, 227.
Denis and Minot (1918). *J. Biol. Chem.* **36**, 58.
Fraser, M. and Gardner (1910). *Proc. Roy. Soc. B.* **82**, 559.
Gardner (1921). *Biochem. J.* **15**, 250.
Gardner and Fox (1921). *Biochem. J.* **15**, 376.
Grigaut (1913). *La Cholesterinémie*, Paris.
Henry (1911). *Feeds and Feeding*, 379.
Magnus-Levy (1910). Quoted by Klein. *Biochem. Z.* **29**, 471.
Menozi (1903). *Atti. R. Acad. Lincei*, v. **12**, 1. 126.
Palmer and Eckles (1914). *J. Biol. Chem.* **17**, 191.
Schmidt-Mülheim (1883). *Pflüger's Arch.* **30**, 384.
Siegfeld (1906). *Milchw. Zentr.* **2**, 1.
Tolmatscheff (1867). *Hoppe-Seyler Med. Chem. Untersuch.* 272.
Wacker and Beck (1921). *Z. Kinderheilk.* **27**, 288.
Wing (1897). *Milk and its Products*, 33.