XX. STUDIES ON THE CHOLESTEROL CONTENT OF NORMAL HUMAN PLASMA. PART I.

BY JOHN ADDYMAN GARDNER AND HUGH GAINSBOROUGH.

From the Biochemical Department, St George's Hospital, London.

(Report to the Medical Research Council.)

(Received November 27th, 1926.)

DURING the past 20 years a very large number of observations have been published on the total cholesterol content of blood or of serum from pathological cases. As a result, the blood picture is described, in any one case, as a hypercholesterolaemia or a hypocholesterolaemia, in comparison to a so-called normal value of total cholesterol in human blood. It is, however, to be noted that relatively few determinations have been made on normal subjects, and that values of the normal, obtained from these sparse data—not too concordant in the different researches—have been quoted from author to author.

It is generally agreed that in certain forms of nephritis, irregularly in diabetes mellitus and also in the later months of pregnancy, a hypercholesterolaemia is found, and that in infective conditions a hypocholesterolaemia occurs. As regards other diseases it is impossible in the present state of our knowledge of the physiological limits of cholesterol content of the blood to decide if there is any constant alteration in the level of this value. Denis [1917] considers that at present the total cholesterol content of the blood is practically of no diagnostic significance.

Though much work has been done on the total cholesterol content of blood or serum, comparatively little attention has been directed to the relative amounts of so-called free or non-esterified cholesterol and cholesterol in ester form. This unfortunate position is to some extent explained by the historical development of our knowledge of this subject. Fat, cholesterol and allied substances were obtained together by the extraction of tissues and fluids of the body by means of ether and other solvents, and in early days no effective methods were available for differentiating, quantitatively at any rate, between free and ester cholesterol. Consequently cholesterol came to be regarded as intimately connected with fat metabolism.

This idea has persisted throughout the history of the subject and has been upheld largely by the French observers. Terroine [1919] considers that there is a constant lipaemic coefficient, *i.e.* a constant ratio of total cholesterol to total fatty acid in individual animals. Chauffard and his school [1920]

consider that "normally there exists a tendency in the organism to preserve the equilibrium between the different elements of the lipaemia, fats, cholesterol and lecithin, and that it is probable that there exists a regulator mechanism which controls at the same time the amounts of the fats and lipins and the relative proportion of these different elements amongst themselves."

Further, Chauffard and his collaborators consider that cholesterol metabolism depends on three factors: (1) alimentation, (2) the glands of internal secretion, particularly the suprarenal cortex, (3) the liver. However, this view does not appear to lay sufficient stress on the fact that sterols are integral constituents of all living cells.

That so much attention has been concentrated on the total cholesterol of blood would seem to imply that the existence of a portion of this total in the form of ester is a purely fortuitous condition of no particular importance. However, a consideration of the different distribution of free and ester cholesterol throughout the body, noticeably in the brain, red blood corpuscles and in the bile would suggest that such an assumption is untenable.

In recent years cholesterol balance experiments by Gardner and Fox [1921, 1, 2; 1925] and by Thannhauser [1923] have shown that in the normal human adult there is always a small negative balance, that is, an excess of output over intake, which indicates that though the food may be a source of cholesterol, nevertheless there must be some synthesis in the body. That such synthesis does take place has been shown by the study of the relative growth and cholesterol content of infants' brain and by experiments with dogs by Beumer and Lehmann [1923], and with rats by Channon [1925] and by Randles and Knudson [1925].

Beumer [1923] goes so far as to advance the view that cholesterol is not to be considered as a food in any sense of the term but that in metabolism it plays a part more comparable with the rôle of the purines, in the sense that the cholesterol excreted by the organism is the true residuum, unrequired by the body, of the metabolic processes and is derived like uric acid from endogenous and exogenous sources. Whatever may be the truth of this conception it does not appear to us to modify the views previously maintained by Gardner in former papers of the nature of the cholesterol cycle.

In considering these different views it does appear to be certain that at the present time we have not the data on which to base a sound theory, nor data enough to criticise these views.

It appeared to us therefore to be of great immediate importance to study the free and ester cholesterol content of the plasma of normal individuals, and to examine the limits of its physiological variations and the conditions that cause such variations, also, if possible, to get evidence of the actual state of existence of the cholesterol in the blood. A general survey of the results hitherto obtained seems to justify the view that changes in the total cholesterol content of blood, or of the ratio of the free to ester forms, take place mainly in the plasma, and that the content of corpuscles is relatively if not

quite constant, and consists mainly of free cholesterol. In this paper we give the results of a number of analyses of the plasma of normal subjects.

TECHNIQUE.

The methods in use for estimating cholesterol and cholesterol esters in various tissues were critically dealt with by Gardner and Williams [1921] and by Gardner and Fox [1921, 1, 1924], and our experience has not led to the modification of the conclusions expressed in these papers. As the analyses of blood and plasma present rather more difficulties than those of other tissues, when both free and ester cholesterol are to be estimated, and rather conflicting statements have been made in recent years, notably by Bloor and Knudson [1916, 1917] and by Richter-Quittner [1920], we think it may be useful to give a more detailed description of our methods than has hitherto been published.

In our experience two methods of extraction are practically equally effective, viz., the method of Fex [1920] and the alcohol-ether method advocated by Bloor. In the case of whole blood about 10 cc. of blood are withdrawn from a vein and treated at once. If the plasma is to be analysed, it is better to draw a sufficient quantity of blood to yield at least 10 cc. of plasma into a tube containing a small quantity of solid potassium oxalate (0.2 %).

The blood is at once centrifuged and the plasma separated and weighed. A convenient quantity is about 10 to 15 g. It has been objected by Richter-Quittner that sodium oxalate and sodium fluoride tend to injure the corpuscles and should not be used. He recommends sodium citrate. In our experience, however, if the blood is centrifuged soon after drawing, the plasma shows no trace of haemolysis.

In Fex's procedure the fluid—whole blood or plasma—is diluted with about 10 to 15 times its volume of 2 % caustic soda, and after standing for several hours is heated for $\frac{1}{2}$ to 1 hour on the water-bath. After cooling the fluid is thoroughly extracted in a separator by ether. This is best done as recommended by Fex; the alkaline fluid is placed in the separator with about half its bulk of ether, and thoroughly shaken at intervals during the day. After standing over night the ethereal solution is separated, replaced by a new quantity of ether and the alkaline fluid and the ether again shaken at intervals during the day. This process is repeated four or more times, until the ether no longer takes up anything from the alkaline fluid. The clear ethereal solution is then distilled to dryness on the water-bath, and the residue taken up in alcohol.

In the other process the weighed blood or plasma is run in a thin stream into about 250 cc. of a mixture of three parts alcohol to one part ether, which is kept gently rotating. The fluid is then heated to boiling on the water-bath. After cooling, the liquid is filtered through a small filter and the proteins very thoroughly washed by decantation with at first the alcohol-ether mixture and then with ether, any protein on the filter being returned at each washing to the flask: the protein is then washed with boiling alcohol and finally again

with ether. The ether-alcohol mixture is then distilled to dryness on the water-bath. The dry residue is boiled out thoroughly with small successive quantities of alcohol and the alcoholic extracts passed through a small filter. In this case it is necessary to filter, as the original ether-alcohol solution contains traces of protein and some of the plasma salts, which are not dissolved by the alcohol. At this stage it is advisable to wash the residue on the filter with ether and put the ether extract into the flask containing the residue insoluble in alcohol. The subsequent hydrolysis of the esters of cholesterol may then be done in this flask. Esters of cholesterol are not very soluble in alcohol, and this procedure obviates any loss of ester.

The free and ester cholesterol in the alcoholic solution, prepared by either method, are estimated by means of digitonin. Our procedure is as follows. The hot alcoholic solution is mixed with an excess of an alcoholic solution of digitonin in a small beaker and brought to boiling point. After cooling, water is added drop by drop until the alcohol is sufficiently diluted for the cholesterol digitonide to begin to deposit. After standing over night the fluid is carefully evaporated to dryness on the top of a water-bath. The contents of the beaker are then treated with three successive portions of 15 to 20 cc. of dry ether and these ether washings filtered through a weighed Gooch crucible and carefully collected. In this way the cholesterol esters along with any resinous matter are obtained in ethereal solution. The digitonide of the free cholesterol is then treated with boiling water to dissolve excess of digitonin, and brought on to the Gooch crucible and the washing completed with hot water. The filtrate should be water-clear, otherwise refiltered until it is. This washing process is complete when the washings, on vigorous shaking, show no trace of frothing. The Gooch crucible and its contents are then dried first at 100° and finally at 110° to constant weight.

The ethereal solution of the esters, etc., is poured into a flask provided with a reflux condenser (for this purpose the flask which held the original ether or ether-alcohol extract is used) and a large excess of an alcoholic solution of sodium ethoxide added, and after standing over night is boiled for at least 8 hours. After cooling, the alcoholic solution is largely diluted with ether and shaken in a separator with water. The aqueous solution so obtained is extracted three times with ether and the ether layer added to the main ether solution. The alkaline fluid is then again extracted in a second separator with a comparatively large quantity of ether, and the alkaline fluid then run off. The main ethereal solution formed by hydrolysis of the esters is then repeatedly shaken with successive portions of water to remove any traces of soap, and the aqueous washings shaken with the ether left in the second separator to avoid any risk of loss of cholesterol. The whole of the ether solution is then distilled to dryness on the water-bath, the residue taken up in alcohol and the cholesterol precipitated with digitonin and treated as described above.

This procedure presents advantages over the original method of Windaus [1910] which is used by most observers, especially in the case of fluids such

as blood which contain only small quantities of cholesterol or cholesterol esters, since any loss of cholesterol digitonide through slight solubility in alcohol is obviated. Such loss in the Windaus process is difficult to allow for, particularly when the quantity of cholesterol digitonide is small. The error due to this solubility is obviously the more serious the smaller the quantity to be weighed.

RESULTS.

In Table I we give the results of a few duplicate analyses, both by Fex's and the alcohol-ether methods, which give an idea of the experimental errors to which the method of analysis is liable when quantities of the order mentioned are dealt with. When the complexity of the operation involved in the analyses is taken into account, we consider these results satisfactory.

Table I.

Method of extraction	Wt. of whole blood or plasma in g.	Wt. of cholesterol digitonide (g.) from		Cholesterol (g.) per 100 g. of whole blood or plasma			Remarks
		(a) Free cholesterol	(b) Ester cholesterol	Free	Ester	Total	
Fex ...	{ 10-3846	0-0378	0-0244	0-0885	0-0571	0-1456	Whole blood from normal male
	{ 10-3802	0-0384	0-0244	0-0899	0-0576	0-1475	
Alcohol-ether	8-3461	0-0402	0-0246	0-1171	0-0716	0-1887	Whole blood taken from a case of mitral stenosis
Fex ...	{ 10-4383	0-0462	0-0320	0-1076	0-0745	0-1821	
	{ 10-3287	0-0453	0-0308	0-1069	0-0727	0-1796	
Alcohol-ether	{ 15-531	0-0414	0-0706	0-0648	0-1105	0-1753	Plasma from a case of myocarditis
	{ 15-531	0-0423	0-0770	0-0662	0-1205	0-1867	
Fex ...	{ 15-531	0-0402	0-0742	0-0629	0-1161	0-1790	
	{ 15-531	0-0389	0-0705	0-0609	0-1104	0-1713	

Though these two methods of extraction give results in practical agreement, and in our experience are the best available when both free and ester cholesterol have to be estimated, nevertheless they are not perfect. Small quantities of cholesterol or cholesterol esters remain persistently in the proteins as the following experiments, selected from many, show.

Alcohol-ether method.

1. The protein collected after extraction from six cases, representing 72.7 g. of plasma, was dissolved in 2 % caustic soda and repeatedly extracted with ether in the manner described above. The ether extract gave 0.0009 g. of compound from free cholesterol, and 0.0011 g. from ester cholesterol. The extracted alkaline protein was then evaporated to dryness and boiled for 4 to 5 hours with glacial acetic acid containing hydrochloric acid. The acid liquor was then neutralised and thoroughly extracted with ether. The ether extract was then hydrolysed by sodium ethoxide to saponify any cholesterol acetate, and again extracted. The ether extract yielded 0.0026 g. of cholesterol digitonide. These figures correspond to 0.00031 % of free cholesterol, and 0.00038 % of ester cholesterol left in the protein and 0.0009 % freed by acid hydrolysis.

2. 7.95 g. of dried protein from 114.3 g. of extracted plasma of nine cases were treated in a similar manner. 0.0045 g. of compound from free cholesterol, 0.0061 g. from ester cholesterol and a trace after acid hydrolysis were obtained. These figures represent 0.00096 % free cholesterol and 0.0013 % ester cholesterol left in the original plasma.

Fex method.

3. 50.15 g. extracted plasma from five cases were very thoroughly re-extracted with ether, and the ethereal extract hydrolysed by sodium ethoxide and again extracted. From this 0.0058 g. of compound was obtained, equivalent to 0.0028 % of cholesterol (free and combined). After acid hydrolysis, as above, an extract was obtained which yielded 0.0012 g. compound equivalent to 0.0006 % cholesterol. The average error in these cases due to inefficient extraction was therefore 0.0034 g. per 100 g.

4. 93.1 g. of extracted plasma from seven cases were very thoroughly re-extracted with ether and the extract hydrolysed. From the extract was obtained 0.0011 g. compound, equivalent to 0.00029 g. cholesterol (free and ester) per 100 g. of original plasma. After acid hydrolysis it yielded 0.0054 g. compound, equivalent to 0.0014 g. cholesterol per 100 g. plasma.

These average errors due to inefficient extraction are obviously negligible, unless perchance they happened to be all concentrated in one sample, which, owing to the care taken, was very improbable, and are probably well within the variations caused by changes in the plasma concentration during the day.

Cholesterol content of plasma of normal individuals.

The subjects of our experiments were drawn chiefly from students in our classes at the London School of Medicine for Women and at St George's Hospital, from nurses, laboratory attendants and colleagues. They were all in normal health, as far as could be judged clinically. The samples of blood were drawn from an arm vein, and were collected in a few cases from fasting patients, *i.e.* before the first meal of the day, but in most cases about 4 to 5 hours after breakfast or a light lunch. In Table II we give essential details and results of our experiments.

DISCUSSION OF RESULTS.

Analyses of the plasma of normal human subjects have been published in recent years by Bloor and Knudson and by Richter-Quittner, who came to rather opposite conclusions. Bloor and Knudson [1917] examined 17 men and 9 women. The total cholesterol in plasma was determined by the colorimetric method of Bloor, and the ester cholesterol by the same method after elimination of free cholesterol by means of digitonin. The colorimetric method of Bloor [1916] is well known to give higher results than the digitonin method, and their average results are, as might be expected, higher than our own. They conclude that in normal human plasma there is a constant relation

Table II.

Women

No. of case	Age of subject	Time after last meal Hours	Nature of meal	Catamenia Between periods	Antico- agulant used	Appearance of plasma	Method of extraction	Wt. of digitonide (g.) from			Amount of cholesterol (g.) in 100 g. of plasma			Ratio free : ester
								Free	cholesterol	cholesterol	Free	Ester	Total	
1	20	4	Breakfast	"	"	Clear, no haemolysis	Fez	11-1515	Lost	0-0457	0-0329	0-0996	—	1:3-97
2	19	4	"	"	"	"	"	10-1138	0-0137	0-0544	0-0329	0-1308	0-1637	1:2-8
3	19	4	"	"	"	"	"	13-2724	0-0112	0-0314	0-0205	0-0575	0-0780	1:3-32
4	21	5	"	"	"	"	"	13-1465	0-0223	0-0738	0-0412	0-1365	0-1777	1:1-52
5	20	3	"	"	"	Yellow, sl. opalescence	"	18-4537	0-0323	0-0489	0-0426	0-0644	0-1070	1:1-42
6	20	4	"	"	"	"	"	14-2678	0-0210	0-0319	0-0358	0-0544	0-0902	1:1-73
7	19	5½	"	"	"	"	"	12-6801	0-0284	0-0509	0-0363	0-0875	0-1538	1:1-54
8	18	4	"	"	"	Clear, v. sl. haemolysis	"	7-4765	0-0210	0-0303	0-0300	0-0867	0-1467	1:1-44
9	25	4	"	"	"	Opalesc., sl. haemolysis	"	9-4117	0-0231	0-0283	0-0597	0-0920	0-1517	1:0-97
10	19	4½	"	"	"	Clear, sl. haemolysis	"	8-464	0-0215	0-0286	0-0628	0-0597	0-1215	1:1-85
11	24	4½	"	"	"	Opalesc., sl. haemolysis	"	9-5207	0-0262	0-0485	0-0599	0-1108	0-1707	1:1-61
12	17	4½	"	"	"	Clear, v. sl. haemolysis	"	6-6325	0-0181	0-0292	0-0663	0-1070	0-1733	1:2-08
13	24	4	Light breakfast	Just before period	"	Clear, no haemolysis	"	10-9658	0-0304	0-0631	0-0675	0-1402	0-2077	1:1-20
14	23	1½	Light lunch	Between periods	Paraffined tube	Yellow, clear	Alcohol-ether	10-5254	0-0328	0-0393	0-0758	0-0908	0-1666	1:1-82
15	22	Imme- diately	Good lunch	Sl. amenorrhoea	"	Brownish, clear, no haemolysis	"	7-5215	0-0172	0-0313	0-0556	0-1012	0-1568	1:1-90
16	20	4½	Light breakfast	Period now on	"	Clear, v. sl. haemolysis	"	12-3943	0-0288	0-0538	0-0565	0-1055	0-1620	1:1-40
17	19	6	Breakfast	Sl. amenorrhoea	"	Clear, no haemolysis	"	13-3648	0-0519	0-0727	0-0944	0-1322	0-2266	1:1-40
18	18	5½	"	Between periods	"	Sl. opalesc., no haemol.	"	12-1697	0-0356	0-0600	0-0713	0-1001	0-1714	1:1-89
19	20	5½	"	Sl. amenorrhoea	"	Clear, no haemolysis	"	14-2066	0-0252	0-0477	0-0431	0-0816	0-1247	1:2-64
20	19	4½	"	Between periods	"	Clear, sl. haemolysis	"	12-1956	0-0243	0-0690	0-0458	0-1302	0-1760	1:2-65
21	18	4½	Light breakfast	"	"	"	"	7-4523	0-0106	0-0281	0-0346	0-0917	0-1263	1:2-65
								Mean 0-0541			0-0986			0-1528

Men

22	24	3 $\frac{3}{4}$	Breakfast	K oxalate	Sl. haemolysis	Fe _x	10-5007	0-0239	0-0602	0-0553	0-1394	0-1947	1:2-50
23	24	4 $\frac{1}{2}$	"	"	"	"	5-7254	0-0029	0-0217	0-0123	0-0922	0-1035	1:7-50
24	22	4	"	"	Yellow, clear, no haem.	"	11-5183	0-0315	0-0258	0-0685	0-0546	0-1211	1:0-82
25	23	3 $\frac{1}{4}$	"	"	Opalescent, no haem.	"	12-0843	0-0052	0-0490	0-0105	0-0866	0-1091	1:9-40
26	23	3 $\frac{1}{4}$	"	"	Clear, sl. haemolysis	"	10-3250	0-0021	0-0444	0-0049	0-1045	0-1094	1:21-3
27	58	Fasting	"	"	Yellow, no haemolysis	"	9-1213	0-0193	0-0526	0-0514	0-1386	0-1900	1:2-69
28	58	2 $\frac{1}{2}$	Heavy lunch	"	Opalescent, no haem.	"	9-8053	0-0163	0-0488	0-0404	0-1210	0-1614	1:2-99
29	33	Fasting	"	"	Clear, no haemolysis	"	10-4763	0-0176	0-0492	0-0418	0-1142	0-1560	1:2-73
30	33	2 $\frac{1}{2}$	Heavy lunch	"	Very milky, green, opalescent	"	10-4885	0-0162	0-0349	0-0375	0-0809	0-1184	1:2-15
31	20-24	4	Breakfast	"	Clear, no haemolysis	"	21-3406	0-0350	0-1149	0-0418	0-1373	0-1791	1:3-29 ⁵
32	20-58	4	"	"	"	"	10-2080	0-0303	0-0510	0-0722	0-1214	0-1936	1:1-68 ⁶
33	19	5	"	"	"	"	10-2368	0-0187	0-0434	0-0444	0-1033	0-1477	1:2-33 ⁷
34	35	4	Light lunch	Paraffin lined tube	"	"	10-6456	0-0310	0-0598	0-0754	0-1454	0-2208	1:1-93
35	22	4	"	"	Yellow, clear, no haem.	"	9-0780	0-0306	0-0526	0-0819	0-1409	0-2228	1:1-72 ⁸
36	58	16	Dinner	K oxalate	Clear, v. sl. haemolysis	"	11-5673	0-0387	0-0546	0-0815	0-1150	0-1965	1:1-41
37	23	5'	Light lunch	Heparin	Clear brown, no haem.	"	9-4307	0-0210	0-0249	0-0541	0-0642	0-1183	1:1-19
38	33	Fasting	"	K oxalate	Clear, no haemolysis	"	16-3950	0-0405	0-0958	0-0801	0-1420	0-2026	1:2-36
39	33	3	Heavy lunch, ample fat	"	Opalescent, no haem.	"	14-5099	0-0365	0-0787	0-0612	0-1319	0-1931	1:2-16
40	23	4	Light lunch	Heparin	Clear, sl. haemolysis	"	11-6176	0-0148	0-0332	0-0310	0-0695	0-1005	1:2-24 ⁹
41	22	2 $\frac{1}{2}$	Moderate lunch	K citrate	Opalescent, sl. haem.	"	12-1277	0-0263	0-0532	0-0527	0-1066	0-1593	1:2-02
42	21	2 $\frac{1}{2}$	Light lunch	"	Clear, sl. haemolysis	"	12-9603	0-0331	0-0379	0-0609	0-0711	0-1320	1:1-17
43	21	3	Very light lunch, chocolate	"	Sl. opalesc., sl. haem.	"	12-7706	0-0294	0-0588	0-0560	0-1119	0-1679	1:2-00
Mean 0-0504 0-1193 0-1694													

¹ The extracted alkaline plasma from these 7 cases, on re-extraction and subsequent hydrolysis, gave 0-0011 g. compound equivalent to 0-0029 g. % plasma. After acid hydrolysis gave 0-0054 g. compound equivalent to 0-0014 g. % plasma.

² Total alkaline plasma from these 5 cases, on thorough re-extraction, yielded 0-0365 g. compound equivalent to 0-0203 g. %. After acid hydrolysis gave 0-0448 g. compound equivalent to 0-0027 g. %.

³ Precipitated protein from these 6 cases dissolved in 2 % NaOH and thoroughly extracted with ether gave 0-0009 g. compound from free cholesterol and 0-0011 g. compound from ester cholesterol equivalent to 0-00391 g. free cholesterol per 100 g. plasma and 0-00038 g. ester cholesterol per 100 g. plasma. After acid hydrolysis gave 0-0026 g. compound equivalent to 0-0009 g. per 100 g. plasma.

⁴ Total alkaline plasma of these 5 cases, after re-extraction and subsequent hydrolysis, gave 0-0058 g. compound equivalent to 0-0028 g. per 100 g. plasma. After acid hydrolysis gave 0-0012 g. compound equivalent to 0-00058 g. per 100 g. plasma.

⁵ Mixed sample from 12 subjects.

⁶ On re-extraction, no free cholesterol but 0-0028 g. compound from ester cholesterol found equivalent to 0-0067 %.

⁷ The protein dissolved in 2 % NaOH and thoroughly extracted. No free cholesterol but 0-0018 g. compound from ester cholesterol equivalent to 0-0048 % plasma.

⁸ Arm somewhat cyanosed on venipuncture.

between free cholesterol and ester cholesterol, the ester cholesterol being 58 % of the total, with a variation from the average in individuals of about 15 % of the average; and further that there is no notable difference in their values between men and women. This constant relation between free and bound cholesterol they consider gives further support to the assumption that cholesterol takes an active part in fat metabolism, and also indicates that for cholesterol esters, as for other "lipoids," there is an efficient regulation. We scarcely think these general conclusions are warranted by their figures, still less by our own. Bloor and Knudson find that the ratio of free to ester cholesterol is on an average 1 : 1.35 in normal men, with individual limits of variation of 1 : 0.84 to 1 : 2.14, and in normal women an average of 1 : 1.49 with limits of 1 : 1.04 to 1 : 2.34. As will be seen from Table II our own average ratios are for men 1 : 2.36, with limits of 1 : 0.82 to 1 : 21.3, or eliminating three cases in which the cholesterol was nearly all in the form of ester, 1 : 2.36 with limits of 1 : 0.82 to 1 : 3.29; and in women an average of 1 : 1.93 with variations of 1 : 0.97 to 1 : 3.97.

On the other hand, Richter-Quittner [1920] on, we think, insufficient normal data, concluded that blood corpuscles contain exclusively free cholesterol and no ester, whereas the *plasma contains exclusively ester and no free cholesterol*. He also draws a distinction between alimentary hypercholesterolaemia and pathological hypercholesterolaemia, but this subject we hope to deal with in another communication.

Richter-Quittner appears to attribute the varying results of other observers to injury to corpuscles in obtaining the plasma, by such causes as too vigorous centrifugalisation, and, particularly, to the use of sodium oxalate or fluoride as anticoagulants, since these salts even in small amounts act as poisons and harm the red cells. In our experiments we were careful to reduce disturbance by centrifugalisation to a minimum. In most experiments, however, we used oxalates in the minimum quantity to prevent coagulation during the short interval between drawing the blood and preparing the plasma. In a great majority of the cases the plasma obtained was yellow and untinged with red, so that, as far as absence of haemolysis is an indication, the red cells were unharmed. However, to test this criticism, we drew the blood in cases 13, 34, 35 into a paraffin lined tube and centrifuged at once without any anticoagulant, in cases 37 and 40 we used heparin (a substitute for hirudin) and in cases 41, 42 and 43 potassium citrate, as recommended by Richter-Quittner. The results, as will be seen from Table II, in no wise differ from those obtained with oxalate.

It is clear from our results that normal individuals differ widely both in the total cholesterol of their plasma, and in the ratio of so-called free cholesterol to cholesterol in the form of esters.

These variations could scarcely be due to the direct absorption of cholesterol from the food into the blood plasma, since, with a few exceptions, the results were obtained from blood after a fast, or several hours after a light

meal, such as breakfast, containing but a small absolute amount of cholesterol; for even if we assume that the food contained 0.15 g. of cholesterol—which is quite an outside figure—and if we assume that the whole of this found its way into the blood stream, this would, in an individual with, say, 6 litres of blood, only increase the cholesterol by 0.00025 g. %, a figure well within the error of experiment.

Red blood corpuscles.

Richter-Quittner is probably right in his contention that the red cells contain mainly free cholesterol, and in this he is in agreement with Bloor and Knudson and other observers. The method usually adopted is to calculate the composition of the red cells from analyses of the whole blood and plasma, together with a determination of the ratio of corpuscles to plasma by the haematocrit or other method. The accumulation of experimental errors involved in such a computation is however too great to indicate, with certainty, the presence of small quantities of ester. A more satisfactory method of proving the presence of ester is a direct analysis of the *thoroughly* washed corpuscles. The cholesterol is more easily extracted from corpuscles than from plasma, and, in this respect, they resemble other tissues. The first thorough and reliable investigation of animal blood was made by Wacker and Hueck [1913] who found that in horse blood the dried red cells contained 0.36 % free and no ester cholesterol, whilst the dried white cells contained 1.729 % free and 0.04 % ester. Beumer and Bürger [1913] also found that human blood cells contain only free cholesterol, though in certain diseases traces of ester appear, but in such small amount that the authors rightly consider the values should be accepted with caution.

We made only one experiment—in the case of mixed blood No. 31—and found after elaborate washing of the corpuscles 0.0036, 0.0018 and 0.0012 % of ester cholesterol in the moist corpuscles; but as the quantities of cholesterol-digitonide weighed were only 2.0, 0.8 and 1.2 mg. respectively we cannot place much reliance on the figures, except that they indicate a small positive precipitate.

An objection might be raised against the direct method that, during the somewhat lengthy process of washing, autolytic changes may have resulted in a loss or gain of ester cholesterol, but this scarcely seems likely as calculation from whole blood and plasma points to the same results. Furthermore, a large mass of thoroughly washed corpuscles which had been kept 4 months in a stoppered bottle, and had undergone considerable autolytic change, was analysed with the following results: 13.8 g. moist material gave 45.4 mg. digitonin-compound from free cholesterol, and 2.2 mg. from ester cholesterol, the latter equivalent to 0.0039 %. Evidently the cholesterol had not been appreciably changed during autolysis. The bulk of the material (73 g.) was extracted, and the cholesterol isolated and converted into benzoate. 0.356 g. of pure benzoate, melting to a turbid liquid at 145°–146° and clearing at 178°, was obtained.

We take this opportunity to thank the Royal Society Grant Committee for help in the purchase of digitonin, and our pupils and colleagues for samples of blood.

REFERENCES.

- Beumer (1923). *Z. ges. exp. Med.* **35**, 328.
Beumer and Bürger (1913). *Arch. exp. Path. Pharm.* **71**, 316.
Beumer and Lehmann (1923). *Z. ges. exp. Med.* **37**, 274.
Bloor (1916). *J. Biol. Chem.* **24**, 227.
Bloor and Knudson (1916). *J. Biol. Chem.* **27**, 107.
—— — (1917). *J. Biol. Chem.* **29**, 7.
Channon (1925). *Biochem. J.* **19**, 424.
Chauffard, Laroche and Grigaut (1920). *Ann. Med.* **8**, 69, 149, 321.
Denis (1917). *J. Biol. Chem.* **29**, 93.
Fex (1920). *Biochem. Z.* **104**, 82.
Gardner and Fox (1921, 1). *Biochem. J.* **15**, 376.
—— — (1921, 2). *Proc. Roy. Soc. Lond. B*, **92**, 358.
—— — (1924). *Biochem. J.* **18**, 1058.
—— — (1925). *Proc. Roy. Soc. Lond. B*, **98**, 76.
Gardner and Williams (1921). *Biochem. J.* **15**, 363.
Randles and Knudson (1925). *J. Biol. Chem.* **66**, 459.
Richter-Quittner (1920). *Wien. Arch. inn. Med.* **1**, 425.
Terroine (1919). Contribution à la Connaissance de la Physiologie des Substances grasses et lipodiques. Masson et Cie., Paris, p. 334.
Thannhauser (1923). *Z. physiol. Chem.* **127**, 278.
Wacker and Hueck (1913). *Arch. exp. Path. Pharm.* **74**, 416.
Windaus (1910). *Z. physiol. Chem.* **65**, 110.