THE EFFECT OF ALLOXAN DIABETES ON EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS IN THE RABBIT*

III. The Mechanism of the Inhibition of Experimental Cholesterol Atherosclerosis in Alloxan-Diabetic Rabbits

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In a previous publication in this series, Duff and McMillan (1) made a comparison of the effects of cholesterol feeding in normal rabbits and in rabbits rendered persistently diabetic by means of alloxan. It was found that in the two groups of animals hypercholesterolemia of comparable degree was induced by the feeding procedure. Nevertheless, the severity of the atherosclerosis of the aorta produced in the diabetic rabbits was much less than in the non-diabetic controls. Only two factors were observed to be consistently associated with this inhibition of the expected morphological effects of cholesterol feeding; namely, the diabetic state and a degree of visible lipemia considerably greater than that observed in the control animals. Because of the occurrence of this greater degree of visible lipemia in the diabetic rabbits, it was thought likely that a study of the main serum lipids, rather than of cholesterol alone, might serve to elucidate the mechanism of the inhibition of cholesterol atherosclerosis in the alloxan-diabetic rabbit. In this paper, such a study is presented. The findings have already been briefly recorded (2).

The lipids of the serum are insoluble in water; nevertheless they are present in the blood serum, an aqueous medium, which normally is clear and limpid. It is obvious, therefore, that there must be some mechanism whereby they are held in suspension in the serum. It is equally obvious that, when this mechanism becomes upset to a greater or lesser degree, then the colloidal stability of the lipids in the serum is impaired. From the study of the lipids in both normal and pathological sera, it has become evident that the serum lipids, as a group, exhibit two important features. In the first place, the different lipid components are known to show an interrelationship which is more constantly maintained and, therefore, of more significance than the concentration of any single component (3). Secondly, there exists a close association between the

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serum lipids and the serum proteins (4). Both of these features have been used to explain the lack of turbidity in normal serum and it is not unlikely that deviations from the usual pattern set by these characteristics can, by reducing the stability of the lipids in the serum, predispose to the precipitation and accumulation of these substances in adjacent tissue spaces, such as the arterial walls. In this study, therefore, these two features of the serum lipids were taken into consideration.

Determinations of the absolute lipid levels can be made with relative ease and accuracy, but the study of the relationship of the lipids to the serum proteins presents many difficulties. Recently, however, Forbes *et al.* (5) have described a reproducible method which was considered likely to reflect the state of binding of the lipids in one or more of the serum lipoproteins. The values obtained by this method are referred to as the "readily extractable" lipid fractions and are thought to represent lipid not bound or only loosely bound to the serum proteins.

The serum lipids in normal rabbits were studied first, in order that a base line might be obtained with which to compare values obtained during the course of the experiments. This was of particular importance in regard to the values obtained by the method of Forbes, since serum total cholesterol is the only lipid constituent that has been studied by this method. The serum lipids in alloxan-diabetic rabbits were then studied and, finally, a study of the serum lipids was made in normal and alloxan-diabetic rabbits that were being fed cholesterol. In addition, the lipids of the aorta in normal and alloxan-diabetic rabbits, before and after a course of cholesterol feeding, were determined.

The study of the serum lipids in normal and alloxan-diabetic rabbits has already been recorded (6). In this study it was found that in the early stages of alloxan diabetes in the rabbit, there may or may not occur a transitory hyperlipemia in which all the serum lipid constituents, but especially the neutral fat, are elevated. In a few cases the hyperlipemia may persist for an indefinite period of time. This elevation of serum lipids is apparently due to mobilization of fat from the tissue fat depots and is related to the severity of the diabetes. In the normal rabbit only a small proportion of the lipid phosphorus and of the total and free cholesterol is "readily extractable;" in the hyperlipemic serum of the alloxan-diabetic rabbit a much greater proportion of these lipid constituents is "readily extractable."

Materials and Methods

Throughout the present experiments young male adult albino rabbits were used. The rabbits were purchased on the open market from different dealers and both long and short eared varieties were included. The animals weighed approximately 2.0 to 3.0 kilos at the start of the experiment. Upon entering the animal house, the rabbits were placed in individual cages, given the standard diet of Purina laboratory chow and water, *ad lib.*, and allowed to acclimatize themselves to their new environment for a period of not less than 7 days. The animals were

then examined for evidence of disease and weighed. Usually at least one control determination of their serum lipid and blood sugar values was made. Blood was drawn by nicking the central artery of the ear with a sharp razor blade. All blood chemical studies of a given series were done in groups, as far as this was possible.

Rabbits were made diabetic by the intravenous administration of alloxan monohydrate (Eastman Kodak Co.) which was prepared as a 5 per cent solution in distilled water. The dose was 200 mg. of alloxan per kilo of the non-fasting weight of the rabbit. The only treatment that was used after this was the administration by stomach tube of about 50 cc. of 20 per cent dextrose on the same day as the injection of alloxan in an effort to counteract the transient hypoglycemia that develops following the use of alloxan (7). The majority of the surviving animals became diabetic, as judged by the elevation of their blood sugar. Only those animals, however, that maintained persistently a blood sugar of about 300 mg. per cent or more with glycosuria were considered diabetic. A small proportion of the animals, after showing initial diabetic manifestations, returned promptly to a normal metabolic state and thereafter showed no overt manifestations of diabetes. Although certain residual defects in carbohydrate metabolism can be demonstrated in such animals by careful studies (8), we have designated them as "alloxan-recovered."

After the inception of the diabetic state, the rabbits were left without any further treatment for a period of about 6 weeks, in order to allow for stabilization of the metabolic processes. The diabetic rabbits, along with normal control animals, were then started on a course of cholesterol feeding.

Pure cholesterol (B.D.H.[1]) dissolved in corn oil as a 5 per cent solution was used. It was found most convenient to dissolve and maintain the 5 per cent solution at 60°C., allowing it to cool somewhat before feeding. A daily dose of 0.75 gm. cholesterol in 15 cc. oil was used. In the early stages of the experiment, the oil solutions were administered by means of a stomach tube. Thereafter, the daily dose of 15 cc. oil (containing 0.75 gm. cholesterol) was mixed with the food.

At the end of the cholesterol feeding period in each experiment, the rabbits were sacrificed by air embolism and autopsied. The heart and aorta were removed *en bloc*. The aorta was carefully opened and the degree of atherosclerosis recorded by means of schematic drawings and graded on an arbitrary scale of 0 to 4 as illustrated by Duff and McMillan in the first paper (1) of this series.

In all the experiments serum lipid determinations were done at intervals of about 2 weeks. The blood was drawn in the non-fasting state and the serum separated. The presence or absence of visible lipemia was noted and graded on an arbitrary scale of 0 to 4. Half of the serum was used immediately for the determination of the absolute levels of the lipid constituents. Total fatty acids were determined by the method of Stoddard and Drury as modified by Man and Gildea (9); lipid phosphorus by the method of Youngburg as modified by Hawk, Oser, and Summerson (10); and free and total cholesterol by the method of Schoenheimer and Sperry as modified by Sperry (11). Neutral fat was calculated as described by Peters and Man (12). The other half of the serum was rapidly frozen and then dried *in vacuo* and extracted overnight with chloroform in the cold according to the method of Forbes (5). The chloroform was then evaporated *in vacuo* at a temperature below 40°C. and determinations of total fatty acids, lipid phosphorus, and free and total cholesterol were made on the residue as above. The values obtained in this latter way will be referred to as the "readily extractable" lipid fractions, representing, according to Forbes, the lipid fractions either not bound or only loosely bound to protein.

Blood sugar content was determined by a modification of Folin's micro method (13) at the time of each lipid analysis.

[1] British Drug Houses.

For the determination of their lipid content, the aortas which had been fixed in formalin were first washed in water. To make the analyses uniform, that portion of the aorta extending from its attachment to the heart down to the point of origin of the iliac arteries was used in every case. The adventitial fat was scraped away, as far as this was possible. The aortas were

TABLE I

Fractionation of Serum Lipids in Cholesterol-Fed Rabbits*

Series	Rabbit No.	Experimental type	Average fatty acids of neutral fat		Average lipid phosphorus		Average cholesterol			
							Total		Free	
			Abs.	R.E.	Abs.	R.E.	Abs.	R.E.	Abs.	R.E.
			m.eq.	*per cent of abs.*	*mg. per cent*	*per cent of abs.*	*mg. per cent*	*per cent of abs.*	*mg. per cent*	*per cent of abs.*
	C34	D	172.6	87	44.2	69	1268	82	557	84
	C35	D	189.6	84	42.7	67	1110	87	544	83
	C36	D	162.2	90	46.1	63	756	87	392	81
	C28	D	64.6	85	39.6	62	2250	91	1037	90
	C30	D	28.9	77	31.4	66	2374	86	645	78
1	C13	AR	13.6	41	10.8	27	492	62	151	54
	C33	AR	12.9	86	13.3	41	642	74	222	73
	C65	C	21.8	88	25.6	47	1513	86	618	77
	C62	C	16.6	55	10.1	27	593	59	182	69
	C64	C	15.5	96	13.6	54	864	72	382	64
	C67	C	16.4	85	21.8	45	1078	80	418	79
	B5	D	51.3	82	14.7	37	207	82	113	81
2	D49	D	23.5	86	21.8	49	1537	84	512	75
	D50	AR	23.8	51	18.9	57	1150	88	310	77
	A98	C	14.5	57	8.2	23	242	67	126	73
	B56	D	31.9	55	36.1	56	2001	89	520	84
3	B74	D	23.6	64	25.9	58	1514	76	553	96
	C52	C	38.0	67	28.7	61	1686	80	605	70
	C68	C	15.2	100	24.5	58	1800	85	762	89
	B3	C	14.9	77	12.1	26	345	48	137	47

Abs. = absolute levels; R.E. = "readily extractable"; D = alloxan-diabetic; AR = "alloxan-recovered"; C = control.

* The average "readily extractable" lipid levels are expressed as percentages of the average absolute lipid values.

then cut into tiny pieces less than 1/8 inch in thickness and dried to constant weight in a vacuum desiccator. Extraction with alcohol-ether was then carried out and determinations of the lipid constituents made as above.

OBSERVATIONS

Tables I and II summarize the results of the study of the serum lipids in normal and alloxan-diabetic rabbits during the course of cholesterol feeding.

The chemical values given are arithmetical averages of biweekly determinations made during the period of cholesterol feeding. Nine diabetic and 3 "alloxan-recovered" rabbits completed a satisfactory course of cholesterol feeding, which was also administered to 8 normal control animals. The duration of cholesterol feeding varied from 48 to 100 days and the total dose of choles-

TABLE II

Serum Lipids in Normal, Alloxan-Diabetic, and "Alloxan-Recovered" Rabbits Fed Cholesterol

Series	Rabbit No.	Experimental type	Duration of cholesterol feeding	Total dose of cholesterol	Average total serum cholesterol	Average serum lipid values expressed in proportion to serum total cholesterol of 100 mg. per cent					Grade of atherosclerosis (0-4)
						Fatty acid of neutral fat	Lipid phosphorus	Cholesterol			
								Total	Free	Ester	
			days	*gm.*	*mg. per cent*	*m.eq.*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	
	C34	D			1268	13.6	3.5	100	44	56	1 (minimal)
	C35	D			1110	17.2	3.9	100	59	51	1 (minimal)
	C36	D			756	21.5	6.1	100	52	48	0
	C28	D			2250	2.9	1.8	100	46	54	3
	C30	D			2374	1.2	1.3	100	27	73	3
1	C13	AR	76	57	492	2.8	2.2	100	31	69	2
	C33	AR			642	2.0	2.1	100	35	65	3
	C65	C			1513	1.4	1.7	100	41	59	4
	C62	C			593	2.8	1.7	100	31	69	2
	C64	C			864	1.8	1.6	100	44	56	2
	C67	C			1078	1.5	2.0	100	39	61	4
	B5	D			207	24.8	7.1	100	55	45	0
2	D49	D			1537	1.5	1.4	100	33	67	3
	D50	AR	64	45	1150	2.1	1.6	100	27	73	4
	A98	C			242	6.0	3.4	100	52	48	1
	B56	D			2001	1.6	1.8	100	26	74	3
3	B74	D	84	63	1514	1.6	1.7	100	36	64	2
	C52	C			1686	2.3	1.7	100	36	64	4
	C68	C	48	36	1800	0.8	1.4	100	42	58	2
	B3	C	100	75	345	4.4	3.6	100	40	60	1

D = diabetic; A.R. = "alloxan-recovered;" C = control.

terol received by each rabbit varied from 36 to 75 gm. However, the animals were divided into 3 series, within each of which the dose, duration, and frequency of feeding of cholesterol were constant. Two control animals did not fall into any series and were grouped together.

There was an increase of all the lipid constituents in each group of animals (Figs. 1 to 4). In the diabetic rabbits, however, the neutral fat and, to a lesser extent, the lipid phosphorus, showed a much greater rise in proportion to the

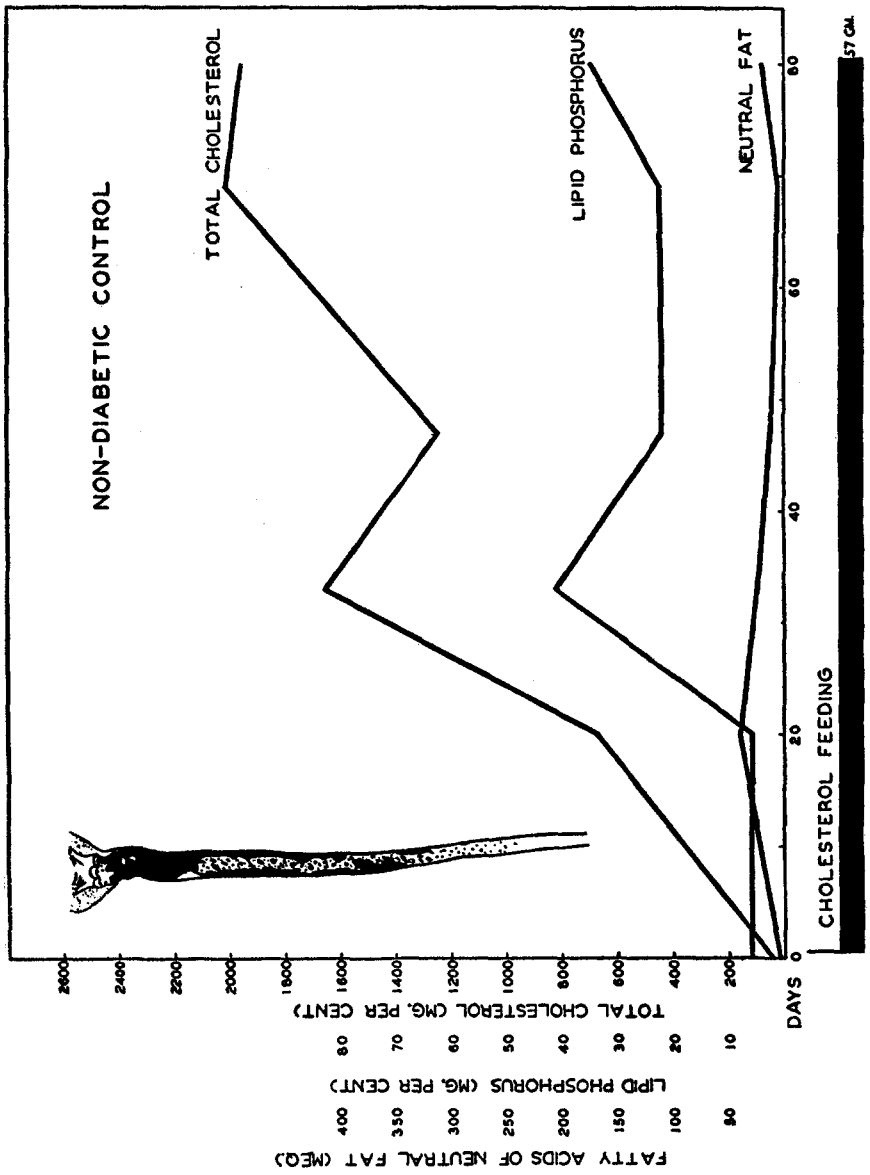

FIG. 1. Diagram and graph illustrating the procedures and findings in non-diabetic control rabbit C65. The aortic atherosclerosis shown was recorded as grade 4.

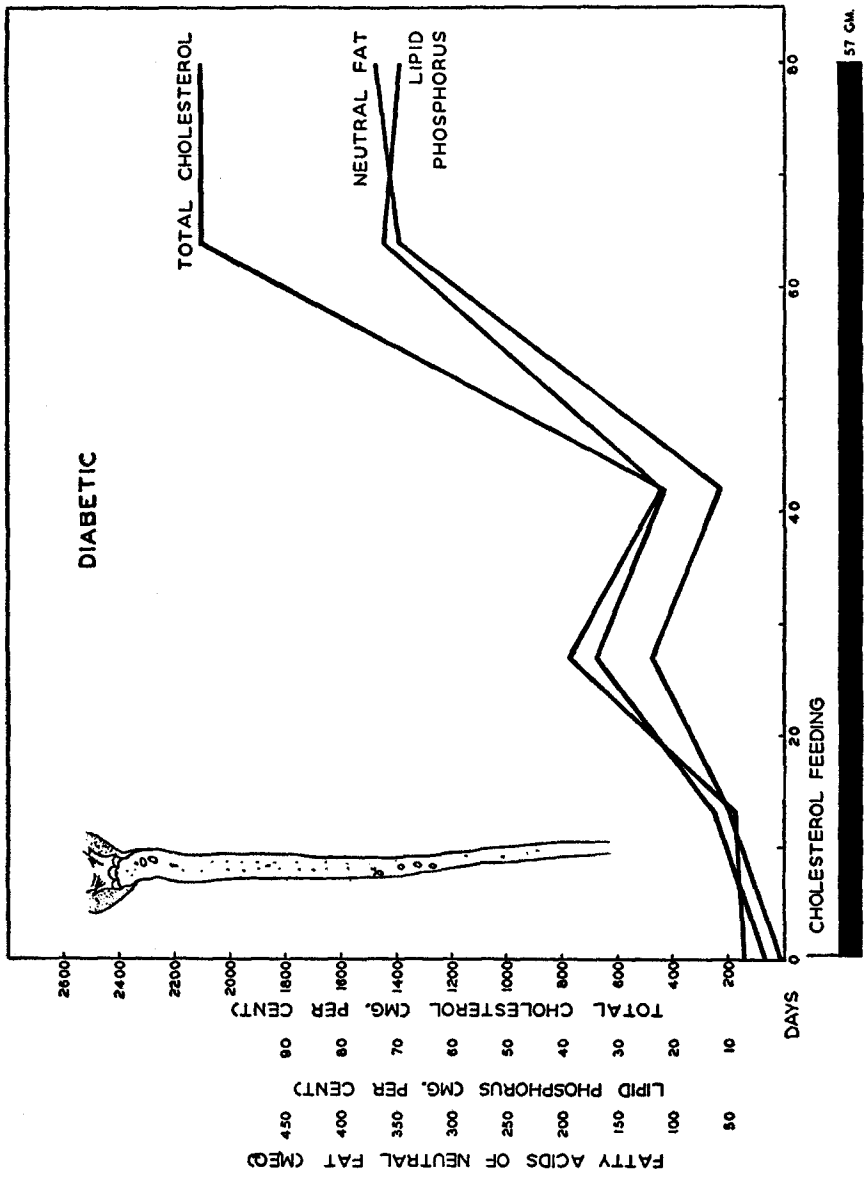

FIG. 2. Diagram and graph illustrating the procedures and findings in diabetic rabbit C35. The aortic atherosclerosis shown was recorded as grade 1 (minimal).

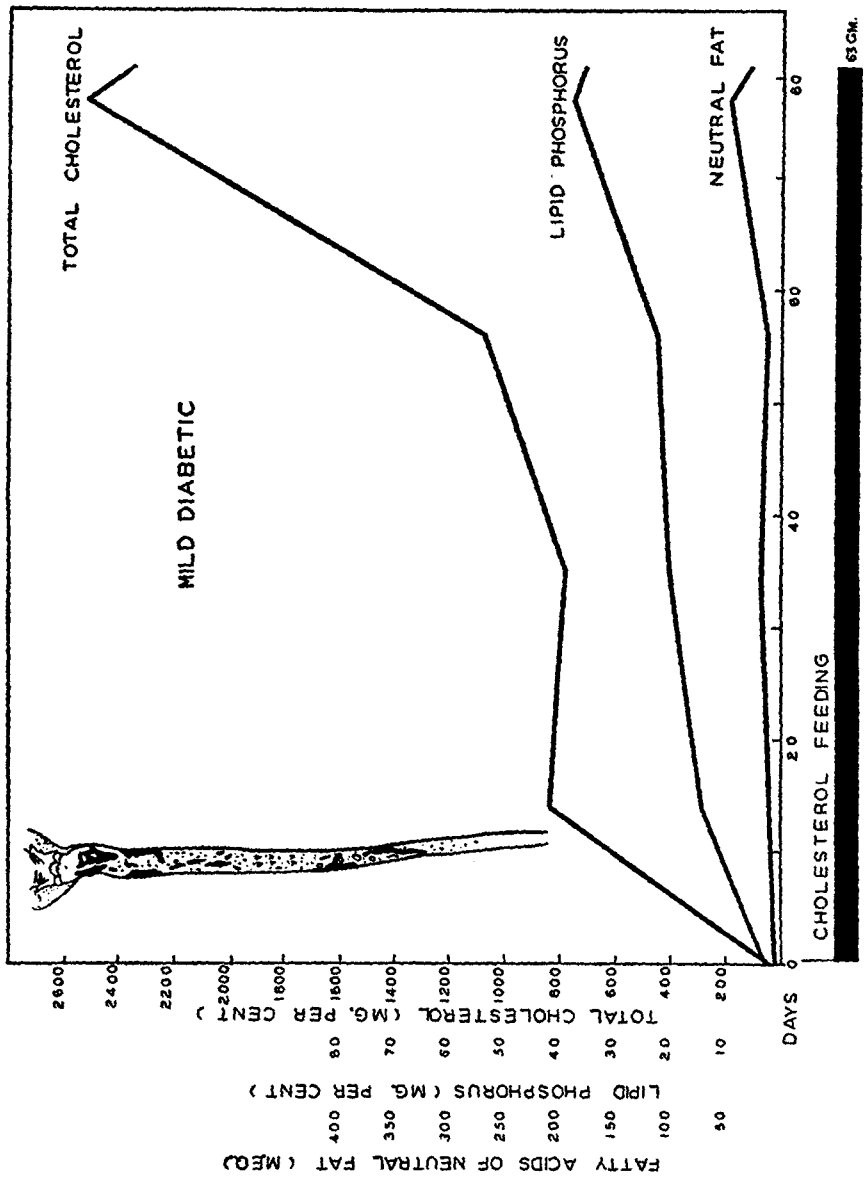

FIG. 3. Diagram and graph illustrating the procedures and findings in mild-diabetic rabbit B74. The aortic atherosclerosis shown was recorded as grade 2.

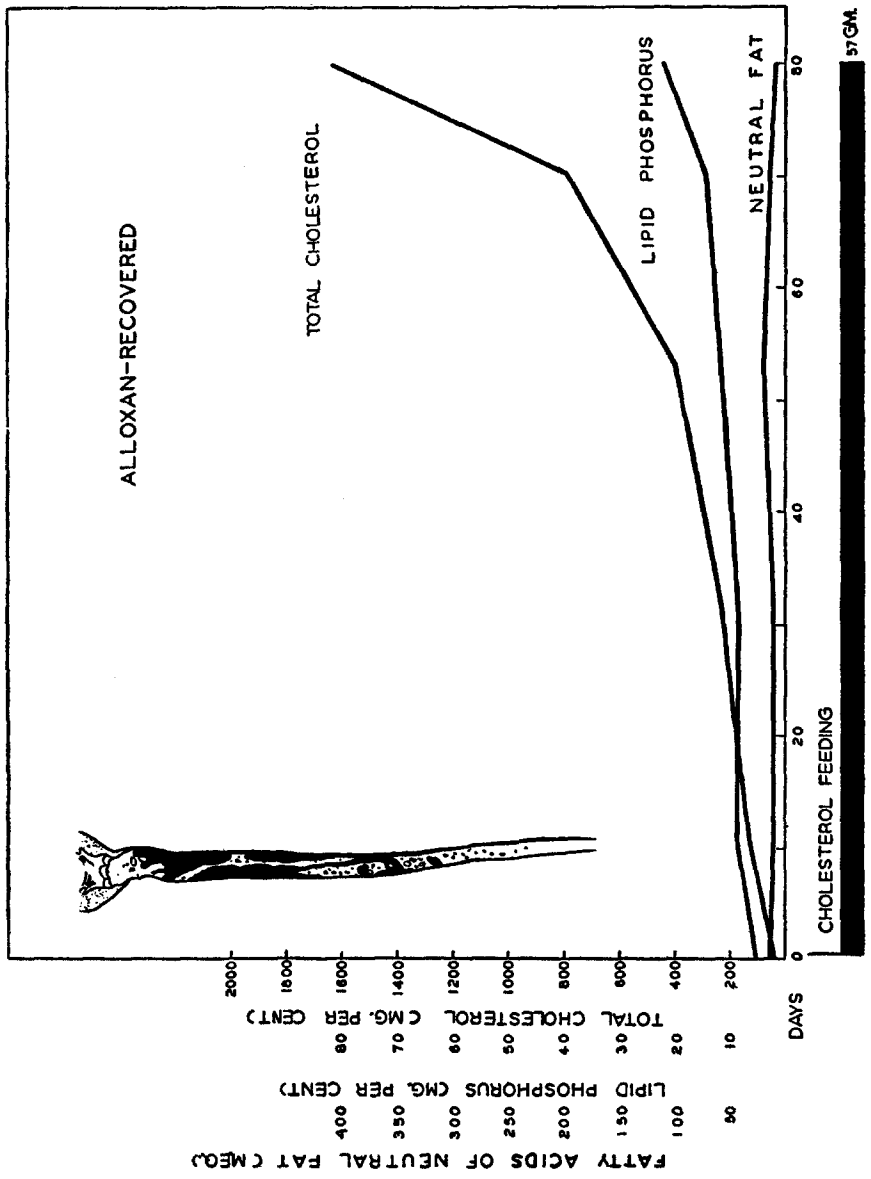

FIG. 4. Diagram and graph illustrating the procedures and findings in "alloxan-recovered" rabbit C33. The aortic atherosclerosis shown was recorded as grade 3.

increase in total cholesterol than they did in the normal or "alloxan-recovered" rabbits. In 5 of the diabetic rabbits, in which the diabetic state was presumably of mild degree (although in all of them there was a persistent elevation of blood sugar with an average value of about 300 mg. per cent), the very marked elevation of neutral fat and lipid phosphorus was not evident.

In Table I, in which the average "readily extractable" lipid levels are expressed as percentages of the average absolute lipid values, it can be seen that, so far as the proportions of "readily extractable" lipids are concerned, there was no detectable difference between the diabetic, "alloxan-recovered," and control rabbits. To take each lipid constituent in turn, it is clear that the proportion of "readily extractable" fatty acids of neutral fat was the same in each group of animals, taking into consideration the numerous sources of error present in its calculation. Similarly, the proportion of "readily extractable," total and free cholesterol was the same in all the animals. In the case of the lipid phosphorus, however, it appears at first glance, that a greater proportion of it was "readily extractable" in the diabetic animals than in the controls, for example, in series 1. But, if the values in the other 2 series are examined, it becomes reasonably clear that the greater percentage of "readily extractable" lipid phosphorus in the diabetic rabbits of series 1 is merely an expression of the higher elevation of the absolute value of this serum constituent in these animals, since the approximation of "readily extractable" and absolute lipid values becomes closer, as the values become more elevated, no matter whether the sera are derived from normal, diabetic, or "alloxan-recovered" rabbits.

However, from examination of the absolute lipid values alone definite positive information was found which could be correlated with the development or failure of development of atherosclerosis. In Table II the average serum lipid values are expressed in proportion to the absolute serum total cholesterol, which has been arbitrarily set at 100 mg. per cent. In this way, the ratios of serum neutral fat and of serum lipid phosphorus to serum total cholesterol in different animals can be readily compared. The most striking feature of the table is that in those diabetic rabbits (series 1, C34, C35, C36; series 2, B5) in which there was a marked elevation of neutral fat and lipid phosphorus in proportion to the increase in total cholesterol, the arteries were protected from the development of atherosclerosis, while in those animals, whether they were diabetics, "alloxan-recovered," or controls, in which the neutral fat and lipid phosphorus were not markedly elevated, the development of atherosclerosis was not inhibited. It should be noted that the level of serum cholesterol in the protected animals was just as high as, or higher than, that in the rabbits that were not protected from the development of severe atherosclerosis.

In summary, in cholesterol-fed rabbits, whether alloxan-diabetic or not, a definite correlation was found between the development of atherosclerosis and an increase of serum cholesterol that was much greater in proportion to

the increase of the serum neutral fat and lipid phosphorus. When these last two lipid constituents rose almost parallel with the serum cholesterol, the development of atherosclerosis was inhibited. A study of the "readily ex-

TABLE III

Lipids of the Aorta*

Experimental type	Rabbit No.	Fatty acids of neutral fat	Lipid phosphorus	Cholesterol			Grade of atherosclerosis (0-4)
				Total	Free	Ester	
		m.eq.	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	
Normal controls (6 animals)	—	6.7–36.9	4.3–12.0	103–164	55–97	35–108	0
Alloxan-diabetic (6 mos.)	A2	3.1	11.8	160	60	100	0
	D75	13.2	9.8	290	98	192	0
"Alloxan-recovered" (6 mos.)	E55	13.9	10.2	133	72	61	0
	E72	38.2	14.2	197	84	113	0
Normal, fed cholesterol	B3	19.9	9.8	269	90	179	1
	C62	21.6	10.6	423	123	300	2
	C64	33.3	8.7	377	155	222	2
	C68	20.5	5.5	314	77	237	2
	C65	35.6	16.2	1203	718	485	4
	C67	21.7	21.0	1068	811	257	4
	A99	32.7	14.2	741	324	417	4
Alloxan - diabetic, fed cholesterol	C34	31.0	6.5	231	78	153	1 (minimal)
	C35	14.1	6.9	111	67	44	1 (minimal)
	C36	4.8	6.3	133	72	61	0
	C28	23.6	10.5	583	149	434	3
	C30	22.1	10.2	680	188	492	3
	D49	12.6	10.1	358	126	232	3
"Alloxan-recovered," fed cholesterol	C13	26.4	7.2	180	62	118	2
	C33	22.5	17.1	917	347	570	3
	D50	18.8	25.5	1628	919	709	4

* The values are expressed per 100 gm. dry weight.

tractable" lipid fractions did not reveal any significant differences in the behavior of these fractions between those animals that developed atherosclerosis and those that did not.

Table III summarizes the data from the lipid analyses of the aortas of normal, alloxan-diabetic, and "alloxan-recovered" rabbits before and after cholesterol feeding. It can be seen that in the group that was not fed cholesterol the

lipids of the aorta were essentially the same, no matter whether they were normal, alloxan-diabetic, or "alloxan-recovered." This was to be expected, since Duff and Wilson (14) in this laboratory have found that neither alloxan-diabetic nor "alloxan-recovered" rabbits develop cholesterol atherosclerosis spontaneously. Indeed, it was because of this work that analyses on more aortas from alloxan-diabetic rabbits were considered unnecessary. After a course of cholesterol feeding, analyses of the atherosclerotic aortas showed an increase of total cholesterol in all of them. In the more severe cases there was also an increase of lipid phosphorus as well. Neutral fat was not appreciably increased. Whether the lesions occurred in alloxan-diabetic, "alloxan-recovered," or normal rabbits, the lipid composition was essentially the same. The amount of cholesterol present was roughly proportionate to the estimated grade of atherosclerosis. In the less severe lesions, the proportion of ester cholesterol was greater than that of free cholesterol, but in the extensive lesions the reverse was true. Analysis of those aortas from alloxan-diabetic rabbits in which atherosclerosis did not develop, in spite of cholesterol feeding, revealed a lipid composition similar to that of the aortas of the rabbits that had not been fed cholesterol.

DISCUSSION

First of all, it should be pointed out that the inhibition of experimental cholesterol atherosclerosis in alloxan-diabetic rabbits has been definitely established (1) and confirmed (15). Therefore, in the work reported here, it was considered unnecessary to use very large numbers of animals. However, in every case in which the serum lipids were studied with a view to understanding the mechanism whereby cholesterol atherosclerosis is inhibited in the alloxan-diabetic rabbit, the serum lipid findings were always considered in the light of the presence or absence of atherosclerotic lesions in that particular rabbit's aorta. In this way actual confirmation, as well as amplification, of the initial premise was achieved.

From the results which have been presented, it is clear that the inhibition of experimental cholesterol atherosclerosis in the alloxan-diabetic rabbit is closely associated with a marked elevation of serum neutral fat and lipid phosphorus in proportion to the increase in serum total cholesterol. More than this, however, the protective effect is apparently independent of the diabetic state, *per se*, since those rabbits that were definitely diabetic, as judged by a persistent elevation of the blood sugar, but did not have a marked increase of serum neutral fat and lipid phosphorus, developed atherosclerosis in a degree comparable with that observed in normal control animals.

It appears, therefore, that, so far as the serum lipids are concerned, the development of experimental cholesterol atherosclerosis in the rabbit depends not merely on the elevation of serum cholesterol, but on an increase of choles-

terol content that is disproportionate to the increase of the other serum lipid fractions. Indeed, studies of the serum lipid pattern in experimental cholesterol atherosclerosis, not only in the rabbit (16) but in the chicken (17) and the dog (18) as well, have shown that in all these animals the development of atherosclerosis is associated with an elevation of serum cholesterol that is much greater than the elevation of the other serum lipids.

As we have already pointed out, the different lipid components of the serum are known to show an interrelationship which, under normal conditions, is more constantly maintained than the concentration of any single component. Because of the relative constancy of the ratio between the lipid phosphorus and the total cholesterol, at least in human serum (19), and because of the hydrophilic properties of the phospholipids (20), it would appear that the latter play an important part in holding the more hydrophobic cholesterol in suspension in the serum—a watery medium. Indeed, the recent experiments of Ahrens and Kunkel (21) in which the polar nature of serum "lecithin" was destroyed by enzymatic hydrolysis, using *Clostridium welchii* lecithinase, support the concept that the phospholipids play an important part in stabilizing the serum lipids. It seems, therefore, that the elevated lipid phosphorus is probably the important factor tending to prevent the deposition of cholesterol in the arterial wall in the alloxan-diabetic rabbit. That the high neutral fat does play some part, however, cannot be denied as yet.

Kellner and collaborators (22) have recently demonstrated that the intravenous injection of synthetic detergents (Tween 80, Triton A 20) in cholesterol-fed rabbits inhibits the development of experimental cholesterol atherosclerosis. In these experiments, the elevation of serum cholesterol was accompanied by a parallel or even greater rise of phospholipids. The conclusion was drawn that the incidence and severity of atherosclerosis were decreased if the blood phospholipid content was elevated concomitantly with the cholesterol. However, one cannot rule out with certainty a direct effect of the detergents employed, independent of the serum lipid changes.

Unlike the experiments with synthetic detergents, alloxan diabetes in the rabbit is a disease in which there is nothing foreign, introduced from outside the body, circulating in the blood stream, other than cholesterol itself which is introduced in the diet. Because of this, the implications of the experiment with alloxan-diabetic rabbits reported above appear much more conclusive. Indeed, so far as we are aware, this is the only experiment thus far reported which clearly bears out the validity of the evidence that experimental cholesterol atherosclerosis develops in the rabbit only when the serum cholesterol is elevated out of all proportion to the elevation of the other serum lipids. For this experiment shows that, if the other serum lipids are all proportionately elevated along with the serum cholesterol, then atherosclerosis does not readily develop.

The study of the "readily extractable" lipid fractions is also highly informative. In all the diabetic, as well as the normal rabbits, that were fed cholesterol and in which the blood lipids were elevated, the greater part of the lipid phosphorus and cholesterol was "readily extractable" in contrast to the small proportion of these substances that is "readily extractable" in normal rabbit sera (6). Nevertheless, many of the diabetic rabbits did not develop atherosclerosis. It would appear, therefore, that, although looseness of linkage with the serum proteins may be conducive to the deposition of cholesterol in the arterial wall, other conditions must also be satisfied before deposition does occur. Some of these conditions have been considered above. The reasons for the postulate that the "readily extractable" fractions represent lipid that is not bound or only loosely bound to protein are not given by Forbes *et al.* (5). However, the following considerations support the validity of the hypothesis.

The freezing and drying of the serum, as employed in Forbes' method of extraction, clearly has to do with the role of water in the binding of the lipids with the serum proteins. In this regard, experiments published in 1942 by McFarlane (23) are very pertinent. Human serum was shaken with ether and the mixture frozen below −25°C. On thawing, considerable quantities of lipid were found in the ether layer. The effect was not obtained when temperatures above −20°C. were used; conversely, temperatures down to −70°C. did not increase the yield. Successive treatment of 1 litre of serum yielded 3.5, 0.35, and 0.2 gm. of lipid; a further 2.5 gm. could be obtained by treatment with alcohol-ether. The only change in the electrophoretic pattern of the serum was a reduction in the concentration of beta globulin. McFarlane found that serum frozen and thawed or dried from the frozen state and reconstituted in the absence of ether became cloudy. His observations suggested the conclusion that the association of lipid with a stabilizing substance depends on the presence of liquid water and is destroyed by freezing. Thus, on subsequent thawing, unprotected lipid is able to aggregate to form visible particles or droplets. It seems very unlikely that the whole of the lipids that McFarlane was able to extract was associated with beta globulin alone; therefore, lipids from other protein fractions must have been involved. Chargaff, Bendich, and Cohen (24) found the same effect of freezing with ether on the integrity of the thromboplastic protein. They stated that once the protective water barrier is frozen away, the uniformity of the ostensibly homogeneous complex disappears owing to the removal of lipids by the ether, and separation into discrete components takes place. Indeed, loosening of the bonds between lipid and protein occurs in both the alpha and beta lipoproteins (25) on freezing to temperatures below −25°C.

It appears, therefore, that in the method of extraction used by Forbes *et al.* the protective water barrier is first removed by the freezing-dehydration process. Extraction with chloroform at a relatively low temperature then re-

moves the lipids that are only loosely associated with protein. Chloroform, of course, is a much weaker extractant than alcohol, which rapidly denatures the protein, and used at a low temperature it would be expected to remove only easily extractable lipid without further alteration of the serum proteins.

At any rate, our previous studies (6) have shown that when this method of extraction is applied to the serum lipid phosphorus as well as to the serum cholesterol in the normal rabbit, only small proportions of these two lipid components (less than 12 per cent lipid phosphorus and usually less than 50 per cent of the total or free cholesterol) are extractable, a result which is in good agreement with the finding of previous investigators that the greater part of these lipid constituents in human serum is closely associated with the serum proteins (25).

The chemical analyses of the aortas showed that in those animals which were not fed cholesterol, there was no difference in lipid composition, no matter whether they were alloxan-diabetic or not. Furthermore, analysis of the aortas of the alloxan-diabetic rabbits that were protected from the development of atherosclerosis, in spite of cholesterol feeding, revealed a similar lipid composition. When atherosclerosis developed following cholesterol feeding, the lipid composition of the lesions was essentially the same in both control and diabetic animals, cholesterol constituting the greater part of the deposited lipid. These results are clearly at variance with those reported by Chaiko[a] *et al.* (17) in the chick. These investigators reported a close correlation between the serum lipid pattern and the lipid composition of the arterial lesions in chicks. However, it should be pointed out that they studied the lipid composition in the arterial wall by means of differential staining—a notoriously unreliable method. Our results show that in the rabbit the deposition of lipids in the arterial walls consists predominantly of cholesterol with only small and fairly constant proportions of other lipids that do not vary significantly, regardless of the quantities of these other lipids present in the circulating blood. In the less severe lesions, the proportion of ester cholesterol was greater than that of free cholesterol, but in the extensive lesions the reverse was true. This latter finding is in agreement with the observations of other workers both in human atherosclerosis (26, 27) and in experimental cholesterol atherosclerosis in the rabbit (16). It has been suggested that the increased proportion of free cholesterol in the severe lesions is due to splitting of cholesterol esters *in situ*.

The discussion so far has dealt with the changes in the serum lipids, without any reference to the factors responsible for these changes. There are, however, certain clues as to how the marked elevation of serum neutral fat and lipid phosphorus occurs in alloxan-diabetic rabbits fed cholesterol. From our study of the serum lipids in alloxan-diabetic rabbits not fed cholesterol (6), evidence was presented that in the transitory lipemia that may occur immediately following the inception of the diabetic state, the elevation of serum lipids was

due to the mobilization of fat from the fat depots and body tissues to meet the excessive demand for fat combustion caused by the disturbance in the regulation of carbohydrate metabolism. Such a concept is supported by the almost total absence of body fat in many diabetic rabbits at autopsy. It appears logical, therefore, to ask whether the marked elevation of serum neutral fat and lipid phosphorus, which may occur in the alloxan-diabetic rabbit fed cholesterol and is closely associated with the failure of development of atherosclerosis in these animals, is not also due to mobilization of body fat. Indeed, it appears quite likely that this is so. However, neither in the original experiment (1) of this series nor in the present study could a correlation between weight loss and inhibition of development of atherosclerosis be demonstrated. It is possible that the loss of body fat is masked by the feeding of cholesterol and oil and by other factors such as skeletal growth, etc. In this connection, it should be pointed out that the recent paper of Firstbrook (28), purporting to show a high net correlation between relative weight gain and severity of atherosclerotic lesions in cholesterol-fed rabbits, is far from conclusive. However, if it should be eventually demonstrated that weight loss in animals on a restricted diet during cholesterol feeding is closely associated with retardation of the development of experimental cholesterol atherosclerosis, the mechanism of this retardation may prove to be the same as that suggested by our experiments. The mobilization of body fat occurring during partial starvation might well produce a disturbance of blood lipid pattern in cholesterol-fed rabbits, similar to, but probably not so marked as, that observed in our alloxan-diabetic rabbits during cholesterol feeding. The problem requires urgent study, particularly since the work of Wilens (29) suggests that in man changes in body weight and nutrition play an important part in the genesis of atherosclerosis.

Finally, from the results which have been presented and discussed, the following conclusions concerning the pathogenesis of experimental cholesterol atherosclerosis in the rabbit may be drawn:—

1. Our experimental results support the evidence that not hypercholesterolemia, *per se*, but rather instability of cholesterol in the blood is the general condition responsible for the deposition of this substance in the arterial walls. For the experiment shows that marked hypercholesterolemia can exist without the development of atherosclerosis, provided that there is a proportionate elevation of serum lipid phosphorus and neutral fat. From the available information, it appears that the elevation of phospholipids is the important factor, tending to keep the increased cholesterol in stable suspension in the blood. Whether the elevated neutral fat plays a part is unknown.

2. The results of the experiments suggest that of the two factors considered to be responsible for the stability of the serum lipids, namely (*a*) their interrelations and (*b*) their association with the serum proteins, the former is probably the more important, at least in so far as the development of experi-

mental cholesterol atherosclerosis is concerned. For, in all the hypercholesterolemic sera studied, the greater proportion of the serum lipids was "readily extractable," irrespective of whether atherosclerosis developed or not. Looseness of linkage of the serum cholesterol with the serum proteins may, by reducing its stability, be conducive to its deposition in the arterial wall. However, a further decrease in the stability of cholesterol, due to derangement of the normal interrelations of the serum lipids, must also occur before deposition of cholesterol in the arterial walls readily takes place.

The bearing of these conclusions upon the pathogenesis of human atherosclerosis may well be important, particularly in view of the recent work of Gofman *et al.* (30). As a result of ultracentrifugal studies, these investigators have presented suggestive evidence that both in experimental cholesterol atherosclerosis in the rabbit and in the naturally occurring human disease, the presence of giant cholesterol-bearing molecules in the serum may be correlated with the development of the disease. It should be noted that these giant molecules appear to have a lower content of protein than the naturally occurring cholesterol-bearing lipoproteins. Moreover, although there was a general trend towards increased frequency of occurrence of such giant molecules in human sera with cholesterol levels over 200 mg. per cent, this was by no means a universal finding. They may occur in sera with normal or low cholesterol values, while sera with an elevated cholesterol content may not show any measurable concentration of them. Such a finding is in keeping with the unsuccessful attempts (31) to correlate the absolute level of cholesterol with the incidence and severity of atherosclerosis. It would be of great interest to have simultaneous studies of the interrelations of the different serum lipid constituents. It may well be that the formation of the giant molecules found in Gofman's study is in some way related to disruption of the normal interrelations of the serum lipids. In this connection, the recent report of Eilert (32) that the administration of estrogen to women caused a sharp reduction in the ratio of total cholesterol to lipid phosphorus and that this effect of estrogen on the serum lipids may have some bearing on the lower incidence of atherosclerosis in women is of interest. Suffice it to say, for the present, that in studying the pathogenesis of atherosclerosis, whether in the experimental animal or in man, it is no longer justifiable to consider only the cholesterol content of the blood. It appears that the relation of cholesterol to the other serum lipids and to the serum proteins is much more important than the absolute level of cholesterol itself.

SUMMARY AND CONCLUSIONS

A study of the serum lipids in normal and alloxan-diabetic rabbits during the course of cholesterol feeding is presented, particular attention being paid to the factors considered to be responsible for the stability of the serum lipids;

namely, (1) their interrelations and (2) their association with the serum proteins.

As far as the interrelations of the lipids were concerned a definite correlation was found between the development of atherosclerosis and an increase of serum cholesterol that was out of all proportion to the increase of serum lipid phosphorus and neutral fat. When these last two lipid constituents rose almost parallel with the serum cholesterol (as they did in some alloxan-diabetic rabbits), then the development of atherosclerosis was inhibited. This correlation was independent of the diabetic state, *per se*. It appeared likely that the marked elevation of serum neutral fat and lipid phosphorus in the diabetic animals was due to mobilization of body fat because of the disturbed carbohydrate metabolism. Because of their hydrophilic and emulsifying properties, it was thought probable that the elevation of the phospholipids was the important factor responsible for the stability of serum cholesterol. That neutral fat played a role, however, could not be denied.

In normal rabbit sera, as we have previously shown, only small proportions of the lipid phosphorus and cholesterol are "readily extractable" (*i.e.*, unattached or only loosely attached to protein). On the other hand, in every case in which the serum lipids were elevated, the greater proportion of the lipid phosphorus and cholesterol was "readily extractable," irrespective of whether atherosclerosis developed or was inhibited.

Analysis of the lipid content of the aorta of rabbits not fed cholesterol, whether diabetic or non-diabetic, and from alloxan-diabetic rabbits fed cholesterol but protected from the development of atherosclerosis, showed that there was no significant difference in lipid content or composition among the animals of these groups. When atherosclerosis developed following cholesterol feeding, the lipid composition of the aortas was essentially the same in both control and diabetic animals. The deposited lipid consisted predominantly of cholesterol with small and fairly constant proportions of other lipids that did not vary significantly regardless of the quantities of these other lipids present in the circulating blood. In the less severe lesions the proportion of ester cholesterol was greater than that of free cholesterol, but in advanced lesions the reverse was true.

The following conclusions are drawn concerning the pathogenesis of experimental cholesterol atherosclerosis in the rabbit:

1. Instability of cholesterol in the blood rather than hypercholesterolemia, *per se*, is the general condition responsible for the deposition of this substance in the arterial walls.

2. Of the two factors considered to be responsible for the stability of the lipids in the blood, the interrelations of the lipids appear to be more important than their relation to the serum proteins, at least in so far as the development of experimental cholesterol atherosclerosis is concerned.

The importance of these conclusions in relation to the pathogenesis of human atherosclerosis is discussed.

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