THE RELATION BETWEEN ATHEROSCLEROSIS AND INGESTED CHOLESTEROL IN THE RABBIT.

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INTRODUCTION.

Earlier investigators who fed diets containing animal protein to herbivora described the occurrence of disease of the aorta that simulated human atherosclerosis. They attributed the experimental lesion to the cholesterol of the diet. Our own earlier experience[1] with diets of this general type led us to conclude that the large amount of protein and not the cholesterol had caused the lesion. We reached this opinion because the cholesterol content of our diet was smaller than the smallest amount alleged to have produced atherosclerosis in the hands of other workers. The data contained in the papers of the earlier workers seemed to us insufficient to prove that cholesterol was the primary factor, for one or more of the following reasons; *viz.*, that in the experiments of Steinbiss,[2] Saltykow,[3] Stuckey,[4] Anitschkow and Chalatow,[5] the duration of the feeding of cholesterol was not extensive enough; the cholesterol was administered to animals on diets which were abnormal and well might have led to metabolic disturbance under any condition; or the cholesterol was fed in conjunction with large amounts of protein. We felt, therefore, that further information was necessary to settle this point.

In order to get an entirely unequivocal answer to this question,

[1] Newburgh, L. H., and Clarkson, S., *Arch. Int. Med.*, 1923, xxxi, 653.

[2] Steinbiss, W., *Virchows Arch. path. Anat.*, 1913, ccxii, 152.

[3] Saltykow, S., *Beitr. path. Anat. u. allg. Path.*, 1914, lvii, 415.

[4] Stuckey, N. W., *Centr. allg. Path. u. path. Anat.*, 1911, xxii, 379; 1912, xxiii, 910.

[5] Anitschkow, N., and Chalatow, S., *Centr. allg. Path. u. path. Anat.*, 1913, xxiv, 1.

we determined to feed cholesterol in different amounts over long periods of time to rabbits that were receiving diets otherwise normal. In this way we hoped to determine: first, whether ingestion of cholesterol at any level is capable in itself of producing vascular disease; and if so, second, how large an amount is required to produce the lesion.

In earlier experiments[1] from this laboratory dealing with this question a diet containing 36 per cent protein was used. The diet resulted in a nitrogen metabolism of about 1 gm. per kilo, roughly four times the normal; and caused the rabbits to ingest from 17 to 31 mg. of cholesterol per kilo daily, according to the size of the animal. Such animals developed advanced intimal disease in 18 to 36 weeks. The atherosclerosis found in these animals was due either to excess of protein or of cholesterol in the diet. If cholesterol were the causative factor it should produce the lesion when added to a normal diet in the amount in which it is present in the above mentioned animal diet. Failure to obtain lesions under these conditions would demonstrate that cholesterol in such amounts was not productive of atherosclerosis; and would justify the conclusion that the excess of protein, which was the only other known abnormal factor, was responsible for the lesion.

The second question which we hoped to answer by means of the experiments to be described was whether cholesterol in any amounts would cause atherosclerosis when it was added to an otherwise normal diet.

When our work was partly completed it became evident that small amounts of cholesterol (such as occur in the high protein diet previously mentioned), when added to a normal diet, have no effect on the aorta or on the level of blood cholesterol. Accordingly, it seemed desirable to discover whether the high protein diet with its very marked effect upon the aorta would change also the blood cholesterol level. This seemed the more necessary after we found that the rabbit could be made to ingest cholesterol in amounts sufficient to induce both arterial disease and hypercholesterolemia.

Methods.

Care of the Animals.—The animals used for the experiments were from the laboratory stock. They had had the usual diet of bread, oats, hay, and greens.

They were from 2½ to 6 months old when treatment was begun. During the experiment they were kept in groups of from four to ten in pens with outside runways. Whenever an animal appeared to be infected it was removed from the series and discarded.

The rabbits were divided into five groups. The first group gave us the control series of blood cholesterols. These rabbits received the stock diet and were bled for the purpose of the experiment, or samples were obtained from the blood of rabbits that were being bled to death in order to supply blood for bacteriological purposes. The other four groups daily received 25, 113, 253, and 507 mg. respectively of cholesterol in capsules. It was the aim of the experiment to administer the amount of cholesterol corresponding to the quantity found in the high protein diet to the second group, and increasingly larger amounts to the other groups. However, it was necessary to regulate the dose to the size of the capsule obtainable. The smallest capsule used held 113 mg. of cholesterol, which was the nearest approximation to the desired eighth of a gram dose. Progressively larger capsules held 253 and 507 mg. respectively. For the first group there was no capsule small enough to hold exactly 25 mg. of cholesterol. Sugar of milk and the cholesterol were mixed in such proportions that a capsule of convenient size would hold the proper dose.[6]

In order to determine whether the ingestion of cholesterol had any effect on the blood level of cholesterol, samples of blood were drawn in some cases from the heart, in some from the ear.

5 cc. of the whole blood collected in an oxalated receptacle were pipetted into a flask containing an alcohol-ether mixture (3–1), with constant agitation. This was heated gently over a water bath for a few minutes, then set aside to cool to room temperature. When cool it was filtered. The filtrate then was made slightly alkaline to litmus with concentrated NaOH, and evaporated to dryness. The residue was taken up several times with hot chloroform. This in turn was evaporated to dryness and taken up with 10 cc. of 95 per cent alcohol, and heated. To this hot solution a 1 per cent digitonin solution was added. Usually only 40 mg. of digitonin were used. If on the addition of the digitonin a heavy precipitate came down quickly, more digitonin solution was added. This precipitate then was filtered on filter paper whose desiccated weight had been determined. The filter paper holding the digitonin cholesteride was then put into the desiccator until the weight became constant.[7]

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[6] To be sure that the rabbits actually got the cholesterol they were placed on their backs, the jaws were kept open by means of a piece of wood with a hole in the center which came just over the gullet, and the capsule was passed down the pharynx by means of a small rubber tube.

[7] Windaus, A., *Z. physiol. Chem.*, 1910, lxv, 110.

DATA.

State of Aorta.—Group II.—Twenty rabbits were fed 25 mg. of cholesterol daily over a period lasting from 51 to 288 days. In no single instance did careful examination of the aortæ reveal an atherosclerotic process. (Table III.)

Group III.—This group received 113 mg. of cholesterol daily from 12 to 302 days. One of these nineteen rabbits, that had ingested cholesterol for 302 days, possessed an aorta which was the site of very slight atherosclerosis. (Table IV.)

Group IV.—Twenty-five rabbits received 253 mg. of cholesterol daily for a period of from 33 to 246 days. Thirteen were fed 147 to 246 days and eight (62 per cent) of the latter animals showed definite atherosclerosis. One other fed 71 days showed very slight abnormal areas which on microscopical examination were found to consist of slight thickening of the intima. Of twelve animals fed 33 to 125 days, eleven (92 per cent) had normal aortæ. When one compares the level of blood cholesterol with the state of the aorta (Table V), one finds that four of the eight atherosclerotic rabbits had abnormally high readings and that in the case of the other four the readings were all well within the normal.

Group V.—507 mg. This group included seven animals, five of which had well marked lesions, but two others showed lesions so minute that even histologically they could not be pronounced positive for atherosclerosis. This group was fed from 47 to 86 days. (Table VI.)

Lesions.—The lesions in the aortæ of rabbits fed cholesterol were identical with those produced by the ingestion of a high protein diet. Grossly the injury appeared as small, yellowish white, slightly raised streaks and plaques. In most instances it consisted of from one to four isolated little plaques, 1 to 3 mm. in diameter. This involvement occurred in the arch of the thoracic aorta, or at the exits of the larger vessels just below the arch. In five cases there were many scattering plaques from the arch nearly to the caudal bifurcation. In several instances the extent of the lesion grossly did not correspond to the microscopical findings; *i.e.*, in the case of one or two plaques of minute size we found the histological picture of an advanced process.

The involvement was primarily in the intima. To get some idea of the extent of involvement microscopically, we have sorted the atherosclerotic aortæ into three groups labeled slight, moderate, and extensive (Table I). Slight indicates that the lesion consists of only a few layers of fatty cells; moderate, the intimal thickening is maximal due to many layers of cells; extensive denotes not only cases of maximal thickening but also those with fibrosis on the lumen side of the lesion, and involvement of the media.

TABLE I.

Extent of Vascular Lesion.

Animal.	Daily dose.	Total dose.	Slight.	Moderate.	Extensive.	Blood cholesterol.
	mg.	*gm.*				*mg. per 100 cc.*
CH73	507.3	43.63		+		382.6
CH157	507.3	40.076		+		249.9
CH16	507.3	28.53		+		Lost.
CH14	507.3	24.25		+		"
CH22	507.3	24	+			"
CH128	253.65	62			+	168.23
CH123	253.65	62	+			65.15
CH116	253.65	56		+		67.3
CH140	253.65	47		+		72.9
CH120	253.65	45		+		Lost.
CH32	253.65	37			+	133.4
CH26	253.65	37	+			126.6
CH30	253.65	37	+			64.8
CH31	253.65	18	+			166.4
CH92	113.2	34		+		70.99

The two cases marked extensive showed a pronounced intimal involvement, in one animal with fibrosis on the lumen side (No. CH32), and in the other with some involvement of the media (No. CH128). Both of these cases occurred in the series receiving 253 mg. daily. There were eight animals which fell into the class designated moderate. Four of these belonged in the group receiving 507 mg. daily. The eighth animal was of the series which ate 113 mg. daily. In the class marked slight, there were five animals. One belonged to the group receiving 507 mg. daily. The other four belonged to the 253 mg. series.

Blood Cholesterol.—Table II contains readings from thirty-six controls. It will be seen that the free cholesterol in this series ranges from 35 to 125 mg. per 100 cc. of whole blood.

TABLE II.

Free Blood Cholesterol in Control Rabbits.

mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
125.44	82.04	67.09	56.15
116.68	77.79	67.09 (fasting).	51.4
116.68	77.79	64.42	51.04
105.9	77.79	64.18	50.7
93.35	75.9	62.2	44.6
87.03	71.1	60.28	43.49
85.57 (fasting).	70.19	59.02 (fasting).	38.8
83.38	69.58	57.6	35.39 (fasting).
83.14	68.55	56.64	

TABLE III.

Animals That Received 25 Mg. Cholesterol Daily.

Animal.	No. of days of feeding.	Total dose.	Blood cholesterol.	Atherosclerosis.
		gm.	*mg. per 100 cc.*	
CH80	288	8	30.63	—
CH101	286	7.9	15.9	—
CH78	282	7.8	Lost.	—
CH112	266	7.5	"	—
CH110	266	7.5	76.8	—
CH84	262	7.2	Lost.	—
CH103	249	6.7	95.2	—
CH53	162	4	92.3	—
CH60	149	3.6	20.9	—
CH57	149	3.6	62.7	—
CH95	141	3.5	42.5	—
CH54	140	3.4	37.4	—
CH64	132	3.2	58.3	—
CH66	132	3.2	59.8	—
CH109	120	3	Lost.	—
CH87	104	2.5	23.9	—
CH56	76	1.9	Lost.	—
CH107	68	1.5	79.7	—
CH58	63	1.5	66.6	—
CH65	51	1.2	88	—

In Table III, the blood cholesterol of twenty animals receiving 25 mg. of cholesterol daily is tabulated in relation to the duration of the feeding. The range is from 16 to 95 mg.

Table IV contains the same information for the rabbits receiving 113.2 mg. daily, with a range of from 23 to 130 mg. The four slightly elevated readings in this group do not occur in the animals that received the largest doses of cholesterol. Furthermore, it should

TABLE IV.

Animals That Received 113.2 Mg. Cholesterol Daily.

Animal.	No. of days of feeding.	Total dose.	Blood cholesterol.	Atherosclerosis.
		gm.	*mg. per 100 cc.*	
CH83	302	34	87.52	—
CH92	302	34	70.98	+
CH96	299	33	78.76	—
CH94	272	30	48.1	—
CH98	225	25	72.9	—
CH81	225	25	Lost.	—
CH108	225	25	22.7	—
CH104	225	25	51.5	—
CH100	225	25	129.8	—
CH35	164	18	Lost.	—
CH42	144	16	130.4	—
CH37	129	14.6	Lost.	—
CH39	127	14.3	128.5	—
CH36	84	9.5	126.1	—
CH34	78	8.8	Lost.	—
CH38	69	7.8	56.5	—
CH41	67	7.4	42.88	—
CH40	60	6.8	77.98	—
CH43	12	1.35	85.08	—

be noted that the animal showing atherosclerosis had a blood cholesterol well within the normal limits.

Table V: The range for this group is from 52 to 251 mg. Of the eighteen readings in Table V there are seven higher than the highest control; but of these seven abnormally high figures, six occurred in animals that were fed less than 150 days, whereas only one high reading is found among ten rabbits fed from 160 to 246 days.

Eight atherosclerotic rabbits in this series had blood cholesterols

determined at the end of the experiment. Four of these showed high readings; *i.e.*, 168, 133, 126, 166, whereas the other four had readings well within the normal; *i.e.*, 65, 67, 72, 64 mg. of free cholesterol per 100 cc. of whole blood. Among the four animals which had high cholesterol levels, two showed lesions of the aorta which were

TABLE V.

Animals That Received 253.65 Mg. Cholesterol Daily.

Animal.	No. of days of feeding.	Total dose.	Blood cholesterol.	Atherosclerosis.
		gm.	*mg. per 100 cc.*	
CH128	246	62	168.22	+
CH123	246	62	65.15	+
CH129	231	58	70.01	—
CH116	222	56	67.3	+
CH115	209	53	52.2	—
CH127	203	51	Lost.	—
CH140	187	47	72.93	+
CH132	187	47	69.04	—
CH120	181	45	Lost.	+
CH131	159	40	103.1	—
CH32	147	37	133.4	+
CH26	147	37	126.6	+
CH30	147	37	64.88	+
CH33	125	31.7	136.18	—
CH136	111	28	98.2	—
CH135	99	25	251.1	—
CH137	80	20.29	Lost.	—
CH31	71	18	166.4	+
CH24	69	17.5	53.19	—
CH122	66	16	Lost.	—
CH124	54	13	63.69	—
CH118	54	13	127.3	—
CH125	46	11.66	Lost.	—
CH25	44	11.1	"	—
CH130	33	8.3	"	—

slight, and two showed extensive lesions. Two other rabbits in this series whose blood cholesterol was abnormally high showed no intimal lesions. It is noteworthy that Rabbit CH135 presented a normal aorta even though the blood cholesterol is recorded at 251 mg. (the highest in the series), whereas Rabbit CH123 with a blood cholesterol of only 65 mg. was found to have an atherosclerotic aorta.

Table VI shows that the ingestion of 500 mg. of cholesterol daily produces a marked hypercholesterolemia. The range here is from 250 to 383 mg.

High Protein Rabbits.—Since the above data make it clear that the cholesterol contained in the high protein diet is not the cause of the

TABLE VI.

Animals That Received 507.3 Mg. Cholesterol Daily.

Animal.	No. of days of feeding.	Total dose.	Blood cholesterol.	Atherosclerosis.
		gm.	*mg. per 100 cc.*	
CH75	87	44.14	286.5	—
CH73	86	43.63	382.6	+
CH157	79	40.08	249.91	+
CH16	55	28.53	Lost.	+
CH19	50	25	"	—
CH22	48	24	"	+
CH14	47	24.25	"	+

TABLE VII.

High Protein Diet.
Relation of the Diet to the Blood Cholesterol and the Vascular Lesion.

Animal.	Blood cholesterol before feeding.	Blood cholesterol at end of experiment.	Atherosclerosis.
	mg. per 100 cc.	*mg. per 100 cc.*	
CH146	51.04	238.24	Moderate.
CH141	77.79	215.32	Extensive.
CH142	64.18	189.62	Moderate.
CH145	64.42	180.00	"
CH144	67.09	126.89	Absent.
CH147	56.64	87.52	"
CH149	83.38	68.07	"
CH150	77.79	36.95	"

vascular lesion produced by this diet it seemed desirable to determine whether this diet had any effect on the blood cholesterol. Accordingly, we fed eight rabbits the high protein diet containing 36 per cent of protein prepared in the same way as that previously used to produce atherosclerosis. A determination of the blood cholesterol was made

in the case of each animal just before it was placed on the experimental diet. After the animals had been on the diet for 6 months second blood cholesterol readings were obtained and the rabbits then were killed so that the aortæ might be examined. Table VII shows the blood cholesterol readings at the beginning and again at the end of the experiment and the condition of the aorta in the case of each animal. It will be seen that the initial blood cholesterols were all well within the normal range. After a period of 6 months half of the rabbits showed blood cholesterol readings very much higher than the normal range. These same rabbits were without exception atherosclerotic, but the other half of the rabbits had normal aortæ and in them the blood cholesterols all fell within the normal range.

DISCUSSION.

In our earlier paper we discussed in detail the work of previous investigators who had studied the effect of various diets on the production of atherosclerosis, and pointed out reasons for not accepting the conclusions of those who believed that they had demonstrated that an excess of cholesterol in the diet was the etiological factor concerned.[8]

We accordingly have planned our experiments with the intention of

[8] Bailey (Bailey, C. H., *J. Exp. Med.*, 1916, xxiii, 69) fed six rabbits cholesterol dissolved in cottonseed oil added to a daily ration of crushed barley. "On an occasional day greens were given with the barley." The daily dose of cholesterol varied from 10 to 500 mg., but the total period of feeding was brief, in no instance lasting as long as 100 days. He found that the rabbit which received 100 mg. daily for 83 days had moderate intimal lesions; one that received 500 mg. daily for 36 days had moderate intimal lesions; one that received from 100 to 500 mg. daily for 97 days had pronounced intimal lesions; and one rabbit that had received cottonseed oil without cholesterol for a longer time than any of the cholesterol-fed rabbits had no vascular disease. It is unfortunate that Bailey did not add the cholesterol to a diet more nearly approaching that which may be considered normal for a rabbit; for one is unable to avoid the question whether sustaining the rabbits on crushed barley in itself may not have given rise to an abnormal metabolic condition. Furthermore, his experiments are too few and too brief to answer the question with which we are mainly concerned; *i.e.*, whether the presence of relatively small amounts of cholesterol such as occur in meat-containing diets when ingested over long periods of time are responsible in any way for the vascular lesions regularly found in rabbits that are sustained by such diets.

getting data adequate to determine the relation of ingested cholesterol to atherosclerosis. We have found that the addition of some 10 to 15 mg. of cholesterol per kilo daily to otherwise strictly normal diets is not productive of vascular disease even though fed over a period considerably longer than that required to produce atherosclerosis with great regularity by means of a diet rich in beef muscle with its accompanying cholesterol. The following calculations will make it clear that the cholesterol of this meat diet is present in amounts which cause rabbits fed this diet to ingest, daily, cholesterol to the extent of 10 to 15 mg. per kilo; *i.e.*, amounts of cholesterol comparable with those which we fed to rabbits of Group II with uniformly negative results.

The urinary nitrogens of two rabbits on this 36 per cent protein diet were determined. The urine was collected for 7 days. Rabbit CH141 weighing 3100 gm. put out 2160 cc. urine, which yielded 25.4 gm. of nitrogen or 3.6 gm. per day. This was at the rate of 1.1 gm. of nitrogen per kilo. Multiplying 3.6 by 6.25 gives 22.5 or the number of gm. of protein ingested by the rabbit daily. Our analysis of different samples of the desiccated beef used showed that it contained 7 mg. of cholesterol per 5 gm.[9] Therefore, in the 22.5 gm. of protein eaten daily, this rabbit at the same time absorbed 31.5 mg. of cholesterol. Rabbit CH149 whose urinary nitrogen was determined also, weighed 2.2 kilos and put out 1.99 gm. of nitrogen per day or 0.9 gm. per kilo. This animal ate 12.5 gm. of protein daily and therefore consumed 17.5 mg. of cholesterol with the meat diet.[10]

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[9] Windaus quoting Neumeister (Windaus, A., *Arch. Pharm.*, 1908, ccxlvi, 117), states that dried muscle contains 0.23 per cent cholesterol.

The analysis of seven different samples of beef gave a mean of 7 mg. of cholesterol for 5 gm. of beef.

Sample	I	5 gm.	yielded	11.7	mg.
"	II	5 "	"	10.6	"
"	III	5 "	"	5.6	"
"	IV	5 "	"	7 5	"
"	V	5 "	"	4.7	"
"	VI	5 "	"	6.1	"
"	VII	5 "	"	3	"
		Mean =		7	mg.

[10] The rabbits were kept in a metabolism cage for 7 days, and the urine collected during the entire period. The bottom of the cage and fine wire matting on which the rabbit rested were covered with a paraffin-ether mixture (10 gm. paraffin were mixed with 8 ounces of ether). The urine was collected in a receptacle that was white and 5 cc. of 3½ per cent HCl were kept in it. Methyl red was

After the fact had been established that these small amounts of cholesterol are without effect upon the rabbit aorta, the next experiments were planned to determine the minimum daily dose of cholesterol when fed for 6 months or more, which would produce atherosclerosis.

We chose the period of 6 months since we previously had learned that our high protein diets caused atherosclerosis within this period of time. Accordingly another series of rabbits was fed 113 mg. daily. Nine of these rabbits took this dose of cholesterol for 9 or more months, but only one of them was found to have a small atherosclerotic patch. Even if this lesion is attributed to the cholesterol in the diet, it is evident that the ingestion of 113 mg. of cholesterol by rabbits, for long periods of time, is harmful to the aorta only as a rare exception. When, however, 250 mg. were fed daily vascular lesions were obtained in nine out of twenty-five rabbits. Thirteen rabbits were fed this dose from 147 to 246 days. Of these, eight (62 per cent) showed vascular disease. Finally, when the daily dose was 507 mg. atherosclerosis was obtained in five of the seven rabbits fed for the comparatively short period lasting from 47 to 87 days. It should be noted that a majority (71 per cent) of the rabbits which received the 500 mg. dose developed aortic disease within a period of time during which the animals receiving 250 mg. of cholesterol rarely developed vascular injury. Specifically, in a series of twelve rabbits

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used as the indicator. A few crystals of thymol were kept in the urine container also. As the urine was voided it was put into a stoppered bottle with some HCl in it, and kept on the ice. The bottom of the cage and the wire matting were rinsed with distilled water into the container. A piece of gauze (two thicknesses) was placed in the bottom of a funnel and the urine and rinse water poured through this. All large particles were retained by the gauze. A few bits of the high protein food were found in the urine container. Since there was a possibility that they might add to the nitrogen content of the urine, a few small pieces of the food were shaken with distilled water, filtered, and then tested for albumin. The test failed to give the albumin reaction. Furthermore, some particles were put into a flask, a few drops of the HCl used for the urine and a few drops of distilled water added. This was allowed to stand overnight at room temperature. The test for albumin again was negative. It seems evident from this that whatever of the food may have reached the urine container was not digested far enough to give a test for albumin and therefore could not add any nitrogen to that of the urine.

that received 250 mg. from 33 to 125 days, there was only one whose aorta was abnormal.

A comparison of the state of the aorta with the daily ingestion of cholesterol, and with the total amount of cholesterol ingested, gives a much closer relationship between the daily dose and atherosclerosis than between the total dose and atherosclerosis. This becomes evident if one selects animals from the third, fourth, and fifth groups, each of which has ingested approximately a total of 25 gm. of cholesterol, and compares the state of their aortæ with the daily dose of

TABLE VIII.

Relation between Size of the Daily Dose and the Intimal Injury.

Animal.	Daily dose.	Total dose.	Atherosclerosis.
	mg.	*gm.*	
CH19	507.3	25	—
CH14	507.3	24.25	+
CH22	507.3	24	+
CH33	253.65	31.7	—
CH136	253.65	28	—
CH135	253.65	25	—
CH137	253.65	20.29	—
CH31	253.65	18	+
CH94	113.2	30	—
CH98	113.2	25	—
CH81	113.2	25	—
CH108	113.2	25	—
CH104	113.2	25	—
CH100	113.2	25	—
CH35	113.2	18	—

cholesterol (Table VIII). By so doing it will be found that 75 per cent of the rabbits of this type (25 gm.) that belong in the 500 mg. series have aortic disease; that 20 per cent of the rabbits in the 253 mg. series which received about 25 gm. as a total dose, were positive; while none of the seven in the 113 mg. series which totalled 25 gm. or thereabouts was positive. A large daily dose quickly produces atherosclerosis. A small daily dose within the time limit of the experiment never produces atherosclerosis regardless of the total amount ingested.

These feeding experiments studied in connection with the earlier high protein series indicate that alimentary atherosclerosis may be produced in rabbits by adding to the diet either an excess of protein or an excess of cholesterol, but the excess of protein necessary to obtain this result is very much less than that necessary for cholesterol. Rabbits whose nitrogen metabolism was 1.1 gm. per kilo, which roughly is four times the normal,[11] became atherosclerotic in 6 months to a year, whereas the rabbit that normally eats no cholesterol[12] must ingest 250 mg. daily to become atherosclerotic.

Lesions.

We already have pointed out that vascular disease caused by ingestion of cholesterol differs in no way from that found in animals fed a high protein diet, and corresponds to the descriptions found in textbooks dealing with disease of the human aortic intima. We use the term atherosclerosis to designate a primary lesion of the intima. "The most conspicuous change is a thickening of the intima accompanied by fat deposits."[13] The process never was far enough advanced in any of our animals to present calcification. Before attributing the aortic lesions under discussion to the cholesterol in the diet, it is necessary to know whether lesions of this type occur spontaneously in the rabbit. The examination of the aortæ of 100 rabbits from the laboratory stock which were killed for the purpose of examination, or were used for bleeding and then examined, never has revealed an example of primary intimal involvement.

However, a certain type of abnormality was not infrequent. This lesion always occurred in the region of the arch. It was uniformly small, being about 1½ mm. in diameter. It was a very white, slightly

[11] The urinary nitrogen of a normal rabbit was determined. It weighed 2.15 kilos and put out 2710 cc. of urine. This amount yielded 3.68 gm. for 7 days, or 0.24 gm. per kilo.

[12] According to the following calculations, a rabbit normally eats about 80 mg. of phytosterol daily. 3 gm. of hay yielded 0.5 mg. of phytosterol. A rabbit eats about 113 gm. of hay. 5 gm. of oats yielded 1.3 mg. of phytosterol. A rabbit eats approximately 225 gm. There are no published data indicating that the ingestion of phytosterol has an effect on the aorta.

[13] MacCallum, W. G., *Physiol. Rev.*, 1922, ii, 70.

raised process with a depression in its center. On microscopic study of this area it was seen to consist of a degenerative change in the media, unaccompanied by fat but often showing marked calcification. This spontaneous lesion corresponds closely to a similar condition found in human arteries as described by Mönckeberg. While this type of lesion sometimes was found in rabbits which in addition showed disease of the intima, the distinction between the spontaneous lesion of the media and the experimental lesion of the intima always was clear and we were satisfied that every rabbit labelled atherosclerotic had developed the experimental lesion regardless of whether the spontaneous medial lesion was present or absent. Hence, we conclude that spontaneous aortic disease is not a source of error in this study.

Blood.

One would expect *a priori* that the ingestion of cholesterol in amounts sufficient to damage the aorta would produce the deleterious effect through the path of hypercholesterolemia. In order to get information on this point a large number of blood cholesterol determinations were made by the Windaus method, on control animals and on animals that had received the various levels of cholesterol for many weeks. Inspection of Table II will show that normal rabbits exhibit a wide range of blood cholesterol readings— from 35.4 to 125.4 mg. with a mean of 71.3 mg. for 100 cc. of whole blood. The animals that received 25 mg. daily had bloods all well within the normal limits, with a mean of 56.7 mg. In the group of animals that received 113 mg. of cholesterol daily, the mean was 80.65 mg. In this series there were four readings just above the highest control, but none of these occurred in those animals that received cholesterol for the longest periods of time; and we accordingly are unable to show that this order of dosage is productive of hypercholesterolemia. Furthermore, it should be pointed out that the only animal of this group with an atherosclerotic aorta had a blood cholesterol of 71 mg. With the animals receiving 253 mg. it again was impossible to establish a relationship between the duration of the feeding and the number of mg. of cholesterol in the blood. Only one of the six animals that were fed more than 200 days had an abnormally high reading. On the other

hand, the highest reading in the whole group occurred in a rabbit that was fed but 99 days. In addition to this, atherosclerotic aortæ were found in animals that had normal as well as high readings, and the animal that exhibited the most extreme hypercholesterolemia had a normal aorta. It would appear then to be true that the prolonged ingestion by rabbits of 250 mg. of pure cholesterol daily does not result in general in hypercholesterolemia; and conversely, that such animals may have a marked hypercholesterolemia without disease of the aorta. When animals receive 500 mg. daily, a well marked hypercholesterolemia occurs. Since five of the seven animals became

TABLE IX.

Effect of Single Dose of Cholesterol on the Blood Level of Cholesterol.

Animal.	Cholesterol ingested.	Blood cholesterol.				
		Before ingestion.	After ingestion.			
			3 hrs.	6 hrs.	12 hrs.	24 hrs.
	gm.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
A	0.5	87.03	56.71			
C	0.5	116.7		119.6	104.3	
G	0.5	67.1		67.3	49.6	
E	0.5	Fasting. 35.4		54.5	44.5	
B	2 empty capsules.	Fasting. 106	117.2	97.3	89	93.4

atherosclerotic (moderately so in four cases) it may be true that there exists a causal relationship between the cholesterol in the blood and the state of the vessel; but we have not sufficient data dealing with this question to be in a position to give a final answer.[14]

Since there is so little correspondence between the amounts of cholesterol ingested and the level of blood cholesterol, we tried to determine what effect if any was produced by the ingestion of a single

[14] In an unpublished communication of Miss Kunde and Professor Carlson of the University of Chicago we learn that the blood of cretin rabbits may run 100 per cent above normal in cholesterol content without any indication of arteriosclerosis.

large dose of cholesterol on the level of the blood for the following 24 hours. Reference to Table IX shows that the blood values were uninfluenced by a single ingestion.[15]

High Protein Rabbits.

The final group of rabbits to which the high protein diet was fed brought out the fact that this diet with a cholesterol content in itself far too small to affect the blood cholesterol does produce marked hypercholesterolemia in those rabbits that become atherosclerotic as a result of the diet. We thus have evidence that this particular type of hypercholesterolemia has its origin in some mechanism other than that of absorption from the digestive tract, and the question immediately arises whether the atherosclerosis is caused by the hypercholesterolemia in this series of rabbits, or whether the two abnormalities occur concommitantly and do not bear the relation of cause and effect to each other, or finally whether the aortic disease occasions the hypercholesterolemia. Since both abnormalities always have occurred together in these experiments it would seem that they bear an intimate relation to each other, but our present information does not permit us to explain this relationship further.

CONCLUSIONS.

1. The range of free cholesterol in the blood of rabbits, as determined by the Windaus method, varies from 35 to 125 mg. with a mean of 71 mg. per 100 cc. of blood.

2. The small amount of cholesterol contained in the high protein diet used by us in earlier work and causing atherosclerosis does not affect the blood cholesterol nor does it cause arterial disease.

3. In order to produce atherosclerosis it is necessary to feed at least ten times that amount of cholesterol.

4. In rabbits receiving such amounts both hypercholesterolemia and atherosclerosis occur, but it is not possible to establish any close

[15] The only other observations in this field which we could find are those of Lehman (Lehman, E. P., *J. Biol. Chem.*, 1913–14, xvi, 495), who claims that a single dose of ingested cholesterol produces cholesterolemia in rabbits, but his published data do not appear to support his contention.

parallelism between the two. High blood readings are found in rabbits with normal aortæ and atherosclerotic rabbits in this series sometimes have shown a normal blood cholesterol.

5. With still greater doses of cholesterol one finally reaches an amount which regularly produces hypercholesterolemia and atherosclerosis within a few weeks.

6. A new series of rabbits fed the high protein diet shows that those rabbits which become atherosclerotic also develop hypercholesterolemia. We attribute this elevation of the blood cholesterol to a metabolic disturbance directly referable to the excess of protein in the diet and not to its cholesterol content.

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