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

I. The Inhibition of Experimental Cholesterol Atherosclerosis in Alloxan Diabetes

II. The Effect of Alloxan Diabetes on the Retrogression of Experimental Cholesterol Atherosclerosis

By G. LYMAN DUFF, M.D., and GARDNER C. McMILLAN, M.D.‡

(From the Department of Pathology, Pathological Institute, McGill University, Montreal)

(Received for publication, October 4, 1948)

The problem of arterial disease associated with diabetes mellitus has loomed larger and larger ever since the discovery of insulin. However, experimental study of this problem has been thwarted by the fact that dogs and cats, in which experimental diabetes can be readily produced by several methods, are notoriously resistant to the development of arterial disease. On the other hand, although experimental arterial diseases of various types can be produced very easily in rabbits, this species cannot be rendered permanently diabetic by anterior pituitary extracts and its pancreatic tissue, like that of other rodents, has an anatomical distribution such as to render total pancreatectomy an operation of extreme technical difficulty. Thus, prior to the recognition of the diabetogenic properties of alloxan, it was not feasible to study experimentally the effects of diabetes in the one mammalian species that appeared most likely to respond to such a metabolic disturbance with the development of some form of arterial disease. The discovery that rabbits can be rendered permanently diabetic by alloxan has made it possible to embark upon a study of the effects of diabetes on the arteries of rabbits with the hope of gathering experimental data that might have significance not only in relation to the problem of arteriosclerosis developing in man in association with diabetes mellitus but also in relation to the larger problem of arteriosclerosis in general.

The pathological changes encountered in the pancreas and in certain other tissues of animals rendered diabetic by pancreatectomy, by the administration of anterior pituitary extracts, and by the injection of alloxan have been reviewed elsewhere(1). In some of the earlier papers dealing with alloxan diabetes as observed in various species, it was stated that no changes in the arteries were found in association with this experimental disease in spite of the coexistence in some instances of marked

* This work was assisted by grants-in-aid from the National Research Council, Canada.

‡ Medical Research Fellow of the National Research Council, Canada.

lipemia, but the duration of these experiments was relatively short. The occurrence of visible lipemia in alloxan diabetes in rabbits lent support to the supposition that atherosclerosis might develop in them after a sufficient period of time, since it is well known that the continued feeding of cholesterol to rabbits is followed by the appearance of lipemia, hypercholesterolemia, and the eventual development of experimental cholesterol atherosclerosis(2–4). Accordingly, Duff and Wilson(5) carried out a series of experiments in which the blood cholesterol levels were followed during the course of prolonged alloxan diabetes in rabbits with a view to determining at the end of the experiments whether any effect had been produced on the arterial system. They found that lipemia was usually an evanescent phenomenon and in some cases did not occur at all. The blood cholesterol was frequently elevated for a time to values of about 350 mg. per cent but it almost always returned to normal in 3 to 6 weeks and so remained for the rest of the animal's life, in spite of the persistence of more or less severe diabetes as judged by the continuance of marked hyperglycemia, glycosuria, polydypsia, polyuria etc. In these experiments they could find no evidence at autopsy of the development of atherosclerosis either in the aorta or other arteries of rabbits that had been diabetic for periods of time ranging from several months up to a maximum of 1 year.

In view of the negative result just described, the experiment reported in Part I of the present communication was undertaken. It was designed to permit of a comparison between the effects of cholesterol feeding in normal rabbits and in rabbits previously rendered diabetic by alloxan. The unexpected result of this experiment was the demonstration that the development of cholesterol atherosclerosis is markedly inhibited in alloxan-diabetic rabbits as compared with non-diabetic control animals, in spite of the fact that hypercholesterolemia of comparable degree is induced in the two groups of animals by cholesterol feeding. This result has already been briefly recorded (6).

The inhibition of the development of experimental cholesterol atherosclerosis in rabbits rendered diabetic by alloxan suggested the importance of determining whether the retrogressive changes that are known to occur in the arterial lesions after the feeding of cholesterol is terminated (2, 3) would be affected by the induction of alloxan diabetes at the completion of an adequate course of cholesterol feeding. An account of an experiment carried out with this end in view is contained in Part II of the present paper.

I. THE INHIBITION OF EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS IN ALLOXAN DIABETES

Materials and Methods

The animals employed were young, adult, domestic white rabbits of both sexes. The ages of these animals were unknown. They weighed from 2 to 3 kilos at the beginning of the experiment, and all were healthy, growing animals. Housed in separate metal cages, they were given a diet of Purina rabbit chow and water *ad libitum*. No dietary supplements were used. The animals were divided into convenient groups or series, and after a period of acclimatization of 1 or 2 weeks determinations of the content of sugar in the blood and of

free and total cholesterol in the serum were made in the fasting state by a modification of Folin's micro method, and by the Schoenheimer and Sperry method respectively. After establishing that these quantities were within normal limits, half of the animals were selected at random and were injected with a freshly prepared 5 per cent aqueous solution of alloxan (Eastman Kodak Co.) in the lateral ear vein. The dose was 200 mg. per kilo of body weight. This treatment was followed by the daily injection of 2 to 6 units of protamine zinc insulin and of about 1 gm. of dextrose for a period of 7 to 14 days. The animals were then left without further treatment for a period of 4 to 5 weeks in order to allow for stabilization of the metabolic processes, after which cholesterol feeding was instituted. Urinary sugar and acetone estimations were made when indicated. Fasting blood sugar and cholesterol values were estimated at approximately biweekly intervals.

The normal control and alloxan-injected animals in each series were fed exactly the same doses of cholesterol on the same days of the experiments and, except for the previously mentioned period of insulin and dextrose therapy, were treated exactly alike. The animals of series 3 received dry powdered cholesterol in gelatin capsules, according to the method of Pollak (7). The animals of all other series received a 3.3 or 5 per cent solution of cholesterol in corn oil dissolved and maintained at 60°C. and fed by means of a stomach tube after cooling. The daily dose of cholesterol varied in the different experimental series from 0.25 to 0.75 gm. The details of the total amounts of cholesterol fed, and the durations of feeding in the various experimental series are shown in Table I.

On completion of the period of cholesterol feeding the surviving animals were killed by air embolism, complete autopsies were performed, and the tissues were fixed and sectioned. The aorta and heart were removed *en bloc*, fixed in formalin, stained in Sudan IV, and the fatty deposits in the intima revealed by this method were recorded on standardized, schematic drawings of the aorta. The aorta was then severed from the heart, small blocks were removed for frozen sections, and the remainder was rolled into a coil and embedded in paraffin. These blocks were sectioned in such a manner that a single microscopic section included the entire length of the aorta. The paraffin sections were stained with hematoxylin and eosin, Verhoeff-Van Gieson stain for elastic tissue, and Mallory's phosphotungstic acid hematoxylin. Frozen sections of the aorta, liver, spleen, and adrenal gland were examined after staining with Sudan IV.

The maximum values accepted as normal were: blood sugar 160 mg. per cent, total cholesterol 80 mg. per cent, and free cholesterol 35 mg. per cent. An animal was regarded as being diabetic if the fasting blood sugar average was 300 mg. per cent or greater, and if most of the obvious manifestations of alloxan diabetes, such as persistent polyuria, glycosuria, polydipsia, polyphagia, and weight loss were present. In addition, the histological demonstration of the characteristic hydropic changes in the pancreas (8) and the lesion of Armanni in the kidney were taken as confirmatory evidence of the existence of a persistent diabetic state. Certain of the animals that had received a diabetogenic dose of alloxan, although they were initially diabetic, reverted to a normal metabolic state by the time that cholesterol feeding was instituted and thereafter showed neither chemical nor obvious evidence of diabetes. Such animals were classed as "alloxan-recovered" (see Table I). The degree of experimental cholesterol atherosclerosis observed in each animal was graded on an arbitrary scale of 0 to 4 as shown in Fig. 1. It is important to note that atherosclerosis of grade 1 severity was recorded even when only a single fleck of lipid deposit was revealed by careful gross examination after Sudan staining of the entire aorta. This gross grading was confirmed by microscopic examination. The amount of sudanophilic, lipid substance in the liver, spleen, and adrenal was graded in a similar arbitrary manner on a scale of 0 to 4 by microscopic examination of stained, frozen sections. A lipemic condition of the blood induced by the diabetic state and/or cholesterol feeding was similarly graded by inspection of the blood

before and after clotting *in vitro*. A lipemic index for each animal was established by averaging the grades of lipemia observed on all the occasions when the blood was sampled.

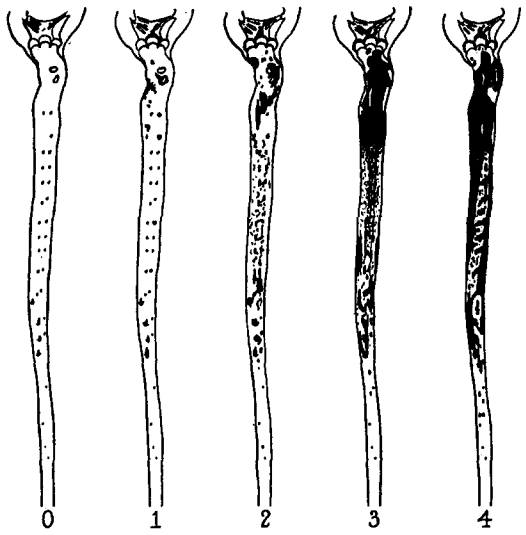

FIG. 1. The standard schematic diagram of the rabbit's aorta used for charting the extent of the atherosclerosis observed at autopsy is illustrated. The five diagrams represent the aortic lesions observed in five animals and show, from left to right, the degrees of atherosclerosis graded as 0, 1, 2, 3, and 4 respectively.

Observations

The observations are summarized in Table I. Over 100 animals were used in this experiment, but the mortality rate from the use of alloxan and from feeding and other accidents was high. Only the 58 animals that completed an experimental course of satisfactory duration are reported upon. These comprised 18 diabetic and 39 non-diabetic animals. In addition, there was one animal (No. 43) that showed a moderate elevation of the blood sugar level in the postprandial state only, and is referred to in the table as mildly diabetic. The 39 non-diabetic animals comprised 13 rabbits that had recovered from the effects of a diabetogenic dose of alloxan ("alloxan-recovered" group), and 26 normal control animals that were subjected to cholesterol feeding alone. The period of cholesterol feeding varied from 52 to 91 days. The total dose of cholesterol fed varied between 16 and 65 gm. and the average daily dose of cholesterol fed varied from 180 to 750 mg. among the different experimental series. The chemical values given in the table are arithmetical averages of biweekly determinations made during the period of cholesterol feeding and of one determination made immediately before cholesterol feeding was begun. The very high maximum values obtained in some of the animals are not shown, but are, nevertheless, indicated by the average values recorded.

The dosage of cholesterol and the duration of cholesterol feeding varied from those that induced neither a hypercholesterolemia nor cholesterol atherosclerosis among the control animals (series 1 and 2) to those that induced in the controls a very severe degree of both, with the formation of confluent atherosclerotic lesions extending over the entire intimal surface of the aorta and measuring from one to one and one-half times the thickness of the underlying media. However, inspection of the data in Table I reveals that there was a remarkable discrepancy in the degree of atherosclerosis induced in the diabetic animals as compared with that induced in the comparable groups of control rabbits fed exactly the same quantities of cholesterol within the same period of time (Figs. 2 and 3). In each experimental group, with the exception of series 1 and 2 in which no atherosclerosis was induced in any of the animals, the diabetic rabbits showed a striking resistance to the induction of experimental cholesterol atherosclerosis of the aorta. On the other hand, the control rabbits and the "alloxan-recovered" group presented the expected incidence and degree of cholesterol atherosclerosis without distinction. The 23 control animals contained in series 3 to 11 inclusive, all presented more or less severe atherosclerosis of the aorta. Of these 23 animals, 8 presented aortic atherosclerosis of grade 1 severity, 4 of grade 2, 4 of grade 3, and 7 of grade 4. The 12 "alloxan-recovered" animals in these same series presented similar findings. Of these 12 animals 1 showed no atherosclerosis, 2 had aortic lesions of grade 1 severity, 4 of grade 3, and 5 of grade 4. In contrast to these results, the 13 diabetic animals in the same groups presented no atherosclerosis in 6 animals, grade 1 aortic lesions (usually minimal) in 6, and aortic atherosclerosis of grade 2 (minimal) in only 1 rabbit. The quantities of sudanophilic lipid material present in the liver, spleen, and adrenals, in general, corresponded closely with that found in the aorta. The diabetic rabbits showed evidence of the same resistance to lipid deposit in the liver, spleen, and adrenals as in the aorta. Indeed, some of the diabetic rabbits were completely protected from the deposition of cholesterol, as judged by careful morphological examination, not only in the aorta but also in these other organs.

Most important is the observation that the diabetic state did not inhibit the development of a hypercholesterolemia that was as high as that induced in the corresponding "alloxan-recovered" and control animals and that was occasionally considerably higher (Figs. 2 and 3). This was also true of both the free and ester fractions of the serum cholesterol; there was no significant alteration in the ratio of ester to total cholesterol content. In addition to exhibiting an equally marked degree of hypercholesterolemia, the diabetic rabbits showed a visible lipemia *in vitro* that was much more marked than that observed in the corresponding non-diabetic animals. The control and "alloxan-recovered" animals did not show more than a moderate to marked opalescence of the serum, while the diabetic animals exhibited a lipemia that was manifest as a milky or creamy appearance that rendered the serum opaque. A further difference in the lipemia of the diabetic and non-diabetic animals was the rapidity with which

TABLE I

Summary of Experimental Data

Series	No.	Sex	Experimental type*	Duration of cholesterol feeding	Total dose of cholesterol	Weight at beginning of cholesterol feeding	Weight at completion of cholesterol feeding	Average blood sugar	Average free serum cholesterol	Average total serum cholesterol	Lipemic index (0–4)	Grade of aortic atherosclerosis (0–4)
				days	*gm.*	*kg.*	*kg.*	*mg. per cent*	*mg. per cent*	*mg. per cent*		
	1	M	D			2.98	2.61	508	68	114	0	0
	2	M	D			2.12	1.89	583	89	111	0	0
1	3	M	D	91	16	2.74	2.92	397	13	32	0	0
	4	M	C			2.56	3.20	109	11	30	0	0
	5	M	AR			3.08	3.51	108	14	32	0	0
	6	M	D			2.53	2.24	501	51	71	0	0
2	7	M	D	90	31	2.09	2.50	473	20	42	0	0
	8	M	C			3.26	4.66	116	11	23	0	0
	9	M	C			3.09	3.75	115	24	55	0	0
	10	F	D			2.45	1.98	517	18	53	0	0
	11	M	D			3.15	2.64	472	45	90	0.8	0
3	12	F	C	91	38	3.54	4.06	96	25	84	0	1
	13	F	C		(dry)	3.08	3.36	96	33	130	0	1
	14	M	C			2.84	2.76	100	27	103	0	1
	15	M	D			2.23	2.05	329	220	726	2	0
4	16	M	C	52	39	2.28	2.63	136	41	120	1	1
	17	M	C			2.41	2.07	153	414	1728	1.3	3
	18	M	AR			3.24	3.58	126	14	48	0	0
	19	M	D			2.22	1.59	481	219	423	3.75	0
	20	M	D			2.37	1.88	427	101	197	2.75	0
5	21	F	C	53	40	4.58	5.15	119	64	210	0.5	1
	22	F	C			3.50	4.59	131	105	316	1	1
	23	F	C			3.02	3.72	129	111	389	1.5	2
	24	M	AR			3.32	3.63	111	32	77	0.25	1
	25	M	D			2.58	1.84	355	192	396	3.7	1
	26	F	D			2.73	2.60	354	57	159	1.7	1
	27	F	C			3.90	4.14	114	155	334	1.7	4
6	28	F	C	90	46	3.62	4.40	123	139	349	2.0	4
	29	M	AR			3.04	3.23	116	104	359	1.8	4
	30	M	AR			3.43	3.78	108	89	232	1.2	4
	31	F	AR			3.55	3.98	115	100	367	1.8	4
	32	M	D			3.48	3.45	452	99	277	2	1
	33	M	C			4.38	5.25	114	15	63	0	2
7	34	M	C	89	48.5	3.40	3.68	118	66	167	1.4	3
	35	M	C			3.29	3.13	111	97	316	1.8	4
	36	M	AR			4.45	4.99	110	59	140	1	3
	37	M	AR			3.94	4.31	116	60	123	1	3

TABLE I—*Concluded*

Series	No.	Sex	Experimental type*	Duration of cholesterol feeding	Total dose cholesterol	Weight at beginning of cholesterol feeding	Weight at completion of cholesterol feeding	Average blood sugar	Average free serum cholesterol	Average total serum cholesterol	Lipemic Index (0–4)	Grade of aortic atherosclerosis (0–4)
				days	*gm.*	*kg.*	*kg.*	*mg. per cent*	*mg. per cent*	*mg. per cent*		
8	38	M	D	76	52	2.47	2.89	375	171	401	4	0
	39	M	C			3.50	4.06	113	121	425	1.75	4
	40	M	C			3.72	4.09	118	70	272	1.25	2
	41	M	AR			3.66	4.23	121	98	340	1.5	3
	42	M	AR			3.12	—	136	159	472	2.25	4
	43	M	MD			3.70	4.00	147	120	309	1.0	1
9	44	M	D	82	52	2.96	3.36	335	459	1970	1.6	2
	45	M	C			2.06	2.83	145	344	632	1.25	1
	46	M	C			2.49	4.10	137	179	334	0.75	3
	47	M	C			2.34	4.17	136	191	491	1.25	4
	48	M	AR			2.94	3.11	177	612	1849	1.5	4
	49	M	AR			3.57	4.50	127	75	357	0.8	1
10	50	M	D	82	60	3.07	3.38	444	505	1075	3.8	1
	51	M	D			3.10	2.19	398	315	703	3.2	1
	52	F	C			4.05	4.20	126	164	562	1.6	4
	53	F	C			3.73	4.51	117	192	602	1.6	2
	54	F	AR			3.85	5.09	115	235	656	2.0	3
11	55	F	D	89	65	2.69	3.31	392	135	346	1.2	1
	56	M	C			2.80	4.17	124	55	221	0	1
	57	M	C			3.17	4.36	128	123	450	1	3
	58	M	C			3.50	4.51	122	143	593	1.3	4

* D = diabetic; C = control; AR = "alloxan-recovered;" MD = mild diabetic.

this phenomenon became apparent after the blood was drawn. Lipemia was apparent in the blood of diabetic animals within 1 to 20 minutes. It was seldom seen in the blood of comparable non-diabetic animals before 30 to 60 minutes after the blood was drawn.

While the data considered above are consistent, the variability of the cholesterol dosage and of the duration of the experiments precludes a detailed analysis of the possible influence of certain other experimental variables, such as sex and change in weight of the animals during the experiment. It is interesting, therefore, to compare the data of individual animals that were similar in as many respects as possible, in order to assess the importance of these factors. The protocols of a number of such animals are given below.

The following protocols are of a diabetic rabbit and of a non-diabetic control

animal that are comparable as to sex and in all other respects, except that in the diabetic animal the degree of lipemia was much greater, and the amount of lipid

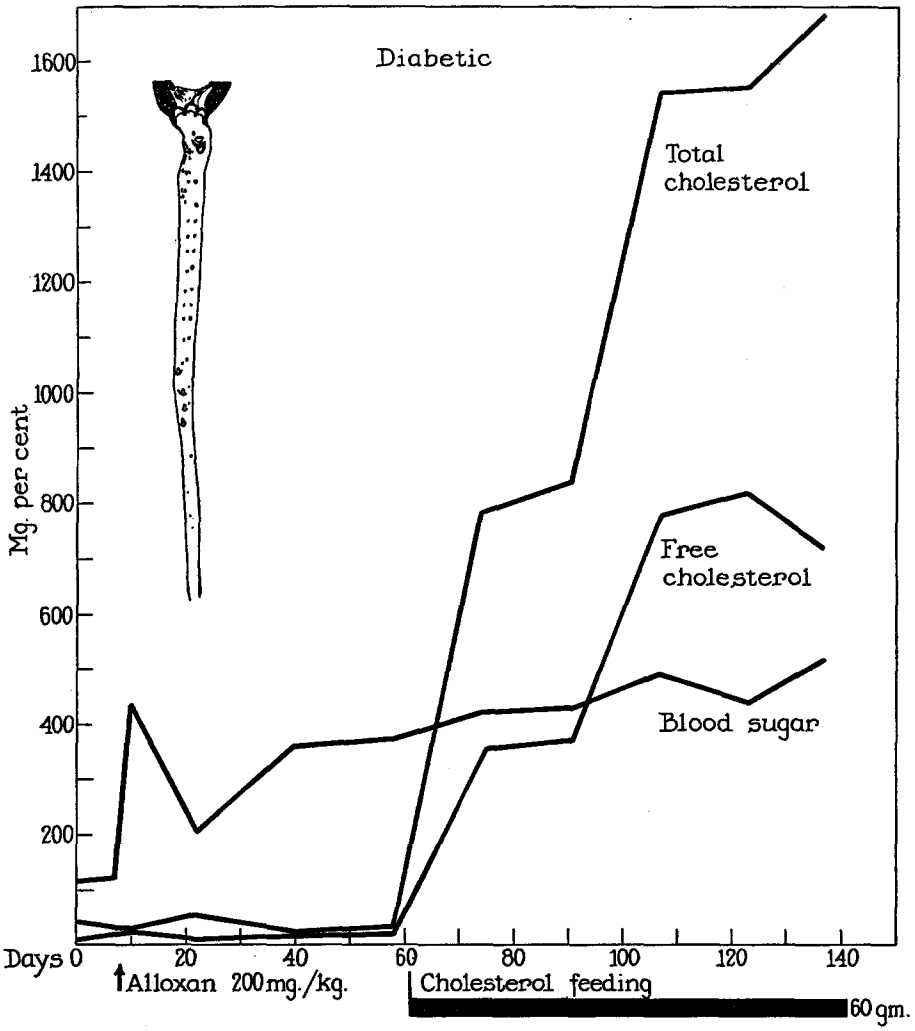

FIG. 2. Diagram and graph illustrating the procedures and findings in diabetic rabbit 50, series 10. The aortic atherosclerosis shown was recorded as grade 1.

deposition in the aorta, liver, spleen, and adrenal was much less than in the control rabbit.

No. 38. Diabetic. Male.—Dose of cholesterol per day, 0.68 gm. Duration of feeding, 76 days. Average total serum cholesterol, 401 mg. per cent. Lipemic index, 4.0. Ather-

osclerosis, grade 0. Liver fat, grade 0. Splenic fat, grade 1. Adrenal fat, normal. Weight gain, 0.42 kilo.

No. 39. Control. Male.—Dose of cholesterol per day, 0.68 gm. Duration of feeding, 76 days. Average total serum cholesterol, 425 mg. per cent. Lipemic index, 1.75. Ather-

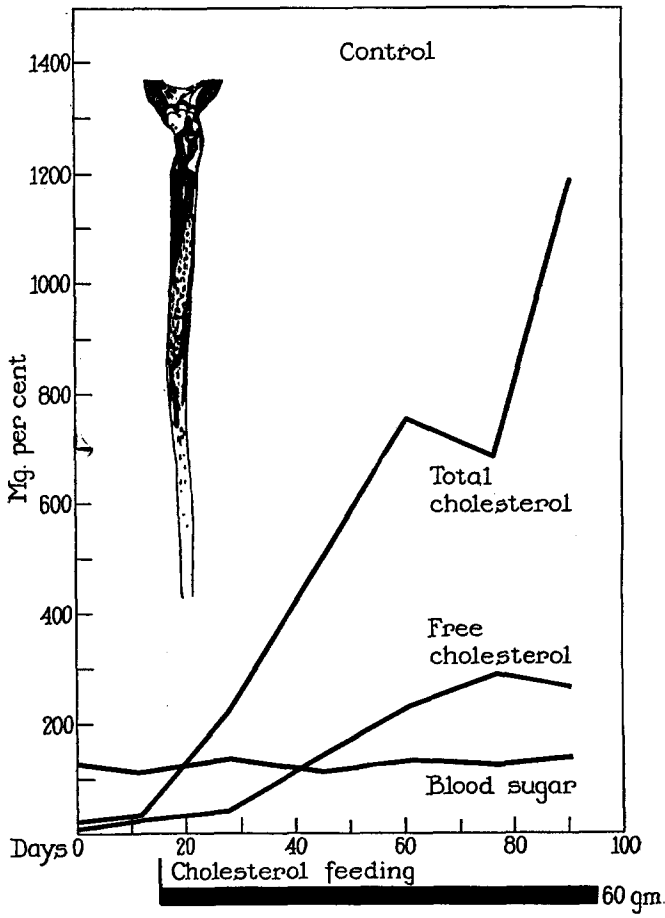

FIG. 3. Diagram and graph illustrating the procedures and findings in non-diabetic control rabbit 52, series 10. The aortic atherosclerosis shown was recorded as grade 4.

osclerosis, grade 4. Liver fat, grade 4. Splenic fat, grade 3. Adrenal fat, grade 4. Weight gain, 0.56 kilo.

The following two groups of protocols in which the comparable diabetic, "alloxan-recovered," and non-diabetic control animals alternate in sex, also indicate that sex is not the determining factor in inhibiting the deposition of lipids in the aorta and elsewhere.

No. 26. Diabetic. Female.—Dose of cholesterol per day, 0.52 gm. Duration of feeding, 90 days. Average total serum cholesterol, 159 mg. per cent. Lipemic index, 1.7. Atherosclerosis, grade 1. Liver fat, grade 1. Splenic fat, grade 1. Adrenal fat, grade 1. Weight loss, 0.13 kilo.

No. 36. "Alloxan-Recovered." Male.—Dose of cholesterol per day, 0.54 gm. Duration of feeding, 89 days. Average total serum cholesterol, 140 mg. per cent. Lipemic index, 1. Atherosclerosis, grade 3. Liver fat, grade 3. Splenic fat, grade 3. Adrenal fat, grade 3. Weight gain, 0.54 kilo.

No. 34. Control. Male.—Dose of cholesterol per day, 0.54 gm. Duration of feeding, 89 days. Average total serum cholesterol, 167 mg. per cent. Lipemic index, 1.4. Atherosclerosis, grade 3. Liver fat, grade 2. Splenic fat, grade 0. Adrenal fat, grade 2. Weight gain, 0.28 kilo.

No. 25. Diabetic. Male.—Dose of cholesterol per day, 0.52 gm. Duration of feeding, 90 days. Average total serum cholesterol, 396 mg. per cent. Lipemic index, 3.7. Atherosclerosis, grade 1. Liver fat, grade 1. Splenic fat, grade 1. Adrenal fat, normal. Weight loss, 0.74 kilo.

No. 29. "Alloxan-Recovered." Male.—Dose of cholesterol per day, 0.52 gm. Duration of feeding, 90 days. Average total serum cholesterol, 359 mg. per cent. Lipemic index, 1.8. Atherosclerosis, grade 4. Liver fat, grade 4. Splenic fat, grade 3. Adrenal fat, grade 4. Weight gain, 0.19 kilo.

No. 31. "Alloxan-Recovered." Female.—Dose of cholesterol per day, 0.52 gm. Duration of feeding, 90 days. Average total serum cholesterol, 367 mg. per cent. Lipemic index, 1.8. Atherosclerosis, grade 4. Liver fat, grade 1. Splenic fat, grade 0. Adrenal fat, grade 2. Weight gain, 0.43 kilo.

No. 27. Control. Female.—Dose of cholesterol per day, 0.52 gm. Duration of feeding, 90 days. Average total serum cholesterol, 334 mg. per cent. Lipemic index, 1.7. Atherosclerosis, grade 4. Liver fat, grade 2. Splenic fat, ? Adrenal fat, grade 2. Weight gain, 0.24 kilo.

No. 28. Control. Female.—Dose of cholesterol per day, 0.52 gm. Duration of feeding 90 days. Average total serum cholesterol, 349 mg. per cent. Lipemic index, 2. Atherosclerosis, grade 4. Liver fat, grade 1. Splenic fat, grade 1. Adrenal fat, grade 2. Weight gain, 0.78 kilo.

Inasmuch as equal daily doses of cholesterol were fed to rabbits of different body weights, it is conceivable that the apparent inhibitory effect of the diabetic state might have occurred because of the chance feeding to the non-diabetic animals of a higher dose of cholesterol per kilo of body weight. In the following protocols the diabetic and non-diabetic animals are matched as to sex, duration of cholesterol feeding, average total serum cholesterol, and, in addition, as to dosage of cholesterol calculated as an average dose per day per kilo of average body weight during the feeding period. It is apparent from examination of these protocols that the experimental result was not determined by actual or relative differences in the dosages of cholesterol.

No. 11. Diabetic. Male.—Dose of cholesterol (dry) per day per kilo, 145 mg. Duration of feeding, 91 days. Average total serum cholesterol, 90 mg. per cent. Lipemic index, 0.8. Atherosclerosis, grade 0. Liver fat, grade 0. Splenic fat, grade 1. Adrenal fat, normal. Weight loss, 0.51 kilo.

No. 14. Control. Male.—Dose of cholesterol (dry) per day per kilo, 150 mg. Duration

of feeding, 91 days. Average total serum cholesterol, 103 mg. per cent. Lipemic index, 0. Atherosclerosis, grade 1. Liver fat, grade 0. Splenic fat, grade 0. Adrenal fat, normal. Weight loss, 0.08 kilo.

No. 44. Diabetic. Male.—Dose of cholesterol per day per kilo, 203 mg. Duration of feeding, 82 days. Average total serum cholesterol, 1970 mg. per cent. Lipemic index, 1.6. Atherosclerosis, grade 2. Liver fat, grade 2. Splenic fat, grade 3. Adrenal fat, grade 2. Weight gain, 0.40 kilo.

No. 48. "Alloxan-Recovered." Male.—Dose of cholesterol per day per kilo, 201 mg. Duration of feeding, 82 days. Average total serum cholesterol, 1849 mg. per cent. Lipemic index, 1.5. Atherosclerosis, grade 4. Liver fat, grade 4. Splenic fat, grade 3. Adrenal fat, grade 4. Weight gain, 0.07 kilo.

Inspection of the data presented in all the groups of protocols given above indicates further that gain or loss of body weight during the course of the feeding period exercised no determining influence on the result of the experiment.

It may be added that it was not possible to select pairs or groups of diabetic and non-diabetic animals matched as in the groups detailed above that yielded evidence contrary to that already presented.

Careful morphological examination, both grossly and microscopically, of the aorta and other organs revealed in varying degrees, as already indicated in Table I, the lesions that have been described by many authors as the characteristic sequelae of prolonged cholesterol feeding in rabbits (2–4). Not only were these lesions characteristic in form and location in the cholesterol-fed control animals, but also in the "alloxan-recovered" and diabetic rabbits. The only distinguishable difference in the morphology of the aortic lesions was a quantitative one as detailed above. This was true also of the lipid deposition in the liver, spleen, and adrenal glands. It should be noted, moreover, that in those parts of the aorta and other arteries that were uninvolved by cholesterol atherosclerosis, no differences could be distinguished microscopically between the diabetic and non-diabetic rabbits. The diabetic animals regularly presented the lesion of Armanni in the kidney and the hydropic changes in the pancreatic islets and ductules that have been described in detail elsewhere as characteristic of prolonged alloxan diabetes (8). The islets of Langerhans and the pancreatic ductules in the "alloxan-recovered" animals lacked hydropic changes but careful study of the islets revealed a peculiar and characteristic disturbance of cell arrangement which was distinctly different from the normal. However, in no other organ, including the thyroid gland, was there any evidence of a histologic difference between the diabetic, "alloxan-recovered," and control animals.

II. THE EFFECT OF ALLOXAN DIABETES ON THE RETROGRESSION OF EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS

Materials and Methods

Eighty-one young, adult, domestic white rabbits of both sexes were employed. Of these 32 completed a satisfactory experimental course, and comprised two experimental groups.

Each of the 25 rabbits in one group was fed 61.5 to 62.5 gm. of cholesterol during 88 to 90 days. In a second group of 7 rabbits each was fed 40.5 gm. of cholesterol during 59 days. The individual daily dose of cholesterol was 0.75 gm. fed by means of a stomach tube as a 5 per cent solution in warm corn oil.

Determinations at intervals of approximately 3 weeks were made of the non-fasting blood sugar, and of the free and total cholesterol content of the serum by a modified Folin micro method and the Schoenheimer-Sperry method respectively.

Immediately following the completion of the period of cholesterol feeding about one-half of the animals of each group received an intravenous injection of 150 mg. of alloxan per kilo of body weight administered as a 5 per cent aqueous solution. No insulin or other therapy was employed. The animals were given an unlimited diet of Purina rabbit chow and water *ad libitum*.

The animals, including diabetic, control, and those that recovered from the effects of a diabetogenic dose of alloxan after a brief period of diabetes ("alloxan-recovered"), were sacrificed at intervals of 1 to 16 weeks after the cessation of cholesterol feeding. Complete autopsies were performed and the tissues were treated in the same manner as described in Part I of this paper. The criteria of the diabetic state and the grading of the degree of aortic atherosclerosis and of the amount of lipid deposited in the liver, spleen, and adrenal were also the same as those used in the experiment described in Part I.

Observations

The experimental procedures and observations are summarized in Table II, in which the average values of serum cholesterol given are those obtained during the cholesterol feeding period, including one determination made the day before cholesterol feeding was begun. The blood sugar values are averages obtained after the cessation of cholesterol feeding. Among the animals that were injected with alloxan, the average of the blood sugar content includes one determination made before alloxan was administered. The period of retrogression was that between the cessation of cholesterol feeding and death of the animal. In the diabetic animals the duration of diabetes was the same as the period of retrogression.

The results as shown in Table II did not demonstrate that alloxan diabetes has any effect on the rate or degree of retrogression of experimental cholesterol atherosclerosis in the rabbit as judged by gross morphological examination of the aorta stained *in toto* with Sudan IV. Neither did microscopic examination of the entire length of the aorta reveal any appreciable differences between the atherosclerotic lesions of comparable diabetic and non-diabetic animals. The diabetic state was not found to alter appreciably the rate or degree of disappearance of abnormal lipid deposits in the liver, spleen, and adrenal cortex as judged by miscroscopic examination of appropriately stained frozen sections. In spite of the fact that in certain of the diabetic animals there occurred shortly after the administration of alloxan a distinct increase in the previously existing hypercholesterolemia, they in common with the other diabetic animals, the control, and "alloxan-recovered" rabbits, showed a return of the blood cholesterol to normal levels within 42 days. The only consistent biochemical differ-

TABLE II

Summary of Experimental Data

Series	No.	Sex	Experimental Type*	Total dose of cholesterol	Time of retrogression	Average blood sugar during retrogression	Average free serum cholesterol	Average total serum cholesterol	Aortic atherosclerosis (0–4)
				gm.	*wks.*	*mg. per cent*	*mg. per cent*	*mg. per cent*	
	1	F	D	61.5		—	90	277	1
1	2	F	D	61.5	1	570	134	382	4
	3	F	C	61.5		122	174	398	3
2	4	F	D	61.5	3	357	163	471	3
	5	F	D	61.5		384	144	426	2
3	6	F	C	61.5	5	134	161	516	4
	7	F	C	61.5		148	107	291	3
4	8	F	D	61.5	6	319	194	472	4
5	9	F	D	61.5	7	425	96	307	3
	10	M	C	61.5		121	—	—	1
6	11	F	D	62.25	9	353	106	261	2
	12	F	C	61.5		121	140	440	4
	13	F	MD	61.5		286	135	430	3
	14	M	D	61.5		410	218	525	4
7	15	M	AR	61.5	11	138	124	318	2
	16	M	AR	61.5		175	81	208	4
	17	M	AR	61.5		163	163	487	2
	18	F	D	61.5		396	183	479	2
	19	F	D	61.5		488	232	480	2
	20	F	C	61.5		133	—	—	2
8	21	M	C	61.5	16	126	83	298	4
	22	M	C	61.5		120	126	371	4
	23	M	C	61.5		111	88	325	2
	24	F	C	61.5		124	143	382	4
	25	F	C	61.5		127	72	240	3
	26	F	D	40.5		309	140	320	3
	27	F	D	40.5		373	88	262	3
	28	F	C	40.5		130	138	424	4
9	29	M	C	40.5	6	120	41	187	3
	30	F	C	40.5		128	145	452	3
	31	F	C	40.5		125	71	214	2
	32	M	AR	40.5		124	83	270	4

* D = diabetic; C = control; AR = "alloxan-recovered;" MD = mild diabetic.

ence observed between the various groups of rabbits was the hyperglycemic state of the diabetic animals. The only morphological differences found to exist between diabetic and non-diabetic animals were the changes in the pancreas and kidney that are associated with the injection of alloxan or with the development of persistent diabetes.

In both the diabetic and non-diabetic animals there was no gross morphological evidence that any appreciable retrogression of the aortic atherosclerosis had occurred during periods of up to 4 months' duration. However, in animals that survived a period of retrogression of 6 weeks or more there was definite microscopic evidence of the disappearance of lipid deposits from the aorta, splenic arterioles, and other small arteries, from the reticulo-endothelial cells of the liver and spleen, and from the parenchymal cells of the liver and adrenal cortex. In the aorta, the gradual disappearance of lipid material was accompanied by a gradual increase in the number of fibroblastic cells in the atherosclerotic lesions.

DISCUSSION

The observations recorded in Part I of this paper demonstrate clearly that, under the conditions described, there was associated with the presence of alloxan diabetes a marked but incomplete inhibition of the development of experimental cholesterol atherosclerosis in cholesterol-fed rabbits. There was also an inhibition of the deposit of sudanophilic lipid substances in the reticulo-endothelial cells of the liver and spleen and in the parenchymal cells of the liver and adrenal cortex. This inhibition occurred in spite of the induction of a marked degree of hypercholesterolemia in many of the diabetic animals, hypercholesterolemia that was usually as high as, and often higher than, that observed in the corresponding control animals.

This inhibitory effect was apparently dependent neither upon the administration of alloxan *per se* nor upon the short initial period of insulin and dextrose therapy, inasmuch as both the diabetic and "alloxan-recovered" animals received such injections before cholesterol feeding was instituted, but only in the diabetic rabbits was the inhibitory effect apparent. The "alloxan-recovered" animals responded to cholesterol feeding exactly as did the control animals. In addition, it was found that the inhibitory effect was not dependent on the sex or weight of the animal, nor upon the daily dosage of cholesterol, the form in which it was administered, nor the duration of cholesterol feeding. The effect was also independent of changes in body weight occurring during the course of our experiments and of the actual degree of hypercholesterolemia induced by the administration of cholesterol. Moreover, there was no gross or histological evidence of a morphological basis for the inhibitory effect either in the aorta or in the other organs in which it was observed. Indeed, the only observed factors consistently associated with the inhibition of the expected morphological effects of cholesterol feeding were the diabetic state and a degree

of visible lipemia considerably greater than that observed in the control animals. A moderate degree of visible lipemia in the control animals was regularly associated with the development of severe atherosclerosis of the aorta. On the contrary, a marked degree of visible lipemia was observed in a large proportion of the diabetic animals that presented at autopsy only a minimal degree of aortic atherosclerosis.

Objection might possibly be raised to our inference that the injection of alloxan *per se,* apart from its diabetogenic effects, was not responsible for the inhibitory effect observed in the diabetic animals on the ground that the "alloxan-recovered" rabbits did not provide a valid control of this possible factor. It could be argued that the "alloxan-recovered" animals were less susceptible to the general effects of alloxan than the diabetic animals as indicated by the very fact that permanent diabetes failed to develop in them. Animals that fail to respond with the development of permanent diabetes to a dose of alloxan that is diabetogenic to a majority of the species are frequently referred to in the literature rather loosely as "alloxan-resistant." However, in our "alloxan-recovered" animals, the diabetogenic effect of alloxan was manifested initially in the production of a temporary diabetic state of mild or moderate severity from which spontaneous recovery occurred within the period of several weeks before cholesterol feeding was started. Clearly, the injection of alloxan was effective, at least to a degree, but these particular rabbits displayed a capacity for recovery that distinguished them from the permanently diabetic animals.

Although we have referred to these animals as "alloxan-recovered" there is reason to believe that such animals have not returned to a strictly normal state. In our present observations, the "alloxan-recovered" animals during the period of cholesterol feeding showed neither obvious nor chemical evidence of diabetes. However, in other experiments (9) more detailed and precise studies have demonstrated that alimentary glycosuria may be present in such animals in spite of normal fasting blood sugar levels and that this is dependent on the occurrence of slight postprandial hyperglycemia. These residual metabolic defects are correlated with the presence of definite histological alterations in the islets of Langerhans which were detectable in the animals of our present experiments months after the injection of alloxan.

There is ample evidence, therefore, to indicate that our "alloxan-recovered" animals did not tolerate the injection of alloxan without suffering from its effects. Accordingly, we are inclined to regard these animals, at least tentatively, as providing a suitable control of the effects of alloxan injection *per se.* Further experiments currently in progress are designed to settle this point definitely by determining whether the inhibitory effect observed in alloxan-diabetic rabbits is abolished by controlling the diabetic state with insulin.

While we are quite unable to offer a specific explanation of the inhibitory

effect observed in the present experiments, it would appear, in view of the considerations set forth in the preceding paragraphs, that it is dependent upon some undefined factor or factors implicit in, or closely associated with, the diabetic state. That the diabetic condition or factors associated with it exercised an influence on the state and stability of the blood lipids in rabbits fed cholesterol in oil is indicated not only by the development of a marked visible lipemia, but also by the inhibition of lipid deposition in the aorta and elsewhere. Since the deposition of lipids in the intima of arteries is an essential feature of the development of experimental cholesterol atherosclerosis, particular interest attaches to any evidence of an alteration of the physicochemical state of the lipids in the blood plasma (*viz.* excessive lipemia) that coexists with protection from the usual effects on the arteries of rabbits associated with hypercholesterolemia.[1]

Almost from the first demonstration of the fact that the feeding of cholesterol is capable of producing atherosclerosis in the arteries of rabbits (10), it was recognized that the development of the arterial lesions is associated with a significant elevation of the cholesterol content of the blood (11). This was confirmed repeatedly by subsequent studies which showed that, in general, the severity of the induced atherosclerosis is correlated with the degree and duration of the induced state of hypercholesterolemia (2–4). It has also been shown that the development of experimental cholesterol atherosclerosis can be inhibited by various modifications of the experimental procedure that prevent the development of the expected degree of hypercholesterolemia (3, 4). On the basis of such data, the concept arose that hypercholesterolemia is the sole factor of importance in the genesis of experimental atherosclerosis. This concept was seriously questioned some years ago by Duff (3) on the basis of the evidence then available and, more recently, certain other investigators have emphasized that factors other than hypercholesterolemia may be important in the development of experimental atherosclerosis, pointing out that animals with comparable levels of induced hypercholesterolemia frequently exhibit widely differing degrees of atherosclerosis (12–15).

The absolute inhibition of the development of experimental cholesterol atherosclerosis in an appreciable number of the diabetic rabbits in our experiments in spite of the presence of marked and prolonged hypercholesterolemia shows clearly that the mere existence of a markedly increased quantity of cholesterol in the circulating blood for a considerable length of time is not in itself capable of causing lesions in the arteries. This conclusion is supported by the demonstration of Steiner (16, 17) and others (18, 19) that the addition of choline to the diet inhibits the development of atherosclerosis in cholesterol-fed

[1] This evidence clearly conflicts with the hypothesis of Moreton (*Science*, 1947, **106**, 190; and 1948, **107**, 371) which postulates that the determining factor in the development of atherosclerosis is the presence in the circulating blood of lipid particles of large size such as are present in abundance in the grossly milky or creamy serum of hyperlipemic states.

rabbits, though it does not prevent the development of marked hypercholesterolemia (16, 17). It is evident, therefore, that the development of experimental cholesterol atherosclerosis is dependent not only upon the occurrence of hypercholesterolemia *per se* but also upon another essential factor or factors as yet undetermined.

We are fully aware that our experimental observations are at variance with the evidence adduced to show that diabetes mellitus in man promotes the development of arteriosclerosis. A logical resolution of this apparent conflict compels consideration of one or more of the following possibilities. First, the conflict may be consequent on species differences. Second, alloxan diabetes in the rabbit may not be metabolically comparable with diabetes mellitus in man. Third, experimental cholesterol atherosclerosis in rabbits may not be comparable with the type of arterial disease to which diabetic patients are prone. Fourth, the impression that occlusive arterial disease in diabetic patients is dependent upon an excessive development of atherosclerosis of the intima of arteries may be erroneous. Obviously, it is impossible in the present state of knowledge to predict which of the possibilities just mentioned may prove to be correct.

The experimental data presented in Part II of this paper clearly fail to demonstrate any difference in the rate of retrogression of experimental cholesterol atherosclerosis in alloxan-diabetic rabbits as compared with control animals. In our experiments the period of retrogression, *i.e.* the period after the feeding of cholesterol was terminated, was limited to 4 months. Whether experiments with longer periods of retrogression would show any differential effect is impossible to say. More prolonged experiments, however, would be technically difficult because of the excessive mortality from alloxan diabetes of long duration in rabbits.

Our negative results are similar to those reported by other investigators who have attempted to influence the retrogression of experimental cholesterol atherosclerosis by the administration of potassium iodide (20, 21). On the other hand, Steiner (22) some years ago brought forward highly suggestive evidence of the ability of choline to bring about some reabsorption of arterial lesions previously induced by cholesterol feeding. Added to this evidence is the recent report of Morrison and Rossi (23) who have described complete reabsorption of the lesions of experimental cholesterol atherosclerosis in 17 out of 23 rabbits given larger daily doses of choline over a period of 182 days after the cessation of cholesterol feeding.

The observations, already cited relative to the effects of choline on the development and retrogression of experimental cholesterol atherosclerosis, coupled with the results of our own experiments on the effects associated with the presence of alloxan diabetes, permit of interesting deductions regarding the process of lipid accumulation in the walls of the arteries of cholesterol-fed rabbits. If

all the observations are correct and correctly interpreted, it is evident that the process of lipid accumulation must represent the resultant of the effects of two separate and distinct sets of factors, those factors the balance of which hinders or promotes the deposit of lipids in the arterial walls, and another set of factors the balance of which hinders or promotes their removal after they are deposited. This is not a new concept but the means of demonstrating its correctness have not hitherto been available. Since alloxan diabetes (or some associated influence) inhibits the development of experimental cholesterol atherosclerosis but has no noticeable effect on the retrogression of the arterial lesions, it follows that the inhibitory effect must be implemented solely or almost solely by interference with the deposit of lipids. On the other hand, the results of Morrison and Rossi (23) indicate that the administration of choline has a powerful effect in facilitating the removal of lipids already deposited in the arterial wall. The effect of choline in inhibiting the development of experimental cholesterol atherosclerosis, as described by Steiner (17) could, therefore, be due either to interference with the deposition of lipids, or to facilitation of their removal as rapidly as they are deposited or to a combination of both effects. However, if choline facilitates the removal of lipids already deposited in the arterial walls, this is the only effect of choline on experimental atherosclerosis that is susceptible of proof by the types of experiment under consideration here. The mechanisms of interference with the deposit of lipids and of facilitation of their removal remain to be investigated and clarified but the means to do so appear now to be at hand.

It is evident that our experimental results find no direct application to the problem of arterial disease in human diabetes. Nevertheless, if it is true that the accumulation of lipids in the intima of arteries is a central feature of human atherosclerosis, as it appears to be of experimental cholesterol atherosclerosis, then the isolation of the factors governing this fundamental biological process in the experimental animal may be expected to help in elucidating the nature of the same process in man.

SUMMARY

A comparison was made of the effects of cholesterol feeding in normal rabbits and in rabbits rendered persistently diabetic by means of alloxan. 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 control animals. Indeed, a large proportion of the diabetic animals presented no atherosclerosis whatever. There was a similar inhibition of the deposit of lipid substances in the liver, spleen, and adrenal glands of the diabetic rabbits.

The inhibition of the development of experimental cholesterol atherosclerosis which was associated with the presence of alloxan diabetes was independent of

the administration of alloxan *per se*. It was not dependent on the sex or weight of the animal, nor upon the daily dosage of cholesterol, the form in which it was administered, nor the duration of cholesterol feeding. It was also independent of changes in body weight occurring during the course of our experiments and of the actual degree of hypercholesterolemia induced by the administration of cholesterol. In addition, there was no gross or histological evidence of a morphological basis for the inhibitory effect either in the aorta or in the other organs in which it was observed.

Only two factors were observed to be consistently associated with the 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.

The inhibitory effect observed in these experiments would appear to depend upon some as yet undetermined factor or factors implicit in the diabetic state or closely associated with it. The experimental data presented demonstrate clearly that hypercholesterolemia is not the sole factor concerned in the genesis of experimental cholesterol atherosclerosis, but that another factor, or factors, rendered inoperative in our experiments must be essential to the production of the arterial lesions.

In view of the inhibitory effect on the development of experimental cholesterol atherosclerosis observed in alloxan-diabetic rabbits, the effect of alloxan diabetes on the retrogression of such arterial lesions was studied in another series of experiments. No effect on retrogression could be demonstrated within periods lasting up to a maximum of 4 months after the cessation of cholesterol feeding.

The results of our two series of experiments, considered together, indicate that the process of deposition of lipids in the arterial walls is governed by factors different from those that are operative in the process of removal of lipids after they have been deposited. The inhibition of the development of experimental chllesterol atherosclerosis in alloxan-diabetic rabbits must depend on interference with the process of deposition of lipids and not on a process of removal of lipids as fast as they are deposited.

Our experimental results find no direct application to the problem of arterial disease in human diabetes. Nevertheless, the experimental procedures that we have employed provide a new basis for the design of further experiments directed toward the elucidation of the nature of the unknown factors that govern the process of lipid deposition in the walls of arteries.

BIBLIOGRAPHY

1. Duff, G. L., *Am. J. Med. Sc.*, 1945, **210**, 381.
2. Anitschkow, N., Experimental arteriosclerosis in animals, in Arteriosclerosis: A Survey of the Problem, (E. V. Cowdry, editor), New York, The Macmillan Company, 1933, chapter 10.

3. Duff, G. L., *Arch. Path.*, 1935, **20,** 81, 259.
4. Hueper, W. C., *Arch. Path.*, 1944, **38,** 162, 245, 350; 1945, **39,** 51, 117, 187.
5. Duff, G. L., and Wilson, D. C., to be published.
6. Duff, G. L., and McMillan, G. C., *Am. Heart J.*, 1948, **36,** 469.
7. Pollak, O. J., *Arch. Path.*, 1944, **37,** 337.
8. Duff, G. L., McMillan, G. C., and Wilson, D. C., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64,** 251.
9. Duff, G. L., Wilson, D. C., and McMillan, G. C., *Nature*, 1947, **159,** 575.
10. Anitschkow, N., and Chalatow, S., *Centr. allg. Path. u. path. Anat.*, 1913, **24,** 1.
11. Wacker, L., and Hueck, W., *Münch. med. Woch.*, 1913, **60,** 2097.
12. Weinhouse, S., and Hirsch, E. F., *Arch. Path.*, 1940, **30,** 856.
13. Hirsch, E. F., and Weinhouse, S., *Physiol. Rev.*, 1943, **23,** 185.
14. Pollak, O. J., *Arch. Path.*, 1945, **39,** 11.
15. Pollak, O. J., *Arch. Path.*, 1947, **43,** 387.
16. Steiner, A., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38,** 231.
17. Steiner, A., *Arch. Path.*, 1948, **45,** 327.
18. Andrews, K. R., and Broun, G. O., *J. Clin. Inv.*, 1940, **19,** 786.
19. Kesten, H. D., and Stilbowitz, R., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49,** 71.
20. Meeker, D. R., Kesten, H. D., and Jobling, J. W., *Arch. Path.*, 1935, **20,** 337.
21. Turner, K. B., and Bidwell, E. H., *Proc. Soc. Exp. Biol. and Med.*, 1937, **35,** 656
22. Steiner, A., *Proc. Soc. Exp. Biol. and Med.*, 1938, **39,** 411.
23. Morrison, L. K., and Rossi, A., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69,** 283.