STUDY ON REGRESSION OF ATHREOSCLEROSIS IN RABBITS

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INTRODUCTION

The question of whether arteriosclerotic lesions regress or not is of fundamental importance in relation to the therapeutic problem of the human arteriosclerosis. The atherosclerotic plaque is, within limits, a reversible lesion. After cessation of cholesterol feeding in rabbits, there is a gradual regression of the cholesterol-induced lesions. Anitschikow[1] investigated on this problem in detail and found that a large plaque, rich in lipid, was gradually transformed into fibrous tissue. Spontaneous regression of cholesterol-induced lesions has been described in the chicken and dog[2][3].

Sigmund and others suggested that the lesions of human atherosclerosis might also undergo regression[4][5]. Many factors are involved in the process of the atherosclerosis, and some of them promote atherogenesis and others slow it down. These factors do not always give the same effect on the development and the regression of the atherosclerosis. For example, it was reported that insulin had no effect on the atherogenesis in the chick, whereas it had an aggravating effect on the regression of the atherosclerosis[6].

The conditions associated with rabbit arterial lesions in post-cholesterol feeding periods are more closely simulated to those in man, in which arterial lipid deposits are not ordinarily associated with marked hypercholesterolemia. Atherosclerosis in man appears to be more closely related to the arterial reaction, degeneration and repair than to the level of serum cholesterol, which is the determinant in the rabbit. The experimental design of the present study might produce atherosclerotic lesions in the rabbit more closely simulated to those in man. Any procedure that would accelerate the regression of lesions in this normocholesterolemic stage would likely act favorably with equal effectiveness on the atherosclerotic lesions of man.

Since 1956, when Sinclair[7] suggested that atherosclerosis might be regarded as a chronic deficiency of arachidonic acid, many studies have been made on the role of essential fatty acids in atherosclerosis. The effect of a diet with polyunsaturated fatty acids on lowering the serum lipid levels has been amply demonstrated by many investigators[8~11]. However, relatively little are known whether essential fatty acids cause any improvement in the diseased patients.

It has been generally held that hypertension has an accelerating as well

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as an aggravating effect upon atherosclerosis[12].

However, the actual mechanism by which hypertension aggravates aortic atherosclerosis is still not thoroughly elucidated.

The relationship between dietary protein and atherosclerosis is yet to be clarified. To our knowledge, investigations have been rarely made concerning the relationship between these factors, *i.e.* dietary protein, essential fatty acids or high blood pressure, and the process of regression of atherosclerosis. The purpose of the present study is to asses the role of these factors on the process of the regression of atherosclerosis in rabbits.

MATERIALS AND METHODS

General Plan of Experiment

Ninty two male white rabbits weighing 2.0-2.3 kg were fed on stock diet for four months to which 2 g of lanolin and 4 g of cottonseed oil were added. At the end of this period, 73 animals that had achieved the most comparable degree of hypercholesterolemia were selected and divided into seven groups.

Group I, consisting of four rabbits, were sacrificed immediately to serve as control for the amount of atherosclerosis developed in four months, while the remaining six groups were taken off the lanolin diet, kept alive on a normal diet and treated as follows:

Group II (Late control), consisting of 12 animals, received a normal diet.

Group III (High protein group), consisting of 13 animals, received a high protein diet containing 37% of protein.

Group IV (Low protein group), consisting 13 animals, received a low protein diet containing 5.2% of protein.

Group V (Hypertension group), consisting of 14 animals in which hypertension was induced with silver cramps by the modified method of Goldblatt, consumed a normal diet[88].

Group VI (Linoleic acid feeding group), consisting of 13 animals, received a diet containing 4 g of purified linoleic acid.

Group VII, consisting of 4 animals, received a normal diet for 4 months and then was maintained on the lanolin diet again.

Animals of Groups II-VI were killed at regular intervals beginning 2 weeks after cessation of lanolin feeding and terminating after 4 months (2, 8, 16 weeks). Animals of Group VII were sacrificed after 2 months following commencement of lanolin refeeding.

Diets used

A normal diet consisted of 350 g of bean extracted refuse, 15 g of bran, and 200 g of cabbage.

A high protein diet consisted of 300 g of bean extracted refuse, 5 g of bran, 13 g of casein and 200 g of cabbage.

A low protein diet consisted of 100 g of sweet potato, 50 g of protein-depleted starch.

Rabbits of linoleic acid feeding group consumed a diet containing 280 g

of bean extracted refuse, 15 g of bran and 200 g of cabbage, and 4 g of linoleic acid. The average daily calorie intake was about the same for each group of rabbits studied.

Chemical studies

All biochemical studies were performed after an overnight fast. On all groups, the levels of serum total cholesterol were measured at the beginning of the experiment, and thereafter at given intervals during the experimental period, and at the time of sacrifice by Kitamura's modification of the Zak-Henly procedure[13]. In the late control group, serum was analyzed periodically at given intervals for hexosamine by the method of Elson and Morgan[14] and post heparin clearing activity was determined at times during the course of the experiment turbidimetrically by the modification of the Yamada's procedure[15]. On all groups except Lanolin refeeding group, serum was analyzed at the time of sacrifice for free and total cholesterol by the method of Sperry and Webb[16], for lipid P by the method of Fiske and Subbarow[17], for lipoproteins by paper electrophoresis according to the method of Kobayashi[18], for hexosamine by the method of Elson and Morgan. Post heparin clearing activity was determined on all groups. On all groups except Linoleic acid feeding and Lanolin refeeding groups, the content of total cholesterol of aorta was measured at the end of the experiment. The concentrations of free and total cholesterol and lipid P in liver were determined after sacrifice in all groups with the exception of Linoleic acid feeding and Lanolin refeeding groups. In the hypertensive animals, the levels of serum urea N were determined at times to check the degree of renal damage. Serum protein was measured on all experimental groups in order to know the state of nutrition.

Anatomical studies

At the time of sacrifice, the aorta, heart, liver, adrenal grand and kidney were removed and weighed. After the aorta was dissected longitudinally, the degree of atheromatosis of the thoracic aorta was graded by judging visually the extent of atheromatosis according to the following schema: zero representing the absence of visible atherosclerosis; +1, minimal but visible plaques; +4, extensive involvement of most of the aorta with numerous raised confluent plaques; and +2 and +3, in intermediate stages.

The blocks of aorta were obtained from ascending portion of the thoracic aorta and fixed in 10% formalin or Carnoy solution and embeded in paraffin. The sections were stained with hematoxylin and eosin, periodic acid Schiff, Van Gieson, Weigert and Colloid-Fe[19].

Frozen sections were prepared from wet formalin tissue cut at 10 μ and stained with Sudan III. The blocks of liver, kidney and heart were excised and placed in 10% formalin. Blocks of the tissues were embeded in paraffin and sectioned at 5–7 micron in thickness.

The alternate cut sections were stained with hematoxylin and eosin.

Estimation of blood pressure

Animals were rested for 5 minutes prior to the measurement of blood

pressure. Determination of blood pressure was made by the ear-capsule technic of Grand and Rothschild[20].

RESULT

General effects of experimental plan

Body weight

Rabbits of the low protein group exhibited marked loss in body weight during the experimental period. Rabbits in the hypertension group also lost weight to the lesser extent. No significant reduction of body weight was observed in rabbits of the other groups.

The weight of heart in animals with hypertension was increased above that of the control group. The heart from animals of the other groups showed no weight gain. The weight of the heart from animals of the low protein group was markedly reduced. The weight of adrenal grand was much less in the low protein group than in the control group, while no significant difference in weight was observed in the other groups. The weight of liver was much lower in the hypertension and low protein groups. The weight of the right kidney in animals with hypertension was much less than in the control animals, whereas that of the left kidney was increased in animals of the hypertensive group.

TABLE 1. Mean Weight of Heart, Liver, Adrenal Grand and Kidney

	Initial body wt. (kg)	Terminal body wt. (kg)	Heart (g)	Liver	Adrenal	Kidney	
						r.	l.
Early control group	3.3		7.2	98	1.03	8.5	8.9
Late control group	3.1	3.0	7.8	76	0.95	8.2	8.1
High protein group	3.0	3.2	7.6	76	1.10	8.0	7.9
Low protein group	3.1	2.5	6.5	67	0.48	6.8	6.6
Hypertension group	3.1	2.8	8.5	61	0.80	6.1	8.6
Linoleic acid	3.2	3.2	7.4	75	0.90	8.0	8.2

Blood pressure

Elevation of blood pressure was noted in 1-2 weeks following the operation of the right renal artery of those animals in which hypertension was induced by means of constriction of bilateral renal arteries, first on the right and two weeks later on the left. Elevation of blood pressure sustained throughout the experimental period.

Serum protein

Serum protein concentrations were significantly lowered in the low protein group. In the hypertension group the levels were decreased below normal. There was no difference in the concentrations of serum protein among the other groups.

Changes of blood elements after cessation of lanolin feeding

Cholesterol

Fig. 1 illustrates the changes of the mean level of serum total cholesterol in the experimental groups during the course of the experiment. After ces-

sation of lanolin feeding the serum cholesterol levels were gradually lowered to the normal level. The levels fell to normal within two weeks and then remained unchanged throughout the rest of the experiment. The average concentrations of serum total and esterified cholesterol for the experimental groups at the time of sacrifice are given in Table 2. No significant difference in the mean levels of serum total and ester cholesterol was observed among the experimental groups. However, as shown in Fig. 1, the decline of the mean level of serum cholesterol in the low protein group was much slower after the cessation of lanolin administration than that in the other groups. Among the other groups, there was no overall difference in the rate at which hypercholesterolemia declined following the discontinuance of lanolin feeding. Linoleic acid feeding did not accelerated the rate of decline.

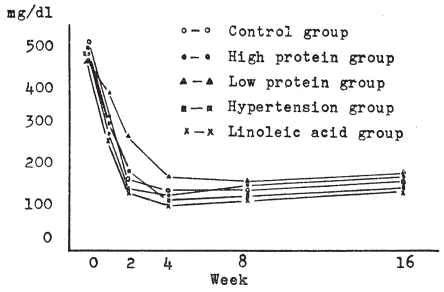

FIG. 1. Change of total cholesterol concentration in serum after cessation of lanolin feeding.

TABLE 2. Terminal Concentration of Lipid in Serum

	Cholesterol			Phospholipid	Lipoprotein index
	Free	Ester (mg/100 ml)	Total		
Early control group	210(192–220)	317(300–331)	527(502–551)	273(261–289)	7.8(7.0–9.0)
Late control group	69(58– 76)	102(93–115)	171(155–191)	166(146–178)	2.2(1.9–2.4)
High protein group	67(58– 81)	92(75–111)	159(147–170)	172(156–182)	2.4(1.8–2.7)
Low protein group	76(67– 87)	98(70–112)	174(137–197)	105(88–116)	3.2(2.8–3.8)
Hypertension group	72(66– 82)	103(92–106)	175(150–193)	153(146–164)	4.7(3.0–6.5)
Linoleic acid group	64(61– 71)	100(85–110)	164(146–181)	166(150–174)	2.2(1.8–2.4)
Normal	72(60– 83)	106(92–118)	178(160–186)	168(158–180)	2.4(1.8–2.6)

Values represent the mean of 4 to 5 rabbits.
Numbers in parentheses indicate range.

Phospholipid

Serum phospholipid concentration were also decreased following the cessation of lanolin feeding and were normal at the end of the experiment. Mean serum level was markedly lowered in the low protein group at the end of the experiment, as illustrated in Table 2.

The average value of serum phospholipid was 105 mg/dl in the low protein group, while in the other groups the mean values were 153 mg/dl or more.

Accordingly, cholesterol: phospholipid ratios were much greater in the low protein group than in the other groups.

Lipoprotein

Beta: alpha lipoprotein ratios were extremely high at the beginning of the experiment and returned to normal upon cessation of lanolin administration.

The terminal values of lipoprotein ratios were much greater in animals of the low protein and hypertension groups than in the other groups. Mean values of lipoprotein ratios in the low protein and hypertension groups were 3.2 and 4.8, respectively. On the other hand, mean values of the other groups were almost alike (2.2-2.4).

Serum hexosamine

Serum hexosamine levels were gradually elevated following the withdrawal of lanolin from the diet, reached a maximum (one and a half of the initial value) in 3 weeks, and then returned to the original levels, as presented in Fig. 2. After the end of the 8th week of off-lanolin there had been no remarkable changes of serum hexosamine levels throughout the rest of the experimental period. At the end of the experiment, as shown in Table 3, there was no significant difference in the mean serum hexosamine concentration among the experimental groups.

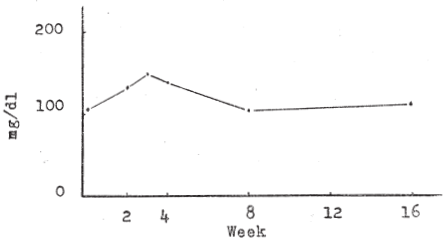

FIG. 2. Change of serum hexosamine level after cessation of lanolin feeding

Post heparin clearing activity

Post heparin clearing activity of rabbits fed lanolin for 4 months were much lower than that of normal rabbits. Upon cessation of lanolin feeding, the level increased gradually toward normal, as shown in Fig. 3. At the time of sacrifice, no significant difference in activity was noted among the experimental groups.

TABLE 3. Terminal Levels of Serum Protein and Hexosamine and Plasma Post Heparin Clearing Activity

	Serum		Plasma activity
	Protein (g/dl)	Hexosamine (mg/dl)	
Early control	6.5	104	0.200
Late control	6.4	115	0.282
High protein	6.4	100	0.287
Low protein	4.9	115	0.270
Hypertension	5.8	95	0.288
Linoleic acid	6.3	102	0.270
Normal	6.4	98	0.302

Values represent the mean of 4 to 5 rabbits

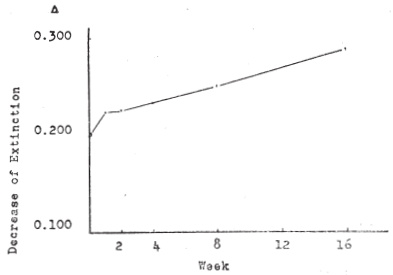

FIG. 3. Change of post heparin clearing activity following withdrawal of lanolin from the diet.

Urea N

Serum urea N concentrations were measured only in rabbits of

the hypertension and control groups.

Urea N concentrations were elevated in rabbits of the hypertensive group.

Changes of lipid content in liver after cessation of lanolin feeding

Concentrations of cholesterol and phospholipid in liver were likewise decreased following the discontinuance of lanolin feeding. At the end of the experiment, a large quantity of cholesterol was accumulated in the low protein group than in the other groups, (6.37 mg/g). On the contrary, the content of phospholipid P was significantly lower in the low protein group. The concentrations of phospholipid P averaged 1.07 mg/g in the low protein group, while no significant difference was observed in the other groups.

There was no remarkable difference in the amount of total and ester cholesterol among the experimental groups, except for the low protein group. The average concentrations of total and ester cholesterol were 5.25 and 2.83 mg/g in the late control group, 5.35 and 2.80 mg/g in the high protein group, and 5.32 and 3.03 mg/g in the hypertension group, respectively (Table 4).

TABLE 4. Terminal Concentrations of Lipid in Liver
(mg/g fresh tissue)

	Cholesterol			Lipid P
	Free	Ester	Total	
Early control	3.89(3.10-3.90)	7.70(7.16-9.12)	11.59(11.00-13.00)	2.72(2.73-3.15)
Late control	2.42(1.95-2.57)	2.83(2.73-3.02)	5.25(4.68- 5.69)	1.57(1.46-1.68)
High protein	2.55(2.17-2.86)	2.80(2.66-3.08)	5.35(4.97- 5.56)	1.68(1.45-1.78)
Low protein	3.16(2.77-3.68)	3.21(2.96-3.52)	6.37(6.11- 6.64)	1.07(0.78-1.54)
Hypertension	2.29(2.09-2.38)	3.03(2.79-3.16)	5.32(5.16- 5.54)	1.66(1.50-1.88)
Normal	1.20(1.10-1.30)	2.00(1.56-2.51)	3.20(2.63- 3.82)	1.41(1.22-1.53)

Values represent the mean of 4 to 5 rabbits
Numbers in parentheses indicate range.

Changes of the content of total cholesterol in aorta and the degree of atheromatosis in aorta and coronary artery (Table 5).

TABLE 5. Terminal Cholesterol Content of Thoracic Aorta and Degree of Atheromatosis in Thoracic Aorta and Coronary Arteries

	Aorta		Total No. of atheromatous coronaries per section	Completely occluded arteries
	Grading*	TC (mg/g)		
Early control	+1.8(1.0-2.0)	5.21(5.02-5.50)	3.0	0
Late control	+1.5(1.0-3.0)	4.90(3.20-5.52)	2.0	0
High protein	+1.4(1.0-3.0)	4.75(2.55-5.82)	2.0	0
Hypertension	+2.2(1.0-3.0)	6.12(5.20-8.07)	3.0	1.3
Low protein	+2.0(1.0-3.0)	7.34(6.30-9.78)	2.7	0.6
Linoleic acid	+1.2(1.0-2.0)			

* Lesions of aorta graded grossly from zero to 4+
Values represent the mean of 4 to 5 rabbits
Numbers in parentheses indicate range

In Table 5 are shown cholesterol content of thoracic aorta in animals of various experimental groups, together with average gross grading of the extent of visible aortic atherosclerosis and mean number of atherosclerotic coronaries. There was no difference in the extent of the atherosclerotic lesions between the early control and the late control. Mean gross grading of the extent of the atherosclerotic lesions in animals of the early control group was 1.8 and that of the late control was 1.5.

On the other hand, cholesterol content of thoracic aorta slightly decreased after cessation of lanolin feeding. Mean cholesterol concentrations of the aortas in animals of the early control and the late control were 5.21 and 4.90 mg/g, respectively. In animals of the high protein group, the extent of atherosclerosis and cholesterol content averaged 1.4 and 4.75 mg/g, respectively. The aortae of the low protein and the hypertension groups revealed much more atherosclerosis than those of the late control, as judged by the gross grading as well as by the cholesterol content. Furthermore, the degree of atheromatosis in both groups exceeded that of the early control, in visual grading as well as in the content of cholesterol. Comparison of the low protein group and hypertension group showed that the mean extent of atherosclerosis was alike in both groups, whereas the content of cholesterol was much greater in the low protein group than in the hypertension group (7.34 and 6.12 mg/g, respectively).

There was no apparent difference in the number of atheromatous coronaries among the experimental groups. However there seemed to be an increased severity of atherosclerosis in the early control, low protein and hypertension groups. Completely occluded arteries were observed only in these groups.

TABLE 6. Effect of Hypertension of Blood Pressure and Urea N. in Serum

	Mean terminal urea N. (mg/dl)	Mean blood pressure	
		Begin (mmHg)	End (mmHg)
Late control	30	72	70
Hypertension group	44	74	110

Values represent the mean of 4 to 5 rabbits

Histological findings

Aorta

Changes of the atheroma after cessation of lanolin feeding

After cessation of lanolin feeding, fibrous tissue was gradually formed from the inner-most part of the atheroma.

In the 2nd week following cessation of lanolin feeding, this fibrous layers were already observed in the inner part of the atheroma (Fig. 4).

Young fibroblasts proliferated in the deeper parts of the atheroma. They were arranged parallel with the intimal surface and growing perpendicular to it. Lipid materials were scanty in those fibrous layers and abundant beneath

these layers (Fig. 5). So-called foam cells were covered with these fibrous layers. Lipid was deposited rather extracellularly than intracellularly. A large amount of PAS-positive substances was accumulated in the fibrous tissue and around fibroblasts. Collagen fibrils, observable with Van Gieson staining, were increased in the intima. Small blood vessels were seen in the atheroma. During 8 weeks following cessation of lanolin administration, the fibrous layers became thicker. Lipid deposit was less pronounced in the 8th week than in the 2nd week of cessation. Lipid droplets tended to be present in intracellular form and spare the surface of the plaques (Fig. 6). Acid mucopolysaccharides were also accumulated in the fibrous tissue in this stage. However, young fibroblasts were decreased in number. In the 16th week, the thickness of the fibrous layers overlying intermediate zone of unorganized debris, was greatly increased. The areas containing unorganized debris, *i.e.* lipid materials, were markedly reduced. Sudan III staining showed small flecks of lipid in the plaques (Fig. 7). Needle-like cholesterin crystals were demonstrated in the intermediate zone (Fig. 8).

Fibrous elements proliferated extensively and the plaques were composed mostly of fibrous elements. Calcification was observed at the base of the plaques (Fig. 9). Reaction for Colloid-Fe was slightly positive only around unorganized debris (Fig. 10). PAS positive substances were seen in the fibrous tissue (Fig. 11). Giant cell formation was never observed in this group.

Effect of a high protein diet

The atheromas of the aortae obtained from the animals of the high protein group that were killed 2 and 8 weeks after institution of a high protein diet showed no different microscopic findings from those of the control. Young fibroblasts were likewise observed at the base of the atheroma. The fibrous layers were produced to the same extent as in the control group (Fig. 12). Colloid-Fe reaction was positive in the fibrous areas and the intensity was the same as in the control.

Sudan III staining revealed a large amount of sudanophilic substances in the atheroma. At the end of the 16th week following cessation of lanolin feeding, the plaques were seen covered with thick fibrous layers. Lipid materials were present in small amounts in the plaques, as illustrated in Fig. 13.

Effect of a low protein diet

It was observed that the formation of the fibrous layers was slowed down in the low protein group after cessation of lanolin feeding.

In the 8th week after commencement of a low protein diet, the fibrous layers were still not observed in the atheroma. The microscopic appearance of the aortic lesions with hematoxylin and eosin as well as with Sudan III staining was quite similar to that of the early control (Fig. 14). Considerable amounts of lipid was still present in the atheroma, including the inner-most part of the intima. Fibrous elements were not prominent. In the 16th week, considerable lipid was seen in the plaques.

Effect of renal hypertension

Also in this group, proliferation of connective tissue was significantly inhibited as in the low protein group.

In the 8th week, the fibrous layers were not prominent in the inner part of the atheroma, where a good deal of lipid was still accumulated (Fig. 15). In the 16th week, the atheroma of this group was not sufficiently replased with connective tissue. Septal structures of the atheroma were partly destroyed and lipid droplets became confluent. Eosinophilic and neutrophilic leucocytes were demonstrated in the atheroma. The qualitative change were suggestive of incipient softening of the plaque and the formation of ulcer (Fig. 16). Fig. 17 illustrated a section of the aorta from an animal that was sacrificed in the 16th week following induction of renal hypertension. There was perivascular infiltration of inflammatory cells with hemorrhage and exudation. The lumen of vasa vasorum was markedly dilated. The wall of vasa vasorum showed marked hyalinization.

Effect of linoleic acid

Two aortae of animals in the linoleic acid group that were sacrificed 2 weeks after starting linoleic acid feeding showed necrosis with multinucleated giant cell reaction in the plaques. The plaque was umbilicated at its centre. Neutrophilic leucocytes were accumulated about these necrotic areas. Lipid substance was scarcely observed in the plaques. These anatomical changes are suggestive of promoted regression of the plaques, *i.e.* central shrinkage of the plaque and histological lipid depletion (Fig. 18, 19).

In the 16th week, Sudan III staining exhibited less amount lipid material than in the control group. Plaques were composed largely of fibrous tissue, as illustrated in Fig. 20. Cholesterin crystals were never observed in the linoleic acid group.

Effect of lanolin refeeding

In Fig. 21 is shown a Sudan III stained section of aorta from an animal that was maintained on lanolin-free diet for 16 weeks and then fed again lanolin for 4 weeks. Needle-like cholesterin crystals were enclosed with dense fibrous tissue. In these areas Sudan III staining revealed the absence of neutral fat. Lipid materials positive for Sudan III staining were infiltrated around these fibrous areas. These fibrous tissue resembled to the so-called cholesterin granuloma. They were located in the deeper part of the atheroma and arranged parallel to the base of the plaque.

Changes of coronary arteries

There was no apparent difference in number of atheromatous coronaries per section among all experimental groups. However, there seemed to be an increased severity of atheromatosis in the early control, low protein and hypertension groups. Fig. 22 illustrated completely occluded coronary artery from a section in the hypertension group.

Other organs

Hematoxylin and eosin staining could not demonstrated any pathological findings, with exception of atrophy of renal tubules in the hypertension group. As lipid staining was not performed, no information is available about difference in amount of lipid accumulated among the experimental groups.

DISCUSSION

There have been experimental reports[4)87)] that the lesions of human atherosclerosis may undergo regression. It has been reported that cholesterin-induced experimental atherosclerosis regresses when the ingestion of cholesterin is discontinued[1)2)3)]. Sequential observation of the process of regression in atherosclerosis was made with atheromatous aortic homograft transplanted in the eyes of rabbits[21)]. Anitschkow stated that cholesterol deposited in the vessel of rabbits disappeared in two or three years after discontinuance of cholesterin ingestion[1)]. Attempts to accelerate the process of regression have been made by several workers[22~26)]. Injected sulfated polysaccharides or phospholipid have been reported to promote the regression of atherosclerosis or arrest the progress of atherosclerosis[27)28)29)]. Stamler has investigated some factors which have influences on the course of regression, finding that intact pancreas plays a role in effecting regression of atherosclerosis and that thyroid hormone, insulin and hydrocortisone inhibited this process[30)].

Estrogenic hormones have been claimed to inhibit the progress of atherosclerosis[31)32)]. Chlorpromazine has been shown to accelerate the fall of cholesterol level after cessation of cholesterol feeding and consequently reduce the amount of lipid accumulated in the atheroma[33)].

As mentioned above, although there are many observations concerning some factors related to the process of preestablished atherosclerosis, relatively little is studied with histological methods on the relationship between dietary factors or hemodynamic factors and preestablished atheroma. The anatomical features of human atherosclerosis differ very much from those of experimental cholesterol atherosclerosis, especially in respect to a large amount of connective tissue component and the tendency of ulceration.

Constantinides showed in rabbits that cholesterol-induced atherosclerotic lesions containing a large number of foam cells were gradually covered with fibrous tissue after cessation of cholesterol feeding and ultimately transformed into the lesions quite similar to those of moderately advanced human aortic atherosclerosis, which consist of thick fibrous cap in the intima and numerous cholesterol clefts or calcification in the depth of the intima[34)]. Atherosclerotic lesions identical with those described by Constantinides, have been likewise reported to be produced by prolonged cholestesterin feeding[35)]. In the present author's opinion, those lesions can be more easily produced by withdrawal of atherogenic diets, for cholesterol has been claimed to inhibit connective tissue proliferation. Atherosclerosis is thought to be an end product of nonspecific reaction of the arterial wall to injury, from whatever cause it may be. When the inciting cause is removed, the repair of the wall is probably complete in the early stage.

The present study also confirmed that moderately advanced atherosclerosis can be brought to undergo partial regression.

Transformation of foam cell atheroma into fibrous plaque should be reasonably regarded as the process of regression of the atherosclerosis, even if this connective tissue repiar has sometimes adverse effect on the organism. As suggested in the present study on lanolin refeeding, connective tissue formed in the atheroma has resistance against another injury (in this case, secondary cholesterol invasion) and restrains further progress of atherosclerosis. Therefore, connective tissue repair may have its significance in arresting atherosclerosis.

However, chemical analysis of cholesterol in the aorta and histologic observation showed that this process of connective tissue repair or regression requires a considerable period of time.

After 4 months of cessation of lanolin feeding, considerable amounts of cholesterol were still present in the aorta.

The histologic findings of the present study were in accordance with those of Constantinides and Kimura. The atherosclerosis is not always a change of the arterial wall which inevitably continues to progress. Regression can occur even in the period of lanolin feeding. Therefore, it is no wonder that Kimura obtained similar histologic findings to ours by prolonged lanolin feeding.

It is of interest that serum hexosamine level reached a maximum in the 3rd week after cessation of lanolin feeding when histological examination showed maximal activities of fibroblasts. Hexosamine is one of the building-stones of ground substance in the arterial wall. Accordingly, increase of serum hexosamine levels might be considered to be a sign of increased fibroblastic activity in the arterial wall or elsewhere.

Among various factors involved in the regression of atherosclerosis, a low protein intake or renal hypertension not only inhibited the process of regression, but also promoted atherosclerosis.

Linoleic acid feeding accelerated the removal of lipid in the atheroma, whereas no effect was noted on regression of atherosclerosis with high protein intake. Further discussion follows below.

Dietary protein and preestablished atherosclerosis

The relationship between dietary protein and the level of serum cholesterol is still not completely known, although experiments with animals in recent years mostly confirm a favorable suppressing effect of a high protein diet on hypercholesterolemia[36~38]. It has been shown that a high protein intake may afford a partial protection against the hypercholesteremic and atherogenic effects of high-fat, high-cholesterol diets[36) 40)], and that hypercholesteremia and atherosclerosis were aggravated in cholesterol-oil-fed animals ingesting a diet low in protein[41)].

In our present study, the fall from hypercholesterolemic state was retarded in the animals of the low protein group. C/P ratios and lipoprotein indices were markedly elevated. Although high fat feeding was stopped at the beginning in this experiment, the same effect of a low protein intake on the hyper-

cholesterolemia as in the previous reports was observed in the period during which hypercholesterolemia sustained. Any effect of a high protein diet was not seen in the present study. Cholesterol deposition in liver remained partially unabsorbed in all experimental groups in the 4th month after cessation of lanolin feeding, especially in the low protein group. As is the case with serum cholesterol, a low protein intake inhibited the resorption of excess cholesterol in liver and aorta. Moreover, a low protein diet induced succesive accumulation of cholesterol in aorta in spite of withdrawal of lanolin from the diet. It has been well known that a low protein intake lowered the concentration of phospholipid in serum and liver, upon which the stability and structure of lipoprotein are dependent.

This was also confirmed in our present study. In contrast, a high protein intake has been demonstrated to reduce serum C/P ratios in the rabbits[42]. It has been said that phospholipid is an essential component of mitochondria and concerned with mitochondrial enzyme system. On the other hand, the activities of many enzymes located principally in mitochondria have been demonstrated in the arterial wall by several workers[43~45]. And also it has been reported that lipid might be metabolized in the arterial tissue[46~49].

Therefore, it is with some reason to assume that phospholipid plays an important role in fat metabolism in the arterial tissue as well as in liver.

Thus, dietary protein plays an important role in the metabolism of the arterial wall by its participation of phospholipid metabolism.

Furthermore, experiments in recent years tended to exhibit that dietary protein is related with fat metablism in another way.

Ohno suggested that the sulfhydril-containing amino acids such as methionine are concerned with synthesis of Co enzyme A, which is essential for lipid metabolism[50].

As discussed above in detail, the derrangement of fat metabolism by a low protein intake may account for impairment of regression of preestablished atheroma and retardation of the fall of hypercholesterolemia in the low protein group. However, another important factor was likewise incriminated for inhibition of regression of atherosclerosis. The connective tissue repair of the arterial wall could be taken as being analogous to wound healing. Any factors which have influence on the protein metabolism may exert the same effects on the arterial wall as on other tissues. Weitzel indicated possible influence of dietary methionine or choline on the connective tissue metabolism in the arterial wall[51]. Immamura, Esaki and Shimizu reported in rabbits that a low protein intake accelerated the atherogenesis and fostered qualitative changes of protein or amino acids in the wall, and that a high protein intake suppressed these adverse qualitative changes of protein in the arterial wall[42][52][53].

Therefore, a low protein diet may inhibit the regression of atherosclerosis in many ways.

Hypertension and preestablished atheroeclerosis

It has been well known that hypertension has an aggravating effect upon atherosclerosis in man. The incidence of atherosclerosis is higher in the hyper-

tensive than in the normotensive[54)56)].

The relationship between atherosclerosis and hypertension have been demonstrated experimentally in rabbits, rats and dogs in which renal or postural hypertension has been induced[57)58~61)].

Dill could produced aortic atherosclerosis in the rabbits by induction of hypertension without supplement of atherogenic diet[62)].

However, the mechanism of this aggravating effect by hypertension is still not clear, although it has been postulated frequently that hypertension allows greater penetration of lipid into the arterial wall. Some investigators have reporetd that a positive correlation existed between hypertension and lipid concentration in serum[56)58)]. Murakami has demonstrated *in vitro* that the amounts of beta-lipoprotein incorporated into the arterial wall increased in proportion to the degree of the pressure exerted upon the arterial wall. High beta-lipoprotein concentration in serum was observed in the hypertension group in the present study, while cholesterol level of serum showed no apparent increase in this group.

Increase of beta-lipoprotein in serum might be responsible for further accumulation of cholesterol in the atheroma in this group.

Murakami reported that the post heparin clearing activities were decreased in the human hypertensive[63)]. In our present study, we could not confirm this fact. Besides the above-mentioned several factors, some local conditions in the arterial wall are concerned with the retardation of regression in the hypertensive state.

Cholesterol content of the aorta was markedly elevated in this group, whereas the amount of cholesterol in liver was not higher than that in the control group. This fact suggested a primacy of the local factor in promoting the atherosclerosis in this group.

The local effect of hypertension was mediated through vascular injury induced by increased pressure and turbulence of the blood.

Following vascular damage, mucopolysaccharides tend to be accumulated in the tissue. It has been well established that intimal deposit of acid mucopolysaccharides is a cardinal change of the early stage in atherosclerosis[64~66)]. Fisher demonstrated the greater amounts or increased polymerization of acid mucopolysaccharides in the aorta of the hypertensive cholesterolized rabbits[57)]. This accumulation of acid mucopolysaccharides was explained by the following mechanism: An increased pressure on the arterial wall decreases the drenching of the wall by way of constriction of vasa vasorum and then decreases the oxygen tension in the wall, especially in the intimal portion. On the other hand, high blood pressure increases the metabolic activity of the wall and demand of the wall for oxygen[67)68)].

Thus, relative shortage of oxygen takes place in the arterial wall, especially in the intima. Low oxygen tension in the tissue upsets the metabolism in the tissue and stimulates fibroblasts to form mucopolysaccharides[69)]. These mucopolysaccharides may play an important role in fat deposition in the wall. Faber and others considered these materials to form protein-complexes with lipoprotein and facilitate the accumulation of lipid[70)71)]. Although, as described just

above, it has been reported that hypertension increases mucopolysaccharides in the arterial wall and fosters intimal fibrosis, it was not the case with preestablished atheroma, in which lipid was already abundantly accumulated. Connective tissue proliferation in the atheroma was not enhanced, but inhibited in the hypertension group.

Lipids, especially cholesterol, were further increased in the preestablished atheroma in the hypertensive state, because of increased penetration of lipoprotein by intensified permeability of the wall or elevated pressure, fatty degeneration following enhanced metabolic disturbance in the wall and hemorrhage from newly formed capillaries in the atheroma, and disturbed disposal of lipid. Lipid substance thus accumulated may inhibit the activity of fibroblasts and impair their maturation. In consequence, fibrosis in the atheroma was possibly inhibited. As shown in Fig. 16, lipid droplets in the atheroma were conglomerated and septal structure of the atheroma was completely destroyed in the final stage. Prior demonstrated the inhibitory effect of cholesterol upon connective tissue repair in the atheroma in the rabbit[72]. Kendall suggested that removal of lipid in the plaque occurs presumably via lymphatic channels around vasa vasorum[3]. Hemorrhagic inflammation of the outer part of the wall, as illustrated in Fig. 17, might disturb this removal of lipid.

Relationship between essential fatty acids and atherosclerosis

The suggested relationship between essential fatty acids and atherosclerosis has been discussed by many investigators in recent years. Sinclair has attributed atherosclerosis to a deficiency of essential fatty acids[7]. Coronary atherosclerosis is not prevalent in the Latin peoples who ingest every day considerable amount of olive oil containing high percentage of essential fatty acids[73].

Kinsell reported a subjective and objective improvement of atherosclerosis in patients by polyunsaturated fatty acid administration[74]. It has been shown that the amount of arachidonic acid in serum cholesterol esters of different species has a correlation with susceptibility to atherosclerosis of the species. Rat and dog, that are known to be resistant to atherosclerosis, have high levels of arachidonic acid in serum cholesterol esters, whereas chicken, rabbit and man, known to be susceptible to the disease, have low levels of arachidonic acid[75].

Kleinsorge and Zielke noted lower serum cholesterol levels and less atherosclerosis in rats fed high-cholesterol diet either with linoleic acid and pyridoxine or with arachidonic acid[76].

Kritschevsky reported that corn oil feeding suppressed atherosclerosis in rabbits fed cholesterol[77]. In the present investigation, this serum cholesterol-lowering effect of essential fatty acid has not been confirmed. Linoleic acid feeding did not accelerate the return of serum cholesterol to the normal level. Lipoprotein index of animals in this group was not lower at the end of the experiment than in the control group.

Histologically, however, sudanophilic substance in the atheroma was much less in this group than in the control group. Cholesterol crystals, as observed

in sections from animsls of the control group, were not demonstrated in this group. The results of this study indicates that linoleic acid may promote the regression of atherosclerosis, acting directly on the arterial wall. The fatty deposits of the plaques are lipid mixtures, which consists of lipids which are liquid at body temperature (solvent) and those which are solid at this temperature (solute). As atherosclerosis progresses, the lipid complex in deposits loses solvent and increases solute (cholesterol).

There may be also a shift in the distribution of cholesterol from the esters into the less soluble free form. A change of the more soluble compound into the less soluble free form will induce crystallization of cholesterol. Cholesterol esters of saturated fatty acid has a higher melting point as compared with those of unsaturated fatty acid esters. These saturated fatty acid esters are considered to be less easily metabolized[78]. As a consequence, cholesterol esters of saturated fatty acids are more easily subjected to the crystalization than those of unsaturated fatty acids.

These crystals act as a foreign body and induced enhanced tissue reaction. Thus, atherosclerosis is augumented.

Concerning the relationship between fatty acid composition of the arterial wall and human atherosclerosis, the published data are at variance with one another. Some indicate that the percentage of polyunsaturated fatty acids in the cholesterol ester fatty acids of atheroma decreases with advance of atherosclerosis, and others assert its increase with atherosclerosis[79~81]. Experimentally, it has been reported in rabbits, chicken and cockerels that polyunsaturated fatty acids concentration of lipid from the atherosclerotic aorta was lower than those of the normal[82~84]. Per Bjoerntrop and Farquhar indicated the possibility that polyunsaturated fatty acid in a diet has an influence on the lipid composition of the atherosclerotic arterial lesion, raising the percentage of polyunsaturated fatty acids in cholesterol esters[85)86)]. The present study suggests the possibility that linoleic acid feeding increases cholesterol esters of linoleic acid in the atheroma and promote the disposal of cholesterol.

Linoleic acid might be concerned with colesterol metabolism by regulating production and degradation of this subtance in tissue.

David demonstated *in vitro* with tissue culture that polyunsaturated fatty acid inhibited intracellular deposition of cholesterol in the fibroblasts from human aorta[39].

Necrosis in the plaque was observed only in the linoleic acid feeding group. However, it will be up to future research to find out whether necrosis in the plaque is specifically due to dierect action of linoleic acid or incidental.

SUMMARY

Atherosclerotic lesions produced in rabbits fed on a lanolin diet for 4 months were capable of regression when the rabbits were taken off the regimen and fed with a normal diet.

Serum cholesterol levels were restored to normal within 4 weeks after cessation of lanolin feeding. Phospholipid content also returned to normal. Serum hexosamine levels were elevated for 8 weeks after cessation of lanolin

feeding, reaching maximum in the 3rd week. Post heparin clearing activity which was low at the cessation of lanolin feeding gradually returned normal.

A high protein diet did not exert influence upon either the course of regression of atherosclerosis or decline of cholesterol level.

A low protein intake retarded the decline of hypercholesteremia and inhibited the regression of atherosclerosis.

Renal hypertension also inhibited the regression of atherosclerosis, but had no influence on the rate of decline of hypercholesteremia.

Linoleic acid feeding did not accelerate the decline of hypercholesteremia, but promoted the resorption of lipid in the atheroma.

By lanolin refeeding an interesting microscopic finding in the atheroma was obtained.

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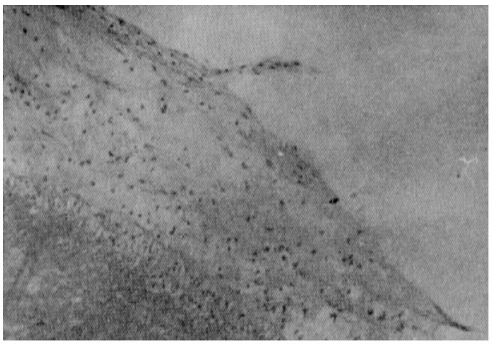

FIG. 4

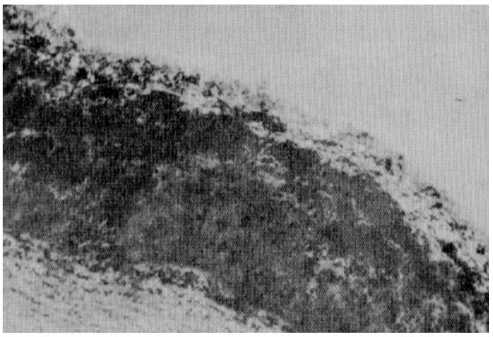

FIG. 5

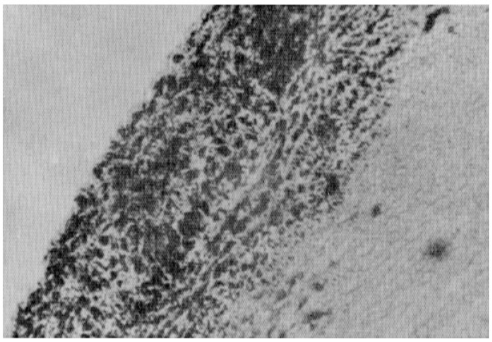

FIG. 6

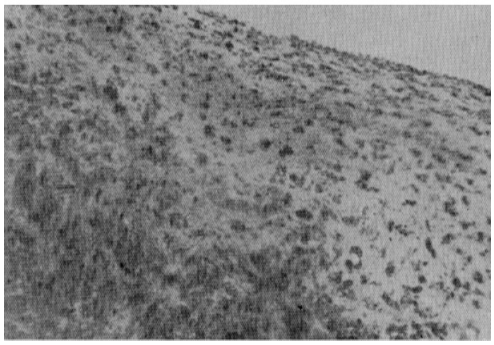

FIG. 7

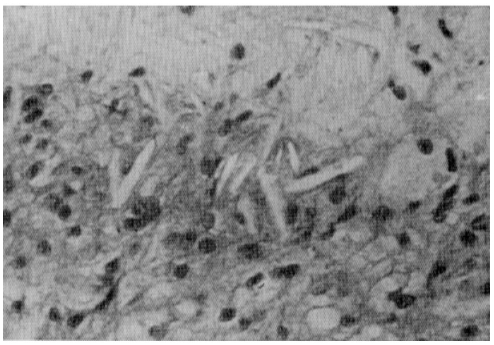

FIG. 8

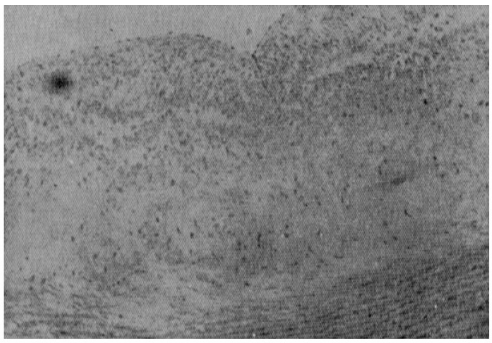

FIG. 9

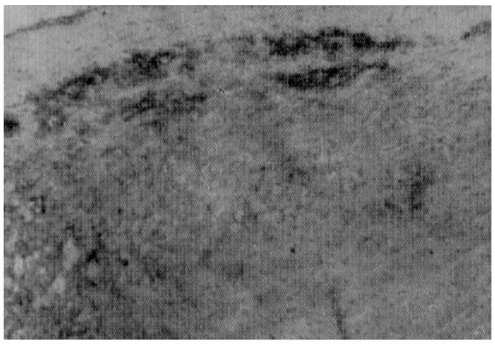

FIG. 10

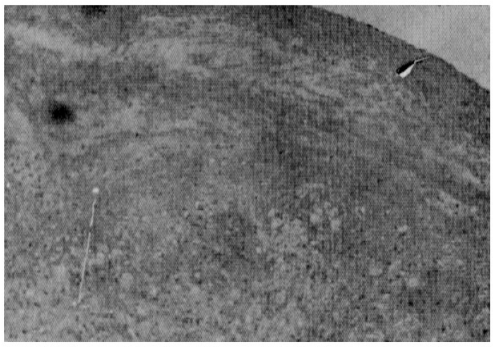

FIG. 11

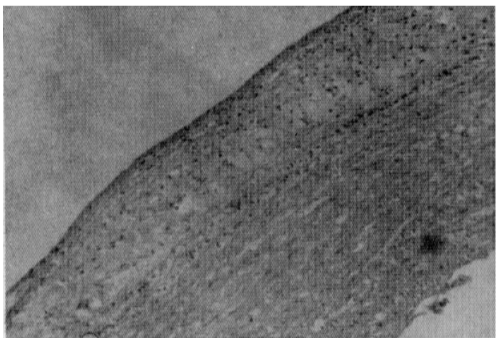

FIG. 12

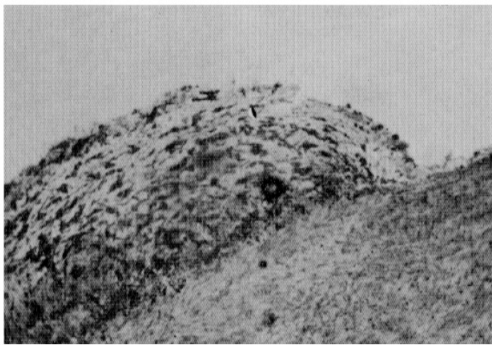

FIG. 13

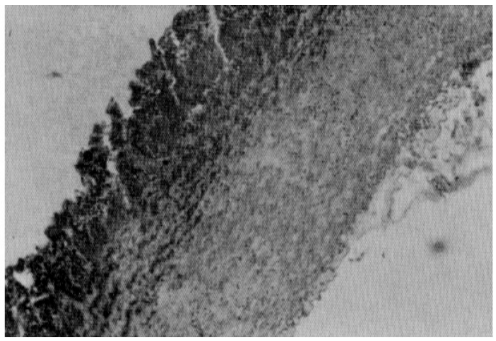

FIG. 14

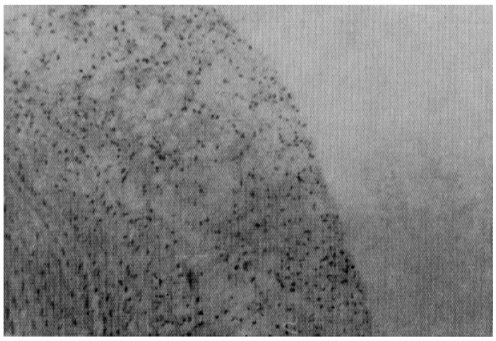

FIG. 15

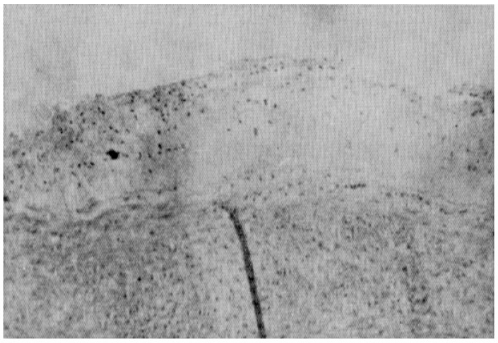

FIG. 16

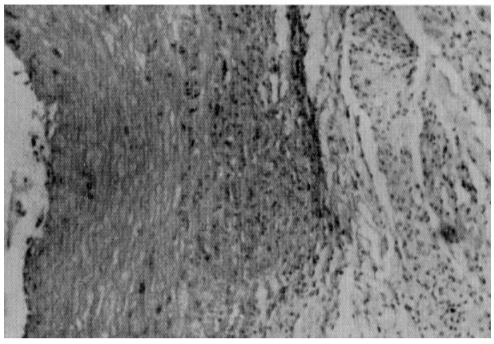

FIG. 17

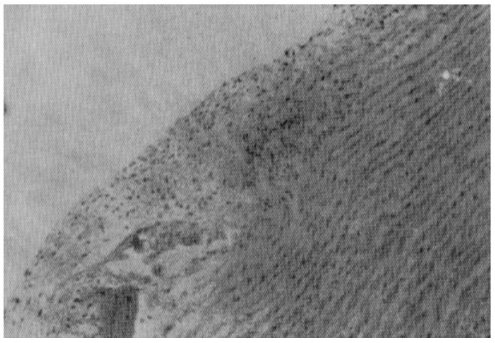

FIG. 18

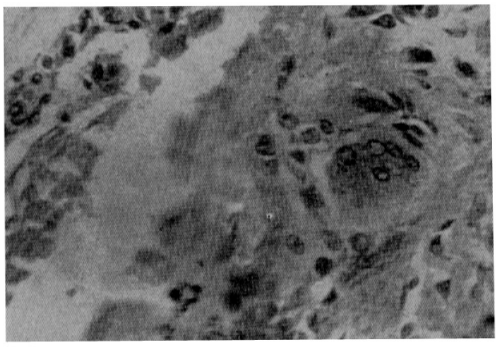

FIG. 19

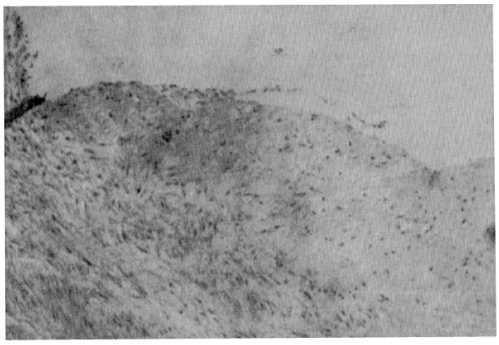

FIG. 20

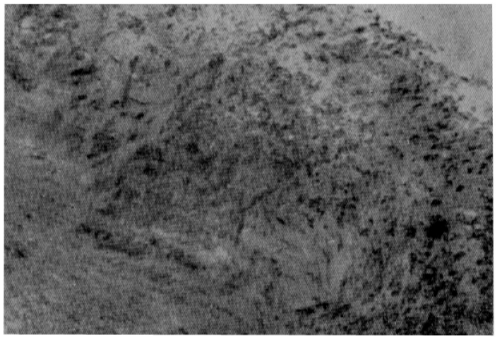

FIG. 21

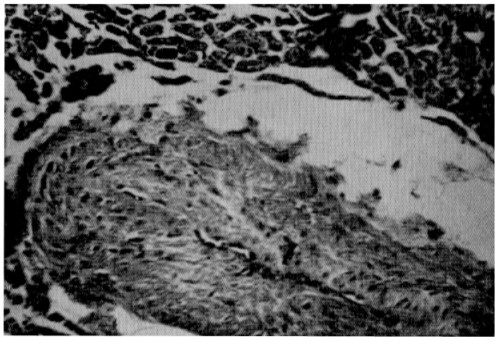

FIG. 22