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PEROXIDES AS A FACTOR OF ATHEROSCLEROSIS

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ABSTRACT

Fatty acid peroxides have been found in the atherosclerotic human aorta by Glavind. A correlation exists between the extent of the atherosclerotic changes and the content of lipid peroxides. It may be inferred that the presence of peroxides in the aorta brings about a disturbance in the function of the arterial wall and gives rise to atherosclerotic changes.

Vitamin E, which prevents the peroxidation of lipids, might suppress the atherogenesis. X-ray irradiation, which induces the auto-oxidation of lipids, might promote the process of the atherogenesis. From these considerations, the present investigation was performed. The determinations of peroxides were carried out on the rabbit aorta by the ammonium thiocyanate method. A correlation existed between the extent of the atherosclerotic changes and the content of lipid peroxides. The aortas with no atherosclerotic changes contained only small amount of peroxides. Peroxides of the rabbit aorta were determined by the method of TBA. Vitamin E injection prevented the lipid peroxidation in the aortas and livers. Furthermore, Vitamin E suppressed the atherosclerotic changes. Peroxides were produced by X-ray irradiation in the aortic wall and liver of the rabbit. Whole body X-ray irradiation promoted the progress of atherosclerotic changes in cholesterol fed rabbit, and produced atheroma-like plaque in the thoracic aorta of the rabbit.

The respiration of the aorta of rat was decreased by peroxides added in the diet. However, the respiration of liver was not influenced. The activity of cytochrome oxidase was more inhibited than that of succinic dehydrogenase. ***In vitro*** effect of peroxides on the respiration of the aorta of rats was also observed.

INTRODUCTION

There have been a variety of theories on the pathogenesis of the atherosclerotic disease. They are divided into two groups; the theories based on the arterial wall, and those based on the mechanical and chemical factors which influence the arterial wall. As seen in the report of Dahne[1], disturbance in arterial wall itself and deposition of substances in the wall which is a passive mechanism due to alteration in plasma composition are different processes. As a matter of course, one gives rise to the other and both processes always coexist.

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Glavind and others reported the presence of peroxidized lipid in the atherosclerotic aorta[2]. Lipid peroxides are produced from unsaturated fatty acids by autoxidation, oxidation by the atmospheric oxygen. Light, heat, oxygen, and iron are proxidants which increase the rate of oxidation by the atmospheric oxygen. Vitamin E prevents the autoxidation of unsaturated fatty acids. Ottolenghi and others[3] reported that certain oxidative enzymes were inhibited in direct proportion to the extent of lipid oxidation. E. D. Wills[4] reported that SH enzymes were found to be more readily inhibited than enzymes not possessing SH groups. Furthermore. peroxides have been demonstrated in certain animal tissue after X-ray irradiation[5].

If the lipid peroxides come to existence in the arterial wall, the activity of oxidative enzymes will be gradually disturbed in the long run, even if lipid peroxides are small in quantity. This may lead to the reduction of ATP formation in the arterial wall. Low energy production is thought to bring about a disturbance in the function of arterial wall. Namely, this low energy production may result in the reduction of the ability of handling the filtrate from serum with chemical or anatomical processes not to be understood by this day. This is thought to be the beginning of the atherosclerotic disease and the basic alteration of the arterial wall itself.

The author's presumption is that oxidative enzyme inhibition is the fundamental moment of the atherosclerotic changes and one thing that is overlooked by various theories on the pathogenesis of the atherosclerotic disease. All factors other than the inhibition of oxidative enzymes by peroxide-like substances are secondary factors for the atherosclerotic changes. The present paper mainly deals with the lipid peroxides as the etiological factor of the atherosclerotic disease from the point of view mentioned above. The studies were performed on the following experiments. 1) Cholesterol Feeding. 2) Vitamin E injection. 3) X-ray irradiation. 4) Influence of peroxides on the oxidative enzyme.

EXPERIMENTS

1) Cholesterol feeding

Material and method. White rabbits weighing about 2.3 kg were divided into three groups by the diet used and the period of feeding as follows: N group; fed on standard diet which was consisted of 350 g of bean extracted refuse, 15 g of bran and 200 g of cabbage. C-4 group; fed on standard diet and 2 g of cholesterol for 4 weeks. C-12 group; fed on standard diet and 2 g of cholesterol for 12 weeks.

N group was sacrificed after 4 weeks from the beginning of the feeding. C-4 group, and C-12 group were sacrificed after 4 weeks and 12 weeks from the beginning of the feeding. The entire thoracic aorta is removed immediately

after sacrifice, and were carefully freed of adventitia and adherent fat and then opened along the longitudinal axes to make a rectangular strip. These strips were rinsed quickly in distilled water, blotted and weighed.

The strips of aortas were observed macroscopically on the extent of atherosclerotic changes in order to examine whether a correlation would be demonstrable between the extent of atherosclerotic changes and the content of lipid peroxides.

The determinations of peroxides were carried out on the specimens of aortas. Peroxide value to 1 kg of fat and 1 kg of aortic tissue was estimated by the method of Hartman and Glavind[6]. All procedures were carried out under the nitrogen atmosphere wherever practicable and sheltered from light. The specimens were extracted with 16 ml of chloroform-methanol mixture (2: 1) for 48 hours. The filtrate was adjusted to 16 ml by adding the mixture and divided into two parts; the one part for total fat, the other for peroxides. Total fat in 8 ml of filtrate was determined by drying it at the constant temperature of 100°C. Fat per g tissue (mg) was calculated. Lipid peroxides were estimated on another 8 ml of filtrate. To the filtrate was added 2 ml of 30% ammonium thiocyanate in absolute methanol and then 0.05 ml of 0.92% $FeCl_2$ in the distilled water. The intensity of the red color was measured in the spectrophotometer at 520 mμ. The peroxide value (POV) was calculated as follows;

$$POV = 0.4145 \times E/G \quad (mE/kg)$$

E: Extinction found

G: Gram of fat or aortic tissue in 8 ml of filtrate.

Results. The wet weight of thoracic aortas, fat in 1 g of aortic tissue and POV to fat extracted from aortas and aortic tissue were shown in Table 1. All the strips of aortic specimens irrespective of the diet used and the period of feeding, were divided into four groups by the percentage of the atherosclerotic areas in the total aortic area; 0%, 1-30%, 31-60%, 61-100%. 0% group was further divided into three groups by feeding conditions which were described above; N group, C-4 group, and C-12 group. Finally the strips of aortas were divided into six groups as follows:

O-N group; no atheromatic change and fed on standard diet.

O-4 group; no atheromatic change and fed on standard diet and 2 g of cholesterol for 4 weeks.

O-12 group; no atheromatic change and fed on standard diet and 2 g of cholesterol for 12 weeks.

1-30% groug; the extent of atheromatic changes was from 1% to 30%.

31-60% group; the extent of atheromatic changes was from 31% to 60%.

61-100% group; the extent of atheromatic changes was from 61% to 100%.

TABLE 1. POV in Aorta of Rabbit

	Wet weight (mg)	F/g tissue (mg)	POV (mE/kg)	
			Fat	Tissue
0-N group (9)	245±69.5	25±10.9	1.4±0.7	0.05±0.03
0-4 group (5)	314±10.7	34± 6.7	2.0±1.5	0.06±0.04
0-12 group (4)	308±23.8	24± 3.1	2.0±0.1	0.05±0.01
1-30% group (4)	322±15.2	51±17.3	2.4±0.3	0.11±0.03
31-60% group (4)	340±22.1	55± 6.9	2.9±0.3	0.15±0.02
61-100% group (4)	360±40.3	79±16.8	3.8±0.6	0.29±0.13

Values: Mean±S.D. (Standard Deviation).
Numbers in parenthesis indicate numbers of case.

The wet weight of aortas and fat in 1 g of aortic tissue were increased according to the extent of the atherosclerotic changes. A correlation existed between the extent of atherosclerotic changes and the content of lipid peroxides. No obvious difference was found among the three groups with no gross atherosclerotic changes. But O-N group seemed a little lower in POV. One can see in the Table 1 that the aortas with no atherosclerotic changes contained a small amount of peroxides.

2) Vitamin E injection

Material and method. The white rabbits were divided into four groups as follows:

N group; fed on standard diet.

E group; fed on standard diet and injected intramuscularly 50 mg of vitamin E every other day.

E+C group; fed on standard diet and 2 g of cholesterol per day. Furthermore, 50 mg of vitamin E injected intramuscularly every other day.

C group; fed on standard diet and 2 g of cholesterol.

All groups were sacrificed 2 weeks and 12 weeks after the beginning of the feeding. The method of measurement was the same as in the experiment 1. Livers removed from the rabbits were also rinsed quickly in the distilled water and blotted, and blocks of liver of about 400 mg in weight were made, on which peroxides measurement was performed. Peroxides in serum was also detected. Peroxides measurements were performed under the nitrogen atmosphere and sheltered from light as strictly as possible by the modified method of TBA[7)8)].

The strips of aortas were examined on the extent of atherosclerotic changes. This was expressed as percentage of the area of atherosclerotic changes in the total aortic area. The specimens were homogenized in 4 ml of cold buffered medium prepared by mixing 100 ml of 0.154 M NaCl, 40 ml of 0.154 M KCl. and 12 ml of 0.1 M Na-K phosphate buffer at pH 7.0. 2 ml of 70% trichloracetic acid and 2 ml of 0.75% thiobarbituric acid (TBA) were added to the

whole volume of homogenate and 2 ml of serum. The tubes were placed in a boiling water bath for 10 minutes and then cooled. The tubes were centrifuged and the optical density was read spectrophotometrically at 525 mμ. Peroxides were expressed as extinction per gram tissue of the aorta and the liver and as extinction per ml of serum (Ext/g, Ext/ml).

Results. The extent of atherosclerotic changes, the wet weight of the aorta and TBA value of the aorta, liver and serum were shown in Table 2.

After 2 weeks of the feeding: In all groups were observed to have no atheromatic change. TBA values of the aotas in C group and E+C group were higher than that in E group and N group. No great difference was observed between the TBA value in N group and that in E group. In the comparison of the TBA value between C group and E+C group, the TBA value in C group was higher than that in E+C group. Its difference was statistically significant ($p<0.01$). The liver showed the same tendency as the aorta. However, no great difference was seen among E+C group, N group and E group. TBA value of serum also revealed no difference among the four groups.

After 12 weeks of the feeding: The extent of atherosclerotic changes were 11% in C group and 4% in E+C group. Its difference was statistically significant ($p<0.01$). However, no atherosclerotic changes were observed in N group and E group. TBA values of the aorta in C group and E+C group were higher than those in E group and N group. Furthermore, TBA value in C group was higher than that in E+C group. But the difference was statistically not significant. There was no difference between the TBA values in E group and in N group. As to the livers, the TBA value in C group was higher than those in the other three groups, among which the TBA values showed no great difference. In serum, TBA value showed no difference among

TABLE 2. The Extension of Atheroma and TBA Value Influenced by Vitamin E Injection

	Group	Per cent of atheroma (%)	Wet weight of Aorta (mg)	TBA Value		
				A (Ext/g)	L (Ext/g)	S (Ext/ml)
2 Weeks	C group (5)	0	365± 86.4	0.376±0.03	0.285±0.08	0.014±0.0004
	C+E group (5)	0	283± 32.9	0.282±0.02	0.235±0.05	0.013±0.0005
	E group (5)	0	288± 58.2	0.240±0.06	0.220±0.03	0.013±0.0002
	N group (5)	0	356± 87.2	0.252±0.06	0.231±0.04	0.014±0.0001
12 Weeks	C group (4)	11+2.4	414±143.8	0.432±0.04	0.348±0.06	0.013±0.0008
	C+E group (4)	4±1.0	405±111.1	0.412±0.03	0.248±0.06	0.013±0.0001
	E group (4)	0	390± 74.2	0.252±0.03	0.216±0.05	0.014±0.0008
	N group (4)	0	415± 78.5	0.261±0.03	0.226±0.08	0.013±0.0005

Values: Mean±S.D. Numbers in parenthesis indicate numbers of case.
Ext/g: Extinction per Gram. Ext/ml: Extinction per ml. A: Aorta
L: Liver S: Serum.

the four groups.

3) *X-ray irradiation*

Material and method. White rabbits weighing about 2.3 kg were divided into four groups as follows:

N group; fed on a standard diet.

X group; fed on a standard diet and irradiated with X-ray (600 r three times at three-week intervals beginning on the 45th day of the experimental period).

C group; fed on a standard diet and 2 g of cholesterol.

C+X group, fed on a standard diet and 2 g of cholesterol. Furthermore, irradiated with X-ray in the same manner as X group.

X-ray was irradiated over the total body. Total dose of X-ray was 1 800 r. On the 90th day of the experiment, all rabbits were sacrificed after an overnight fast. The determinations of peroxides were carried out on the aortic wall, the liver and the serum by the method of TBA mentioned in the experiment of vitamin E injection. For the histological study, a fragment of aorta was taken from the ascending portion, fixed in 10% formalin and embedded in paraffin. The sections were stained with hematoxylin and eosin and Sudan III.

Results. The number of white blood cells was reduced to 2 000–3 000 by the irradiation of 600 r. 2 weeks were needed for recovery to the normal level of white blood cell. Losing of hair from the back was also observed.

The extent of the atherosclerotic changes, the wet weight of the aortas and TBA value of the aortas, the livers and the serum were shown in Table 3. TBA value of the serum was almost equal in all of the four groups. The extent of atherosclerotic changes was 1% in X group, 5% in C group and 17% in C+X group. The difference between C group and C+X group was statistically significant ($p<0.05$). TBA value was highest in X group, which was followed by X+C group and C group. The difference between TBA value in X+C group and that in C group was statistically significant ($p<0.05$). TBA value of the liver showed the same tendency as the aorta; highest in X group,

TABLE 3. The Extension of Atheroma and TBA value Influenced with X-ray Irradiation

	Per cent of Atheroma (%)	Wet weight of Aorta (mg)	TBA Value		
			A (Ext/g)	L (Ext/g)	S (Ext/ml)
X group (5)	1±1.7	238±27.8	0.812±0.08	0.628±0.13	0.014±0.0009
X+C group (5)	17±7.5	343±16.1	0.626±0.05	0.589±0.07	0.014±0.0007
C group (5)	5±3.2	339±55.7	0.344±0.03	0.485±0.06	0.013±0.0008
N group (5)	0	240±24.7	0.270±0.01	0.246±0.01	0.013±0.0004

Values: Mean±S.D.

Numbers in parenthesis indicate numbers of case.

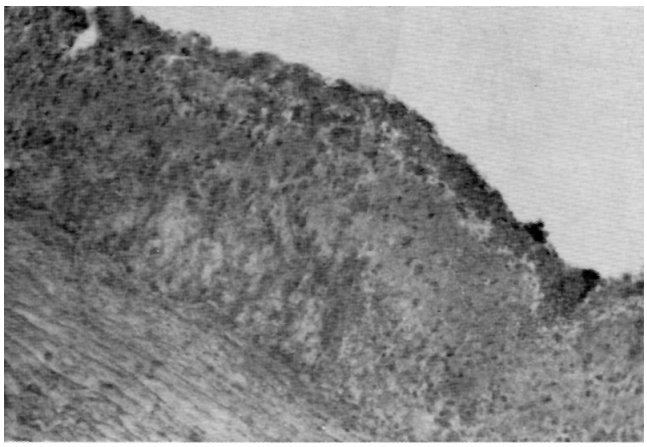

FIG. 1. Sudan III Staining.

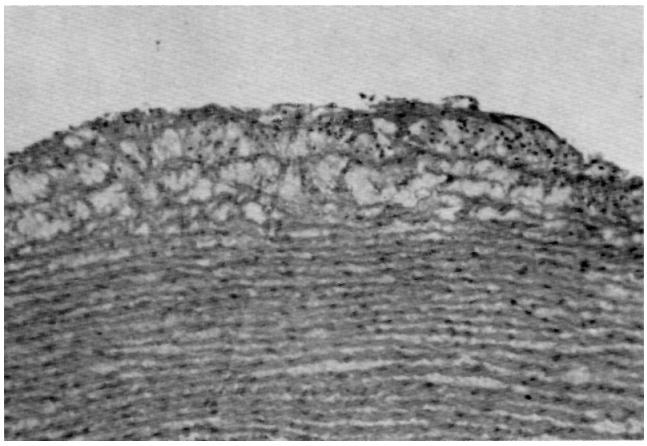

FIG. 2. Hematoxylin and Eosin Staining.

second in X+C group, and third in C group. As a whole, TBA value in the livers was lower than that in the aorta. Histological study revealed the Sudan III positive substances in the subendothelial layer of X group, as shown in Fig. 1. The section stained with hematoxylin and eosin indicated the elevation of endothelium, as shown in Fig. 2.

4) Influeuce on oxidative enzymes

Materials and method. Male rats weighing 120 g were used as experimental animals. They were divided into three groups as follows:

P group; fed on stock diet and 1 ml of oxidized fish oil per day (POV: 500 mE/kg).

F group; fed on stock diet and 1 ml of non-oxidized fish oil per day (POV: 15 mE/kg).

N group; fed on stock diet.

All rats were fed for 12 weeks. Rats were sacrificed by brain concussion. Immediately after removal from the animal, the entire thoracic aorta was cut free from the heart and abdominal aorta, carefully freed of adherent fat and connective tissue, and then opened along the longitudinal axes so as to provide a strip. Two strips from the same group were paired for the convenience of experiment. The wet weight of aortas was weighed with a torsion balance. Studies were carried out on the following articles; respiration of aorta using glucose and succinate as substrate, respiration of liver homogenate using glucose as substrate, succinic dehydrogenase activity of aorta, cytochrome oxidase activity of aorta, and the *in vitro* effect of oxidized methyl esters of unsaturated fatty acids on respiration of aorta.

Respiration of aorta: The specimens of aortas were transferred to the Warburg flasks. Constituents were as follows:

(1) Central well; 0.2 ml 5 M KOH
(2) Main vessel; Krebs-Ringer-Phosphate Buffer 2.7 ml
0.2 % glucose 0.1 ml or sodium succinate 0.08 mM
pH 7.4

The flasks were placed in the Warburg apparatus which had been maintained at constant temperature of 38°C in the atmospheric air. Fifteen minutes were needed for temperature equilibration. Manometer reedings were made at fifteen minutes intervals.

Succinic dehydrogenase activity of aorta: The manometric method of Slater, as described by Malinow[9], was adapted as follows:

(1) Central well; 0.2 ml 5 M KOH
(2) Main vessel; 0.1 M KCN (0.1 mM)
1/20 M TRIS (0.025 mM)
(3) Lateral Vessel; Methylene Blue 0.003 mM
sodium succinate 0.08 mM

Total volume 3.0 ml pH 7.4

Cytochrome oxidase activity of aorta: The method of Schneider and Potter[10] was adapted as follows:

(1) Central well; 0.2 ml 5 M KOH
(2) Main vessel; 1/20 M TRIS (0.025 mM)
(3) Lateral vessel; cytochrome C 3 mg
ascorbic acid 0.03 mM

Total volume 3.0 ml pH 7.4

Tipping was performed after ten minutes of equilibration. Readings were made every 15 minutes for one hour, 38°C and with air as gas phase. Results

were corrected for endogenous respiration.

In vitro effect of oxidized methyl esters of unsaturated fatty acids on respiration of thoracic aorta: In order to investigate the influence of oxidized methyl esters of unsaturated fatty acids on respiration of thoracic aorta, the following experiments were done with Warburg manometer in the same manner as respiration of aorta. Constituents placed in Warburg flask were as follows:

(1) Central well; 0.2 ml 5 M KOH

(2) Main vessel; Krebs-Ringer-Phosphate Buffer 2.1 ml
sodium succinate 0.08 mM
pH 7.4
Furthermore, in P case, 0.5 ml of 0.18% methyl ester of unsaturated fatty acids (POV: 1 400 mE/kg) in 1% alcohol solution was added to main vessel. In F case, non oxidized methyl esters of unsaturated fatty acids were added to main vessel. In N case as normal, 0.5 ml of 1% alcohol solution was added to main vessel.

Respiration of liver homogenate: Liver was homogenized. Its concentration was 10% of liver in Krebs-Ringer-Phosphate Buffer at pH 7.4. The homogenate was transferred to the Warburg flasks, and its respiration was measured as on the aorta.

Constituents were as follows:

(1) Central well; 0 2 ml 5 M KOH

(2) Main vessel; 10% liver homogenate of Krebs-Ringer-Phosphate Buffer 2.0 ml
2% glucose 0.1 ml
pH 7.4

Results. Respiration of thoracic aorta (Substrate; Glucose):

The oxygen consumptions in μl per 100 mg of wet weight of aorta per hour were shown in Table 4. The oxygen consumption of aorta in P group was much lower than that in F group and N group. The difference between

TABLE 4. Respiration of Thoracic Aorta of Rat
(Substrate: Glucose)
Oxygen Consumption in μl per 100 mg of Wet Weight of Tissue in 60 min

	P group (5)	F group (5)	N group (5)
O_2 Consumption	7.02±2.2	18.12±7.3	20.03±6.1

Values: Mean±S.D.
Nunbers in parenthesis indicate numbers of case.

N group and P group was statistically significant ($p<0.05$). No significant difference between the oxygen consumption in F group and that in N group was observed during 60 minutes.

Respiration of thoracic aorta (Substrate; Succinate):

The oxygen consumptions in μl per 100 mg of wet weight of aorta were shown in Table 5. The oxygen consumption in 120 minutes in P group was the lowest among the three groups. However, the control group F showed lower oxygen consumption than that in N group.

Succinic dehydrogenase activity of thoracic aorta:

The activity of succinic dehydrogenase of thoracic aorta was expressed as oxygen consumption/hr/100 mg of wet weight. The oxygen consumptions of aorta in one hour were shown in Table 6. The oxygen consumption in P group is slightly lower than that in F group and in N group. However, no great difference in consumption of oxygen was observed between P group and that in F or N group.

Cytochrome oxidase activity of thoracic aorta:

The activity of cytochrome oxidase of thoracic aorta was expressed as oxygen consumption/hr/100 mg of wet weight. The oxygen consumption of aorta in one hour were shown in Table 7. The oxygen consumption in P group was much lower than that in F group or in N group. The difference in the oxygen consumption between P group and N group was statistically significant ($p<0.05$). With the comparison between the oxygen consumption in F group and that in N group, no significant difference was observed.

In vitro effect of oxidized methyl esters of unsaturated fatty acids on

TABLE 5. Respiration of Thoracic Aorta of Rat
(Substrate: Succinate)
Oxygen Consumption in μl per 100 mg of Wet Weight of Tissue in 60 min

	P group (3)	F group (3)	N group (3)
O_2 Consumption	76.1±11.8	92.4±5.2	121.4±32.0

Values: Mean±S.D.
Numbers in parenthesis indicate numbers of case.

TABLE 6. Succinic Dehydrogenase Activity of Thoracic Aorta of Rat
Activity Expressed as Oxygen Consumed/hr/100 mg of Wet Weight

	P group (4)	F group (4)	N group (4)
O_2 Consumption	51.5±6.0	53.7±6.7	58.8±5.2

Values: Mean±S.D.
Numbers in parenthesis indicate numbers of case.

TABLE 7. Cytochrome Oxidase Activity of Thoracic Aorta of Rat
Activity Expressed as Oxygen Consumed/hr/100 mg of Wet Weight

	P group (5)	F group (5)	N group (5)
O_2 Consumption	135.8±19.5	173.9±4.7	167.4±13.4

Values: Mean±S.D.
Numbers in parenthesis indicate numbers of case.

TABLE 8. *In vitro* Effect of Oxidized Unsaturated Fatty Acids on Respiration of Thoracic Aorta of Rat
(Substrate: Succinate)
Activity Expressed as Oxygen Consumed/hr/100 mg of Wet Weight

	P case (4)	F case (4)	N case (4)
O_2 Consumption	105.8±3.1	147.8±20.2	154.0±9.7

Values: Mean±S.D.
Numbers in parenthesis indicate numbers of case.

TABLE 9. Respiration of Liver Homogenate
(Substrate: Glucose)
Oxygen Consumption in μl per 100 mg of Wet Weight of Tissue in 60 min

	P group (5)	F group (5)	N group (5)
O_2 Consumption	41.5±6.4	39.3±8.0	37.6±8.1

Values: Mean±S.D.
Numbers in parenthesis indicate numbers of case.

respiration of thoracic aorta:

The oxygen consumptions in one hour were recorded in Table 8. The oxygen consumption in P group was lower than that in F group or in N group. The difference in the oxygen consumption between P group and that in F group was statistically significant ($p<0.05$). No obvious difference in the oxygen consumption between F group and N group was observed.

Respiration of liver homogenate (Substrate; Glucose)

The oxygen consumptions in μl/100 mg of wet weight of liver were shown in Table 9. The oxygen consumptions for 60 minutes indicated no obvious difference in the respiration of aorta.

DISCUSSION

Cholesterol feeding. The question was recently arisen if the formation of lipid peroxides has any etiological significance in the development of the

atherosclerotic changes. Glavind and others[2] reported that a correlation was found between the severity of the atherosclerotic changes and the peroxides content of the lipid in the aorta. They thought that the peroxidation should be secondary to deposition of lipid as the normal aorta did not contain peroxides. However, it is by no means unlikely that fatty acid peroxides are naturally formed in a small quantity from unsaturated fatty acids in animal body, and they play a certain role in the metabolism since the peroxides have biological nature to inhibit oxidative enzymes. As shown in the present experiment, lipid peroxides were detected in small amounts in the lipid of the aortas of rabbits in which no visible atherosclerotic changes were observed. It seemed to be possible that lipid peroxides had a role in inhibiting oxidative enzymes in the aorta at the earliest stage of atherosclerosis when no gross changes were observed. If the deposition of lipid started in the aortic wall and resulted in atherosclerotic lesions, peroxidation of lipid in the atheroma would be accelerated by the chain reaction. It was thought to be the result of peroxidation of lipid in the aortic wall that peroxide value of lipid in the aorta and that per wet weight of the aortic tissue increased in accordance with the extent of the atherosclerotic changes. In the comparison between peroxides value of lipid in the aorta and that of the aortic tissue, the increase in the latter was more rapid than that in the former. One may speculate that the development of the atherosclerotic changes is strongly influenced by the secondary chemical or mechanical factors after the primary deposition of lipid has taken place in the aortic wall with the basic alteration of the aorta itself or the disturbance of oxidative enzymes. In the case of cholesterol feeding, the secondary factor is hypercholesteremia.

Vitamin E injection. TBA value in the aorta and the liver was higher in C group than in C+E group after 2 weeks from the beginning of the vitamin E injection. Zalkin and Tappel[11] reported that lipid peroxidation was found *in vivo* in the tissue of vitamin E deficient rabbit. They also reported in another paper[12] that added tocopherol was effective in inhibiting lipid peroxidation in isolated mitochondria. Dam and Granados[13] have proposed that the fundamental role of vitamin E involves inhibition of unsaturated fat autoxidation. The present experiment suggests that lipid peroxidation in the aorta and the livers is inhibited by injected vitamin E. Twelve weeks after the beginning of the vitamin E injection, the atherosclerotic changes were observed in both C group and C+E group, but the extent of the changes was lesser in the latter than in the former. From this fact, vitamin E is thought to protect against the basic alteration of aorta itself caused by the lipid peroxidation which is considered to affect oxidative enzymes in the aortic wall. It is logical to believe that the difference of peroxides content between C group and C+E group gave rise to the difference in the extent of the

atherosclerotic changes, even if the secondary factors to influence the aorta were alike. The inhibitory action of vitamin E on lipid peroxidation was more active in the liver than in the aorta, which was clearly shown after 12 week injection of vitamin E. The serum TBA value was low and show no difference among the four groups. The present experiment suggests that the strength of neutralization of peroxides and the anti-oxidant ability were different among various kinds of tissues.

X-ray irradiation. When doses of irradiation are used, there may occur oxidation of double bond of fatty acids in the organism and reactions which lead to the complete destruction of the molecules. Recent investigation have focussed attention on peroxide as possible intermediates in the production of biological irradiation damage. It has been shown that peroxides give effects similar to those caused by irradiation to some bacteria and bacteriophages, and that peroxides of fatty acids administered to animal cause toxic effect which,, in many respects, closely resemble to those caused by irradiation[14]. Stuart Lindsay and others[15] reported that arteriosclerosis was developed at the site of X-ray irradiation. According to their report, X-ray irradiation may selectively cause injury to the internal elastic membrane and this degenerative phenomenon is followed by the development of intimal fibrosis and plaque formation. The present experiment of X-ray irradiation to the total body caused deposition of lipid in the subendothelial layer of the aorta and formation of atheroma in rabbits. As shown in Table 3, TBA value of the aorta was highest in X group, in which, however, the extent of atherosclerotic changes was 1% of the total aortic area. The atherosclerotic changes was severer in X+C group than in C group. In X group, the initial alteration of the aorta itself by the peroxides produced by X-ray irradiation might be the strongest among the three groups, but, the secondary factors to influence the aortic wall was small. This suggests that the development of the atherosclerotic changes is greatly influenced by the factors to affect the aorta secondarily, hypercholesterolemia in this case. In X+C group, two of the factors for development of atherosclerotic changes coexisted in the aortic wall; one is the peroxides which was thought to induce the basic alteration of the aortic wall itself or the inhibition of oxidative enzymes, the other is hypercholesterolemia to affect the aortic wall as a secondary chemical factor.

A similar tendency was seen in the liver. In liver, X-ray irradiation was considered to promote the production of lipid peroxides. In the serum, TBA value indicated no difference among the four groups; N group, X group, X+C group and C group. This may suggest that blood is strongly protected from the toxic effect of X-ray irradiation. Albert A. Barber[16] reported that lipid peroxide formation in incubated rat brain homogenate was inhibited by any of the vertebrate serum tested. In the experiments with vitamin E injection

and with X-ray irradiation, TBA value of the serum was nearly constant and very low. Moreover, TBA value of the livers was lower than that of the aorta. It is thought from these facts that the ability to protect oxidative enzymes from action of the lipid peroxides varied with the kind of tissue.

Influence on oxidative enzymes. It has been known for about twenty years that the arterial wall is a metabolically active organ[17]. The rate of glycolysis of aortic tissue is comparatively high, and most of its metabolic energy is probably derived from carbohydrate. In contrast to most tissues, however, the presence of oxygen causes only a relatively small decrease of pyruvate or lactate production from glucose. In the aorta only about 20–30% of the glucose seems to be utilized in oxidation, even in the presence of oxygen. It is, however, important to realize that fatty acids and amino acids are also oxidized via the citric acid cycle. The inner layer of the arterial wall is relatively avascular and has a low oxygen supply. The energy metabolism of the artery is much less favourable than that of most organs, and a failure of such a poor production of energy could have, under some circumstances, deleterious effects upon the balance between health and disease. Human arteriosclerotic or aging aortic wall have been extensively assayed for enzyme activities and the deficiency of the following enzymes was uncovered: glyoxalase[18], succino oxidase[19], cytochrome oxidase[19], and so forth. Shock[20] reported that with an increase in age, there was a significant reduction in basal heat production and carbon dioxide elimination and that there was a significant increase in pO_2 and a decrease in pCO_2 in expired air. In the present investigation on respiration of the aorta of rat, it was shown that the oxygen consumption in P group was lower than that in N group and F group. This was thought that oxidative enzymes in the aortic wall of rat were influenced by the peroxides added in the diet. The difference between the respiration using glucose as substrate and that using succinate as substrate was not clearly understood, but may suggest that succinoxidase was influenced by non-oxidized fatty acid. In the case with glucose as substrate, the oxygen may be consumed in a place or places in TCA cycle and H_2 transport system other than succinoxidase system. These may be the reason why the oxygen consumption in F group show no difference from N group in the experiment using glucose as substrate. Wills[4] reported that succinoxidase and cytochrome oxidase were inhibited to a greater extent by unsaturated fatty acids than saturated fatty acids. The inhibition of enzymes of this group by unsaturated fatty acids is likely to be a combined effect of two distinct conditions, one is combination of negative charged fatty acids with the basic protein groups, and the other presence of unsaturated bonds in the fatty acids. Inhibition of the enzymes by unsaturated fatty acids may be explained on the basis that it is these peroxides which are responsible.

The present experiment showed that the activity of succinic dehydrogenase

was not markedly suppressed by peroxide feeding and that cytochrome oxidase activity was depressed by peroxide feeding. The activity of succinic dehydrogenase was thought to be inhibited by peroxide, as the succinic dehydrogenase contained -SH group in the molecule. Cytochrome oxidase is normally not regarded as an -SH enzyme. Wills[21] reported that some inhibition occurred even when -SH groups were blocked and this fact, coupled with observations on the inhibition of some non-SH enzymes, indicated that changes other than simple oxidation of -SH to -S-S- should take place. Bernheim and others[22] also reported that incubation of washed tissue suspension with ascorbic acid inactivated a number of enzymes including succinoxidase, cytochrome oxidase, and choline oxidase with a concomitant increase of TBA value. Tappel and Zalkin[23] found that liver mitochondria took up oxygen in the absence of added substrate and that this oxygen uptake resulted in the formation of lipid peroxide. During formation of these peroxides there was a concomitant destruction of some of the mitochondrial enzymes, *e.g.* succinic oxidase and DPNH-cytochrome c reductase. In general, the -SH group of enzymes is likely to be vital targets for peroxide inhibition on account of (a) the nature of the enzymes inhibited and (b) the fact that -SH compounds such as cysteine or glutathione are protective. It is considered that the fact that several of the enzymes are oxidative is incidental. The inhibition of cytochrome oxidase in the present experiment was considered to be due to some action of peroxides other than simple oxidation of -SH to -S-S-. The inhibition of succinic dehydrogenase activity would be observed in another stage.

In liver, the oxygen consumption did not indicate any difference among three groups. Tissue of aorta may be influenced more easily than that of the liver by the peroxide added in the diet. This difference may depend on the level of metabolism in the aorta and in the liver. The oxidation in the liver is more active than that in the aorta. However, the respiration of the liver may be also influenced by the peroxides added in the diet in a very long time. Considering from the *in vitro* effect of oxidized methyl esters of unsaturated fatty acids on the respiration of the aorta, peroxides should inhibit directly the oxidative enzymes in the aorta. Through the present experiments the true role of the inhibitory effect of peroxides of fatty acids *in vivo* is not easy to be established, but nevertheless, it is possible that this may be of considerable importance in metabolic control. These peroxides may then slow a vital metabolic path by inhibiting oxidative enzymes, *e.g.* cytochrome oxidase and the respiration of tissue. Located in important membranes around or within the cell, probably as constituents of phosphatides, it appears possible that unsaturated fatty acids may be well sited to exert an important metabolic control. Christie and Dahl[24] reported that as a rat aged the respiration of abdominal aorta fell more sharply than that of the thoracic aorta. This result may provide an explanation for the relatively high incidence of atherosclerosis

in abdominal aorta. And it was very interesting that with aging the activity of cytochrome oxidase was more decreased than that of succinic dehydrogenase in the kidney of rat[25]. Wertheimer and other[26] reported that the oxygen and glucose uptake by rat aortas decreased with aging of the animals.

These lipid peroxides which are more or less concerned with aging slow down the production of energy in the arterial wall. This disorder of artery leads to the reduction of ability to control the factors to affect the artery secondarily. As revealed in the present experiment of X-ray irradiation, peroxides are considered to accelerate the atherosclerotic changes. Vitamin E prevents the progress of atherosclerotic changes. The failure of a energy supply caused by inhibition of oxidative enzymes should take place in the arterial wall before the onset of deposition of lipid. This alteration of artery itself is considered to be fundamental pathogenesis of the atherosclerosis.

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