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SH COMPOUNDS AND ATHEROSCLEROSIS

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ABSTRACT

Fatty acid peroxides have been found in the atherosclerotic human aorta. Glavind found a correlation between the extent of the atherosclerotic changes and the content of lipid peroxides. These lipid peroxides are very toxic to the tissues. There are several compounds in the tissues to counteract the toxicity of peroxides *in vivo, i.e.* SH compounds, ascorbic acid, or tocopherol.

It may be inferred that an increase of peroxides and a decrease of SH compounds occur in the experimental atherosclerotic aorta and that the administration of such reducing substances as ascorbic acid or SH compounds reactivates the respiration of the aorta inhibited by peroxides and retards the atherogenesis. It may be also supposed that a decrease of SH compounds occurs in the human atherosclerotic aorta. From these considerations the present investigations were performed. In the rabbits fed cholesterol, a decrease of nonprotein SH content and an increase of TBA reactive substances were observed in the aorta at the stage when the reduction of respiratory activity was not revealed. Furthermore, a decrease of total SH content was observed in the serum, while total SH, nonprotein SH contents and SDH activity were normal in the liver. In the rabbits fed cholesterol with concomitant administration of ascorbic acid or SH compounds, the grade of atherosclerosis and the abnormality of total cholesterol and total SH contents were improved. The development of atherosclerosis was retarded as compared with the control, while the abnormality of lipids in quantity and of fatty acid composition was not improved.

Nonprotein SH content in the human atherosclerotic abdominal aorta decreased as compared with the normal aorta.

From these results, it may be concluded on atherogenesis that a damage of protein induced by nonenzymatic peroxidizing lipid-protein reaction exists in the serum and aorta.

INTRODUCTION

There have been only a few reports concerned with the relationship of lipid peroxides to atherosclerosis. In 1952 Glavind and others[1] first found that the atherosclerotic human aortae contained lipoperoxides and the content of the peroxides paralleled with the degree of severity of the atherosclerosis, while the normal aortae were free from lipoperoxides. Fukuzumi[2] and Woodford[3] also determined lipid peroxides in the lipid extract of the

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atherosclerotic human aorta. Lipid peroxides are produced from unsaturated fatty acid by free radical chain reaction involved in autoxidation.

On the other hand, Burt[4] found that the incidence of acid-fast pigment (ceroid) in the human aortic atherosclerosis was directly proportional to the degree of initial thickening. By Hartroft's investigation[5] abundant deposits of acid-fast and sudanophilic pigment resembling ceroid could also be demonstrated in every lesion of the human aortae which exhibited atheroma of advanced stages. The ceroid pigment was believed to be formed by autoxidation of unsaturated fat *in vitro*[6].

It was recently demonstrated by Fukuzumi[7] that the atherosclerotic human aorta contained lipid-protein complexes, in which oxidized lipids containing transisomers existed. *In vitro*, the hydroperoxide of methyl linoleate extensively denatured the isolated human low density lipoproteins, and an initial or primary association of hydroperoxide of methyl linoleate with low density lipoproteins was noted prior to its denaturation[8]. Therefore, if lipids of lipoproteins are autoxidized *in vivo*, the lipoprotein might be denatured to be hardly metabolized and accumulated in the arterial tissue. Kayahan[9] claimed that denatured proteins in the intima of the human atherosclerotic subjects was responsible for an increase in lipid-binding capacity of the aorta.

These lipid peroxides are very toxic to the tissue. Bernheim and others[10] showed that fatty acid peroxides inhibited some oxidative enzymes such as succinic dehydrogenase. Tappel[11] has reported that lipoperoxides formed in rat liver *in vitro* or after feeding tocopherol-deficient diet destroy the cell membrane system or the electron transfer system and that this leads to cellular damage consequently.

Therefore, if the lipid peroxides come to existence in the arterial tissue, they might disturb the tissue metabolism in the long run, even if small in quantity. No experimental atherosclerosis has yet been developed by feeding lipid peroxides for a long period. So it remains unexplained up to now that lipid peroxides may be a atherogenic factor.

There are several compounds in the organism which counteract the toxicity of peroxides *in vivo*, *i.e.* SH compounds, tocopherol, ascorbic acid, or quinone. Oya[12] found that total tocopherol content per total lipids in the rabbit aorta after cholesterol feeding decreased as compared with the normal rabbit. He also found, *in vitro*, that tissue respiration of the rat aorta was reduced by addition of lipid peroxides, and this reduction of the respiration could be protected by addition of tocopherol. Iwakami[13] showed that on the aorta of cholesterol- and tocopherol-fed rabbit, TBA value (lipoperoxides content) was significantly lower at the second week and the development of atherosclerosis was significantly less at the 12th week as compared with the cholesterol-fed rabbit. Dubouloz and Fondarai[14] found that ethyl oleate peroxide was capable

of oxidizing the -SH groups of proteins to -S-S- and to further oxidation products. In general, -SH enzymes, for example succinic dehydrogenase, were found to be more readily inhibited than enzymes not posessing -SH groups and protected by sulfhydryl compounds such as glutathione or cysteine[15]. SH compounds is water-soluble and its action as antioxidant is different from tocopherol. However, it was assumed from the point of view mentioned above that an increase of peroxides and a decrease of SH compounds might occur in experimental atherosclerosis and that the administration of the reducing substances such as ascorbic acid or SH compounds might reactivate the respiration of the aorta inhibited by peroxides and retard the atherogenesis. It was also supposed that a decrease of SH compounds might occur in the human atherosclerotic aorta. There is no certain method to determine an extremely small amount of lipoperoxide in the organism. Therefore, it is considered to be necessary that the observation of antioxidants, especially SH compounds, of which determination may be certain, is performed together with the observation of lipoperoxides in the organism. The present investigations consisted of three parts. Experiment 1, was performed to determine the changes of peroxides and SH compounds in the tissues of cholesterol-fed rabbits and Experiment 2, to determine the change of nonprotein SH in the human atherosclerotic aortae. Experiment 3 was done to study the effect of administration of some antioxidants such as ascorbic acid, cysteine and α-mercaptopropionyl-glycine on the rabbits fed cholesterol.

MATERIALS AND METHODS

Experiment 1

White male rabbits weighing about 2.0 kg were divided into three groups by the diets and the period of feeding as follows;

N group: 27 rabbits fed on standard diet for 4 weeks.

C-4 group: 28 rabbits fed on cholesterol diet for 4 weeks.

C-12 group: 3 rabbits fed on cholesterol diet for 12 months.

Diets used: A standard diet consisted of 350 g of bean extracted refuse, 15 g of bran and 200 g of cabbage per day. A cholesterol diet consisted of standard diet and 1 g of cholesterol per day.

All groups were sacrificed at the end of the period respectively. The aorta and liver were analyzed for thiobarbituric acid reactive substances, nonprotein sulfhydryl, succinic dehydrogenase, and total nitrogen. The heparinized plasma was analyzed for TBA reactive substances. Because of the paucity of materials, SDH activity and TBA value of the aorta were not estimated on C-12 Group. SDH activity and nonprotein SH content of the liver were not measured on C-12 group.

Total nitrogen: 20 mg of the frozen dried materials was homogenized in 3.8 ml of distilled water and diluted to 500 volume of the initial weight. 0.5

ml of the aliquot was heated to ash in 1 ml of 50% H_2SO_4 with KOH bath and then 1 ml of the Nessler's reagent was added. The volume was adjusted to 10 ml with distilled water. The optical density was read at 513 mμ in a spectrophotometer.

Nonprotein SH: 100 mg of the frozen dried material was homogenized in 3 ml of phosphate buffer pH 7.4 and allowed to stand for 30 minutes. And then 3 ml of 5% sulfosalycilic acid was added. SH content of the filtrate was estimated by the amperometric titration method with 0.217×10^{-3} mol $AgNO_3$ titration solution.

Succinic dehydrogenase (SDH) activity: The aorta was sliced 0.5 mm of width. The liver was homogenized at 10% in phosphate buffer (0.1 M, pH 7.4). SDH activity was measured by direct method of Warburg. 100 mg of wet weight of the aorta slices or 0.3 ml of the liver homogenate was incubated with constant shaking in 3.0 ml of the following medium at 37°C in an atmosphere of air.

Central vessel: 0.2 ml of 10 N NaOH
Side vessel : 0.2 ml of 1/10 N Na-succinate
Main vessel : 0.5 ml of 0.03% phenadine metosulfate
0.3 ml of 0.1 mol KCN
1.8 ml of 0.1 mol phosphate buffer pH 7.4
0.2 ml of distilled water

Manometric readings were made at fifteen minutes intervals after fifteen minutes of the thermal equilibration from addition of the substrate. Results were corrected for endogenous oxygen consumption.

TBA reactive substances: Three strips of the aortae were freed from adherent fat and adventitia and pooled for the estimation. One gram of the aorta and liver were homogenized in 3 ml of cold phosphate buffer pH 7.4. The homogenate or 2 ml of plasma was shaken in a water bath at 38°C in an atmosphere of air for 60 minutes, and then 2.5 ml of 20% TCA was added. After removal of protein, 2 ml of the supernatant was heated at 100°C for 30 minutes with 1.25 ml of 0.75% thiobarbituric acid. The resultant pink color was determined at 532 mμ in a spectrophotometer.

Experiment 2

Thirty-five samples of the thoracic aorta and twenty-nine samples of the abdominal aorta were obtained at autopsy at the Nagoya University Hospital and immediately used for the experiment. Whenever the samples could not be used immediately, they were placed in the freezer and kept at −40°C with dry ice. The ages of the individuals from whom the samples were taken were between 43 and 76 years. The subjects had died from miscellaneous causes. The samples were divided into four groups by the degree of the macroscopic severity of the atherosclerotic changes as follows: normal group, slightly

atherosclerotic group, moderately atherosclerotic group, and severely atherosclerotic group. After removal of adventitia and media the intima layers of the samples were analyzed for nonprotein sulfhydryl. The content of nonprotein sulfhydryl was determined by the same method as used in the experiment 1.

Experiment 3

A) A preliminary experiment. The mitochondria from 2 g of male rat liver was prepared by Schneider's differential centrifugation method[16] and resuspended in 10 ml of phosphate buffer pH 7.4. This mitochondrial suspension was divided into five equal parts. Each part was transferred to the flask as follows:

A group: 2 ml of the mitochondrial suspension, 0.2 ml of the emulsion of ethyl linolate-peroxide (POV: 42.5 mE/1) and 1 ml (50 mg) of α-mercaptopropionylglycine (M.P.G.) were mixed in a Warburg flask.

B group: 2 ml of the mitochondrial suspension, 0.2 ml of the emulsion of ethyl linoleate-peroxide, and 1 ml of distilled water were mixed.

C grouo: 2 ml of the mitochondrial suspension, 1 ml of M.P.G. and 0.2 ml of distilled water were mixed.

D group: 2 ml of the mitochondrial suspension and 1.2 ml of distilled water were mixed.

E group: 2 ml of the mitochondrial suspension, 0.1 ml of M.P.G., 0.2 ml of the emulsion of ethyl linoleate-peroxide, and 0.9 ml of distilled water were mixed. Each mixture was incubated with constant shaking for 30 minutes at 37°C. 0.3 ml of the mixture of each flask was transferred in the reactive mixture as follows:

0.1 mol phosphate buffer pH 7.3	1.5 ml
0.01 mol ATP	1.5 ml
0.02 mol $MgCl_2$	1.0 ml
0.1 mol succinate	0.5 ml
10^{-4} mol cytochrome C	0.2 ml

The respiration of liver mitochondria of each group was determined by oxygen electrode.

Furthermore, TBA value of the mitochondrial mixture, before the determination of the respiration, was measured in A and B groups. After incubation at 37°C for 30 minutes, 2 ml of 20% trichloroacetic acid was added to 2 ml of the mitochondrial mixture. After centrifugation for 15 minutes, 3 ml of the supernatant was heated at 100°C for 30 minutes with 1.25 ml of 0.75% thiobarbituric acid. The resultant pink color was determined at 532 mμ spectrophotometrically.

B_1) White male rabbits weighing about 2.0 kg were divided into five groups by the diet used and the administration of the reducing substances as follows:

N group: 13 rabbits fed on the same standard diet as used in the experiment 1.

Control group: 12 rabbits fed on the same choresterol diet as used in the experiment 1 and were injected with 1 ml of distilled water subcutaneously every day.

Vitamin C group: 13 rabbits fed on cholesterol diet and were injected with 100 mg of ascorbic acid subcutaneously every day.

Cysteine group: 12 rabbits fed on cholesterol diet and were injected with 100 mg of cysteine-glucose subcutaneously every day.

M.P.G. group: 13 rabbits fed on cholesterol diet and were injected with 100 mg of α-mercaptopropionyl-glycine (M.P.G.) subcutaneously every day.

All groups were fed for 3 months and sacrificed 24 hours after the last administration. The entire thoracic aortae were removed and opened to make a rectangular strip. The severity of atherosclerosis on each strip of aortae was observed macroscopically. Furthermore, the aorta, liver, and serum were analyzed for total cholesterol and total sulfhydryl. In the serum, phospholipids were also estimated.

The degrees of severity of atherosclerosis: The extent of the atherosclerotic changes on each strip of aortae was scrutinized and the severity of atherosclerosis was graded on a scale of 0 to 4 by McMillan's method[17].

Total cholesterol: The levels of total cholesterol in the aorta, liver, and serum were measured by Kitamura's modification[18] of the Zak-Henly's procedure.

Total sulfhydryl: The determination was carried out at room temperature by amperometric titration with an apparatus constructed as described by Benesch *et al.*[19] with a modification as follows:

Titration was carried out in 200 ml beakers containing 50 ml tris buffer solution (pH 7.8). In this solution were immersed the rotating platinum electrode (800 r.p.m.) and the bridge from the reference electrode (Hg–HgO saturated $Ba(OH)_2$ electrode of Samuelson and Brown). The electrode was standarized monthly by means of the known mercaptan solution. The galvanometer sensitivity was order of 0.04 to 0.06 μÅ per minute. For the titration solution was used 2×10^{-3} $AgNO_3$.

Phospholipids: Concentrations of phospholipids in the serum were determined by the method of Fiske and Subbarow[20].

B_2) White male rabbits weighing about 2.0 kg were divided into five groups by the diet used and the administration of the reducing substances as follows:

N group: 8 rabbits fed on the same standard diet as used in the experiment 1.

Control group: 5 rabbits fed on standard diet and 0.5 g of cholesterol per day. In addition, 1 ml of distilled water was injected subcutaneously every day.

Vitamin C group: 5 rabbits fed on standard diet and 0.5 g of cholesterol per day. In addition, 50 mg of ascorbic acid was injected subcutaneously every day.

Cysteine group: 5 rabbits fed on standard diet and 0.5 g of cholestrol per day. In addition, 50 mg of cysteine-glucose was injected subcutaneously every day.

M.P.G. group: 5 rabbits fed on standard diet and 0.5 g of cholesterol per day. In addition, 50 mg of M.P.G. was injected subcutaneously every day.

All groups were fed for three months and sacrificed 24 hours after the last administration. The aorta, liver, and serum were analyzed for fatty acid composition of total lipids.

Fatty acid composition of total lipids: Gas liquid chromatography was used[21]. Aorta, liver, and serum lipids were extracted in ether-methanol mixture refluxing for 30 minutes at 60°C, and saponification of extracted lipids was carried out by refluxing with 10 ml of 0.2 N KOH-methanol solution for 30 minutes at 60°C. After the removal of the unsaponificable substances, the fatty acids obtained by acidification were methylated with diazomethane. The methylated fatty acids were then taken up in ethyl ether, concentrated to the desired volume, and injected into the evaporization chamber of a gas liquid chromatograph of Yanagimoto Seisakusho Co., Ltd., model GCG-3 D attached with hydrogen flame ionization detector GCF-100 using 15% diethyl-eneglycol succinate polyester on Celite 545 (80 to 100 mesh) column.

Column temperature: 200°C

Carrier gas N_2: 40 cc/min.

Carrier gas H_2: 60 cc/min.

The percentage of fatty acids was obtained by measuring the area under each peak with a planimeter.

RESULTS

Experiment 1

In this experiment total nitorogen content, nonprotein SH content, SDH activity, and TBA value in the rabbits following cholesterol feeding were investigated.

Total nitrogen content of the aorta was not changed in the C-4 group and significantly lowered in the C-12 group as compared with the normal group. That of the liver was not altered on 4th week (Table 1).

Normal ranges of nonprotein SH content were 53.5 ± 18.5 in the aorta and 1066 ± 47 μg/100 mg GSH equivalent in the liver. The concentration of non-protein SH in the aorta tended to decrease in the C-4 group and significantly decreased in the C-12 group as compared with the N group (Table 2). That in the liver of the C-4 group was not different from that of the N group. The ratios of nonprotein SH to total nitrogen were demonstrated in Table 3. The ratio in the aorta tended to decrease in the C-4 group and was not changed in the C-12 group as compared with the N group. The ratio in the liver of the C-4 group was not different from that of the N group.

TABLE 1. Total nitrogen contents in rabbit tissues

	Normal	4th week of cholesterol feeding	12th month of cholesterol feeding
Aorta mg/100 mg dry weight of tissue	16.5±2.8 (5)	14.7±1.3 (5)	11.4±0.2 ## (3)
Liver mg/100 mg dry weight of tissue	10.2±2.2 (5)	10.5±4.0 (5)	

± Standard deviation
Difference from normal value significant ($p<0.05$)
The figures in parenthesis indicate the number of samples in each group.

TABLE 2. The concentrations of nonprotein SH in rabbit tissues

	Normal	4th week of cholesterol feeding	12th month of cholesterol feeding
Aorta μg/100 mg GSH equiv.	53.5±18.5 (5)	28.9±12.3 # (5)	27.9±8.2 ## (3)
Liver μg/100 mg GSH equiv.	1066±47 (5)	1137±122 (5)	

± Standard deviation
Difference from normal value significant ($p<0.06$)
Tendency to differ from normal value ($0.05<p<0.1$)
Each value is based on the initial dry weight.
The figures in parenthesis indicate the number of samples in each group.

TABLE 3. The ratios of nonprotein SH/total nitrogen in rabbit tissues

	Normal	4th week of cholesterol feeding	12th month of cholesterol feeding
Aorta	3.32±1.28 (5)	1.92±0.69 # (5)	2.44±0.62 (3)
Liver	111.3±32.9 (5)	116.3±39.8 (5)	

± Standard deviation
Tendency to differ from normal value ($0.05<p<0.1$)
The figures in parenthesis indicate the number of samples in each group.

Succinic dehydrogenase activity in the aorta of the C-4 group had a tendency to increase as compared with that of the N group, but statistically not significant. That of the liver in the C-4 group was not different from that of the N group (Table 4).

The concentration of thiobarbituric acid reactive substances in the aorta was significantly increased in the C-4 group. Those in the liver and

TABLE 4. Succinic dehydrogenase activity in rabbit tissues

	Normal	4th week of cholesterol feeding
Aorta μl/100 mg dry weight of tissue/hrs	263.8±28.2 (4)	324.8±27.7 # (5)
Liver μl/100 mg wet weight of tissue/hrs	139.3±8.9 (4)	115.2±18.8 (4)

± Standard deviation
Tendency to differ from normal value ($0.05<p<0.1$)
The figures in parenthesis indicate the number of samples in each group.

TABLE 5. The contents of TBA reactive substances in rabbit tissues

	Normal	4th week of cholesterol feeding	12th month of cholesterol feeding
Aorta Ext./g wet weight of tissue	0.173±0.060 (6)	0.258±0.068 ## (6)	
Liver Ext./g wet weight of tissue	0.123±0.020 (6)	0.126±0.025 (4)	0.165±0.015 ## (3)
Serum Ext./ml	0.066±0.007 (8)	0.068±0.008 (5)	0.091±0.004 ## (3)

± Standard deviation
Difference from normal value significant ($p<0.05$)
The figures in parenthesis indicate the number of samples in each group.

plasma were not altered in the C-4 group and significantly increased in the C-12 group as compared with those of the N group (Table 5).

Experiment 2

In this experiment was studied nonprotein SH content in the human aorta. As indicated in Table 6, nonprotein SH content of the abdominal aortae

TABLE 6. The concentrations of nonprotein SH in human aorta

	Normal group	Slightly atherosclerotic group	Moderately atherosclerotic group	Severely atherosclerotic group
Thoracic aorta μg/100 mg GSH equiv.	21.3±7.8 (4)	20.3±9.2 (13)	21.9±7.4 (16)	19.7±3.2 (2)
Abdominal aorta μg/100 mg GSH equiv.	28.9±6.3 (4)	24.5±8.5 (8)	21.0±8.0 ## (9)	16.5±8.6 # (5)

± Standard deviation
Difference from normal value significant ($p<0.05$)
Tendency to differ from normal value ($0.05<p<0.1$)
Each value is based on the initial dry weight.
The figures in parenthesis indicate the number of samples in each group.

significantly decreased in the moderately atherosclerotic group and tended to decrease in the severely atherosclerotic group as compared with the normal group. No difference of that of the thoracic aortae was observed among the four groups.

Experiment 3

In this experiment the effect of the administration of some reducing substances on atherogenesis on cholesterol-fed rabbits was investigated.

A) The preliminary experiment on α-mercaptopropionyl-glycine was carried

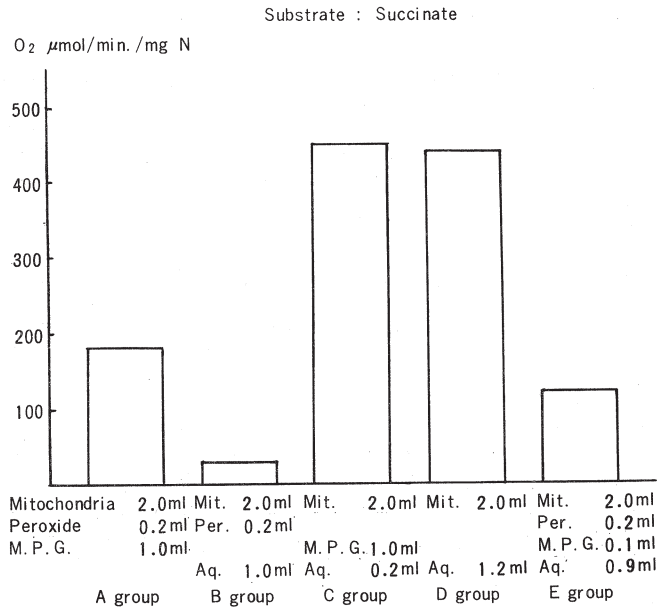


FIG. 1. Respiration of liver mitochondria after 30 min. incubation at 37°C.

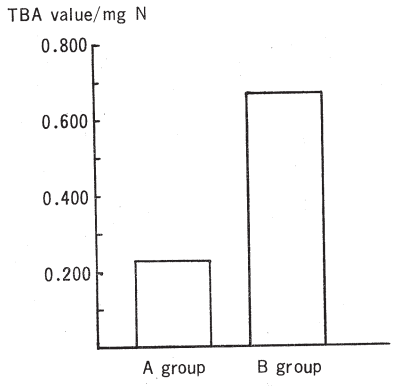


FIG. 2. TBA value of liver mitochondria after 30 min. incubation at 37°C.

out. As demonstrated in Figs. 1 and 2, *in vitro*, the peroxide of ethyl linoleate suppressed the respiration of liver mitochondria remarkably, and M.P.G. could protect the respiration from being supressed by the peroxide. This protective action was observed even in the E group in which the addition of M.P.G. was 1/10 times in the A group. TBA value of the material analyzed for the respiration, after incubation at 37°C for 30 minutes, was distinctly lower in the A group than in the B group.

B) On the cholesterol feeding rabbits, to which some reducing substances were administered simultaneously with cholesterol feeding, the degree of severity of atherosclerosis on the entire thoracic aorta was demonstrated in Table 7. The number of cases in grades 1 and 2 was greater in each group administered with ascorbic acid, cysteine, or M.P.G. than in the control group respectively.

As given in Table 8, total cholesterol content of the aorta significantly decreased in the vitamin C group, tended to decrease in the cysteine group, and was not altered in the M.P.G. group as compared with that in the control group. No difference was observed in the levels of total cholesterol of the serum and liver among the other four groups except the normal group.

TABLE 7. The degree of severity of atherosclerosis of rabbit aorta (by McMillan's method)

	Control group	Ascorbic acid group	Cysteine group	M.P.G. group
Grade 0	0	0	0	0
Grade 1	1	0	3	1
Grade 2	3	7	3	7
Grade 3	5	5	5	4
Grade 4	3	1	1	1
Total	12	13	12	13

TABLE 8. The concentrations of total cholesterol in rabbit tissues

	Control group	Vitamin C group	Cysteine group	M.P.G. group	Normal group
Aorta mg/g wet weight of tissue	25.4±12.0 (12)	16.0±8.0 ## (11)	18.0±7.5 # (11)	20.9±12.2 (12)	
Liver mg/g wet weight of tissue	30.1±17.9 (6)	28.3±7.4 (6)	28.1±8.2 (6)	32.2±8.1 (6)	4.9±0.5 ## (6)
Serum mg/100 ml	1314±517 (8)	1385±499 (8)	1524±331 (7)	1153±414 (8)	60±14 ## (5)

± Standard deviation

Difference from control value significant ($p<0.05$)

Tendency to differ from control value ($0.05<p<0.1$)

The figures in parenthesis indicate the number of samples in each group.

The content of phospholipids in the serum was not different among the other four groups expect the normal group (Table 9).

Fatty acid composition of the lipids extracted from standard diet used in this experiment was shown in Table 10. Fatty acid composition of total lipids in the aorta was shown in Table 11. Cholesterol feeding caused a significant decrease in percentages of palmitic and linoleic acids and then a significant increase in percentage of arachidonic acid as compared with the normal. No significant changes of other fatty acids in the aorta were observed between the control group and the normal group. No difference of fatty acid composition of the aorta lipids was noted also among the other four groups except the normal group.

As demonstrated in Table 12, percentage of liver palmitic acid tended to decrease in the cholesterol feeding rabbits as compared with the normal. No

TABLE 9. The concentrations of phosphlipids in rabbit serum

	Control group	Vitamin C group	Cysteine group	M.P.G. group	Normal group
Serum mg/100 ml	509±226 (8)	487±60 (7)	488±131 (6)	383±106 (18)	93±20 ## (11)

± Standard deviation

Difference from control value significant ($p<0.05$)

The figures in parenthesis indicate the number of samples in each group.

TABLE 10. Fatty acid composition of the lipids extracted from standard diet

	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:4}$
%	12.4	4.2	22.6	53.2	6.7	0

TABLE 11. Fatty acid composition of aorta lipids of rabbit

	Control group (5)	Vitamin C group (5)	Cysteine group (5)	M.P.G. group (5)	Normal group (4)
$C_{16:0}$ %	14.3±2.3	13.8±1.6	16.1±2.6	13.7±2.4	##21.8±3.9
$C_{18:0}$ %	9.6±2.2	8.2±1.0	8.3±1.6	9.7±2.0	8.6±2.8
$C_{18:1}$ %	24.7±4.6	23.5±2.4	25.3±4.2	25.2±3.4	20.7±2.5
$C_{18:2}$ %	28.2±3.7	31.3±3.7	28.4±4.2	28.1±9.9	##34.1±1.1
$C_{18:3}$ %	4.6±1.4	6.8±2.3	4.9±0.7	5.2±1.6	4.7±0.2
$C_{20:4}$ %	7.8±2.0	5.5±2.7	6.7±2.1	6.5±3.1	## 2.8±0.7

± Standard deviation

Difference from control value significant ($p<0.05$)

The figures in parenthesis indicate the number of samples in each group.

TABLE 12. Fatty acid composition of liver lipids of rabbit

	Control group (3)	Vitamin C group (2)	Cysteine group (3)	M.P.G. group (3)	Normal group (3)
$C_{16:0}$ %	13.4±1.4	18.8±2.9	17.2±4.2	12.7±0.8	#17.8±1.9
$C_{18:0}$ %	14.6±3.5	16.8±0.5	14.1±3.1	14.8±1.5	16.7±3.6
$C_{18:1}$ %	19.5±2.2	#13.0±1.7	18.2±4.3	18.3±2.1	12.8±4.9
$C_{18:2}$ %	35.4±0.2	33.4±4.6	34.8±1.1	36.1±4.9	38.1±2.4
$C_{18:3}$ %	3.2±0.8	3.3±0.1	3.0±0.8	4.3±1.1	3.7±1.5
$C_{20:4}$ %	7.3±0.5	##9.8±0.1	6.2±0.7	7.0±1.8	7.8±3.1

± Standard deviation
Difference from control value significant ($p<0.05$)
Tendency to differ from control value ($0.05<p<0.1$)
The figures in parenthesis indicate the number of samples in each group.

TABLE 13. Fatty acid composition of serum lipids of rabbit

	Control group (2)	Vitamin C group (3)	Cysteine group (4)	M.P.G. group (2)	Normal group (8)
$C_{16:0}$ %	20.4±0.7	18.0±2.9	21.4±3.2	26.5±6.4	#16.3±2.4
$C_{18:0}$ %	16.6±0.7	15.1±1.1	14.2±1.6	17.6±0.1	13.5±2.6
$C_{18:1}$ %	12.1±0.5	11.7±1.5	16.6±5.3	10.3±1.3	#17.0±3.0
$C_{18:2}$ %	38.8±2.0	41.6±1.2	36.8±2.5	34.0±3.2	40.1±2.3
$C_{18:3}$ %	3.3±0.8	3.6±1.1	2.9±1.3	2.5±1.1	3.5±1.1
$C_{20:4}$ %	3.4±0.3	4.2±0.3	3.3±1.5	4.1±0.4	2.8±1.2

± Standard deviation
Tendency to differ from control value ($0.05<p<0.1$)
The figures in parenthesis indicate the number of samples in each group.

significant changes of other fatty acids in the liver were observed between the control and the normal. In the liver of the vitamin C group a tendency to decrease in percentage of oleic acid and a significant increase in percentage of arachidonic acid were noted. No difference of fatty acid composition of the liver lipids was observed among the control group, the cysteine group, and the M.P.G. group.

As indicated in Table 13, in the serum of the cholesterol feeding rabbits percentage of palmitic acid tended to increase and percentage of oleic acid tended to decrease as compared with the normal ones. No significant changes of other fatty acids in the serum were noted between the control group and the normal group. No difference of fatty acid composition of the serum lipids was also noted among the other four groups expect the normal group.

As indicated in Table 14, total SH content of the aorta was lowered below the normal level by cholesterol feeding. That content of the aorta then

TABLE 14. The concentrations of total SH in rabbit tissues

	Control group	Vitamin C group	Cysteine group	M.P.G. group	Normal group
Aorta μg/100 mg wet weight of tissue	7.73±0.74 (5)	10.30±1.10 ## (5)	8.82±1.27 (5)	8.88±0.92 # (5)	9.86±3.16 ## (5)
Liver μg/100 mg wet weight of tissue	52.4±8.7 (5)	47.5±11.2 (5)	49.3±6.1 (5)	51.2±4.7 (5)	51.5±7.9 (5)
Serum μmol/100 ml	43.3±9.4 (5)	51.3±7.8 (8)	45.7±5.1 (7)	53.7±7.2 # (8)	58.6±10.3 ## (14)

± Standard deviation
Difference from control value significant ($p<0.05$)
Tendency to differ from control value ($0.05<p<0.1$)
The figures in parenthesis indicate the number of samples in each group.

increased in the vitamin C group, tended to increase in the M.P.G. group and was not changed in the cysteine group as compared with that in the control group. However, no difference was observed in the content of liver total SH among these five groups. Total SH content of the serum in the control group was lower than in the normal group and tended again to increase in the M.P.G. group as compared with the control. There was no difference in that content of the serum among the control group, the vitamin C group, and the cysteine group.

DISCUSSION

1) Changes of peroxides and SH compounds in the rabbits following cholesterol feeding

Glavind and others[1] showed that atherosclerotic human aorta contained lipo-peroxides and that the content of the peroxides was in parallel with the degree of severity of atherosclerosis while normal aorta was free from lipo-peroxides. Since then there has been an increasing interest on the role of lipid peroxides in the atherogenesis. Woodford and others[3] determined lipid peroxides in the lipid extracts of the human aorta of varying degree of atherosclerosis. Fukuzumi[2] also confirmed the existence of hydroperoxides in the lipid of the atherosclerotic human aorta and could not find it in the lipid of the normal aorta.

The thiobarbituric acid (TBA) reaction has been widely used as a measure of lipid peroxidation in tissue slices and homogenates, because the content of TBA reactive substances may be believed relatively well to represent the content of the peroxides. However, TBA value is considered to be influenced both by autoxidation of lipids in the materials and by the content of reducing substances. Iwakami[13] in our laboratory also reported an increase of TBA reactive substances in the aortic wall of the rabbits following cholesterol

feeding. Manifestation of atheroma-like lesions and a remarkable increase of TBA reactive substances were also observed in the aorta of the rabbits after X-ray irradiation that was considered to increase lipo-peroxides in the organism.

In the present experiment, TBA reactive substances in the aorta increased already on 4th week following cholesterol feeding when those in the liver and serum did not increase. Since TBA reactive substances in the aorta of the normal rabbits were about 3 times those in the serum and higher than in the liver, it is supposed that the increase of TBA reactive substances only in the aorta of cholesterol-fed rabbits, probably increase of lipoperoxides, may result from some condition in which the peroxides increase in the aortic wall itself without increasing those infiltration from blood flow. Some condition to increase lipoperoxides has been mentioned in the following several reports. Bernheim and others[10] showed that incubation of washed tissue suspension or mitochondria with ascorbic acid inactivated a number of enzymes including the succinoxidase, cytochrome oxidase, and choline oxidase, and that the extent of inactivation paralleled with the amount of oxidized fatty acid. Ottolenghi and others[22] reported that succinoxidase and choline oxidase were inhibited in direct proportion to the extent of lipid oxidation produced by the incubation of mitochondria with ascorbic acid and by ultraviolet irradiation of mitochondria. Tappel and Zalkin[23] found that liver mitochondria took up oxygen in the absence of added substrate, that this oxygen uptake resulted from the formation of lipid peroxides and that during formation of these peroxides there was a concomitant destruction of some of the mitochondrial enzymes, *e.g.* succinoxidase and DPNH-cytochrome c reductase. Willis[15] found that -SH enzymes were more readily inhibited by emulsions of oxidized unsaturated fatty acids than enzymes not possesing -SH groups and were protected by sulfhydryl compounds such as glutathione. Lewis and Willis[25] reported that sulfhydryl groups of proteins and amino acids such as cysteine, glutathione, and papain were destroyed by oxidized linoleic acid emulsion, that the rate of destruction was proportional to the peroxide value of the emulsion and that the peroxides themselves were destroyed during the destruction of -SH groups. Thus oxygen, ascorbic acid or ultraviolet rays is regarded as a factor to increase lipoperoxides in the tissues. Because SH compounds among them were considered to be decreased in the case of the increase of peroxides in the tissues, the author determined nonprotein SH and succinic dehydrogenase (one of SH enzymes) in the rabbit tissues following cholesterol feeding.

There is no report of nonprotein SH content in the arterial wall, so far as I know. Concerning glutathione, main component of nonprotein SH, Wang and Kirk[25] reported an increase of total glutathione in the arterial wall with increasing ages. On age-dependent changes in reduced glutathione, however, there was no comment in the report. By means of the amperometric titration used in this experiment, only reduced form of SH compounds was measured.

From the decrease of total nonprotein SH in the arterial wall of the rabbit following cholesterol feeding, it is inferred that reduced glutathione might be lowered in the wall. Nonprotein SH content and ratio of nonprotein SH/total nitrogen tended to decrease already on the 4th week when those in the liver did not change. These changes were in inverse proportion to the changes of TBA content of the aorta mentioned above. Nonprotein SH content of the aorta was lower than one-tenth of that of the liver.

There has been several studies concerning the effect of cholesterol feeding on the respiratory activity of the aorta. Munro and others[26] reported that cholesterol feeding resulted in a lower oxygen uptake of the aorta in the cockerel and in a higher oxygen uptake in the rat. Mrhová and others[27] found a significant decrease of the activity of the aortic SDH system in the rabbit as early as 4 weeks after starting cholesterol feeding, when no pathological changes were to be seen in the aorta. They found also a significant decrease of that on 10th week when atherosclerotic changes were to be seen. Fisher and Geller[28] observed a depression of respiratory activity in rabbit cholesterol atherosclerosis. By Maier and Haimovici's study[29], at the early stage in the atherosclerotic process in rabbits, the oxidative capacity of both succinoxidase system and cytochrome oxidase system was increased in the atherosclerotic intima-media layer, while it was decreased at the later stage. The liver, in spite of the site of massive deposition of lipids, showed no enzymatic changes at any time during the administration of the atherogenic regimen. On the contrary, Whereat[30] reported that the intima from the rabbits rendered atherosclerotic by cholesterol feeding showed a much higher rate of oxygen consumption than the controls when succinate was used as a substrate.

In the present experiment, SDH activity of the aorta tended to increase on 4th week after cholesterol feeding, while that of the liver showed no changes. These suggest that SDH activity of rabbit aorta might be impaired at the early stage of atherosclerotic process locally in the wall and increased with mesenchymal reaction of the arterial tissue to the injury and then decreased with extensive tissue damage of the arterial wall.

2) Nonprotein SH content of the atherosclerotic aortae in the human subjects

As mentioned above, there has been no report on nonprotein sulfhydryl content in the arterial tissue. Concerning glutathione, main component of nonprotein SH in the organism, Wang and Kirk[25] reported an increase of total glutathione of the normal human aortic tissue with increasing age. However, they found no difference between the total gluththione contents of atherosclerotic and normal tissue proteins in the human aorta.

In the present experiment, nonprotein SH content of the human abdominal aorta significantly decreased in the moderately atherosclerotic group and tended to decrease in the severely atherosclerotic group as compared with the normal

group. No difference of the thoracic aortae was observed between the normal and the atherosclerotic. By means of the amperometric titration used in this experiment, only reduced form of SH compounds was measured. Therefore, from the decrease of nonprotein SH, it is inferred that reduced glutathione might be lowered in the human atherosclerotic abdominal aorta. It is very interesting that the results in this experiment are correspondent to the relatively higher incidence of human atherosclerosis in abdominal aorta than in thoracic aorta. The difference of the respiration rate between abdominal and thoracic portions in the human aorta is regarded as a cause of the difference of development and progression of atherosclerosis between those portions. Christie and Dahl[31] observed that the abdominal portion of the rat aorta respired less actively than the thoracic portion. Priest[32] observed in the rat that the oxygen consumption of the thoracic aorta was significantly greater than that of the abdominal aorta per unit of DNA and about one fifth that for the liver. Then Whereat[30] reported that the most atherosclerotic area (the aortic arch) showed a higher oxygen consumption than the less atherosclerotic areas (the thoracic and abdominal areas) in the cholesterol feeding rabbits. Maier and Haimovici[33] found in the dog a falling gradient of cytochrome coxidase activity from the thoracic to abdominal portions and a remarkable decrease of SDH activity in the abdominal portion as compared with the thoracic portion. These different patterns of changes in the constituents in the course of atherosclerosis between in the thoracic and abdominal aortas might be related with the difference in those susceptibility to the atherosclerosis.

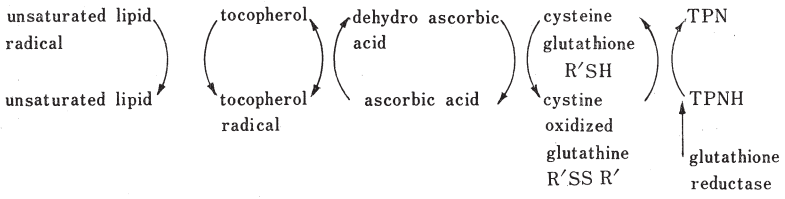
3) Effect of administration of ascorbic acid, cysteine, or α-mercaptopropionylglycine on the tissues of cholesterol feeding rabbits

Numerous articles have been devoted to the effect of ascorbic acid on atherosclerosis. It was reported that ascorbic acid administration decreased cholesterol levels in the blood of cholesterol fed rabbits[34] and in the blood of the patients with atherosclerosis[35]. In rabbit experiments, ascorbic acid prevents the development of alimentary atherosclerosis[36)~39]. Ascorbic acid deficiency gives rise to atherosclerosis in guinea pigs in spite of the normal blood cholesterol levels[40]. Ascorbic acid content of the human arterial tissue tends to be lower in segments susceptible to atherosclerosis as compared with normal segments and higher in adjacent segments where atherosclerosis is rare[41].

On the other hand, there have been few reports concerning the effect of SH compounds on atherosclerosis. However, cholesterol-lowering effect of methionine has been reported by several investigators in rats[42)~45], in chicks[46], and in monkeys[47]. l-Cysteine is only partially effective in preventing the alimentary hypercholesterolemia in cebus monkeys[47], and cebus monkeys deficient in cysteine lose their efficiency in removing cholesterol and have a

resultant elevation of the plasma level of cholesterol[48]. In the rat, addition of methionine to a high fat and low protein diet markedly prevents the production of thrombotic and atherosclerotic lesions and reduces the hypercholesterolemia[42].

Tappel[49] has mentioned about interrelationships of sulfur amino acids, ascorbic acid, vitamin E, and free radical peroxidation as follows. (1) Sulfur amino acids could act as antioxidants somewhat similar to vitamin E by (a) reacting with free radical intermediates of lipid peroxidation, thereby breaking the chain reaction, and (b) decomposing lipid peroxides. If free radical lipid peroxidation does proceed, the sulfur amino acids protect cellular constituents by the reactions mentioned (1 a) and (1 b). (2) Cysteine, glutathione, and sulfhydryl proteins are a large and reactive pool of redox compounds for reduction of vitamin E. (3) Reduction of tocopherols can be coupled with a number of biological redox substances as shown in the next table.



Ascorbic acid is known to form powerful synergistic antioxidant combination with tocopherols. Reduction of a transitory tocopherol semiquinone radical by ascorbate is likely the reaction. The ascorbic-dehydroascorbic redox system is in redox balance with glutathione. Glutathione in turn is in redox equilibrium with a large pool of sulfhydryl compounds in the animal tissue.

Siegel and Halpern's experiments[50] indicated that the injurious effect of peroxides was reduced by certain antioxidants, especially indoles, tocopherol and thiols. Schneider and others[51] found that high concentration of ascorbate prevented peroxidation and swelling-lysis by an antioxidant action while with low concentrations of it lipid peroxidation occured gradually in the experiment of rat liver mitochondria. Ascorbic acid is a strong reducing agent and its oxidation-reduction potential is higher than that of most biological compounds[52].

In the present experiment on rabbits, the severity of the cholesterol-induced atherosclerosis in the aorta could be retarded by administration of asorbic acid, cysteine or α-mercapto propionyl-glycine respectively. Furthermore, total cholesterol content in the aorta decreased significantly in the ascorbic acid group and tended to decrease in the cysteine group as compared with the control group, while not altered in the serum and liver. The content of cholesterol in the aorta has been regarded to a certain extent as a useful index of the severity of atherosclerosis[17].

The content of phospholipids in the rabbit serum was not changed by administration of ascorbic acid, cysteine, or M.P.G.

Some investigators[53)54)] found in rabbits that saturated fatty acids decreased, and oleic and linoleic acids increased in percentage of fatty acid composition of the aorta lipids following cholesterol feeding. In the present experiment in rabbits, saturated fatty acids significantly decreased in percentage of fatty acid composition of the aorta lipids, while unsaturated fatty acids were changed differently from the reports mentioned above. Fatty acid composition of the serum lipids was not significantly changed by cholesterol feeding. No significant influence on fatty acid composition of the aorta and serum lipids of cholesterol feeding rabbits was induced by administration of ascorbic acid, cysteine, or M.P.G.

In the present experiment, the severity of atherosclerosis could be retarded, and the cholesterol content could be decreased in the cholesterol-fed rabbits with administration of some antioxidants such as ascorbic acid, cysteine, or M.P.G., while no difference of the aorta lipids could be found in quality. Total SH content was lowered below the normal level in the aorta, while not changed in the liver by cholesterol feeding. The decrease of total SH content in the aorta and serum to be induced by cholesterol feeding could be almost prevented by administration of ascorbic acid, cysteine, or M.P.G. Total SH compounds determined by the amperometric titration method used in the experiment 3 consist of protein SH radicals, SH enzymes and nonprotein SH (almost reduced glutathione) in the tissues and protein SH radicails in the serum. And total protein SH radicals determined by this method include all of SH radicals even in the inner parts of protein particle itself. Therefore, the decrease of protein SH radicals may signify the change of protein particle in three dimensional structure. And the decrease of total SH content in the rabbit serum following cholesterol feeding may indicate the damage of serum protein.

Noticeable points to be considered concerning the relation of SH compounds to atherogenesis in the experiments 1, 2 and 3 are as follows:

a) Decreases of reduced glutathione, total SH contents, and SDH activity in the aorta.

b) Prevention of the decrease of total SH content in the aorta by administration of ascorbic acid.

c) Prevention of cholesterol atherosclerosis by administration of SH compounds.

d) A decrease of total protein SH content in the serum. These changes may be produced by some factors to change -SH radicals to -S-S- groups in the aorta and serum. In serum protein this change may take place in the inner parts of protein particle. The damage of serum protein may be probably induced by nonenzymatic reactions rather than by the disturbance of protein metabolism, since total SH content and SDH activity of the rabbit liver are normal after

cholesterol feeding. Furthermore, this reaction can occur more easily with increasing lipid content such as in the rabbits fed cholesterol. The most probable reaction may be a peroxidizing lipid-protein reaction.

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REFERENCES

1) Glavind, J., Hartmann, S., Clemmensen, J., Jessen, K. E. and Dam, H., Studies on the role of lipoperoxides in human pathology. 11 the presence of peroxidized lipids in the atherosclerotic aorta, *Acta Path. Microbiol. Scand.*, **30**, 1, 1952.
2) Fukuzumi, K. and Tanaka, T., Lipids of atherosclerotic artery. 1. Breast aorta, *Yukagaku*, **10**, 659, 1961 (in Japanese).
3) Woodford, F. P., Böttcher, C. J. F., Oette, K. and Ahrens, Jr. E. H., The artifactual nature of lipid peroxides detected in extracts of human aorta, *J. Atheroscler. Res.*, **5**, 311, 1965.
4) Burt, R. C., The incidence of acid-fast pigment (ceroid) in aortic atherosclerosis, *Amer. J. Clin. Path.*, **22**, 135, 1952.
5) Hartroft, W. S., Pathogenesis and significance of hemoceroid and hyaloceroid, two types of ceroidlike pigment found in human atheromatous lesions, *J. Gerontal.*, **8**, 158, 1953.
6) Casselman, W. G. B., The *in vivo* preparation and histochemical properties of substances resembling ceroid, *J. Expt. Med.*, **94**, 549, 1951.
7) Fukuzumi, K. and Iwata, Y., Lipids of atherosclerotic artery. 11. Dialysis of lipids of abdominal aorta and lipids in lipid-protein complexes existing in the aorta, *Yukagaku*, **12**, 93, 1963 (in Japanese).
8) Nishida, T. and Kummerow, F. A., Interaction of serum lipoproteins with the hydroperoxide of methyl linoleate, *J. Lipid Res.*, **1**, 450, 1960.
9) Kayahan, S., Cholesterol-binding capacity of normal and atherosclerotic intimas, *Lancet*, **1**, 223, 1959.
10) Bernheim, F., Wilbur, K. M. and Kenaston, C. B., The effect of oxidized fatty acids on the activity of certain oxidative enzymes, *Arch. Biochem.*, **33**, 177, 1952.
11) Tappel, A. L., Vitamin E and selenium in the *in vivo* lipid peroxidation, *in* Lipids and their Oxidation, edited by Schulz, H. W., p. 372, The AVI publishing Campany, inc., 1962.
12) Oya, T., Significance of Vitamin E for atherogenesis, *J. Nagoya Med. Ass.*, **90**, 15, 1967 (in Japanese).
13) Iwakami, M., Peroxides as a factor of atherosclerosis, *Nagoya J. Med. Sci.*, **28**, 50, 1965.
14) Dubouloz, P. and Fondarai, J., Sur le métabolisme des peroxydes lipidiques. 11. Action des peroxydes lipidiques sur les groupements thiols protéiques, *Bull. Soc. Chim. Biol.*, **35**, 819, 1953.
15) Wills, E. D., Effect of unsaturated fatty acids and their peroxides on enzymes, *Biochem. Pharmacol.*, **7**, 7, 1961.
16) Schneider, W., Intracellular distribution of enzymes. 1. The distribution of succinic dehydrogenase, cytochrome oxidase, adenosine-triphosphatase, and phosphorus compounds in normal rat tissues, *J. Biol. Chem.*, **165**, 585, 1946.
17) McMillan, G. C., Horlick, L. and Duff, G. L., Cholesterol content of aorta in relation to severity of atherosclerosis, *Arch. Path.*, **59**, 285, 1955.
18) Kitamura, M., Study on direct determination of serum total cholesterol by ferric reaction, *Jap. J. Clin. Path.*, **6**, 202, 1958 (in Japanese).
19) Benesch, R. E., Lardy, H. A. and Benesch, R., The sulfhydryl groups of crytalline

proteins. 1. Some albumins, enzymes, and hemoglobins, *J. Biol. Chem.*, **216**, 663, 1955.
20) Fiske, C. H. and Subbarow, Y., The colorimetric determination of phosphorus, *J. Biol. Chem.*, **66**, 375, 1925.
21) Kikuchi, S. and Ito, M., Fatty acid compositions of human serum cholesterol esters, *Saishin-Igaku*, **18**, 2430, 1963 (in Japanese).
22) Ottolenghi, A., Bernheim, F. and Wilbur, K. M., The inhibition of certain mitochondrial enzymes by fatty acids oxidized by ultraviolet light or ascorbic acid, *Arch. Biochem*, **56**, 157, 1955.
23) Tappel, A. L. and Zalkin, H., Lipid peroxidation in isolated mitochondria, *Arch. Biochem.*, **80**, 326, 1959.
24) Lewis, S. E. and Wills, E. D., The destruction of -SH groups of proteins and amino acids by peroxides of unsaturated fatty acids, *Biochem. Pharmacol.*, **11**, 901, 1962.
25) Wang, I. and Kirk, J. E., The total glutathione content of arterial tissue in individuals of various ages, *J. Gerontol.*, **15**, 35, 1960.
26) Munro, A. F., Rifkind, B. M., Liebescheutz, H. J., Campbell, R. S. F. and Howard, B. R., The effect of cholesterol feeding on the oxygen consumption of aortic tissue from the cockerel and the rat, *J. Atheroscler. Res.*, **1**, 296, 1961.
27) Mrhová, O., Zemplényi, T. and Lojda, Z., The effect of cholesterol-fat feeding on the activity of rabbit aorta dehydrogenase systems, *Quart. J. Exp. Physiol.*, **48**, 61, 1963.
28) Fisher, E. R. and Geller, J. H., Effect of cholesterol atherosclerosis, hypertension and cortisone on aortic oxygen consumption in rabbit, *Circ. Res.*, 8, 820, 1960.
29) Maier, N. and Haimovici, H., Oxidative capacity of atherosclerotic tissue of rabbit and dog, with special reference to succinic dehydrodenase and cytochrome oxidase, *Circ. Res.*, **16**, 65, 1965.
30) Whereat, A. F., Oxygen consumtion of normal and atherosclerotic intima, *Circ. Res.*, **9**, 571, 1961.
31) Christie, R. W. and Dahl, L. K., Dissimilarity in oxygen consumption between the thoracic and abdominal aorta in rats, *J. Exp. Med.*, **106**, 357, 1957.
32) Priest, R. E., Consumption of oxygen by thoracic and abdominal aorta of the rat, *Amer. J. Physiol.*, **205**, 1200, 1963.
33) Maier, N. and Haimovici, H., Metabolism of arterial tissue. Oxidative capacity of intact arterial tissue, *Proc. Soc. Exp. Biol. Med.*, **95**, 425, 1957.
34) Myasnikov, A. L., Influence of some factors on development of experimental cholesterol atherosclerosis, *Circulation*, **17**, 99, 1958.
35) Tiapina, L. A., L'effect de l'acide ascorbique sur les lipides sanguins dans l'hypertension essentielle et l'athérosclérose, *Cor Vasa*, **3**, 98, 1961.
36) Chakravarti, R. N. and Mukerji, B., Studies in experimental atherosclerosis. Part V. Therapeutic effect of ascorbic acid and Vitamin B_{12} in cholesterol atherosclerosis, *Indian J. Med., Res.*, **45**, 315, 1957.
37) Davis, O. and Oester, Y. T., Experimental atherosclerosis: Inhibitory effects of ascorbic acid and inositol, *Proc. Soc. Exp. Biol. Med.*, **81**, 284, 1952.
38) Zaitev, V. F., Myasnikov, L. A. and Sheikman, M. B., Effect of ascorbic acid on distribution of cholesterol-4-C^{14} in tissues of animals with experimental atherosclerosis, *Fed. Proc.*, **24**, T 971, 1964.
39) Zeitsev, V. F., Myasnikov, L. A., Kasatkina, L. V., Lobova, N. M. and Sukasova, T. I., The effect of ascorbic acid on experimental atherosclerosis, *Cor vasa*, **6**, 19, 1964.
40) Willis, G. C., The reversibility of atherosclerosis, *Canad. Med. Ass. J.*, **77**, 106, 1957.
41) Willis, G. C. and Fishman, S., Ascorbic acid content of human arterial tissue, *Canad. Med. Ass. J.*, **72**, 500, 1955.
42) Renaud, S., Prevention by methionine feeding of atherosclerosis and thrombosis in hyperlipemic rats, *Proc. Soc. Exp. Biol. Med.*, **121**, 452, 1966.

43) Seidel, J. C. and Harper, A. E., Effects of ethionine and methionine on serum lipids and lipoproteins, *Proc. Soc. Exp. Biol. Med.*, **111**, 579, 1962.
44) Seidel, J. C. and Harper, A. E., Diet and cholesterolemia: VII. Effects of methionine, ethionine, and p-fluorophenylalanine, *J. Lipid Res.*, **4**, 75, 1963.
45) Seidel, J. C., Nath, N. and Harper, A. E., Diet and cholesterolemia: V. effects of sulfur-containing amino acids and protein, *J. Lipid Res.*, **1**, 474, 1960.
46) Johnson, D. Jr., Leveille, G. A. and Fisher, H., Influence of amino acid deficiencies and protein level on the plasma cholesterol of the chick, *J. Nutr.*, **66**, 367, 1958.
47) Mann, G. V., Andrus, S. B., McNally, A. and Stare, F. J., Experimental atherosclerosis in cebus monkeys, *J. Exp. Med.*, **98**, 195, 1953.
48) Mann, G. V., Cysteine deficiency and cholesterol metabolism in primates, *Circ. Res.*, **18**, 205, 1966.
49) Tappel, A. L., Vitamin E as the biological lipid antioxidant, *Vitamins Hormones*, **20**, 493, 1962.
50) Siegel, S. M. and Halpern, L. A., Effects of peroxides on permeability and their modification by indoles, Viamin E, and other substances, *Plant Physiol.*, **40**, 792, 1965.
51) Schneider, A. K., Smith, E. E. and Hunter, F. E., Correlation of oxygen consumption with swelling and lipid peroxide formation when mitochondria are treated with the swelling-inducing agents Fe^{2+}, glutathione, ascorbate, or phosphate, *Biochemistry*, **3**, 1470, 1964.
52) Rivers, J. M., Ascorbic acid in metabolism of connective tissue possible role, *New York J. Med.*, **65**, 1235, 1965.
53) Kimura, N., Nishimoto, S., Mori, F., Kitamura, M., Mizuguchi, T. and Yoshinaga, M., The fatty acid composition in various tissues of the normal and cholesterol fed rabbits, *Jap. Circ. J.*, **29**, 1171, 1965.
54) Anan, K. and Yasuzaki, K., Total content and fatty acid composition of abdominal aorta lipids in rabbits fed cholesterol, *Ochanomizu Med. J.*, **13**, 185, 1965 (in Japanese).