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STUDIES ON ANTIOXIDANTS IN ATHEROSCLEROSIS

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ABSTRACT

Since Glavind reported the existence of peroxides in the atheroma, many investigatiors have become concerned with the studies of peroxides in atherogenesis. But the investigation of this problem is not yet complete. There exist in the living body SH compounds and antioxidants which inhibit the production and increase of lipoperoxides.

In this paper, the changes of the antioxidant in the living body were investigated whether lipoperoxides have a role in atherogenesis or not. I investigated in rabbits the changes of SH compounds and ascorbic acid in the aorta, liver, and serum following cholesterol feeding and analysed the polarographic protein wave activity of the serum for the estimation of SH compounds. Topographical investigation on SH compounds and ascorbic acid were made with histochemical method on the atherosclerotic rabbit aorta. The changes of these compounds in the serum were investigated on the atherosclerotic patients.

In the aorta and serum of the rabbits, SH content was decreased after the cholesterol feeding. The content of ascorbic acid was not changed in the serum, but increased in the aorta. The decrease in polarographic protein wave activity was observed in the serum. SDH activity decreased in the aorta. The decrease of SDH activity in the liver followed the decrease in the aorta. In the liver of the cholesterol-fed rabbit, no significant difference of SH and ascorbic acid content was observed compared with the control rabbit. Staining intensity of SH compounds and SDH was decreased in the intima and the inner media, but that of ascorbic acid was increased. In the serum of the atherosclerotic patients, SH content tended to decrease. No significant difference of ascorbic acid content was found between the atherosclerotic ahd the nonsclerotic subjects.

It is assumed that in the course of atherosclerosis the denaturation of the proteins in the aorta and serum might be induced. This denaturation might be caused by nonenzymatic action of lipoperoxides.

INTRODUCTION

Atherosclerosis is a state of so-called plaque formation caused by the cholesterin or lipid deposition, of the thickening of the intima, and of the increase of the connective tissue. Most of cholesterin and the lipid in atheroma comes from the blood lipoprotein that infiltrats into the intima and deposits there.

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In the serum of patients of coronary sclerosis and apoplexy, the increase of β-lipoprotein is observed in many cases. Therefore, the relation between atherosclerosis and the lipid of β-lipoprotein has been intensively studied. On the other hand, there have been reports on the autoxidation products of the lipid in atheroma[1)2)].

In 1952, Glavind[3)] found the existence of lipo-peroxides in the atherosclerotic human aorta and reported that an interrelation existed between the degree of atherosclerosis and peroxide content in the lipid. Iwakami[4)] reported that larger amount of peroxides was found in the atherosclerotic rabbit aorta than in the normal and that vitamin E injection prevented lipid peroxidation. According to Nishida and Kummerow[5)], β-lipoprotein of the human serum is easily denaturated *in vitro* by methyl linolate hydroperoxide, whereas other lipoprotein is resistant to lipoperoxides. These denaturated lipoproteins is not easily metabolized because of the firm lipid-protein complex formations. Fukuzumi[6)] proved the existence of lipid-protein complex in the atherosclerotic aorta and of the lipid-portion complex contained oxidized fat. He explained the mechanism of the initiation of the lipid deposition in the arterial wall as follows: Polyunsaturated fatty acid of the blood lipoprotein are autoxidized, and lipoperoxides ensue. The lipoprotein containing lipoperoxides enters into the arterial wall by filtration and are combined firmly with the protein of the tissue. Atherosclerosis was induced based on these peroxide-protein complex. X-ray irradiation induces the autoxidation of the lipid. When X-ray was irradiated over the total body of the rabbit, atherosclerotic lession was found in the aorta, and TBA value of the aorta was increased.

It is generally said that lipoperoxides destroy the cell membrane system and inactivate SH enzymes. Because of such toxic effects, peroxides are considered to damage the arterial tissue even though the amount is little.

There exist in the human body antioxidants which have the opposite effects. Phenols, quinones, tocopherols, gallic acid, ascorbic acid are regarded as important antioxidants. SH compounds are among the things most susceptible to peroxides. With regard to the investigation of sulfhydryl (hereinafter represented as SH) compounds, in 1945 Barron[7)] reported the rssults of his investigation on protein-SH, and since then, we have seen a remarkable progress made in this field. SH is classified into non-protein-SH, of which glutathione is a example, and protein-SH which is closely related to the enzymatic activity. It has become an established fact that, non-protein SH and cell respiration have some relation, and that SH enzymes play an important role in the metabolism of the carbohydrate, protein, and lipid. SH compounds are the most effective preventive substances to the deleterious effects of peroxides and of X-ray irradiation.

It is our assumption that the decrease of SH compounds may occur in the arterial wall with the accumulation of peroxides in the course of the athero-

sclerosis. On the other hand, decrease in SH compounds of long standing might also bring about the accumulation of peroxides.

Ascorbic acid changes easily into dehydroascorbic acid in the body and forms powerful synergic antioxidant-combinations with tocopherols[8]. Ascorbic acid redox system is in redox balance with glutations. Schneider[9] reported in the study of the liver mitochondria of the rat that lipid peroxidation was inhibited by ascorbic acid, and this action had a close relation with the concentration of ascorbic acid.

With the above-mentioned facts, in mind, the following investigations were made. In the first experiment, changes of SH compounds and ascorbic acid were investigated with rabbits on the aorta, liver, and serum following cholesterol feeding, and also polarographic protein wave of the serum was analysed for the estimation of SH and SS compounds. In the second experiment, the topographical investigations on SH compounds and ascorbic acid were made with histochemical method on the aorta of the cholesterol-fed rabbit. In the third experiment, the changes of those compounds in the serum were investigated on the atherocslerotic patients.

MATERIALS AND METHODS

Experiment 1. Experiment on cholesterol-fed rabbits

A. SH content, SDH activity, and ascorbic acid content in the aorta, liver, and serum of rabbits

White rabbits weighing about 2–3 kg were divided into four groups by the diet used and the period of feeding as follows:

E.N. group (early normal group) fed on standard diet for 2 weeks.

L.N. group (late normal group) fed on standard diet for 12–16 weeks.

E.C. group (early cholesterol group) fed on cholesterol diet for 2 weeks.

L.C. group (late cholesterol group) fed on cholesterol diet for 12–16 weeks.

Each animal group consisted of 9 rabbits.

Diet used:

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

A cholesterol diet consisted of standard diet and 2 g of cholesterol.

All groups were sacrificed at the end of the feeding period. The entire thoracic aorta was immediately removed and carefully freed of adventitia and adherent fat and then opened along the longitudinal axis to make a rectangular strip. These strips were rinsed quickly in distilled water, blotted, and weighed. Livers removed from rabbits were also rinsed and blotted.

SH determination of the aorta, liver, and serum:

The strip of the aorta, 200 mg weight, was homogenized in 4 ml of distilled mater and used for SH determination. Homogenate of the liver block, about

100 mg in weight, was made in 3.0 ml of distilled water, and SH measurement was performed. One ml of the serum was used for the determination. The amperometric titration method was used for the determination which Benesch and Benesch[10] adopted for the protein. Titration was carried out in 200 ml beakers containing 50 ml tris buffer solution (pH 7.8). In this solution were immersed the rotating platimum electrode (800 r.p.m.) and the bridge from the reference electrode (Hg-Hgo saturated $Ba(OH)_2$ electrode of Samuelson and Brown[11]). The electrode was standardized monthly by means of the standard mercaptan solution.

The galvanometer sensitivity is 0.04 to 0.06 μA per. mm.

The titration solution: 2×10^{-3} $AgNO_3$

Measurement of succinic dehydrogenase activity in the aorta and liver:

Aorta slices used for the measurement of succinic dehrdrogenase (hereinafter represented as SDH) activity were cut by tangential slicing with a sharp scalpel. Liver homogenate (2%) was used. The polarographic determination[12] was used for the measurement of SDH activity. The rate of the oxygen consumption was calculated from the rate of fall of the PO_2 in the incubation

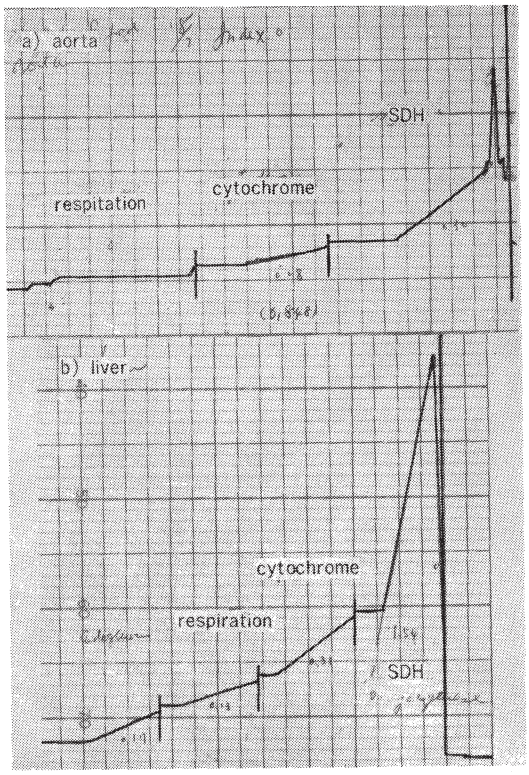

FIG. 1. Charts of oxygen consumption by polarographic method.

medium. The oxygen electrode was inserted vertically into the solution to the depth of 10 mm below the liquid surface. The incubation vessel was fitted with a water jacket through which warm water was promped to maintain the incubation medium at 20°C. Changes in PO_2 were follwed over periods of 3–4 min after an initial period of 1 min to achieve thermal eqilibrium (Fig. 1).

Incubation medium:

0.1 M succinate	0.1 ml
0.05 M tris buffer (pH 7.6)	10.0 ml
0.5 M phenadin methosulfate	0.2 ml
0.1 M KCN	0.1 ml

Dry weight: After incubation, the tissue was gently blotted with hardened filter paper and transferred to a tared weighing bottle which was reweighed after drying in air at over 105°C for not less than 3 hours.

Ascorbic acid determination in the aorta, liver, and serum:

After sacrifice, the thoracic aorta and liver were removed as soon as possible. The thoracic aorta, 300 mg weight, and blocks of the liver, 100 mg weight, were removed and homogenated in 4.0 ml of 10 percent metaphosphoric acid. Ascorbic acid content was determined by the DNP method which incorporated by the modification described by Ogawa and Kishigami[13], of the original

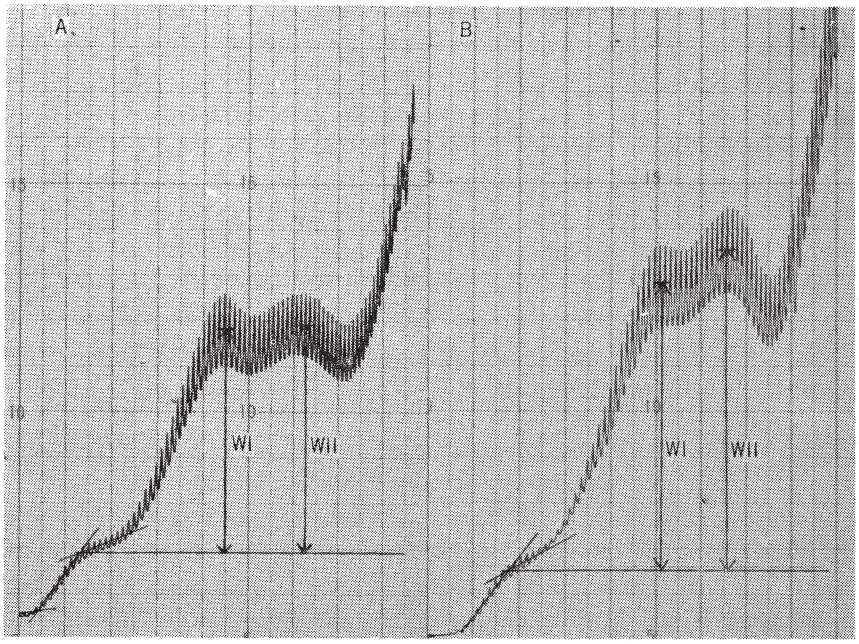

FIG. 2. The determination of the height of the protein double wave. Denative reaction by KOH for 5 min.
Curve A, rabbit serum of L.C. group; Curve B, rabbit serum of L.N. group.

method of Rue-Kueither. Takahashi's modification[14] of Rue-Kueither DNP method was used for determination of serum ascorbic acid content.

B. Polarographic protein wave activity of the rabbit serum

White rabbits weighing about 2–3 kg were divided into two groups of L.N. and L.C. group by the diet used and the period of feeding as same as in experiment A. The rabbit serum was prepared for the measurement of polarographic protein wave activity. For this study, the native reaction and the denative reaction by KOH were performed. Sensitivity of polarograph (Yanagimoto Pa 102) is 0.4 μA per mm. 600 μF. Drop time of the dropping mercury electrode in 4 seconds. Temperature is 20°C. The supporting electrolyte: 2.0 ml 0.01 M $CoCl_2$, 2.5 ml 5 M NH_4OH, and distilled water 18 ml. Native reaction: 0.1 ml of 1/10 diluted serum was added as to cobaltous solution and polarographed.

Denative reaction: 0.1 ml of the rabbit serum was added to 8.0 ml distilled water containing 1 M KOH 1.0 ml. The mixture was kept 5°C for 5 min. One ml of this solution was added to cobaltous solution and subjected to polarographic analysis. The height of the double wave of the proteins was measured from the diffusion current for the reduction of divalent cobalt to the median point of the maximum (Fig. 2).

Experiment 2. Histochemical examination of SH, SDH, and ascorbic acid in the atherosclerotic rabbit aorta

Experimental animals were divided into four groups of E.N. group, L.N. group, E.C. group, and L.C. group as same as in experiment 1 A. Each group consisted of 3–5 rabbits. The thoracic aorta were obtained from ascending portion. Preparation of section: Frozen sections were cut in pair with the normal control in the Pearse cryostat[15] and kept at −20°C. Then they were attached to a thinner slide glass by momentary thawing by the heat of finger. Dihydroxy dinaphthyl disulfide (DDD) method as described by Barnett and Seligman[16] was used for the demonstration of SH activity. SDH activity was demonstrated using slightly modified Nitro BT method after Nachles[17]. (incubation time is 1 hour). Ascorbic acid content of the thoracic aorta were demonstrated by the Sosa's method[18].

Experiment 3. SH content, ascorbic acid content, and polarographic protein wave activity in the atherosclerotic human serum

Nonsclerotic control and atherosclerotic subjects were selected among the patients hospitalized in Nagoya University Hospital and Seto Tosei Hospital. Diagnosis of atherosclerosis was made on clinical and laboratory examinations which included X-ray, ECG, and fundoscopy, etc. Nonsclerotic control subjects were convalescencing from such diseases as peptic ulcer, gastritis, and acute bronchitis. It was ascertained that their brains, hearts, and kidneys were not affected, and their ages were above fifty. The patients given vitamin prepa-

ration were excluded for the measurement. The atherosclerotic subjects consisted of fifteen cerebral atherosclerotic and five coronary atherosclerotic patients. The determinations of SH content, ascorbic acid content, and polarographic protein wave activity of the atherosclerotic human serum were carried out. SH content of the serum was determined by Benesch's modification of amperometric method. At the same time, the total serumprotein was measured by protein refractometer. Measurements of ascorbic acid content of the serum were performed by Takahashi's modification[14] of DNP method. Polarographic protein wave activity of the human serum was measured. For this measurement, native reaction, and denative reaction by KOH were performed.

Supporting electrolyte: 1 ml 0.01 M $[Co(NH_3)_6]Cl_3$, 0.2 ml 5 M NH_4OH, 0.2 ml 5 M NH_3, 23.6 ml distilled water.

Native reaction: 0.1 ml of 1/10 diluted serum was added to cobaltic solution and polarographed.

Denative reaction: 0.5 ml serum was treated with 0.75 ml of 1 M KOH kept 5°C for 5 min or 30 min, and 0.1 ml of this solution was added to cobaltic solution and subjected to polarographic analysis.

RESULT

Experiment 1

A. The difference are expressed as follows: when statistically calculated.

$P<0.01$	significant.
$0.01<P<0.05$	tendency to increase or to decrease.
$P>0.05$	not significant.

Table 1 shows SH content of the rabbit tissues. The tendency to decrease in SH content in the aorta of E.C. group was observed in the second week in comparison with E.N. group. SH content of the aorta of L.C. group in the twelfth week significantly decreased as compared with L.N. group. In the liver no significant difference was seen between E.N. and E.C. groups, and also in the twelfth week no significant difference of SH content was observed between L.N. and L.C. groups. SH content in the serum of E.C. group tended to decrease in comparison with that in E.N. group, and that of L.C. group in the twelfth week significantly decreased in comparison with the late normal.

TABLE 1. Total SH Contents of Thoracic Aorta, Liver, and Serum of Rabbit

	Thoracic Aorta (μg/dl)	Liver (μg/dl)	Serum (μMol/dl)
E.N. group (9)	5.49±0.90	41.0±5.1	53.4±8.3
E.C. group (9)	4.73±0.66	42.7±7.1	43.0±8.8
L.N. group (9)	*5.37±0.99	46.4±7.3	*50.3±6.5
L.C. group (9)	*4.01±0.94	48.0±5.6	*41.4±8.6

Values: Mean±S.D.
Numbers in parenthesis indicate numbers of case.
*, Difference is statistically significant, between L.N. and L.C. group.

SDH activity is expressed by oxygen consumption γ O_2/min/mg (Table 2). In the aorta the oxygen consumption of E.C. group tended to decrease in comparison with that of N. group. The rate of the oxygen consumption of L.C. group became lower than that of N. group, and a significant difference was observed between N. and L.C. groups. In the liver no significant difference was seen between N. and E.C. groups, and the oxygen consumption of L.C. group showed a significant decrease in comparison with that of N. group. Table 3 shows ascorbic acid content of rabbit tissues. Ascorbic acid content of the aorta of E.C. group tended to increase in comparison with E.N. group, and a significant increase was also observed in L.C. group in comparison with L.N. group. In the serum and the liver no significant difference was observed between E.N. and E.C. groups and between L.N. and L.C. groups.

TABLE 2. Succinic Dehydrogenase Activity of Thoracic Aorta and Liver of Rabbit. Activity expressed as oxygen consumed γO_2/min/mg dry tissue

	N. group	E.C. group	L.C. group
Thoracic Aorta	0.054±0.012 (5)	0.038±0.015 (7)	*0.037±0.018 (6)
Liver	0.564±0.124 (5)	0.574±0.102 (6)	*0.274±0.072 (5)

Values: Mean±S.D.
Numbers in parenthesis indicate numbers of case.
*, Difference is statistically significant between N. and L.C. group.

TABLE 3. Total Ascorbic Acid of Thoracic Aorta, Liver and Serum of Rabbit (mg/dl)

	Thoracic Aorta	Liver	Serum
E.N. group (7)	1.13±0.33	17.6±2.4	0.74±0.12
E.C. group (7)	1.22±0.49	25.6±5.9	0.67±0.24
L.N. group (7)	*0.86±0.21	22 1±4.8	0.74±0.23
L.C. group (7)	*1.22±0.46	29.9±5.4	0.74±0.28

Values: Mean±S.D.
Numbers in parenthesis indicate numbers of case.
*, Difference is statistically significant between L.N. and L.C. group.

TABLE 4. Polarographic Protein-wave Activity of Rabbit Serum

	Native Reaction		Denative Reaction	
	Wave I	Wave II	Wave I	Wave II
L.N. group (9)	27.4±3.1	29.8±2.6	*30.3±3.4	*32.2±3.8
L.C. group (9)	27.8±2.4	30.1±3.1	*26.9±2.0	*29.7±1.6

Values: Mean±S.D.
Numbers in parenthesis indicate numbers of case.
*, Difference is statistically significant between L.N. and L.C. group.

B. The polarographic protein wave activity is represented by the height of the waves, and the results were shown in Table 4.

In the rabbit serum: No signifiant difference in the height of the waves of L.N. and L.C. group was seen in the native reaction and a significant decrease in the height of both wave I and wave II of L.C. group in the denative reaction was seen in comparison with that of L.N. group.

Experiment 2

SH staining: The shade of pink color indicated the local concentration of SH compounds. In the normal control, diffuse pink color staining was observed from the intima to the outer media and pink color was not confined in the cells but observed in the elastic fibre and the interstitium. The same saining intensity in the section of the early cholesterol group in the second week was observed in comparison with the normal control. The reduction of the staining intensity was observed in the intima and the inner media of the section with no atheroma in the twelfth week following cholessterol feeding (Fig. 3).

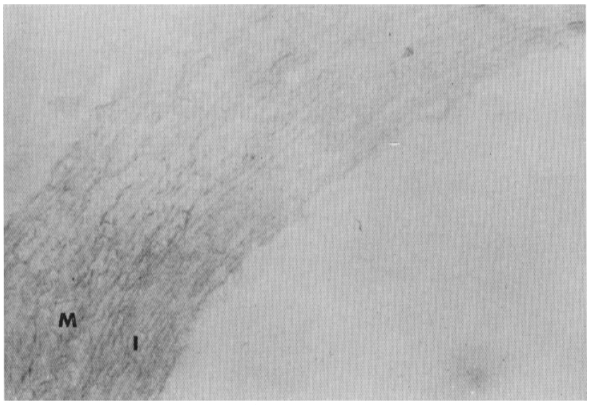

FIG. 3. SH content in the aorta with no atheroma in the twelfth week following cholesterol feeding.
Pink color indicates SH concentration. (by DDD method ×100) M: media, I: intima.

In the section of the late cholesterol group, where a remarkably advanced formation of atheroma was found, the staining intensity of SH compounds decreased significantly in atheroma and decreased remarkably in the inner media (Fig. 4).

SDH staining: The brown colored deposition of formazan glanules revealed the sites of intracellular SDH activity. In the normal control section of the aorta, the brown granules were observed gathered in many insular groups in every layer and the deposition of formazan was confined in the cells. In the

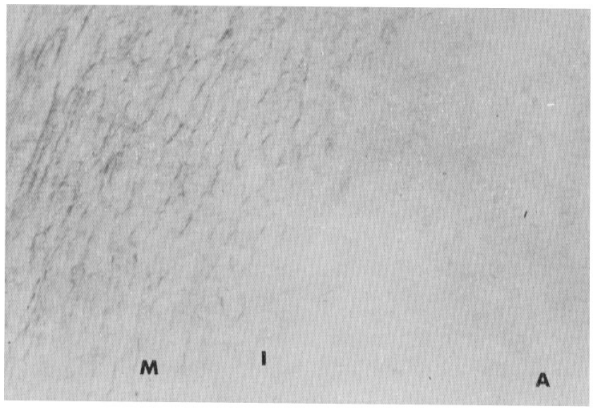

FIG. 4. SH content in the aorta where a formation of the atheroma was found, in the twelfth week following cholesterol feeding.

Pink color indicates SH concentration. (by DDD method ×100). M: media, I: intima, A: atheroma.

section of the early cholesterol group, increase of the staining intensity was observed in some cases and a decrease of the brown color in other cases compared to the normal section. Thus no difference was observed between E.N. and E.C. group. In the section of the late cholesterol group, the decrease of the staining intenstity was found in the intima and the inner media, although large groups of brown granules were observed in atheroma (Fig. 5).

Ascorbic acid staining: Brown granules of silver nitrate indicated the presence of reduced ascorbic acid. Spotty brown granules were observed in

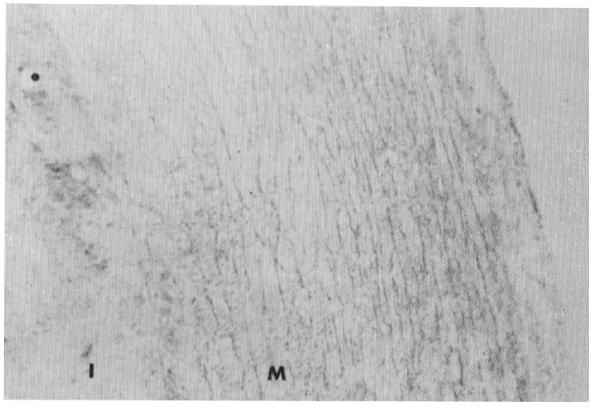

FIG. 5. SDH activity in the aorta of the rabbit in the twelfth week following cholesterol feeding.

Brown diformazan deposite indicate sites of SDH activity. (by Nitro BT method ×100). M: media, I: intima.

every layer in the normal control section. In the second week, no difference of the staining intensity was shown between E.N. and E.C. groups. In the section of the late cholesterol group, increase of the staining intensity was found in the intima and the inner media, and number of the brown granules was increased there (Fig. 6).

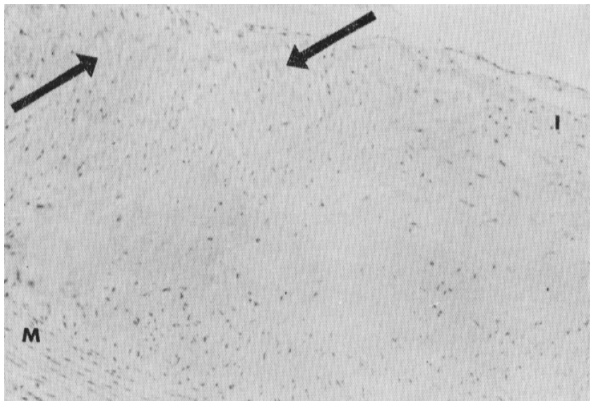

FIG. 6. Ascorbic acid content in the aorta of the rabbit in the twelfth week following cholesterol feeding.

Brown granules (arrows) indicate the presence of reduced ascorbic acid (by Sosas method ×100). M: media, I: intima.

Experiment 3

The atherosclerotic subjects consisted of fifteen cerebral and five coronary atherosclerotic group. SH content of atherosclerotic subjects tended to decrease in comparison with that of the non-sclerotic control group (Table 5). No relations exist between SH content and the amount of the serum protein (Fig. 7).

TABLE 5. Total SH Contents of Human Serum

Nonsclerotic (20)	55.5±6.7 μMol/dl
Atherosclerotic (20)	50.6±8.8 μMol/dl

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

In the human serum no significant difference in ascorbic acid content was observed between the non-sclerotic control and the atherosclerotic groups, as shown in Table 6. Polarographic protein wave activity of the human serum was shown in Table 7. In the native reaction the height of wave 1, no difference was observed between the non-sclerotic and the atherosclerotc groups. In wave II, a significant decrease was observed in the atherosclerotic group. In

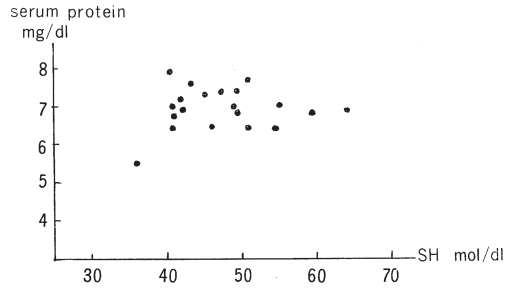

FIG. 7. Relation between SH content and serum protein of atherosclerotic human serum.

TABLE 6. Total Ascorbic Acid of Human Serum

Nonsclerotic (20)	0.64±0.21 mg/dl
Atherosclerotic (20)	0.56±0.29 mg/dl

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

TABLE 7. Polarographic Protein-wave Activity of Human Serum

	Native Reaction		Denative Reaction 5 min		Denative Reaction 30 min	
	Wave I	Wave II	Wave I	Wave II	Wave I	Wave II
Nonsclerotic (20)	18.3±1.55	*24.4±1.9	*21.4±1.2	*29.8±2.5	23.4±1.4	37.5±2.8
Atherosclerotic (20)	17.0±2.28	*21.6±2.9	*19.1±1.8	*26.2±3.0	21.2±1.6	35.0±3.5

Values: Mean±S.D.
Numbers in parenthesis indicate numbers of case.
*. Difference is statistically significant between nonsclerotic and atherosclerotic.

5 minutes' denative reaction a significant decrease was observed in both wave I and wave II of the atherosclerotic group, and in the 30 minutes' denative reaction, a tendency to decrease was observed in both wave I and wave II of the atherosclerotic group.

DISCUSSION

Such SH compounds as SH enzymes, proteins, and glutathione exist in the living body. SH enzymes were investigated by Barron and Singer[7] and more than fifty of them have been found. They stated that SH enzymes play an important role in the metabolism. Wills[19] reported that such SH enzymes as choline oxidase, urease, glyoxalase, papain, and SDH, etc., were generally more susceptible to peroxides than non-SH enzymes. Bernheim and others[20] also stated that some oxidative enzymes were inhibited by fatty acid peroxides.

Ethyloleate peroxides had the capacity of oxidizing SH compounds into SS and further into SO_2 or SO_3[21].

The protein molecules in the body consist of many and various polypeptide combined by SH with one another. The changes of solid structure of the proteins are considered to correlate with the changes of protein-SH. There are many reports on the relation between changes of the proteins constituting the structure of the arterial wall and atherogenesis. Using electrophoretic equipment, Esaki[22] revealed that amino acids of the aortic wall in atherosclerosis of the human being and rabbit increase or decrease significantly in comparison with the control. According to Imamura[23], in the aorta of the rabbit fed lanolin for a few weeks, an increase of aspargic acid and glutamic acid and decrease of alanin and glycine were observed. This result was also said to be consistent with that of the age-dependent changes of the constitution of the amino acids contained in the elastin of the arterial wall. Shimizu[24] reported that in the experiment of the amino acids by Ninhydrin-Schiff staining, Ninhydrin-Schiff positive substances of the fibre and interstitium in the media and elastic lamella decreased in the atherosclerotic aorta of the rabbit. From the results mentioned above, it has been made clear that at the early stage of atherosclerosis, the structural changes of the protein constituting the aortic tissue take place. However, it has not yet clear how such changes are induced. To our knowledge there is one report on glutathione in the atherosclerotic aorta by Kirk[25]. He reported that the glutathione content in the human aorta increase with age. The glutathione content in the liver and blood red corpuscle of old rats increases in comparison with that of young rat. This is because oxidized glutathione increases. Atherosclerosis is found among comparatively aged human beings, and it may be based on the changes with the aging of the arterial wall. Accordingly, the glutatione content in the arterial wall are considered to have some relation with the course of atherosclerosis.

In this study, SH content was measured using amperometric method and histochemical method in order to reveal the influence of cholesterol feeding on the proteins in the aorta of the rabbit through changes of SH content. The total SH content measured by amperometric method is the sum of protein-SH and nonprotein-SH. Most of nonprotein-SH in the living body exists as reduced glutathione. SH stained by histochemical method is mainly protein-SH. In the present experiment, the tendency to decrease total SH was observed biochemically in the second week following cholesterol feeding in the aorta, wheareas histochemical investigation did not show any difference in SH content between two-weeks cholesterol-fed group and the normal group. From these results, it may be assumed that nonprotein-SH might be at first influenced following cholesterol feeding and that the changes of protein-SH followed. In the twelfth week a significant decrease of SH content was observed in the aorta of the cholesterol-fed rabbit in comparison with the control. In the intima and the

innermedia, the decrease of SH content was shown histochemically. However, it may be considered that the structural protein in the aorta may be affected at the comparatively early stage of atherosclerosis following cholesterol feeding, due to the reduction of the staining intensity observed in the intima and the inner media of the section with no atheroma in the twelfth week following cholestrol feeding. The decrease of the staining intensity was not confined in the cells, but also observed in the elastic fibre and the interstitium. This shows that the alteration in the structural protein takes place also in these places. With recent use of the electron microscope in the medical experimental field, the structure of intracellular organellae became clear. In the intima and the inner media, the swelling of the mitochondria of the smooth muscle cells and the enlargement of the endoplasmic reticulm of the smooth muscle were observed in atherosclerosis[26]. Kato[27] reported that the low electron density of the mitochondria substratum, and the decrease in the cristae in the intima cells of the atheroseclrotic rabbit aorta were observed. These morphological changes of the wall are reflected functionally by the changes of the enzymatic ativities and metabolic rates of the tissues. There are many reports[18)~40)] on the metabolism of the arterial wall. It became clear that the metabolism of arterial wall is much more active than ever realized.

There have been many reports on SDH activity, one of SH-enzymes, in the aorta of rabbits. Zemplenyi and others[31] reported that in the case of rabbits fed cholesterol every day, SDH activity increased in about one week and then decreased gradually below the normal level. In our former experiment[32] of the rabbit fed 1 g of cholesterol per day, the tendency of increase of the activity in the fourth week and decrease in the twelfth week was observed. In the present experiment of the rabbit fed 2 g of cholesterol per day there was decrease of the activity in the second week and significant decrease in the twelfth week. Histochemically no changes of the activity in the second week and the significant decrease in the twelfth week were observed. It was found that in the second week no remarkable organic changes are produced because of significant decrease of SDH activity if we consider SDH activity as indicator of the advancemeat of arteriosclerosis. The thickening of the intima is not observed in the second week. From the above-mentioned histochemical point of view, if we consider the reports and results of atherosclerosis, it is assumed that the SDH activity of the rabbit aorta might be partially impaired at the early stage of atherosclerosis and increased as mesenchymal cell reaction of the arterial wall to the injury and decreased with advance of extensive damage of the arterial tissue.

The second week after cholesterol feeding when the decrease of protein-SH was not observed in the aorta, the decrease of protein-SH was already noted in the serum. This shows that the qualitative changes of the serum protein had occurred, before the changes of the structural protein of the wall did not

take place. As mentioned above, SH radical is oxidized to S-S. In order to investigate total SH and S-S content of the serum, protein waves of the serum was investigated with aid of polarography. It has become an established fact that the double wave of the serum protin is produced in polarograph by the compounds containing SH radical in them. In reference to the significance of the first and second waves, some authors concluded that the second wave depended on cysteine and cystine, and others believed that the first wave was most likely dependent on the bonding of the amino acids in the polypeptide chain[33]. Thus the investigation of this problem is not yet completed. But there is no objection to the origin of the second wave. In the native reaction, only free-reactive-SH is concerned with the determination of the wave height. In the denative reaction free-reactive-SH and sluggish-SH participate in the determination of the wave height. Accordingly, the polarographic protein wave activity is influenced by the structure of the proteins and SH/SS ratio in the denative reaction. In the rabbit serum, the native reaction did not show any difference between the cholesterol-fed group and the normal. This shows that SH of the surface of the protein is not altered remarkably. On the other hand, it is inferred from the significant low level of the wave height after KOH denaturation as compared to the normal the protein in the serum is qualitatively denatured, same as the inference from the result of the amperometric titration.

For the purpose of knowing whether such alteration of the serum proteins in the rabbit is observed in the human, we compared the serum of cardiac or cerebral atherosclerotic patients with the serum of the normal control group of same age. Total SH content was decreased in the cordiac or cerebral atherosclerotic patient group. Measurements of SH content by amperometric and polarographic methods are influenced by the amount of the total protein and AG ratio. In this case, no relations exist between the SH content and the amount of the proteins. Accordingly, not only quantitative changes but also qualitative changes are supposed to take place. Wakabayashi[34] reported that the reduction of the wave height in polarograph of the serum was induced by X-ray irradiation whose toxic effect is similar to that of lipoperoxides.

No difference of ascorbic acid content in the aorta was observed in the second week between in the cholesterol-fed rabbit and in the control rabbit. The significant increase of ascorbic acid in the aorta of the cholesterol-fed rabbit was shown in the twelfth week in comparison with the control. Regarding to the relation of ascorbic acid with the atherosclerosis, Myasnikov[35] stated that ascorbic acid kept the disease from advancing, whereas, vitamin D accelerated its advance. Wills[36] also reported that atherosclerotic lesions were found in the aorta of the scorbutic guinea pigs. In one point of view, ascorbic acid is needed in the synthesis of collagen and plays an important role in the wetabolism[37)~39]. The significant increase in ascorbic acid of the aorta ap-

parantly is to repair the tissues. This fact was observed more clearly by histochemical experiment. As shown in Fig. 6, brown granules of ascorbic acid significantly increase in atheroma. The decrease in ascorbic acid of atherosclerotic human serum was reported. In the present experiment, no significant difference in ascorbic acid was observed between the nonsclerotic control and atherosclerotic groups. The same result was observed in the serum of the cholesterol-fed rabbit. Ascorbic acid has the nature of being antioxidant by its active-H. On the other hand it promotes lipid autoxidation as catalyzer with hematin and Mn. It can be said that the increase of ascorbic acid observed in this experiment is favorable to the peroxide production of the lipid in the aorta.

Almost tenfold amount of SH and SDH activity is contained in the liver compared with that in the aorta. The changes in SH content and SDH activity in the liver take place later in comparison with the aorta. This is considered to be due to the fact that the liver contained the large amount of SH compounds.

It is said that the fundamental form of atherosclerosis from the pathological and histological standpoint is a hyalinosis of the intima. Hyalinosis of the intima is caused by infiltration of the serum proteins. It has been not yet clarified, how the serum proteins are denaturated and deposited there. In the present experiment SH content of the serum of the patients suffered from atherosclerosis has no relation with the total protein content in the serum. SH content and SDH activity in the liver of the rabbit was normal in the second week. Accordingly, the decrease of SH in the serum protein may be due to nonenzymatic effect of toxic agent on the proteins rather than due to disturbance of protein metabolism in the liver. The characteristics of this agent are as follows: It converts easily SH of glutathione, SDH and the proteins into SS, and advances chain reaction of SH to SS in the protein molecules. In this experiment decrease of SH content in the serum was considered to be induced by hyperlipemia. Therefore, this agent has relation with the lipid. The influential substance possessing the above-mentioned effects may be peroxides as reported by Tappel and Fukuzumi. Sinex[41] revealed that the fluorescent substances of the elastin from the arterial wall originates from peroxides and indicated the possibility of the production of peroxides in the organism. Considering the liability of SH compounds to the oxidative degradation by peroxides, it may be considered that peroxides are concerned with the decrease of SH compounds in the arterial wall.

The living body exists always under the influence of oxygen, radial-ray, heat and metal ion which cause the autoxidation of the lipid, especially unsaturated fatty acids. The disturbance of the protein in the serum and aorta induced by lipoperoxides was observed in the course of atheroselerosis, and this disturbance was shown in the serum earlier than in the aorta. The aorta

contains a very little amount of SH compounds and proteins in comparison with the liver. Accordingly, the fact that the aorta loses SH is exceedingly serious to itself. In consequence, it is my opinion that in order to prevent the occurrence and advance of atherosclerosis I should use the antioxidant and the medicament which counteract the deleterious effects of peroxides, in order to prevent peroxide production and extirpate peroxides in the serum.

CONCLUSION

The studies were done on SH compounds and ascorbic acid in the aorta, liver, and serum of cholesterol-fed rabbits, and in the serum of atherosclerotic patients. Results were obtained as follows:

1) In the aorta of cholesterol-fed rabbits, decrease of SH content, decrease of SDH activity, and increase of ascorbic acid were observed in comparison with the control rabbits. Histochemically, these changes were observed in the intima and the inner media.

2) SH content in the serum of cholesterol-fed rabbits and of atherosclerotic patients tended to decrease compared with the control. Polarographic protein wave activity decreased in denative reaction.

3) No significant difference in the liver SH content of cholesterol-fed rabbits was observed and decrease of SDH activity was shown after decrease in the aorta.

In the course of atherosclerosis, denaturation of the proteins in the aorta and serum was observed. This denaturation was considered to be caused mainly by nonenzymatic effect of lipoperoxides.

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