

Acta Medica Okayama

Volume 36, Issue 3

1982

Article 1

JUNE 1982

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Yoshimi Higuchi*

*Okayama University,

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Yoshimi Higuchi

Abstract

Measurement of lipid peroxides and alpha-tocopherol was undertaken in rats with streptozotocin-induced diabetes. In sera and livers in diabetic rats, the lipid peroxides increased but alpha-tocopherol decreased. To study the effect of vitamin E deficiency in the diabetic state, diabetes was induced in rats maintained on a vitamin E deficient diet. Serum lipid peroxides increased greatly but alpha-tocopherol decreased. Lipid peroxides and alpha-tocopherol increased in the liver of vitamin E deficient states. In the liver, vitamin E deficient diabetic rats had lower lipid peroxides levels but higher alpha-tocopherol levels than vitamin E deficient non-diabetic rats. On the basis of the present experiments, it was considered that the decrease of alpha-tocopherol might be due to consumption as an antioxidant as lipid peroxides increased in sera and livers. The decrease of lipid peroxides in the liver was thought to play an important part of the increase in serum lipid peroxides.

KEYWORDS: lipid peroxides, α -tocopherol, diabetes mellitus

*PMID: 7113741 [PubMed - indexed for MEDLINE]

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LIPID PEROXIDES AND α -TOCOPHEROL IN RAT STREPTOZOTOCIN-INDUCED DIABETES MELLITUS

Yoshimi HIGUCHI

*Department of Internal Medicine, Okayama University Medical School, Okayama 700, Japan
(Director : Prof. I. Kimura)*

Received October 8, 1982

Abstract. Measurement of lipid peroxides and α -tocopherol was undertaken in rats with streptozotocin-induced diabetes. In sera and livers in diabetic rats, the lipid peroxides increased but α -tocopherol decreased. To study the effect of vitamin E deficiency in the diabetic state, diabetes was induced in rats maintained on a vitamin E deficient diet. Serum lipid peroxides increased greatly but α -tocopherol decreased. Lipid peroxides and α -tocopherol increased in the liver of vitamin E deficient states. In the liver, vitamin E deficient diabetic rats had lower lipid peroxide levels but higher α -tocopherol levels than vitamin E deficient non-diabetic rats. On the basis of the present experiments, it was considered that the decrease of α -tocopherol might be due to consumption as an antioxidant as lipid peroxides increased in sera and livers. The decrease of lipid peroxides in the liver was thought to play an important part of the increase in serum lipid peroxides.

Key words : lipid peroxides, α -tocopherol, diabetes mellitus.

In vivo peroxide synthesis has been thought to be closely related to aging and various other diseases (1-5). In 1952, Glavind *et al.* first demonstrated peroxidized lipids in the wall of human atherosclerotic aorta (2). This finding has been confirmed by many researchers (6). Dormandy *et al.* suggested a close correlation between the peroxide content of erythrocytes in patients with intermittent claudication (including diabetic patients) and worsening of the peripheral circulation (7).

Fogelman *et al.* proposed that modification of low density lipoproteins (LDL) with malondialdehyde may be a prerequisite for the accumulation of cholesterol esters within cells involved in the atherosclerotic reaction (8). Kibata *et al.* reported that serum lipid peroxide levels were high in patients with stroke and myocardial infarction but that α -tocopherol levels were low (9, 10). In the previous paper, I showed that lipid peroxide concentrations in sera, heart muscle and liver obtained from rats with experimentally induced myocardial necrosis (MN rat) increased, whereas α -tocopherol levels decreased (11). It has been reported that levels of serum lipid peroxide were elevated in patients with diabetes (12) and this disease is known to be complicated by atherosclerosis.

In the present study, serum lipid peroxide in rats with streptozotocin-induced diabetes and the lipid peroxide content of the rat livers were measured. α -

Tocopherol in the rat sera and livers was also assayed.

MATERIALS AND METHODS

Patients. Sixty-seven diabetic patients (25 male, 42 female) were included in this study. They were hospitalized or treated at outpatient clinics in the Department of Medicine, Okayama University Medical School or its affiliated hospitals. The age range was from 28 to 75 years. Eighty-three people (age range 29 to 71 years) who were found to have no abnormalities at an in-hospital physical checkup (so-called "Human Dock") performed at either the Okayama Red Cross Hospital or the Mizushima First Hospital served as healthy controls. Healthy controls consisted of 58 males and 25 females. Blood was collected in the morning fasting state.

Animals. Male Wistar rats weighing 150 to 200 g were used and were maintained on laboratory chow pellets and water *ad libitum*. Groups of rats were maintained on a vitamin E deficient diet.

Chemicals. All chemicals were of reagent grade and were obtained from the following sources: 2-thiobarbituric acid and 1.1.3.3-tetraethoxypropane were from Wako Pure Chemical Industries, Osaka, Japan. Phosphotungstic acid was from Nakarai Chemical, Kyoto, Japan, and dl- α -tocopherol was a gift from Eisai Pharmaceutical Co., Tokyo, Japan. Streptozotocin was from Sigma Chemical Co., St. Louis, U.S.A.

Streptozotocin diabetes in rats. Adult male rats, fasted for 12 h, were injected i.v. with 75 mg/kg body weight of streptozotocin prepared in 0.05 M citrate buffer (pH 4.5) immediately before injection. Injections were done *via* a tail vein under mild ether anesthesia. Rats were segregated into 3 groups and rats from each group were killed by venesection under deep ether anesthesia one week, two weeks and 20 weeks after injection.

Streptozotocin diabetes production in vitamin E deficient rats. Male Wistar rats weighing 50 to 60 g were fed with a vitamin E deficient diet (obtained from Eisai Pharmaceutical Co., Tokyo, Japan). When the rats had grown to 150 to 200 g body weight, streptozotocin (75 mg/kg body weight) was administered to certain groups of rats *via* a tail vein and all rats were maintained on the vitamin E deficient diet. Controls included vitamin E deficient rats without streptozotocin injections and untreated normal rats. Sixteen weeks after streptozotocin injection, all rats were killed in order to collect blood and livers.

All rats were fasted 12 h before killing and blood was collected *via* the inferior vena cava. Sera were separated from blood samples and glucose, lipid peroxides, α -tocopherol, cholesterol, triglyceride, GOT and GPT were measured. After extensive perfusion with 1.15 % KCl solution, the livers were removed and homogenized with the same solution at 4 °C. Liver homogenates were kept at -20 °C until measurements of lipid peroxides, α -tocopherol and protein contents were done.

Measurement of serum lipid peroxides. Lipid peroxides were measured as a thiobarbituric acid reactive substance (TBARS). Malondialdehyde, a breakdown product of peroxidized lipids, was determined according to the method of Yagi *et al.* (13), described in detail in the previous paper (11). Briefly, serum protein-lipid complexes were precipitated with 1/12 N sulfuric acid and 10 % phosphotungstic acid and this precipitate was incubated with 0.67 % TBA reagent for 60 min at 95 °C. The extracted mixtures were centrifuged at 3000 rpm for 10 min and a portion of the n-butanol phase was removed for fluorometric measurement at 515 nm excitation and 553 nm emission. For a standard solution, tetraethoxypropane was used. Measurements were done with a Shimadzu RF-510 fluorospectrophotometer.

Measurement of liver TBARS. Measurement of TBARS in the liver was done according to the method of Masugi *et al.* (14) with slight modification. Liver homogenates were dissolved with 7% sodium dodecylsulfate (SDS) solution and 0.1 N HCl, then the mixtures were precipitated with 10% phosphotungstic acid. The precipitate was incubated with 0.67% TBA reagent for 45 min at 95 °C, then the mixtures were extracted with n-butanol. The n-butanol phase was removed for spectrophotometry at a wave length of 532 nm. Measurements were done with a Shimazu UV-100-01 Spectrophotometer. Tetraethoxypropane was used for a standard.

Measurement of serum α -tocopherol. Serum α -tocopherol level was measured according to the method of Abe and Katsui (15). Serum 0.2 ml, one ml of distilled water and one ml of absolute ethanol were mixed and the mixture was extracted with n-hexane. The hexane phase was removed for fluorometric measurement at 286 nm excitation and 330 nm emission. α -Tocopherol solution, which was prepared by addition of absolute ethanol to a final concentration of 2 ng/ml, was used as an external standard.

Measurement of liver α -tocopherol. Liver α -tocopherol measurement was done by the method of Tayler *et al.* (16). Liver homogenate was mixed with absolute ethanol and 25% ascorbic acid, then the mixture was preincubated for 5 min at 70 °C. Following addition of one ml of 10 N KOH, the mixture was incubated for 30 min at 70 °C then extracted with n-hexane. The extracted mixture was centrifuged and the hexane phase was removed for fluorometric measurement. Analytical procedures and the external standard were similar to those used in the measurement of serum α -tocopherol.

Liver protein measurement. Measurement of the protein concentration in the liver was done by the Biuret method (17).

Protein concentration was determined according to the standard protein curve obtained using albumin solutions.

RESULTS

TBARS and α -tocopherol levels in diabetic patients. Serum TBARS level was $4.59 \pm$

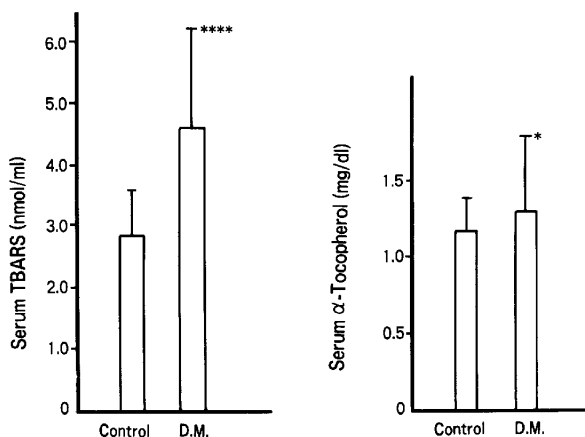


Fig. 1. (*left*) Serum lipid peroxide (TBARS) levels in diabetic patients and healthy controls (mean + S.E.). **** indicates $p < 0.001$.

Fig. 2. (*right*) Serum α -tocopherol levels in diabetic patients and healthy controls (mean + S.E.). * indicates $p < 0.05$.

1.63 nmol/ml for diabetic patients and 2.84 ± 0.77 nmol/ml for healthy controls (Fig. 1). There was a significant difference ($p < 0.001$). The serum α -tocopherol level was 1.30 ± 0.49 mg/dl for diabetic patients and 1.17 ± 0.21 mg/dl for healthy control. The difference was statistically significant ($p < 0.05$) (Fig. 2).

TBARS and α -tocopherol levels in diabetic rats. Rats injected with streptozotocin (75 mg/kg body weight) developed polydipsia, polyuria and glucosuria. The mean of the blood sugar level was 135 mg/dl for the control rats, 274 mg/dl for rats injected with streptozotocin one week before [DM (1 w)], 284 mg/dl for rats injected two weeks before [DM (2 w)] and 339 mg/dl for rats injected 20 weeks before [DM (20 w)] (Fig. 3). These results indicate that the streptozotocin injection definitely induced diabetes in the rats.

The serum TBARS level was 3.12 ± 0.66 nmol/ml for DM (1 w), 2.80 ± 0.28 nmol/ml for DM (2 w), 2.77 ± 0.39 nmol/ml for DM (20 w) and 2.39 ± 0.29 nmol/ml for control rats (Fig. 4). Serum TBARS levels of DM rats were higher than those of control rats and the differences between DM (1 w) or DM (2 w) and the control were statistically significant ($p < 0.05$).

The serum α -tocopherol level was 0.49 ± 0.11 mg/dl for DM (1 w), 0.64 ± 0.19 mg/dl for DM (2 w), 0.40 ± 0.08 mg/dl for DM (20 w) and 0.54 ± 0.04 mg/dl for control rats (Fig. 5). The mean α -tocopherol level of DM (2 w) rats was higher than that of control rats but DM (1 w) rats and DM (20 w) rats had lower α -tocopherol levels than control rats. The difference between DM (20 w) and the control was statistically significant ($p < 0.01$).

The TBARS content of the liver was 0.36 ± 0.04 nmol/mg prot. for DM (1 w) rats, 0.33 ± 0.04 nmol/mg prot. for DM (2 w), 0.35 ± 0.04 nmol/mg prot.

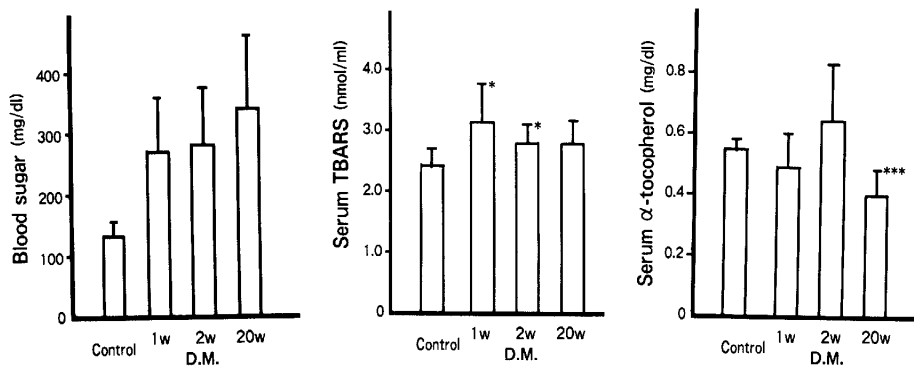


Fig. 3. (left) Blood sugar levels in streptozotocin treated (DM) rats and normal controls (mean + S.E.).

Fig. 4. (middle) Serum TBARS levels in DM and normal control rats (mean + S.E.). * indicates $p < 0.05$.

Fig. 5. (right) Serum α -tocopherol levels in DM and normal control rats (mean + S.E.). *** indicates $p < 0.01$.

for DM (20 w) and 0.29 ± 0.04 nmol/mg prot. for control rats (Fig. 6). Compared to the control, DM (1 w) and DM (20 w) had statistically significant higher values ($p < 0.02$ and $p < 0.05$).

The α -tocopherol content of the liver was 0.054 ± 0.007 μ g/mg prot. for DM (1 w), 0.052 ± 0.015 μ g/mg prot. for DM (2 w), 0.005 ± 0.003 μ g/mg prot. for DM (20 w) and 0.061 ± 0.013 μ g/mg prot. for control rats (Fig. 7). Diabetic rats had a lower content of α -tocopherol in the liver and the difference between the control and DM (20 w) was statistically significant ($p < 0.001$).

Table 1 shows serum cholesterol and triglyceride levels. Diabetic rats had higher serum cholesterol levels than control rats. The difference between DM (1 w) and the control was statistically significant ($p < 0.05$). The difference in triglyceride levels between diabetic rats and control rats did not reach statistical significance. Table 1 also shows values of serum GOT and GPT. Transaminases were slightly elevated in diabetic rats.

Fig. 8 shows α -tocopherol-cholesterol ratios. The value was 0.87 ± 0.09

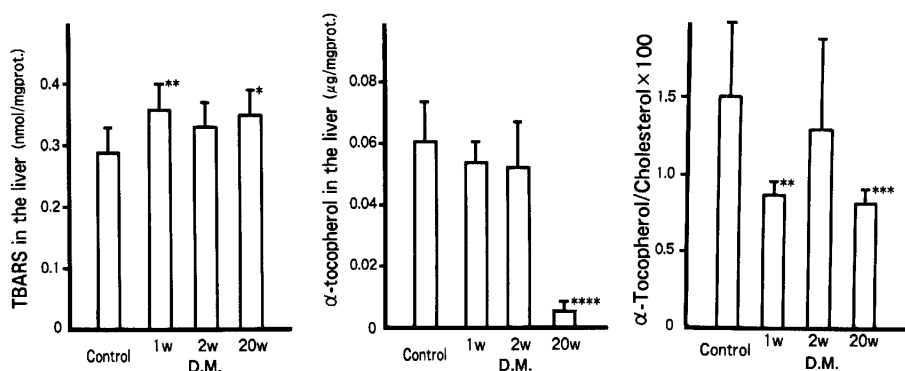


Fig. 6. (left) Liver TBARS contents in DM and normal control rats (mean + S.E.). * indicates $p < 0.05$. ** indicates $p < 0.02$.

Fig. 7. (middle) Liver α -tocopherol contents in DM and normal control rats (mean + S.E.). **** indicates $p < 0.001$.

Fig. 8. (right) Serum α -tocopherol-cholesterol ratios in DM rats and control rats (mean + S.E.). ** indicates $p < 0.02$. *** indicates $p < 0.01$.

TABLE 1. SERUM CHOLESTEROL, TRIGLYCERIDE, GOT AND GPT LEVELS IN DM AND CONTROL RATS

	No. of rats	Cholesterol mg/dl	Triglyceride mg/dl	GOT U/L	GPT U/L
Control	5	43.6 ± 8.6	66.0 ± 13.7	128.0 ± 22.0	45.2 ± 3.2
D.M. 1w	6	$56.3 \pm 9.4^*$	105.8 ± 47.8	204.0 ± 104.6	74.0 ± 33.2
D.M. 2w	5	49.8 ± 11.6	82.4 ± 16.0	172.5 ± 20.2	106.3 ± 15.5
D.M.20w	6	49.0 ± 6.1	70.3 ± 23.3	255.8 ± 176.3	132.5 ± 81.5

* indicates $p < 0.05$.

(Mean \pm S.E.)

for DM (1 w), 1.35 ± 0.59 for DM (2 w), 0.82 ± 0.09 for DM (20 w) and 1.51 ± 0.49 for control rats. When compared to the value for the control, DM (1 w) and DM (20 w) had statistically significant lower values ($p < 0.02$ and $p < 0.01$).

TBARS and α -tocopherol in vitamin E deficient diabetic rats. The mean of the blood sugar level was 121 mg/dl for the non-deficient control, 118 mg/dl for the vitamin E deficient control and 346 mg/dl for vitamin E deficient DM (Fig. 9).

TBARS and α -tocopherol were measured in rats maintained on the vitamin E deficient diets. The serum TBARS level was 8.13 ± 2.30 nmol/ml for vitamin E deficient DM, 8.70 ± 1.80 nmol/ml for the vitamin E deficient control and 3.46 ± 0.41 nmol/ml for the non-deficient control (Fig. 10). Vitamin E deficient diabetic and vitamin E deficient control rats had higher serum TBARS levels than the non-deficient control. The difference between vitamin E deficient diabetic rats and non-deficient controls and that between vitamin E deficient and non-deficient controls were statistically significant ($p < 0.01$, $p < 0.001$). However, the difference between vitamin E deficient diabetic and vitamin E deficient control did not reach statistical significance.

The serum α -tocopherol level was 0.06 ± 0.06 mg/dl for vitamin E deficient diabetic rats, 0.10 ± 0.01 mg/dl for the vitamin E deficient control and 0.54 ± 0.12 mg/dl for the non-deficient control (Fig. 11). Both vitamin E deficient diabetic rats ($p < 0.001$) and deficient controls ($p < 0.001$) had lower levels than non-deficient control rats. However the difference between vitamin E deficient diabetic rats and deficient controls did not reach statistical significance.

The TBARS content of the liver was 0.77 ± 0.23 nmol/mg prot. for vita-

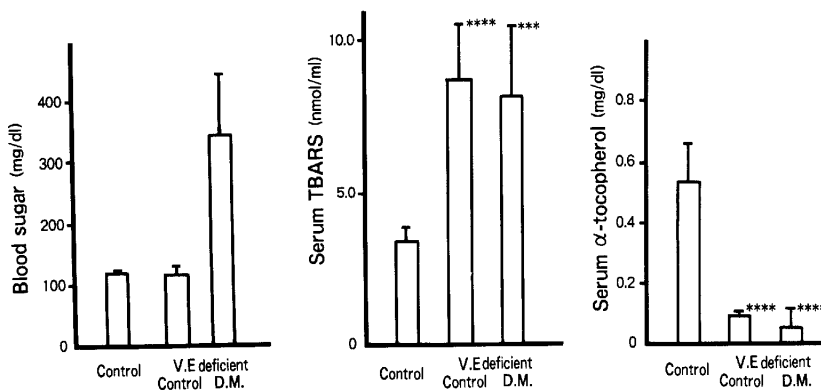


Fig. 9. (left) Blood sugar levels in vitamin E deficient diabetic, vitamin E deficient control, and normal control rats (mean + S.E.).

Fig. 10. (middle) Serum TBARS levels in vitamin E deficient diabetic, vitamin E deficient control and normal control rats (mean + S.E.). *** indicates $p < 0.01$. **** indicates $p < 0.001$.

Fig. 11. (right) Serum α -tocopherol levels in vitamin E deficient diabetic, vitamin E deficient control, and normal control rats (mean + S.E.). **** indicates $p < 0.001$.

min E deficient diabetic rats, 2.60 ± 0.76 nmol/mg prot. for vitamin E deficient controls and 0.44 ± 0.11 nmol/mg prot. for non-deficient control rats (Fig. 12). Both the difference between vitamin E deficient DM and the non-deficient control and the difference between vitamin E deficient controls and non-deficient controls were statistically significant ($p < 0.02$, $p < 0.001$). Vitamin E deficient control rats had a higher TBARS content in the liver than vitamin E deficient diabetic rats ($p < 0.001$).

The α -tocopherol content of the liver was 0.167 ± 0.077 μ g/mg prot. for vitamin E deficient diabetic rats, 0.041 ± 0.01 μ g/mg prot. for vitamin E deficient controls and 0.017 ± 0.004 μ g/mg prot. for non-deficient control rats (Fig. 13). Both groups of vitamin E deficient rats had a higher α -tocopherol content in the liver than non-deficient control rats (diabetic $p < 0.01$, non-diabetic $p < 0.001$). Vitamin E deficient controls had lower values than vitamin E deficient diabetic rats ($p < 0.001$).

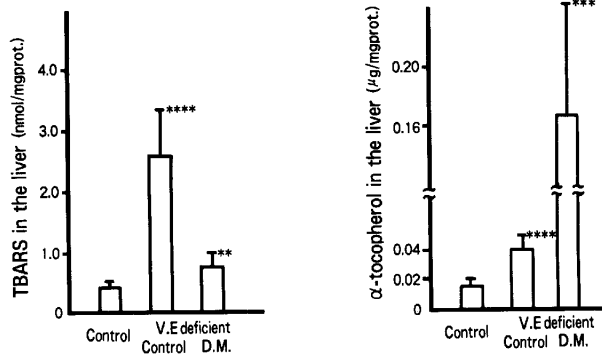


Fig. 12. (left) Liver TBARS contents in vitamin E deficient diabetic, vitamin E deficient control, and normal control rats (mean + S.E.). ** indicates $p < 0.02$. **** indicates $p < 0.001$.

Fig. 13. (right) Liver α -tocopherol contents in vitamin E deficient diabetic, vitamin E deficient control, and normal control rats (mean + S.E.). *** indicates $p < 0.01$. **** indicates $p < 0.001$.

TABLE 2. SERUM CHOLESTEROL, TRIGLYCERIDE, GOT AND GPT LEVELS IN VITAMIN E DEFICIENT DIABETIC, VITAMIN E DEFICIENT CONTROL AND NORMAL CONTROL RATS

	No. of rats	Cholesterol mg/dl	Triglyceride mg/dl	GOT U/L	GPT U/L
Control	5	67.0 ± 2.8	87.4 ± 27.1	127.4 ± 34.2	48.6 ± 10.5
V.E deficient Control	6	$103.7 \pm 7.8^*$	102.3 ± 20.2	300 \uparrow	96.0 ± 22.1
V.E deficient D.M	7	$91.0 \pm 9.5^*$	102.9 ± 15.8	186.7 ± 53.8	78.1 ± 29.8

* indicates $p < 0.001$.

(Mean \pm S.E.)

As seen in Table 2, the serum cholesterol value in vitamin E deficient controls and diabetic rats was higher than that of non-deficient control rats (both $p < 0.001$). On the other hand, as for serum triglyceride levels, the differences between vitamin E deficient rats and non-deficient rats were not significant.

Serum transaminases were elevated in both vitamin E deficient rat groups.

DISCUSSION

It has been reported that serum lipid peroxides levels of diabetic patients were higher than those of normal individuals (12). This was confirmed in the present experiments, which also showed that serum α -tocopherol levels of diabetic patients increased when compared to healthy controls. Similar findings have been reported by others (18). These elevations can be explained on the basis of the frequent complication of hyperlipidemia with diabetes. α -Tocopherol was found in the VLDL, LDL and HDL fractions of lipoprotein (19). In various hyperlipidemias, serum α -tocopherol levels were elevated corresponding to elevation of the VLDL and LDL fractions.

The diabetic state in rats was induced by streptozotocin (20). Various measurements were done at one week, two weeks and 20 weeks after streptozotocin injection. Serum lipid peroxides levels increased in diabetic rats. The lipid peroxides content of the liver increased in diabetic rats when compared to control rats. This increase might be due to metabolic disorder in the liver resulting from the diabetic state or to liver damage from streptozotocin. Although, streptozotocin has been reported to have only a mild toxic effect on the liver (21). Serum transaminases were higher in diabetic rats than in control rats, although there were no significant differences.

Shigeta *et al.* showed that the release of lipid peroxides into the medium from a cultured hepatocyte cell line which had originated from the liver of a rat with streptozotocin-induced ketotic diabetes increased and that the increase was inhibited successfully by addition of insulin to the culture medium (22). The increase in the production of lipid peroxides in the liver seemed to be related closely to insulin deficiency. Therefore, it is conceivable that, in diabetes, chronic metabolic disturbances due to insulin deficiency cause increase in lipid peroxides in the liver and other organs and result in the elevation of serum lipid peroxides.

In the present experiments, serum α -tocopherol levels were not elevated in the rats with experimentally induced diabetes. Moreover, serum lipid levels in diabetic rats were not higher than those of control rats. It was reported that serum α -tocopherol levels were related to serum lipid concentration and more closely to cholesterol levels (23), thus the α -tocopherol-cholesterol ratio might be a useful index for the evaluation of *in vivo* α -tocopherol concentrations. Diabetic rats had lower ratios than control rats (Fig. 8). This finding implies an absolute

decrease of α -tocopherol in diabetic rats. The mean serum α -tocopherol level in human diabetic patients was higher than that in healthy subjects. α -Tocopherol levels of diabetic patients varied greatly mainly due to differences in nutritional status and to the degree of control of diabetes and hyperlipidemia. Further experiments should be carried out to clarify the meaning of this experimental gap. The α -tocopherol content of the liver in DM (1 w) and DM (2 w) was slightly lower than that of the control, and DM (20 w) was definitely low (Fig. 7). In contrast to the increase in lipid peroxides, serum and liver α -tocopherol concentration decreased. These findings suggest that α -tocopherol might be consumed as an antioxidant. It has been shown that antioxidant and membrane stabilizing effects decreased in the vitamin E deficient state (24, 25) but that lipid peroxides increased (26). To assess these findings, lipid peroxides and α -tocopherol in rat sera and livers were measured in a vitamin E deficient diabetic state.

In the first step, vitamin E deficient non-diabetic rats and age-matched control rats were compared. The former had significantly higher serum and liver lipid peroxides than the latter. The serum α -tocopherol level of vitamin E deficient non-diabetic rats was lower than that of the latter presumably because of the vitamin E deficient diet. On the other hand, the liver α -tocopherol content in vitamin E deficient non-diabetic rats increased.

In the next step, vitamin E deficient diabetic and vitamin E deficient non-diabetic rats were compared and there were no significant differences in serum lipid peroxides and α -tocopherol levels. Vitamin E deficient diabetic rats had lower liver lipid peroxides but higher levels of liver α -tocopherol than the vitamin E deficient controls. These findings were not in accord with the results of the comparison of vitamin E deficient non-diabetic and normal rats. This discrepancy can be explained by postulating an increase in liver lipids in the vitamin E deficient state (27) which would result in the increase in liver α -tocopherol. Furthermore, in the diabetic state the increase in liver lipids was higher than in the non-diabetic state (28), resulting in higher α -tocopherol levels in vitamin E deficient diabetic rats than in vitamin E deficient non-diabetic rats. These postulations remain unproven since liver lipids were not measured in the present experiments.

Serum transaminases increased in both diabetic and non-diabetic vitamin E deficient rats. These increases might be due to liver damage in the vitamin E deficient state. More experiments should be carried out to clarify the cause.

In the previous paper (11), it was concluded that the increase in liver lipid peroxides plays a major role in the elevation of serum lipid peroxides in rats with experimentally induced myocardial necrosis. In the present experiments, serum and liver lipid peroxides increased in diabetic and vitamin E deficient rats. Therefore the increase in the production of lipid peroxides in the liver is thought to play an important part in the increase in serum lipid peroxides.

Acknowledgments. The author wishes to thank Professor I. Kimura and Dr. M. Kibata for suggestions. Further thanks are due to Mr. M. Osada and Miss. U. Ueno for technical assistance.

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