

# Ascorbic Acid Metabolism in Diabetes Mellitus

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In contrast to normal subjects diabetic patients had very low plasma ascorbic acid and significantly high ( $p < 0.001$ ) dehydroascorbic acid irrespective of age, sex, duration of the disease, type of treatment, and glycemic control. However, there was no significant difference between the mean leukocyte ascorbate concentrations of the two populations. The *in vitro* rates of dehydroascorbate reduction in the hemolysate and the erythrocyte reduced glutathione levels and the glucose-6-phosphate dehydrogenase activities, which regulate the dehydroascorbate reduction, were similar in normal and diabetic subjects. The turnover of ascorbic acid was higher in the diabetics than that in the normal volunteers. Experiments with diabetic rats indicated that the increased turnover of ascorbic acid was probably due to increased oxidation of ascorbate to dehydroascorbate in tissue mitochondria. Ascorbic acid supplementation at a dose of 500 mg per day for a brief period of 15 days resulted in an increase in the plasma ascorbate level temporarily, but it did not lower the blood glucose level of the diabetic patients.

**I**N A PREVIOUS PAPER<sup>1</sup> it was reported that in contrast to normal subjects, diabetic patients had low ascorbic acid (AA) and high dehydroascorbic acid (DHA) levels in plasma. However, in that paper the patient population was not defined as to their treatment and glycemic control. Also, data from normal subjects having family history of diabetes were not given. Therefore, further study was made to verify the earlier observation and to explore the cause of diminution of plasma ascorbate level in diabetes. Plasma AA/DHA ratio would be a reflection of AA metabolism in tissues and erythrocytes, particularly with respect to oxidation of AA to DHA and reduction of DHA back to AA. Oxidation of AA takes place in mitochondrial fractions of different tissues.<sup>2-4</sup> So, we compared the rate of oxidation of AA to DHA in tissue mitochondrial fractions from normal and diabetic rats. We also compared the rates of reduction of DHA to AA in erythrocytes from normal and diabetic subjects. Since the reduction of DHA is coupled with reduced glutathione (GSH), glucose-6-phosphate dehydrogenase (G6PD EC 1.1.1.49) and glutathione reductase (GR, EC 1.6.4.2),<sup>5-6</sup> we made a comparative assay of these parameters in erythrocytes from normal and diabetic subjects. Incidentally, there are controversial reports regarding erythrocyte GSH and G6PD in diabetes.<sup>7-14</sup> We were further interested to see the effect of AA supplementation on the plasma AA status and the hyperglycemic condition of the diabetic patients.

## MATERIALS AND METHODS

### Subjects

The diabetic subjects were volunteers from the Diabetic Clinic of N.R.S. Medical College and Hospital, Calcutta. They were all confirmed diabetics of the maturity onset type. The duration of the disease varied from 2 mo to 25 yr. During our investigation, 20 subjects were diagnosed as diabetics for the first time and they were grouped as newly diagnosed untreated diabetics. Unless and otherwise mentioned, the normal and diabetic subjects did not get any vitamin C supplementation other than that consumed through food. The calculated dietary ascorbic acid intake of both the normal and diabetic subjects was between 20–40 mg per day. In a separate experiment 12 normal subjects and 12 diabetics were supplemented with 500 mg vitamin C per day for 15 days besides the usual dietary intake.

### Methods

The collection of blood, estimation of blood glucose and estimation of AA and DHA in plasma were made according to the methods published before.<sup>1</sup> In the case of diabetic patients, whose plasma AA was rather low, 0.4 ml of 30% metaphosphoric acid was added to 2.0 ml plasma. In this extract AA could be estimated at a level as low as 0.12 mg/dl plasma. DHA was identified in the diabetic plasma spectrophotometrically.<sup>1</sup> AA in leukocytes was measured by the method of Denson et al.<sup>15</sup>

For estimation of blood glucose, blood was drawn from the insulin treated patients after overnight fasting. In the case of normal volunteers and diabetic subjects taking oral hypoglycemic drugs, blood glucose was estimated 2 hr after taking meal rich in carbohydrate diet. In the case of newly diagnosed diabetics, blood glucose was estimated 2 hr after feeding 1.25 g glucose per kg body weight after overnight fasting.

GSH was estimated in the erythrocyte by the method of Beutler.<sup>16</sup> Erythrocyte G6PD and GR were assayed as described elsewhere.<sup>17</sup>

The rate of reduction of DHA in the erythrocyte hemolysate was measured using an incubation system consisting of 1.0 ml of 1:9 hemolysate, 33.4  $\mu$  moles Sørensen buffer, pH 7.0, 2  $\mu$  moles G6P, 0.075  $\mu$  mole NADP, 0.5  $\mu$  mole oxidised glutathione (GSSG) and 2.84  $\mu$  moles DHA in a total volume of 2.0 ml. The details of the procedure and identity of the AA formed were described before.<sup>5</sup>

Oxidation of AA in rat tissue: Female albino rats (Charles Foster strain) of body weight 200–225 g were made diabetic by a single intra-peritoneal injection of streptozotocin at a dose of 8 mg/100 g body weight. The rats were sacrificed one month after streptozotocin injection. The *in vitro* rate of oxidation of AA in the tissue mitochondria from normal and diabetic rats was measured as follows. Mitochondria were isolated from tissue homogenates in isotonic KCl solution and suspended in definite volumes of 0.025 M

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phosphate buffer, pH 7.3, so that 5 ml of the suspension contained mitochondria from approximately 1 g tissue. Protein was estimated by Folin's method. The incubation system contained 0.5 ml mitochondrial suspension, 0.3 ml of 0.025 M phosphate buffer, pH 7.3, 0.28  $\mu$  mole of AA, 0.01  $\mu$  mole of ferricytochrome C in a total volume of 1 ml. Incubation was carried out at 37°C for 5–60 min in a Dubnoff shaking incubator. Reaction was stopped by adding 1 ml of 10% HPO<sub>3</sub> solution. AA was estimated in the extract as described elsewhere.<sup>1</sup> The product of oxidation of AA was identified as DHA.<sup>1</sup>

RESULTS

Table 1 shows that in contrast to normal subjects, the mean plasma AA level of maturity onset diabetic patients is very low. In 93 cases out of 134 diabetics, the plasma AA content was nil. The mean plasma AA level of normal subjects (age 18–70 yr) was 0.52 mg/dl  $\pm$  0.25 S.D. Table 1 also shows that normoglycemic offsprings of diabetic parents have normal mean plasma AA level. The results were comparable to that obtained from normal controls having similar age groups (21–23 yr) and dietary status. Table 1 further shows that the mean plasma DHA content of the diabetics is significantly higher ( $p < 0.001$ ) than that of the normal subjects. DHA was practically absent in the plasma of normal subjects and all the vitamin C present in the plasma was in the form of AA. On the contrary, only traces of AA were present in the plasma of most of the diabetic patients and whatever vitamin C present in the plasma was in the form of DHA. The plasma AA level of the diabetics remained low irrespective of whether the blood was drawn in the fasting or post-prandial condition. Also, age, sex, duration of the disease and type of treatment had no influence on the plasma AA level of the patients (Table 1). Similar results were obtained from newly diagnosed untreated

diabetics and those treated with insulin or sulfonylurea (Table 1).

The results presented in Table 1 indicate that the plasma AA status was not correlated with the blood glucose levels of the diabetics. Plasma ascorbate was low in patients with apparently uncontrolled hyperglycemia (mean blood glucose 366 mg/dl  $\pm$  46 S.D., ser No.8, Table 1) as well as in those with controlled blood glucose level (109 mg/dl  $\pm$  20 S.D., ser No. 9, Table 1). This was further substantiated by the observation that plasma AA status of a group of newly diagnosed untreated patients did not improve after the patients were euglycemic by effective method of therapy (ser Nos. 4 and 5, Table 1).

The low plasma AA content and the accumulation of a significant level of DHA in the plasma of diabetic patients might be due to (i) increased turnover of AA to DHA and/or (ii) decreased rate of reduction of DHA to AA in the tissues and erythrocytes. However, the rate of reduction of DHA in the erythrocytes was similar in normal and diabetic subjects. The mean rate of reduction expressed in  $\mu$ moles AA formed per g Hb  $\pm$  S.D. was in normal ( $n = 27$ ): 5 min, 7.09  $\pm$  1.26; 10 min, 11.12  $\pm$  1.68; 15 min, 14.42  $\pm$  2.43; and 30 min, 20.89  $\pm$  3.27. The corresponding values in diabetic subjects ( $n = 27$ ) were 5 min, 7.6  $\pm$  2.00; 10 min, 12.12  $\pm$  1.82; 15 min, 14.86  $\pm$  1.93; and 30 min, 19.94  $\pm$  2.93.

Table 2 shows that there is no significant difference in the mean GSH level and G6PD activity between erythrocytes of normal and diabetic subjects. However, the mean erythrocyte GR activity of diabetic patients was significantly higher ( $p < 0.001$ ) than that of normal subjects. Incidentally, the mean erythrocyte

Table 1. Plasma AA and DHA Levels of Normal and Diabetic Subjects

Sl No	Subjects	Age Yr	No of Subjects & Sex	Treatment	Blood Glucose	Plasma AA	Plasma DHA
1	Normal*	18–70	96M/17F	—	74 $\pm$ 8	0.52 $\pm$ 0.25	0.04 $\pm$ 0.06
2	Normal*	21–23	19M/7F	—	71 $\pm$ 6	0.54 $\pm$ 0.31	0.08 $\pm$ 0.10
3	Normal†	21–23	18M/17F	—	76 $\pm$ 5	0.53 $\pm$ 0.32	0.08 $\pm$ 0.14
4	Diabetic‡	21–67	12M/8F	Before treatment	183 $\pm$ 49	0.11 $\pm$ 0.16	0.18 $\pm$ 0.07
5	Diabetic‡	21–67	12M/8F	After treatment	121 $\pm$ 29	0.10 $\pm$ 0.14	0.17 $\pm$ 0.10
6	Diabetic§	20–62	13M/13F	Insulin	261 $\pm$ 88	0.07 $\pm$ 0.11	0.19 $\pm$ 0.09
7	Diabetic§	21–75	39M/18F	Oral**	198 $\pm$ 64	0.07 $\pm$ 0.14	0.19 $\pm$ 0.10
8	Diabetic	37–75	6M/5F	Insulin/Oral**	366 $\pm$ 46	0.09 $\pm$ 0.14	0.21 $\pm$ 0.08
9	Diabetic¶	36–74	10M/10F	Oral**	109 $\pm$ 20	0.07 $\pm$ 0.17	0.16 $\pm$ 0.07

Values represent mean  $\pm$  SD. In the case of insulin treated diabetic patients, blood was drawn in the fasting condition. In all other cases two hours post prandial values are reported.

\*Without family history of diabetes and without prehistory of organic diseases.

†Normoglycemic offsprings of diabetic parents (either one or both) but without prehistory of organic diseases.

‡Newly diagnosed. Sl No 4, untreated; Sl No 5, same patients, after 3 mo of treatment with insulin or oral sulfonylureas.

§Blood samples were collected at random, glycemic control of the patients not known.

||Uncontrolled diabetics, mean blood glucose values 3 months earlier was 332  $\pm$  51 mg/dl.

¶Controlled diabetics, mean blood glucose value 3 months earlier was 151  $\pm$  42 mg/dl.

\*\*Tolbutamide or chlorpropamide.

Table 2. Erythrocyte GSH, G6PD and GR of Normal and Diabetic Subjects

Subjects	Age Yr	No of Subjects and Sex	Blood Glucose** mg/dl	GSH mg/dl RBC	G6PD IU	GR IU
Normal*	18-50	54M/3F	78 ± 5	57.46 ± 7.72	4.77 ± 1.25	0.46 ± 0.24
Normal†	21-23	18M/16F	72 ± 8	59.12 ± 7.50	4.08 ± 1.42	0.87 ± 0.26
Diabetic‡	21-67	4M/3F	185 ± 52	56.33 ± 4.32	5.49 ± 2.15	1.98 ± 0.67
Diabetic§	20-62	9M/7F	251 ± 86	53.92 ± 9.67	4.45 ± 1.14	1.49 ± 0.64
Diabetic	26-75	23M/8F	187 ± 51	57.80 ± 13.46	5.21 ± 1.33	1.31 ± 0.63

Values represent mean ± SD.

\*Without family history of diabetes and without prehistory of organic diseases.

†Normoglycemic offsprings of diabetic parents but without prehistory of organic diseases.

‡Newly diagnosed, untreated.

§Insulin treated.

||Chlorpropamide/tolbutamide treated.

\*\*Fasting value in insulin treated patients; 2 hr postprandial in all other cases.

GR activity of normoglycemic subjects having family history of diabetes was also significantly higher ( $p < 0.001$ ) than that of the normal subjects without family history of diabetes (Table 2).

To see whether low plasma AA level accompanied by a significant content of DHA was an effect of high turnover of AA, AA was supplemented to both normal and diabetic volunteers at a daily dose of 500 mg for 15 days. Table 3 shows that though 24 hours after the last dose of AA there is a similar increase, above basal, in the mean plasma AA levels of normal and diabetics, the increase in the diabetics is only temporary. Seven days after discontinuation of vitamin C supplementation, the mean plasma AA level remained high in the normal subjects, whereas in the diabetics it fell almost to the low level observed before AA administration. It was also observed that after feeding AA for a brief period of 15 days there was no significant change in the mean blood glucose level of the diabetic patients (Table 3).

We considered that the increased turnover of AA in the diabetic subjects might be due to increased oxidation of AA in the tissues. In vitro experiments indi-

cated that the rates of oxidation of ascorbic acid by the mitochondrial fractions of liver, kidney, pancreas and adrenal from streptozotocin induced diabetic rats were significantly higher ( $p < 0.001$ ) than that of normal rats (Fig. 1). However, the mean plasma AA level of pair-fed diabetic rats ( $0.59 \text{ mg/dl} \pm 0.10 \text{ S.D.}, n = 9$ ) was not significantly lower than that of normal controls ( $0.65 \text{ mg/dl} \pm 0.07 \text{ S.D.}, n = 9$ ). This is probably because rat can synthesize AA and make up the plasma level. There was no significant change in the plasma AA level of the diabetic rats when the mean blood glucose level was lowered from  $297 \pm 9 \text{ mg/dl}$  to  $113 \pm 12 \text{ mg/dl}$  ( $n = 6$ ) by treatment with bovine insulin (10 units b.d. for 7 days).

Since plasma ascorbate level may not always fully reflect the tissue store of the vitamin, we also estimated the leukocyte AA contents of the diabetic subjects. Table 4 shows that although the mean plasma total ascorbate level is significantly lower ( $p < 0.001$ ) in the diabetic patients than that of the normal subjects, there is no significant difference in the mean leukocyte ascorbate concentrations between the two populations.

Table 3. Effect of Vitamin C Supplementation on Plasma AA, DHA and Blood Glucose Levels of Normal and Diabetic Subjects

Subjects	Age Yr	No of Subjects and Sex	Ascorbic Acid Supplementation†	Blood   Glucose mg/dl	Plasma AA mg/dl	Plasma DHA mg/dl
Normal	25-40	12M	Before	74 ± 9	0.54 ± 0.24	0.03 ± 0.04
			After‡	73 ± 7	1.01 ± 0.23	0.04 ± 0.02
			Discontinued§	74 ± 6	0.73 ± 0.22	0.04 ± 0.04
Diabetic*	30-50	10M/5F	Before	178 ± 58	0.06 ± 0.12	0.18 ± 0.08
			After‡	183 ± 64	0.57 ± 0.37	0.32 ± 0.14
			Discontinued§	185 ± 70	0.10 ± 0.12	0.19 ± 0.08

Values represent mean ± SD.

\*Chlorpropamide/tolbutamide treated.

†500 mg per subject per day orally for 15 days.

‡24 hr after last dose of AA.

§7 days after discontinuation of AA supplementation.

||2 hr postprandial values.

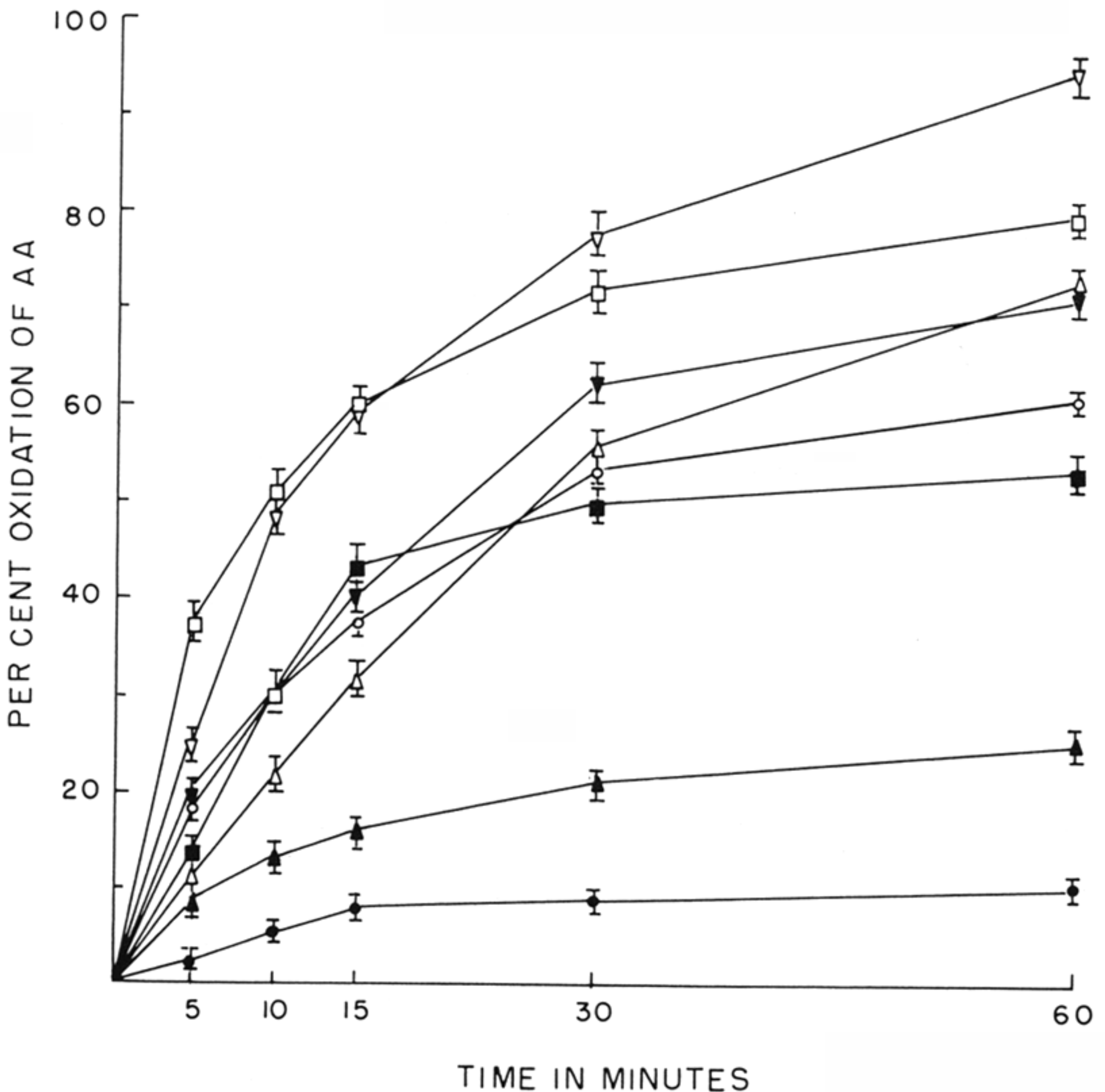


Fig. 1. Rate of oxidation of AA in tissue mitochondrial fractions from normal and diabetic rats. Open symbols represent diabetic rats and closed symbols represent normal rats. ○●, pancreas; △▲, adrenal; □■, liver; ▽▼, kidney. The values represent mean  $\pm$  S.D. from 6 diabetic (blood glucose  $345 \pm 26$  mg/dl) and 6 normal rats (blood glucose,  $78 \pm 3$  mg/dl).

#### DISCUSSION

The results presented in this communication indicate that plasma AA level is very low in maturity onset diabetes irrespective of age, sex, duration of the disease and type of treatment. Also, the plasma AA status is independent of the blood glucose levels of the diabetics. The plasma AA is low in patients with uncontrolled hyperglycemia as well as in those whose blood glucose is controlled by effective method of therapy. This would indicate that the low plasma AA

is due to some metabolic disorder, other than hyperglycemia, associated with diabetes mellitus per se. The nature of the disorder is yet to be known.

The function of AA in the body is not clear. It is presumed that the function is associated with its oxidation to DHA. However, DHA is not detected in the plasma of normal subjects. DHA is reduced to AA in the tissues and erythrocytes by GSH and this reduction is mainly dependent on G6PD and to some extent on GR.<sup>5</sup> The low AA level and the relatively high DHA in plasma of diabetic patients are not due

Table 4. Plasma and Leukocyte Total Ascorbic Acid Contents of Normal and Diabetic Subjects

Subjects	Age Years	No of Subject & Sex	Treatment	Blood Glucose (mg/dl)	Total Ascorbate	
					Plasma (mg/dl)	Leukocyte ( $\mu\text{g}/10^8$ cells)
Normal*	25-50	18M	—	79 $\pm$ 10	0.63 $\pm$ 0.24	25 $\pm$ 7
Diabetic	18-50	8M/10F	Insulin	268 $\pm$ 69	0.24 $\pm$ 0.09	25 $\pm$ 6
Diabetic	36-58	12M/14F	Oral†	196 $\pm$ 58	0.25 $\pm$ 0.07	28 $\pm$ 8

Values represent mean  $\pm$  S.D. Glucose and ascorbic acid measurements were done using blood drawn after overnight fasting.

\*Without family history of diabetes and without prehistory of organic diseases.

†Chlorpropamide/Tolbutamide.

to lack of DHA reduction. The in vitro rate of DHA reduction in the hemolysate and the erythrocyte GSH level and G6PD activity are similar in normal and diabetic subjects. Eppes et al.<sup>8</sup> have also observed that erythrocyte G6PD activity is not altered in diabetes. This is in contrary to the observations of Chanmugam and Frumin<sup>7</sup> and Drel and Shelest<sup>9</sup> who have reported decreased G6PD activity in diabetes mellitus. Contradictory reports regarding the blood GSH level of diabetic patients are also found in the literature. Seltzer<sup>11</sup> reported a decreased blood GSH level in diabetes, whereas Caren et al.<sup>10</sup> did not find any correlation between the blood GSH level and glucose level. Some investigators reported that GSH level in blood was decreased only in diabetes with ketosis.<sup>12-14</sup> The erythrocyte GR activity was found to be significantly higher ( $p < 0.001$ ) in diabetes than that in normal subjects. This was also reported by others.<sup>18,19</sup> However, the high GR activity was not expected to influence the DHA reduction markedly because the latter was not that much dependent on the GR activity as on the G6PD.<sup>5</sup>

The low plasma AA level in diabetes is apparently due to high turnover of AA in the body (Table 3). The high turnover is possibly due to increased oxidation of AA to DHA, because the rate of oxidation of AA is greatly increased in tissue mitochondrial fractions of diabetic rats. In case of increased oxidation of AA to DHA in the body, one would expect a high level of DHA in the plasma of diabetic subjects. Though the mean plasma DHA level of diabetic patients is significantly higher ( $p < 0.001$ ) than that of normal subjects, the value is relatively low (Table 1). This may be explained by the fact that erythrocytes have no permeability barrier to DHA and the uptake of DHA by the red cells is very rapid.<sup>20,21</sup> We also observed that 5 min after addition of 25  $\mu\text{g}$  DHA to 1 ml blood, 85% of the DHA disappeared from the plasma and accumulated in the cells. This would explain that, even in the case of increased oxidation of AA to DHA, plasma DHA level would remain low.

The low plasma total ascorbate level in diabetes

would show that the diabetics were apparently deficient in vitamin C. However, the leukocyte AA concentration was not less in diabetic patients (Table 4). One group of authors<sup>22,23</sup> consider that the leukocyte AA concentrations are more closely related to the tissue status of the vitamin than have the serum levels. On the other hand, others<sup>24,25</sup> have suggested that leukocyte AA levels do not reliably reflect tissue status and that both serum and leukocyte ascorbate levels should be used to do this. Nevertheless, according to the guidelines suggested by the Interdepartmental Committee on Nutrition for National Defense, the Ten State Nutrition Survey, and the Nutrition Survey of Canada, the low plasma total ascorbate level (Table 4) do indicate a poor nutritional status<sup>26-29</sup> of the diabetic subjects. Since the leukocyte AA concentration was not low in diabetes, the low plasma ascorbate level, as mentioned earlier, is apparently due to increased turnover of AA, which is considered to be a metabolic defect associated with diabetes mellitus. Supplementation of AA at a dose of 500 mg per day resulted in an increase in the plasma AA level temporarily. We, therefore, consider that the diabetic patients should be given AA supplementation to maintain the nutritional status. However, further experiments are needed to establish the minimum daily allowances for the diabetics. In variance with the earlier reports,<sup>30,31</sup> AA supplementation did not lower the blood glucose level of the diabetic patients (Table 3). Also, high doses of AA caused a temporary rise in the plasma DHA of the diabetics (Table 3). Though it is not known whether this level of plasma DHA would be harmful to the diabetic patients, yet pharmacological doses of DHA are reported to be neurotoxic<sup>32</sup> and diabetogenic<sup>33</sup> in rats.

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