THE RELATION BETWEEN MELANIN FORMATION AND ASCORBIC ACID

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The relation between ascorbic acid and melanin pigment has been recognized clinically. Experimentally, it has been shown that ascorbic acid has the action of effecting reversion of the process of forming melanin from tyrosine and of inhibiting dopachrome, an intermediate product of the tyrosine-tyrosinase reaction to form melanin. Treatment of pigmented anomaly with massive doses of ascorbic acid has recently come to attract attention. Before reporting its effect, we would like to present preliminarily the results of the studies carried out experimentally on (a) the role of ascorbic acid in the formation of melanin from tyrosine, and (b) the amount of ascorbic acid detected in the skin following administration of massive doses of the acid.

EXPERIMENTAL

Methods

1. Tyrosine-Tyrosinase Reaction

Addition of tyrosinase to tyrosine in buffer solution leads to the formation of dopachrome after a lag period, followed by the formation of 5,6-dihydroxyindole which, in turn, is converted into melanin. As dopachrome is red in color, inhibition by ascorbic acid can be determined colorimetrically (1). The reaction system consisted of 1 ml of 0.01 mg/ml L-tyrosine, 3 ml of *M*/100 phosphate buffer, pH 7.2, and 1 ml of 0.05 mg/ml tyrosinase (Wortington Biochemical Corp.). To this reaction system was added 0.1 to 0.5×10^{-6} *M* sodium ascorbate, and Coleman Spectrophotometer with filter 430 was used for colorimetric determinations.

2. Estimation of Ascorbic and Dehydroascorbic Acids.

Estimation of ascorbic and dehydroascorbic acids was carried out by the 2,4-dinitrophenyl hydrazine method of Roe-Teruuchi (2). Total ascorbic acid is the sum of ascorbic and dehydroascorbic acids.

3. Histochemical Investigation of Ascorbic Acid in the Skin.

Histochemical investigation of ascorbic acid in the skin was carried out by Dean's method (3).

4. Dopa Reaction

Sections of fresh skin, fixed for 2 to 3 hours in 5 % formalin, were examined for dopa reaction with 1 % dopa in *M*/15 phosphate buffer, pH 7.4.

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RESULTS

1. *Interference of Ascorbic acid in Melanin Formation from Tyrosine.*

Formation of Dopachrome and Ascorbic acid — Addition of 0.05 mg/ml tyrosinase to the solutions of 0.1 to $3 \times 10^{-6}M$ sodium ascorbate and 0.25 mg/ml tyrosine in phosphate buffer showed that the lag period was apparently prolonged in the samples containing less than $0.3 \times 10^{-6}M$ ascorbate as compared with the control, and that the formation of dopachrome did not take place in 24 hours in the samples containing more than $0.5 \times 10^{-6}M$ ascorbate (Fig 1).

FIG. 1 Amounts of Ascorbic Acid Interfering in the Development of Dopachrome

Reaction system: 1 ml of 0.01 mg/ml tyrosine, 1 ml of 0.05 mg/ml tyrosinase and 1 ml of pH 6.8 phosphate buffer
 Δ , $0.3 \mu M$ Na ascorbate; $\times$, $0.2 \mu M$ Na ascorbate; $\circ$, $0.1 \mu M$ Na ascorbate; $\bullet$, control; $\square$, 3.0, 2.0, 1.0, $0.5 \mu M$ Na ascorbate.

Inhibition of the Formation of Dopachrome and of Dehydroascorbic Acid.

— $1 \times 10^{-6}M$ sodium ascorbate was mixed with the tyrosine solution, and tyrosinase was added to the mixture. Dehydroascorbic acid and dopachrome were determined at the same time (Fig. 2).

Ascorbic acid was oxidized to dehydroascorbic acid at the early stages of the reaction, 70 per cent being oxidized in 10 minutes and 100 per cent in 30 minutes. The formation of dopachrome began after a lag period of 10 minutes. Addition of ascorbic acid to the tyrosine-tyrosinase reaction system prolonged the lag period in the formation of dopachrome, an intermediate product of the reaction, while most of the ascorbic acid added was oxidized to dehydroascorbic acid. It seems to indicate that ascorbic acid interferes in the formation of dopachrome from dopaqui-

FIG. 2 *Oxidation of Ascorbic Acid During Formation of Dopachrome in Tyrosine-Tyrosinase Reaction*

Reaction system: 1 ml of 0.01 mg/ml tyrosine, 1 ml of 1.2 mg/ml tyrosinase, 1 ml of 0.198 mg/ml sodium ascorbate and 1 ml of S³⁵-lipoic acid, 0.1-2.0 mg.

○, Total ascorbic acid; ×, dehydroascorbic acid; ●, dehydroascorbic acid (control); ---, dopachrome.

none which is thought to proceed as follows: $\text{dopa} \rightleftharpoons \text{dopaquinone} \longrightarrow \text{dopachrome}$. As Rotman (4) has stated that ascorbic acid increases the amount of dopa, it is not likely that the acid is involved in the formation of dopaquinone from dopa, but it acts possibly as the reducing agent in the oxidation of dopaquinone to dopachrome. It does not seem, therefore, to act on tyrosinase. Both thiamine and lipoic acid were shown to exhibit the same kind of interference in the reaction (5).

Inhibition of Dopachrome Formation and of Thiamine Disulfide Formation.

— It has been recognized histochemically or shown by the results of quantitative estimation that thiamine disulfide abounds in the pigmented areas of the skin and the pigment of the hair (5). Addition of thiamine-HCl to the tyrosine tyrosinase reaction system prolonged, as did the addition of ascorbic acid, the lag period and suppressed the consumption of oxygen, whereby thiamine was converted into a disulfide type (Fig. 3).

Inhibition of Dopachrome Formation and of the β -Lipoic Acid Formation

— Like ascorbic acid and thiamine, lipoic acid inhibited also the formation of dopachrome. When more than 1.2 mg of lipoic acid was added to the reaction system, dopachrome formation did not take place within 24 hours, while the formation began after a prolonged lag period when less than 1 mg was added (100 minutes with 1 mg and 50 minutes with 0.1 mg) (Fig. 4). The reaction was suspended after the formation of dopachrome, and the solution was subjected to freeze drying, to which alcohol was added. The alcohol portion was evaporated *in vacuo* at 40° and after dissolution in water it was subjected to paper chromatography using water-saturated butanol as a developing solvent system. It was found that the α -lipoic acid added had turned into β -lipoic acid as shown in Fig. 5.

FIG. 3 Amount of Thiamine Interfering with the Development of Dopachrome

○, 10 µg; △, 50 µg; □, 100 µg thiamine was added; ●, control.

FIG. 4 Lipoic Acid and Formation of Dopachrome

Reaction system: 1 ml of 0.01 mg/ml tyrosine, 1 ml of 0.05 mg/ml tyrosinase and 2 ml of pH 6.8 phosphate buffer. S^{35} -lipoic acid, 0.1–2.0 mg

×, 0.1 mg; ○, 0.2 mg; △, 0.4 mg; □, 0.6 mg; ▲, 1.0 mg; +, 1.2 mg, 1.5 mg, 2.0 mg, lipoic acid; ●, control.

FIG. 5 Formation of Lipoic Acid and its Oxidation Product (?)

Reaction system: 1 ml of 0.01 mg/ml tyrosine, 1 ml of 0.05 mg/ml tyrosinase and 2 ml of pH 6.8 phosphate buffer. S^{35} -lipoic acid, 0.6–1.5 mg.

Effect of Glutathione on the Formation of Dopachrome — Inhibition of tyrosine-tyrosinase reaction by glutathione is said to be due to the inactivation of copper in the enzyme by combining with glutathione. In order to compare the inhibition of glutathione against the formation of dopachrome with that of ascorbic acid, various amounts of glutathione were added to the reaction system. It was shown that complete inhibition took place at concentrations higher than $0.8 \times 10^{-6} M$ glutathione (observed for 120 minutes) and the prolongation of the lag time at higher than $0.02 \times 10^{-6} M$ (Fig. 6). The efficiency as expressed in

FIG. 6 Amounts of Glutathione Interfering with the Development of Dopachrome

Reaction system: 1 ml of 0.01 mg/ml tyrosine, 1 ml of 0.05 mg/ml tyrosinase and 3 ml of pH 6.8 phosphate buffer. Glutathione, 0.02–3 μM .

$\times$, 0.04; Δ , 0.06; $\square$, 0.1; $\blacktriangle$, 0.2; +, 3.0, 2.0, 1.0, 0.8 μM glutathione; $\bullet$, control.

the molar concentration for inhibiting the formation of dopachrome in a decreasing order was as follows: glutathione, ascorbic acid, thiamine, lipoic acid.

Effect of Ascorbic Acid on the Skin Dopa Reaction — Fig 7 shows the results of the test in which the sections of the skin were treated with ascorbic acid before carrying out the dopa reaction tests. It will be seen that 0.8 μM ascorbic acid inhibited the dopa reaction.

2. Absorption of Ascorbic Acid Given Orally in Large Doses

The doses of ascorbic acid given in the treatment of pigmented anomaly range from 1 to 2 g per day orally. Ascorbic acid concentration in the blood and its amount excreted in the urine were investigated following the administration in massive and small doses in order to evaluate the effectiveness of administering massive doses.

Total Ascorbic Acid Levels in the Blood — Following oral administration of 200 mg ascorbic acid, the blood total ascorbic acid levels in four normal subjects

averaged 1.6 mg per 100 ml 3 hours after administration, but the values given 1,000 to 2,000 mg averaged 2.4 mg per 100 ml (Table I, Fig. 7).

FIG. 7 Blood Level of Total Ascorbic Acid after Oral Administration of 1,000 mg Ascorbic Acid Three Times Daily
Arrows show administration of 1,000 mg ascorbic acid.

TABLE I
Blood Level of Ascorbic Acid after Oral Administration
of 0.2, 1.0 and 2.0 g

AsA	No.	Before adm.	1 hour	2 hours	3 hours
g					
0.2	1	0.8	1.0	2.0	2.0
	2	0.8	1.0	2.0	2.2
	3	0.4	0.4	0.8	0.8
	Average	0.7	0.8	0.9	1.6
1.0	1	1.0	1.4	1.8	2.2
	2	1.0	1.2	1.8	2.4
	3	1.2	1.2	2.6	2.6
	4	1.0	1.0	2.4	2.4
	5	0.8	1.0	2.2	2.0
	Average	1.0	1.1	2.4	2.4
2.0	1	0.4	0.6	1.2	1.2
	2	0.8	1.0	2.6	2.6
	3	0.6	1.2	2.4	2.6
	4	1.2	1.6	2.6	2.6
	5	1.0	1.2	2.0	2.2
	Average	0.8	1.1	2.4	2.4

After withdrawing the blood samples early in the morning, 1,000 mg ascorbic acid was administered orally and the blood total ascorbic acid levels were measured after 2, 3 and 5 hours. Then another dose of 1,000 mg was given orally, followed by still another dose of 1,000 mg after 3 hours. The blood total ascorbic acid levels were estimated 3 and 12 hours after the last dose was given. The results show that, as indicated in Fig. 7, the blood total ascorbic acid levels did not rise above 2.0 mg per 100 ml even after frequent administrations of large doses. There seems to be a certain upper limit in the blood level.

Normal subjects were given 2,000 mg ascorbic acid orally three times a day for about 60 consecutive days, and the blood samples were withdrawn at 10:00 a. m. (3 hours after 1,000 mg was given) on the 7th, 14th, 21st, 28th, 35th, 42nd, 49th and 56th days (Fig. 8). The highest level of 2.6 mg per 100 ml was shown

FIG. 8 Blood Level of Total Ascorbic Acid after Oral Administration of 2,000 mg Ascorbic Acid
○, A; ×, K; ●, T.

in case A after 2 weeks, and the values found in the cases K and T remained in the range of 1.8 to 2 mg during the same period.

TABLE II
Amount of Ascorbic Acid in Pigmented and Non-pigmented Skin

No. of cases	Pigmented skin	No. of cases	Non-pigmented skin
	mg/100 g		mg/100 g
2-7	Average 4.8	3-6	Average 4.0

Excretion of Total Ascorbic Acid in the Urine — Following oral administration of 200 mg ascorbic acid, samples of urine were obtained every hour for 10 hours. The amounts of total ascorbic acid excreted by 4 normal subjects in 24-hour urine were 23, 41, 89 and 16 mg, which corresponded to 11, 20, 44 and 8 per cent, respectively, of the amount administered (Fig. 9). The highest level was reached 4 hours after administration.

When 2,000 mg ascorbic acid was administered orally for 30 consecutive days, the amounts excreted in 24-hour urine on the 7th, 21st and 30th days did not show a marked increase (Fig. 10), as compared with the amount excreted before administration, though there was a slight increase. A series of experiments on the excretion of ascorbic acid in the urine seems to indicate that, though a large amount of ascorbic acid was absorbed, the blood total ascorbic acid level did not rise above a certain level, a considerable amount ingested being decomposed in the body, judging from the amounts of total ascorbic acid excreted in the urine (diketogulonic acid was not estimated).

3. Ascorbic and Dehydroascorbic Acids in the Skin Following Administration of Ascorbic Acid in Massive Doses

The results of the experiments showed that ascorbic acid interfered in the formation of the melanin pigment *in vitro* and that 0.1×10^{-6} M ascorbic acid was capable of prolonging the lag period involved in the formation of dopachrome. The question is what proportion of the amount of ascorbic acid administered can reach the skin.

The ascorbic acid levels in the non-pigmented and pigmented areas (dark and

FIG. 9 Urinary Ascorbic Acid after Loading with 2,000, 1,000 and 200 mg
 ×, 2,000 mg; ●, 1,000 mg; ○, 200 mg ascorbic acid.

FIG. 10 Urinary Excretion of Total Ascorbic Acid after Daily Intake of 2,000 mg, as Observed for One Month

brown) of the skin of the guinea pig were estimated. The results showed that there were no marked differences between the amount of the total ascorbic acid in the pigmented and the non-pigmented areas in the same animal, the values found in the former being from 2 to 7 (with an average of 4.8) and in the latter from 3 to 6 mg per 100 g (with the average of 4) (Table II).

The ascorbic acid in the skin was detected histochemically largely in the form of ascorbic acid. It was demonstrated over the wide area extending from the

TABLE III
Amount of Total Ascorbic Acid in the Pigmented and Non-pigmented Skin after Intramuscular Injection of 0.3 g to Rabbits

No. of cases	Pigmented skin	No. of cases	Non-pigmented skin
	mg/100 g		mg/100 g
20-41.5	Average 32.5	32-47	Average 40.3

epithelium to the corium. It was not shown, however, that ascorbic acid was concentrated in the basal layer (Fig. 11).

FIG. 11 *Ascorbic Acid in the Skin (histochemically observed)*

Following the intramuscular injection of 500 mg ascorbic acid to 4 guinea pigs and 4 rabbits the specimens of the skin, weighing 1 to 2 g, were obtained after 1, 2, 3 and 24 hours to estimate the ascorbic and dehydroascorbic acid levels in them (Fig. 12). In guinea pigs, the highest ascorbic acid level (50 to 60 mg per 100 ml) was found in the skin 1 hour after intramuscular administration, and low levels (5 to 10 mg per 100 ml) were found even after 24 hours. Ascorbic acid occupied the majority of the amount increased, and dehydroascorbic acid showed a slight increase in the skin 2 hours after administration. Similar results were obtained in rabbits.

Following the intramuscular injection of 300 mg ascorbic acid to guinea pigs, the samples of the pigmented and non-pigmented areas of the skin were obtained after 2 hours to estimate ascorbic and dehydroascorbic acids (Table III). The results showed that the value found in the pigmented area was not greater than that found in the non-pigmented area, the former being 32.5 and the latter 40.3 mg per 100 ml.

Two patients with osmidrosis were injected intravenously with 5,000 mg ascorbic acid 1 hour before operation. The skin removed by surgery 1 hour later was freed of fat and was divided into the epithelium and the corium with a razor to measure ascorbic acid. The total ascorbic acid levels were found to be 7 in the epithelium and 16.7 mg per 100 g in the corium.

FIG. 12 Amount of Total Ascorbic Acid and Dehydroascorbic Acid in the Skin of Guinea Pigs and Rabbits after Intramuscular Injection of 500 mg of Ascorbic Acid

A, guinea-pig; B, rabbit.

●, total ascorbic acid; ○, dehydroascorbic acid.

DISCUSSION

The effectiveness of ascorbic acid in the treatment of pigmented anomaly has been recognized. In recent years massive doses of ascorbic acid, 1 to 2 g, have come to be given to patients with this condition with success. In order to determine its effectiveness, we are now studying the lightness and tone of color of the skin with photoelectric colorimeter. In this experiments, relation of ascorbic acid to the formation of melanin pigment was studied and the following results were obtained.

- (a) Clinically, ascorbic acid has a therapeutic effect.
- (b) Experimentally, it has a reducing action on the tyrosine-tyrosinase reaction.
- (c) It reduces the formed melanin pigment.

Rientis (6) and Sealock *et al.* (7) have found that there is a derangement of metabolism of tyrosine or L-phenylalanine in animals deficient in ascorbic acid, and have inferred that ascorbic acid may be concerned in the process of melanin formation. Rothman (4) has reported that irradiation with ultraviolet rays results in the reduction of ascorbic acid in the skin, and has claimed that ascorbic acid accelerates the conversion of tyrosine into dopa but inhibits the formation of dopaquinone from dopa. The results of our experiments on addition of ascorbic acid to the tyrosine-tyrosinase reaction system *in vitro* led us to infer that ascorbic

acid might continue to arrest the oxidation process of the reaction system until it undergoes oxidation. In the results of the experiments in which the reaction of the skin for dopachrome was tested, Togashi (8) assumed the combination of ascorbic acid with the copper in tyrosinase which resulted in the inhibition of the tyrosine-tyrosinase reaction. Thiamine and lipoic acid, which can act as an oxidizing and reducing agent in the same manner as ascorbic acid, also inhibited the formation of dopachrome. Of these three substances, it was ascorbic acid that interfered in this reaction at the lowest molar concentration, $0.1 \times 10^{-6} M$.

The skin ascorbic acid concentration is about 1 mg per 100 g in normal persons and 5 mg per 100 g in rabbits. Ascorbic acid in the skin has been found largely as ascorbic acid, but there is no irrefutable evidence that it concerns in the formation of the melanin pigment in the skin. It is thought that ascorbic acid in the skin is concerned with the formation of collagen in the corium and other mechanism, as has been proved histochemically. Since $0.1 \times 10^{-6} M$ ascorbic acid inhibited the formation of dopachrome *in vitro*, $0.01 \times 10^{-6} M$ ascorbic acid which is contained in 1 g of the normal skin must be said to be too small in amount to play a role in the formation of melanin in the normal skin. In the light of this observation, administration of massive doses of ascorbic acid seems to be clinically desirable, for it will reach the skin in necessary amounts.

The results of the experiments carried out in animals (rabbits and guinea pigs) to study the concentration of ascorbic acid in the skin following administration of massive doses of the acid showed that the total ascorbic acid in the skin 1 to 2 hours after administration ranged from 30 to 60 mg per 100 g. In view of the fact that the total ascorbic acid representing an increase over the normal concentration in amount was made up largely of ascorbic acid, it was supposed that it could inhibit *in vivo* the formation of dopachrome, as it did *in vitro*. Our experiments did not show, however, that the ascorbic acid administered found its way to the pigmented area and was retained there in larger amount than to the non-pigmented area, as the experiments conducted by Conbleet (9) and Shigematsu (10) did. It would seem that massive doses of ascorbic acid administered would, considering the amount excreted in the urine, be absorbed in a large amount. However, the facts that the blood total ascorbic acid concentration did not rise above a certain limit and that the amount excreted in the urine did not reach 20 per cent of the amount administered (2 g given orally) even if the administration was continued for over a month, would seem to indicate that ascorbic acid is decomposed in the body at the early stages. Hellman and Burn (11) have reported that, when L-ascorbic acid- $1-C^{14}$ was administered to humans, ascorbic acid and diketo-L-gulonic acid represented 20 per cent of the amount excreted in the urine, but oxalic acid accounted for 40 per cent.

SUMMARY

- 1 Ascorbic acid continued to arrest the oxidation process of tyrosine-tyrosinase system until it undergoes oxidation.
2. The efficiency as expressed in molar concentrations for inhibiting the formation of melanin was, in a decreasing order, as follows: glutathione, ascorbic acid,

thiamine, lipoic acid. The lowest molar concentration of ascorbic acid needed for inhibition was 0.1×10^{-6} M.

3. The skin ascorbic acid level is 1 in normal persons and 5 mg per 100 g in rabbits. After intramuscular injection of 0.5 g ascorbic acid the level was raised to 30 to 60 mg per 100 g in rabbits in 1 to 2 hours, consisting mostly of ascorbic acid rather than dehydroascorbic acid.

4. The highest level of blood ascorbic acid after repeated oral administration, 1 g three times daily, was 2.6 mg per 100 ml.

5. Urinary excretion of ascorbic acid was 10 to 20 per cent of the amounts administered. When 2 g ascorbic acid was administered orally for 30 consecutive days, urinary ascorbic acid in 24 hours on the 7th, 21st and 30th days did not show a marked increase.

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