

Ceruloplasmin in Monkey Plasma<sup>1)</sup>

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An abnormally greenish tint appeared in the blood plasma of adult rhesus monkeys that had been daily administered an intramuscular injection of 1-methyl-2-( $\beta$ -naphthyl)-aziridine or chlorpromazine.

This greenish component in the monkey plasma could not be extracted with cyclohexane, 1,2-dichloroethane or *n*-butyl alcohol, and the greenish tint was scarcely changed by the addition of ethylenediaminetetraacetate. The greenish monkey plasma showed a visible absorption band at 610 nm like the blue fraction separated from it. These absorption bands at 610 nm, both in the greenish monkey plasma and the separated blue fraction, disappeared with the addition of ascorbic acid and reappeared with oxygen, as with human ceruloplasmin (Cp). The electron spin resonance spectrum of the greenish monkey plasma was similar to that of human Cp. Furthermore, the major portion of copper in monkey plasma was in the blue fraction, and the total plasma copper and *p*-phenylenediamine oxidase activity (PPD activity) increased with deepening of the green tint. These results suggested that the abnormal greenish tint of the monkey plasma is due to the presence of the blue copper protein Cp in the plasma.

Based on examination of the relationships between total plasma copper and PPD activity in monkey and rat plasma, monkey Cp activity was compared with those of other mammalian species.

**Keywords**—ceruloplasmin; greenish monkey plasma; 1-methyl-2-( $\beta$ -naphthyl)-aziridine; total plasma copper; *p*-phenylenediamine oxidase activity

An abnormally greenish tint appeared in the blood plasma of adult rhesus monkeys that had been daily administered an intramuscular injection of 1-methyl-2-( $\beta$ -naphthyl)aziridine (I) or chlorpromazine (II). However, such an abnormal greenish tint was scarcely observed in rat plasma.

No reports on an abnormal greenish tint of plasma in human and animals could be found.

We tried to analyze this phenomenon in the monkey plasma by studying the blue copper protein ceruloplasmin (Cp).

## Experimental

**Apparatus**—Copper in blood plasma was measured using a Toshiba-Beckman NF-1B atomic absorption spectrophotometer and a Hamamatsu TV copper hollow cathodelamp.

The combustion of blood plasma for the copper determination was done using a Yanagimoto LT-2S low temperature asher and specially made quartz boats (20 × 35 × 10 mm).

All spectrophotometric measurements were done using a Hitachi 356 and EPU-2A spectrophotometers. Electron spin resonance (ESR) spectra were measured with a Varian V4502 spectrometer. Ultrafiltration of blood plasma was done using Centrifo ultrafilters (Amicon Co., Ltd.). The membrane CF-50A essentially does not retain for molecules of molecular weight below 50000.

**Materials**—Chemicals: The standard copper solution was prepared by dissolving 160.0 mg of high purity copper in 2.5 ml of 7.5 N nitric acid, and diluting this accurately with water to 100 ml. The copper content of this solution was 1.60 mg per ml. A series of standard solutions for the calibration curves were made up from this solution by dilution with water.

A solution of 0.2% (w/v) 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline (bathocuproine) in isoamyl alcohol was used.

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2) Location: Sagisu, Fukushima-ku, Osaka 553, Japan.

Commercial *p*-phenylenediamine dihydrochloride was purified by recrystallization from water and the white crystals were kept in a refrigerator. The solution of *p*-phenylenediamine dihydrochloride (0.5% w/v) was prepared just before use.

**Biological materials:** Greenish monkey plasma was obtained from rhesus monkeys given daily administration of 2.5 and 7.5 mg of I/kg/day for 2 and 4 weeks or 3.5 mg of II/kg/day for 4 weeks. Control monkey plasma was obtained from rhesus and Japanese monkeys. Rat plasma was obtained from Wistar strain. These plasma samples were frozen until use and the supernatants were used after centrifugation.

**Method**—The blue fraction was separated from greenish monkey plasma by the method of Holmberg and Laurell,<sup>3)</sup> as shown in Chart 1. The greenish monkey plasma was condensed by ultrafiltration with the membrane CF-50A. Next, the pH of the greenish concentrate (S-1) was adjusted with 0.1 N acetic acid to 6.2 then to 5.5. The white precipitates (P-1 and P-2) from the solution were isolated by centrifugation. The greenish solution (S-4) was cooled to about 0° in ice-water then precooled 10% (v/v) ethanol was added. The blue precipitate (P-3) was centrifuged at low temperature then dissolved in 0.9% (w/v) sodium chloride. The faint blue solution was condensed by ultrafiltration again, and an equal volume of a mixture of 90% (v/v) ethanol and chloroform (9:1) was added to the concentrate (S-6). The sky-blue fraction (S-9) was obtained by extraction of the blue precipitate (P-4) with a minimal amount of saline solution.

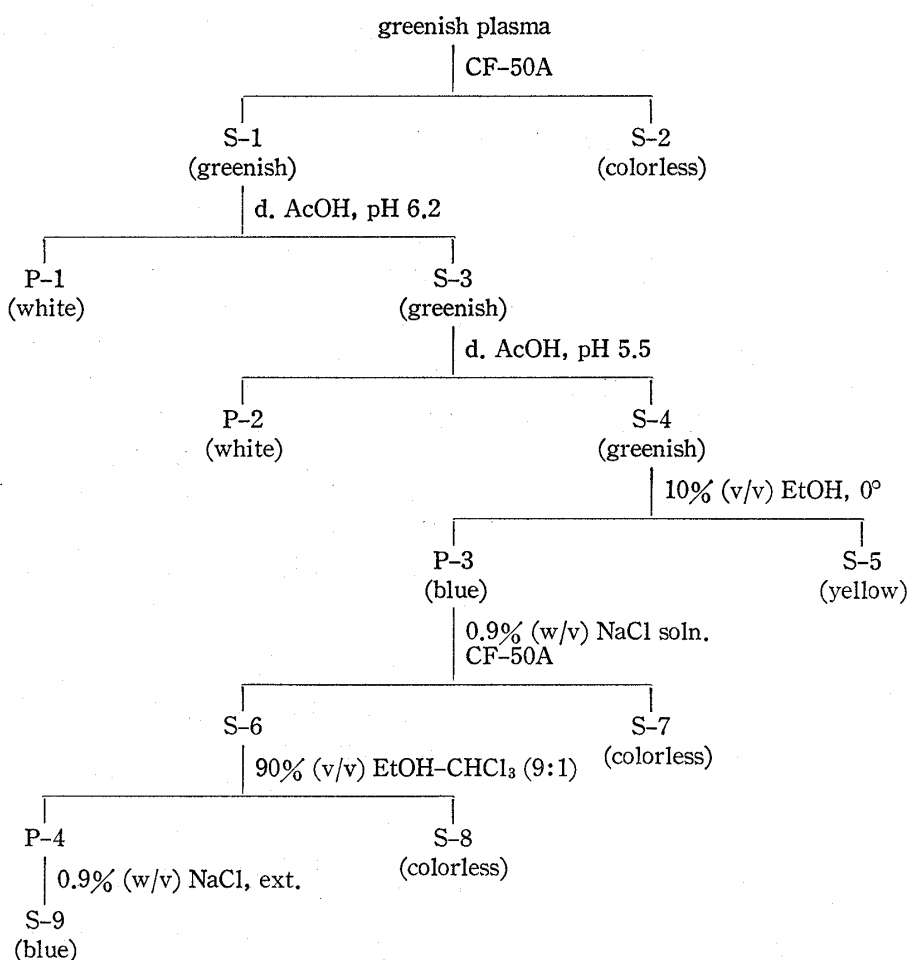


Chart 1

Plasma copper was determined by atomic absorption spectrometry using the modified Bogart and Beinert's colorimetric method.<sup>4)</sup> A half ml of the plasma was placed on the quartz boat and allowed to stand overnight under reduced pressure in a desiccator with phosphorus pentoxide as the drying agent. The dried sample was ashed in a low temperature asher for 15 hr at 50 W of power with an oxygen flow of 5 ml/min, and a pressure of 3–4 mmHg. After combustion, the ash was dissolved in 0.1 ml of concentrated hydrochloric acid and then transferred to a test tube with 3.0 ml of wash water. A half ml of 0.4% (w/v) hydroxylamine hydrochloride was added and the solution was mixed well. Three ml of 0.2% (w/v) bathocuproine

3) C.G. Holmberg and C.B. Laurell, *Acta Chem. Scand.*, **2**, 550 (1948).

4) M.V.D. Bogart and H. Beinert, *Anal. Biochem.*, **20**, 325 (1967).

in isoamyl alcohol was added to the tube, followed by 2.5 ml of saturated sodium acetate. The mixture was shaken for 15 min and allowed to stand for about 30 min. The extract containing the bathocuproine-copper complex was measured by atomic absorption spectrometry at 3247 Å with a lamp current of 6 mA, an acetylene flow of 2 l/min, and an air flow of 10 l/min. The copper content in the sample solution was found from the calibration curve obtained by treating the standard copper solution in a similar manner. The calibration curves (0.08–1.60 µg/ml) were linear under these conditions.

*p*-Phenylenediamine oxidase (PPD) activity in the plasma was determined by the method of Ravin.<sup>5)</sup> Plasma (0.1 ml) was placed in three tubes. One ml of 0.5% (w/v) sodium azide was added to one test tube as an enzyme-inhibited control. Eight ml of 0.4 M acetate buffer (pH 5.5) was added to each tube, followed by 1.0 ml of 0.5% (w/v) *p*-phenylenediamine dihydrochloride. The test tubes were shaken well and warmed for 1 hr at 37°. Next, 1.0 ml of 0.5% (w/v) sodium azide was added to each reaction mixture, except the control. The absorbancy of the sample solution at 530 nm was read *vs.* the control. PPD activity was represented by absorbancy at 530 nm.

The degree of the greenish tint in the plasma, namely the blue due to CP, was evaluated by the difference in the plasma absorbancy at 610 nm before and after the reversible reaction with ascorbic acid and oxygen. One ml of blood plasma was placed in a quartz cell (4×10×30 mm). The absorbancy of the plasma at 610 nm decreased with decolorization of the tint by addition of 30 µl of 0.5 M ascorbic acid. After the absorption peak at about 610 nm had disappeared, the reduced plasma was bubbled slowly with oxygen gas. Oxidation reached equilibrium quickly and the absorbancy at 610 nm was recovered quantitatively with the reappearance of the greenish tint.

## Results and Discussion

The greenish component, which was retained on the CF-50A membrane, could not be extracted from the monkey plasma with cyclohexane, 1,2-dichloroethane, or *n*-butyl alcohol. The greenish tint was scarcely changed by the addition of ethylenediaminetetraacetate.

The greenish monkey plasma had a visible absorption band at 610 nm corresponding to blue. The absorption peak of the blue fraction separated from the greenish plasma was also observed at 610 nm. These absorption bands at 610 nm, both in the greenish monkey plasma and the separated blue fraction, disappeared with ascorbic acid and reappeared with oxygen, as shown in Fig. 1. Accordingly, the blue component in the fraction and the greenish

component in the plasma were considered to be identical. Moreover, this behavior in the reversible reaction with ascorbic acid and oxygen was the same as that of human Cp.<sup>6)</sup>

Absorption peaks at 540, 578, and 550 nm in the plasma spectra as shown in Fig. 1, depended on the hemolysis of red cells in the plasma, and consequently, these peaks were probably due to oxyhemoglobin and hemoglobin.

The copper content in each fraction separated from the greenish monkey plasma was determined and listed in Table I. The major part of the copper in the monkey plasma was in the blue fraction. The data in Table I suggests that the blue component in the fraction is closely related to the plasma copper.

The ESR spectrum of the greenish monkey plasma in 9 GHz at 77 °K resembled that of human Cp.<sup>7)</sup> A narrow hyperfine splitting, Type I Cu<sup>2+</sup>, and a broader hyperfine splitting,

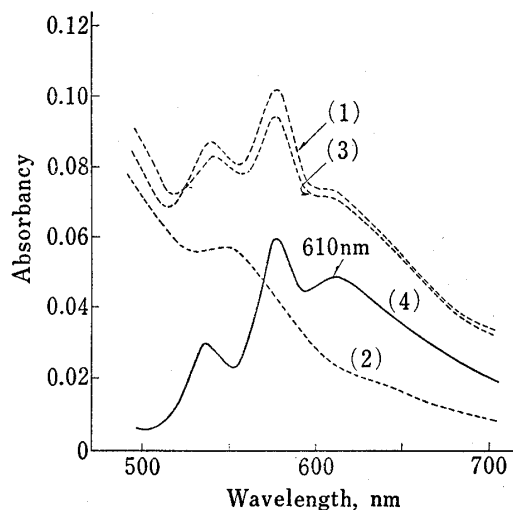


Fig. 1. Absorption Spectra

- (1) Greenish monkey plasma (No. 21, 7.5 mg of I/kg/day, *i.m.* daily administration, 4 weeks).
- (2) Decolorization with ascorbic acid.
- (3) Recovery with oxygen.
- (4) Difference spectrum, (1)–(2).

5) H.A. Ravin, *J. Lab. Clin. Med.*, **58**, 161 (1969).

6) W.E. Blumberg, J. Eisinger, P. Aisen, A.G. Morell, and I.H. Scheinberg, *J. Biol. Chem.*, **238**, 1675 (1963).

7) L.E. Andréasson and T. Vänngård, *Biochem. Biophys. Acta*, **200**, 247 (1970).

TABLE I. Distribution of Copper in Greenish Monkey Plasma

| Fraction <sup>a)</sup> |                 | Copper               |       |
|------------------------|-----------------|----------------------|-------|
|                        |                 | ( $\mu\text{g/ml}$ ) | (%)   |
| S-2,                   | Colorless, soln | 0.17                 | 6.9   |
| P-1, P-2,              | White, ppt.     | 0.14                 | 5.8   |
| S-5,                   | Yellow, soln.   | 0.78                 | 31.9  |
| P-3, <sup>b)</sup>     | Blue, ppt.      | 1.36                 | 55.4  |
| total                  |                 | 2.45                 | 100.0 |

a) The fractions are shown in Chart 1.

b) This fraction includes the those corresponding to S-6 to S-9 and P-4 in Chart 1.

TABLE II. ESR Parameter of Greenish Monkey Plasma and Human Cp

|                          |                           | Greenish monkey plasma <sup>a)</sup> | Human Cp <sup>b)</sup> |
|--------------------------|---------------------------|--------------------------------------|------------------------|
| Type I $\text{Cu}^{2+}$  | $g_{\perp}$               | 2.03                                 | 2.05                   |
|                          | $g_{\parallel}$           | 2.20                                 | 2.208                  |
|                          | $ A_{\parallel} $ (gauss) | 74.7                                 | 79.15                  |
| Type II $\text{Cu}^{2+}$ | $g_{\perp}$               | 2.02                                 | 2.04                   |
|                          | $g_{\parallel}$           | 2.25                                 | 2.258                  |
|                          | $ A_{\parallel} $ (gauss) | 195.3                                | 203.23                 |

a) The spectrum was measured in about 9 GHz at 77 °K.

b) From L.E. Andréasson and T. Vänngård (reference 7).

Type II  $\text{Cu}^{2+}$ , which were detected in human Cp were observed in the greenish monkey plasma, as shown in Table II. However, such hyperfine splitting peaks were difficult to find in the control monkey plasma.

These results suggested that the abnormal greenish tint of the monkey plasma is due to the presence of the blue copper protein Cp in the plasma.

Cp containing 7–8 atoms of copper per mole incorporates from 56 to 99% of the total plasma copper in mammalian species,<sup>8)</sup> and is only one component which PPD activity in blood plasma.<sup>5)</sup> Accordingly, the variation of Cp in the plasma can be explained quantitatively based on the total plasma copper, PPD activity and the degree of plasma tint (absorbancy at 610 nm).

To evaluate the effects of the administration of I or II on Cp in monkey and rat plasma, the total plasma copper, PPD activity and plasma absorbancy at 610 nm were determined. Table III shows the increasing ratio of these three factors to the control in monkey and rat plasma. In monkey plasma, as shown in Table III, the total plasma copper, PPD activity and absorbancy at 610 nm were closely related to each other. Furthermore, the increasing ratios of the three factors, which were approximately constant in the monkey plasma regardless of the amount and period of dosage, suggested that the Cp increase is limited.

Fig. 2 shows the relationships between total plasma copper and PPD activity in monkey and rat plasma. For 53 samples of control monkey plasma and 17 samples of plasma from daily administered monkeys, the relationships were represented by Equations (1) and (2) in Fig. 2, respectively. For 38 samples of rat plasma, the relationship was represented by Equation (3) in Fig. 2. Statistical analysis showed highly significant differences between Equations (1) and (2) and Equations (1) and (3).

The mean values of nonceruloplasmin copper in control monkey and rat plasma were indicated by the intersections ( $\text{Cu}=0.26, 0.07 \mu\text{g/ml}$ ) of Equation (1) and (3) lines on the

8) G.W. Evans and R.E. Wiederanders, *Am. J. Physiol.*, **213**, 1183 (1967).

TABLE III. Increasing Effects<sup>a)</sup> of Daily Administration of I and II on Total Plasma Copper, PPD Activity and Absorbancy at 610 nm in Monkey and Rat Plasma

|    | Dose  | Monkey          |              |                | Rat             |              |
|----|---|-----------------|--------------|----------------|-----------------|--------------|
|    |   | Total plasma Cu | PPD activity | Abs. at 610 nm | Total plasma Cu | PPD activity |
| I  | 2.5 mg/kg/day, <i>i.m.</i> , <sup>b)</sup> 2w <sup>d)</sup> | 2.2             | 2.9          | 2.6            |                 |              |
|    | 4w <sup>d)</sup>  | 2.1             | 2.8          | 3.1            |                 |              |
|    | 7.5 mg/kg/day, <i>i.m.</i> , <sup>b)</sup> 2w <sup>d)</sup> | 2.1             | 2.7          | 2.9            |                 |              |
|    | 4w <sup>d)</sup>  | 2.2             | 2.8          | 2.8            |                 |              |
| II | 50 mg/kg/day, <i>s.c.</i> , <sup>c)</sup> 4w <sup>d)</sup>  |                 |              |                | 1.6             | 1.6          |
|    | 3.5 mg/kg/day, <i>i.m.</i> , <sup>b)</sup> 4w <sup>d)</sup> | 1.9             | 2.7          | 1.9            |                 |              |

a) Values represent the ratio to each control.

b) Intramuscular injection.

c) Subcutaneous injection.

d) Weeks.

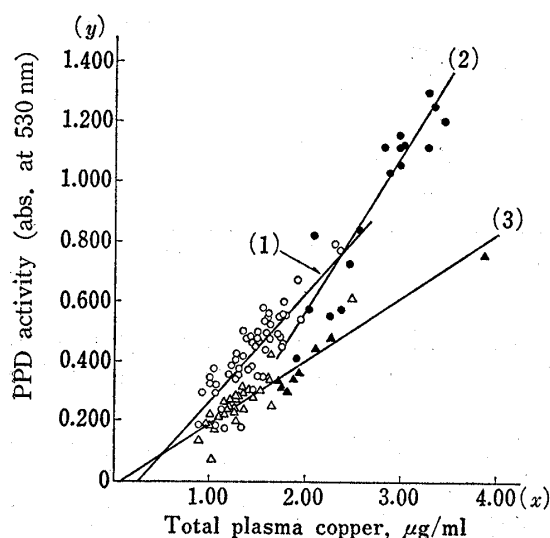


Fig. 2. Relation between Total Plasma Copper and PPD Activity in Monkey and Rat Plasma

(1) Monkey: control, (○); correlation coefficient,  $r=0.8482^{**}$ ; regression equation,  $y=0.3663x-0.097$ ; standard deviation,  $s=0.072$ ; variation coefficient,  $cv.=16.5\%$ ; number of plots,  $n=53$ .

(2) Monkey: daily administration of I and II, (●);  $r=0.9421^{**}$ ;  $y=0.5229x-0.471$ ;  $s=0.093$ ;  $cv=9.8\%$ ;  $n=17$ .

(3) Rat: control, (△); daily administration of I, (▲);  $r=0.9248^{**}$ ;  $y=0.2129x-0.018$ ;  $s=0.046$ ;  $cv=15.6\%$ ;  $n=38$ .

abscissa. From these values and the mean values of total plasma copper in control monkey and rat, the percentages of Cp-bonded copper in the total plasma copper were calculated to be 82% and 95% in monkey and rat, respectively.

The slopes of Equations (1) and (3) show the Cp activity in monkey and rat plasma, respectively. The ratios of these slopes indicate that the monkey Cp activity was about 1.7 times higher than that of rat Cp. Accordingly, the activity value of monkey Cp was calculated to be about 10.9 from that of rat Cp, 6.35,<sup>8)</sup> and was estimated to be nearly equal to that of pig Cp, 10.6,<sup>8)</sup> or higher. In other words, monkey Cp has higher activity than other known mammalian Cp.<sup>8)</sup> Moreover, the higher slope of Equation (2) compared with that of Equation (1) suggested that the Cp activity in monkey plasma increased with daily administration of I or II.

In rat plasma, the amount of Cp increased with daily administration of I. However, the activity of rat Cp remained normal, and the abnormal greenish tint of the plasma was scarcely observed.

TABLE IV. Total Plasma Copper, Cp-bonded Copper, PPD Activity and Cp Activity in Control Plasma of Monkey and Rat

|        | $n^a)$ | Total plasma Cu           |        | Cp-bonded Cu         |     | PPD Activity |        | Ratio of Cp activity |
|--------|--------|---------------------------|--------|----------------------|-----|--------------|--------|----------------------|
|        |        | mean ( $\mu\text{g/ml}$ ) | $s^b)$ | ( $\mu\text{g/ml}$ ) | (%) | mean         | $s^b)$ |                      |
| Rat    | 30     | 1.31                      | 0.30   | 1.24                 | 95  | 0.266        | 0.094  | 1.0                  |
| Monkey | 53     | 1.45                      | 0.31   | 1.19                 | 82  | 0.436        | 0.134  | 1.7                  |

a) Number of samples. b) Standard deviation.

The differences of Cp activity in monkey and rat may depend upon the molecular structure of the Cp, *i.e.* the number of intramolecular copper (II) atoms and their coordinations. The abnormal greenish tint of the blood plasma also seems to be governed by the differences of Cp activity among mammalian species.

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