# The Contribution of Citrate to the Ferroxidase Activity of Serum* (34009)

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Osaki and co-workers (1–4) found that the rate at which transferrin is formed from ferrous ion and apotransferrin *in vitro* is markedly accelerated by the serum copper protein, ceruloplasmin. Ceruloplasmin appears to exert its effect by catalyzing the oxidation of iron from the ferrous to the ferric form, after which ferric ion joins with apotransferrin to form the characteristic, colored transferrin complex. On the basis of these studies, the above investigators proposed that ceruloplasmin be classified as a ferro-$O_2$-oxidoreductase, with the designation "serum ferroxidase."

The existence of a nonceruloplasmin serum constituent which catalyzes iron oxidation was suggested by Schen and co-workers (5, 6). When human serum was subjected to immunoelectrophoresis, two bands were detected which stained with the Prussian blue reaction after incubation with a solution of ferrous iron. One of these bands corresponded in electrophoretic mobility and immunologic reactivity to ceruloplasmin. The other was located in the region of the $\beta$-lipoproteins. This second substance was not identified, but its resistance to inactivation by heat suggested that it was not a protein.

The purposes of the present report are to present additional evidence for a nonceruloplasmin serum factor that accelerates transferrin formation and to identify the factor as citrate.

*Materials and Methods.* All blood samples were collected and subsequently analyzed in specially cleaned glassware (7). Blood was obtained from women in the third trimester of pregnancy, from the umbilical vein of newborn infants, from normal human subjects, and from patients with Wilson's disease. A partially purified human ceruloplasmin preparation was provided by the American Red Cross. A further degree of purification was accomplished by chromatography on Sephadex G-200.

Serum ferroxidase activity was measured by the method of Osaki *et al.* (2). Three ml of incubation mixture contained 1 ml of appropriately diluted (1:10–1:100) serum, apotransferrin (30 $\mu M$), ferrous ammonium sulfate (30 $\mu M$), and ascorbic acid (300 $\mu M$) in 0.0133 $M$ sodium phosphate buffer, pH 6.7. After the addition of the iron–ascorbate solution, the rate of increase in absorbance at 460 m$\mu$ was measured at room temperature in a Cary recording spectrophotometer, model 15. The determined values were corrected by subtracting the nonenzymatic rate of transferrin formation, which was measured in a cuvette containing all the above reagents except serum.

Methods published by other workers were employed for the assay of serum *p*-phenylenediamine (pPD) oxidase activity (8), the measurement of serum citrate (9), serum immunoelectrophoresis (10), and Prussian blue staining of immunoelectrophoretic plates (5). Serum copper was determined, after deproteinization with 2 $N$ HCl and 20% trichloroacetic acid, with oxalyldihydrazide as the colorimetric reagent (11). Serum iron was measured by a method described elsewhere (7).

*Results. Evidence for a nonceruloplasmin serum factor with ferroxidase activity.* Ferroxidase activity and pPD oxidase activity were found to be inhibited by azide, both in normal human serum samples and in purified ceruloplasmin solutions (Fig. 1). The pPD oxidase activity of serum and of ceruloplasmin was inhibited to the same extent by increasing concentrations of azide (Fig. 1A). Similarly, the ferroxidase activity of ceruloplasmin was inhibited progressively by in-

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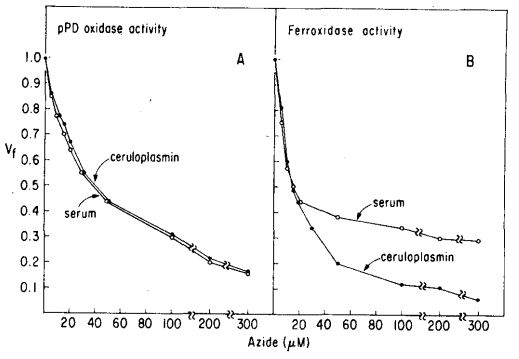


FIG. 1. The effect of azide on *p*-phenylenediamine oxidase activity (A) and on ferroxidase activity (B) in serum and in purified ceruloplasmin solutions. Ferroxidase activity in serum is resistant to further inhibition by azide at concentrations greater than 30 $\mu M$. $V_f$: fractional velocity.

creasing concentrations of azide (Fig. 1B). However, in serum, ferroxidase activity was inhibited progressively as the azide concentration was increased to 30 $\mu M$, but at greater concentrations only a slight further decline in activity was observed. This observation suggested that some of the ferroxidase activity of serum was due to a component which differed from ceruloplasmin in that it was not inhibited by azide.

Sera with wide variations in ceruloplasmin content were collected from four groups of human subjects, namely, pregnant women, normal individuals, newborn infants, and patients with Wilson's disease. The relation between ferroxidase and pPD oxidase activity in these sera was compared with the relation between the two activities in solutions of purified human ceruloplasmin (Fig. 2). A linear relation between ferroxidase activity and pPD oxidase activity was found both in sera and in ceruloplasmin solutions; however, the two curves were not superimposable. At any given level of pPD oxidase activity, the ferroxidase activity in serum was greater than that in ceruloplasmin solutions. The ceruloplasmin curve could be extrapolated through zero, but the curve for sera intersected the ordinate at a point equivalent to a ferroxidase activity of 13 μmoles/ml/hr. Thus, the ferroxidase activity of human serum exceeded that which could be accounted for by its ceruloplasmin content.

After immunoelectrophoresis of normal human serum, two bands which accelerated the oxidation of ferrous ion could be seen. One band corresponded to ceruloplasmin in location and immunologic reactivity. The other was found at the site occupied by β-lipoproteins. When either azide or cyanide was added during the staining procedures, the ferrous-oxidizing activity of ceruloplasmin was abolished, but that at the β-lipoproteins locus was unaffected. Immunoelectrophoretic plates made with sera from newborn infants and patients with Wilson's disease exhibited a faint or absent ceruloplasmin band, but the band at the β-lipoprotein locus was as intense as with preparations of normal serum.

*Identification of citrate as the nonceruloplasmin serum factor with ferroxidase activity.* In the following studies, serum that contained no detectable ceruloplasmin was obtained from a patient (D.H.) with Wilson's disease. This serum was used as a source of

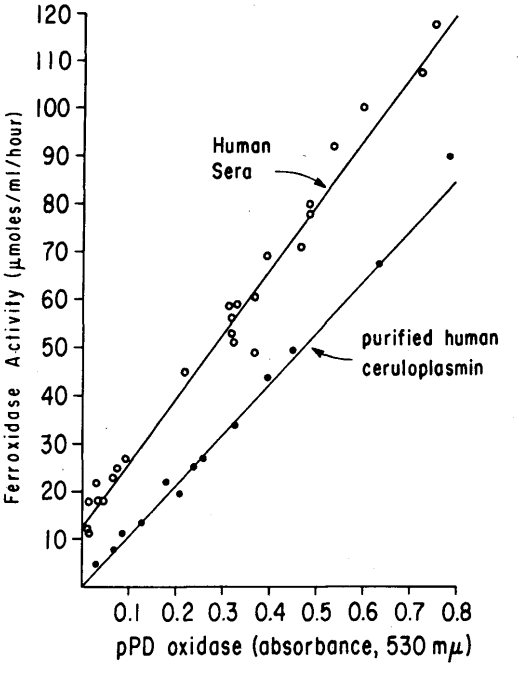


FIG. 2. Relation of ferroxidase activity to *p*-phenylenediamine (pPD) oxidase activity in serum and in purified ceruloplasmin solutions. The relation is linear in both cases, but serum contains more ferroxidase activity than can be accounted for by its ceruloplasmin content.

TABLE I. The Effect of Ultrafiltration on Serum Ferroxidase Activity.

| Sample | Vol | Ferroxidase Concentration (μmoles/ml/hr) | Ferroxidase Total (μmoles/hr) | Recovery (%) |
|---|---|---|---|---|
| Whole serum | 25 | 17.5 | 440 | (100) |
| Serum after dilution and concentration | 25 | 6.9 | 170 | 38 |
| Ultrafiltrate | 225 | 1.25 | 280 | 64 |
| Total | | | 450 | 102 |

the nonceruloplasmin factor.

The effect of dialysis on ferroxidase activity was assessed by dialyzing Wilson's disease serum against 200 vol of 0.9% NaCl at 4° for 12 hr. Ferroxidase activity decreased from 13.6 to 3.5 μmoles/ml/hr. Dialysis was continued for 48 hr with two changes of dialysis medium, after which ferroxidase activity was 1.3 μmoles/ml/hr. This loss of activity after dialysis suggested that the nonceruloplasmin factor was removed by the procedure.

To pursue this suggestion further, the effect of ultrafiltration on ferroxidase activity was evaluated. Twenty-five ml of Wilson's disease serum was diluted to 250 ml with 0.9% NaCl. The diluted serum was concentrated to its original volume of 25 ml in an ultrafiltration apparatus with a diffusion membrane which retained substances with a molecular weight greater than 10,000.[2] The ferroxidase activity of the ultrafiltrate and the diluted and concentrated serum were compared with that of the original serum sample (Table I). Most of the ferroxidase activity was found in the ultrafiltrate. The dilution and concentration procedure was repeated and a further loss of activity from 6.9 to 1.9 μmoles/ml/hr was observed. Thus, the factor appeared to have a molecular weight of less than 10,000.

Wilson's disease serum was diluted 1:10 with 0.04 *M* phosphate buffer, pH 6.7. Five ml of the diluted serum was applied to each of two 1 × 2-cm chromatographic columns, one containing Dowex 2-X8, an anion exchange resin and the other containing Dowex 50W, a cation exchange resin. Ferroxidase activity was measured in the original serum sample and in the column eluates. Exposure to Dowex 2-X8 removed almost all of the ferroxidase activity whereas almost none was removed by Dowex 50W.

The above observations suggested that the nonceruloplasmin factor is of small molecular weight and that it behaves as an anion at physiologic pH. That the factor might be citrate was adopted as a working hypothesis, because citrate is one of the prominent serum anions (12), and because citrate is known to catalyze the auto-oxidation of iron (13). Accordingly, the effect of citrate on transferrin-formation from ferrous ion was measured. Solutions of sodium citrate in various concentrations were used instead of diluted serum in the assay for ferroxidase activity. Citrate was found to accelerate transferrin formation (Fig. 3). The relation between citrate concentration and ferroxidase activity was nonlinear, and no additional increase in the rate of transferrin formation occurred with concentrations greater than 5 μg/ml. The effect

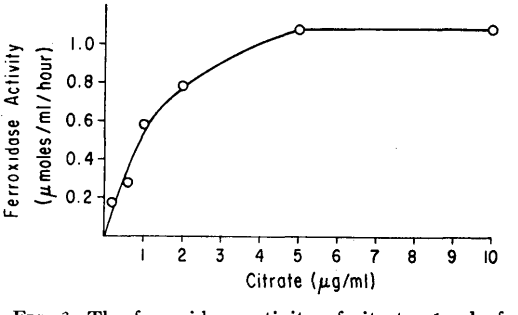


FIG. 3. The ferroxidase activity of citrate: 1 ml of citrate solution was used instead of diluted serum in the assay for ferroxidase activity.

[2] Diaflo ultrafiltration cell, model 400, with a 3-in. UM-1 membrane, manufactured by Amicon Corporation, Cambridge, Massachusetts.

TABLE II. The Effect of Ion Exchange Chromatography on Ferroxidase Activity of Serum.

| Resin | Column fraction | Serum[a] | | Citrate solution | |
|---|---|---|---|---|---|
| | | Ferroxidase ($\mu$moles/ml/hr) | Citrate ($\mu$g/ml) | Ferroxidase ($\mu$moles/ml/hr) | Citrate ($\mu$g/ml) |
| | Before chromatography | 19.4 | 36 | 14 | 21 |
| Dowex 2-X8 | Eluate | 1.4 | 0 | 0.5 | 0.5 |
| | Phosphate wash | 0.4 | 0 | 0.3 | 0 |
| | 5% NaCl wash | 14 | 18 | 12 | 11 |
| Dowex 50W | Eluate | 18 | 22 | 9 | 6 |
| | Phosphate wash | 7 | 6 | 4 | 4 |
| | 5% NaCl wash | 0 | 0 | 0 | 0.5 |

[a] From a patient with Wilson's disease.

of citrate was not inhibited by azide.

The experiments with ion exchange chromatography on Dowex resins were then repeated, and column eluates were analyzed for both citrate concentration and ferroxidase activity (Table II). When serum was added to Dowex 2-X8 columns, only traces of citrate were found in the elutates and in phosphate washes, and these fractions had little or no ferroxidase activity. The citrate could be eluted with 5% NaCl and such elutates contained most of the ferroxidase activity of the material applied to the column. When Dowex 50W was used as the exchange resin, most of the citrate as well as most of the ferroxidase activity was found in the first eluate and the phosphate washes. When a citrate solution was applied to the columns, the results were identical (Table II, columns 3 and 4).

Paper chromatography of serum was performed in a butanol–propionic acid–water (2:2:1) system on Whatman no. 1 filter paper. The solvent front was allowed to ascent 20 cm over a period of about 6.5 hr. After chromatography, the paper was cut into 12 equal segments and each segment was eluted with 5 ml of 0.9% NaCl. Eluates were analyzed for citrate and for ferroxidase activity. Citrate and ferroxidase activity migrated with an $R_f$ of 0.4 in this sytem, and ferroxidase activity was found only in eluates of those segments which contained citrate.

In order to determine whether citrate accounted for the second iron-oxidizing band seen in immunoelectrophoretic preparations, the following experiments were performed. Serum from patient D.H. with Wilson's disease was subjected to dilution and concentration by ultrafiltration 5 times, as previously described. Prior to the procedure, serum citrate was 30.5 $\mu$g/ml, serum ferroxidase activity was 16 $\mu$moles/ml/hr, and a blue, iron-positive band at the $\beta$-lipoprotein locus was found after immunoelectrophoresis. After dilution and concentration, citrate was 0.5 $\mu$g/ml, ferroxidase activity was 1.6 $\mu$moles/ml/hr, and no blue band was present on immunoelectrophoresis. When sufficient citrate was added to make a concentration of 60 $\mu$g/ml, reappearance of the iron-positive band was observed on immunoelectrophoretic

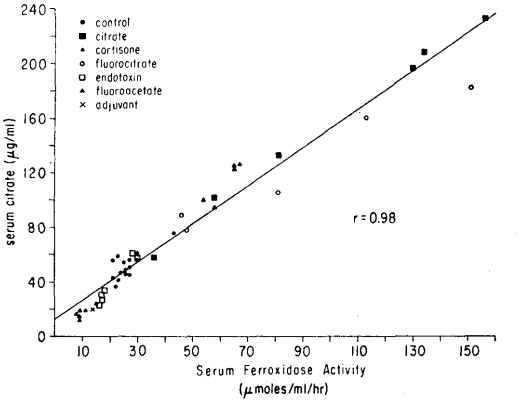


FIG. 4. The relation between citrate and ferroxidase activity *in vivo*. Rats were treated with various agents, as indicated by the symbols, in order to vary the serum citrate concentration.

plates. Thus, citrate appeared to be essential to the demonstration of iron-oxidizing activity at the β-lipoprotein locus.

The effect of *in vivo* changes in serum citrate on ferroxidase activity was assessed in rats. Induction of adjuvant arthritis (14), treatment with cortisone (15), and the intraperitoneal injection of bacterial endotoxin (100 μg of *E. coli* lipopolysaccharide) resulted in reduced serum citrate levels. Increased serum citrate levels were induced by citrate injection and by treatment with fluoroinhibitors (16). In all cases, the change in serum citrate correlated with a similar change in serum ferroxidase activity (Fig. 4). The correlation coefficient ($r$) calculated from these data was 0.98.

*Discussion.* The above studies indicate that the serum contains two factors which accelerate the rate of transferrin formation from apotransferrin and ferrous iron. One of these is ceruloplasmin. The other can be distinguished from ceruloplasmin in that it is dialyzable, and is not inhibited by azide. It appears to be identical to a ferrous-oxidizing substance that can be separated from ceruloplasmin by immunoelectrophoresis (6). Although the factor appears to be bound to the β-lipoproteins during immunoelectrophoresis, the bond is a loose one since it can be ruptured by dialysis. The nonceruloplasmin factor accounts for about 15% of the total ferroxidase activity in normal man and for nearly 100% of the activity in patients with Wilson's disease.

The following observations establish the identity of this factor with citrate: (i) Pure solution of citrate have transferrin-forming activity (Fig. 3). (ii) The serum factor behaves in a fashion identical to citrate on ion exchange chromatography (Table II). (iii) The factor and citrate migrate with the same $R_f$ value in a paper chromatographic system. (iv) Citrate added to dialyzed serum restores the iron-oxidizing properties of the β-lipoprotein band on immunoelectrophoretic plates. Finally, (v) induced alterations in serum citrate in rats *in vivo* are correlated with similar changes in ferroxidase activity (Fig. 4).

Osaki *et al.* (1–4) proposed that ceruloplasmin exerts its effects on the rate of transferrin formation by accelerating the oxidation of ferrous ion to ferric ion, after which the extremely rapid reaction of ferric ion with apotransferrin occurs. Since citrate is known to accelerate the auto-oxidation of ferrous ion in pure solutions (13), its effect on transferrin formation may be similar to that proposed by Osaki for ceruloplasmin.

A possible additional effect of citrate is an acceleration of the rate of reaction of ferric ion with transferrin. Bates *et al.* (17) found that the rate of transfer of iron from ferric citrate to transferrin increased as the ratio of citrate to iron increased, up to a citrate: iron molar ratio of 20:1. They proposed that a transferrin–iron–citrate complex formed an intermediate in the reaction. Of course, such a mechanism would not apply to the effect of citrate in the immunoelectrophoretic system, since transferrin formation is not required.

*Summary.* The ferroxidase activity of ceruloplasmin has been demonstrated by other workers. In the present investigation, the ferroxidase activity of human serum was greater than that which could be accounted for by its ceruloplasmin content. The additional activity was due to a substance which was dialyzable, heat stable, separable from ceruloplasmin by immunoelectrophoresis, and not inhibitable by azide. The chromatographic properties of this substance were identical with those of citrate. Ferroxidase activity of citrate was demonstrated in the absence of other plasma factors. Furthermore, induced changes in serum citrate levels *in vivo* were correlated with changes in serum ferroxidase activity. Thus, citrate accounts for the nonceruloplasmin ferroxidase activity of serum.

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## Isolation and Growth of Rat Cytomegalovirus *in Vitro* (34010)

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Cells characteristic of cytomegalovirus (CMV) infection have been observed histologically in salivary glands of guinea pigs, rats, mice, hamsters, moles, dogs, monkeys, and man (1). The cytomegaloviruses of mice, guinea pigs, horses, monkeys, and man have been grown *in vitro* in cell cultures and the viruses have shown marked species specificity with the exception of monkey CMV which grows in human as well as monkey cells, and the equine CMV which grows in rabbit cells (1–3).

In 1934, Kuttner and Wang described cytomegalic inclusion disease in the salivary glands of 50% of wild rats trapped in Peking, China and they were able to transmit the disease to other rats with cell-free filtrates (4). We have been unable to find any reports of isolation of the rat CMV in culture.

During an investigation of the prevalance of trypanosomes in roof rats (*Rattus rattus*) in the province of Panama, Republic of Panama, 116 adult rats were trapped during the first 7 months of 1968 and brought to Gorgas Memorial Laboratory, where they were autopsied after one or more bleedings. Blocks of submaxillary, sublingual, and parotid glands were obtained from 84 rats (46 females and 38 males) and histopathologic evidence of CMV infection was found in 41 (23 females and 18 males). Characteristic intranuclear and intracytoplasmic inclusions were found in the submaxillary glands of all 41, the sublingual glands of 11, and the parotid glands of 2. (Fig. 1). The kidneys, lungs, and brains of the rats were examined and no CMV lesions were noted. The present report describes the isolation and growth of rat CMV *in vitro* from salivary glands of these wild Panamanian rats.

*Materials and Methods. Collection of specimens.* The right salivary glands of 15 rats were dissected with sterile instruments, transferred to sterile containers, and frozen at −70°. The left salivary glands were examined histopathologically and 6 of the 15 had evidence of CMV infection. The frozen

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