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SERUM AZIDE-RESISTANT FERROXIDASE ACTIVITY

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SUMMARY

The azide-resistant ferroxidase activity of fresh, undialyzed human serum, either normal or from patients with Wilson's disease, can be accounted for by citrate and by the incomplete inhibition of ceruloplasmin ferroxidase activity in 1 mM concentrations of NaN_3.

Prolonged dialysis of serum at pH 5.5 resulted in the development of appreciable ferroxidase activity when the dialyzed serum was assayed at pH 6.0 in acetate buffer. This activity could not be accounted for by either citrate or ceruloplasmin and corresponds to the ferroxidase II activity reported by others. Since this activity was not detected in human serum under physiologic conditions and its activity was a function of the pH, duration and temperature of dialysis, its physiologic role in iron metabolism has not been established.

INTRODUCTION

Catalysis of the oxidation of Fe(II) to Fe(III) by ceruloplasmin (ferroxidase, EC 1.16.3.1) has been demonstrated both in vitro [1, 2] and in vivo [3–5]. Azide inhibits the oxidation of iron by ceruloplasmin but does not inhibit a second iron-oxidizing substance that can be found in serum when ferroxidase activity is measured at pH 6.7 in phosphate buffer [6]. This azide-resistant factor is dialyzable, heat stable, separable from ceruloplasmin by immunoelectrophoresis, and chromatographically identical with citrate. The iron-oxidizing activity of citrate is shown in the absence of other factors. Finally, induced changes in serum citrate levels in vivo are correlated with changes in serum ferroxidase activity. Therefore, we concluded that the azide-resistant ferroxidase activity of serum is due to citrate [6].

Topham and Frieden [7] reported that human serum, previously equilibrated to pH 5.5 by dialysis against acetate buffer and assayed at pH 6.0 in acetate buffer, contained appreciable azide-resistant ferroxidase activity. They attributed the activity to a copper-containing serum lipoprotein, ferroxidase II, that was inhibited by heat but not by azide and had a ferroxidase activity that was one-sixth that of ceruloplasmin. They concluded that the azide-resistant ferroxidase activity of serum was due to ferroxidase II and not to citrate.

The purpose of this report is to present the results of further studies on the azide-resistant ferroxidase activity of human serum in order to resolve these apparent differences.

MATERIALS AND METHODS

Blood was collected from normal human subjects and from patients with Wilson's disease in specially cleaned glassware [8] and allowed to clot at room temperature. The serum was used within several hours. Purified human ceruloplasmin was obtained from Miles-Yeda, Ltd., Rehovoth, Israel. Human apotransferrin was obtained from Certified Blood Donor Service, Inc., Woodbury, New York. Ceruloplasmin and apotransferrin solutions were dialyzed against 0.15 M NaCl before use.

Serum ferroxidase activity was measured by the method of Johnson et al. [9]. The reaction was carried out at pH 6.7 in 1.0 ml of a mixture containing 0.2 ml of appropriately diluted (1:5–1:50) serum, apotransferrin (30 μM), ferrous ammonium sulfate (30 μM) and ascorbic acid (300 μM) in 0.0133 M sodium phosphate buffer. After the addition of the iron–ascorbate solution, the rate of increase in absorbance at 460 nm was measured at room temperature in a Cary recording spectrophotometer, Model 15. When the reaction was carried out at pH 6.0, 1.0 ml of reaction mixture contained 0.2 ml of diluted serum, apotransferrin (30 μM) and ferrous ammonium sulfate (30 μM) in 0.2 M sodium acetate buffer. The reaction was begun by the addition of the iron solution. Values were corrected by subtracting the nonenzymatic rate of transferrin formation.

Methods published by other workers were employed for the assay of *p*-phenylenediamine oxidase activity [10] and the measurement of serum citrate [11].

RESULTS

Ferroxidase activity in human serum

Fresh human serum, either from normal subjects or from patients with Wilson's disease, contained appreciable amounts of azide-resistant ferroxidase when assayed at pH 6.7 in the presence of 100 μM NaN_3 (Table I). In normal serum, 68% of the activity was abolished by azide and was therefore accounted for by ceruloplasmin. About 32% of the activity was azide-resistant. In the Wilson's disease serum, 82% of the activity was azide-resistant.

The total ferroxidase activity of both normal human and Wilson's disease serum was less in acetate buffer at pH 6.0 than in phosphate buffer at pH 6.7, primarily

TABLE I

FERROXIDASE ACTIVITY IN FRESH HUMAN SERUM

Assays were carried out in 0.0133 M phosphate buffer, pH 6.7, or 0.2 M acetate buffer, pH 6.0, in the absence and presence of 100 μM NaN_3. NHS, normal human serum. WD, Wilson's disease serum. The values represent mean $\pm$ S.E.

Serum	Assay conditions	Number of subjects	Ferroxidase (μmoles/ml per h)	
			Total	Azide-resistant
NHS	pH 6.7, phosphate buffer	31	53.0 $\pm$ 1.22	16.8 $\pm$ 0.95
WD	pH 6.7, phosphate buffer	7	12.8 $\pm$ 0.70	10.2 $\pm$ 0.90
NHS	pH 6.0, acetate buffer	15	23.0 $\pm$ 1.21	1.6 $\pm$ 0.05
WD	pH 6.0, acetate buffer	7	1.0 $\pm$ 0.29	0.4 $\pm$ 0.03

because the azide-resistant fraction was reduced. At pH 6.0, the azide-resistant ferroxidase of normal human serum constituted 7% of the total activity, whereas that of Wilson's disease serum accounted for 40% of the total activity.

Contribution of ceruloplasmin to azide-resistant ferroxidase activity

The ferroxidase activity of fresh, undialyzed normal human serum and of purified human ceruloplasmin was measured at pH 6.0 in the presence of increasing concentrations of NaN_3 (Fig. 1). Complete inhibition of activity was not achieved with either preparation. 13% of the ferroxidase activity remained at an azide concentration of 100 μM and 5% of the activity remained at 1 mM concentration.

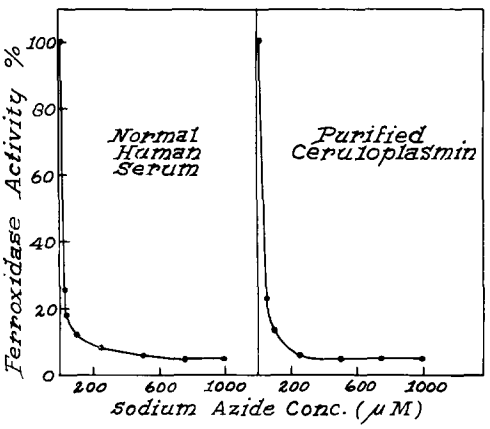

Fig. 1. Effect of increasing NaN_3 concentration on ferroxidase activity of fresh normal serum and purified human ceruloplasmin. The ferroxidase assays were performed at 30 °C, pH 6.0, in 0.2 M acetate buffer.

An average of 5% (0.7 μmole/ml per h) of the ferroxidase activity of six normal human serum specimens was resistant to inhibition by 1 mM NaN_3 at pH 6.0 while little or no ferroxidase activity (less than 0.1 μmole/ml per h) was detected in serum from patients with the ceruloplasmin deficiency of Wilson's disease.

Contribution of citrate to azide-resistant ferroxidase activity

The azide-resistant ferroxidase activity of fresh serum from a patient with Wilson's disease was measured at pH 6.7 in phosphate buffer and at pH 6.0 in acetate buffer (Table II). The concentration of citrate in the serum was 30 μg/ml. Azide-resistant ferroxidase activity was detected at pH 6.7 but not at pH 6.0. The serum was then dialyzed against 0.15 M NaCl to remove the citrate. No azide-resistant ferroxidase activity was detected when the dialyzed serum was assayed at pH 6.7. Citrate was then added to an aliquot of the dialyzed serum and the azide-resistant ferroxidase activity, at pH 6.7, was restored.

The contribution of citrate to the ferroxidase activity of serum depended upon pH. The iron-oxidizing activity of citrate was barely detectable at pH 6.0, but its activity increased progressively as pH was increased. Iron oxidation was greater in phosphate buffer (Fig. 2, C) than in acetate buffer (Fig. 2, D). In contrast, ferroxidase activity of ceruloplasmin was maximal at about pH 6.5, whether in phosphate (Fig.

TABLE II

CONTRIBUTION OF CITRATE TO AZIDE-RESISTANT FERROXIDASE ACTIVITY

All assays were performed on a single serum sample from a patient with Wilson's disease. Dialysis was carried out at 22 °C for 20 h against 200 vol. 0.15 M NaCl. The assays were carried out in the presence of 100 μM NaN_3.

Preparation of serum	Final citrate concentration (μg/ml)	Assay conditions	Azide-resistant ferroxidase (μmoles/ml per h)
Fresh	30	pH 6.7, phosphate buffer	13
		pH 6.0, acetate buffer	0
Dialyzed	1	pH 6.7, phosphate buffer	0
		pH 6.0, acetate buffer	0
Dialyzed and citrate added	36	pH 6.7, phosphate buffer	17
		pH 6.0, acetate buffer	0

2, A) or acetate (Fig. 2, B) buffers. Thus, at physiologic pH, citrate activity equaled or exceeded that of ceruloplasmin.

Development of azide-resistant ferroxidase (ferroxidase II) activity in human serum

Fresh normal human serum, in which azide-resistant ferroxidase activity could not be detected at pH 6.0 in acetate buffer, was dialyzed against acetate buffer at pH 5.5. In confirmation of the observations of Topham and Frieden [7], azide-resistant ferroxidase activity of appreciable magnitude was detected after dialysis

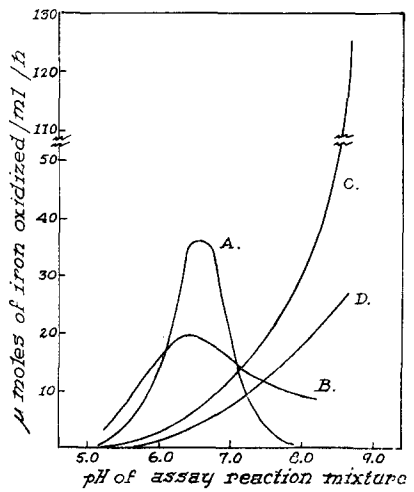

Fig. 2. Influence of pH on the rate of iron oxidation by ceruloplasmin and by citrate. The curve labeled A (ceruloplasmin) was obtained by dialyzing normal human serum against 0.15 M NaCl for 24 h to remove the citrate and then performing the assay in 0.0133 M phosphate buffer adjusted to the indicated pH. The curve labeled B was obtained by dialyzing normal human serum against 0.15 M NaCl for 24 h to remove citrate and performing the assay in 0.2 M acetate buffer adjusted to the indicated pH. The curve labeled C was obtained on undialyzed serum from a patient with Wilson's disease; the ferroxidase activity was measured in 0.0133 M phosphate buffer adjusted to the indicated pH. The curve labeled D was obtained on undialyzed serum from a patient with Wilson's disease; the ferroxidase activity was measured in 0.2 M acetate buffer adjusted to the indicated pH.

TABLE III

DEVELOPMENT OF AZIDE-RESISTANT FERROXIDASE (FERROXIDASE II) ACTIVITY IN NORMAL HUMAN AND WILSON'S DISEASE SERA

All assays were carried out in 0.2 M acetate buffer, pH 6.0, in the presence of 100 μM NaN_3 following dialysis against 300 vol. of 0.05 M acetate buffer adjusted to the pH specified.

Dialysis conditions		Azide-resistant ferroxidase (μmoles/ml per h	
		Normal Human	Wilson's disease
Not dialyzed		0.0	0.0
pH 5.5, 22 °C	24 h	19.4	23.8
	48 h	21.6	54.0
	72 h	23.8	48.6
pH 5.5, 36 h	22 °C	12.8	
	37 °C	35.6	
37 °C, 36 h	pH 5.5	35.6	
	pH 7.4	3.8	

(Table III). Most of the development of azide-resistant ferroxidase activity occurred during the first 24 h of dialysis. More activity was apparent when the dialysis was carried out at 37 °C than at 22 °C. The development of activity during the dialysis was optimal at pH 5.5. Dialysis at pH 7.4 resulted in the development of only about 10% as much activity as at pH 5.5 (Table III). In other studies, activity developed in phosphate buffer as well as in acetate buffer at pH 5.5, and therefore was not acetate-dependent. Development of azide-resistant ferroxidase activity after dialysis at low pH was also observed in Wilson's disease serum.

Storage of serum at acid pH resulted in development of azide-resistant ferroxidase activity, even if dialysis was not performed (Table IV). In these experiments, serum was adjusted to pH by dropwise addition of buffer and allowed to stand at room temperature for 48 h. Azide-resistant ferroxidase activity did not appear in whole serum or in aliquots adjusted to pH 7.4 with 0.1 M HCl and acetate buffer respectively. In contrast, azide-resistant ferroxidase activity developed in samples adjusted to pH 5.5 with either 1.0 M HCl or acetate buffer.

TABLE IV

DEVELOPMENT OF AZIDE-RESISTANT FERROXIDASE ACTIVITY (FERROXIDASE II) WITHOUT DIALYSIS

All assays were carried out in 0.2 M acetate buffer, pH 6.0, in the presence of 100 μM NaN_3.

Sample	Azide-resistant ferroxidase (μmoles/ml per h)	
	0 h	48 h
Whole serum	2.4	0.5
pH 7.4, adjusted with 0.1 M HCl	2.7	1.6
pH 7.4, adjusted with acetate buffer	2.7	1.1
pN 5.5, adjusted with 1.0 M HCl	1.4	21.1
pH 5.5, adjusted with acetate buffer	0.8	24.4

DISCUSSION

The ferroxidase activity of serum in the presence of azide is accounted for by 3 factors: (1) incomplete inhibition of ceruloplasmin, (2) the effect of citrate, and (3) an activity that develops only after a period of exposure of serum to acid pH. The relative importance of these three factors depends upon the assay conditions (pH and buffer), and upon the conditions of storage and dialysis of the serum samples. In fresh, undialyzed specimens, assayed in phosphate buffer, pH 6.7, the azide-resistant activity is related to citrate. At pH 6.0, citrate has little effect and any activity present results from incomplete inhibition of ceruloplasmin.

Dialysis removes the activity of the citrate. However, if the dialysis has been performed at acid pH, an azide-resistant ferroxidase develops. This new activity appears to be identical to that described by Topham and Frieden [7], and named by them "ferroxidase II". The activity develops both in normal and Wilson's disease serum. It can be detected in either acetate buffer, pH 6.0 or in phosphate buffer, pH 6.7. Ferroxidase II activity could not be detected in fresh, undialyzed serum either from normal subjects or from Wilson's disease patients. Therefore, it is unlikely that it plays a physiologic role in iron metabolism. For the same reason, ferroxidase II activity would not compensate for the deficiency of ceruloplasmin ferroxidase activity in Wilson's disease as suggested by Topham and Frieden [7]. On the other hand, the iron-oxidizing activity of citrate increases with increasing pH (Fig. 2) while that of ceruloplasmin at pH 7.4 is only about one-seventh of the activity at pH 6.7 in phosphate buffer. Thus, at physiologic pH, the relative role of citrate in oxidizing iron, particularly in the absence of ceruloplasmin, may be of importance.

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