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Biological Variability of Superoxide Dismutase, Glutathione Peroxidase, and Catalase in Blood

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We studied the biological variability of blood superoxide dismutase (SOD; EC 1.15.1.1), glutathione peroxidase (GPX; EC 1.11.1.9), and catalase (CAT; EC 1.11.1.6) in a sample of 1836 apparently healthy subjects, ages 4–97 years. SOD and GPX activities were assayed in plasma (P) and erythrocytes (E) by automated methods, and CAT was measured in erythrocytes by a manual technique. No statistically significant variation of these antioxidant enzyme activities according to gender was demonstrated, except for E-GPX, which was slightly but significantly higher in women than in men ($P < 0.001$). Activities appear rather stable in adults <65 years old, but decrease for most enzymes in the elderly. There is no evidence that weight, blood pressure, or menopause influences the antioxidant enzymes' activities. In girls ages 10–14 years, E-SOD activity is reduced by 16% ($P < 0.05$) after menarche. Variations related to smoking and alcohol consumption are slight and concern only P-SOD and P-GPX, respectively. Conversely, intake of some drugs (e.g., anti-inflammatory agents, antidepressants, and thyroid hormones) modifies activity of some of the three enzymes. E-SOD positively correlates with P-SOD ($r = 0.216$, $P < 0.001$) and E-CAT ($r = 0.123$, $P < 0.001$), and E-GPX with P-GPX ($r = 0.218$, $P < 0.001$). Finally, we propose reference intervals for activities of the three antioxidant enzymes in blood in individuals <65 years old.

Additional Keyphrases: *variation, source of · enzyme activity · sex- and age-related effects · peroxidation · reference intervals*

Free radicals and peroxides are clearly involved in physiological phenomena such as synthesis of prostaglandins and thromboxanes (*1*), and in pathogenesis of various diseases, including atherosclerosis, inflammatory diseases, and cancer (reviewed in ref. *2*), and are thought to participate in aging processes (reviewed in ref. *3*). The biological effects of these highly reactive compounds are controlled in vivo by a wide spectrum of antioxidative defense mechanisms: vitamins E and C, carotenoids, metabolites such as glutathione and uric acid, and antioxidant enzymes (*1, 4, 5*). Among these enzymes, superoxide dismutase (SOD; EC 1.15.1.1) catalyzes dismutation of the superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2), catalase (CAT; EC 1.11.1.6) detoxifies H_2O_2, and glutathione peroxidase (GPX; EC 1.11.1.9) both detoxifies H_2O_2 and converts lipid hydroperoxides to nontoxic alcohols.[3] In some recent clinical studies, one or several of these antioxidant enzymes were measured in blood as possible biological indicators, especially concerning hyperlipidemia and atherosclerosis (*6*), alcoholism (*7–13*), diabetes (*14, 15*), Down syndrome (*16, 17*), cancer (*18, 19*), and Alzheimer disease (*20*). However, data concerning the biological variability of these enzymes in blood of apparently healthy subjects are scarce and have often been obtained from restricted populations (*21–23*). Therefore, our aim was to describe the main factors of biological variation of SOD, GPX, and CAT in plasma and erythrocytes of a large population (about 1800 subjects), including the possible effects of tobacco, alcohol, and drugs, to quantify the effects of these factors and to examine possible interrelationships between all of these enzyme activities.

Materials and Methods

Population Samples

Our sample included 1782 individuals selected without known bias (860 males and 922 females), ages 4–65 years, attending the Center of Preventive Medicine of Vandoeuvre-Lès-Nancy, France, for health examinations. These individuals were identified from the files of the state health insurance fund and were invited in family groups. A more detailed description of the population is given elsewhere (*24*). Information regarding smoking, alcohol consumption, and drug intake was obtained by questionnaires. In addition, 54 totally autonomous and apparently healthy subjects, ages 65–97 years (16 men and 38 women), were recruited in a convalescent home.

Blood Samples

Venous blood samples were collected into heparinized tubes between 0800 and 0900 h. The blood was centrifuged at 1000 × *g* for 15 min and plasma was separated. The erythrocytes were carefully sampled from the bottom of the tubes to minimize contamination with leukocytes; they were washed three times with isotonic saline solution, and lysed by addition (1/6, by vol) of doubly distilled water containing 5 mL/L Triton X-100, followed by vigorous vortex-mixing and storage on ice for 10 min. Membrane-free hemolysate was obtained by centrifugation at 10 000 × *g* for 5 min. Hemoglobin

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[3] Nonstandard abbreviations: SOD, superoxide dismutase; GPX, glutathione peroxidase; CAT, catalase; and E, erythrocytes.

concentration was determined with a Coulter Counter T660 (Coulter Electronics, Margency, France). For assay of SOD, hemoglobin was extracted from hemolysate by addition of an equal volume of chloroform/ethanol (15/25, by vol). Hemolytic, icteric, or turbid samples were discarded.

Analytical Methods

Assay of SOD activity: SOD activities of both plasma and hemolysate were measured at 30 °C by a modification of the method of L'Abbé and Fischer (*25*) in a Cobas-Bio centrifugal analyzer (Hoffmann-La Roche, Basle, Switzerland). Concentrations of xanthine oxidase (EC 1.1.3.22; grade III, from buttermilk; Sigma Chemical Co., St. Louis, MO) and cytochrome *c* (Type III, from horse heart; Sigma) in the reaction mixture were 26 U/L and 2 μmol/L, respectively; these concentrations were consistent with those proposed by McCord and Fridovich (*26*). The final reaction volume was 390 μL and the sample volume of plasma (undiluted) or hemolysate (diluted 10-fold) was 4 μL. Absorbance was monitored at 415 nm for 3 min. For each plasma sample, we analyzed a sample blank (the reaction mixture contained all components except xanthine oxidase) separately and subtracted the results from the overall reaction rate. Standards (0–30 Sigma units/mL) were prepared by diluting a commercial SOD preparation from human erythrocytes (cat. no. S-9636; Sigma). One unit of SOD activity was defined as the amount of enzyme that inhibits the rate of cytochrome *c* reduction by 50% in the present conditions of measurement. One Sigma unit was equivalent to 25 of our units.

Assay of GPX activity: GPX activity in both plasma and hemolysate was determined with Ransel kits (cat. no. RS 505; Randox Labs., Crumlin, North Ireland). We measured activities at 37 °C in a Cobas-Bio centrifugal analyzer. This assay, based on the method of Paglia and Valentine (*27*), requires cumene hydroperoxide as a substrate. The final concentrations of reagents in the assay were those recommended by the manufacturer. The sample volume was 5 μL of plasma diluted fourfold, or 5 μL of hemolysate diluted fivefold. The total reaction volume was 265 μL. Absorbance was monitored at 340 nm for 3 min.

Assay of CAT activity: CAT activity was measured in hemolysates at 30 °C with a Shimadzu UV-160 spectrophotometer (Velizy, Villacoublay, France) by the method of Aebi (*28*). The decomposition of the substrate H_2O_2 was monitored spectrophotometrically at 240 nm. Activity was expressed as *K*/g of hemoglobin [*K:* rate constant of the first order reaction as defined by Aebi (*28*)].

Statistics

Statistical analyses were performed by using the BMDP® software (BMDP Statistical Software, Los Angeles, CA). We used standard stepwise multiple-regression analysis to study interrelationships between enzyme activities and their potential determinants. The regression matrix included 1836 rows and the following columns: intercept, I (gender), age, age-squared, I (menopause), I (elderly: ages >65 years), I (adolescent: ages between 10–14 years), weight, blood pressure, alcohol consumption, cigarette consumption, and number of I (drug) corresponding to drugs taken by >10 individuals. In the analysis, I ( ) is a binary indicator function. In all statistical analyses, the values of P-SOD were log-transformed.

We studied partial correlations of the various enzyme activities after adjustment for the effects of the confounding factors. The absence of outliers was verified by use of scatter plots involving the various possible couples of enzyme activities (data not shown).

We established reference intervals by using symmetrical empirical percentiles (2.5% and 97.5%) for GPX and CAT, and nonsymmetrical percentiles (0.5% and 95.5%) for P-SOD, the distribution of which shows asymmetry toward the upper end of the range of values (*29*).

Results

Analytical Variations

We assessed the precision of measurements by repeated assay of pools of plasma or hemolysates that were stored at −40 °C. The coefficients of variation (CV) for within-day repeatability and day-to-day reproducibility are shown in Table 1. Precision ranged between 3.4% and 8.1% for the various enzyme activities.

Erythrocyte activities were expressed as a function of hemoglobin concentration; however, no real difference in the results and their statistical significance was observed when these activities were expressed as a function of hematocrit or the number of erythrocytes.

Table 1. Precision of SOD, GPX, and CAT Measurements in Plasma (P) and Erythrocytes (E)

	P-SOD	E-SOD	P-GPX	E-GPX	E-CAT[a]
Within-day repeatability					
n	24	24	24	24	10
Mean	46.0 U/mL	35.3 U/mg of Hb	676 U/L	44.2 U/g of Hb	321 *K*/g of Hb
CV,%	3.4	4.2	3.5	3.6	4.4
Day-to-day reproducibility					
n	16	15	20	18	15
Mean	44.3 U/mL	32.4 U/mg of Hb	626 U/L	41.6 U/g of Hb	363 *K*/g of Hb
CV,%	5.2	5.2	7.1	7.5	8.1

[a] See text for definition of units.

Biological Variations

Gender: No statistically significant variation of enzyme activities related to gender could be demonstrated except for E-GPX, which is significantly higher in women than in men ($P < 0.001$) (Table 2).

Age: Age has a statistically significant effect on all antioxidant enzyme activities (Table 2). P- and E-GPX correlate positively with age and negatively with age-squared, and are decreased in the elderly; in addition, P-GPX is decreased in adolescents. E-CAT and E-SOD correlate negatively with age-squared, E-CAT being slightly diminished in the elderly. P-SOD correlates negatively with age. Scatter plots of P-GPX, E-GPX, E-CAT, E-SOD, and P-SOD activities against age (Figure 1) illustrate the results of the multiple-regression analysis. For P- and E-GPX, scatter plots appear somewhat parabolic, whereas a decrease with age is more linearly related for E-CAT and P- and E-SOD.

Menopause, menarche, weight, and blood pressure: No statistically significant effect of menopause, weight, or blood pressure on enzyme activities was observed. Conversely, E-SOD mean activity is decreased by 16% ($P < 0.05$) in menstruating girls ages 10–14 years, in comparison with pre-menarche girls of the same age range.

Drugs and other xenobiotics: Multiple-regression analysis shows slight but statistically significant effects of alcohol consumption on P-GPX and of smoking on P-SOD activity (Table 2). The effects of drug intake on the antioxidant enzyme activities are also listed in Table 2. Antidepressants and thyroid hormones clearly increase P-GPX and E-SOD activities, respectively. Less pronounced are the influences of hypolipidemic agents on P-GPX; vasculotropics and anti-inflammatory drugs on E-GPX; antiarthritics and antihistaminic agents on E-SOD; and cardiotonics and thyroid hormones on P-SOD.

Correlations between antioxidant enzyme activities: We found statistically significant positive correlations between E- and P-SOD ($r = 0.216$, $P < 0.001$), between E-CAT and E-SOD ($r = 0.123$, $P < 0.001$), and between E- and P-GPX ($r = 0.218$, $P < 0.001$). We found no statistically significant correlation between GPX and SOD or CAT.

Reference Intervals

To establish reference intervals, we excluded subjects presenting a pathological state or taking drugs showing a significant influence. Individuals with P-SOD activity >105 U/mL were also excluded. Because of the limited number of subjects >65 years, we did not calculate reference intervals for the elderly. The partition criteria were age, and in some cases, gender and menarche. The reference intervals we obtained for subjects <65 years are presented in Tables 3–5. Of course, these intervals are valid only for the particular assays used.

Discussion

Because of the obvious lack of data concerning the biological variability of the three antioxidant enzymes in blood of healthy subjects, we attempted to identify the factors affecting activities of SOD, GPX, and CAT. Because erythrocytes and plasma contain different isoenzymes of SOD (*30, 31*) and GPX (*32*), we measured activities of these enzymes in both biological media. However, the organs from whence these plasma enzymes originate are not known with certainty. We did not measure P-CAT in this study because P-CAT activity is 3600-fold lower than the activity found in erythrocytes, and its determination cannot be performed by the method of Aebi (*28*) but requires the use of more-

Table 2. Predictors of Antioxidant Enzyme Activities in Blood by Multiple-Regression Analysis

Variables	P-GPX, U/L	E-GPX, U/g of Hb	E-CAT, kU/g of Hb	E-SOD, U/mg of Hb	P-SOD,[a] U/mL
Gender (1 male, 2 female)	—	2.02 (0.51)[b,d]	—	—	—
Age (years)	3.96 (0.80)[d]	0.23 (0.05)[d]	—	—	−0.0005 (0.001)[e]
Age-squared (years^2)	−0.04 (0.01)[d]	−0.002 (0.001)[e]	−0.004 (0.001)[f]	−0.0006 (0.0001)[d]	—
Elderly[c]	−148.63 (28.32)[d]	−8.39 (2.04)[d]	−28.18 (12.32)[f]	—	—
Adolescent	−42.06 (13.48)[e]	—	—	—	—
Alcohol consumption (g/d)	0.31 (0.14)[f]	—	—	—	—
Tobacco consumption (g/d)	—	—	—	—	0.0008 (0.0004)[f]
Antidepressant use[c]	103.89 (41.11)[e]	—	—	—	—
Cardiotonic agent use[c]	—	—	—	—	0.08 (0.04)[e]
Hypolipidemic agent use[c]	41.35 (20.03)[f]	—	—	—	—
Thyroid hormone use[c]	—	—	—	6.53 (1.88)[d]	0.009 (0.03)[f]
Vasculotropic agent use[c]	—	−3.15 (1.33)[f]	—	—	—
Anti-inflammatory drug use[c]	—	3.85 (1.53)[f]	—	—	—
Antiarthritic agent use[c]	—	—	—	4.37 (2.00)[f]	—
Antihistaminic agent use[c]	—	—	—	4.27 (1.93)[f]	—
Intercept	591.35	34.85	461.65	29.37	1.58
Multiple R^2	0.098	0.052	0.014	0.022	0.012

[a] Log transformation used to increase normality.
[b] Regression coefficient (and standard error).
[c] Indicator function (1 = yes, 0 = no).
Significance of regression: [d] $P < 0.001$, [e] $P < 0.01$, [f] $P < 0.05$; all others nonsignificant (—).

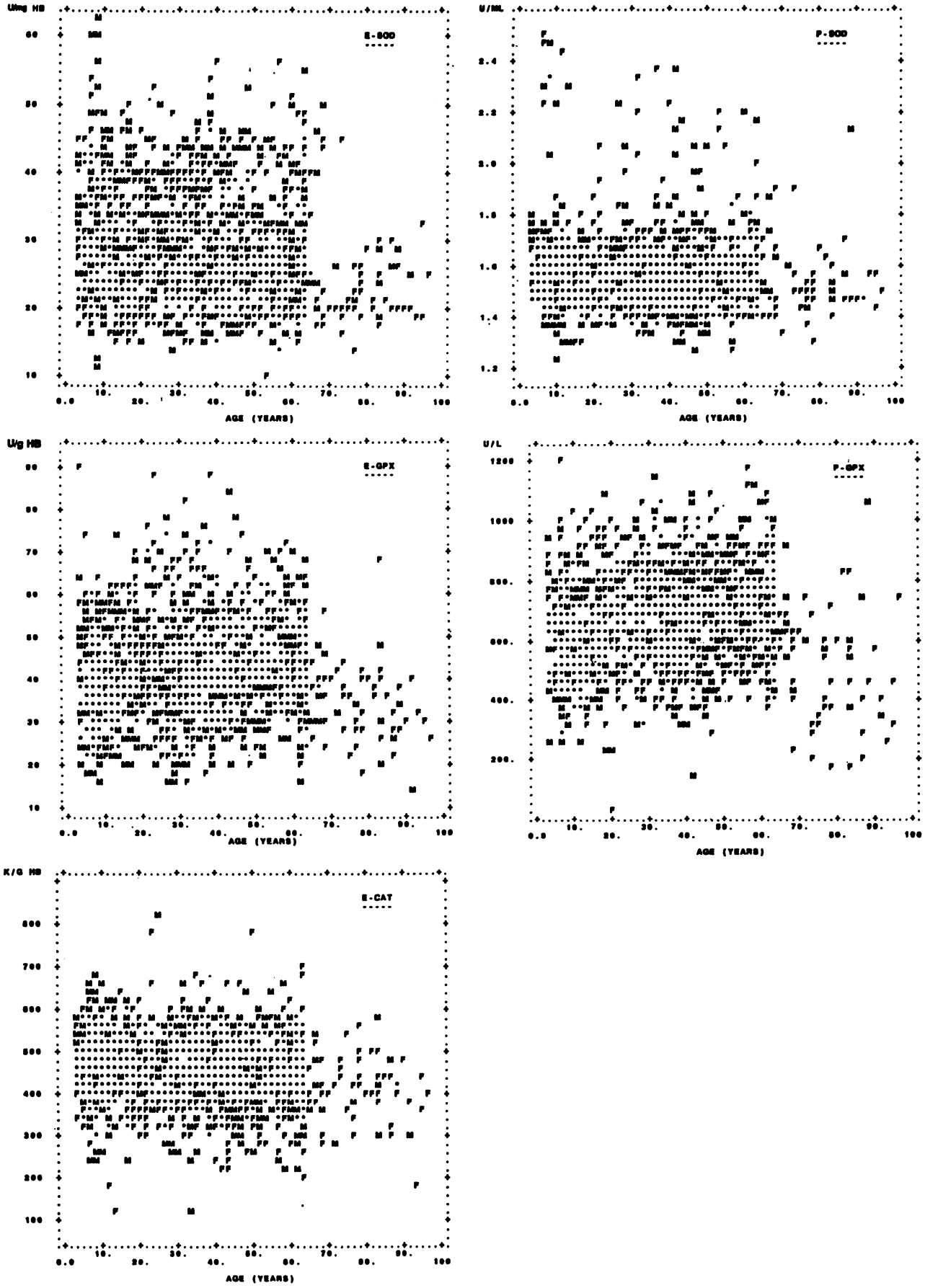

Fig. 1. Scatter plots of P-GPX, E-GPX, E-CAT, E-SOD, and P-SOD activities against age
M, male; F, female; *, more than two points

Table 3. Reference Intervals for P-SOD, P-GPX, and E-CAT, Both Sexes

Age, years	No. of subjects	P-SOD, U/mL[a]	P-GPX, U/L[b]	E-CAT, K/g Hb[b]
<20	512	20.0–55.9	355–934	465–608
20–65	1242	21.0–57.0	398–987	295–587

[a] 0.5th and 95.5th centiles.
[b] 2.5th and 97.5th centiles.

Table 4. Reference Intervals for E-SOD by Sex: 2.5th and 97.5th Centiles

	No. of subjects		E-SOD, U/mg of Hb	
Age, years	Males	Females	Males	Females
4–10	115	100	15.8–57.3	18.0–47.4
10–14	88	50[a]	17.7–46.0	16.7–40.8[a]
		22[b]		13.6–38.6[b]
14–20	72	65	19.2–44.6	16.1–47.4
20–65	1242[c]		17.1–45.0[c]	

[a] Before menarche.
[b] After menarche.
[c] Men and women combined.

Table 5. Reference Intervals for E-GPX by Sex: 2.5th and 97.5th Centiles

	No. of subjects		E-GPX, U/g of Hb	
Age, years	Males	Females	Males	Females
<20	275	237	19.8–59.5	23.3–62.3
20–65	569	673	22.5–67.0	24.4–68.1

sensitive methods such as polarographic techniques (*33*).

We found no statistically significant variation of the antioxidant enzyme activities according to gender, except for E-GPX, which was slightly higher in women than in men ($P < 0.001$). The variation we found is consistent with that described recently by McMaster et al. (*23*), except those authors also observed a higher activity of P-GPX in men. Conversely, Nève et al. (*22*) found no difference according to gender either for E-GPX or P-GPX in a more restricted population of 145 healthy individuals.

Important changes in the enzyme activities associated with aging are recorded in this study: activities appear rather stable in adults <65 years old, but decrease for most enzymes in the elderly; this simultaneous diminution of the three main antioxidant enzyme activities in the blood of the elderly had not been reported previously. Schäfer and Thorling (*34*) also described a reduction of E-GPX in the elderly, as did Jozwiak and Jasnowska (*21*) for E-SOD in such individuals. Unlike us, the latter authors found that E-GPX and E-CAT were higher in subjects ages 65–80 years than at ages 25–50 years; they also observed that E-GPX was lower in children ages 4–14 years than in this group of adults <50 years, whereas we found a decrease in adolescents only for P-GPX.

In 1986, Toth et al. (*35*) hypothesized that the antioxidant activities and protective abilities of erythrocytes from cigarette smokers might be greater than in erythrocytes from nonsmokers. They found that erythrocytes from smokers contained more CAT than did those from nonsmokers, but contained the same activities of GPX. We did not confirm these results; our multiple-regression analysis showed a slight but significant effect of cigarette consumption only on P-SOD activity ($P < 0.05$).

The role of free radicals and hydrogen peroxide in the metabolism and toxicity of alcohol is supported by many studies (reviewed in *36*). Therefore, many authors have tried to use the enzymes metabolizing these highly reactive chemical compounds as biological markers of alcoholism. Tanner et al. (*7*) and Bjorneboe et al. (*8*) found no variation of E-GPX and E-CAT, respectively, in alcoholics. On the other hand, several groups described an increase in E-SOD (*9–12*) and P-SOD (*13*) in such subjects. Conversely, Kubota et al. (*11*) found a decrease in P-SOD in the case of alcoholic liver disease, whereas others observed no modification of E-SOD related to alcoholism (*8, 12, 13*). These somewhat conflicting results are probably related to heterogeneity of the populations studied with regard to alcohol consumption or especially to the kind and the extent of the alcoholic liver troubles. Thus, Ledig et al. (*12*) observed no variation of E-SOD in alcoholics without liver disease, the activity being increased only in steatosis or cirrhosis. In our population of apparently healthy subjects, we found a statistically significant effect of alcohol consumption only on P-GPX activity ($P < 0.05$), which seems to be increased in drinkers.

As far as we know, there are no reports in the literature concerning the influence of drugs on antioxidant enzymes in blood. However, the strong effects we observed in therapy by antidepressants or thyroid hormones require confirmation in more extensive populations. Finally, we found a positive correlation between SOD and CAT in erythrocytes. This appears logical because CAT is thought to account for a notable part of the destruction of H_2O_2, which is partly generated by SOD (*37*). However, the reasons why E-SOD correlates with P-SOD and not with E-GPX (another enzyme catabolizing H_2O_2 in the erythrocyte) remain unclear.

In conclusion, we have investigated various possible sources of biological variation of SOD, GPX, and CAT activities in plasma and erythrocytes. Some of our findings, e.g., those concerning alcohol consumption, are inconsistent with data of other authors. This may be partly explained by large differences in the type and size of the populations studied. In the absence of standardized methods for the determination of these enzymes, differences in the assay conditions (e.g., types of substrates used and precision of measurements) may also affect the results and the degree of significance. Finally, our work demonstrates the existence of a concomitant decrease in most of the antioxidant enzymes in blood of

the elderly. This fits fairly well with the hypotheses of the free radical theory of aging (*3*). However, the study of the status of pro-oxidants and antioxidants in elderly subjects requires further investigation.

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