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Age Dependence of the Level of the Enzymes Involved in the Protection against Active Oxygen Species in the Rat Brain (42526)

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Abstract. Levels of Cu, Zn superoxide dismutase (CuSOD), Mn superoxide dismutase (MnSOD), catalase, and glutathione peroxidase (GPx) were assessed in the rat brain cortex. The concentrations of Cu- and MnSOD were found to increase linearly with the logarithm of the age of the animal from 3 days before birth to 30 months, both in the whole cortex tissue and in its cytoplasmic fraction. Catalase and GPx levels showed different trends; in particular, GPx, which appears to play a key role in detoxification of hydrogen peroxide, after an initial fall increases steadily with age. The enhancement of the levels of SOD and GPx could be related to protection against an increased production of reactive oxygen species in the aging process.

Measurement of the activity of specific enzymes in the brain during the life span is an important approach in brain aging studies. In particular, assessment of the activity of Cu, Zn superoxide dismutase (CuSOD), Mn superoxide dismutase (MnSOD), catalase, and glutathione peroxidase (GPx) is of interest because these enzymes are considered to be specifically involved in the defense of the cell against active oxygen species such as superoxide ion, hydrogen peroxide, $OH^{\bullet}$ radical. These species may produce tissue injuries in several pathological processes, and according to many authors, they are one of the possible causes of the aging process (1, 2).

Data on the level of SOD, GPx, and catalase in brain have been the object of many papers, but the different methods utilized for their determination, the number of samples analyzed, the life period considered (usually the developmental period), the brain regions selected, and the subcellular fractionation of the biological material have given rise to conflicting results, particularly in regard to old age (3–7). Homogeneous data, obtained with reliable techniques, may help one understand the role of these enzymes and as a consequence the role of active oxygen species in the aging process. In this paper we report the levels of CuSOD, MnSOD, catalase, and GPx measured in the rat brain cortex during the whole life span, particularly in old animals. In particular the NMR method, which we utilized for the determination of CuSOD and MnSOD (8), appears to be free of interference and therefore particularly suitable to carry out measurements in brain homogenates. To avoid loss of enzyme activity during tissue manipulation and fractionation, the enzyme levels were measured in crude homogenates.

Materials and Methods. All the chemicals used were reagent grade. Wistar rats were killed by decapitation. The brain was immediately removed and dissected, and the cortex was placed in cold PBS buffer and homogenized. A Teflon pestle Potter–Elvehjem homogenizer, clearance 0.10–0.15 mm, was used to obtain homogenates containing intact mitochondria. The measurements of the enzyme levels were carried out on these homogenates (about 0.2 g tissue/ml solution) in the absence and in the presence of 1% Triton X-100. Detergent was added to obtain the release in the solution of the enzymes partially bound to membranes or contained in subcellular organelles. In this way a relatively rough but still significant separation of the enzymes present in different cell compartments was achieved. That is, in the absence of the detergent, the activity of the cytoplasmic enzymes should mainly be measured, while in the presence of detergent the enzymes of the whole brain cortex contribute to the measured activities.

Under our experimental conditions the

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contamination of homogenates by blood was negligible (Hb content below 1 mg/g tissue). Cu- and MnSOD were determined according to an NMR method utilizing the fluoride ion as a probe of the level of MnSOD and CuSOD (8). In fact, the presence of Cu- or MnSOD in tissue homogenates increases the relaxation rate of $^{19}F^-$ many orders of magnitude. Cu- and MnSOD were distinguished by measuring the relaxation time of $^{19}F^-$ added to the crude homogenate before and after the addition of 2 m*M* NaCN, which makes the contribution of CuSOD to the $^{19}F^-$ relaxation rate negligible. A pulse NMR spectrometer operating at 16 MHz with $\frac{\pi}{2}$–τ–$\frac{\pi}{2}$ sequence used. Controls of CuSOD levels were also carried out by polarography (9). Catalase, glutathione peroxidase, and glutamate dehydrogenase activities were determined spectrophotometrically. In particular, catalase activity was measured following the decay of the absorbance of hydrogen peroxide at 240 nm (10). The activity of glutathione peroxidase was determined according to Little *et al.* (11) by measuring the oxidation of NADPH coupled to GSH via GSSG reductase at 340 nm. The measurement of glutamate dehydrogenase activity was carried out using 2-oxoglutarate and NH_4^+ as substrates and NADH as coenzyme; the oxidation of NADH was followed at 340 nm (12). No significant effect of Triton X-100 on the activity of these enzymes and on the relaxation times of Cu- and MnSOD was found.

Results. *MnSOD and CuSOD levels.* The concentrations of MnSOD and CuSOD were measured in the crude homogenates in the absence and in the presence of Triton X-100. In the first case the SOD present in the intact mitochondria and nuclei did not contribute to the measured enzyme level, while in the presence of the detergent the whole SOD content of the tissue was measured.

Evidence that in crude homogenates prepared in the absence of Triton X-100 mitochondria are mostly intact was obtained by measurement of the activity of glutamate dehydrogenase, a soluble enzyme located within the mitochondrial membrane. According to these measurements the ratio of the activities of this enzyme in the homogenates, prepared in the presence and in the absence of Triton X-100, was on average 5.

The concentration of MnSOD measured by

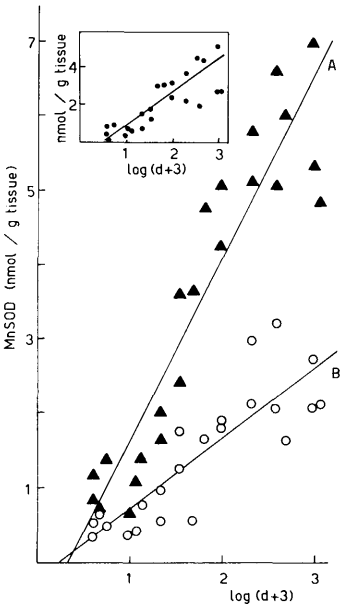

FIG. 1. MnSOD concentration in rat brain as a function of age. The MnSOD was measured by NMR, in the presence (▲) and in the absence (○) of Triton X-100, in homogenates of rat brain cortex. In the insert the difference between the concentrations of plots A and B is reported. The straight lines represent the best fit to experimental data.

NMR in the homogenates, either with or without Triton X-100, was found to increase monotonically with age. In particular, good linear relationships between the concentration of this enzyme and the logarithm of the days of postnatal age (d) plus 3 days, that is, log (d + 3), were calculated for either type of homogenate (see Fig. 1, plots A and B). The equation of the regression line A of Fig. 1, presence of the detergent, was [MnSOD] = $-0.803 + 2.47 \log (d + 3)$, correlation coefficient 0.93, where −0.803 and 2.47 are the intercept and the slope of the straight line, respectively. The equation of curve B, absence of detergent, was [MnSOD] = $-0.224 + 0.948 \log (d + 3)$, and the correlation coefficient was 0.85. The difference in SOD levels found in rats of the same age, see Fig. 1, should be ascribed mainly to animal variability since the standard deviation of the NMR method is about 4%. The above data indicate that MnSOD is evenly distributed between the cytoplasmic fraction and the fraction solubilized by Triton X-100. Furthermore, the concen-

tration of MnSOD in the latter fraction, calculated as the difference between the data of plots A and B of Fig. 1, increases linearly during the life span of the rat (see Fig. 1 insert). The regression line was [MnSOD] = −0.517 + 1.496 log (d + 3), correlation coefficient 0.82. The lower value of correlation coefficient of this plot with respect to those of plots A and B of Fig. 1 is easily explained since the data reported in the insert were calculated as the difference between the experimental data of line A and of line B.

From Fig. 1 it also appears that the MnSOD concentration increases during the life span of the rat by a factor of about 5 both in the cytoplasm and in the whole cortex tissue.

The CuSOD concentrations at different animal ages, measured in homogenates in the presence and in the absence of Triton X-100, are reported in Fig. 2, curves A and B, respectively. Also, in this case the best linear relationships were obtained when the logarithm of the postnatal age plus 3 days was considered. The equations of the regression straight lines A and B, reported in Fig. 2, are [CuSOD] = 0.023 + 0.830 log (d + 3), correlation coefficient 0.91, and [CuSOD] = 0.073 + 0.482 log (d + 3), correlation coefficient 0.85, respectively. From Fig. 2 it appears that CuSOD

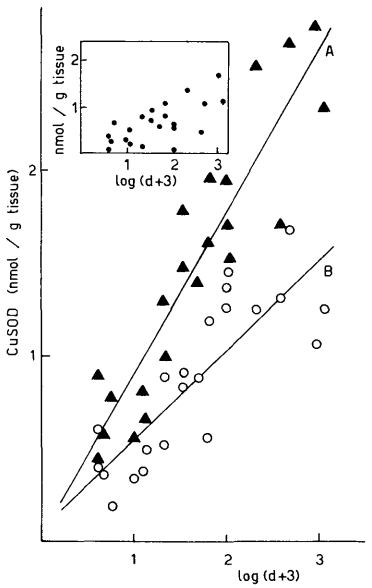

FIG. 2. CuSOD concentration in rat brain as a function of age. For the conditions see Fig. 1.

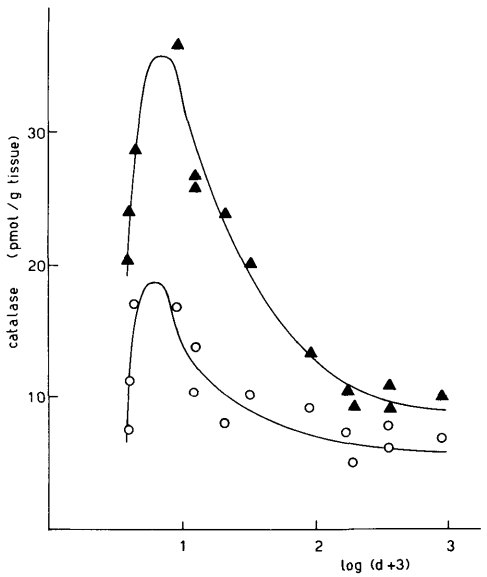

FIG. 3. Catalase concentration in rat brain as a function of age. The enzyme concentration was measured by spectrophotometry (see text) in crude homogenates in the presence (▲) and in the absence (○) of Triton X-100.

is mainly localized in the cytoplasmic fraction, where it increases by a factor of about 4 during the life of the rat. As a consequence, our data on the concentration of CuSOD in the fraction solubilized by Triton X-100 show a large variability with age because they were calculated as the difference between two sets of data which are relatively close (see Fig. 2 insert).

Since the relaxation time of CuSOD, in brain homogenates, is particularly sensitive to the copper oxidation state (13), controls of the CuSOD level measured via NMR were performed by polarography (9). A linear relationship with a slope equal to 1 was found between the two sets of data, correlation coefficient of 0.98, indicating the good reliability of the NMR method.

Catalase and glutathione peroxidase level. The dependence of the catalase level on age is reported in Fig. 3. The data of this figure indicate an increase in the activity of this enzyme in the first days of postnatal life and a drop in the activity by a factor of about 3 during the next 3 months to a practically constant level. Figure 3 also shows that catalase is evenly distributed between the cytoplasmic fraction and the fraction solubilized by Triton X-100.

The contribution of the catalase present in the blood to the catalase concentration measured in the brain cortex was negligible because the contamination of the homogenates by blood was lower than 0.3%. According to our data the catalase levels are at least two orders of magnitude lower than the sum of the concentrations of MnSOD and CuSOD. In particular, in old rats the ratio between the SOD concentration and the catalase concentration is on the order of 10^3. This low level of catalase points out the fundamental role of GPx in the detoxification of hydrogen peroxide in the brain.

The dependence of the activity of glutathione peroxidase on age is reported in Fig. 4. From this figure it appears that the activity of GPx, after a drop occurring in the 1st week of life, which was also reported by other authors (14), increases steadily with age. This figure indicates no substantial difference between the levels of GPx measured in the presence and in the absence of Triton X-100. Similar results were also obtained for the supernatants of crude homogenates after centrifugation at 50,000*g* for 30 min. No glutathione peroxidase activity was measured when the pellets obtained by the centrifugation of the crude homogenates were resuspended in PBS buffer containing Triton X-100. These experimental data seem to indicate that, in the rat brain cortex, glutathione peroxidase is mainly localized in cytoplasm.

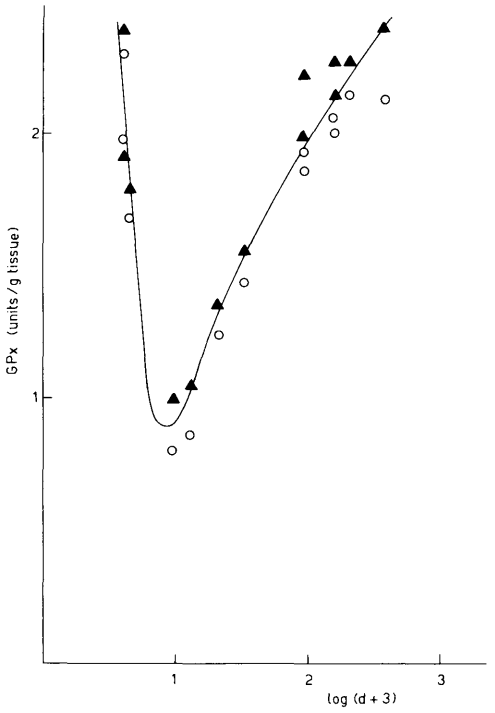

FIG. 4. Glutathione peroxidase concentration in rat brain as a function of age. The enzyme activity was measured spectrophotometrically in crude homogenates in the presence (▲) and in the absence (○) of Triton X-100.

Finally, we have tentatively evaluated the relative activities of catalase and GPx present in the brain by measuring the competition between these two enzymes for hydrogen peroxide. Experiments carried out by adding brain homogenates to solutions containing 0.1 m*M* hydrogen peroxide and 2 m*M* GSH, in either the presence or the absence of 0.4 m*M* CN^- as catalase inhibitor, showed no difference in the GSH oxidation rate and in the total amount of GSH consumed. This result seems to point to the key role of GPx in the scavenging of hydrogen peroxide in the brain cortex.

Discussion. The continuous increase in the levels of CuSOD and MnSOD with age in crude homogenates of rat brain cortex contrasts with the dependence of the concentrations of total protein and enzymes on age. The reports on this topic usually describe a fast increase in the enzyme content during the early period of life to a level which remains constant or decreases slowly with age (15, 16). According to the hypothesis of superoxide ion-mediated oxygen toxicity (17), the increased level of superoxide dismutases could represent a defensive response of brain to an augmented production of superoxide with age.

It is interesting to note that the best correlation coefficients for the regression analysis of Mn- and CuSOD were obtained when the levels of these enzymes were plotted against log (d + 3). This fact indicates that the SOD levels increase continuously, in a logarithmic way, starting at a few days before the parturition. That is, we are in the presence of a "preparation for birth," with an SOD maturational pattern that begins for both enzymes in the last days of fetal life. These data support the findings of Frank and Groseclose (18).

The complementary trends of catalase and GPx during the first weeks of life seem to in-

dicate a correlation between these enzymes. However, because of the negligible contribution of catalase in the control of the hydrogen peroxide level, glutathione peroxidase appears to play a key role in the detoxification of hydrogen peroxide, the production of which may increase parallel to that of the superoxide ion. Therefore the rise in the GPx level, after the sharp drop observed during the first week of life, may also be considered in terms of protection from the active oxygen species.

However, hydrogen peroxide could be also generated in brain by different mechanisms from superoxide dismutase catalysis. For instance, large amounts of hydrogen peroxide may be generated during oxidative deamination of catecholamines by monoamine oxidases.

The increase of the level of CuSOD, MnSOD, and GPx with brain age is particularly interesting since in other tissues such as blood and lung (19), the SOD concentration does not change with age. This fact may reflect a peculiarity of the brain metabolism and function. If superoxide ion and hydrogen peroxide are directly or indirectly toxic to the brain tissue, it appears reasonable to assume that in the brain the apparatus of protein synthesis may be programmed to elevate the level of these protective enzymes in response to an increase of reactive oxygen species, as has been demonstrated in other tissues (20).

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