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Superoxide dismutase, glutathione peroxidase and catalase in developing rat brain

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The specific activities of Cu,Zn- and Mn-superoxide dismutases, of glutathione peroxidase and of catalase, the enzymes considered to be specifically involved in the defence of the cell against the partially reduced forms of oxygen, were determined as the function of postnatal age in the early (up to 60 days) period of rat brain development. The enzymes were assayed in the cytoplasmic fraction, in the crude mitochondrial fraction including peroxisomes, and in the mitochondria. The results show that the temporal changes of these enzymes cannot be correlated with each other, thus indicating that they do not concertedly parallel the increasing activity of aerobic brain metabolism during development. Specifically the cytoplasmic fraction shows a gradual increase of the Cu,Zn-superoxide dismutase activity with age, whereas the glutathione peroxidase activity is constant from birth. Furthermore the increase of the mitochondrial Mn-superoxide dismutase as a function of postnatal age is more remarkable than that of the cytoplasmic Cu,Zn-enzyme. Higher activities of catalase in adult animals are detectable only in the subcellular fraction containing peroxisomes, because of the modest catalase activity of the brain. These results indicate independent regulation of the expression of these enzyme activities in the process of brain differentiation and point to a relative deficiency of enzymic protection of the brain against potentially toxic oxygen derivatives. This situation is similar to the pattern already described in the rat heart and in rat and mouse ascites-tumour cells, at variance with the much more efficient enzyme pattern present in rat hepatocytes.

It has been established that aerobic metabolism produces potentially toxic oxygen derivatives, such as the superoxide anion radical ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2). Their cytotoxicity is likely to be due to the secondary production of singlet oxygen ($^1O_2$) or $OH^\bullet$ radicals in redox reactions between each other or with transition-metal ions, such as dismutations or the so-called Haber–Weiss reaction (Fridovich, 1974). The enzymes involved in the cell defence against oxygen cytotoxicity have been repeatedly proposed to be superoxide dismutases, catalase and peroxidases (Halliwell, 1974). It seems reasonable to assume that their action is concerted, as superoxide dismutases catalyse $O_2^{-\bullet}$ dismutation producing H_2O_2 whereas catalase or peroxidases remove it. However, it is also apparent that the presence of catalase is restricted in most eukaryotic cells to isolated compartments such as peroxisomes, whereas superoxide dismutase and peroxidases (essentially glutathione peroxidase in animal cells) are present in the mitochondrial and cytoplasmic fractions (Chance *et al.*, 1979). Furthermore, superoxide dismutase is represented by two types of enzymes, namely the Cu,Zn- and the Mn-superoxide dismutase, with seemingly preferential location in the cytoplasm and the mitochondrion respectively (Tyler, 1975).

In a previous paper (Mavelli *et al.*, 1978) we reported the gradual increase of superoxide dismutase activity in the developing rat brain as compared with the behaviour of the same enzyme in other tissues. In the present work we have determined, as a function of postnatal age, the activities of both Cu,Zn- and Mn-superoxide dismutases, and also of glutathione peroxidase and catalase, with the aim of establishing a more detailed developmental pattern for these enzymes, and also in relation to different subcellular localization.

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The system chosen, i.e. the developing brain of an animal, such as the rat, born neurologically immature, seemed particularly appropriate for an attempt to establish a correlation between the activities of enzymes involved in preventing oxygen-mediated biological damage and the extent of aerobic metabolism. In fact the aerobic metabolism increases as the brain maturation proceeds, and the parallel increment of some enzymes related to the cellular activation of oxygen, such as succinate dehydrogenase, citrate synthase and pyruvate dehydrogenase, has been reported (Booth *et al.*, 1980).

Materials and methods

All the chemicals used were reagent grade and were purchased from Merck. NADPH and the kit for the determination of glutathione peroxidase were obtained from Boehringer, and t-butyl hydroperoxide was from Merck–Schuchardt.

Wistar rats of different postnatal ages were killed by decapitation. Brains were excised and washed with 0.32M-sucrose and were then homogenized without prior separation of white matter and grey matter in the same solution in a Potter–Elvehjem glass homogenizer. The subcellular fractions were prepared from homogenates (pooled homogenates from three animals for each age) by the procedure of Gray & Whittaker (1962). The recovery of mitochondria from crude fractions of different developmental ages was monitored by measuring the cytochrome oxidase activity, which is reported to parallel the increase of mitochondrial population during brain maturation (Caley & Maxwell, 1971). The recovery was found to be the same for different ages, on this basis. Rat hepatocytes and Ehrlich and Yoshida ascites-tumour cells were prepared as previously described (Bozzi *et al.*, 1981).

Protein was determined by a modification of the Lowry procedure (Lees & Paxman, 1972), with bovine serum albumin as standard. Enzyme assays on particulate fractions were made after sonication for 5min in bursts of 30s with cooling at 0°C. Catalase activity was assayed by a u.v. method (Lück, 1963). Glutathione peroxidase activity was determined by a colorimetric method with t-butyl hydroperoxide as substrate (Beutler *et al.*, 1977). Cytochrome oxidase activity was measured by a spectrophotometric method (Cooperstein & Lazarow, 1951). Units of these three enzymes are expressed as substrate (M) transformed/min per mg of protein. In the mitochondrial fraction the catalase and glutathione peroxidase activities were measured in the presence of 0.1% Triton X-100, to obtain optimal release of the enzymes. Cu,Zn- and Mn-superoxide dismutases were determined by a polarographic method (Rigo *et al.*, 1975) at pH 10, with the use of 1.25mM-KCN to discriminate the CN^--insensitive Mn-enzyme from the CN^--sensitive Cu,Zn-superoxide dismutase (Rigo *et al.*, 1975). This particular method was selected, firstly because it is free of interference from biological materials, and secondly because a complete inhibition of the Cu,Zn-enzyme by CN^- can be obtained only at alkaline pH (Rotilio *et al.*, 1972). Superoxide dismutase contents are reported as μg of enzyme/mg of protein, with reference to the activity in the same conditions of standard samples of purified proteins, which were the bovine Cu,Zn-enzyme and the *Bacillus stearothermophilus* Mn-superoxide dismutase. The catalytic constant of the latter enzyme as measured by polarography was found to be $1.3 \times 10^8 M^{-1} \cdot s^{-1}$, in fairly good agreement with that measured by pulse radiolysis at pH 10.2 (McAdam *et al.*, 1977). In the absence of a purified rat Mn-superoxide dismutase, the use of a well-characterized bacterial enzyme as reference protein was considered to be valid, at least preliminarily, since superoxide dismutases of the same metal class do not show significant variations of the catalytic constant. To compare the two dismutase activities in terms of catalytic efficiency, it should be kept in mind that, although at the pH of the assays the rate constant of the Cu,Zn-enzyme is 10 times that of the Mn-enzyme, the two rate constants are comparable at neutral pH (Forman & Fridovich, 1973).

Results

Fig. 1 reports the changes of activity of glutathione peroxidase and superoxide dismutase in the cytoplasmic fraction of the rat brain during the first 2 months of postnatal development. This interval was chosen because previous work on superoxide dismutase (Mavelli *et al.*, 1978) had shown that no further significant increase of the enzyme activity was observed later than about 2 months after birth. It appears that the two enzymes have very different developmental profiles: there is a regular increase with age in Cu,Zn-superoxide dismutase activity and an age-independent value for glutathione peroxidase activity. Fig. 2 shows the pattern of glutathione peroxidase and superoxide dismutase activities in the mitochondrial fraction during the same period. The superoxide dismutase activities are expressed as a function of total protein in Fig. 2, and of cytochrome oxidase activity in Fig. 3. Both Figures document the very remarkable and specific increase in Mn-superoxide dismutase activity. In contrast, the specific activities of Cu,Zn-superoxide dismutase and of glutathione peroxidase in the mitochondrial fraction display almost no change during development.

The changes of catalase activity over the same

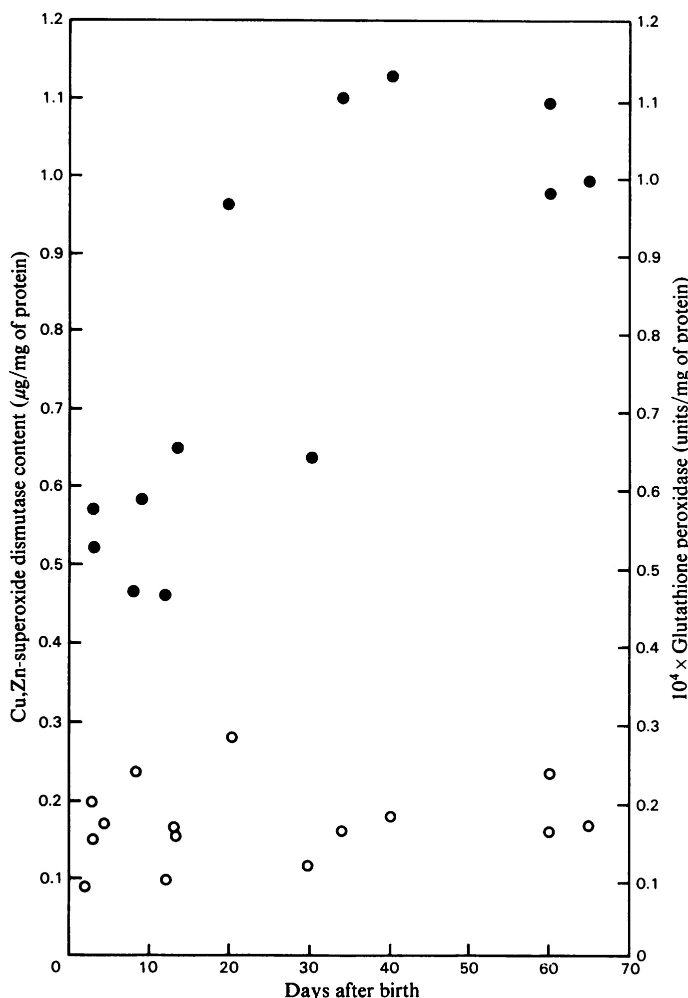

Fig. 1. *Changes in Cu,Zn-superoxide dismutase* (●) *and in glutathione peroxidase* (○) *activities in the cytoplasmic fraction during rat brain development*
For experimental details see the text.

period were hardly detectable because of the low content of the enzyme in the brain. Table 1 reports some results obtained in either total homogenates or crude mitochondrial fractions (containing peroxisomes) of the neonatal and adult (60 days) rat brain. An increase with age was evident in peroxisomes, but in total homogenates less catalase was measured in the adult than in the newborn brain with reference to the total cell protein.

Table 1 also reports, for comparison, the activities of glutathione peroxidase and Cu,Zn-superoxide dismutase in rat hepatocytes and rat brain of newborn and adult animals. The activities of the same enzymes in Ehrlich and Yoshida ascites-tumour cells are reported in Table 1 as well.

Discussion

The results obtained in the present work show that the specific activities of superoxide dismutase, catalase and glutathione peroxidase do not uniformly parallel the development of aerobic metabolism during the postnatal maturation of rat brain. The behaviour of Cu,Zn-superoxide dismutase and glutathione peroxidase in the cytoplasmic fraction (Fig. 1) has to be discussed in relation to the hypothesis of a functional correlation between the former enzyme, which catalyses H_2O_2 production from superoxide liberated by cellular processes into the cytoplasm, and glutathione peroxidase, which is the H_2O_2-removing enzyme of the cytoplasm (Rotilio *et al.*,

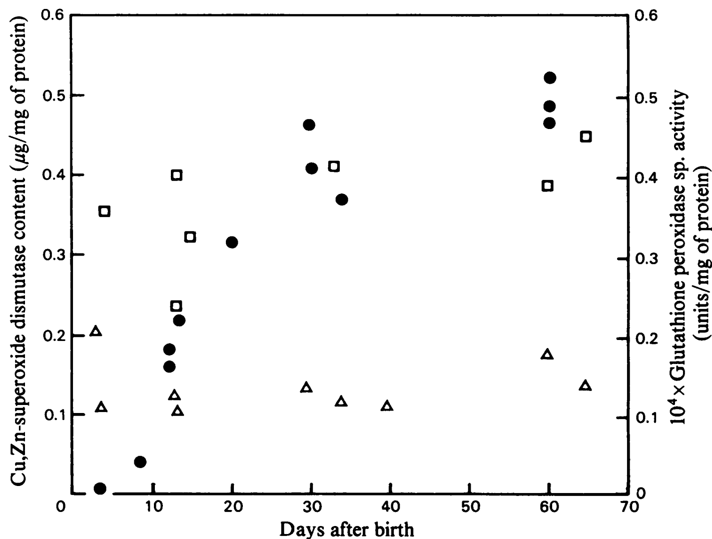

Fig. 2. *Changes in Cu,Zn-superoxide dismutase* (□), *Mn-superoxide dismutase* (●) *and glutathione peroxidase* (△) *activities in the mitochondrial fraction during rat brain development*
For experimental details see the text.

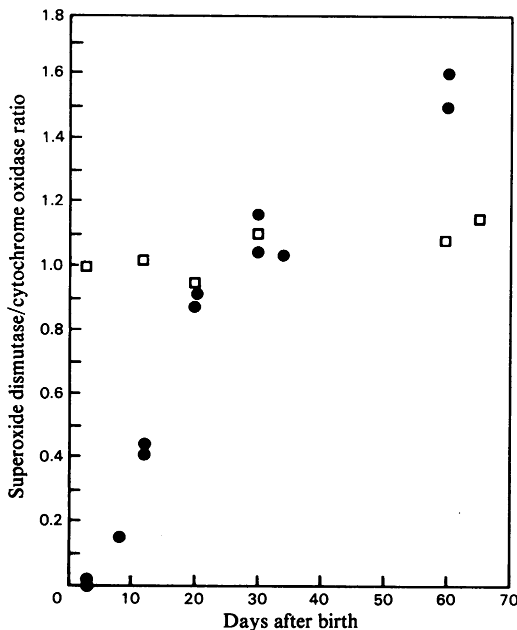

Fig. 3. *Relationship between the changes in superoxide dismutase and cytochrome oxidase activities in developing rat brain*
□, Cu,Zn-superoxide dismutase; ●, Mn-superoxide dismutase. For experimental details see the text.

1973). The unequal rates at which the two enzymes approach the value typical of the adult rat brain are noteworthy in this respect. It is apparent that a relative increase of superoxide dismutase activity over that of H_2O_2-removing enzymes within the same cellular compartment might not result in a greater capability of defence against reactive oxygen derivatives (Rigo & Rotilio, 1980). In view of these considerations, the developing rat brain can be regarded as a useful model *in vivo*, as it provides a variable temporal pattern of the ratio between different enzyme activities involved in the defence against oxygen cytotoxicity. Table 1 shows that the ratio of glutathione peroxidase to Cu,Zn-superoxide dismutase, which is the relevant one as far as the cytoplasm is concerned, decreases with age, in contrast with the behaviour of liver cells. From these data the brain appears to be relatively deficient in such enzymes, and this situation compares with that already described in the rat heart (Nohl & Jordan, 1980) and with that of Ehrlich ascites-tumour cells. Both the rat heart and Ehrlich ascites-tumour cells are sensitive to quinone antibiotics such as adriamycin and daunomycin, which produce H_2O_2 from $O_2^{-\bullet}$ through intracellular activation (Myers *et al.*, 1977; Bozzi *et al.*, 1981), whereas Yoshida ascites-tumour cells are almost as resistant as liver cells. Such a sensitivity has been related to deficiency of the glutathione peroxidase system (Bozzi *et al.*, 1981). Analogously the toxicity of hyperbaric oxygen on the central nervous system, which probably involves the generation of $O_2^{-\bullet}$ (Clark & Fisher, 1977), may be associated with the same enzymic deficiency in the brain, as compared, for instance, with the adult liver (Table 1). In fact, exogenous catalase was found to protect against convulsions provoked in mice by hyperbaric oxygen, whereas exogenous superoxide dismutase did

Table 1. *Specific activities of catalase, glutathione peroxidase and Cu,Zn-superoxide dismutase in different cell types*
For experimental details see the text.

	$10^4 \times$ Catalase sp. activity (units/mg of protein)	$10^4 \times$ Glutathione peroxidase activity (units/mg of protein)	Cu,Zn-superoxide dismutase content (μg/mg of protein)	Glutathione peroxidase/ Cu,Zn-superoxide dismutase ratio
Rat hepatocytes (total homogenates)				
Newborn	1.30 ± 0.10	0.29 ± 0.05	0.40 ± 0.07	0.73
Adult	3.00 ± 0.60	2.81 ± 0.63	1.74 ± 0.42	1.61
Rat brain (total homogenates)				
Newborn	0.07 ± 0.01	0.08 ± 0.01	0.27 ± 0.09	0.30
Adult	0.40 ± 0.005	0.10 ± 0.01	0.50 ± 0.05	0.20
Rat brain (subcellular fractions)				
Newborn	0.05 ± 0.02	0.18 ± 0.02	0.40 ± 0.05	0.45
Adult	0.25 ± 0.05	0.20 ± 0.02	1.00 ± 0.01	0.20
Ehrlich ascites-tumour cells (total homogenates)	<0.01	0.13 ± 0.01	0.25 ± 0.01	0.52
Yoshida ascites-tumour cells (total homogenates)	<0.01	0.36 ± 0.02	0.51 ± 0.02	0.71

not (Hilton *et al.*, 1980). The absence of cerebral symptoms during adriamycin anti-cancer therapy as compared with the established heart damage (Myers *et al.*, 1977) can be explained by the fact that the drug is unlikely to reach the central nervous system.

Another interesting result is the remarkable increase with time of the specific activity of Mn-superoxide dismutase in the mitochondrial fraction (Figs. 2 and 3). It is clear from Fig. 3 that such an increase is not due to an unspecific increase in total mitochondrial protein, since it is still remarkable when related to the cytochrome oxidase activity. On the other hand, the increase does not appear to be correlated with either cytochrome oxidase and glutathione peroxidase activity, or even with that of the mitochondrial Cu,Zn-enzyme (Figs. 2 and 3). It should also be noted that the activity of the Mn-enzyme, as compared with that of the Cu,Zn-enzyme in both soluble and mitochondrial fractions, is practically absent up to 10 days after birth. This may indicate that its activity is very low in the prenatal period as well. In any case, it can be concluded that the two types of dismutases, which are both coded for in the nucleus (Weisiger & Fridovich, 1973), respond with a distinct 'time constant' to the differentiation of the same tissue of residence, pointing to distinct mechanisms of regulation. As far as the nature of such mechanisms is concerned, the present data do not permit us to say whether the increase of enzyme activities is due to increased protein synthesis or to better availability of the active metals for apoproteins.

The amount of Cu,Zn-superoxide dismutase detected in the mitochondrion (as well as that of Mn-enzyme measured in the cytoplasm) was found to be age-independent. It is at present difficult to say whether these findings involve contaminating enzyme activities from other subcellular compartments during fractionation or reflect a local control of the activation of certain enzymes. It should be noted that the Cu,Zn-enzyme of mitochondria just parallels the behaviour of cytochrome oxidase (Fig. 3), and this may suggest a close correlation of the Cu,Zn-superoxide dismutase activity with the rate of oxygen reduction by brain mitochondria.

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