Rate of tryptophan hydroxylation in brain nuclei of Lyon Hypertensive (LH), Normotensive (LN) and Low Blood Pressure (LL) rats

Age	5 weeks		9 weeks		21 weeks	
C_1 area						
LH	1.73 ± 0.12*	(9)	1.09 ± 0.15**	(10)	2.32 ± 0.14	(9)
LN	2.47 ± 0.29**	(10)	1.57 ± 0.20	(11)	2.44 ± 0.24	(9)
LL	1.60 ± 0.14	(10)	1.68 ± 0.17	(11)	2.34 ± 0.21	(9)
C_2 area						
LH	2.52 ± 0.32	(9)	3.64 ± 0.32	(10)	3.62 ± 0.32	(9)
LN	2.19 ± 0.31	(10)	3.63 ± 0.32	(11)	3.07 ± 0.32	(9)
LL	2.17 ± 0.26	(10)	3.13 ± 0.28	(11)	3.39 ± 0.41	(9)
Raphe dorsalis						
LH	30.57 ± 3.54	(9)	46.06 ± 4.05	(10)	33.28 ± 3.78	(9)
LN	24.03 ± 2.86	(10)	45.79 ± 3.31	(10)	29.57 ± 3.90	(9)
LL	30.82 ± 2.90	(10)	44.46 ± 2.73	(11)	31.46 ± 3.07	(9)
Raphe centralis						
LH	33.19 ± 3.61	(9)	19.45 ± 3.56	(10)	13.11 ± 1.22	(9)
LN	33.90 ± 6.18	(10)	18.53 ± 3.31	(10)	10.08 ± 1.79	(9)
LL	31.87 ± 2.90	(10)	21.59 ± 2.98	(11)	13.78 ± 2.32	(9)
Raphe magnus						
LH	5.90 ± 1.05	(9)	8.94 ± 0.92*	(10)	9.21 ± 1.36	(9)
LN	4.67 ± 0.58	(10)	6.26 ± 0.58	(10)	8.77 ± 1.82	(9)
LL	6.92 ± 1.09	(10)	8.14 ± 1.14	(11)	10.13 ± 2.00	(9)

Data (ng of L-5-HTP formed/30 min per mg of protein) are means ± SEM. The number of animals in each group is in brackets. Statistical differences between animals of the same age are indicated:
* $p < 0.05$ vs LN rats; ** $p < 0.05$ vs LL rats.

been made to discuss the data as a function of aging, since rats of different ages were killed and analyzed in different runs of assays.

As indicated in the table, the rate of tryptophan hydroxylation of the C1 area was found to be decreased in 5 week-old LH rats (-30%, $p < 0.05$) when compared to LN, but not when compared to LL rats; in 9 week-old LH rats, it was found to be decreased as compared to LN (-30%) and also significantly decreased as compared to LL rats (-35%, $p < 0.05$); at 21 weeks of age, there was no difference between the three strains. There was no significant difference in the C2 area, the nuclei raphe dorsalis, centralis and magnus between the LL, LN and LH rats at the three ages studied, except for an increase in the nucleus raphe magnus of 9 week-old LH rats when compared to LN rats only ($+42\%$, $p < 0.05$). Similarly, no difference was found in the posterior and anterior hypothalamus (data not shown), a result which is similar to the data obtained on Japanese spontaneously hypertensive rats[11,12].

In the present work, the rate of tryptophan hydroxylation was estimated in various brain areas of rats from a genetically hypertensive (LH) or from two control (LN and LL) strains. It might be stated that if a difference is found between LH and LN rats, the same modification must also exist between LH and LL rats to be considered as possibly related to the hypertension. This was not the case for the changes found in LH rats in the nucleus raphe magnus at 9 weeks of age and in the C1 area in 5 week-old animals. Therefore it can be assumed that these alterations are unrelated to the high blood pressure of the LH strain.

On the contrary, the rate of tryptophan hydroxylation of the C1 area was decreased in 9 week-old LH, as compared to both LN and LL rats. Therefore, this change might be related to the difference in the blood pressure levels between these strains. However, it remains to be determined whether there is a link between the hypertension and this transient decrease in the rate of tryptophan hydroxylation in the C1 area. Recent results could support such a link, since serotonergic neurons originating from this medullary area are likely to play an important role in vasomotor control[13].

1 Acknowledgment. The authors wish to thank Dr M. F. Belin, Dr J. F. Pujol and Mrs J. Sacquet for their help during this study. This work was supported by the Fondation pour la Recherche Médicale Française and the C.N.R.S.

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0014-4754/85/040478-02$1.50 + 0.20/0

Involvement of reactive oxygen species in the microsomal S-oxidation of thiobenzamide

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Summary. Superoxide dismutase, catalase and methional proved capable of inhibiting the microsomal oxidation of thiobenzamide, which is most probably catalyzed by the flavin-containing monooxygenase. This indicates that excited oxygen species (e.g. $\cdot O_2^-$, H_2O_2, $\cdot OH$) are involved in the catalytic cycle of this enzymatic reaction. CO, which inhibits the cytochrome P-450-dependent oxygen radical formation, had no effect on the oxidation reaction, suggesting that the source of the reactive oxygen species is not the microsomal mixed-function oxidase.

Key words. Superoxide; hydrogen peroxide; hydroxyl radicals; flavin-containing monooxygenase.

The microsomal flavin-containing monooxygenase (MFMO) is an enzyme capable of oxidizing a wide variety of amine- and sulfur-containing xenobiotics[1,2], leading to the formation of S- or N-oxides which, in many cases, are toxic metabolites. Several reports have revealed the participation of activated oxygen species like superoxide ions or hydroxyl radicals in the catalytic cycles of various oxidases and oxygenases, many of which are flavoproteins[3,4]. As oxygen radicals were reported to be involved

Table 1. Inhibition of the microsomal oxygenation of thiobenzamide by scavengers of reactive oxygen species. Values are means and their SE ($\bar{x} \pm$ SEM, n = 3–4 each)

Scavenger	Concentration (mg/ml)	Oxygen species scavenged	Enzyme[a] activity	% inhibition
None	–	–	20.15 ± 3.22	0
Superoxide dismutase	0.1	$\cdot O_2^-$	12.00 ± 2.17	40.5
Superoxide dismutase	0.2	$\cdot O_2^-$	7.62 ± 1.09	62.2
Heat-denatured superoxide dismutase	0.2	–	17.43 ± 2.89	13.5
Catalase	0.1	H_2O_2	7.63 ± 3.01	62.1
Superoxide dismutase + catalase	0.1 each	$\cdot O_2^-/H_2O_2$	3.31 ± 0.05	83.6
Methional	1.0	$\cdot OH/\cdot OR/RCOO\cdot/R\cdot$	0.33 ± 0.10	98.4
Diethyldithiocarbamate	0.1[b]		8.57 ± 1.22	57.5
(+)-Catechin	0.1[b]		8.50 ± 1.00	57.8

[a] nmol thiobenzamide oxidized to thiobenzamide-S-oxide/min per mg protein; [b] mmol/l.

Table 2. Effect of CO on the microsomal oxidation of thiobenzamide. Values are means and their SE ($\bar{x}$ + SEM, n = 4 each)

Incubation atmosphere	nmol thiobenzamide-S-oxide formed min · mg protein
O_2/N_2 (20:80%)	17.03 ± 0.96
O_2/CO (20:80%)	17.55 ± 2.02

both in microsomal mixed-function oxidase-catalyzed reactions [3,5] and in the oxidation of diamines by the appropriate oxidase[6], it was of interest to investigate whether they also mediate the MFMO activity which leads to the oxidation of xenobiotics carrying an amino or a thio group.

Materials and methods. All chemicals employed were of analytical grade purity. Catalase (Boehringer, Mannheim; 65,000 U/mg protein) and superoxide dismutase (Sigma, München; 3000 U/mg protein) were dialyzed against phosphate buffer (0.1 mol/l, pH 7.4) before use. Microsomes were isolated from the livers of male Wistar rats (220–250 g, breeder: Winkelmann, Borchen) according to Remmer et al.[7]. The microsomal oxygenation of thiobenzamide, which is supposed to be catalyzed by the flavin-containing monooxygenase[8], was assayed in the following manner: reaction mixtures were composed of phosphate buffer (0.1 mol/l, pH 7.4), an NADPH-regenerating system (66.4 µmol/l NADP, 288 µmol/l glucose-6-phosphate and 140 mU/ml glucose-6-phosphate dehydrogenase), microsomes (approx. 1 mg protein/ml) and Mg SO_4 (5.5 mmol/l). The reaction was started by addition of thiobenzamide in 10 µl of acetonitrile to yield a final concentration of 1 mmol/l. The formation of thiobenzamide-S-oxide was followed at 370 nm in a Beckmann 3600 double-beam spectrophotometer against a blank containing 10 µl of acetonitrile instead of thiobenzamide, and calculated using a molar absorption coefficient of 2930 M^{-1} cm^{-1} [8].

In the experiments concerning the effect of CO on the oxidation of thiobenzamide, Warburg flasks were used. The reaction mixture was incubated in a shaking water-bath for 10 min and continuously gassed by a mixture of either O_2/CO or O_2/N_2 (20:80%). After 10 min the reaction was stopped by addition of 0.5 ml of the incubate to 1 ml of trichloroacetic acid. After centrifugation, the absorbance of the product was measured at 370 nm. All solutions were pregassed with the appropriate gas mixture before the start of the experiment.

Results and discussion. Addition of superoxide dismutase, which catalyzes the disproportionation of superoxide ions, to rat liver microsomes led to a concentration-dependent inhibition of thiobenzamide S-oxidation, which was suggested to be catalyzed by the microsomal flavin-containing monooxygenase[8] (table 1). This indicates that superoxide anion radicals are produced during this catalytic cycle, and seem to mediate the oxidation reaction. When superoxide dismutase was heat-denatured, it lost its inhibitory activity on the reaction (table 1), thus excluding the possibility that the observed inhibition of the reaction by native superoxide dismutase could have been due to a protein effect. Catalase, which scavenges H_2O_2, also suppressed the enzymic oxidation of thiobenzamide (table 1).

Simultaneous addition of both superoxide dismutase and catalase had a more marked inhibitory effect on the S-oxidation than had the addition of either enzyme alone, suggesting that both activated oxygen species play a role in the reaction (table 1). $\cdot O_2^-$ and H_2O_2 may interact, leading to the formation of ·OH- or ·OH-like radicals[3,4,9]. It should be mentioned, however, that hydroxyl radical formation from $\cdot O_2^-$ and H_2O_2 is still a matter of debate; but it was recently shown that superoxide in fact reacts with hydrogen peroxide[10], although a long chain reaction was proposed yielding first of all HOO· and HOO^-. HOO^- reacts then with H_2O_2, yielding $\cdot O_2^-$ and ·OH[10]. Methional (β-methylthiopropionic aldehyde), a potent scavenger of ·OH-radicals, even totally suppressed the oxidation of thiobenzamide (table 1). As methional is not specific for ·OH, but reacts also with other oxidizing radicals (·OR, RCOO· and R·, but not $\cdot O_2^-$)[11], the possibility that other radicals are involved besides or instead of ·OH cannot be excluded. Hence, it seems that in fact superoxide and H_2O_2 are produced during the microsomal S-oxidation of thiobenzamide, and that they interact, yielding ·OH-radicals and/or related species which might be the ultimate oxidizing species.

The involvement of free radicals in the microsomal oxygenation of thiobenzamide-catalyzed reactions is further substantiated by the inhibitory activity exerted by two non-discriminative free-radical scavengers, diethyldithiocarbamate and (+)-catechin[12]. As the cytochrome P-450 systems is the major source for superoxide ions and hydrogen peroxide in liver microsomes[13], the effective inhibition by superoxide dismutase of the S-oxidation of thiobenzamide might be due to a limited specificity of the reaction. To exclude this possibility, the reaction was run in the presence of CO, which is known to inhibit the cytochrome P-450-dependent oxygen radical formation[13]. CO, however, had no effect on the S-oxidation of thiobenzamide (table 2), suggesting that, in fact, the source of reactive oxygen species in this system is not cytochrome P 450.

In summary, activated oxygen species (e.g. $\cdot O_2^-$, H_2O_2, ·OH) most probably mediate the S-oxidation of thiobenzamide thought to be catalyzed by the microsomal flavin-containing monooxygenase; the ultimate oxidizing species are most probably ·OH or related free radicals.

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0014-4754/85/040479-03$1.50 + 0.20/0

Substance P-like immunoreactivity in the frog dorsal root ganglia

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Summary. The distribution of substance P-like immunoreactivity was studied in the thoracic dorsal root ganglia of the frog *Rana esculenta* by immunohistochemistry. Substance P-like immunoreactivity was contained in approximately 50% of primary sensory neurons. The immunoreactive fibers arising from the cell bodies are collected in small bundles within the ganglia neuropil before entering the central and peripheral roots.
Key words. Substance P; immunohistochemistry; spinal ganglia; frog.

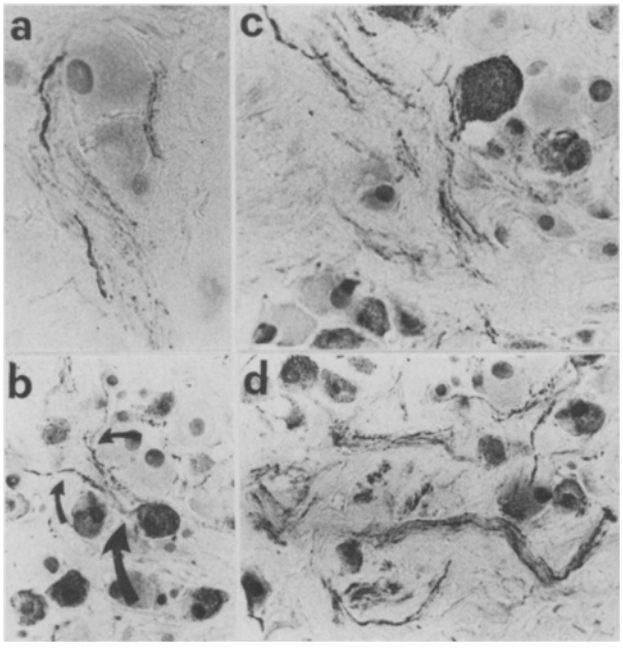
Substance P (SP) is a polypeptide which is thought to play a role in sensory systems, particularly in those involved in the processing of pain information (for review see Nicoll et al.[1]). In fact, immunohistochemical studies have demonstrated the presence of SP in primary sensory neurons of mammalian dorsal root ganglia (DRG)[2,3] as well as in the dorsal horn of the spinal cord of cats[3], rats[2], monk[4] and frogs[5,6]. There is also experimental evidence that immunoreactive SP in the dorsal originates from DRG[7-10] and physiological studies have provided evidence for the role of SP as a transmitter of primary sensory neurons in rats[11] and in frogs[12].

In view of the above and of the fact that the morphological features of immunoreactive SP elements in DRG of the frog have not yet been shown, we investigated immunohistochemically the distribution of SP in DRG in the frog *Rana esculenta*. Two frogs, anesthetized with tricaine methanesulphonate (MS 222, 1:3000), were perfused transcardially with 10 ml of saline (NACl 0.64%) followed by 15 ml of fixative (0.1 M phosphate-buffered 4% formaldehyde, pH 7.4) at 4°C. The DRG (thoracic 1–2) were removed, placed in the fixative for an additional 3 h and then incubated for a few days at 4°C in buffer containing 20% sucrose. The ganglia were cut into transverse sections 12 µm thick in a cryostat and put on slides pretreated with potassium chrome sulphate gelatine. Sections were incubated with SP antiserum, diluted 1:50 to 1:500, for 20 h at 4°C and later processed according to the unlabeled peroxidase-antiperoxidase technique[13] using diaminobenzidine as chromogen. Control sections were processed in parallel but incubated with diluted antiserum preabsorbed (1 mg/ml) with SP or without being exposed to SP antiserum.

The DRG of the frog consist of large neuronal cells and small satellite cells[14]. Although the existence of post-ganglionic sympathetic neurons 'without central processes' has been reported[15], recent anatomical results do not support these data[14]. Presumably all DRG neurons of the frog are primary sensory neurons.

In our results substance P-like immunoreactivity was localized in some of the large neuronal cells (figure, b and c) suggesting that it is contained only in a portion of the ganglion cell population. In 10 sections 200 SP positive neurons were counted of a total number of 400 neurons. We may say that substance P-like immunoreactivity is contained in approximately 50% of primary sensory neurons. Since in the frog thoracic spinal cord somatostatin-like immunoreactivity appeared to be contained in primary afferents[6], some of the neurons which have negative immunoreactivity to substance P may contain somatostatin.

The process arising from the SP-like immunoreactive cell body is clearly visible in the figure, c, where the unipolar or pseudounipolar nature of the neuron is apparent. We presume that the diverging fiber branches appearing in the figure, b (arrows) originate both from the same single process arising from the nearby dark neuron which is positive to SP-like immunoreactivity.



Frog dorsal root ganglion showing the distribution of substance P-like immunoreactivity. *a* Fibers showing a positive SP-like immunoreactivity collected in bundles run between non-immunoreactive cells. ×250. *b* The small arrows indicate SP-like immunoreactive diverging fibers both belonging to the process originating from the SP positive cell (large arrow). ×180. *c* SP-like immunoreactive neuron is apparent close to other neurons negative to SP. The process emerging from one pole of the cell runs in a bundle of fibers which show SP-like immunoreactivity. × 220. *d* SP-like immunoreactive bundles of fibers are shown in the neuropil of the spinal ganglion. ×180.