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# PREVENTION BY *GINKGO BILOBA* EXTRACT (EGb 761) AND TROLOX C OF THE DECREASE IN SYNAPTOSOMAL DOPAMINE OR SEROTONIN UPTAKE FOLLOWING INCUBATION

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**Abstract**—Prolonged incubation of synaptosomes in Krebs–Ringer oxygenated medium in the presence of ascorbic acid ($10^{-4}$ M) led, after 20 min, to a decrease in [$^3$H]dopamine (DA) (synaptosomes prepared from the striatum) and [$^3$H]serotonin (5HT) (synaptosomes prepared from the cortex) uptake. The decrease was progressive and uptake was virtually abolished after a 60 min incubation period. A concentration-dependent (from $5 \times 10^{-6}$ M) role of ascorbic acid in the decrease of [$^3$H]DA or [$^3$H]5HT uptake was demonstrated. This decrease was potentiated by $Fe^{2+}$ ions and prevented by the ferrous chelating agent desferrioxamine. Thus, the progressive decrease in synaptosomal uptake of either [$^3$H]-DA or [$^3$H]5HT could depend on the generation of free radicals by the association of ascorbic acid with $Fe^{2+}$ ions. The decrease in synaptosomal uptake was prevented, in a concentration-dependent manner, by the *Ginkgo biloba* extract EGb 761 (4–16 μg/mL) and the vitamin E analog trolox C ($10^{-4}$ M). The terpenic fraction of EGb 761, Bn 52063 (up to 0.5 μg/mL), did not prevent the reduction of [$^3$H]amine uptake. In contrast, the flavonoidic fraction, Cp 202, was effective (from 1 μg/mL) and its efficacy was shared by the flavonoid quercetin (from 0.1 μg/mL). The prolongation of the ability of synaptosomes to take up [$^3$H]amine elicited by EGb 761, in particular its flavonoidic fraction, as well as by trolox C could be due to their free radical scavenger properties.

The usual composition of incubation medium used for measuring the uptake of $^3$H-labelled neurotransmitters has been selected to allow optimum function during a brief (few minutes) period of incubation. Most of the components are present at concentrations close to those encountered endogenously. In the first part of this study we aimed to determine the time course of the ability of synaptosomes to take up amines. We observed that, after 20 min incubation under such conditions, amine uptake decreased progressively. This prompted us to determine the components responsible for this accelerated loss in uptake function. Ascorbic acid is commonly present in these media in order to prevent oxidation of catecholamines ([$^3$H]dopamine (DA§), [$^3$H]norepinephrine) [1] which results from oxygen bubbling, intended to ensure energetic metabolism. In addition, these media unavoidably contain micromolar concentrations of ferrous ions, as a common contaminant of most of the constituents of these preparations [2, 3]. Thus, $Fe^{2+}$, $O_2$ and ascorbic acid, which are critically involved in free radical generation [4], are simultaneously present and could generate free radicals during a prolonged incubation at 37° [5]. These free radicals could alter DA synthesis [6]. They could also peroxidize membrane lipids [7], reducing [$^3$H]adenosine [8] or [$^3$H]GABA binding [9, 10], or altering various synaptosomal functions such as [$^3$H]choline [11] or [$^3$H]DA uptake [12–14]. The critical role of the ascorbic acid–ferrous ions couple suggested the involvement of a peroxidative mechanism. To substantiate this hypothesis, we determined the effectiveness of the reference free radical scavenger trolox C [15] in preventing the loss of amine uptake ability. We tested also the *Ginko biloba* extract (EGb 761) since it has been claimed that it displays free radical scavenger properties [16] which should be linked to its flavonoid glucorhamnoside components. Our interest in EGb 761 is strengthened by its potential use in the treatment of cerebral disorders resulting from ischemia and anoxia, and cerebral degenerative diseases [17, 18] in which peroxidative processes could operate.

## MATERIALS AND METHODS

*Animals.* Male Swiss albino mice 25–30 g, CD1 (Charles River, Saint Aubin lès Elbeuf, France) were used. The animals were housed in a well-ventilated room, at an ambient temperature of 22°, with a 12 hr light–dark cycle. Food and water were available *ad lib.*

*Synaptosomal preparations.* A crude synaptosomal fraction (S1) was obtained by homogenization (Potter–Elvehjem, clearance 80–130 μm) of the striatum or cortex in 10 vol. of ice-cold 0.32 M sucrose [19] containing pargyline ($10^{-4}$ M) followed by centrifugation (1000 *g*, 10 min, 2°).

‡ Corresponding author.

§ Abbreviations: DA, dopamine; 5HT, serotonin.

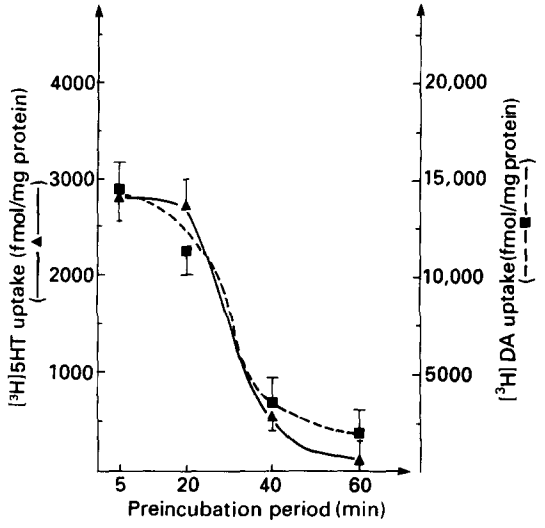


Fig. 1. Time course of the ability of synaptosomes to take up [$^3$H]5HT or [$^3$H]DA after different periods of incubation. Synaptosomes prepared from the cortices or striata of mice were incubated (37°) for different periods of time (indicated on abscissa) before the addition of [$^3$H]5HT (20 nM final concentration) or [$^3$H]DA (20 nM final concentration). The incubation was stopped 5 min ([$^3$H]5HT) or 3 min ([$^3$H]-DA) later. Values are means ± SEM of three experiments performed in triplicate.

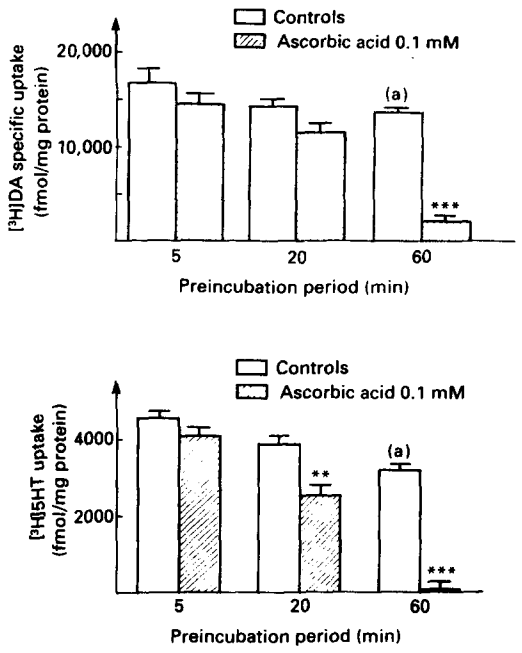


Fig. 2. Effect of removal of ascorbic acid from the incubation medium on the time course of the ability of synaptosomes to take up either [$^3$H]5HT or [$^3$H]DA. Upper panel: synaptosomes, prepared from the striata of mice, were incubated (37°) with or without ascorbic acid ($10^{-4}$ M) for various periods of time (indicated on abscissa) before the addition of [$^3$H]DA (20 nM final concentration). The incubation was stopped 3 min later. Lower panel: similar experiment to previous one except that synaptosomes were prepared from the cortex and the incubation was stopped 5 min after the addition of [$^3$H]5HT (20 nM final concentration). Values are means ± SEM of three experiments performed in triplicate. (a) $P < 0.05$ as compared with controls of the 5th min of preincubation. $^{**}P < 0.01$; $^{***}P < 0.001$ as compared with controls (without ascorbic acid) after the same preincubation period.

*Incubation conditions*. The Krebs–Ringer phosphate buffer (NaCl 103 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM, $KH_2PO_4$ 1 mM, $NaHCO_3$ 27 mM, glucose 5.4 mM) was gassed (95% $O_2$, 5% $CO_2$) for 30 min prior to use. Aliquots of the supernatant (50 μL) were preincubated (37°) with 900 μL of buffer. Then, [$^3$H]DA 20 nM final concentration (50 μL) or [$^3$H]-5HT 20 mM final concentration (50 μL) was added.

*Synaptosomal uptake*. After incubation in the presence of the amine (3 min for [$^3$H]DA or 5 min for [$^3$H]5HT) uptake was stopped by dilution with ice-cold Krebs–Ringer buffer (4 mL) followed by vacuum filtration through filters (Whatman GF/B), soaked previously with Krebs–Ringer phosphate buffer. Each tube was rinsed and the filters were washed twice with 4 mL ice-cold Krebs–Ringer buffer and dried for 1 hr in a ventilated incubator (60°). Filters were put in minivials containing 5 mL Aqualyte® (J. T. Baker Chemical, Deventer, The Netherlands). The radioactivity was determined by liquid scintillation spectrometry. Non-specific uptake was determined at 0° under similar conditions. The specific uptake was expressed as fmol/mg of protein. The protein concentration was determined by the method of Lowry *et al.* [20].

*Drugs*. The dried extracts, *Ginkgo biloba* (EGb 761) Cp202 (corresponds to EGb 761 extract devoid of terpenic substances) and BN 52063 (corresponds to EGb 761 extract devoid of flavonoidic substances) were prepared by the Henri Beaufour Institute from leaves of *Ginkgo biloba*. The final extract of EGb 761 is standardized to contain 24% of flavonoid glycosides (ginkgo flavone glycosides) and 6% terpene lactones (ginkgolides, bilobalides) [21]. These dried extracts were dissolved in Krebs–Ringer phosphate buffer.

Trolox C and quercetin were purchased from Aldrich (Saint Quentin Fallavier, France) and Sigma (L'Isle d'Abeau Chesne, France), respectively. Desferrioxamine was obtained from Ciba-Geigy (Basel, Switzerland). [$^3$H]DA (20 Ci/mmol) and [$^3$H]5HT (9.3 Ci/mmol) were obtained from Amersham (Les Ulis, France).

*Statistics*. Differences between groups were determined either by analysis of variance (two-way ANOVA) or by Dunnett's *t*-test.

## RESULTS

### *Influence of the preincubation period on* [$^3$H]*5HT or* [$^3$H]*DA uptake*

In the usual oxygenated (95% $O_2$, 5% $CO_2$) incubation medium (with $10^{-4}$ M ascorbic acid) both the [$^3$H]5HT uptake by synaptosomes from the cortex and the [$^3$H]DA uptake by synaptosomes from the striatum decreased from the 20th min and

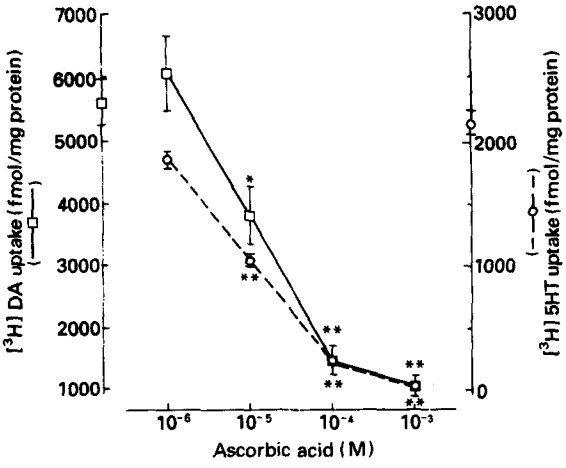


Fig. 3. Ability of synaptosomes to take up [$^3$H]DA after a 60 min incubation period in the presence of increasing concentrations of ascorbic acid. Synaptosomes, prepared from the striata of mice, were incubated for 60 min with increasing concentrations of ascorbic acid (indicated on abscissa) before the addition of [$^3$H]DA (20 nM final concentration), the incubation being stopped 3 min later. The specific [$^3$H]DA uptake in controls was 5619 ± 361 fmol/mg protein. The results obtained for the various ascorbic acid concentrations were compared by Dunnett's *t*-test. *$P < 0.05$ as compared with controls (without ascorbic acid); **$P < 0.01$ as compared with controls (without ascorbic acid). Values are means ± SEM of three experiments performed in triplicate.

were virtually abolished after 60 min incubation (Fig. 1).

*Influence of ascorbic acid on* [$^3$H]*5HT and* [$^3$H]*DA uptake*

Omission of ascorbic acid from the incubation medium prevented the progressive decrease in the uptake of either [$^3$H]DA or [$^3$H]5HT (Fig. 2, upper or lower panel). The effects of ascorbic acid on the reduction of the synaptosomal uptake of [$^3$H]DA or [$^3$H]5HT were dose dependent from $10^{-5}$ to $10^{-3}$ M (Fig. 3).

*Influence of ferrous ions on* [$^3$H]*DA or* [$^3$H]*5HT uptake*

Addition of the ferrous chelating agent desferrioxamine ($10^{-4}$ M) to the incubation medium resulted in complete prevention of the ascorbic acid-induced reduction in synaptosomal uptake ability for both [$^3$H]DA and [$^3$H]5HT (Table 1). In contrast, in an incubation medium with a low ascorbic acid concentration ($5 \times 10^{-6}$ M) that decreased only moderately the ability of synaptosomes to take up either [$^3$H]DA or [$^3$H]5HT, the addition of ferrous ions, at the $10^{-6}$ M final concentration, resulted in a marked reduction in amine uptake (Table 1). At this concentration, ferrous ions also moderately decreased amine uptake in the absence of ascorbic acid.

*Effect of EGb 761 or trolox C on the ascorbic acid-induced reduction in synaptosomal* [$^3$H]*DA or* [$^3$H]*5HT uptake*

EGb 761 as well as trolox C ($10^{-4}$ M) completely prevented the reduction in synaptosomal uptake elicited by 60 min preincubation in the presence of ascorbic acid ($10^{-4}$ M) (Table 2). When EGb 761 was added to the incubation medium an increase in [$^3$H]5HT uptake as compared with controls was observed, at each tested time between the 5th and the 60th min of incubation (Fig. 4). The protective effect of EGb 761 was dose dependent (Fig. 4, inset).

*Attempt to reverse the decrease in* [$^3$H]*DA uptake by EGb 761*

Synaptosomes, prepared from the striata of mice, were incubated with ascorbic acid ($10^{-4}$ M). EGb 761 (20 μg/mL) was added at the 30th min of this incubation and [$^3$H]DA was added 20 min later. The [$^3$H]DA uptake by synaptosomes exposed to EGb 761 was increased compared with that observed without EGb 761, but it was decreased compared with that of synaptosomes incubated in the absence of ascorbic acid. Thus, EGb 761 did not reverse the

Table 1. Effects of desferrioxamine or $FeSO_4$ on the decrease in synaptosomal uptake of [$^3$H]DA or [$^3$H]5HT elicited by ascorbic acid

| | [$^3$H]DA uptake (fmol/mg protein) | Statistical analysis of interactions | [$^3$H]5HT uptake (fmol/mg protein) | Statistical analysis of interactions |
|---|---|---|---|---|
| Controls | 8406 ± 290 | $P < 0.001$ | 2213 ± 63 | $P < 0.001$ |
| Desferrioxamine ($10^{-4}$ M) | 9366 ± 780 | | 3025 ± 307 | |
| Ascorbic acid ($10^{-4}$ M) | 2778 ± 241 | | 226 ± 14 | |
| Desferrioxamine + ascorbic acid | 9648 ± 340 | | 2880 ± 188 | |
| Ascorbic acid ($5 \times 10^{-6}$ M) | 7034 ± 270 | $P < 0.001$ | 1346 ± 140 | $P < 0.001$ |
| $FeSO_4$ ($10^{-6}$ M) | 7613 ± 463 | | 1548 ± 168 | |
| Ascorbic acid + $FeSO_4$ | 3507 ± 747 | | 443 ± 172 | |

Synaptosomes, prepared from the striata or cortices of mice, were incubated for 60 min with or without ascorbic acid before the addition of [$^3$H]DA (20 nM) final concentration) or [$^3$H]5HT (20 nM final concentration).
The interactions between ascorbic acid and desferrioxamine were analysed by two-way ANOVA.
Values are means ± SEM of three experiments in triplicate.

Table 2. Effects of EGb 761 or trolox C on the decrease in synaptosomal uptake of [$^3$H]DA or [$^3$H]5HT elicited by ascorbic acid

| | [$^3$H]DA uptake (fmol/mg protein) | Statistical analysis of interactions | [$^3$H]5HT uptake (fmol/mg protein) | Statistical analysis of interactions |
|---|---|---|---|---|
| Controls | 9477 ± 624 | $P < 0.001$ | 2138 ± 93 | $P < 0.001$ |
| EGb 761 (10 μg/mL) | 9202 ± 545 | | 3208 ± 141 | |
| Ascorbic acid ($10^{-4}$ M) | 2813 ± 698 | | 233 ± 11 | |
| EGb 761 + ascorbic acid | 9195 ± 615 | | 3133 ± 404 | |
| Controls | 9897 ± 660 | $P < 0.001$ | 2082 ± 88 | $P < 0.001$ |
| Trolox C ($10^{-4}$ M) | 12,130 ± 412 | | 2977 ± 317 | |
| Ascorbic acid ($10^{-4}$ M) | 2550 ± 601 | | 244 ± 10 | |
| Trolox C + ascorbic acid | 11,000 ± 607 | | 2816 ± 293 | |

Synaptosomes, prepared from the striata or cortices of mice, were incubated for 60 min with or without ascorbic acid before the addition of [$^3$H]5HT (20 nM final concentration) or [$^3$H]DA (20 nM final concentration) and the incubation was stopped 5 min later.

The interactions between ascorbic acid and the different drugs were analysed by two-way ANOVA.

Values are means ± SEM of three experiments in triplicate.

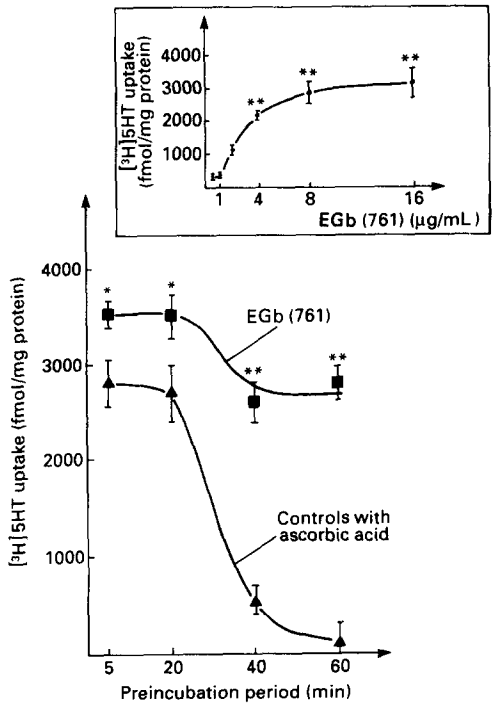


Fig. 4. Time course of the ability of synaptosomes to take up [$^3$H]5HT during incubation with ascorbic acid ($10^{-4}$ M); modification by EGb 761. Synaptosomes prepared from the cortices of mice were preincubated (37°) with or without EGb 761 for different periods (indicated on abscissa) before the addition of [$^3$H]5HT (20 nM final concentration). The incubation was stopped 5 min later. Inset: the synaptosomes were preincubated for 60 min with increasing concentrations of EGb 761 (indicated on abscissa) before the addition of [$^3$H]5HT (20 nM final concentration). The incubation was stopped 5 min later. Values are means ± SEM of three experiments performed in triplicate. * $P < 0.05$ relative to respective controls (with ascorbic acid) without EGb 761; ** $P < 0.01$ relative to respective controls (with ascorbic acid) without EGb 761. Differences between groups were determined by Dunnett's *t*-test.

Table 3. Effects of EGb 761 on the decrease in synaptosomal uptake of [$^3$H]DA elicited by ascorbic acid

| | [$^3$H]DA uptake (fmol/mg protein) | |
|---|---|---|
| | 30th min | 50th min |
| Controls | 15,580 ± 506 | 13,800 ± 445 |
| Ascorbic acid | 10,426 ± 417 | 4384 ± 665* |
| Controls (+EGb 761 at 30th) | | 15,607 ± 600 |
| Ascorbic acid (+EGb 761 at 30th) | | 6740 ± 804† |

Synaptosomes, prepared from the striata of mice, were incubated for 50 min with ascorbic acid ($10^{-4}$ M). EGb 761 (20 μg/mL) was added at the 30th min of incubation and [$^3$H]DA (20 nM final concentration) was added 20 min later. The uptake was stopped 5 min after the addition of [$^3$H]DA.

* $P < 0.001$ relative to respective controls (without ascorbic acid); † $P < 0.05$ relative to respective ascorbic acid group.

The statistic interactions were analysed by Student's *t*-test.

Values are means ± SEM of three experiments in triplicate.

alteration in the ability of synaptosomes to uptake amines once it was established (Table 3).

*Effect of different fractions of EGb 761 and quercetin on [$^3$H]5HT or [$^3$H]DA uptake*

We tested several fractions of EGb 761 to determine which was responsible for the observed protection of amine uptake function. The Cp 202 fraction was effective from 1 μg/mL for a 40 min preincubation period and from 5 μg/mL for a 60 min preincubation period (Figs 5 and 6, upper panel). This effect was shared by quercetin, from 0.1 or 0.5 μg/mL, for preincubation periods of 40 and 60 min, respectively (Figs 5 and 6, middle panel). Finally, for each preincubation period considered,

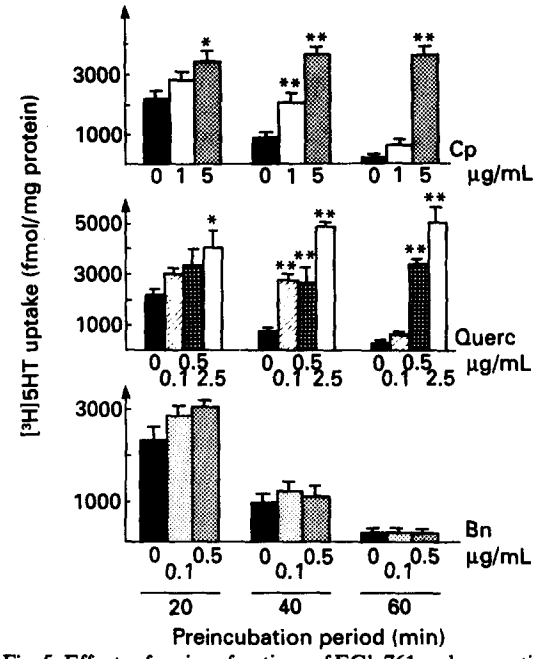


Fig. 5. Effects of various fractions of EGb 761 and quercetin on the ability of synaptosomes to take up [$^3$H]5HT in the presence of ascorbic acid ($10^{-4}$ M). Synaptosomes prepared from the cortices of mice were preincubated (37°) for different periods (indicated on abscissa) with or without different concentrations of Cp 202 (upper panel, Cp), quercetin (middle panel, Querc) and Bn 52063 (lower panel, Bn). The uptake was stopped 5 min after the addition of [$^3$H]5HT (20 nM final concentration). Values are means ± SEM of three experiments performed in triplicate. * $P < 0.05$ relative to respective controls (with ascorbic acid); ** $P < 0.01$ relative to respective controls (with ascorbic acid). Differences between groups were determined by Dunnett's $t$-test.

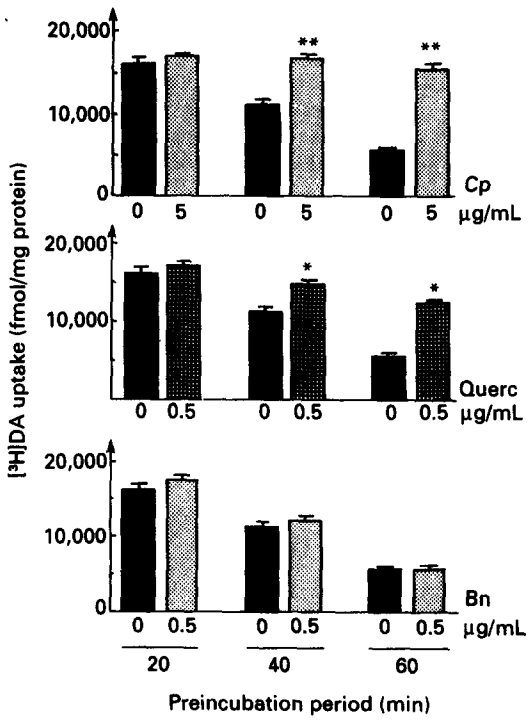


Fig. 6. Effects of various fractions of EGb 761 and quercetin on the ability of synaptosomes to take up [$^3$H]DA after various periods of incubation (indicated on abscissa) in the presence of ascorbic acid ($10^{-4}$ M), with or without different concentrations of Cp 202 (upper panel, Cp), quercetin (middle panel, Querc) and Bn 52063 (lower panel, Bn). The uptake was stopped 3 min after the addition of [$^3$H]-DA (20 nM final concentration). Values are means ± SEM of three experiments performed each in triplicate. * $P < 0.05$ relative to respective controls (with ascorbic acid); ** $P < 0.01$ relative to respective controls (with ascorbic acid). Differences between groups were determined by Dunnett's $t$-test.

the Bn 52063 fraction was ineffective at all tested concentrations between 0.1 and 0.5 µg/mL (Figs 5 and 6, lower panel).

## DISCUSSION

A dramatic decrease in [$^3$H]amine uptake occurred following long term incubation of synaptosomes with ascorbic acid, in the usual oxygenated Krebs–Ringer medium. The decrease in uptake was observed for both [$^3$H]DA from striatal synaptosomes and [$^3$H]-5HT from cortical synaptosomes. Both effects had similar kinetics, since the maximal decreases occurred between the 20th and 40th min of incubation. Although the composition of the radioactivity retained by synaptosomes was not analysed, it probably corresponds mainly to the amine taken up, but not metabolized, on account of the monoamine oxidase inhibition operated by pargyline at the earliest time point of the synaptosomal preparation and also of the short period of amine exposure to synaptosomes: 3 min ([$^3$H]DA) or 5 min ([$^3$H]5HT). Various mechanisms could be responsible for the decrease in the ability of synaptosomes to take up these amines. We considered the possible involvement of ascorbic acid as it has been reported to induce, at concentrations similar to those reached in the usual incubation medium ($10^{-4}$ M), a peroxidative process, which could disturb amine uptake. In our study, the critical role of ascorbic acid was verified by the almost complete preservation of synaptosomal uptake ability following ascorbic acid suppression. The peroxidative process elicited by ascorbic acid is claimed to depend critically on the presence of ferrous ions [5]. The influence of $Fe^{2+}$ ions was verified by the increase in the loss in uptake ability exerted by a low concentration of ascorbic acid ($5 \times 10^{-6}$ M) caused by the addition of $Fe^{2+}$ ions, at a concentration of $10^{-6}$ M. Furthermore, desferrioxamine, which chelates [22] the $Fe^{2+}$ ions unavoidably brought to the incupation medium by most of the constituents, prevented ascorbic acid-induced peroxidation. However, it has been claimed that desferrioxamine displays intrinsic antioxidant properties. Even if this is true it supports the involvement of a peroxidative process in the loss of uptake ability. In addition, the kinetic profile of the decreased amine uptake is compatible with such a

process [4]. The first 20 min of incubation could correspond to the generation of $H_2O_2$, whereas the second 20 min period corresponds to the peroxidation process, as evidenced by Laudicina and Marnett [3] through an increase in malondialdehyde, which reached a plateau after 60 min of incubation at 37°. The decrease in synaptosomal [$^3$H]DA uptake occurring under peroxidative conditions reported here is in contrast with the increase observed by Pastuszko *et al.* [12], Dabrowiecki *et al.* [13] and Rafalowska *et al.* [14]. This difference may be explained by the incubation period considered by these authors which was shorter (5–6 min) than that in our study (20–60 min). The preventive effect of trolox C, a vitamin E analog without the lateral hydrocarbon chain which is a well-documented free radical scavenger [15], is also in keeping with the suggestion of a peroxidative process. The *Ginkgo biloba* extract, EGb 761, has been demonstrated to have antioxidant properties [16] which we were prompted to try to evidence on this model. The prevention of the synaptosomal decrease in [$^3$H]DA uptake by EGb 761 is due to the flavonoidic fraction (Cp 202). Quercetin, which in a glucorhamnoside form is the main component of Cp 202 and EGb 761 extracts, prevented the decrease in [$^3$H]DA uptake. Its effective concentrations were roughly similar to the concentrations at which it is found in the Cp 202 fraction and EGb 761 extract. Quercetin might operate either through its iron chelating effect [23] or through its free radical scavenger properties [24, 25]. Flavonoids could limit lipoperoxidation either by scavenging hydroxyl radicals [26] or superoxide anions [27], or by preventing the formation of lipid peroxy radicals [23]. EGb 761 prevented the loss in ability of synaptosomes to take up amines but did not reverse the alteration once it was established. EGb 761, added to synaptosomes after a 30 min incubation period, stopped the alteration at the level reached at this time, but did not return it to the control level.

The DA and 5HT carriers, both recently cloned, display obvious structural similarities [28, 29] with 12 transmembrane domains which probably form a channel. The functioning of this channel could depend on membrane fluidity that may be decreased by membrane peroxidation. We have evidenced a decrease in membrane fluidity through an increase in the fluorescence (measured in polarized light) of synaptosomes incubated with the diphenyl 1,6 hexatriene probe under peroxidizing conditions (ascorbic–$Fe^{2+}$). The increased fluorescence was parallel to the loss in [$^3$H]DA uptake ability, suggesting a link between these events [30].

The ascorbic acid concentrations which displayed, in this study, deleterious effects on [$^3$H]DA or [$^3$H]-5HT uptake are in the same range (2 mM) as that measured in rat brain by Rajalaksmi and Patel [31]. In other respects, a role for iron has been suggested in the degeneration of the nigro-striatal dopaminergic pathway associated with Parkinson's disease [32–34]. The effects of the ascorbic acid–ferrous ions couple shown in this study substantiate the hypothesis of an involvement of free radicals in this disease [13]. Therefore, the protective effect of EGb 761 towards peroxidative alterations induced by the ascorbic acid–ferrous ions couple could be of value in trying to prevent the neurodegenerative process. This view is strengthened by the prevention exerted in mice by EGb 761 of the toxic effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine on nigro-striatal dopaminergic neurons [36].

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