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Prevention of lipofuscin development in neurons by anti-oxidants*

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Summary. It was documented that ageing is associated with a progressive and highly significant proliferation of the total number of light microscopically visible lipofuscin granules in the grey substance of sections of the cervical spinal cord of Balb/c mice. The mean total numbers ($\pm$standard errors) of lipofuscin granules in standard sections of the glutaraldehyde-osmium fixed, epon embedded spinal cords that were examined with a phase contrast light microscope in 1 week, 1 month, 8 months and 18 months old mice were 0, 269 ± 56, 1101 ± 82 and 2464 ± 318, respectively. The population densities of multiglobular lipofuscin units as seen with the electron microscope in random spinal cord neurons of the same 4 age groups corresponded well with the above quantitative, light microscopic data.

Continuous treatment for 8 months with either the natural anti-oxidant Vitamin E (α-tocopherol) at 40 mg/mouse/week or the synthetic anti-oxidant butylated hydroxytoluene at about 100 mg/mouse/week diminished significantly the proliferation of lipofuscin granules in spinal cord neurons that developed during that period of ageing. No toxicity of any sort was caused by these two treatments.

These results provide support for the peroxide theory of lipofuscin biogenesis and encourage further exploration of the possibilities of obtaining greater anti-lipofuscin effects with less molecular bulk of anti-oxidants.

Key words: Tocopherol – Butylated hydroxytoluene – Anti-oxidants – Ageing – Lipofuscin – Neurons

Introduction

Lipofuscin is a complex pigmented material that has been known for a long time to progressively accumulate with age in the cells of the nervous

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system, the heart and many other organs of human and mammalian tissues. It consists of a largely insoluble conglomerate of polymerised and oxidised fatty acids and their aldehydic derivatives cross-linked with phospholipids, proteins, triglycerides, nucleic acids and some iron, and it also contains some lysosomal enzymes. According to prevailing evidence, (Constantinides 1984) it is thought to develop in the following manner: Various peroxides and other oxygen-containing free radicals oxidise the unsaturated fatty acids of the phospholipids of the internal membranes of cells and turn them into lipoperoxides by attaching next to their double bonds; the lipoperoxides, in turn, are transformed into aldehydes (notably malonaldehyde) and the latter cross-link many cell membrane and cytosol components such as phospholipids, proteins, triglycerides and nucleic acids into an insoluble lipofuscin complex. The cross-linking effect of aldehydes here seems similar to that by which formaldehyde and glutaraldehyde fix protoplasm.

Support for the above oxidative scenario of lipofuscin biogenesis can be found in the repeatedly established fact that chronic deficiency of the natural anti-oxidant Vitamin E (tocopherol) in experimental animals causes in numerous tissues massive accumulation of a lipofuscin that is identical histochemically and morphologically with that which accumulates spontaneously during ageing in animals and man (Gedigk and Fischer 1959; Sulkin and Srivanij 1960; Nishioka 1966; Miyagishi et al. 1967; Swensen and Telford 1973).

Electron microscopically, lipofuscin units consist of clusters of globules of varing densities in the cytoplasm that proliferate and seem to grow in size with the passage of time (Gedigk and Fischer 1979). Lysosomes often fuse with these globular clusters and attempt to digest them through their enzymes by autophagocytosis without success. The lipofuscin units are indigestible by lysosomal enzymes, and while hepatocytes and renal tubule cells can get rid of them by ejecting them through exocytosis into the extracellular space, the neurons, the heart cells and the smooth muscle cells seem unable to eject them. Thus, since in some neurons lipofuscin is continuously produced but never removed, it gradually accumulates with ageing in the cytoplasm, displacing most organelles (Constantinides 1984).

There is now considerable evidence that excessive accumulation of lipofuscin can seriously damage and even destroy the cells in which it occurs (Gedigk and Wessel 1964; Mann et al. 1978; Brizzee and Ordy 1979; Heinsen 1981; Davies and Fotheringham 1981; Dowson 1982). Thus, lipofuscin-filled neurons have been found to lose RNA, specific organelles, dendrites and specific functions in areas such as the hypothalamus, the hippocampus, the visual cortex and the cerebellum. Furthermore, extreme lipofuscin packing, whether induced spontaneously by ageing in neurons, or experimentally by chronic Vitamin E deficiency in uterine smooth muscle, has been found to lead to disintegration and disappearance of some of the cells it afflicts.

In view of the evidence for the role of lipid peroxidation in the genesis of lipofuscin, one might expect that chronic treatments of animals with antioxidant chemicals would inhibit the development of this material in neurons with ageing. Unfortunately, however, the results of the 3 quantita-

tive experimental studies that have so far investigated this possibility have proved conflicting and inconclusive: The first study by Tappel et al. (1973) found that anti-oxidant treatment had *no significant effect*, the second study by Blaubeer et al. (1979) that it *increased*, and the third by Kruk and Enesco (1981) that it *decreased* the accumulation of an extractable fluorescent component of lipofuscin in brain homogenates of ageing mice.

Nevertheless, the extractable fluorescent component of lipofuscin that was used as a chemical endpoint in the above 3 investigations – a Schiff base resulting from the cross-linking of some amines with malonaldehyde (Blaubeer et al. 1979) – has been recently shown to represent only a tiny fraction of the whole lipofuscin complex (Tappel et al. 1973; Bieri et al. 1980), a fraction that is not necessarily representative of its whole morphological mass. No assessment whatsoever of microscopically visible lipofuscin granules was undertaken in these studies. And furthermore, the conditions under which the anti-oxidant treatments were administered to the animals in the first 2 of these investigations resulted in serious toxicity such as completely arrested body growth, catabolism and extremely high mortality (Tappel et al. 1973; Blaubeer et al. 1979).

For all these reasons we considered it worthwhile to re-investigate the effect of anti-oxidants on lipofuscin development in ageing neurons using a quantitative *morphological* endpoint, namely the total count of microscopically visible lipofuscin granules in all neurons of a cervical spinal cord section. We also tried to find out whether a chronic anti-oxidant treatment can be given daily without producing any toxicity or mortality.

Materials and methods

Two experiments were done, using male mice of the Balb/c strain. In the *first experiment*, we assessed the histological lipofuscin content of the spinal cord of the following 3 groups: (*1*) 1 week old mice, (*2*) 18 months old mice, and (*3*) 18 months old mice treated with the natural anti-oxidant Vitamin E (α-tocopherol), given subcutaneously at the relatively low dosage of 16 mg/mouse/week (once weekly) during the first 10 monts of their life.

In the *second experiment*, we assessed the histological lipofuscin content of the spinal cord in the following 4 groups: (*1*) 1 month old mice, (*2*) 8 months old mice, (*3*) 8 months old mice treated continuously with Vitamin E, given by stomach tube at the relatively high dosage of 40 mg/mouse/week (in 5 daily instalments of 8 mg each week), starting at the end of the first month after birth, and (*4*) 8 months old, treated continously with the synthetic ani-oxidant butylated hydroxy-toluene (BHT), given in the diet at about 15 mg/mouse daily, starting at the end of the first post-natal month.

The morphologic assessment of the total number of microscopically visible lipofuscin granules per section of spinal cord in all groups was accomplished as follows: A 2 mm long cylindrical segment of the cranial end of the cervical spinal cord was removed immediately after killing each animal with ether, fixed in glutaraldehyde and osmic acid and embedded in epoxy resin, following the routine procedure of tissue preparation for electron microscopy. One micron thick unstained transverse sections of the whole spinal cord were then cut from the cranial end of every specimen and examined in a light microscope under phase contrast. In such preparations, we found that every lipofuscin granule appeared as a discrete black dot in the cytoplasm of neurons, and the total number of all such granules in all neurons within the butterfly-shaped grey substance of one spinal cord section from each animal was counted. Random ultra-thin sections were also cut from most spinal cord specimens and examined electron microscopically.

To completely eliminate subjective bias in counting, all counts were performed "blind" on coded slides, i.e. without knowledge of the identity of the spinal cord section that was being counted. After decoding, the mean counts $\pm$ standard errors (ε) for each group were computed and the significance of the results was evaluated statistically.

All mice were weighed initially, terminally and at monthly intervals throughout the duration of this study, and they were subjected to a detailed autopsy after death.

In the first experiment we used 6 mice per group. In the second experiment we used 10 mice in the 8 month control group, and 8 mice in every other group. All animals survived for the duration of their experiments.

Results

The total lipofuscin granule count per spinal cord section increased progressively and highly significantly with time after birth, as can be seen by comparing the counts of 1 week, 1 month, 8 months and 18 months old untreated mice from both experiments (*see Table 1*). The P value for the difference between the mean counts of any two age groups was <0.001.

There was a close correlation between the total number of light microscopically visible lipofuscin granules and the average concentration of electron microscopically visible lipofuscin complexes in random sections of spinal cord neurons from the various age groups that we studied. Thus, both light and electron microscopically lipofuscin granules were completely absent 1 week after birth, first began to appear in small numbers as early as 1 month after birth, had developed in appreciable numbers in most neurons 8 months after birth, and had proliferated into large packs in many neurons 18 months after birth. Ageing did not only cause a great proliferation of the numbers of light-microscopically visible lipofuscin granules but, as shown by the electron microscope, it also induced certain qualitative changes in them; they tended to somewhat increase in size, to become more multiglobular and to expand their electronlucent component with the passage of time (see Figs. 1–4).

When Vitamin E was given at the relatively low dosage of 16 mg/mouse/week in the first experiment, it had no significant effect on the accumulation of lipofuscin granules in spinal cord neurons during 18 months of post-natal life (*see Table 2*).

When, however, Vitamin E was given at the higher dosage of 40 mg/mouse/week in the second experiment, it significantly reduced the accumulation of lipofuscin granules in 8 months of post-natal life by about 20%, with a P value of 0.02 (*see Table 3*). Similarly, the synthetic anti-oxidant BHT also reduced lipofuscin granule accumulation significantly by about

Table 1. Average total count of lipofuscin granules $\pm\varepsilon$ per cervical spinal cord section in 4 age groups of Balb/c mice. The differences between the values of any two age groups are significant with a P value of <0.001

1 week old mice	1 month old mice	8 months old mice	18 months old mice
0	269 ± 56	$1{,}101 \pm 82$	$2{,}462 \pm 318$

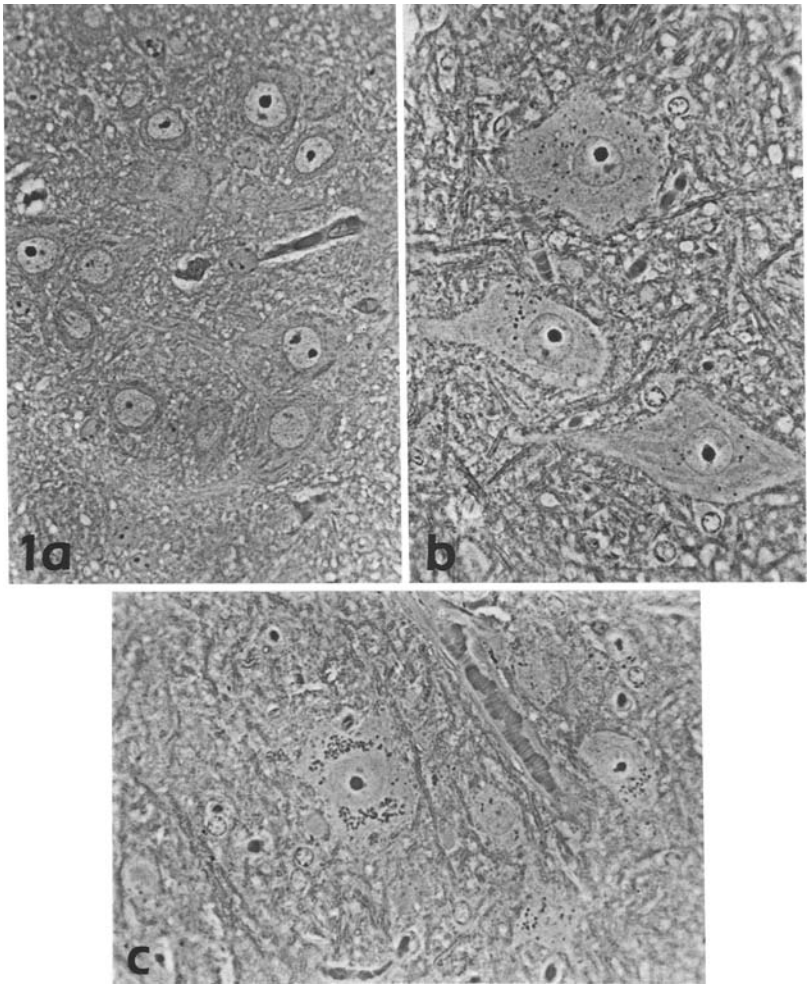

Fig. 1. a Typical cervical spinal cord neurons from 1 week old mice. No lipofuscin granules have yet developed in the cytoplasm of the relatively small young neurons that are scattered among the as yet unmyelinated axons. (Phase contrast light micrograph, ×400). **b** Typical cervical spinal cord neurons from 8 months old mice. Appreciable numbers of lipofuscin granules (visible as small black dots) have materialised in the cytoplasm of most neurons which have grown in size and are now surrounded by myelinated axons. (Phase contrast light micrograph, ×400). **c** Typical spinal cord neurons from 18 months old mice. The lipofuscin granules are now more numerous and often form tightly packed aggregates in the cytoplasm, as in the case of the neuron slightly left of center. (Phase contrast light micrograph, ×400)

20%, with a P value of 0.02 (*see Table 3*). No difference in granule size was seen between 8 months old treated and untreated mice.

None of the treatments used in this study produced any toxicity whatsoever. All Vitamin E and BHT treated groups grew as well, displayed the same vigor and vitality, and presented the same normal autopsy findings upon sacrifice as the untreated control groups.

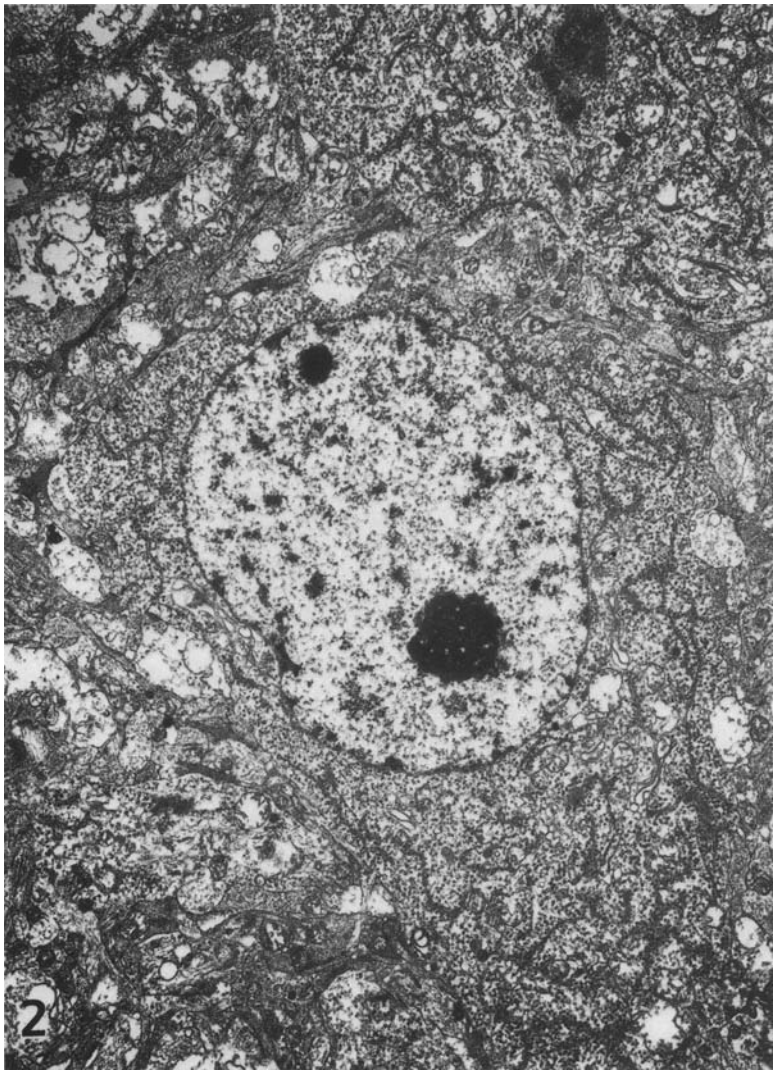

Fig. 2. Electron micrograph of a typical spinal cord neuron from 1 week old mice. Not a single lipofuscin complex has yet appeared in the neuronal cytoplasm. (× 2,400)

Discussion

Our results show that chronic daily treatment at a certain dosage level with both the natural anti-oxidant Vitamin E and the synthetic anti-oxidant butylated hydroxy-toluene (BHT) will prevent partially but significantly the proliferation of microscopically visible lipofuscin granules in spinal cord neurons that develops during 8 months' ageing, without causing any toxicity to the animals that receive this treatment. Since we compared light microscopically the numbers of lipofuscin granules in untreated and treated ani-

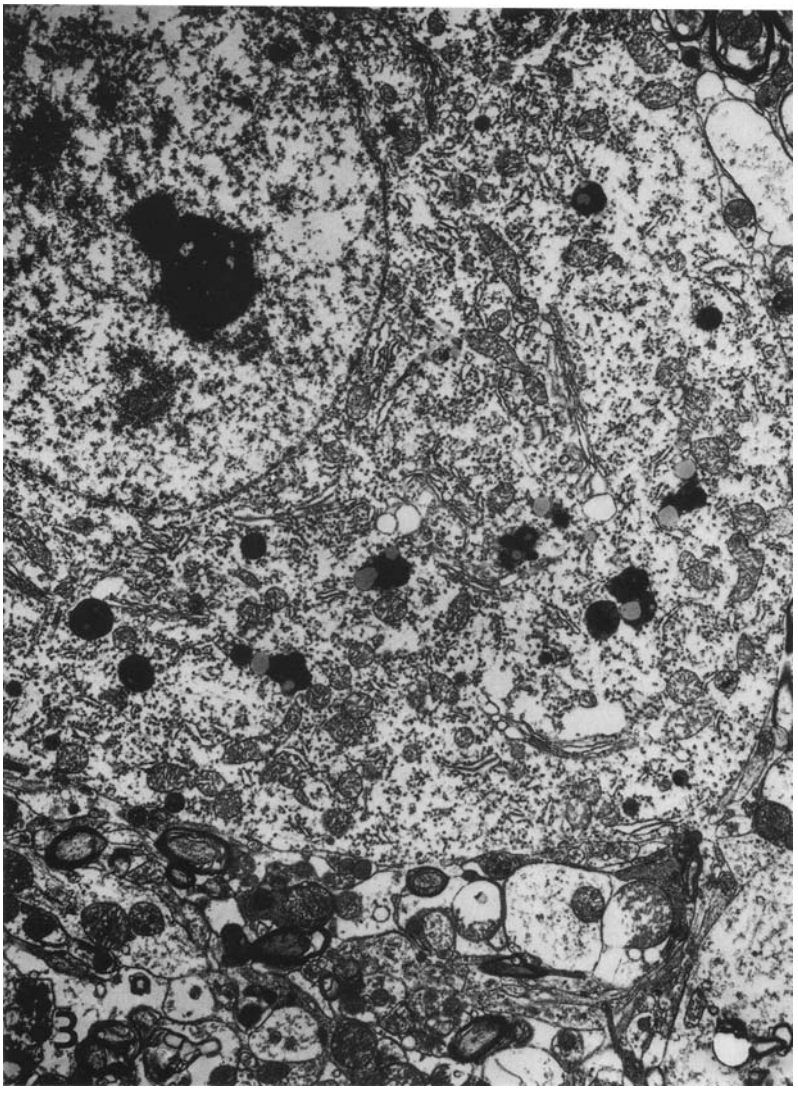

Fig. 3. Electron micrograph of a typical spinal cord neuron from 8 months old mice. Several lipofuscin complexes have materialised in the neuronal cytoplasm (× 2,400)

mal groups that were killed at exactly the same time interval after birth (i.e. at precisely the same stage of the ultrastructural size evolution of their granules) it seems permissible to conclude that the above treatments also diminished the expansion of the approximate total morphological mass of neuronal lipofuscin that is associated with ageing.

Our conclusions, based on a morphological quantitation of the total numbers of the whole, microscopically visible lipofuscin granules in neurons are thus different from those of Tappel et al. (1973) and Blaubeer et al. (1979), but in agreement with those of Kruk and Enesco (1981) – all three

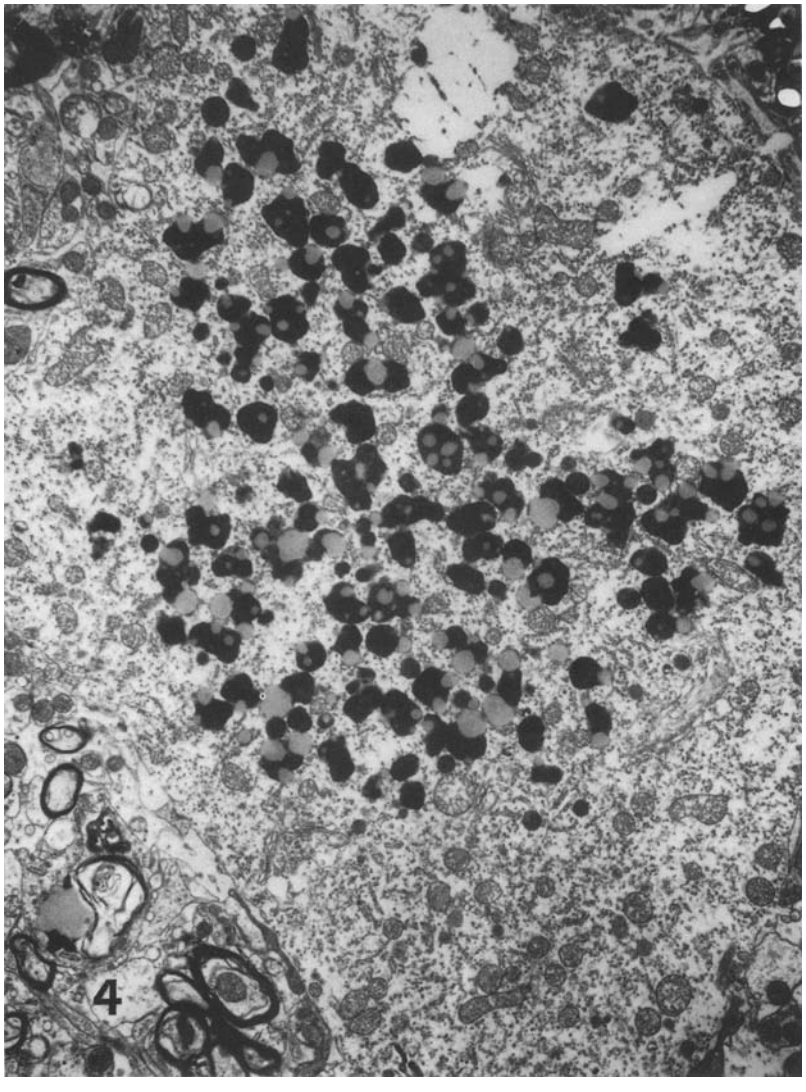

Fig. 4. Electron micrograph of a typical tightly packed aggregate of lipofuscin complexes in the cytoplasm of a spinal cord neuron from 18 months old mice. The lipofuscin complexes are not only more numerous but also somewhat larger and more muliglobular than those seen in 8 months old mice. (× 2,400)

of which were based on the chemical study of a very small extractable fluorescent fraction of lipofuscin in brain homogenates.

The following comments seem appropriate in correlating our results with those of the above mentioned 3 previous studies:

Tappel et al. (1973) found that a diet containing a mixture of 5 different anti-oxidants, given to mice at a very high dosage, starting with their 9th month of life, had *no effect* on the rise of the extractable fluorescent fraction

Table 2. Average total count of lipofuscin granules $\pm\varepsilon$ per cervical spinal cord section in untreated controls and in animals treated with Vitamin E at 16 mg/mouse/week (First Experiment). There is no significant difference between the value of the animals treated with the above relatively low dosage of Vitamin E and that of the 18 months old untreated controls

1 week old mice	18 months old mice	18 months old mice plus Vitamin E
0	2,462 ± 318	2,579 ± 401

Table 3. Average total count of lipofuscin granules $\pm\varepsilon$ per cervical spinal cord section in untreated controls and in animals treated with Vitamin E at the higher dosage of 40 mg/mouse/week or Butylated Hydroxytoluene (BHT) at about 100 mg/mouse/week (Second Experiment). The difference between the values of both the Vitamin E and BHT treated animals and the 8 months old untreated controls is significant with a P value of 0.02

1 month old mice	8 months old mice	8 months old mice plus Vitamin E	8 months old mice plus Butylated Hydroxytoluene (BHT)
269 ± 56	1,101 ± 82	882 ± 59	899 ± 27

of lipofuscin in their brains during the subsequent 14 months of their life. However, it should be emphasized that the regime used by these workers caused marked toxicity since it completely arrested the growth of their animals, induced catabolism in many, and diminished their survival by 50% as compared to the untreated controls. Apart from the fact that these authors collected no data on the whole microscopically visible lipofuscin granules, the lack of an effect on the fluorescent lipofuscin fraction in that study could have been due to any or all of the following: (*1*) The toxicity and protein catabolism induced by their treatments that would be bound to affect many important parameters such as food intake and intermediate metabolism, (*2*) possible mutually neutralising interactions (because of incompatibilities) between the five anti-oxidants used, (*3*) the very late beginning of the treatment (at the 9th post-natal month), and (*4*) the exceedingly high dosage of the Vitamin E used (about 3 times the highest level used in the present study), a fact of some importance in view of the findings of Pongracz (1973) that at very high dosages Vitamin E exhibits oxidant rather then anti-oxidant properties (Pongracz 1973).

Blaubeer et al. (1979), on the other hand, fed mice for 2 years a diet very high in unsaturated fatty acids (10% safflower oil) and supplemented one group with a very high Vitamin E dosage and another with Vitamin E plus ascorbic acid. They found that that the treatment with Vitamin E alone killed most (76%) of their animals so that they could not collect any significant data in that group, and that the less toxic combined Vitamin

E plus ascorbic acid treatment *increased* the fluorescent lipofuscin fraction in the pooled brains of their animals 2.5 times compared to untreated controls. In attempting to evaluate the results of Blaubeer et al. (1979) we have consider the following: (*1*) As already stated, changes in the small extractable fluorescent fraction of lipofuscin do not necessarily represent changes of the main non-extractable lipofuscin mass, (*2*) the high unsaturated fatty acid content of the diet would have likely increased lipofuscin production over and above that achieved by ageing alone, and (*3*) an interaction between the highly oxidisable – by air exposure – unsaturated fatty acids and Vitamin E in the diet could well have peroxidised the Vitamin E itself so as not only to abolish its anti-oxidant activity but even to reverse it, as reported by Chow and Draper (1979).

Finally, Kruk and Enesco (1981) gave 2 months old mice only a single intraperitoneal injection of Vitamin E and 3 months later found that the fluorescent lipofuscin fraction of their brains was significantly *lower* than that of untreated 5 months old controls. It is very interesting that these workers who were the only ones to obtain a significant inhibition of the development of a chemical lipofuscin fraction with a natural anti-oxidant (in agreement with our own finding that were obtained using whole lipofuscin counts as endpoint) were also the only ones whose anti-oxidant treatment was given under conditions that did not cause any toxicity, catabolism or mortality (as in our own present study).

On the whole then, the findings of the present study provide support for the peroxide theory of lipofuscin biogenesis during ageing and encourage further in vivo investigation of chronic anti-oxidant treatments at moderate dosage levels and under conditions that do not produce any toxicity, for possible therapeutic applications in man.

The problem with anti-oxidant therapy in humans at this moment is the relatively large molecular bulk of Vitamin E or BHT that seems to be necessary in order to achieve a moderate suppression of lipofuscin accumulation in neurons during ageing. If human lipofuscin is as susceptible to anti-oxidant treatment as mouse lipofuscin, our data suggest that humans would have to consume approximately 10 grams/day Vitamin E and about twice as much BHT for a moderate slowdown of lipofuscin development in their neurons. Such quantities seem unrealistically large and expensive for prolonged human use.

For this reason, we are now exploring various possibilities of obtaining greater anti-lipofuscin effect with less molecular bulk of anti-oxidant. One of the alternatives now under investigation in this laboratory is the action of various non-toxic combinations of compatible anti-oxidants, in the hope of achieving potentiating effects that may lead to effective treatment with smaller total anti-oxidant doses. Another alternative under study is to find out whether the blood-brain barrier for anti-oxidants can be in some way diminished so as to make it possible for a greater percentage of ingested anti-oxidant molecules to penetrate into neurons.

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