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Effect of thyme oil and thymol dietary supplementation on the antioxidant status and fatty acid composition of the ageing rat brain

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The present study measured changes in antioxidant enzyme activity in, and the phospholipid fatty acid composition of the ageing rat brain and tested whether dietary supplementation with thyme oil or thymol could provide beneficial effects. There were significant declines in superoxide dismutase (*EC* 1.15.1.1) and glutathione peroxidase (*EC* 1.11.1.9) activities and the total antioxidant status in the untreated rats with age, while thyme-oil- and thymol-fed rats maintained significantly higher antioxidant enzyme activities and total antioxidant status. The proportions of 18 : 2*n*-6, 20 : 1*n*-9, 22 : 4*n*-6 and 22 : 5*n*-3 in the brain phospholipids resulting from all three dietary treatments were significantly higher in 28-month-old rats than in 7-month-old rats. Only 20 : 1*n*-9 levels in 28-month-old thyme-oil- and thymol-treated rats were significantly higher than in the age-matched control. The proportion of 22 : 6*n*-3 in brain phospholipids, which declined with age in control rats, was also significantly higher in rats given either supplement. This latter finding is particularly important as optimum levels of 22 : 6*n*-3 are required for normal brain function. These results highlight the potential benefit of thyme oil as a dietary antioxidant.

Thyme oil: Thymol: Ageing: Polyunsaturated fatty acids: Docosahexaenoic acid

Biological membranes are the sites of enzymes, hormonal receptors and transport systems for ions and metabolites. Major components of these membranes are phospholipids containing polyunsaturated fatty acids (PUFA) which (by chain length and degree of saturation) influence membrane functions (Stubbs & Smith, 1984; Wahnon *et al.* 1989).

PUFA contain methylene-interrupted double bonds, which make them highly vulnerable to free-radical attack (Halliwell & Gutteridge, 1989). In living organisms, free-radical production tends to increase with age while tissue antioxidant defences tend to decline (Harman, 1995; Socci *et al.* 1995; Cao *et al.* 1996) causing numerous deleterious effects (Stubbs & Smith, 1984; Cohen & Zubenko, 1985; Spector & Yorek, 1985; Neuringer *et al.* 1988; Salem, 1989).

One of the most important tissues in which appropriate concentrations of PUFA are essential is the brain; its concentration of docosahexaenoic acid (DHA; 22 : 6*n*-3) is particularly important. Numerous reports have shown that, during the early development of the rat brain, the liver provides DHA which is then tenaciously retained (Neuringer *et al.* 1988; Salem, 1989; Scott & Bazan, 1989). However, the detrimental effects of ageing, especially increased free-radical production, cause concentrations of DHA to decline (Barja de Quiroga *et al*. 1990, 1992; Deans *et al*. 1994; Youdim, 1997). Alterations in levels of PUFA, especially DHA, are likely to have pronounced implications for brain function (Lamptey & Walker, 1976; Yamamoto *et al*. 1991; Okuyama, 1992; Chalon *et al*. 1998; Kaplan & Greenwood, 1998; Suzuki *et al*. 1998).

Previous studies have shown that dietary supplementation with plant essential oils protects and maintains levels of PUFA in cell membranes (Deans *et al.* 1993*a*,*b*, 1994; Recsan *et al.* 1997). These essential oils are present within glandular structures of plants, notably oil cells, glands, glandular hairs, oil or resin ducts. It is commonly assumed that these volatile essential oils are produced within the secretory cells of the oil gland (Deans & Svoboda, 1990). One particular essential oil of interest in our laboratory is that extracted from thyme (*Thymus vulgaris* L.) which contains various terpenoids such as α-pinene, myrcene, p-cymene, γ-terpinene, linalool, thymol and carvacrol. The antioxidant activities of these components have recently been characterized (K. A. Youdim and S. G. Deans, unpublished results). The present study investigated the effect of thyme oil and its major component thymol, which makes up almost 49 % of the total oil, as dietary antioxidant supplements during the rat life span on age-related changes in brain: (1) superoxide dismutase (*EC* 1.15.1.1; SOD) and glutathione peroxidase (*EC* 1.11.1.9; GSHPx) activities,

Abbreviations: DHA, docosahexaenoic acid; GSHPx, glutathione peroxidase; PUFA, polyunsaturated fatty acid; SOD, superoxide dismutase; TAS, total antioxidant status.

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(2) total antioxidant status (TAS) concentration, (3) fatty acid composition in membrane phospholipids.

Materials and methods

Animals, diets and housing

Male Wistar rats (B&K Universal Ltd, Hull, UK) (*n* 150, 7 months old) were randomly allocated to three equal groups and housed four rats to a cage measuring 220 × 320 × 500 mm. Temperature was maintained between 21° and 22° with a light–dark cycle of 13 h : 11 h. Fresh water was available *ad libitum* at all times. All rats were fed on a standard laboratory pelleted diet (RM 3(E); Special Diet Services, Witham, Essex, UK; Table 1), with thyme oil (F.D. Copeland & Sons, London, UK) or thymol (Sigma, Poole, Dorset, UK) administered with the diet at 42·5 mg/kg body weight per d. This level of administration is considerably lower than reported values for the median lethal doses of these two components. For thyme oil and thymol these have been found to be 1200 mg/kg and 980 mg/kg respectively in rats (Clark, 1995) compared with our dosage of 42·5 mg/kg. The level of administration chosen was similar to that used in previous trials, in which these compounds protected against the decline in PUFA levels in livers from aged rodents (Deans *et al.* 1993*a*,*b*, 1994).

Sample collection, storage and homogenization

Five rats from each diet group were killed every 3 months, using hypriam–midizolam (1·0 ml/300 g). Body and tissue weights were recorded at each sampling date. Brains were excised, washed with ice-cold 0·25 M-sucrose solution, blotted dry, then wrapped in foil and immediately placed in liquid N_2 and stored at −80° for subsequent analysis.

The following day after organ removal, brain tissues were thawed in ice water at 4° for analysis of enzyme activities and lipid extraction. Portions of brain tissue were initially weighed then homogenized in 0·25 M-sucrose buffer pH 7·2 (1 : 10, w/v) using a Ystral T1500 homogenizer (Ystral Ltd, UK, Hemel Hempstead, Herts., UK). Homogenates were then centrifuged using a Camlab alc4239R High Speed Refrigerated Centrifuge (Camlab, Cambridge, Cambs., UK) for 10 min at 2500 ***g***. The cytosolic supernatant fractions were collected by centrifuging the 2500 ***g*** supernatant fraction for a further 60 min at 100 000 ***g*** at 4° using a Centrikon T-11X0 ultracentrifuge (Kontron Instruments, Watford, Herts., UK). All samples were stored in ice during analysis to prevent undesirable loss in activity.

Analytical techniques

Tissue SOD and GSHPx activities and the TAS were determined in the 100 000 ***g*** supernatant fraction using commercial test kits supplied by Randox Laboratories, Crumlin, UK. All measurements were performed on a Pye Unicam spectrophotometer (Model SP8-500; Cambridge, Cambs., UK).

Samples of thawed tissue were weighed, homogenized and the total lipid extracted in chloroform–methanol (2 : 1, v/v) with subsequent aqueous washing according to the standard procedures of Christie (1982). Phospholipid fractions were isolated by TLC on silica gel G using a solvent system of hexane–diethyl ether–formic acid (80 : 20 : 1, by vol.). Following transmethylation by refluxing with methanol–toluene–sulfuric acid (20 : 10 : 1, by vol.) (Gurr *et al.* 1969), the fatty acid compositions of the phospholipid fractions were determined by GLC using a capillary column system (Carbowax, 30 m × 0·25 mm, film thickness 0·25 μm: Alltech, Carnforth, Lancs., UK) in a CP9001 instrument (Chrompack, Middleburg, The Netherlands). The column was operated at a head pressure of 71·8 kPa using the following temperature programme. The initial column temperature was 150°, which increased to 200° at 15°/min, followed by an increase to 250° at 2°/min. The column temperature was maintained at 250° for an additional 10 min to facilitate the elution of the longer chain fatty acids. The identities of the peaks were verified by comparison with the retention times of standard fatty acid methyl esters (Sigma Chemical Co.). Data analysis, using an EZ Chrom Data System (Scientific Software Inc., San Jose, CA, USA), enabled the calculation of the fatty acid composition.

Statistical analysis

A balanced factorial ANOVA was used to assess the effects of supplementing the diets of rats with thyme oil or thymol during their lifetime. Tests of significant differences between 7-month-old and 28-month-old rats and differences between dietary effects in 28-month-old rats were performed using Student's *t* test, taking $P < 0{\cdot}05$ as the limit of significance.

Results

Brain superoxide dismutase and glutathione peroxidase activities

Significant changes in SOD and GSHPx activities were observed in rats during their life span as a result of all

Table 1. Composition of the experimental diet*

Ingredient	g/kg
Casein, alcohol extracted	189·6
L-Cystine	2·8
Maize starch	450·2
Maltodextrin 10	118·5
Sucrose	94·8
Cellulose, BW200	47·4
Soyabean oil	42·7
Salt mix†	9·5
$Ca_3(PO_4)_2$, dibasic	12·3
$CaCO_3$	5·2
Potassium citrate	15·6
Vitamin mix (V13401)‡	9·5
Choline bitartrate	1·9

* The fatty acid composition of the whole diet was (g/100 g total fatty acids): 14 : 0 1·43, 16 : 0 16·4, 18 : 0 3·10, 18 : 1*n*-9 18·4, 18 : 1*n*-7 1·98, 18 : 2*n*-6 45·1, 18 : 3*n*-3 4·69, 20 : 5*n*-3 2·54, 22 : 6*n*-3 2·06.

† Contained (g/kg salt mix): NaCl 259, MgO 41·9, $MgSO_4.7H_20$ 257·6, $CrKSO_4.12H_2O$ 1·925, $CuSO_4$ 1·05, KI 0·035, Fe citrate 21·0, $MnSO_4$ 12·25, $ZnSO_4$ 5·6, sucrose 399·6.

‡ Contained (g/kg vitamin mix): retinyl palmitate (150 mg retinol/g) 0·8, cholecalciferol (2·5 mg/g) 1·0, menadione sodium bisulfate (62·5 % menadione) 0·08, biotin (1·0 %) 2·0, cyanocobalamin (0·1 %) 1·0, pteroylmonoglutamic acid 0·2, nicotinic acid 3·0, calcium pantothenate 1·6, pyridoxine-HCl 0·7, riboflavin 0·6, thiamin-HCl 0·6, sucrose 988·42.

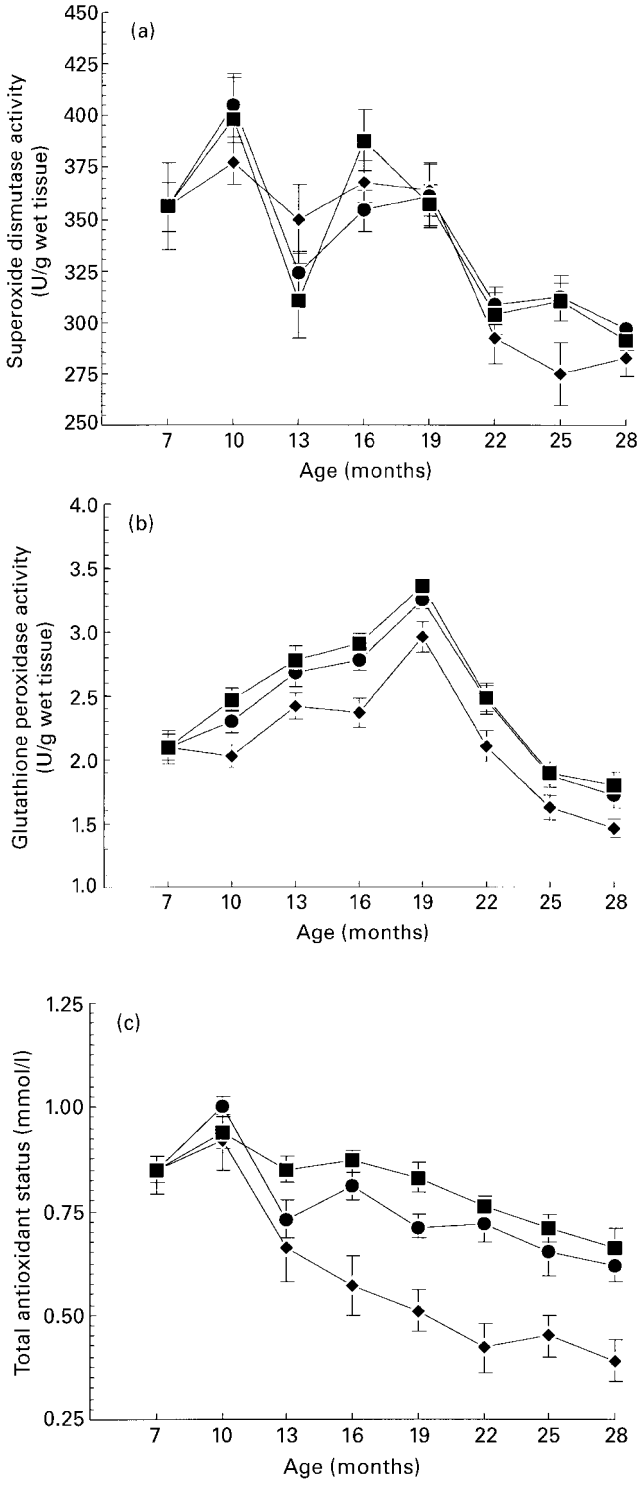

Fig. 1. Age-related changes in (a) superoxide dismutase activity, (b) glutathione peroxidase activity and (c) the total antioxidant status in brains from rats fed on a control diet (♦), a thyme-oil-supplemented diet (■) or a thymol-supplemented diet (●). Values are means for five rats, with standard deviations represented by vertical bars.

three dietary treatments ($P < 0{\cdot}001$) (Fig. 1(*a*)). SOD activity in rats from all three dietary groups was found to be significantly lower by approximately 19 % in 28-month-old rats compared with 7-month-old rats ($P < 0{\cdot}05$ or $P < 0{\cdot}01$). No significant differences between the SOD activities following the three treatments were observed in old rats. In contrast, GSHPx activity increased as a result of all three treatments between 7-and 19-months after which it declined (Fig. 1(*b*)). In 28-months-old control, thyme-oil and thymol-treated rats GSHPx activities were all significantly lower than in 7-month-old rats ($P < 0{\cdot}001$, $P < 0{\cdot}01$ and $P < 0{\cdot}05$ respectively). In 28-month-old control rats, activity was lower by 30 %, considerably more than in thyme-oil- and thymol-treated rats of the same age, in which it was lower by only 18 % and 14 %. Despite the decreases in enzyme activity in the thyme oil and thymol treatment groups, the levels were significantly higher by 18 % ($P < 0{\cdot}05$) and 24 % ($P < 0{\cdot}01$) respectively than in the age-matched control.

Brain total antioxidant status

There was a significant decline in the TAS as a result of all three treatments during the rat life span ($P < 0{\cdot}001$) (Fig. 1(c)). The TAS in brains from 28-month-old control rats fell by approximately 55 % ($P < 0{\cdot}001$), almost twofold more than the declines exhibited in thyme-oil- and thymol-treated rats ($P < 0{\cdot}05$). Despite the decline in the TAS in 28-month-old thyme-oil- and thymol-treated rats, it was found to be 61 % ($P < 0{\cdot}01$) and 71 % ($P < 0{\cdot}01$) higher than that in the age-matched control.

Fatty acid composition in the brain phospholipid fraction

Changes in fatty acid composition in the brain phospholipid fraction with age for the three treatments are shown in Figs. 2 and 3. There were significant changes ($P < 0{\cdot}01$ or $P < 0{\cdot}001$) in the proportions of all fatty acids except arachidonic acid (20 : 4*n*-6) during the rat life span as a result of all treatments. The proportions of palmitic acid (16 : 0) in 28-month-old control, thyme-oil- and thymol-treated rats were all significantly lower compared with those in 7-month-old rats ($P < 0{\cdot}01$). However, there were no significant differences in the proportion of 16 : 0 between treatments in old rats. In contrast, the proportions of linoleic acid (18 : 2*n*-6), eicosenoic acid (20 : 1*n*-9), docosatetraenoic acid (22 : 4*n*-6) and docosapentaenoic acid (22 : 5*n*-3) in all three dietary treatments were significantly higher in 28-month-old rats than in 7-month-old rats. In rats (28 months) given thyme oil or thymol, the proportions of 20 : 1*n*-9 differed significantly from the age-matched control ($P < 0{\cdot}05$), being approximately 30 % and 26 % higher respectively. There were also notable changes in the proportion of DHA (22 : 6*n*-3) with age for the three treatments. In 28-month-old control rats, the proportion of 22 : 6*n*-3 had declined significantly ($P < 0{\cdot}001$), falling by 17 %, whilst in 28-month-old thymol treated-rats it fell by 4 % and in thyme-oil-treated rats it had remained unchanged. The proportions of DHA in 28-month-old thyme-oil- and thymol-treated rats were 22 % and 16 % higher ($P < 0{\cdot}001$, $P < 0{\cdot}001$) than in the age-matched control. Finally, although no significant differences were observed between proportions of 20 : 4*n*-6 in 7-month and 28-month-old rats, 28-month-old thyme-oil- and thymol-treated rats exhibited

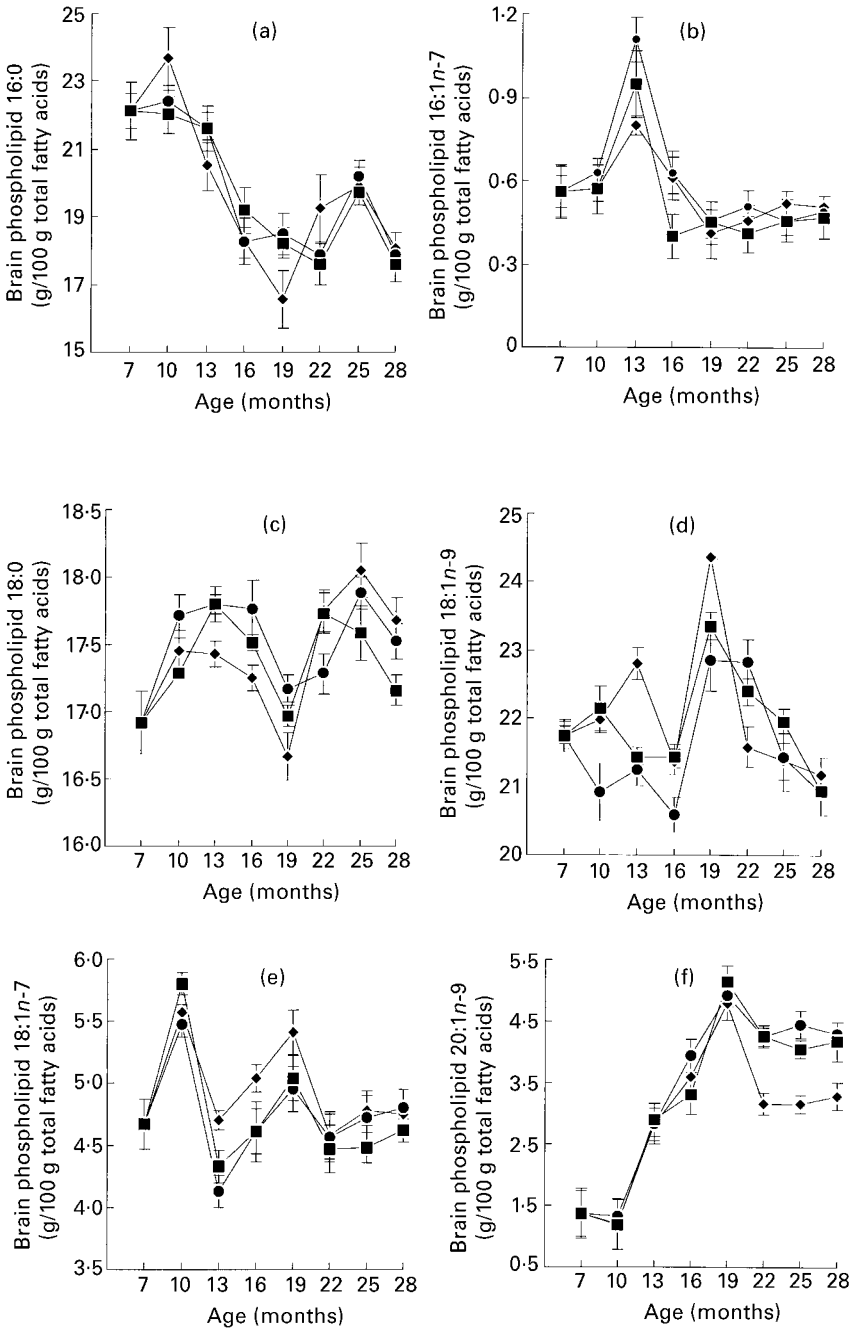

Fig. 2. Changes with age in the proportions of saturated and monounsaturated fatty acids in the brain phospholipid fraction (g/100 g total fatty acids) from rats fed on a control diet (♦), a thyme-oil-supplemented diet (■) or a thymol-supplemented diet (●). Values are means for five rats, with standard deviations represented by vertical bars.

proportions of 20 : 4*n*-6 10 % higher ($P < 0{\cdot}05$) than in the age-matched control.

Discussion

Despite the numerous antioxidant systems present in cells that are utilized to combat free radicals, there appears to be an imbalance between the production of radicals and their removal with age (Harman, 1995). These free radicals attack PUFA components of the cell membranes, consequently reducing membrane fluidity and therefore functionality (Stubbs & Smith, 1984).

The age-related decrease in brain SOD activity was found to agree with earlier studies (Benzi *et al.* 1988; Vanella *et al.* 1989; Barja de Quiroga *et al.* 1990; Rao *et al.* 1990), although others have shown it to be unchanged (Reiss & Gershon, 1976; Kurobe *et al.* 1990; Semsei *et al.* 1991) or even increased (Danh *et al.* 1983). In the case of brain GSHPx

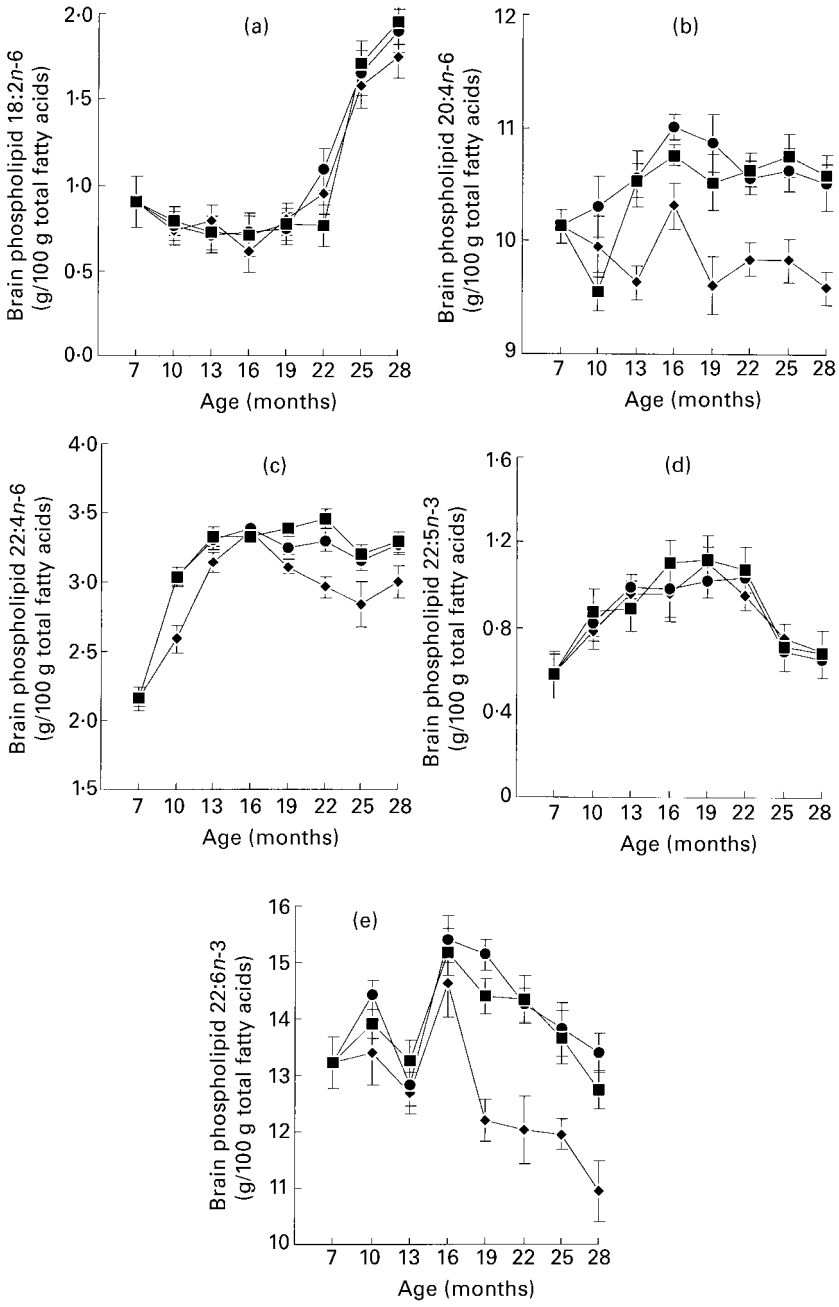

Fig. 3. Changes with age in the proportions of polyunsaturated fatty acids in the brain phospholipid fraction (g/100 g total fatty acids) from rats fed on a control diet (♦), a thyme-oil-supplemented diet (■) or a thymol-supplemented diet (●). Values are means for five rats, with standard deviations represented by vertical bars.

activity, this appears to be the only study that has shown brain GSHPx activity to be lower in aged rats (28 months) compared with young rats (7 months), whilst the majority of other investigations report activity to be unchanged (Mizuno & Ohta, 1986; Ansari *et al.* 1989; Barja de Quiroga *et al.* 1990, 1992) or even increased (Vitorica *et al.* 1984; Benzi *et al.* 1988). Dietary supplementation with thyme oil or thymol was not found to have influenced SOD activity in 28-month-old rats in comparison with the age-matched controls. In contrast, GSHPx activities in thyme-oil- and thymol-treated rats at 28 months were significantly higher than activity in control rats at the same age. By far the most dramatic effect of thyme oil and thymol was that on the TAS. Whilst in control rats the TAS concentration declined during the rat life span, both dietary supplements were found to inhibit this process. Whether this was due to their effects on GSHPx activity is not known. However, it is possible that the higher TAS in 28-month-old rats was a consequence of

the antioxidant properties of the supplements (Youdim, 1997) and/or an increase in other endogenous antioxidants that were not measured in the present study.

The present study also found that in the phospholipid fraction, concentrations of 18 : 2*n*-6, 20 : 1*n*-9, 22 : 4*n*-6 and 22 : 5*n*-3 increased with age. Interestingly, the concentrations of these PUFA (except 22 : 5*n*-3) were found to be higher in rats whose diets were supplemented with either thyme oil or thymol. The brain is one of the most important tissues for which maintaining appropriate concentrations of PUFA is essential; its concentration of DHA is particularly important. Reports have shown that the detrimental effects of ageing, especially increased free-radical production, cause concentrations of DHA to decline (Barja de Quiroga *et al.* 1990, 1992; Deans *et al.* 1994; Youdim, 1997) in agreement with the findings of the present study. However, the significant decline in the phospholipid DHA concentration with age was only observed in control rats, not those with diets supplemented with thyme oil or thymol. Similarly, 20 : 4*n*-6 was also maintained at significantly higher concentrations in old thyme-oil- and thymol-treated rats.

The concentration of DHA is particularly high in the cerebral cortex (grey matter). Among subcellular fractions of brain tissue, the highest levels of DHA are found in synaptosomes, synaptic vesicles, mitochondria and microsomes (Onuma *et al.* 1984; Neuringer & Connor, 1986; Neuringer *et al.* 1988; Salem, 1989; Scott & Bazan, 1989) all of which can be characterized as fluid and metabolically active. The protection of PUFA levels, especially that of DHA, is likely to have pronounced implications for the functional operations of the brain (Lamptey & Walker, 1976; Yamamoto *et al.* 1991; Okuyama, 1992; Chalon *et al.* 1998; Kaplan & Greenwood, 1998; Suzuki *et al.* 1998). As far as the nervous system is concerned, DHA has been shown to influence certain electrophysiological variables as well as learning functions, and to a great extent optimum levels of DHA ensure harmonious cerebral development (Bourre *et al.* 1993). It has also been reported that deficiencies in DHA affect aspects of memory, motor skills and behaviour (Lamptey & Walker, 1976; Yamamoto *et al.* 1987, 1991; Okuyama, 1992; Chalon *et al.* 1998; Kaplan & Greenwood, 1998; Suzuki *et al.* 1998).

The results presented here show that both thyme oil and thymol provided beneficial effects on the antioxidant status of the rat brain, which may in turn have influenced the concentrations of PUFA, especially DHA. This result is particularly important in light of the reported functional roles of DHA, discussed earlier. Neither supplement appeared to be more effective than the other and in the light of the fact that the thymol composition of thyme oil was less than 50 %, this observation suggests that whilst the majority of the antioxidant potency exhibited by thyme oil is as a result of its thymol composition, other possible antioxidant components within the oil, such as carvacrol (Youdim, 1997) may also be contributing to its effect.

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