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Tissue-specific differences in antioxidant distribution and susceptibility to lipid peroxidation during development of the chick embryo

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Abstract

The purpose of this study was to determine the tissue-specific acquisition of antioxidant capacity during chick embryo development and to assess the effectiveness of this process in the prevention of lipid peroxidation. The transfer of α-tocopherol, carotenoids and ascorbic acid from the yolk/yolk sac membrane (YSM) to the developing chick embryo and the distribution of these antioxidant compounds between the embryonic tissues were investigated. The concentrations of α-tocopherol and carotenoids in the yolk decreased between day 15 of development and hatching at day 21, concomitant with an increase in the levels of these antioxidants in the YSM. The concentration of both these lipid-soluble antioxidants in the liver increased dramatically between day 18 of embryonic development and day 1 after hatching. The adipose tissue content of α-tocopherol also increased markedly during the late embryonic/early neonatal period. However, the levels of α-tocopherol in the liver were far higher than in any other tissue with particularly low levels observed for the brain. Also, carotenoids were undetectable in the developing brain. Ascorbic acid was not present in the initial yolk but high levels of this water-soluble antioxidant were detected in the YSM, particularly at the early stages of development. The concentration of ascorbic acid in the embryonic brain was far higher than in any other tissue. Homogenates of brain tissue were extremely susceptible to lipid peroxidation during incubation in vitro whereas extracts of liver, yolk and YSM were relatively resistant to lipid peroxidation, particularly in the absence of exogenous Fe^{2+}.

Keywords: Antioxidant; Lipid peroxidation; α-Tocopherol; Ascorbic acid; Carotenoid; (Chick embryo)

1. Introduction

During the development of the chick embryo, lipids are transferred from the yolk via the yolk sac membrane (YSM) and the embryonic circulation for distribution to the developing tissues [1,2]. A proportion of the fatty acids derived from the yolk lipids are utilised by the tissues for energy production via the β-oxidation pathway [3]. The remainder, which are incorporated into the tissue lipids of the embryo are, in the case of several tissues, characterised by a relatively high degree of unsaturation when compared with the original lipids of the yolk [1–7]. This increase in the proportion of polyunsaturated fatty acids may result from a combination of selective uptake by the tissues,

Abbreviations: BHT, butylated hydroxytoluene; DBI, double bond index; HPLC, high-performance liquid chromatography; TBARS, thiobarbituric acid reactive substances; YSM, yolk sac membrane; VLDL, very low density lipoprotein

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preferential β-oxidation of the more saturated fatty acids and the action of desaturation/elongation systems in the YSM and liver [3,7,8]. In several of the developing tissues, specific roles for polyunsaturated fatty acids have been suggested; for example, the high levels of docosahexaenoic ($22:6n-3$) and arachidonic acids ($20:4n-6$) in the phospholipids of the brain and retina [3–5,7,8] appear to be necessary for the optimal development and function of these tissues [9–13].

Notwithstanding such important functions, a potentially deleterious consequence of the accumulation of polyunsaturates in the tissue lipids will be an increased susceptibility of the tissues to peroxidative damage [14–16]. The hatching and early neonatal stages may represent a particularly critical period in this regard, due to the exposure of the chick to atmospheric oxygen, the onset of pulmonary respiration and the dramatic increase in metabolic rate which occur at this time [17]. The brain may be especially at risk from free radical attack since, at least in mammals, this tissue has been shown to generate a greater amount of free radicals per gram of tissue than any other organ [18]. Thus the development of an effective antioxidant capacity [19–21] in the tissues of the chick embryo may be vital not only for protection against peroxidative damage during embryogenesis but also as a preparation for the increase in oxidative stress over the neonatal period.

The various components of the avian embryo's antioxidant system may be classified into two groups. The first category includes those components such as α-tocopherol, carotenoids and selenium which are pre-packaged in the initial yolk [22]; the subsequent tissue-specific distribution of these components within the embryo will be dependent on the transport and uptake mechanisms which function in this system. The second category encompasses those antioxidant components which are synthesised in the embryonic tissues during development; typical examples of this group include ascorbic acid [23] and the enzymes glutathione peroxidase, superoxide dismutase and catalase [24].

In a recent paper from this laboratory [25], we reported that the concentrations of α-tocopherol, retinol and selenium and the activities of glutathione peroxidase and superoxide dismutase were very low in the brain of the chick embryo, whereas much higher levels of all these components were present in the liver. These findings raised the question of the mechanism by which adequate antioxidant protection is afforded to the developing brain. The present paper compares the distribution of α-tocopherol, carotenoids and ascorbic acid between a range of tissues throughout development and reports differences in the susceptibility of these tissues to lipid peroxidation.

2. Materials and methods

2.1. Embryos

Fertilised eggs of the Ross-1 broiler-breeder strain were obtained from a commercial hatchery and incubated at 37.8°C and 60% relative humidity in a forced draught incubator with automatic egg turning. The chicks hatched after 21 days and were maintained in the incubator for 1 day with access to drinking water but with no food provision. At each developmental stage studied, 5 embryos or chicks were used. Samples of brain, liver, adipose tissue, heart, lung, kidney, YSM and residual yolk were collected and weighed. The determination of ascorbic acid content and the lipid peroxidation measurements were performed immediately on portions of fresh tissue. Samples of tissue destined for the determination of α-tocopherol, carotenoids and fatty acid composition were stored at -20°C under nitrogen for up to 2 weeks before use.

2.2. Fatty acid analysis

Tissue portions were extracted for total lipid by standard procedures following homogenisation in a suitable excess of chloroform/methanol (2:1, v/v) [26]. Portions of the total lipid were subjected to transmethylation [27] and the composition of the resultant fatty acid methyl esters was determined by gas–liquid chromatography using a capillary column system (Carbowax, 30 m $\times$ 0.25 mm, film thickness 0.25 μm; Alltech, Carnforth, UK) in a CP9001 instrument (Chrompack, Middelburg, The Netherlands). Integration of the peaks and subsequent data handling was performed using an EZ Chrom Data System (Scientific Software, San Jose, CA, USA) enabling

the fatty acid composition and the total amount of derived fatty acid from each sample to be quantified. The identities of the peaks were verified by comparison with the retention times of standard fatty acid methyl esters

The tissue concentrations of fatty acyl double bonds were expressed in terms of a double bond index (DBI) defined by the equation:

$$\begin{aligned}\mathrm{DBI} = F[&\mathrm{p16{:}1} + \mathrm{p18{:}1} + 2(\mathrm{p18{:}2}) + 3(\mathrm{p18{:}3}) \\ &+ 4(\mathrm{p20{:}4}) + 5(\mathrm{p20{:}5}) + 5(\mathrm{p22{:}5}) \\ &+ 6(\mathrm{p22{:}6})]\end{aligned}$$

where $F = \mu$mol total fatty acyl groups/g tissue and p16:1 etc. = the molar proportion of that fatty acid.

2.3. Determination of α-tocopherol

α-Tocopherol was determined by the method of McMurray et al. [28] as modified by Gaál et al. [25]. In brief, the samples were saponified with ethanolic KOH in the presence of pyrogallol and then α-tocopherol was extracted from the mixture with petroleum spirit. The extract was dried under nitrogen, redissolved in methanol and injected onto a Spherisorb type S30DS2, 3μ C_{18} reverse-phase HPLC column, 15 cm × 4.6 mm (Phase Separations, Clwyd, UK). Chromatography was performed using a mobile phase of methanol/water (97:3, v/v) at a flow rate of 1.1 ml/min. Fluorescence detection of α-tocopherol utilised excitation and emission wavelengths of 295 and 330 nm respectively. Calibration was performed using standard solutions of α-tocopherol in methanol.

2.4. Determination of carotenoids

The tissue or yolk sample was homogenised in 1 vol. of H_2O and 2 ml of the homogenate was mixed with 6 ml of ethanol/H_2O (2:1, v/v). Hexane (5 ml) was then added and the mixture was shaken vigorously for 5 min. The hexane phase, containing the carotenoids, was separated by centrifugation and collected. Analysis of the carotenoids extracted from the initial yolk and also from the YSM and liver at day 19 of development was performed by HPLC using a Spherisorb type S30DS2, 3μ C_{18} reverse-phase column, 25 cm × 4.6 mm (Phase Separations) with a mobile phase of acetonitrile/dichloromethane/methanol (7:2:1, v/v) using UV detection. Comparison of the peaks with carotenoid standards (lutein, zeaxanthine, β-crypoxanthin, lycopene and β-carotene) indicated that lutein comprised more than 90% of the total carotenoid content of yolk, YSM and liver. As a consequence, the yolk and tissue carotenoid concentrations reported in this paper were determined from the absorbance at 442 nm of the hexane extracts using lutein as a standard.

2.5. Determination of ascorbic acid

The ascorbic acid content of the tissues was determined spectrophotometrically by the method of Omaye et al. [29] based on the oxidation of ascorbic acid and the reaction of the oxidation products with 2,4-dinitrophenylhydrazine to form the derivative bis-2,4-dinitrophenylhydrazone. The latter was determined by its absorbance at 520 nm. The reaction was performed in the presence of thiourea to provide a mildly reducing medium in order to prevent interference from non-ascorbic acid chromogens.

2.6. Estimation of lipid peroxidation

In order to estimate the extent of endogenous lipid peroxidation in the tissues at the time of removal from the embryo, samples were homogenised in 9 vol. of 0.01 M sodium phosphate buffer (pH 7.4), containing 1.15% (w/v) KCl. The concentration of thiobarbituric acid reactive substances (TBARS) in the samples was determined immediately after homogenisation according to the method of Ohkawa [30] using 1,1,3,3-tetramethoxypropane as a standard. Prior to the reaction, butylated hydroxytoluene (BHT) was added to the homogenates to give a concentration of 0.01% (v/v) as recommended by Pikul et al. [31].

In order to measure the in vitro susceptibility of the homogenates to lipid peroxidation, the homogenates were incubated for 20 min at 37°C without BHT. In some incubations, $FeSO_4$ was present at a concentration of 0.1 mM. At the end of the incubation, BHT was added to give a concentration of 0.01% (v/v) and TBARS were determined as described above.

2.7. Chemicals

Fatty acid methyl ester standards, 2,4-dinitrophenylhydrazine, 1,1,3,3-tetramethoxypropane, BHT and lycopene were obtained from Sigma, Poole, UK. α-tocopherol, lutein and β-carotene were obtained from Fluka (Gillingham, UK). Zeaxanthin and β-cryptoxanthin were obtained from Apin (Abingdon, UK).

2.8. Expression of results

Results are presented as mean ± S.E. of measurements on tissue from five replicate embryos at each developmental stage studied. Statistical analysis was performed by Student's *t*-test.

3. Results

3.1. Fatty acid composition of the liver and brain of the newly-hatched chick

The major fatty acyl component of the total lipid of the liver of the day old chick was 18:1*n* − 9 followed by 18:2*n* − 6 with lower proportions of 20:4*n* − 6 and 22:6*n* − 3 (Table 1). In contrast, the lipids of the brain were characterised by much greater proportions of 20:4*n* − 6 and 22:6*n* − 3. Thus on the basis of percentage composition of fatty acids the brain lipids exhibited the more highly polyunsaturated profile. However, because of the greater concentration of total lipid (expressed as μmol fatty acyl groups/g tissue) in the liver, this tissue displayed a 2.7-fold higher concentration of acyl double bonds per g tissue (expressed as the DBI) in comparison with the brain.

Table 1
Fatty acid composition of total lipid and the tissue concentration of double bonds in the liver and brain from day-old chicks

	Liver	Brain
Fatty acid (mol%)		
16:0	8.3 ± 0.1	33.3 ± 0.5
16:1*n* − 7	1.7 ± 0.1	0.9 ± 0.1
18:0	9.0 ± 0.1	20.0 ± 0.4
18:1*n* − 9	58.1 ± 0.5	16.4 ± 0.7
18:2*n* − 6	11.5 ± 0.1	2.5 ± 0.2
18:3*n* − 3	0.3 ± 0.1	–
20:4*n* − 6	5.2 ± 0.2	9.3 ± 0.2
20:5*n* − 3	0.6 ± 0.0	–
22:5*n* − 3	0.3 ± 0.1	0.8 ± 0.2
22:6*n* − 3	4.6 ± 0.1	16.8 ± 0.4
F	258.8 ± 1.8	80.3 ± 1.1
DBI	355.1	132.1

Values are means ± S.E. of measurements on five livers or brains. *F* = μmol fatty acyl groups/g tissue; DBI = double bond index as defined in Section 2.

3.2. Concentrations of α-tocopherol, carotenoids and ascorbic acid in the yolk and tissues during development

The initial concentration of α-tocopherol in the yolk prior to incubation was 60.8 ± 5.4 μg/g yolk for the batch of eggs used in this experiment. The concentration of this vitamin in the yolk decreased during development to a value of 15.9 ± 1.4 μg/g yolk by 1 day after hatching, with a rapid decrease occurring between days 15 and 18 of embryonic development (Fig. 1). This same period, between days 15 and 18, was also characterised by a 3.0-fold increase in the concentration of α-tocopherol in the YSM whereas the major increase (4.2-fold) in the concentration of this vitamin in the liver occurred somewhat later, between days 18 and 22 (Fig. 1). Concomitant with this increase in the liver, the level

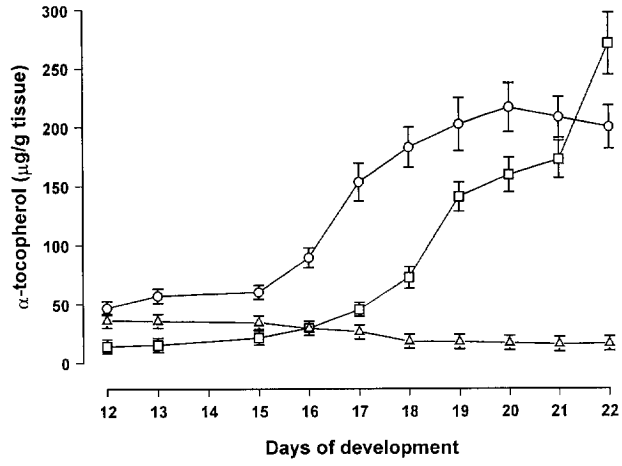

Fig. 1. Changes in the concentration of α-tocopherol in the yolk (△), YSM (○) and liver (□) during chick embryo development. Values are the means of measurements on 5 replicate embryos at each stage with the SE indicated by the error bars.

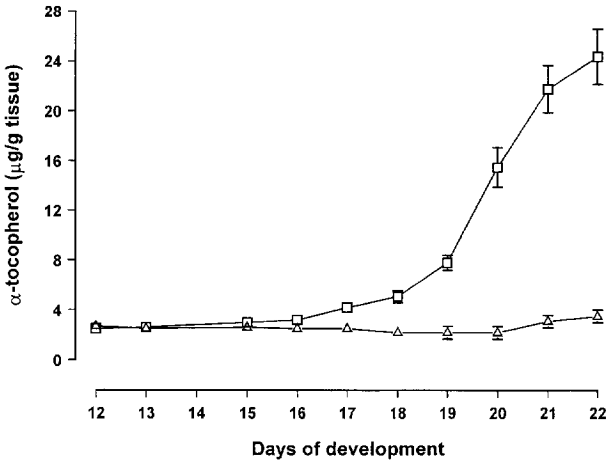

Fig. 2. Changes in the concentration of α-tocopherol in adipose tissue (□) and brain (△) during chick embryo development. Details as for Fig. 1.

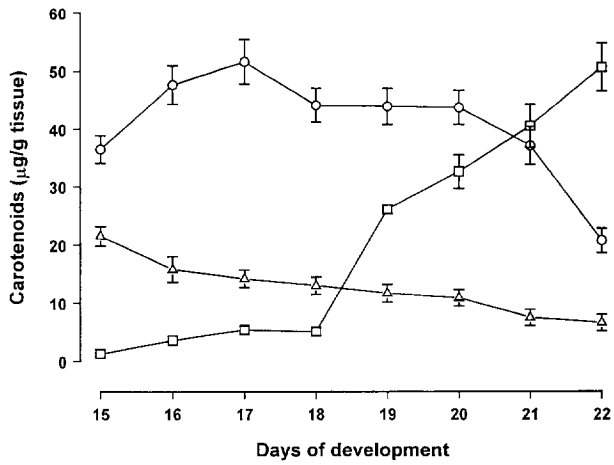

Fig. 3. Changes in the concentration of carotenoids in the yolk (△), YSM (○) and liver (□) during chick embryo development. Details as for Fig. 1.

of α-tocopherol in adipose tissue also increased markedly, by 5.3-fold, between days 18 and 22 (Fig. 2).

In contrast, the concentration of α-tocopherol in the brain was maintained at a low level throughout this period (Fig. 2); for instance, just prior to hatching at day 20, the concentration of α-tocopherol in the liver was 73-fold higher than in the brain. However, the level of this vitamin in the brain did increase significantly ($P < 0.01$), by 1.5-fold (i.e. from 2.2 ± 0.2 μg/g tissue at day 20 to 3.4 ± 0.2 μg/g tissue at day 22), over the hatching period (Fig. 2). Not shown in the figures are the α-tocopherol levels in the kidney, lung and heart of the embryo: between days 17 and 22 the tissue concentrations (μg/g tissue) increased from 3.4 ± 0.3 to 11.4 ± 1.2 in the kidney; from 3.2 ± 0.3 to 9.2 ± 0.9 in the lung; and from 5.9 ± 0.4 to 8.4 ± 0.7 in the heart.

The initial concentration (μg/g yolk) of total carotenoids in the yolk prior to incubation was 24.4 ± 2.2 and this value decreased during development to 6.6 ± 0.4 by the first day after hatching (Fig. 3). The concentration of carotenoids in the YSM increased to a maximal value by day 17 of embryogenesis and then decreased by more than 50% over the hatching period (Fig. 3). The level of carotenoids in the liver increased dramatically, by 10-fold, between days 18 and 22; i.e., in parallel with the increase in the α-tocopherol content of this tissue (Fig. 3; cf., Fig. 1). In marked contrast, carotenoids were undetectable in the brain at all developmental stages studied.

The tissue concentration of ascorbic acid in the YSM was highest at the earliest developmental stage studied, i.e. day 8, and decreased continuously thereafter (Fig. 4). The brain displayed a marked increase in ascorbic acid content between days 8 and 13 with a slight decrease thereafter (Fig. 4). In fact the concentrations of ascorbic acid in the brain during the final week of embryonic development were far higher than in any other tissue studied; respectively 4.0-, 5.2-, 6.0-, 6.7- and 14.3-fold higher than in liver (Fig.

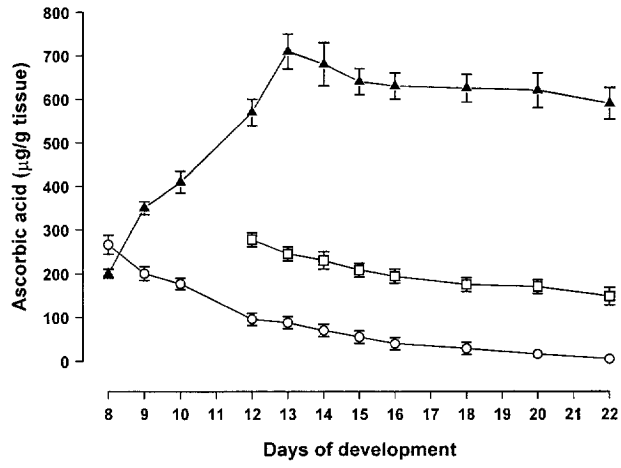

Fig. 4. Changes in the concentration of ascorbic acid in the YSM (○), liver (□) and brain (▲) during chick embryo development. Details as for Fig. 1.

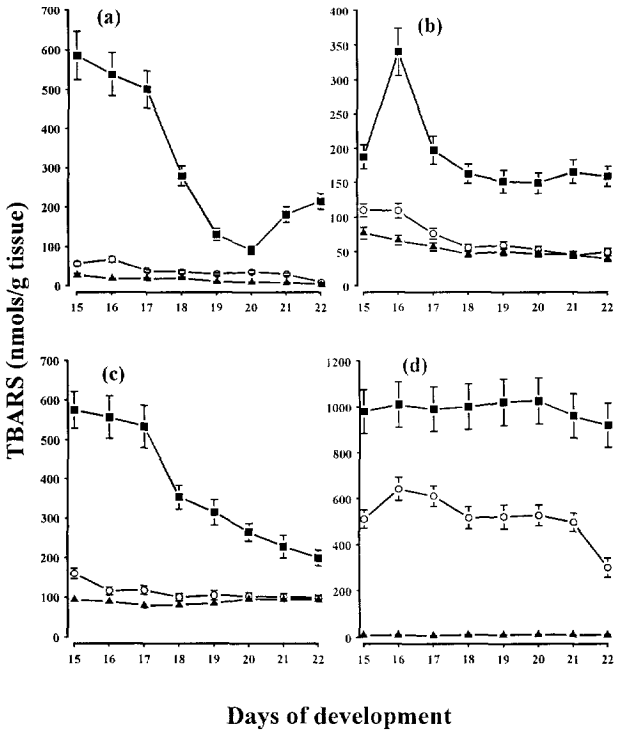

Fig. 5. Susceptibility of the yolk (a), YSM (b), liver (c) and brain (d) to lipid peroxidation. TBAR concentrations were determined in fresh tissue (▲) and following incubation of tissue homogenates for 20 min at 37°C in the absence (○) and presence (■) of $FeSO_4$ (0.1 mM). Details as for Fig. 1.

4), kidney, lung, heart, and thigh muscle (data not shown) by one day after hatching. Ascorbic acid was not detectable in the initial yolk.

3.3. Susceptibility of embryonic tissues to lipid peroxidation

The results in Fig. 5 indicate that the extent of lipid peroxidation in the brain at the time of removal from the embryo was very low at all developmental stages in terms of the endogenous levels of TBARS in the fresh tissue. In fact much higher endogenous levels of TBARS were detected in the liver and YSM. However, brain homogenates were extremely susceptible to lipid peroxidation during in vitro incubation even in the absence of exogenous Fe^{2+}, with a further major increase in lipid peroxidation observed when Fe^{2+} was added. In contrast, very little lipid peroxidation occurred during incubation of homogenates of yolk, YSM and liver without exogenous Fe^{2+}; substantial levels of TBARS were only observed after addition of Fe^{2+}. Moreover, the extent of Fe^{2+}-stimulated lipid peroxidation in homogenates of yolk, YSM and liver varied greatly according to the stage of development. In particular, the susceptibility to Fe^{2+}-induced lipid peroxidation in the yolk and liver decreased dramatically after day 17 of development.

4. Discussion

4.1. Degree of unsaturation of the lipids of the liver and brain of the newly-hatched chick

The brain of the day old chick is highly enriched in C_{20} and C_{22}-polyunsaturated acyl groups with $20{:}4n-6$ and $22{:}6n-3$ present as major components. In contrast, $18{:}1n-9$ is the predominant acyl moiety of the liver lipids with significant levels of $18{:}2n-6$ also present. Thus, on the basis of the molar proportions of the different fatty acids in these two tissues, the brain displays the more highly polyunsaturated profile. However, as a consequence of the much higher tissue level of acyl lipids in the liver, the concentration of acyl double bonds per g tissue, expressed as the DBI, is considerably greater in the liver than in the brain (Table 1).

The consequences of such differences in the degree of lipid unsaturation between the two tissues with regard to their potential susceptibilities to lipid peroxidation are difficult to assess in quantitative terms. The major complicating factor is the lack of a simple linear relationship between the number of double bonds in a fatty acid and its susceptibility to peroxidation. Thus monounsaturates are relatively resistant to peroxidation with dramatic exponential increases in susceptibility with increasing levels of polyunsaturation [15,16]. The fact that $18{:}1n-9$ is the major contributor to the high DBI value in the liver, whereas $22{:}6n-3$ is the major source of acyl double bonds in the brain, suggests that a simple consideration of the tissue concentration of acyl double bonds will tend to underestimate the relative susceptibility of the brain to lipid peroxidation. Moreover, it should be noted that there are pronounced differences in both the molecular and the subcellular locations of the acyl double bonds between the two

tissues; most of the 18:1$n-9$ in the liver is found esterified as cholesteryl ester droplets in the cytoplasm of the hepatocytes whereas the C_{20} and C_{22} polyunsaturates of the brain are primarily located in the neuronal membranes as components of phospholipid [6,7].

4.2. Transfer of antioxidant components from the yolk and YSM to the embryonic tissues

The development of the characteristic highly polyunsaturated tissue lipid profiles in the chick embryo, as exemplified above by the liver and brain, clearly require the concomitant acquisition of antioxidant capacity by the tissues. A major aim of the present study was to describe the transfer of antioxidant components from the yolk/YSM and to quantify their distribution between the developing tissues. The results indicate that different tissues display distinct developmental strategies with regard to the expression of antioxidant capacity. For example the liver of the chick embryo is characterised by the accumulation of very high levels of α-tocopherol whereas the brain, by comparison, was relatively deficient in this vitamin (Fig. 1 and Fig. 2). This dramatic difference between the brain and the liver may be explained partly by tissue-specific disparities in storage capacities for the vitamin and partly as a result of the transport mechanisms which function in the embryo. The chick embryo liver accumulates massive amounts of lipid in the form of cytoplasmic droplets [32], thus providing a suitable intracellular milieu for the storage of large amounts of a lipid-soluble vitamin such as α-tocopherol. Within the brain, however, it is likely that the main sites of α-tocopherol storage will reside in the phospholipid bilayers of the cell membranes. Although this vitamin is regarded as the major chain-breaking antioxidant in cell membranes, its concentration in the membranes is usually very low; less than 1 mol α-tocopherol per 1000 mol of phospholipids [33]. Thus it is likely that the capacity of the brain to store this vitamin will be very limited.

In mammals, dietary α-tocopherol is packaged with lipids into chylomicrons by the intestinal epithelial cells and released into the circulation [34,35]. It appears that the action of lipoprotein lipase on the chylomicrons facilitates the uptake of the vitamin by the adjacent peripheral tissues [34–36]. However, a major proportion of the vitamin remains associated with the resultant chylomicron remnants which are then taken up by the liver [34,35]. An analogous process has been suggested to function in the chick embryo [25] where yolk lipid droplets containing α-tocopherol are taken up by the YSM and processed into lipoprotein particles which are released into the embryonic circulation [3,37,38]. The action of adipose tissue lipoprotein lipase [6] on the lipoproteins may explain the marked increase in the concentration of α-tocopherol observed in this tissue between days 18 and 21 of development (Fig. 2). The major increase in the level of this vitamin in the liver at this time (Fig. 1) is most likely due to the subsequent uptake of lipoprotein remnants by this tissue [32]. The absence of lipoprotein lipase in the brain of the chick embryo [6] may limit the ability of this tissue to acquire α-tocopherol via lipoprotein hydrolysis.

The concentration of carotenoids in the liver of the embryo also increases dramatically from day 18 onwards (Fig. 3); i.e. in parallel with the marked accumulation of α-tocopherol in the tissue at this time. Thus it may be suggested that, as with α-tocopherol, the carotenoids of the yolk are incorporated into plasma lipoproteins following uptake by the YSM, and large proportions of the yolk-derived carotenoids are eventually deposited in the liver as components of lipoprotein remnants. The observation that the carotenoid content of the embryonic brain was below the detectable limits of the assay (< 0.2 μg/g tissue) may again be a reflection of the absence of lipoprotein lipase in this tissue.

Ascorbic acid is absent from the initial yolk [23] but is synthesised in the YSM of the chick embryo [39]. In the present study, the level of ascorbic acid in the YSM was maximal at day 8, the earliest developmental stage studied, and decreased thereafter (Fig. 4). The concentration of ascorbic acid in the brain increased markedly between days 8 and 13 and was maintained at a high level throughout development (Fig. 4) in agreement with the results of Wilson [23,40]. The liver contained much lower levels of ascorbic acid than the brain (Fig. 4) and a key result of the present study is that the concentration of ascorbic acid in the brain is far higher than in any other tissue studied. Primary cultures of brain cells from the chick embryo have been shown to express a high-affinity saturable uptake mechanism for ascorbic

acid [23]. Thus the transport and uptake mechanisms which serve to deliver antioxidant components from the yolk/YSM to the embryonic tissues result in a marked degree of tissue-specific partitioning, with ascorbic acid being preferentially supplied to the brain whereas the liver is the major destination for α-tocopherol and carotenoids.

4.3. Antioxidant systems of the brain of the chick embryo

The brain, with its precarious combination of high levels of peroxidation-susceptible acyl groups such as $20{:}4n-6$ and $22{:}6n-3$ together with especially high rates of metabolic activity and O_2 utilisation, would appear to be at great potential risk from peroxidative damage [18]. In the case of the developing avian brain, the hatching and early neonatal period may be a particularly hazardous time as the emergent chick is exposed to atmospheric O_2 and also displays a marked increase in metabolic rate [17]. Peroxidative damage to the central nervous system at this crucial period of neural differentiation and behavioural adaptation [41] could conceivably cause irreversible impairments of brain function as exemplified in the chicken by encephalomalacia induced by vitamin E deficiency [42]. It may seem somewhat surprising then that the brain's endowment with a range of major antioxidant components is not particularly generous. The levels of α-tocopherol (Fig. 2), carotenoids (Fig. 3), selenium [25], glutathione peroxidase [24,25] and catalase [24] are very low in the brain in comparison with the liver and the other major tissues of the embryo. The one exception to this pattern is provided by ascorbic acid which, in total contrast to the other major antioxidants, is present in the developing avian brain at much higher concentrations than in the liver (Fig. 4) and in the other major tissues. These observations suggest that ascorbic acid may play a key role in the antioxidant defence system of the avian brain.

The α-tocopherol content of the brain, although present at a low concentration, may nevertheless afford an effective protection against peroxidation providing the tocopheryl radical, produced as a consequence of free radical quenching, can be efficiently recycled back to the active form of the vitamin, as proposed by Packer [33,43]. It is well recognised that radical-scavenging antioxidants act in vivo not individually, but rather cooperatively or synergistically with each other. In particular, ascorbic acid is able to reduce the tocopheryl radical to regenerate the active form of the vitamin [33,43–45]. Thus, in the brain of the chick embryo, the combination of a low level of α-tocopherol with a high concentration of ascorbic acid may provide an efficient means of protection against lipid peroxidation by maintaining a high rate of antioxidant recycling. Important roles for ascorbic acid in providing antioxidant defence to the brain have also been described for a range of reptilian [46] and mammalian [47–49] species.

4.4. Susceptibility of embryonic tissues to lipid peroxidation

The endogenous concentration of TBARS, used as a marker of lipid peroxidation, was very low in fresh brain tissue (Fig. 5). At day 20, the basal concentration of TBARS in the brain was considerably lower than in the liver, with values for both tissues similar to those reported by Stock et al. [50]. Endogenous levels of TBARS were also higher in the yolk and YSM than in the brain. Thus it appears that the antioxidant system of the brain operates in vivo with particular efficiency. Previous studies have indicated that, in vivo, the antioxidant systems of the brain and other tissues of the chick embryo are able to mount a robust defence against attempts to induce peroxidation by hyperoxia [50] or exposure to Pb [51].

In contrast to the in vivo situation, homogenates of brain tissue were extremely susceptible to lipid peroxidation during in vitro incubation without added Fe^{2+} with a further major increase in peroxidation in the presence of Fe^{2+}. Homogenates of liver, YSM and yolk were very resistant to lipid peroxidation when incubated without exogenous Fe^{2+}; high rates of peroxidation were only observed when Fe^{2+} was added. These results are consistent with the findings of Meydani et al. [52] which indicated that homogenates of rat brain were very sensitive to lipid peroxidation. It is significant that those embryonic tissues with a high α-tocopherol content such as liver, YSM and yolk exhibit resistance to in vitro lipid peroxidation when Fe^{2+} is absent, whereas the brain with its very low α-tocopherol content is highly susceptible. It is also of note that the extent of in vitro peroxidation (Fe^{2+} absent) was significantly

($P < 0.01$) lower when using brain homogenates from the newly hatched chick (day 22, Fig. 5d) compared to samples from the late embryonic period, possibly reflecting the increased α-tocopherol level in the brain after hatching (Fig. 2). Kornbrust and Mavis [53] demonstrated that differences in the susceptibility of microsomes, prepared from different mammalian tissues, to in vitro lipid peroxidation were clearly related to variation in the α-tocopherol contents of the microsomes.

The results in Fig. 5 also indicate that the susceptibility of homogenates of liver and residual yolk to Fe-induced peroxidation decreases markedly after day 17 of development. Whereas in the liver this period is characterised by the accumulation of large amounts of α-tocopherol and carotenoids, the opposite situation is displayed by the yolk where the levels of lipid-soluble antioxidants decrease at this time (Fig. 1 and Fig. 3). A possible explanation may be that the recycling of bile components to the yolk [3] may confer antioxidant protection to the residual yolk lipids.

In conclusion, the present study suggests that antioxidant protection in the brain of the chick embryo is primarily provided by the action of α-tocopherol which is present in the cell membranes at a low concentration but which may be continuously recycled by the presence of a very high level of ascorbic acid. This mechanism appears to work well in vivo, although brain homogenates are very susceptible to peroxidation in vitro.

4.5. The antioxidant status of the neonatal chick

The later stages of avian embryo development are characterised by the dramatic accumulation of both cholesteryl ester [32] and lipid-soluble antioxidants (Fig. 1 and Fig. 3) in the liver, presumably as a consequence of the hepatic uptake of lipoprotein remnants. During the first few days after hatching the cholesteryl ester is rapidly cleared from the liver via excretion in the bile [3]. However, the α-tocopherol and carotenoids in the liver represent a valuable resource for the chick with the potential to provide protection against the oxidative stress of neonatal life. The level of α-tocopherol in the liver has been shown to decrease by 10-fold during the first 2 weeks after hatching [54] as a result of transfer of the vitamin to other tissues. Thus, the liver functions as a site of accumulation of α-tocopherol during embryonic life and then as a centre of redistribution of the vitamin after hatching.

In mammals, dietary vitamin E is delivered to the liver as a component of chylomicron remnants and a proportion of the vitamin is then incorporated into very low density lipoprotein (VLDL) and re-secreted from the liver. Within the hepatocyte, the transfer of vitamin E from the chylomicron remnant to the site of VLDL assembly is mediated by a specific tocopherol-binding protein. This protein is highly specific for the *RRR*-α-tocopherol stereoisomer, which is therefore preferentially incorporated into VLDL whereas other forms of the vitamin are excreted in the bile [35]. The dramatic changes in the hepatic levels of α-tocopherol during the late fetal/early neonatal period of avian development suggests that the chick may be a useful model system for investigating the mechanism involved in vitamin E distribution both within the hepatocyte and from the liver to the peripheral tissues.

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