INVESTIGATION OF THE UPTAKE, METABOLISM AND DELIVERY OF POLYUNSATURATED FATTY ACIDS IN THE DEVELOPING AVIAN EMBRYO.

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DECLARATION

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CONTENTS

Abbrevations and nomenclature

ACAT	----------	Acyl-CoA: cholesterol acyl-transferase
apo	----------	apolipoprotein
CE	----------	Cholesteryl ester
CPT1	----------	Carnitine palmitoyltransferase I
Cx	----------	Fatty acid(s) with x carbons
DG	----------	Diacylglycerol
DGAT	----------	Diacyl glycerol Acyltransferase
FFA	----------	Free fatty acid
GC	----------	Gas Chromatography
GPAT	----------	Glycerol phosphate acyltransferase
G.3.P.	----------	Glycerol 3-phosphate
HDL	----------	High density lipoprotein
HPLC	----------	High Performance Liquid Chromatography
IDL	----------	Intermediary density lipoprotein
IS	----------	Internal standard
LDL	----------	Low density lipoprotein
LPL	----------	Lipoprotein Lipase
Lyso PC	----------	Lyso-phosphatidylcholine
M-CoA	----------	Malonyl CoA
MG	----------	Monoacylglycerol
MGAT	----------	Mono acyl glycerol acyltransferase
PC	----------	Phosphatidylcholine
PE	----------	Phosphatidylethanolamine
PI	----------	Phosphatidylinositol
PL	----------	Phospholipid
PLA_2	----------	Phospholipase A_2
PS	----------	Phosphatidylserine
PUFA(s)	----------	Polyunsaturated fatty acid(s)
RT	----------	Retention Time
TG	----------	Triacylglycerol

TLC	----------	Thin layer Chromatography
VLDL	----------	Very low density lipoprotein
15:0	----------	pentadecanoic acid
16:0	----------	palmitic acid
16:1	----------	palmitoleic acid (*n-7*)
18:0	----------	stearic acid
18:1	----------	oleic acid (*n-9*)
18:2	----------	linoleic acid (*n-6*)
18:3	----------	linolenic acid (*n-3*)
20:4 or AA	----------	arachidonic acid (*n-6*)
20:5 or EPA	----------	eicosapentaenoic acid (*n-6*)
22:5	----------	docosapentaenoic acid (*n-3*)
22:6 or DHA	----------	docosahexaenoic acid (*n-3*)

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The avian embryo exhibits a range of unique features which favour its suitability as a model for investigating polyunsaturated fatty acid (PUFA) metabolism and transport. Recent evidence has shown that metabolic remodelling of yolk fatty acid moieties occur in the yolk complex, especially the transfer of docosahexaenoic acid (DHA) from phospholipid (PL) fraction in the yolk to triacylglycerol (TG) fraction in the blood. Moreover a preferential disappearance of yolk DHA associated with the phospholipid fraction specifically phosphatidylethanolamine, had been described at around day 12. A phospholipase A_2 was found to be involved in this process coincident with an increase of DHA within the free fatty acid fraction in the yolk. Fatty acid metabolism in the yolk sac membrane during embryonic development has been studied. The activities of key regulatory enzymes of acyl glycerolipid synthesis and oxidation were measured; carnitine palmitoyltransferase I, monoacylglycerol acyltransferase, glycerolphosphate acyltransferase and diacylglycerol acyltransferase. Maximal CPT I activity was found at day 12 and significantly declined to day 18. The MGAT activity remained constant throughout development suggesting that this pathway of glycerolipid biosynthesis may provide a mechanism to selectively retain essential PUFAs during a period of intense fatty acid oxidation. The measured activity of microsomal GPAT was noticeably lower compared to MGAT, with peak activity at day 12. The presence of DGAT activity completes the TG synthetic pathway which is released into the embryonic blood as part of very low density lipoproteins (VLDL). Characterisation and analysis of lipoprotein lipid in the blood at day 14 and day 22 extended the knowledge regarding the delivery system of DHA in the avian embryo. The VLDL TG was hydrolysed by lipoprotein lipase with the accompanying uptake of fatty acids into the adipose tissue. Hydrolysis of TG containing DHA by LPL appeared to be less active towards other TG species resulting in an enrichment of DHA in the TG of resulting lipoproteins which would be cleared by the liver. The results suggest that the yolk sac membrane has an important role in the regulation of DHA delivery to the developing embryo. Moreover the supply of DHA to the neural tissues in the developing embryo appears to be co-ordinated by both the adipose tissue and liver.

LITERATURE REVIEW

INTRODUCTION

The chick embryo provides a very useful model for the study of fatty acid metabolism. Yolk lipids are the primary nutritive source supporting embryogenesis and the absence of maternal and embryonic interactions means that the avian embryo is an essentially closed system except for exchange of respiratory gases (CO_2 and O_2) and water. This system provides a useful tool for the investigation of lipid transport and the metabolism of polyunsaturated fatty acids (PUFA) especially since significant amounts of essential fatty acids such as docosahexaenoic acid (DHA) or arachidonic acid (AA) are present in the yolk.

Fatty acid metabolism in the developing chick embryo can be divided into two parts. Firstly, avian development between day 0 and day 12 is associated with a low mobilisation of the yolk lipids, described in detail in several articles (Romanoff, A.L. 1960; Slack, J.M.W. 1991; Bellairs, R. 1992). In contrast the last ten days of embryogenesis is characterised by the mobilisation of yolk lipid for energy and the transfer of polyunsaturated fatty acid to the embryo (Noble, R.C. & Cocchi, M. 1990; Maldjian, A. 1996). This study aims to further our understanding of fatty acid uptake, metabolism and transport in avian embryo during development.

1.1 EGGS COMPONENTS

1.1.1 Composition and structure of the egg.

The nutrients required for the formation and growth of the embryo, including lipids, protein, vitamins and trace elements, are pre-packaged in the egg by the time of laying. The average 60g newly laid egg can be divided into four parts: the shell (6g), the shell membranes and air space, albumen (34g) and the yolk (20g). The growth of the embryo depends on the exchange of respiratory gases (CO_2 and O_2) and moisture

through microscopic eggshell pores. The shell also provides a barrier which prevents microbial contamination of the egg (Board, R.G. & Sparks, N.H.C. 1991) Moreover mobilisation of calcium from the shell occurs to meet the needs of the developing embryo, this is mediated by the chorioallantoic membrane (Tuan, R.S. 1987). On approach to hatching, the piping of the shell by the chick is facilitated by the thinning of the shell as a result of this calcium mobilisation (Noble, R.C. & Tullet, S.G. 1989b).

The albumen fraction consists essentially of water and a small amount of solid matter 4% (w/w) with a significant amount of carbohydrate. Several proteins (at least 13 have been identified) are present; the major one being ovalbumen (54 % of the total, w/w). The albumen is ingested orally by the chick embryo from day 12 of development, and represents a major nutritional protein resource for the embryo (Palmer, B.D. & Guillette Jr, L.J. 1991).

The yolk consists of a single massive cell called the oocyte. It is composed of 50 % water and 50 % solids. Lipids are by far the principal components, making up about 66 % (w/w) of the dry matter, protein represents almost 33 % (Romanoff, A.L. 1960). The yolk is essentially an emulsion of large floating lipid droplets and dense proteineous granules. Following high speed centrifugation of the yolk three distinct fractions are produced: 1/ A floating fat layer consisting of triacylglycerol (TG)-rich lipoproteins: 2/ A pellet consisting of dense granule fraction containing some associated lipid: 3/ An infranatant containing water-soluble proteins. The majority of these components are not synthesised by the ovary but are secreted into the bloodstream from the liver and deposited in the oocyte. More than 90% of the total yolk lipids are synthesised by the adult liver as specialized type of very low density lipoprotein (VLDL). The VLDL fraction provides 60 % of the yolk solids, 22 % of the protein. The VLDL particles are small and regular in size (30 nm) and contain two apoproteins, namely apoB (m.w. 500 kDa) and apo-VLDL II (homodimer, subunit m.w. 9.5 kDa). The granule fraction, also synthesised by the liver, contributes 25 % of the solids, 48 % of the protein but only 7 % of the yolk lipids. The granules are formed from vitellogenin and consists of two proteins: phosphovitin (m.w. 30

kDa) and lipovitellin (m.w. 340 kDa) products of proteolysis. The formation and the deposition of these components in the oocyte (yolk) by the parent bird are well documented (Griffin, H.D., Perry, M.M. & Gilbert, A.B. 1984; Noble, R.C. & Cocchi, M. 1990; Burley, R.W., Evans, A.J. & Pearson 1993). Following uptake into the oocyte, the apoproteins are proteolytically modified: apoB is modified to apovitellins III, IV, V and VI and apo-VLDL-II is converted to apovitellin I. On the other hand, the composition of the oocyte lipids remains the same as that secreted by the maternal liver (Noble, R.C. & Cocchi, M. 1990; Griffin, H.D. 1992; Etches, R.J. 1996). Within the oocyte, the endocytotic vesicles packed with yolk precursor particles undergo a series of fusion to form large membrane bound yolk spheres. These consist of tightly packed VLDL particles (Griffin, H.D. *et al.* 1984; White, H.B. 1991; Griffin, H.D. 1992; Stevens, L. 1996). The fatty acid compositions of the TAG, PL and CE of the yolk of the domestic chicken egg are given in ***Table 1***.

Table 1: Fatty acids compositions of the lipids of the initial yolk. The fatty acids listed account for the majority of the fatty acids present. The remaining small percentage consists of C_{14} , C_{15}, C_{17} and C_{20} acids (Noble, R.C. & Cocchi, M. 1990).

	CE	TG	PE	PS	PC	Sph	FC
wt. % of total lipid	1.3	63.1	7.10	0.81	20.5	0.3	4.9
Fatty acids, wt. % of total							
Palmitic	29.1	24.5	21.7	33.6	33.7	41.7	
Palmitoleic	1.0	6.6	1.1	5.4	1.0	6.5	
Stearic	9.5	6.4	30.1	27.3	15.8	17.6	
Oleic	40.1	46.2	15.3	15.9	27.7	23.7	
Linoleic	18.0	14.7	9.2	7.3	14.1	9.1	
Linolenic	0.3	1.1	<0.5	<0.5	<0.5	<0.5	
Arachidonic	0.9	0.3	13.2	8.5	4.4	<0.5	
Docosahexaenoic	0.5	<0.5	8.4	1.2	1.8	<0.5	

By far, the principal lipid class is triacylglycerol (TG) followed by phospholipid (PL), and then free cholesterol. Cholesteryl ester (CE) and free fatty acids (FFA) are also present but are minor components. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) represents the majority of the phospholipid in the yolk, about 70 % and 25 % respectively. Oleic acid (18:1n-9) is found to be the major fatty acid in the CE, TG and total PL fractions. Palmitic (16:0) and stearic acid (18:0) together represent more than a third of the fatty acids in each fraction. Linoleic acid (18:2n-6) is the major polyunsaturated fatty acids, whereas linolenic acid (18:3n-3) is present only at low concentrations, at least in eggs from hens maintained on current commercial diets. The C_{20-22} fatty acids, 20:4n-6 (arachidonic acid; AA) and 22:6n-3 (docosahexaenoic acid; DHA) are found predominantly associated with the phospholipid fraction with only trace amounts of these long chains PUFAs detectable in TAG and CE fraction. An extensive structural analysis has been performed on the triacylglycerol and phospholipid fractions of the yolk (Noble, R.C. & Cocchi, M. 1990) to establish the position and disposition of the major fatty acids. A summary of their findings is shown in ***Table 2.***

Table 2: Stereospecific analysis of yolk triacylglycerol and phospholipid in the initial yolk (Noble, R.C. & Cocchi, M. 1990). -- Not detected.

	Triacylglycerol			Phospholipid	
POSITION	**1**	**2**	**3**	**1**	**2**
Palmitic	71.5	5.1	6.5	71.8	2.0
Palmitoleic	6.0	2.6	5.7	--	--
Stearic	4.1	1.4	8.7	18.1	0.5
Oleic	14.7	56.0	74.6	7.3	44.2
Linoleic	2.3	33.5	3.5	0.6	27.8
Linolenic	0.8	1.2	0.6	--	6.9
Arachidonic	--	--	--	--	1.5
Docosahexaenoic	--	--	--	--	13.8

The majority of 20:4n-6 and 22:6n-3 are found in the PE fraction. As in most animal tissues, saturated fatty acid are predominantly associated in position 1, and unsaturated in position 2, the fatty acid 20:4n-6 and 22:6n-3 are attached in second position.

1.2 FATTY ACID METABOLISM DURING EMBRYOGENESIS.

Once the egg has been laid and incubation initiated, embryonic development goes on within a closed system apart from the exchange of heat, respiratory gases and water vapour. The nutrition of the embryo during the early stages of development is poorly understood, but it has been claimed that glucose and amino acids provide the main energy source at this time (Romanoff, A.L. 1967; Noble, R.C. & Cocchi, M. 1990). During the latter half of development, which represents the major growth period, the energy metabolism of the embryo is characterised by the utilisation of yolk lipid.

The formation and the growth of the embryo and the extra-embryonic organs are discussed in several articles (Romanoff, A.L. 1960; Slack, J.M.W. 1991; Bellairs, R. 1992). The early stages of development see the growth of extraembryonic structures such as the amniotic, the chorioallantoic and the yolk sac membrane. The chorioallantoic structure is found inside the surface of shell and consists of a highly vascularised membrane. The blood flowing in the blood vessel picks up oxygen and delivers it to the developing embryo. Carbon dioxide is removed from the embryo and carried in the blood to the chorioallantoic membrane when it then diffuses through the eggs shell pores. The rates of O_2 uptake and CO_2 release by the egg increase dramatically from about day 10, reaching a plateau level by day 18 of incubation. This may reflect the increased rate of β-oxidation of yolk derived lipids by embryonic tissues (Romanoff, A.L. 1967; Noble, R.C. & Tullet, S.G. 1989b). The second major role of this organ is the mobilisation of the Ca^{2+} from the shell to supply the needs of the embryo. This mechanism is based on the conversion of CO_2 to carbonic acid catalysed by carbonic anhydrase. This reaction and the transport of calcium into the blood circulation involve the presence of a chorioallantoic Ca^{2+} binding protein (Tuan, R.S. 1987).

The yolk sac membrane structure is formed from the dissolution of the continuous and the extra vitellines membranes. The yolk sac membrane consists of an outer mesoderm consisting of flattened cells to form a supportive membrane and an inner

endoderm consisting of simple columnar cells containing the usual range of subcellular components and through which yolk absorption occurs (Romanoff, A.L. 1960). During the first week of development, the embryo grows on the surface of the yolk, at the same time the yolk sac membrane grows out to encapsulate the yolk which is complete by the fifth day.

The major role of the yolk sac membrane is the uptake of yolk materials and the transfer of these components into the embryonic circulation. The absorptive side of the membrane forms elaborate folds or villi which increases the membrane surface in contact with the yolk. At the same time, the yolk sac membrane undergoes extensive vascularization through the development of a network of capillaries emanating from within the villi. The blood flowing from these capillaries passes into the portal system of the embryo by several major vitelline veins that enter the embryo through the yolk stalk. In some respects this membrane is analogous to the mammalian placenta and shares some characteristics with the small intestine.

1.2.1. Lipid uptake by the yolk sac membrane.

The transfer of yolk lipid during the incubation period has been extensively studied in the chicken (Romanoff, A.L. 1960; Noble, R.C. & Moore, J.H. 1964; Noble, R.C. & Moore, J.H. 1967b; Maldjian, A. 1996). The mean weights of total lipid associated with the yolk contents, yolk sac membrane and tissue of the chick embryo at different stages of incubation are shown on the ***Figure 1.***

The amount of lipid transferred from the yolk during the first 13 days is minimal. From day 13 until hatching period, the lipid uptake increases dramatically. The amount of yolk lipid decreases from around 5 g at day 13 to 1 g at day 21 (hatching) i.e. an overall loss of 400 mg per day. Between days 13 and days 17 of incubation, the uptake of lipids is accompanied by its rapid accumulation in the yolk sac membrane, such that by day 17 the membrane contains as much lipid as the yolk contents (Noble, R.C. & Moore, J.H. 1967b). From day 17 to hatching there is

gradual loss of lipid from the membrane and an associated increase in lipid associated with the embryo (Noble, R.C. & Cocchi, M. 1990).

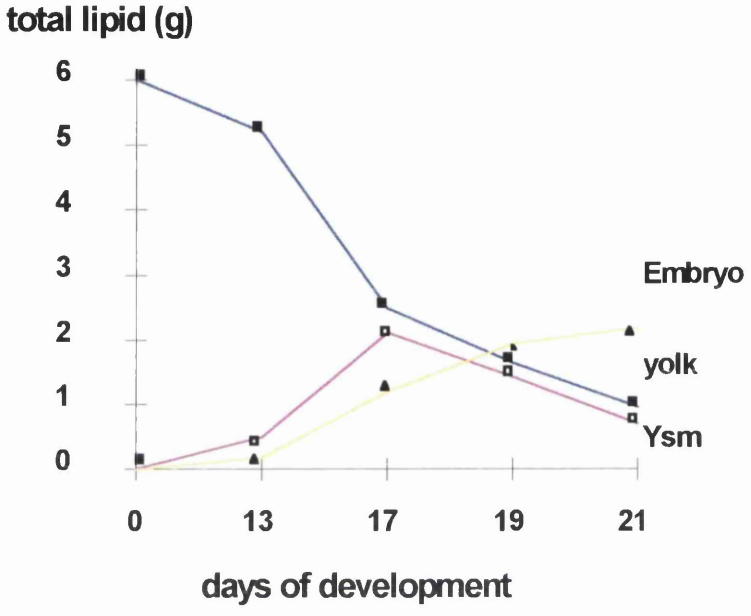


***Figure 1*: The relative movement of lipid from the yolk contents into the chick embryo through the yolk sac membrane. Weights of total lipid in the yolk contents, yolk sac membrane and embryonic tissues during the last week of incubation (Noble, R.C. & Cocchi, M. 1990).**

The proportions of the major lipid classes of the yolk complex during development are shown in ***Table 3*** (Noble, R.C. & Cocchi, M. 1990). The proportions of the major lipid classes of the yolk remain approximately constant throughout development. However the PC/PE ratio increased from around 3 in the unincubated egg yolk to almost 9 at day 21 of incubation.

Table 3: **Lipid changes in the yolk during development (Noble, R.C. & Cocchi, M. 1990).**

	Yolk content		
Days of incubation	***0***	***13***	***21***
Total lipid, g	6.11	4.93	0.80
Lipid composition, % of total lipid			
CE	1.3	0.42	0.50
Free cholesterol	4.9	6.11	5.00
TG	63.1	71.8	75.9
PL	29.7	21.4	18.2
Phospholipid composition, % of total phospholipid			
PE	23.9	14.0	8.30
PS	2.7	1.21	3.43
PC	69.1	75.4	71.7
sph	1.0	1.79	2.67

This result has also been found previously where a preferential uptake of the PE fraction, especially the molecular species of PE that contains DHA, from the yolk occurred from around day 12 (Noble, R.C. & Moore, J.H. 1967b), **see *Table 4.***

***Table 4*: Preferential uptake of PE and DHA from yolk lipid (Noble, R.C. & Moore, J.H. 1967b).**

	Yolk day 0		**Yolk day 15**		**Yolk day 19**	
	PC	**PE**	**PC**	**PE**	**PC**	**PE**
wt % of total PL	69.1	**23.9**	80.6	**14.2**	80.8	**12.4**
Fatty acid (wt % of total lipid)						
16:0	33.5	21.2	31.1	20.6	31.8	22.4
18:0	15.7	29.5	16.1	26.0	15.9	27.6
18:1n-9	27.5	15.0	28.0	20.5	27.9	19.3
18:2n-6	14.0	9.0	15.2	11.4	15.5	10.1
20:4n-6	4.3	12.9	4.6	11.7	4.8	12.3
22:6n-3	1.7	8.2	1.5	3.1	1.0	1.9

The transfer of 16:0, 18:0, 18:1, 18:2, 18:3 and AA followed the same kinetic profile throughout development. In contrast, the transfer of DHA was shown to have a different profile. Suggesting that DHA is transported differently from other yolk lipids (Maldjian, A., Farkas, K., Noble, R.C., Cocchi, M. & Speake, B.K. 1995). **See *Figure 2.***

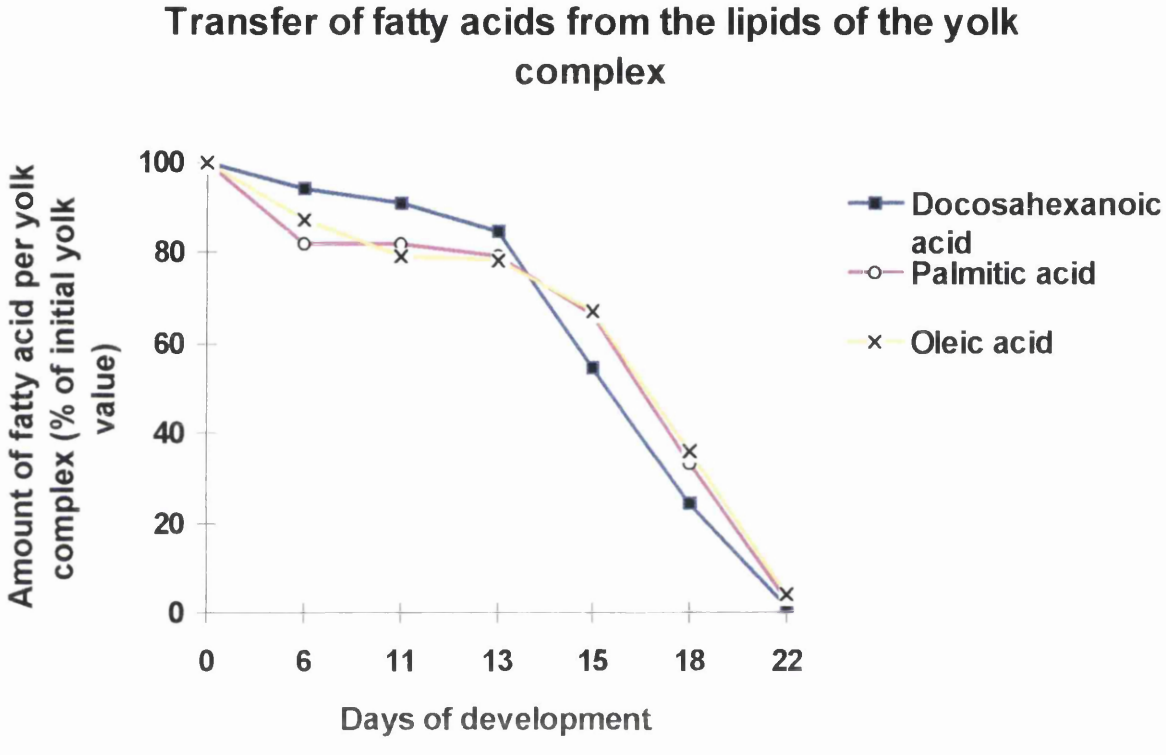


***Figure 2*: Transfer of fatty acids from the lipids of yolk complex. Changes in level of oleic acid (×), Palmitic acid (O) and DHA (■) are shown during incubation. Values are expressed as percentages of the amounts in the initial yolks before incubation and are the means of 4 replicates. (Maldjian, A. *et al.* 1995).**

There is no real evidence for extracellular digestion having a role in the uptake of the major lipids into the membrane. Nevertheless, low levels of lipid breakdown products

were identified within the yolk during the most intense period of lipid uptake. Free fatty acid, glycerol, lyso-phospholipid have been observed in the yolk (Rhodes, D.N. & Lea, C.H. 1957; Romanoff, A.L. 1960; Noble, R.C. & Cocchi, M. 1990). This suggests that there may be a lipase present which hydrolyses yolk lipid moieties.

The bulk transfer of yolk lipid by the endodermal cells (apical surface) has been shown from electron microscopy studies to occur by a process of phagocytosis. These studies have shown that lipid droplets are surrounded by the microvilli of the yolk sac membrane and incorporated directly inside the cell. This process result in the accumulation of large lipid droplets that often occupy the whole cytoplasmic space (Noble, R.C. & Cocchi, M. 1990). Electron microscopy studies have also provided important evidence that extensive modification of the lipid droplet occurs in the yolk sac membrane, ultimately resulting in the formation of lipoprotein particles that are secreted by exocytosis from the basal surface of the endoderm cells. The presence of lipid rich vesicles in the cisternae of the reticulum endoplasmic and Golgi have been identified. The yolk sac membrane is well equipped for the assembly of yolk derived lipids into lipoprotein for secretion into the blood stream (Lambson, R.O. 1970).

Previous work has demonstrated that the fatty acids that accumulate in the embryonic lipids are almost entirely derived from the yolk. It had been found that up to 80 % of the C_{16} and C_{18} fatty acids transferred from the beginning of the incubation to the first day after hatching are oxidized for energy, indeed about 90% of the total energy requirements for embryogenesis is derived from the β-oxidation of fatty acids. (Maldjian, A. *et al.* 1995).

Table 5 **Recovery of yolk lipids of the newly hatched chick. The amount of each fatty acid present in total lipid per day-old chick is expressed as a percentage of the amount transferred from the initial yolk between the first day of embryogenesis and the first day post hatching. Taken from Maldjian (1996).**

	amount in lipids of day-old chicks as % of that transferred from yolk
16:0	20.5
18:0	26.5
18:1n-9	19.0
18:2n-6	25.3
20:4n-6	74.6
22:6n-3	60.6

DHA (22:6n-3) and arachidonic acid (20:4n-6) are not oxidized to the same extent as other fatty acids with 60.6 % and 74.6 %, of the original yolk content being transferred to the chick respectively. Moreover other works have showed that the proportion of DHA and AA are progressively increasing during transit from the yolk to the embryonic plasma (Yafei, R. & Noble, R.C. 1990).

The major source of DHA in the yolk is phospholipid, whereby DHA comprises approximately 8% w/w of total PE fatty acids. In contrast only trace levels (less than 0.2 %) of DHA are present in yolk triacylglycerol (Noble, R.C. & Cocchi, M. 1990). Analysis of embryonic plasma, however, has shown that DHA is predominately associated with the TG fraction up to 11 % ***Table 6***.

Table 6: **Changes in the fatty acid compositions of triacylglycerol and phospholipid during transfer between the yolk, the yolk sac membrane and the embryonic plasma. (Yafei, R. & Noble, R.C. 1990).**

	Yolk (day 0)		YSM (day 12)		Plasma (day 12)	
Lipid class	**TG**	**PL**	**TG**	**PL**	**TG**	**PL**
% w/w of total lipid	64.6	28.3	68.6	24.6	42.8	25.6
wt % of total lipid						
16:0	24.6	27.0	27.7	24.5	25.6	32.7
18:0	6.0	16.8	6.3	18.5	8.0	15.8
18:1n-9	44.7	25.1	41.0	24.1	34.4	15.3
18:2n-6	15.3	15.2	16.1	15.4	10.9	13.9
20:4n-6	0.2	6.5	0.7	9.7	3.5	17.8
22:6n-3	0.2	5.7	1.3	4.5	11.5	3.1

Therefore, following uptake of DHA from the yolk into the yolk sac membrane, and its subsequent secretion into the blood, DHA is transferred from the phospholipid moiety and incorporated into triacylglycerol, this TG is then presumably assembled into lipoprotein particles and secreted into the embryonic circulation. The distribution of arachidonic acid between the plasma lipid classes differs sharply from the pattern displayed by DHA. Although significant levels of arachidonic acid are found in the plasma triacylglycerol, much higher proportions are present in the phospholipid fraction.

This pattern of fatty acid redistribution is also a characteristic found in phospholipid and triacylglycerol of chylomicron during absorption of lipid by the mammalian intestine. Arachidonic acid is preferentially incorporated into chylomicron phospholipid (Nilson, A., Landin, B., Jensen, E. & Akesson, B. 1987; Nilson, A., Hjeltje, L. & Strandvik, B. 1992), whereas DHA and other n-3 polyunsaturated fatty acids exhibit a greater tendency for incorporation into chylomicron triacylglycerol (Li,

J., Wetzel, M.G. & O'Brien, P.J. 1992). It should be noted however, that some embryonic fatty acids may be derived from *de novo* synthesis due to the presence of a desaturation/elongation enzyme system. The presence of the Δ^6-desaturase in the yolk sac membrane and tissues of the embryo may catalyse the conversion of linoleic acid (18:2n-6) to arachidonic acid (20:4n-6) (Noble, R.C. & Cocchi, M. 1990). The *de novo* synthesis of DHA does not appear to have an important role in the supply of this essential fatty due to the low availability of linolenic acid (18:3n-3) the required substrate.

In summary, DHA is taken up preferentially from the yolk by the yolk sac membrane compared to the other fatty acids. The major proportion of DHA is present as a component of PE, and this phospholipid species disappears preferentially from the yolk. DHA is associated predominantly with the triacyglycerol fraction in the blood in contrast to that found in the yolk. Arachidonic acid, however is found to be associated with the plasma phospholipid fraction. DHA appears to be preserved in the developing embryo against a background of high fatty acid oxidation. There therefore must exist a system or a mechanism whereby PUFAs are remodelled during transport across the yolk sac membrane, and are protected from fatty acid oxidation in the mitochondria.

1.2.2. Formation and release of plasma lipoprotein by the yolk sac membrane.

The principle of lipoprotein formation has been studied using cell lines derived from the liver and the intestine of adult mammals (Maria, C.L. 1991). In the chick embryo, the yolk sac membrane is responsible for the synthesis and release of lipoproteins (Griffin, H.D. 1992; Shand, J.H., West, D.W., Noble, R.C. & Speake, B.K. 1994). A direct link between the rapid conversion of yolk-derived cholesterol to cholesteryl ester and its requirement for the assembly of stable lipoprotein particles to enable lipid transfers to the embryo have been strongly suggested (Shand, J.H. *et al.* 1994). Cholesteryl esters are formed by the presence, on the cytoplasmic face of the endoplasmic reticulum (ER), of acyl-CoA: cholesterol acyl-transferase (ACAT). This

enzyme has been analysed and its activity quantified. Yolk sac membrane ACAT activity increased 4-fold between days 9 and 16 of development which suggests that formation of lipoproteins maybe of importance during this period. A major step of lipoprotein assembly is the association of nascent apoB with newly synthesised neutral lipid in the lumen of the endoplasmic reticulum. ApoB is the major structural protein of chylomicrons and VLDL (Converse, G. & Skinner, E.R. 1992). Synthesis of apoB together with the synthesis of TG and CE for lipoprotein assembly take place by the co-ordinated actions of diacylglycerol acyl transferase (DGAT) and ACAT located on the cytoplasmic face of the ER. The presence of DGAT activity in the yolk sac membrane has not been determinated.

1.2.3. Delivery of plasma lipids to the tissues. The role of lipoprotein lipase.

Lipids are transported in the blood associated with lipoprotein particles. Lipoproteins are classified according to increasing density: chylomicrons, chylomicron remnants, very low density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Each of these particles consists of a core of hydrophobic lipids surrounded by a shell of polar lipids and apoproteins. Triacylglycerols and cholesterol are transported into the blood predominantly associated with the VLDL. Triacylglycerol in VLDL are processed by the action of lipoprotein lipase (LPL, e.c. 3.1.1.34). This enzyme is present at the luminal surface of the blood capillaries of specific tissues where it catalyses the hydrolysis of triacylglycerols present in the plasma VLDL. The fatty acids thus released can be taken up and used by the adjacent tissue (Maria, C.L. 1991). The presence of this enzyme in specific tissues of the developing chick was investigated previously (Speake, B.K., Noble, R.C. & McCartney, R.J. 1993) **See *Table 7.*** Lipoprotein lipase (LPL) was characterised by its sensitivity to inhibition by NaCl and its requirement for apoC-II. LPL activity was found to be highest in the adipose tissue. Moreover the activity was found at around day 14 of incubation in parallel with the high rate of transfer of fatty acid from the yolk (Speake, B.K. *et al.* 1993; Maldjian, A. *et al.* 1995).

Table 7: **Lipase activity characteristics of chick's embryo at day 14 of incubation (Speake, B.K. *et al.* 1993).**

	Lipase activity (units/g tissue)		
	Standard assay	**+NaCl**	**-apoC-II**
Adipose tissue	87.4	3.4	7.1
Heart	30.2	2.5	2.1
Thigh muscle	5.8	2.1	2.0
Breast muscle	1.3	0.8	0.8
Liver	6.0	5.5	4.6
Brain	4.6	4.7	4.5

It has been suggested that the high activity of embryonic LPL in adipose tissue will favour the diversion of a significant proportion of the triacylglycerol-fatty acids into the adipocytes for storage. However the significant activity of LPL in the embryonic heart suggests an important role of fatty acids in energy provision in this tissue. It has been suggested that LPL may have a direct role in determining the composition of fatty acid taken up by the embryonic tissues, notably adipose tissue and liver (Speake, B.K. *et al.* 1993). Studies have indicated that TG species containing C_{20} and C_{22} PUFA exhibit a degree of resistance to LPL action (Nilson, A. *et al.* 1987; Melin, T., Qi, C., Bengtsson-Olivecrona, G., Akesson, B. & Nilsson, A. 1991). Free fatty acid release from plasma chylomicron TG by LPL action contained a lower proportion of C_{20} and C_{22} PUFA compared to the initial chylomicron TG. Moreover the residual TG was enriched in C_{20} and C_{22} PUFA. In the chick embryo, a significant amount of DHA was incorporated into adipose tissue in accordance with the high LPL activity (Speake, B.K. *et al.* 1993). However the residual TG was found to be enriched in DHA suggesting that TG species containing DHA are somewhat resistant to the action of LPL compared to other TG species. Following the hydrolysis of a large proportion of the lipoprotein TG by LPL, the resultant remnant particles, IDL, consisting mainly of CE can be taken up by the liver, or converted into LDL. LDL are

the major carrier of cholesterol in the blood, they contain a single copy of apoB, which is recognised by target cells. LDL is known to have several roles: transport of cholesterol to periphery and regulation of the cholesterol metabolism. HDL particles are derived from surplus surface material (phospholipids, free cholesterol and apoproteins) of triacylglycerides-rich lipoproteins during lipolysis as described above. However it is incorrect to regard HDL as a single homogeneous species, since it is known to be a mixture of particles that differ in size, in lipid and apolipoprotein composition.

1.2.4. The role of the adipose tissue

It has been suggested that adipose tissue may play a pivotal role in the delivery of DHA to the developing tissues (Maldjian, A. 1996). DHA is associated with the triacylglycerol fraction of plasma lipoproteins and will therefore be susceptible to hydrolysis by LPL. Adipose tissue has a very high LPL activity and although it may prefer other TG species, LPL will exhibit some activity towards DHA containing TG thereby supplying adipose tissue with this fatty acid. Analysis of the fatty acid composition of adipose tissue in the developing avian embryo (Maldjian, A. 1996) showed that the amounts of the major C_{18} fatty acids in the tissue TG changed in parallel with the profile of TG accumulation in the depot, with a continuous increase between days 12 and 19, then remaining constant over the hatching period. However a totally different pattern of accumulation was found for DHA. The amount of DHA found in the TG increased to day 16, and plateaued and then decreased dramatically over the hatching period. **See *Figure 3.***

Moreover studies looking at the release of polyunsaturated fatty acid from adipose TG both *in vivo* and *in vitro*, have shown that $C_{20\text{-}22}$ polyunsaturated fatty acids are preferentially mobilised from the adipose tissue and released into the circulation as free fatty acids (Speake, B.K., Cerolini, S., Maldjian, A. & Noble, R.C. 1997) over the hatching period.

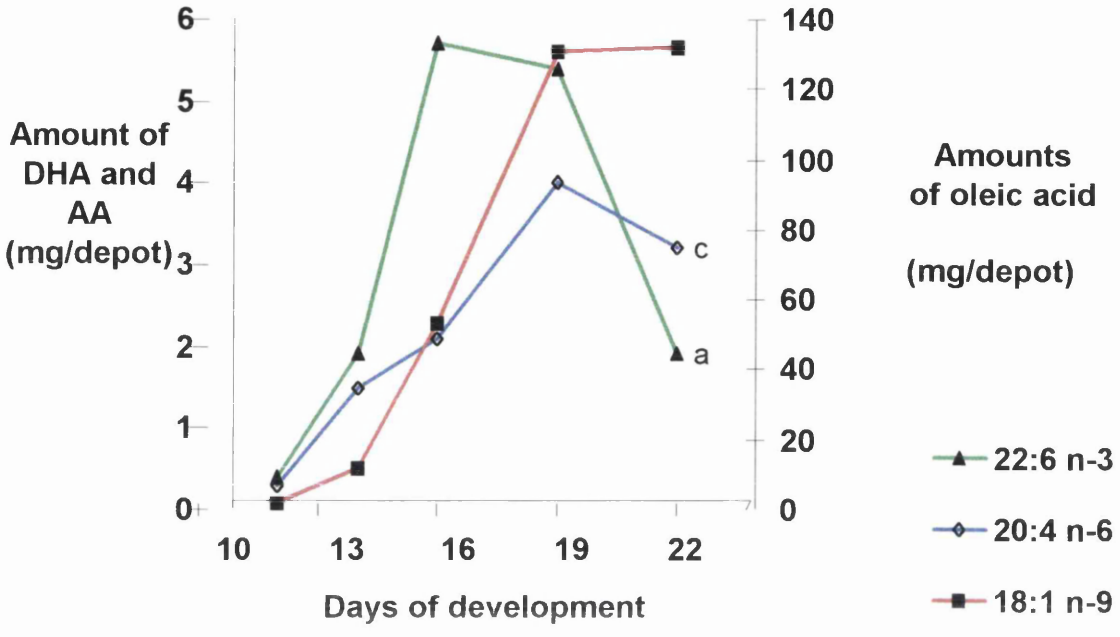


***Figure 3*; Amounts of DHA (▲), arachidonic acid (◊) and oleic acid (■) in adipose triacylglycerol, the amounts (mg/depot) are shown. S.E.M. were always less than 10 %, comparison between days 22 and 19: $^{a}P<0.001$, $^{c}P<0.02$, (Farkas, K., Ratchford, I.A.J., Noble, R.C. & Speake, B.K. 1996).**

1.2.5. The role of the liver.

During the latter half of avian development, the liver is being supplied with lipoprotein remnants which consist largely of CE. Analysis of the lipid composition of the liver during avian development was done previously (Noble, R.C. & Moore, J.H. 1967a; Noble, R.C. & Cocchi, M. 1990; Shand, J.H. *et al.* 1994; Maldjian, A. 1996) **See *Table 8.*** The lipid composition of the liver bears no resemblance to that of the lipid absorbed from the yolk. The yolk lipid consists in the main of TG (70-75%), PL (20-25%) and free cholesterol (6-7%), whereas the accumulation of lipid in the liver

during the last week of development is almost wholly accounted for by cholesteryl ester. (Noble, R.C. & Moore, J.H. 1967a; Shand, J.H. *et al.* 1994).

***Table 8*: Lipid and fatty acid composition change in the liver during last week of incubation (Noble, R.C. & Cocchi, M. 1990).**

	CE		TG		PL	
day of incubation	**13**	**21**	**13**	**21**	**13**	**21**
Lipid concentration (mg/liver)	1.63	80.2	15.1	3.58	51.4	14.2
Fatty acid composition (%, w/w of total lipid)						
16:0	2.31	1.95	23.4	23.0	22.4	17.8
18:0	2.04	2.74	8.81	11.1	24.6	28.4
18:1n-9	77.2	79.6	30.4	35.7	7.29	7.97
18:2n-6	12.7	12.5	8.32	8.23	9.94	13.8
20:4n-6	2.24	1.47	3.52	2.92	26.2	20.0
22:6n-3	1.91	0.42	20.1	11.6	6.45	9.12

Previous work has showed that there is a transfer of DHA from TG to PL in the liver and this transfer appeared to be specific for DHA and is not exhibited by other fatty acids (Noble, R.C. & Cocchi, M. 1990; Maldjian, A. 1996). In some studies done on neonatal rodents, it was suggested that the liver may have a major role in the mediation of DHA transport to the brain and retina (Li, J. *et al.* 1992). The unique profile of DHA metabolism in the developing avian embryo suggests that the avian liver may also be an important regulator of this essential PUFA delivery.

1.2.6. Delivery of DHA into the brain.

The essential role of dietary n-3 fatty acid in the development of the brain and retina is well documented (Neuringer, M., Anderson, G.J. & Connor, W.E. 1988; Anderson, G.J., Connor, W.E. & Corliss, J.D. 1990; Makrides, M., Neumann, M., Simmer, K., Pater, J. & Gibson, R. 1995). DHA is a major PUFA in the brain and retina and deficiencies of this fatty acid in the diet lead to disturbances in behaviour, reduced IQ in human infants and impaired visual acuity. Very high levels of DHA are present in the photoreceptor cells of the retina and in the brain (the retinal membranes have a high content of phospholipid with DHA comprising up to 65 % of the total fatty acids of PE and PS). In the chick embryo similar results were found in the brain by Maldjian *et al* (1996) **See *Table 9.***

Table 9: **The fatty acid profiles of the major PL classes of brains from day 14 chicken embryos (Maldjian, A. *et al.* 1996).**

	PC	PE	PS	PI
wt % of total PL	**40.8**	**34.6**	**19.5**	**5.4**
Fatty acid (wt % of total lipid)				
16:0	61.4	13.5	10.1	3.5
18:0	3.8	21.4	37.4	37.6
18:1n-9	23.8	9.0	7.0	9.0
18:2n-6	2.6	0.9	1.4	0.6
18:3n-3	0.2	0.2	0.2	--
20:4n-6	3.0	13.0	3.2	45.0
22:6n-3	4.1	40.5	39.7	3.8

DHA is an essential fatty acid which can be supplied directly through dietary means or indirectly via biosynthesis from linolenic acid. However, it has been shown that

provision of n-3 fatty acids to mammalian embryos in the form of linolenic acid is a very inefficient means of supplying the target tissues with their DHA requirements compared with the provision of preformed DHA (Noble, R.C. & Cocchi, M. 1989a). The precise role of DHA in brain development remains to be ascertained, however evidence has accumulated which emphasizes the importance of an adequate supply of DHA to the neural tissues at the appropriate stages of development.

The route and mechanism of DHA delivery to the neural tissues are unknown at this time. Controversy exists over the form of the DHA-containing lipid species taken up by the brain. Studies in mammals have shown that the brain has the ability to uptake DHA in the form of free fatty acid bound to albumin (Anderson, G.J. & Connor, W.E. 1988). In which case the adipose tissue of the developing avian embryo may have a central role in DHA provision via its specific mobilisation of C_{20}-C_{22} fatty acids (Speake, B.K. *et al.* 1997). However remnant uptake by the liver results in the hepatic accumulation of TG which is highly enriched in DHA. During the hatching period, this DHA is transferred from TG to PL in the liver. It is possible that liver synthesis of very low density lipoproteins (VLDL) could incorporate this DHA associated with PL which is subsequently secreted into the bloodstream. As previous work suggested, the brain could obtain its supply of DHA from phospholipid secreted by the liver (Scott, B.K. & Bazan, N.G. 1989). Alternatively, studies have shown that DHA is preferentially taken up by the brain in the form of sn-2 lysoPC DHA compared to the unesterified form (Thies, F., Pillon, P., Moliere, Lagarde, M. & Lecerf, J. 1994). Mammalian liver has been shown to secrete lysoPC moreover there was a preferential release of unsaturated fatty acyl species of lysoPC (Graham, A., Zammit, V.A. & Brindley, D.N. 1988; Robinson, B.S., Baisted, D.J. & Vance, D.E. 1989). Therefore the liver may direct DHA towards LysoPC synthesis and subsequent secretion. In any case, the liver does appear to have a pivotal role in the delivery of this essential fatty acid to the developing tissues.

1.3. TRANSFER OF POLYUNSATURATED FATTY ACIDS FROM THE YOLK COMPLEX TO THE EMBRYONIC TISSUES.

The aim of this study was to investigate the metabolic pathways that may be involved in the uptake, remodelling and delivery of polyunsaturated fatty acids in the chick during embryonic development. As discussed previously, there as been a number of studies which have detailed fatty acid compositional changes which occur at various intervals during chick development. However, the fundamental metabolic processes which determine these processes have not been examined. How are fatty acids transferred from the yolk into the yolk sac membrane ? Which mechanisms are involved in the remodelling of some fatty acids between lipid moieties from the yolk to the plasma ? Are some fatty acids less sensitive to β-oxidation ? How are essential fatty acids delivered to the developing tissues such as liver or brain ? The aim of the present study was to investigate the metabolic processes in the yolk sac membrane and the liver which may regulate the fatty acid compositional changes previously found. The study of delivery of fatty acid to the embryonic tissues was carrying out by analysing the fatty acid composition of embryonic plasma lipoproteins at different stages of development.

1.3.1. Uptake of polyunsaturated fatty acid.

The transfer of fatty acid into the yolk sac membrane occurs via a non-specific phagocytosis (Noble, R.C. & Cocchi, M. 1990). Previous work has demonstrated that the majority of yolk DHA is associated with the PE fraction, and this is preferentially removed from the yolk (Noble, R.C. & Moore, J.H. 1967b). Others have shown that the transfer of DHA follows a unique and different pattern from that of other fatty acids (Maldjian, A. *et al.* 1995). Since DHA is found esterified at the 2 position of PE and an increased in percentage of DHA in the free fatty acid fraction in the yolk was found during development (B.K. Speake, unpublished studies) an investigation of phospholipase A_2 activity was carried out.

Phospholipase A_2 (PLA_2; e.c. 3.1.1.4.) catalyses the hydrolysis of the 2-sn acyl ester bond of a sn 3-phosphoglycerol (phospholipids and its lyso forms)

Phospholipids + action of PLA_2 ⟶ Free fatty acid + lysophospholipids.

This enzyme is found in snake venom, and mammalian exocrine glands, where it serves a digestive role. The enzyme is small, water-soluble with a molecular weight about 13,000-15,000 Da, they are unusually stable and require Ca^{2+} for optimal activity. The properties of this extracellular enzyme have been well documented (Verheij, H.M., Slotboom, A.J. & De Haas, G.H. 1981; Waite, M. 1987). Regarding work done on physical state of the PLA_2 substrate, lipids droplets and dense proteaneous granules in the yolk maybe particularly suitable for to PLA_2 action (Dennis, E.A. 1983; Reynolds, L., Washburn, Deems, W.N. & R.A. Dennis 1991).

1.3.2. Fatty acid metabolism.

The overall metabolism of fatty acids involves a partitioning between esterification and oxidation ***See Diagram 1.*** Acylglycerol synthesis involves esterification of fatty acids to the glycerol backbone which can occur by two distinct pathways, each pathway ultimately resulting in the formation of diacylglycerol. Diacylglycerol formation has been shown to occur either by acylation of glycerol 3-phosphate, catalysed by glycerol phosphate acyl transferase (GPAT), or acylation of monoacylglycerol catalysed by mono-glycerol acyltransferase (MGAT). Diacylglycerol plays a pivotal role in the synthesis of neutral and polar lipids. It can be converted into triacylglycerol, by further acylation catalysed by diacylglycerol acyltransferase (DGAT) or directed towards phospholipid synthesis. The oxidation of fatty acid occurs by β-oxidation in the mitochondria, the rate limiting enzyme of β-oxidation is carnitine palmitoyl transferase I (CPT I).

Previous work (Maldjian, A. *et al.* 1995) has shown that in the avian embryo polyunsaturated fatty acids (PUFA), are transferred between lipid classes during

transport from the yolk to the developing embryonic tissues. In particular, docosahexanoic acid (DHA) is found associated with the phospholipid fraction in the yolk then associated with the plasma triacylglycerol fraction. The present study was to investigate the mechanism of this PUFA remodelling, during transport across the yolk sac membrane. The activities of key regulatory enzymes involved in fatty acid oxidation (CPT I) and esterification (GPAT, MGAT and DGAT) were investigated throughout avian development.

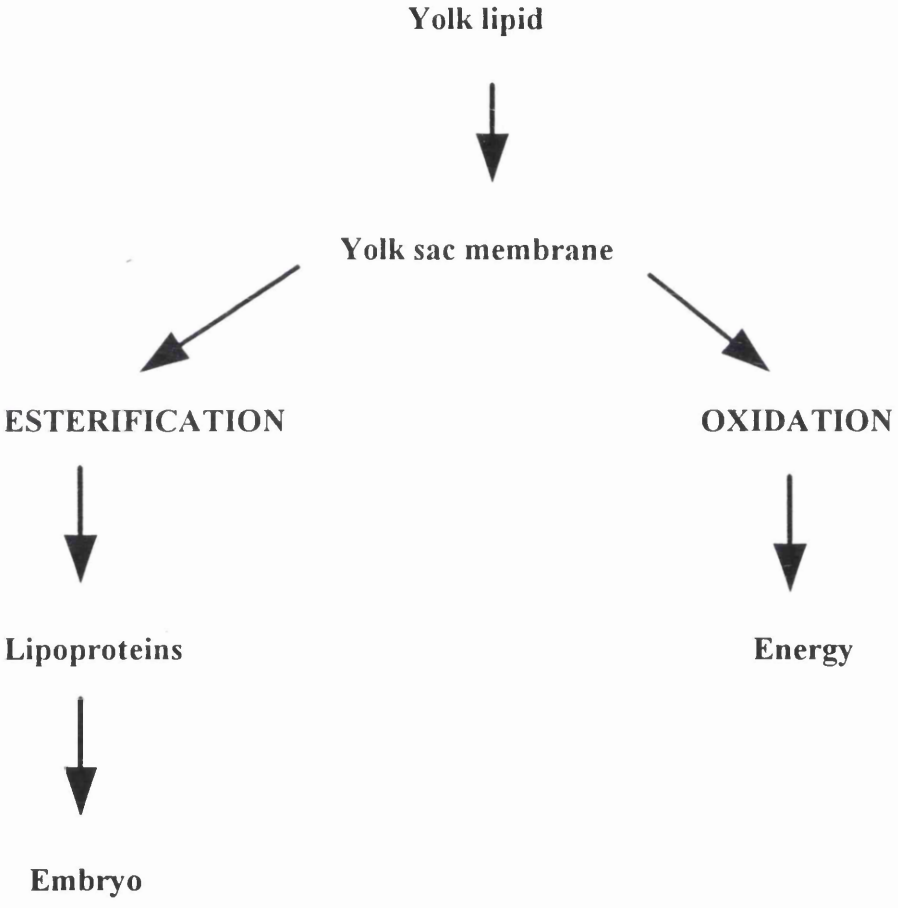


Diagram 1 : **lipid metabolism in yolk sac membrane**

1.3.2.1. Esterification of fatty acid

The synthesis of membrane lipids, steroids and lipoprotein involves the process of esterification. **See *Diagram 2.*** As described earlier, esterification of fatty acids can occur either by the glycerol 3-phosphate or mono acylglycerol pathway. The rate limiting enzyme of glycerol 3-phosphate pathway is glycerolphosphate acyltransferase (GPAT; e.c. 2.3.1.15). This enzyme catalyses the following reaction:

Sn-Glycerol 3-phosphate + Acyl-CoA ⟶ 1-acyl-sn-glycerol 3-phosphate + CoA.

The enzyme converts water-soluble glycerophosphate to a lipid product and catalyses the committed step in the biosynthesis of phosphoglycerides and triacylglycerols from glycerophosphate. In most mammalian organs, GPAT is present in both the endoplasmic reticulum (microsomes) and the mitochondrial outer membrane. (Bates, E.J. & Saggerson, E.D. 1979).

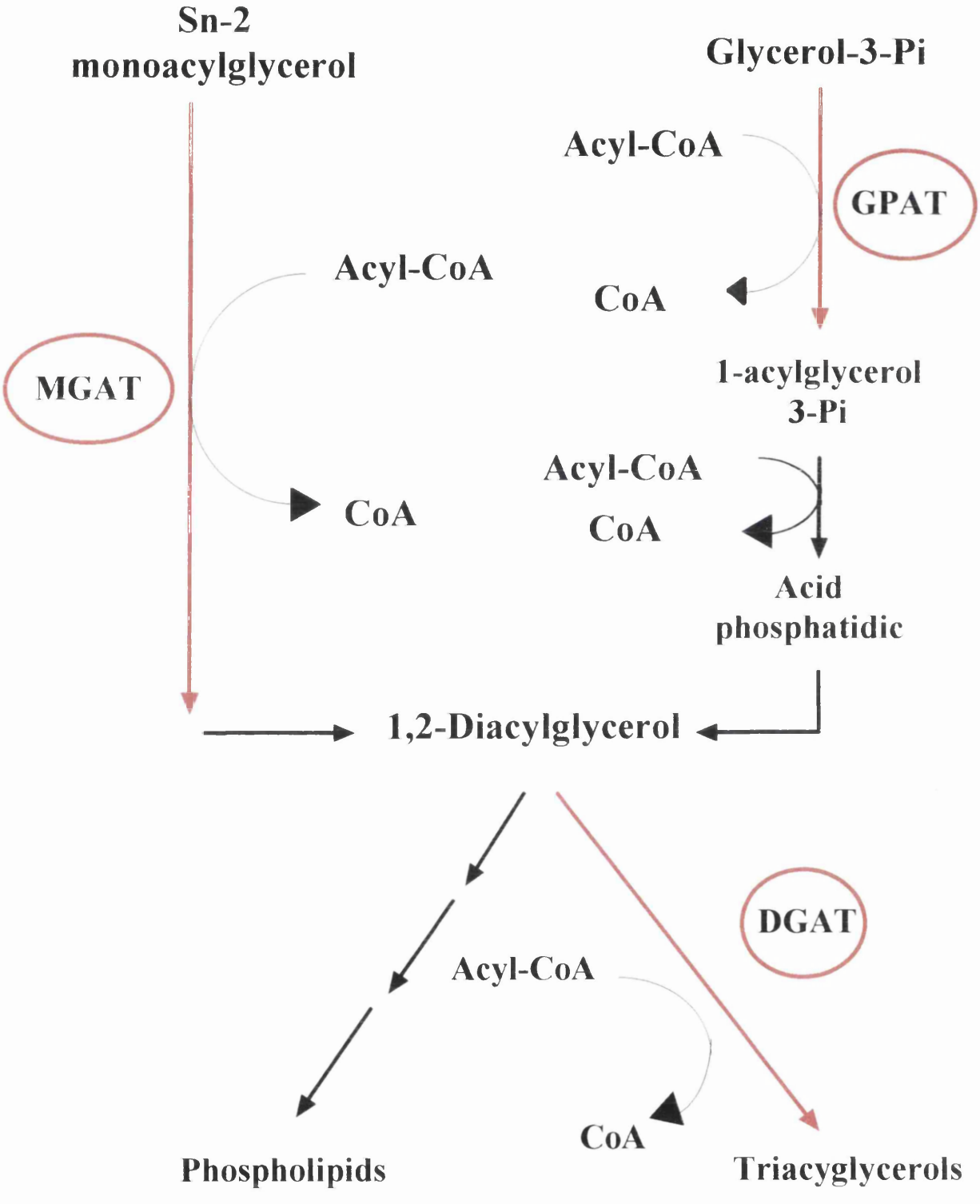
The rate limiting enzyme of the mono-acylglycerol pathway is monoacylglycerol acyltransferase (MGAT e.c.2.3.1.22). This enzyme is present within the membrane of the microsomal fraction, and catalyses the synthesis of sn-1,2-diacylglycerol from 2-monoacylglycerol and long chain fatty acyl-CoAs (Coleman, R.A., Walsh, J.P., Millington, D.S. & Maltby, D.A. 1986).

2-Monoacylglycerol + Acyl-CoA ⟶ sn-1,2-diacylglycerol + CoA

It is well documented that during periods of intense lipid oxidation, for example suckling rat, hibernating marmot and chick embryo a method of protecting essential fatty acids from oxidation is via esterification of sn-2-monoacylglycerols by MGAT (Tian Xia, Nadia Mostafa, B., Ganesh Bhat, Gregory L. Florant & Coleman, R.A. 1993). The existence of such a mechanism in the yolk sac membrane may protect essential fatty acids from oxidation.

Diagram 2.: **Biosynthetic pathway of triacylglycerols and phospholipids: GPAT (glycerol phosphate acyltransferase). MGAT (monoacylglycerol acyltransferase). DGAT (diacylglycerol acyltransferase).**

FATTY ACID ESTERIFICATION



Diacylglycerol acyltransferase (DGAT e.c.2.3.1.20) catalyses the committed step in the formation of triacylglycerol and possibly has a regulatory role in the supply of TG for lipoprotein synthesis (Owen, M.O., Corstophine, C.C. & Zammit, V.A. 1997). 1.2 diacylglycerol acyltransferase (DGAT) is associated with a complex, bound to the surface of the endoplasmic reticulum membrane. It catalyses the following reaction:

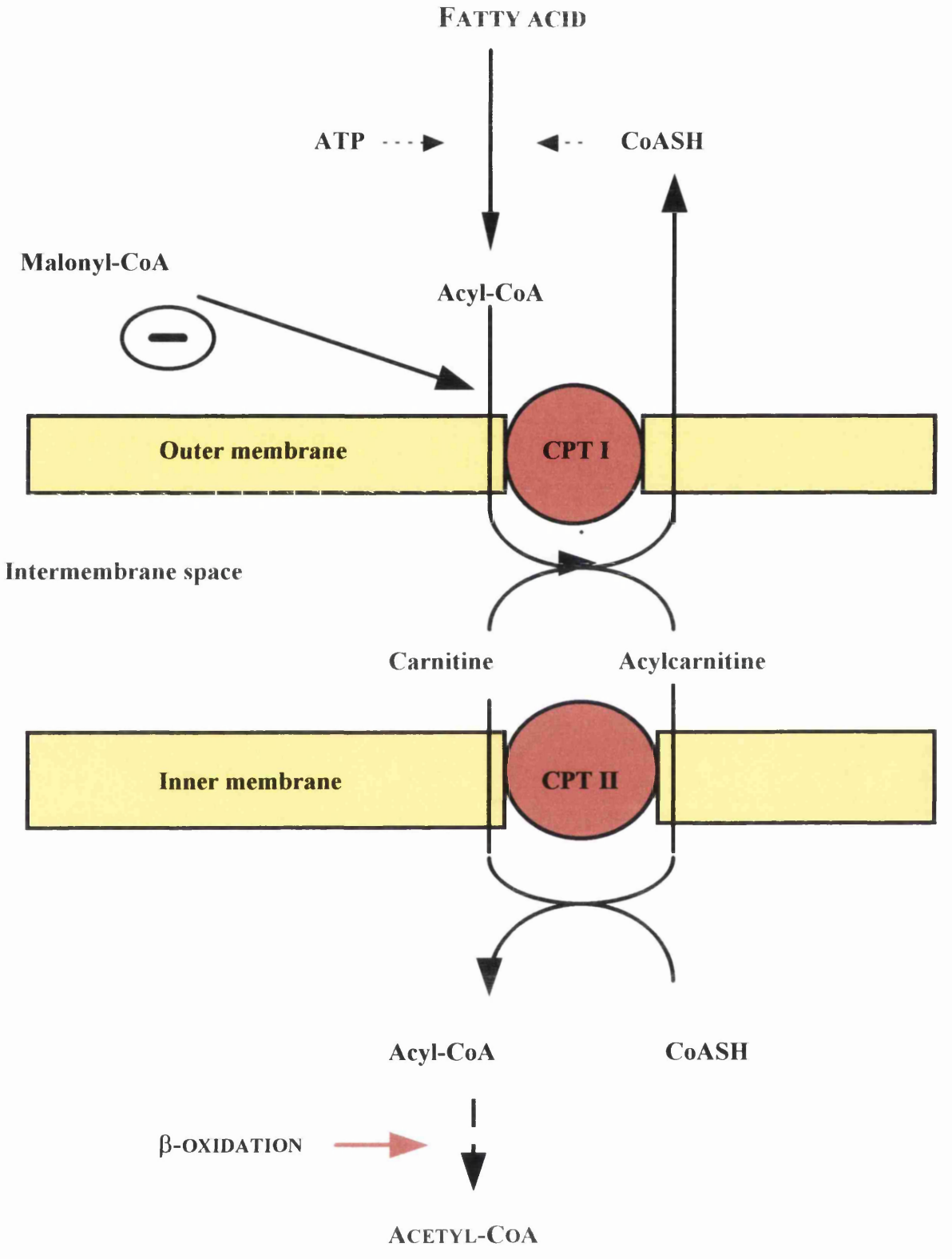
Diacylglycerol + Acyl-CoA ⟶ **triacylglycerol + CoA**

Investigation of this enzyme in the yolk sac membrane and the liver was carried out to provide information on the ability of these tissues to synthesis triacylglycerol a prerequisite for lipoprotein assembly.

1.3.2.2. Oxidation.

Fatty acids are a major source of energy for the heart and for skeletal muscle. In the chick during the second period of embryogenesis when there is a high transfer of fatty acid from the yolk to the embryo, the energy required is provided by fatty acids oxidation (Noble, R.C. & Cocchi, M. 1990). The role of carnitine palmitoyl transferase I (CPT I) and its regulation by malonyl CoA has been extensively investigated, (for review McGarry, J.D. & Brown, N.F. 1997). Long chain fatty acids esterified to CoA in the extramitochondrial compartment of the cell gain access to the enzymes of oxidation in the mitochondrial matrix by a carrier system that uses carnitine. The rate limiting reaction of this carrier system is catalysed by carnitine palmitoyl transferase I. The products of this reaction are acyl-carnitine and free CoA. The liberated CoA is transported back into the cytosol and the acyl-carnitine is carried through the second membrane and reacts with a CoA in the matrix. This second reaction is catalysed by CPT II. **See *diagram 3***. The released carnitine is transported to the extramitochondrial compartment. The newly formed acyl-CoA can now enter the oxidation cycle (Eaton, S., Bartlett, K. & Pourfarzam, M. 1996).

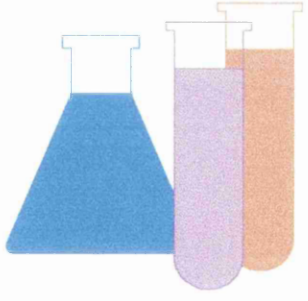
***Diagram 3*: Roles of carnitine palmitoyltransferase I and II (CPT I and CPT II) in the mitochondrial transport of fatty acid. Inhibitor, Malonyl CoA (MCoA). Diagram inspired from McGarry *et al.* (McGarry, J.D., Esser, A.S.V., Woeltje, K.F., Weis, B. & Foster, D.W. 1991).**


FATTY ACID
ATP
CoASH
Malonyl-CoA
Acyl-CoA
Outer membrane
CPT I
Intermembrane space
Carnitine
Acylcarnitine
Inner membrane
CPT II
Acyl-CoA
CoASH
β-OXIDATION
ACETYL-COA

1.3.3. PUFA transport.

Synthesis and secretion of lipoproteins by the yolk sac membrane in the developing chick embryo has been well described (Noble, R.C. & Cocchi, M. 1990; Shand, J.H., West, D.W., McCartney, R.J., Noble, R.C. & Speake, B.K. 1993). During the earliest period of transfer of fatty acids, lipids are assembled into lipoproteins by the yolk sac membrane and secreted. In the liver, a store of cholesteryl ester (CE) accumulates during the last period of embryonic life (Shand, J.H. *et al.* 1994) and after hatching is secreted as a component of a lipoprotein (Noble, R.C. 1987; Tarugi, P., Nicolini, S., Marchi, L., Ballarini, G. & Calandra, S. 1994). As development proceeds the yolk sac membrane lipoprotein secretory role diminishes in line with decreased yolk content and decreased rate of lipid transfer. At around hatching it is the liver which is the major producer of plasma lipoproteins. No detailed analysis of fatty acid composition has been performed on the different plasma lipoproteins at different stages of development but results from Maldjian, A. (1996) showed that high transfer of DHA occurs from day 12 to day 15, on the other hand at hatching all the DHA had been transferred to the embryo. As discussed, yolk PUFAs are delivered to the embryo as part of a lipoprotein particle, therefore analysis of different plasma lipoproteins at different period would provide key information on how the embryo metabolises these PUFAs.

MATERIALS AND METHODS



2.1. PREPARATION OF THE TISSUES

2.1.1. Embryos.

Fertile eggs from Ross 1 broiler-breeder parents were obtained from a commercial poultry supplier (Ross poultry: Thornhill, Scotland). They were incubated at 37.5°C and 60% relative humidity in a force draught incubator with automatic egg turning. Hatching occurred after 21 days of incubation and chicks were maintained for one day with access to drinking water but no food provision.

2.1.2. Sample collections for enzyme assay.

At each developmental stage studied, several eggs were opened and the embryos were gently dissected from their yolk complex (i.e., yolk plus yolk sac membrane). The liver was removed and washed with sucrose buffer (Sucrose, 250 mM, TRIS, 5 mM, EGTA 1 mM, pH 7.4 @ 0 °C). The yolk sac membrane was washed extensively with cold NaCl 0.85% to remove residual yolk. Tissue samples were kept on ice prior to subcellular fractionation. Yolks were transferred carefully without any presence of blood into plastic vials and stored at -80°C prior to further analysis.

Tissue samples were finely chopped using scissors and gently homogenised in sucrose buffer using a glass/teflon homogeniser. Subcellular fractionation was carried out by differential centrifugation. Homogenised samples were centrifuged at 400 g for 10 minutes at 4°C. The resulting supernatant was centrifuged at 4000 g, for 10 min to isolate mitochondria. The yolk lipid, cell debris and blood cells were discarded. The supernatant was centrifuged in an ultracentrifuge (Centrikon T.11XO, Kontron Instruments) at 100,000 g for 1 hour to prepare microsomes. The mitochondrial pellet was washed in sucrose buffer and recentrifuged at 4000 g for 10 min. Mitochondrial and microsomes were finally resuspended in sucrose buffer and stored

at -80°C. Mitochondria for the CPT 1 assay, were resuspended in KCl buffer (KCl 150 mM, TRIS 5 mM, EGTA 1 mM, pH 7.4 at 0 °C) and placed on ice.

2.1.3. Extraction of plasma lipoproteins.

2.1.3.1. Blood collection for lipoprotein analysis.

On day 14 of incubation, 20 embryos were sacrificed and blood was collected from the blood vessel associated with the embryonic chorioallantoic membrane which lies just beneath the shell. The anti coagulant EDTA (Diaminoethanetetra-acetic acid disodium salt) was added to the blood at a final concentration of 3 mg/ml. The blood collected was pooled, due to the small volume obtained per embryo. Blood was also collected on day 22 (1 day post hatch). This blood sample was obtained from 20 chicks by decapitation. The blood samples were left at 4 °C for 1 hour.

2.1.3.2. Isolation of plasma lipoproteins.

Blood samples were centrifuged at 3000 g at 4°C, for 20 min. in a benchtop centrifuge (ALC, Camlab). Blood plasma was collected and the volume measured. The following reagents were added to the plasma: Phenylmethylsulfonyl fluoride (0.015%), EDTA (0.04%) to prevent lipoprotein modification and Sodium Azide (0.05%) to prevent bacterial growth (all final concentration).

Lipoproteins were separated by density gradient ultracentrifugation. A solution of NaCl/KBr (density 1.346 g/ml) was used to adjust the plasma salt density to the appropriate level to allow the separation of the different lipoprotein species. VLDL, IDL, LDL and HDL were isolated from plasma at density 1.006 g/ml, 1.019 g/ml, 1.063 g/ml and 1.21 g/ml respectively. The plasma was transferred into centrifugation tubes and laid over with saline at an equal density to create a discontinuous gradient.

Samples were centrifuged at 100,000 g for 18 hours at 12 °C in a ultracentrifuge (Centrikon T.11XO, Kontron Instruments) using a horizontal rotor. Centrifugation gave sequential isolation of the lipoprotein fraction by flotation to the top of the tube. Each lipoprotein fraction was removed, stored under N_2 at -20 °C.

2.1.4. Sample collection for lipid analysis.

As previously, embryos were sacrificed, embryos were excised from the eggs and carefully separated from their associated yolk complexes. A maximum volume of yolk was collected without any blood contamination.

2.1.5. Protein analysis.

Mitochondrial and microsomal protein concentration were determined by the method of Lowry (Lowry, O.H., Rosebrough, N.J., Fan, A.L. & Randall, R.J. 1951) with bovine serum albumin (BSA) as standard.

2.1.5.1. Assay of lipoprotein protein.

Lipoprotein was determined following the method of Lowry *et al.* (1951) with a few modifications. A mixture consisting of 50 mls of reagent A (2% disodium carbonate, 0.4% sodium hydroxide, 0.16% potassium tartrate and 1% sodium dodecyl sulphate), and 0.5 ml of reagent B (4% cupric sulphate) was prepared and 3 ml of this solution added to duplicate lipoprotein samples total volume 0.2 ml. Tubes were incubated at room temperature for 10-60 min., after which, 0.3 mls of diluted Folins and Ciocalteu reagent (1:1 v/v) was added. The tubes were incubated for further 45 min.. The absorbance was read against blank sample at 660 nm.

2.2. ENZYME ASSAYS

2.2.1 Glycerolphosphate acyltransferase (GPAT e.c. 2.3.1.15).

The enzyme converts water-soluble glycerophosphate to a lipid product and catalyses the committed step in the biosynthesis of phosphoglycerides and triacylglycerols from glycerophosphate. GPAT was assayed in both mitochondrial and microsomal fractions. N-ethylmaleimide was used to inhibit microsomal GPAT (Bates, & Saggerson, E.D. 1979).

2.2.1.1. Analytical method.

GPAT was assayed radiochemically at 30°C by following the incorporation of [^{14}C]-glycerol 3-phosphate into butanol-soluble products. Assays were performed in a final volume of 0.25 ml containing 50 mM TRIS/HCl buffer pH 7.4, KCl 120 mM, 0.2 mM dithiothreitol (freshly prepared), defatted B.S.A. 4 mg/ml, palmitoyl-CoA 100 μ M, 0.2 μCi [^{14}C]-glycerol-3-phosphate, unlabelled sn-glycerol 3-phosphate (G.3.P.) 3 mM. A concentration of 3 mM G.3.P. was found to be saturating for GPAT activity in the yolk sac membrane. This concentration was also used for measuring GPAT activity in the liver since the activity in this tissue was lower.

Assay samples were preincubated for 3 min at 30°C on a heated block. The reaction was started with 50 μg of either microsomal protein or mitochondrial protein from the yolk sac membrane and 50 - 100 μg microsomal protein from the liver. Assay of GPAT activity in the yolk sac membrane was terminated after 5 minutes. Liver GPAT assay was terminated after 10 minutes. This was due to the lower activity of the liver enzyme compared to the yolk sac membrane enzyme and also due to the limitations of quantity of microsomal protein available. The reaction was stopped by adding 2.0 ml of water saturated 1-butanol to each tube, vortexed, and placed in ice. A volume of 0.75 ml of butanol-saturated-water was added, vortexed, and the mixture centrifuged at 600 g in a bench top centrifuge for 10 min. at room temperature. The

upper butanol layer was transferred to fresh tubes and was washed with 1.3 ml butanol-saturated-water. The wash was repeated twice, the tubes were vortexed and centrifuged for 3 min. at 600 g. 1 ml of the washed butanol layer was transferred to a scintillation vial and 10 ml of Opti-fluor (Packard company) added. Radioactivity associated with the organic phase was determined using a scintillation counter (1900 TR, Liquid Scintillation Analyser, Packard company).

The results are expressed in nmols/min/mg of either microsomal or mitochondrial protein.

2.2.2. Diacylglycerol acyltransferase (e.c. 2.3.1.20).

As described below, DGAT activity was measured by following the esterification of [^{14}C]-palmitoyl CoA into [^{14}C]-triacylglycerol. The products were extracted into heptane, using the method described by Jamdar & Cao (Jamdar, S.C. & Cao, W.F. 1995).

2.2.2.1. Analytical method:

DGAT was assayed in the presence of 1,2 diacyl-sn-glycerol dispersed in 0.1% Tween-20 and [^{14}C]-palmitoyl CoA. The standard assay was in a final volume of 0.5 ml, containing 50 mM TRIS-HCl pH 7.5, 10 mM Mg SO_4, 1.2 mM diacylglycerol, 0.04 mg/ml dithiothreitol, 1.25 mg B.S.A. fat free, 100 μM palmitoyl CoA with 0.1 μ Ci of [^{14}C]-palmitoyl CoA (Ci/mole). The substrate concentration used was saturating, and the reaction was linear with time. The reaction was started with the addition of enzyme and the samples were incubated at 37°C for 10 minutes. The reaction was terminated by adding 0.75 ml of a "stop mixture" containing 2-propanol/ heptane/ water (80:20:2 v/v/v), the tubes were vortexed briefly and placed on ice. After 5 minutes, 0.5 ml of heptane and 0.25 ml of distilled water was added to each tube. The tubes were centrifuged at 600 g for 5 min. in a bench top centrifuge. The

heptane layer (upper phase) was transferred into fresh test tubes, and washed twice with 1 ml of "wash mixture" containing 0.5 N NaOH/ ethanol/ water (10:50:50 v/v/v) and centrifuged at 600 g for 5 min. following the first wash and for 10 min. following the second wash. The radioactivity associated with the organic phase was measured using a scintillation counter. A volume of 0.15 ml of heptane was transferred to scintillation vials, and 10 ml of opti-fluor was added.

The reaction products formed in the DGAT assay were identified as di- and triacylglycerol following thin-layer chromatography of 0.25 ml of the heptane phase. The major reaction product formed in this assay was identified as [^{14}C]-triacylglycerol. The specific activity of DGAT was calculated on the basis of radioactivity in the TG fraction minus the radioactivity present in the monoacylglycerol and diacylglycerol fraction.

2.2.3. Monoglycerol acyltransferase (MGAT e.c.2.3.1.22).

Monoacylglycerol acyltransferase (e.c.2.3.1.22) present within the membrane of the microsomal fraction catalyses the synthesis of sn-1,2-diacylglycerol from 2-monoacylglycerol and long chain fatty acyl-CoAs (Coleman, R.A. *et al.* 1986). MGAT activity was determined by measuring the conversion of [^{14}C]-palmitoyl CoA to [^{14}C]sn-1.2 diacylglycerol as described by Jamdar & Cao (1995).

2.2.3.1. Analytical method:

MGAT was assayed in the presence of 2-monoolein dispersed in 0.1 % Tween 20 (v/v). The reaction mixture contained 24 mM TRIS/HCl buffer pH 7.5, 50 mM KCl, 8.0 mM $MgSO_4$, 0.25 mM 2-monoolein, 0.75 mM dithiothreitol, 1.25 mg/ml of free fat B.S.A., 25μM [^{14}C]-palmitoyl CoA (0.02 μCi/mole) and 20 μl of the phospholipid mixture (15 μg of PC/ 15 μg of PS 1:1, w/w), in a final volume of 0.5 ml.

The reaction mixture was preincubated at 37 °C for 5 min. and the reaction started by addition of 2 μg microsomal protein in a volume of 20 μl. The reaction was terminated after 5 min. by the addition of 0.75 ml of "stopping mixture" containing 2-propanol/ heptane/ water (80:20:2 v/v/v). After 5 min., 0.5 ml of heptane and 0.25 ml of water was added and the tubes were centrifuged at 600 g for 5 min. The heptane layer was removed to fresh tubes, and washed twice with 1 ml of "wash mixture" containing 0.5 N NaOH/ ethanol/ water (10:50:50 v/v/v) and centrifuged at 600 g for 5 min. (as DGAT). A volume of 0.25 ml of heptane was removed and the radioactivity measured using a scintillation counter. The remaining heptane was dried and the lipids separated by TLC in a heptane/ isopropyl ether/ acetic acid (60:40:4 v/v/v) solvent system. Carrier lipids (monoacylglycerol/ diacylglycerol/ triacylglycerol) were added to help visualisation. Radioactivity associated with the DG and TG fractions were measured using a scintillation counter.

The specific activity of the MGAT pathway was calculated on the basis of radioactivity present in the DG fraction plus $^1/_2$ of the radioactivity in the TG fraction, (as 2 moles of palmitoyl-CoA were required to form TG from 2-monoacylglycerol).

2.2.4. Carnitine palmitoyl transferase I (CPT1).

CPT I activity was measured as described Kolodziej (Kolodziej, M.P., Crilly, P.J., Corstorphine, C.G. & Zammit, V.A. 1992). The reaction was measured by following the conversion of [^{3}H]-carnitine to [^{3}H]-palmitoyl-carnitine. The product was extracted from [^{3}H]-carnitine using water saturating butanol. The activity of CPT I can be inhibited by the presence of malonyl CoA (McGarry, J.D., Esser, A.S.V., Woeltje, K.F., Weis, B. & Foster, D.W. 1991). The sensitivity of CPT I to malonyl-CoA was measured by assaying CPT I activity in the presence of increasing concentrations of malonyl-CoA.

2.2.4.1. Analytical method:

CPT I was assayed in a reaction mixture containing: 5 mM TRIS/ HCl pH 7.4, 150 mM KCl 1 mM EGTA, 0.02 g of B.S.A. fat free, 5 mM $MgCl_2$, 5 mM ATP, 1 mM of dithiothreitol, 2 µg/ml antimycin A, 4 µg/ml rotenone in a final volume of 1 ml. Maximum CPT I activity was measured in the presence of 250 µM of palmitoyl-CoA. Malonyl CoA sensitivity was measured in the presence of 35 µM palmitoyl CoA.

The reaction was started by addition of 25 µl of sample, concentration usually 10 mg/ml protein. The tubes were mixed and incubated for 2 min. at 37 °C. Following this incubation period 25 µl of [^{3}H]-carnitine (0.5 mM, 0.35 Ci/mol) was added to each tubes and the samples incubated for 2 min. The reaction was stopped by addition of 300 µl of 6N HCl. The tubes were then mixed and placed immediately on ice. The [^{3}H]-palmitoylcarnitine formed was quantified as described below. Distilled water (1 ml) and 4.5 ml of water saturated butanol was added to each tube. Each tube was shaken manually for at least 3 min. and then centrifuged at 600 g using a bench top centrifuge. The upper butanol layer was transferred into fresh tubes using a Pasteur pipette. The butanol phase containing [^{3}H]-palmitoylcarnitine was washed 4 times with 3 ml of butanol saturated water. A 3 ml aliquot of the butanol phase was transferred into scintillation vials with 10 ml of Opti-fluor (Packard company).

Maximum CPT I activity was expressed as µmoles [^{3}H]-palmitoylcarnitine formed per minute per mg of mitochondrial protein. Sensitivity to malonyl-CoA was expressed as the concentration of malonyl-CoA (µmole) which illicited 50 % inhibition.

2.2.5. Phospholipase A_2 assay using pyrene-labeled acyl chains on eggs' components.

2.2.5.1 Analytical method:

Samples were prepared from the egg yolk and whole yolk sac membrane from day 10 to day 16 of development. The fluorescent phospholipid used was 1-hexadecanoyl-2-(1-pyrendecanoyl)-sn-glycero-3-phosphoethanolamine (β-py-C_{10}-HPE). In an attempt to form vesicles with the same composition as lipid droplets present in the yolk, phospholipids were extracted from egg yolk and used to produce liposomes. A standard curve was made by measuring the light intensity of different concentrations of 1-pyrenedecanoic acid, the product of hydrolysis.

2.2.5.2. Liposomes preparation.

Liposomes were prepared according to the procedure of Batzri & Korn (Batzri, S. & Korn, E.D. 1973). Liposomes were formed from a mixture of yolk phospholipids (average of 760 g/mole corresponding of the major phospholipids present in the yolk fraction) and β-py-C_{10}-HPE (m.w.: 808.05 g/mole), final concentration (in cuvette) of 1 mmole and 5 μmole, respectively. Phospholipids and substrate were dried under nitrogen and suspended in methanol/ ethanol (2:1, v/v) to obtain a concentration between 20-40 μmoles of phospholipid/ml (Batzri, S. & Korn, E.D. 1973).

The ethanoloic lipids were rapidly injected into an Amicon filtration cell containing 10 ml of saline buffer (40 mM TRIS-HCl, pH 7.4, 2 mM $CaCl_2$) to form liposomes. Liposomes were concentrated to approximately 2 ml volume under 10 psi pressure. The liposomes were washed in a further 10 ml of warm buffer and filtered down to approximately 1-2 ml. Filtrates were discarded and the liposome preparation, which appeared with a slight translucent blue-brown colour was diluted up to 6 mls using the appropriate buffer. Liposome preparation were stable for up to one week at

temperatures below 4 °C and light protected. The substrate was very sensitive to the light.

2.2.5.3. Preparation of the samples.

Yolk protein was isolated as an acetone/diethyl ether-dried preparations (Parkin, S.M., Speake, B.K. & Robinson, D.S. 1982). Yolk and whole yolk sac membrane protein were blended in distilled water at 4°C (1/1, v/v) and 15 ml of cold acetone was added to the sample. The homogenates were centrifuged at 800 g for 20 min. at 4 °C. The acetone layer was discarded and the protein pellets were washed a second time with 15 ml of acetone, centrifuged at 800 g for 20 min at 20 °C and the top layer discarded. The pellets were resuspended in 15 ml of diethyl ether and dried down under vacuum. The defatted dried residue was resuspended in $CaCl_2$ buffer and 0.1 % Triton X-100, to disperse the sample.

2.2.5.4. The assay.

Liposomes were resuspended in appropriate volume of $CaCl_2$ buffer (40 mM TRIS, 2 mM $CaCl_2$, pH 7.4) in a heated (37.5°C) and magnetically stirred (low speed to avoid bubble formation) cuvette. Excitation wavelength of 341 nm and emission wavelength of 376 nm were selected by Perkins Helmer software (Instrument control V.4,0). The fluorescence measurement was carried out using a Luminescence spectrophometer (Perkin Helmer, Beaconsfield, U.K.). The fluorescence spectrum of the substrate is shown on ***Figure 4.*** The corresponding slits were 2.5 nm. The fluorescence of the reaction medium (Blank) was recorded and the reaction was initiated by the addition of the protein sample. Optimal assay conditions, i.e. $CaCl_2$ concentration, pH optimum, protein concentration were determined. All PLA_2 activity assayed was found to be Ca^{2+} dependant, a concentration of 2 mM was used to assay yolk samples and 4 mM of $CaCl_2$ was used for yolk sac membrane samples. A protein concentration of 200 μg was assayed for the determination of whole yolk

sac membrane PLA_2 activity, 100 μg was used to assay yolk PLA_2 activity. The activity was expressed in μmole per minute per mg of protein. The enzyme activity was determined by measuring the slope of the curve representing the increase of fluorescence versus time. see *Figure 5.*

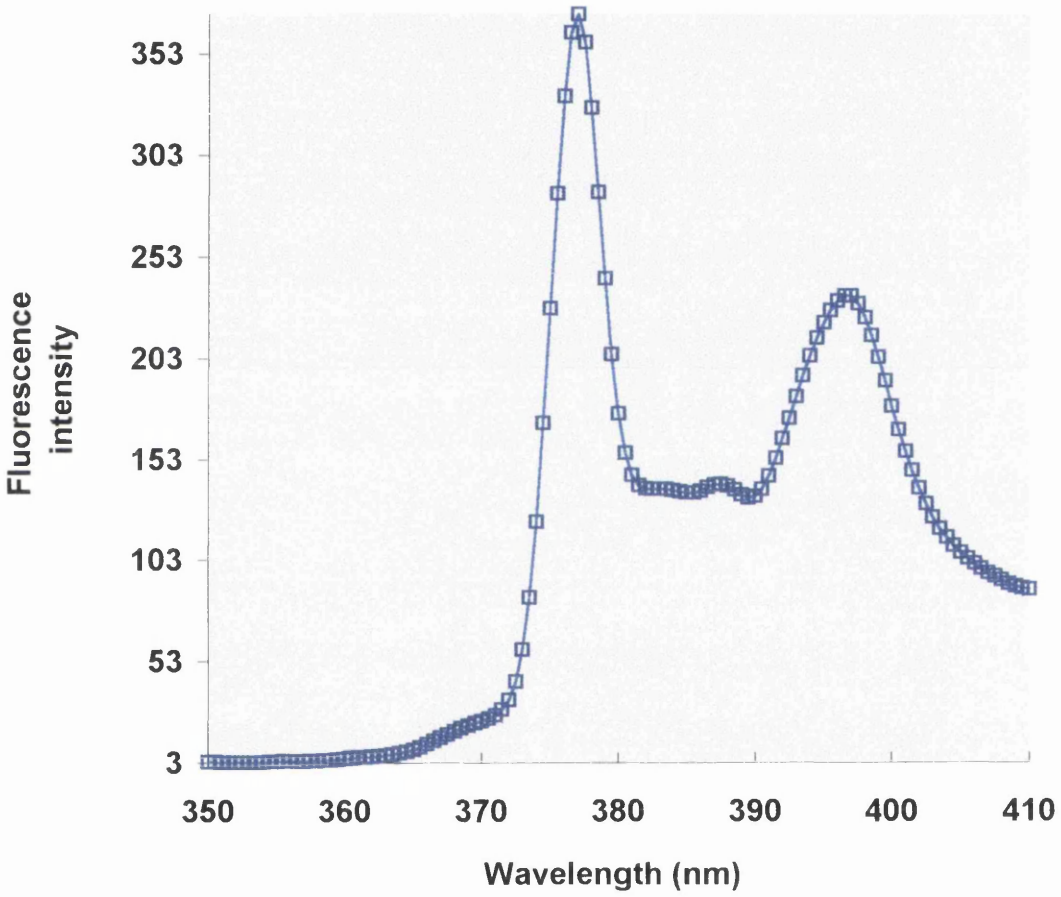


***Figure 4*: Fluorescence emission spectra of β-py-C_{10}-HPE. Emission spectra were recorded in 40 mM TRIS, 2 mM of $CaCl_2$, pH 7.5, containing 5 μmole of substrate inserted in 1 mmole forming liposome. Excitation wavelength sets at 341 nm, slit 2.5.**

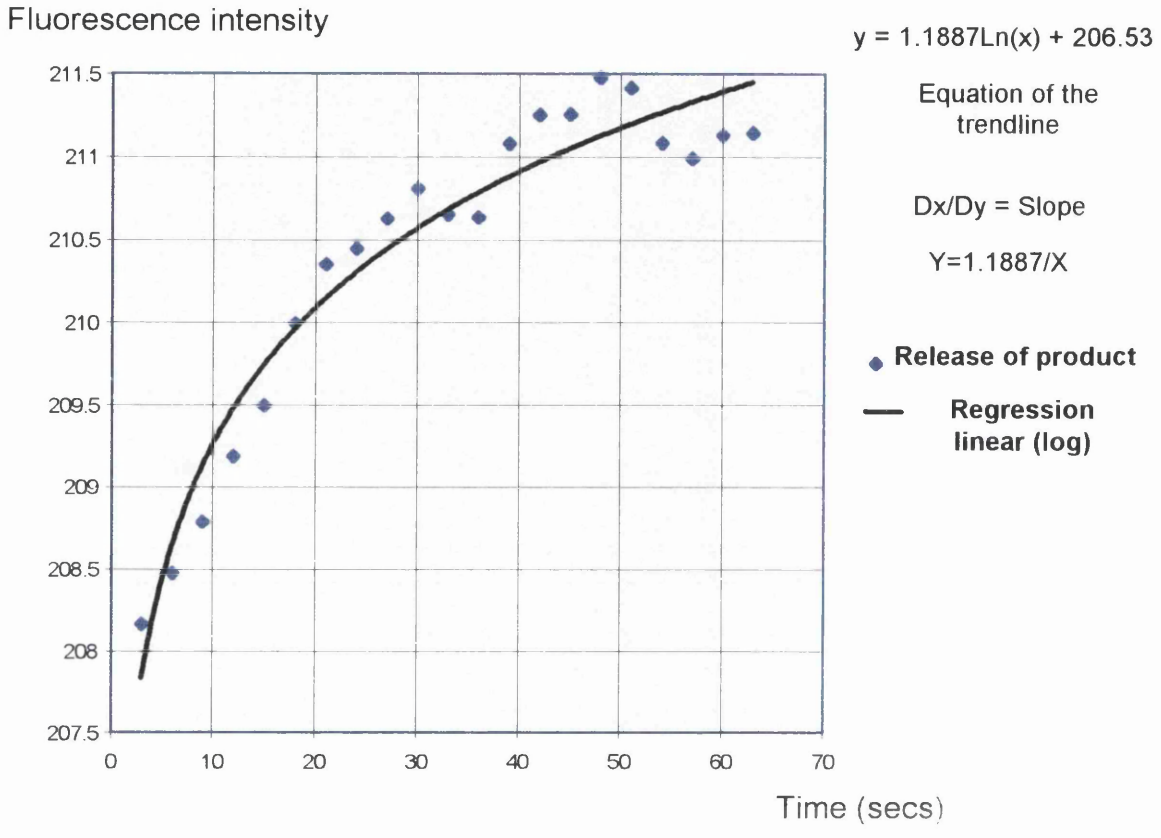


Figure 5: **Fluorescence emission spectra of sample with mitochondria protein. The assay was performed by injecting 200 μg of mitochondrial protein into the cuvette. the reading was made at 376 nm. The slope of the curve was determined by deriving the function given (Excel 5). The activity was measured at the maximal rate.**

2.3. LIPID ANALYSIS

2.3.1 Lipid extraction.

Lipid extraction was carried out as described by Christie (Christie, W.W. 1982). Yolks were homogenised in chloroform/ methanol (2:1, v/v). The homogenates were then filtered through a Whatman filter paper (reference n°1, Whatman labscales Ltd, Kent, U.K.) into measuring cylinders. The deposits on the filter papers were washed with an excess of solvent. Such chloroform/ methanol extracts are known to contain sugars, urea, salts and amino acids in addition to lipids, these were removed by addition of an aliquot of KCl (0.88%, w/v). After shaking, the mixture was left to stand overnight, to allow separation of aqueous and organic phases. The upper "aqueous phase" was discarded and the lower "organic phase" containing the lipids was transferred to a round bottom flask and the solvents evaporated using a rotary evaporator (in a water bath at 55 °C). The lipid extracts were then dissolved in a known volume of chloroform (10 ml). Addition of a small amount of methanol was sometimes necessary if a small amount of water was still present in the samples. In such cases, samples were dried again and the operation was process until the lipid extract in chloroform was perfectly clear. The final chloroform extract was transferred into glass vials and stored in the fridge. A aliquot (usually half) of the chloroform extract was dried in preweighed round bottom flasks to determine the weight of total lipid in the sample. An aliquot of the remainder of the lipid extract (i.e. that which was not dried for gravimetric determination) was used for the determination of the fatty acid composition of the total lipid and to extract individual lipid classes by thin layer chromatography.

2.3.2 Separation of lipid classes by thin layer chromatography (TLC)

20 × 20 cm glass plates were cleaned with cotton wool dampened with hexane. The stationary phase was Kieselgel 60 G (Merck, ATG, Darmstadt, Germany). 22.5 g of

silicate K 60 in 50 ml of distilled water was required to spread 5 plates. This phase was applied to the plates using an aluminium spreader which was adjusted to deliver a layer of silica gel of defined thickness (0.25 mm). Plates were allowed to dry for 30 min at room temperature and were then transferred to an oven for a minimum period of 30 min at 110°C. Plates were left to cool at room temperature before sample application. The eluting solvent was hexane/ diethyl ether/ formic acid (80:20:1, v/v/v), prepared fresh. 200 ml of this eluting solvent was added to the chromatank, which was lined with filter paper and sealed with a glass cover, and the system was allowed to equilibrate for 30 min before beginning the chromatography. Lipid dissolved in chloroform was applied to the plate at 1 cm from the base, using a 500 µl glass syringe. The applications were allowed to dry at room temperature before the plates were placed carefully into the chromatank. When the solvent front reached 1 cm from the top of the plate (after approximatly 45 min.) plates were removed from the chromatank and air dried for a few seconds. They were then sprayed with Trifluorofluorescence and the bands representing the lipid classes visualised. The following ***Diagram 4*** shows the lipid class separation obtained under these conditions:

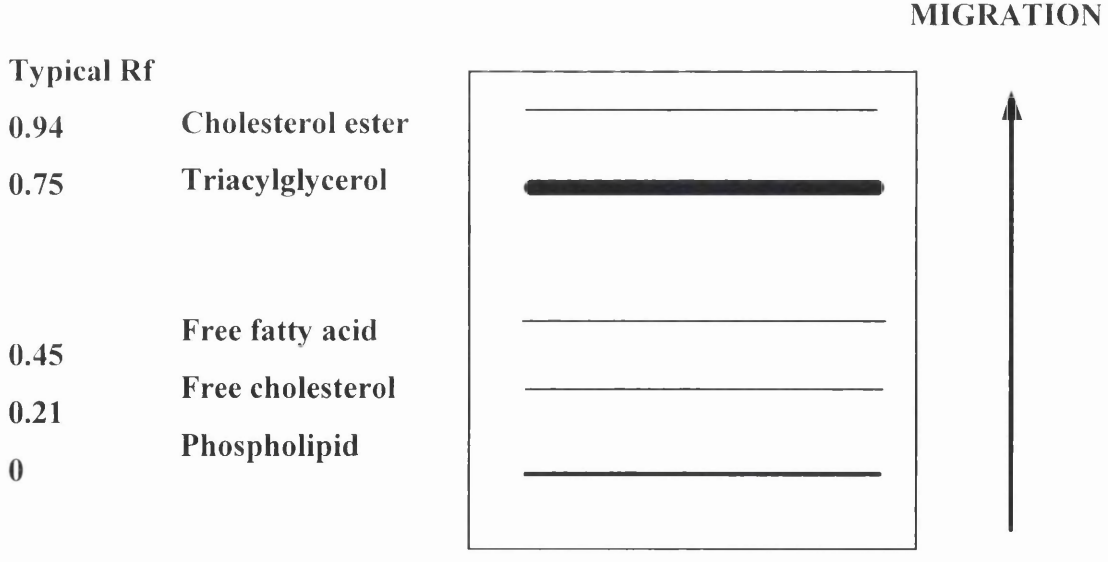


***Diagram 4*: Separation of lipid classes by thin layer chromatography.**

Bands were scraped from the plates and the lipid extracted using diethyl ether, the phospholipid was extracted using methanol. The lipid extract was centrifuged at 600 g for 2 min. At this stage, the extracted lipids were either methylated for fatty acid composition analysis or transferred to a glass vial of known weight, to determine the amount of each lipid class. Free cholesterol was determined by an enzymatic/colourmetric method using a Boehringer Kit System (Lewes, East Sussex, England).

2.3.3. Methylation of fatty acids

A volume of 1 ml of pentadecanoic (C_{15}) standard was added to each lipid extract. This allows quantification of the fatty acid peaks by comparison of their retention times with that of the C_{15} standard using gas chromatography (GC). Lipids fractions were transferred to round bottom flasks and reduced to dryness, 4 ml of the methylating agent (methanol/ toluene/ sulphuric acid: 20:20:1 v/v/v) was added. The samples were refluxed for at least 30 min and then allowed to cool. Once cold, 10 ml of water and 10 ml of hexane were added. The tubes were shaken vigourously and samples poured into B19 test tubes. Two layers formed rapidly; the upper hexane layer was transferred into clean test tubes containing a small amount of the drying agent, sodium sulphate/ sodium hydrogen carbonate (4:1 w/w). The tubes were left to stand for a minimum of 30 min.. The hexane was then transferred into smaller tubes and left until GC preparation and analysis.

2.3.4. Gas chromatography analysis

Samples were concentrated by drying under gas nitrogen, and then dissolved in a minimum volume of hexane. Samples were analysed using a autosampler CP9001 GC (Chrompack, Middleburg, The Netherlands). It was equipped with a 30 m by 0.25 mm capillary column fitted with a 3.0 m by 0.25 μm retention gap (Carbowax, Alltech associates, Carnforth, U.K.). Temperature was set at 185 °C for 2 min, then

raised to 230 °C at a rate of 5°C/min. The helium carrier gas was maintained at a flow rate of 25cm/sec. Injection volume was 50 µl and the split ratio was 1:50. The detector was a flame ionisation detector with a hydrogen flow of 30 ml/min. Air flow was 300 ml/min. The make up flow of nitrogen was 30 ml/min. The injector and FID temperature was 250 °C. Identities of the peaks were verified by comparison with the retention times of the standard fatty acid methyl ester and with proprietary methyl ester mixtures of known composition (Sigma Chemical LTD, Pool, U.K.).

2.4. EXPRESSION OF THE RESULTS.

All data are expressed as the mean ± S.E.M. of at least 3 replicate samples. Where appropriate, statistical analysis were performed using an unpaired Student's t-test. Significant differences where expressed as following: $^{a}P<0.001$; $^{b}P<0.005$; $^{c}P<0.01$; $^{d}P<0.025$; $^{e}P<0.05$.

2.5. MATERIALS

Chemicals were purchased from Sigma Chemical LTD. Palmitoyl-CoA from Pharmacia (Biotech, U.S.A.) [^{14}C] glycerol-3-phosphate di-sodic (ethanol/water 1:4 v/v) purchased from I.C.N. Bovine Serum Albumin fat free (B.S.A.) from Advanced Protein Product LTD. [^{14}C]palmitoyl CoA, (sodium acetate pH 5.9-6.0) and [^{3}H]-Carnitine (L-(methyl-^{3}H) carnitine hydrochloride) were from Amersham, U.K. 1-hexadecanoyl-2 (1-pyrendecanoyl) sn-glycero-3-phosphoethanolamine (β-py-C_{10}-HPE) and 1-pyrenedecanoic acid from Molecular Probes Company.

RESULTS

INTRODUCTION

As discussed previously a number of key features of PUFA metabolism occurs from around day 12 to day 16 of avian development. We therefore decided to investigate a number of metabolic parameters from day 10 to day 16. In some cases obtaining sufficient material was our limiting factor and this precluded detailed analysis at some developmental points.

3.1. FATTY ACID ANALYSIS IN THE EGG YOLK DURING DEVELOPMENT.

Previous work has analysed the fatty acid composition of the yolk complex which comprises both the yolk and the yolk sac membrane. The present analysis was performed on yolk lipid only during embryonic development (***Table 10***). The majority of fatty acids were associated with the triacylglycerol fraction, with 63.9 ± 0.3 % (% w/w of total lipid). Phospholipid (PL) was the second major lipid class with 29.4 %. The other lipid classes, cholesteryl ester (CE), free cholesterol (FC) and free fatty acids (FFA) were present but at a lower percentage. During development, the percentage of TG increased from day 0 to day 16, whereas the percentage of PL slightly decreased. The proportion of CE and FC remained similar throughout development. The percentage of ratio of FFA increased from 0.79 ± 0.1 to 1.62 ± 0.3 %.

3.1.1. Fatty acid composition of yolk triacylglycerol (*Table 11*).

At day 0, oleic acid was found to be the major fatty acid (44.1 ± 1.4 %, % w/w of total fatty acid) associated with the TG fraction following by palmitic acid with 25.8 ± 0.5 %. Linoleic acid was the major polyunsaturated fatty acid (PUFA) present in the

the TG fraction with 14.1 ± 0.5 %. Linoleic, arachidonic (AA; 20:4) and docosahexaenoic acid (DHA; 22.6) percentage were below 1 %.

3.1.2. Fatty acid composition of yolk phospholipid (*Table 12*).

At day 0, palmitic and oleic acid were the major fatty acids present under the PL fraction with 27.4 ± 0.9 and 22..5 ± 1.4 % (% w/w of total fatty acid) respectively. Linoleic acid was the major PUFA in PL with 17.5 + 1.0 %. In agreement with earlier findings (Noble, R.C. & Cocchi, M. 1990) DHA and AA were present in egg yolk PL with 6.16 ± 0.2 and 5.41 ± 0.4 % respectively. From day 0 to day 16 of development the percentage of the major fatty acids increased slightly whereas the percentage of DHA decreased by more than 60 % from 6.16 ± 0.2 to 2.32 + 0.1 %. This pattern was not found for the other PUFA.

3.1.3. Fatty acid composition of yolk free fatty acid (*Table 13*).

At day 0, oleic and palmitic acid were the major fatty acid with 32.9 ± 0.5 and 18 ± 2.0 (% w/w of total lipid) respectively. Linoleic acid was the major PUFA with 17.5 ± 1.0 %. DHA and AA were present at less than 5 %. The free fatty acid (FFA) composition was found to change during development. The percentage of stearic and oleic acid slightly decreased whereas the percentage of palmitic, palmitoleic and linoleic increased. Interestingly the percentage of arachidonic acid and DHA in the FFA fraction increased to day 10 then decreased towards day 16. This was particularly evident for DHA, as illustrated in ***Figure 6***. The decrease of DHA within the PL fraction was associated with an increase of DHA in FFA fraction. No significant differences were found within the cholesteryl ester class (CE) and the triacylglycerol class (TG).

Table 10: **Yolk lipid class composition (% w/w of total lipid) during development. Values are means ± S.E.M., n=4**

Days of development	0	10	12	14	16
Lipid class (%, w/w of lipid content)					
Triacylglycerol	63.9 ± 0.3	63.4 ± 0.6	65.8 ± 0.4	66 ± 0.7	67.1 + 0.7
Phospholipid	29.4 ± 0.2	28.3 ± 0.4	27.2 + 0.3	27.2 + 0.6	25.5 ± 0.7
Free fatty acid	0.79 ± 0.1	2.04 ± 0.5	1.31 ± 0.3	1.63 ± 0.4	1.62 + 0.3
Cholesteryl ester	1.21 ± 0.1	2.35 + 0.4	1.05 ± 0.1	0.92 + 0.0	1.33 ± 0.1
Free cholesterol	4.73 ± 0.4	3.91 ± 0.4	4.62 ± 0.1	4.29 + 0.1	4.44 + 0.0

Table 11: **Fatty acid composition (% w/w of total lipid) of yolk triacylglycerol. Values are means ± S.E.M., n=4**

Days of development	0	10	12	14	16
Fatty acid (% w/w of lipid content)					
Palmitic	25.8 ± 0.5	26.5 ± 0.5	25.3 ± 0.6	26.6 ± 0.2	27 ± 0.7
Palmitoleic	3.06 ± 0.1	3.54 ± 0.2	3.43 ± 0.6	3.89 ± 0.5	3.93 ± 0.3
Stearic	7.17 ± 0.3	7.13 ± 0.2	6.74 ± 0.4	6.76 ± 0.3	7.01 ± 0.3
Oleic	44.1 ± 1.4	43.3 ± 1.6	45.2 ± 2.1	42 ± 1.2	43.4 ± 1.5
Linoleic	14.1 ± 0.5	14.1 ± 1	13.9 ± 1.2	15.1 ± 0.6	13.2 ± 0.8
Linolenic	0.67 ± 0.1	0.64 ± 0.1	0.63 ± 0.1	0.67 ± 0.1	0.54 ± 0.0
Arachidonic	0.33 ± 0.0	0.27 ± 0.0	0.28 ± 0.0	0.29 ± 0.0	0.25 ± 0.0
Docosahexaenoic	0.3 ± 0.1	0.18 ± 0.0	0.18 ± 0.0	0.19 ± 0.0	0.2 ± 0.0

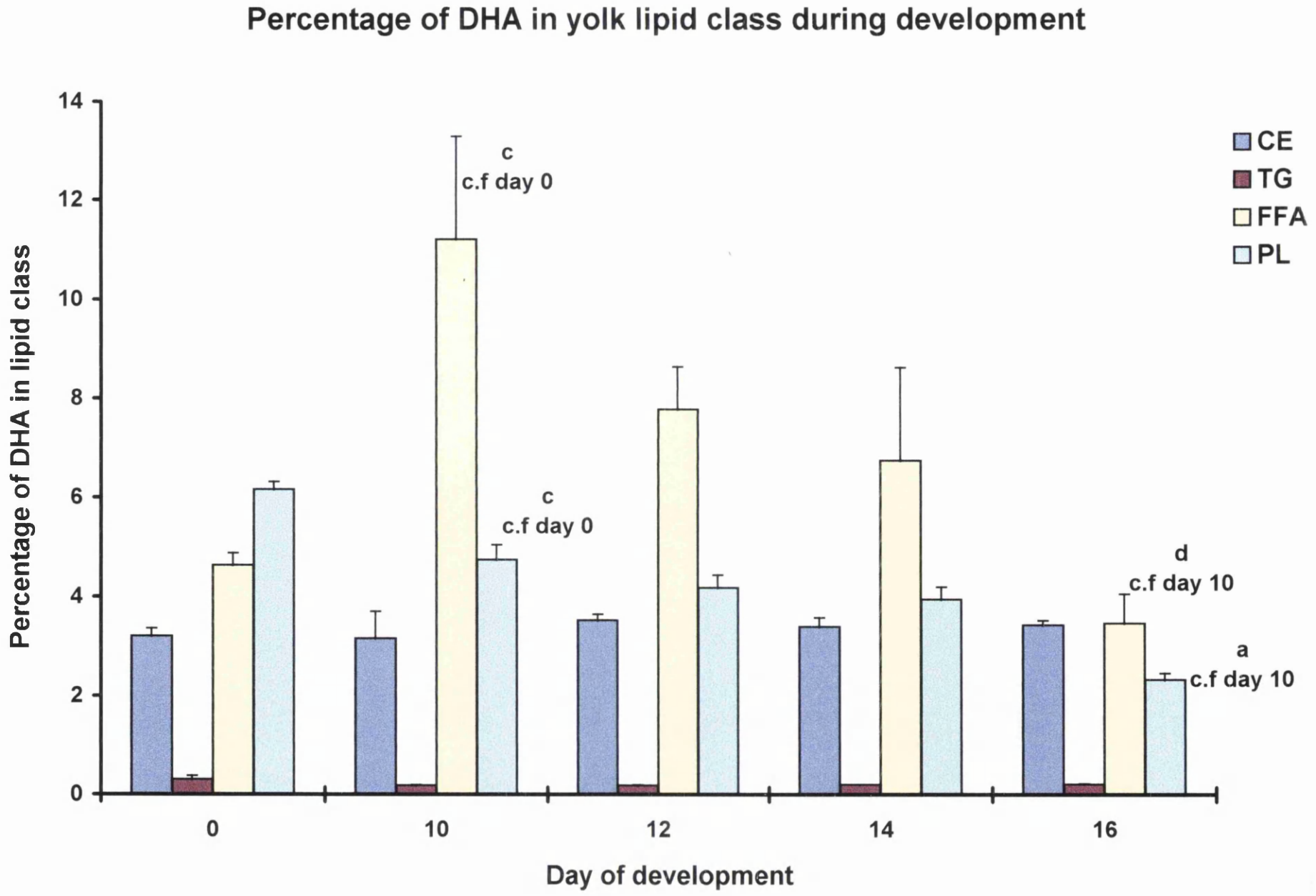
Table 12: **Fatty acid composition (% w/w of total lipid) of yolk phospholipid. Values are means ± S.E.M., n=4**

Days of development	0	10	12	14	16
Fatty acid (% w/w of total lipid)					
Palmitic	27.4 ± 0.9	28.7 ± 0.4	27.6 ± 0.2	29.1 ± 0.3	28.7 ± 0.3
Palmitoleic	0.93 ± 0.1	1.14 ± 0.1	1.06 ± 0.2	1.24 ±0.1	1.17 ± 0.1
Stearic	17.1 ± 1.3	15.9 ± 0.2	17.3 ± 0.3	16.3 ± 0.2	18.1 ± 0.4
Oleic	22.5 ± 1.4	23.4 ± 0.9	24.9 ± 0.9	24.4 ± 0.6	25.3 ± 0.8
Linoleic	15.2 ± 0.8	15.7 ± 0.6	14.8 ± 0.8	15.1 ± 0.3	15.4 ± 0.6
Linolenic	0.15 ± 0.0	0.17 ± 0.0	0.11 ± 0.0	0.13 ± 0.0	0.14 ± 0.02
Arachidonic	5.41 ± 0.4	5.33 ± 0.2	5.04 ± 0.1	4.91 ± 0.1	4.64 ± 0.1
Docosahexaenoic	6.16 ± 0.2	4.74 ± 0.3	4.18 ± 0.3	3.94 ± 0.3	2.32 ± 0.1

Table 13: **Fatty acid composition (% w/w of total lipid) of yolk free fatty acid. Values are means ± S.E.M., n=4**

Days of development	0	10	12	14	16
Fatty acid (% w/w of total lipid)					
Palmitic	18 ± 2.0	14.2 ± 0.9	19 ± 0.6	19.4 ± 1.5	19.6 ± 0.9
Palmitoleic	2.45 ± 0.3	1.51 ± 0.3	4.08 ± 0.9	4.46 ± 0.5	5.19 ± 0.8
Stearic	13.8 ± 1.4	11.9 ± 1.9	8.9 ± 1.6	6.87 ± 1.1	6.34 ± 1.3
Oleic	32.9 ± 0.5	28.6 ± 1.2	27.2 ± 1.5	27.3 ± 1.9	28.2 ± 2.3
Linoleic	17.5 ± 1.0	16.1 ± 0.9	19.8 ± 2.8	21.6 ± 0.8	22.6 ± 2.1
Linolenic	1.34 ± 0.0	1.33 ± 0.1	2.73 ± 0.5	3.27 ± 0.3	3.4 ± 0.1
Arachidonic	4.58 ± 0.4	6.68 ± 0.9	3.7 ± 0.8	2.86 ± 0.6	2.07 ± 0.8
Docosahexaenoic	4.63 ± 0.3	11.2 ± 2.1	7.78 ± 0.9	6.75 ± 1.9	3.47 ± 0.6

Figure 6. **Percentage of docosahexaenoic acid (DHA) (% w/w of total lipid) in the egg yolk lipid class during embryonic development. Results are means of 4 replicates + S.E.M. Statistical analysis was performed on each consecutive day, between day 0 and day 10 and between day 10 and day 16. $^{d}P<0.025$, $^{c}P<0.01$, $^{a}P<0.001$. Cholesteryl ester (CE), triacylglycerol (TG), free fatty acid (FFA), phospholipid (PL).**


Percentage of DHA in yolk lipid class during development
Percentage of DHA in lipid class
14
12
10
8
6
4
2
0
CE
TG
FFA
PL
c
c.f day 0
c
c.f day 0
d
c.f day 10
a
c.f day 10
0
10
12
14
16
Day of development

3.2. INVESTIGATION OF PHOSPHOLIPASE A_2 ACTIVITY DURING EMBRYONIC DEVELOPMENT.

An investigation of PLA_2 activity was carried out to establish the mechanism whereby DHA is transferred from the PL fraction to the FFA fraction in egg yolk and in the yolk sac membrane during development. The investigation was performed between day 10 and day 16 of development, coinciding with the highest rate of fatty acid transfer from the yolk to the yolk sac membrane. Results were expressed as µmole of fatty acid released per minute per mg of tissue.

3.2.1. Phospholipase A_2 activity in the yolk.

The PLA_2 activity within the yolk is shown in ***Figure 7***. At day 0 no PLA_2 activity was detected in the yolk. A significant increase in PLA_2 activity was found however on day 10. This activity remained high throughout the remaining developmental period.

3.2.2. Phospholipase A_2 activity in the whole yolk sac membrane.

The PLA_2 activity within the yolk sac membrane is shown in ***Figure 8***. PLA_2 activity was present at all stages examined, the activity was found to be low (6.80 ± 1.50 µmole of fatty acid release/min/mg of protein) at day 10. The PLA_2 activity then increased significantly from day 10 reaching a plateau on day 12 which was maintained to at least day 16.

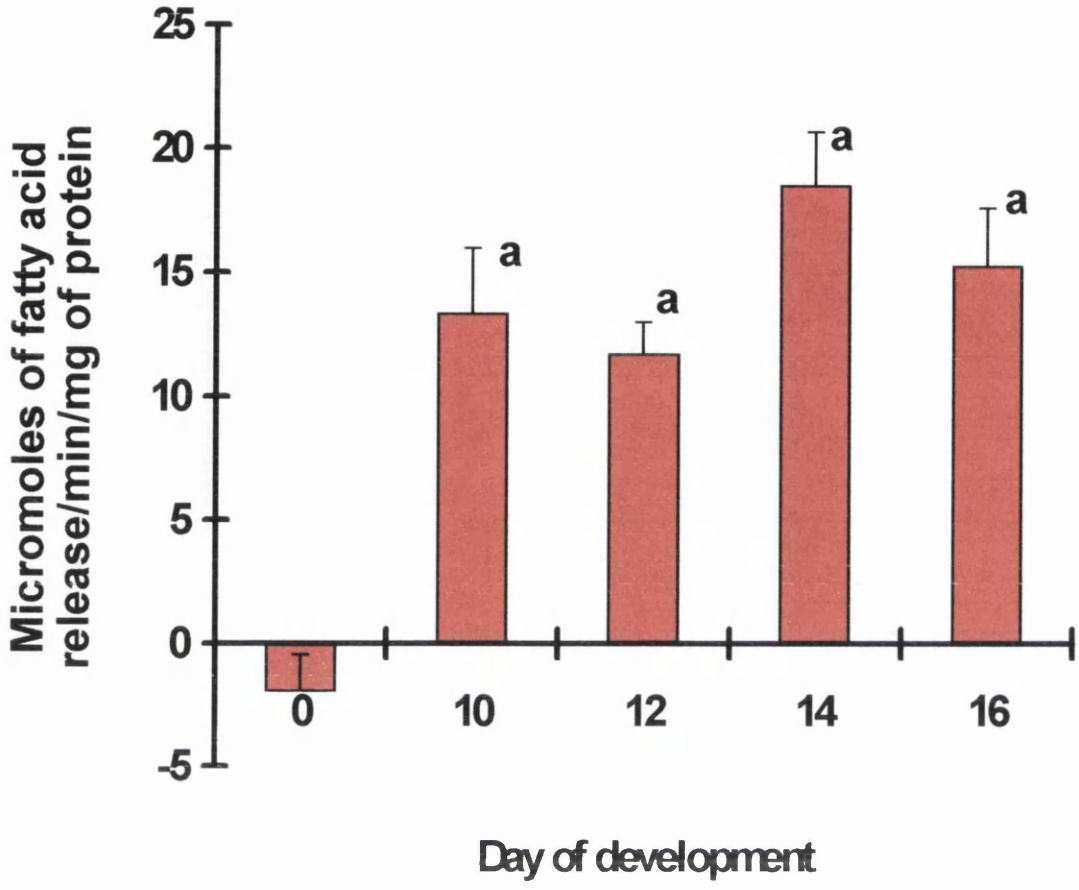


Figure 7: **Phospholipase A_2 activity (μmoles of fatty acid release/ min/ mg of yolk protein) in the yolk during embryogenesis. Values are means + S.E.M., of at least five replicate sample. Statistical analysis was performed by comparison to day 0. $^{a}P<0.001$.**

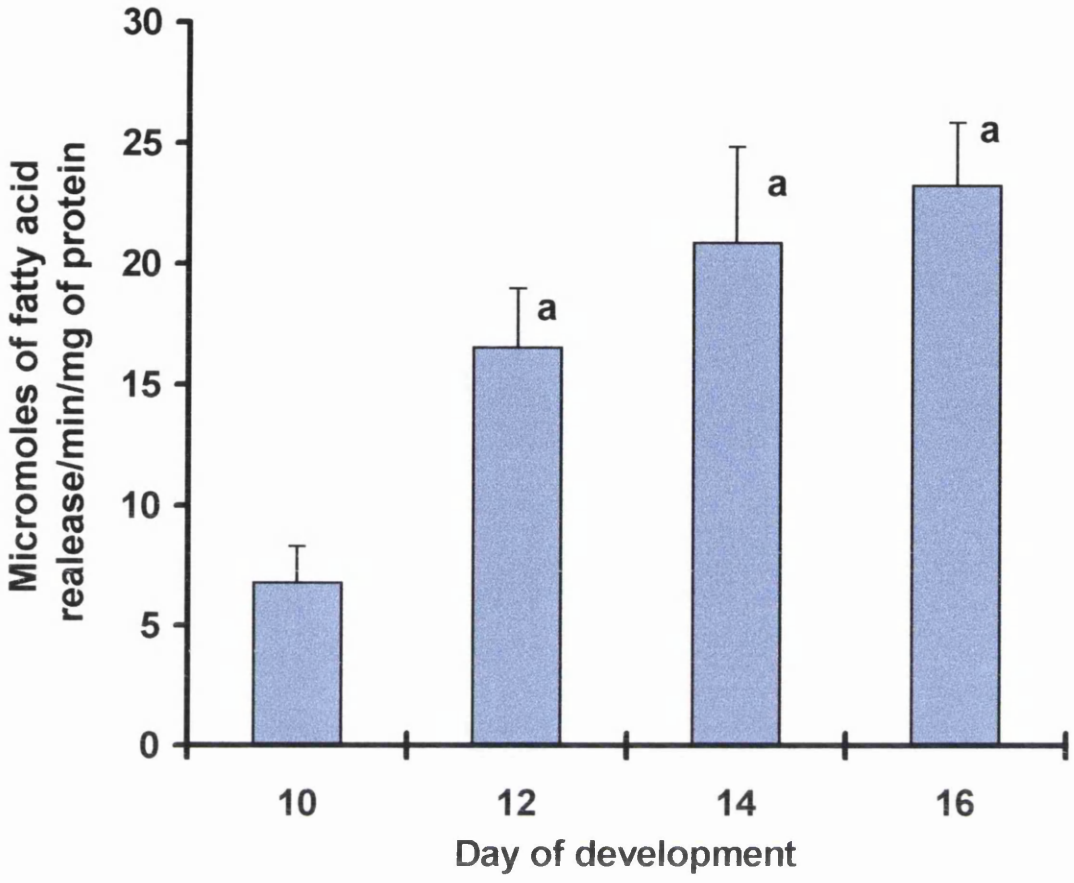


Figure 8: **Phospholipase A_2 activity (μmoles of fatty acid release/ min/ mg of yolk sac membrane protein) in the whole yolk sac membrane. Values are means + S.E.M. of at least 4 replicates. Statistical analysis was performed by comparison to day 10. $^{a}P<0.001$.**

3.3. ACTIVITIES OF SOME KEY ENZYMES INVOLVED IN LIPID METABOLISM IN YOLK SAC MEMBRANE AND LIVER.

An investigation of the fatty acid metabolism of the yolk sac membrane and liver during embryonic development has been undertaken. This study involved examining the activities of the following key regulatory enzymes of oxidation and acyl glycerolipid synthesis: carnitine palmitoyltransferase (CPT I), monoglycerol acyltransferase (MGAT), glycerolphosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT).

3.3.1 CPT I activity in mitochondria of the yolk sac membrane during embryonic development and sensitivity to malonyl-CoA.

As shown on ***Figure 9*** CPT I activity was determined between day 10 and day 18 of embryonic development. This period corresponded to the high rate of lipid transfer from the yolk to the yolk sac membrane. Maximal CPT I activity was found on day 12 of development, 145 nmoles/min/mg of protein. CPT I activity then significantly declined to 69 nmoles/min/mg of protein on day 18. The sensitivity of CPT I to inhibition by malonyl CoA is shown in ***Table 14***. CPT I remain relatively insensitive to malonyl CoA throughout development. However there was a significant increase in its sensitivity to malonyl CoA on day 14.

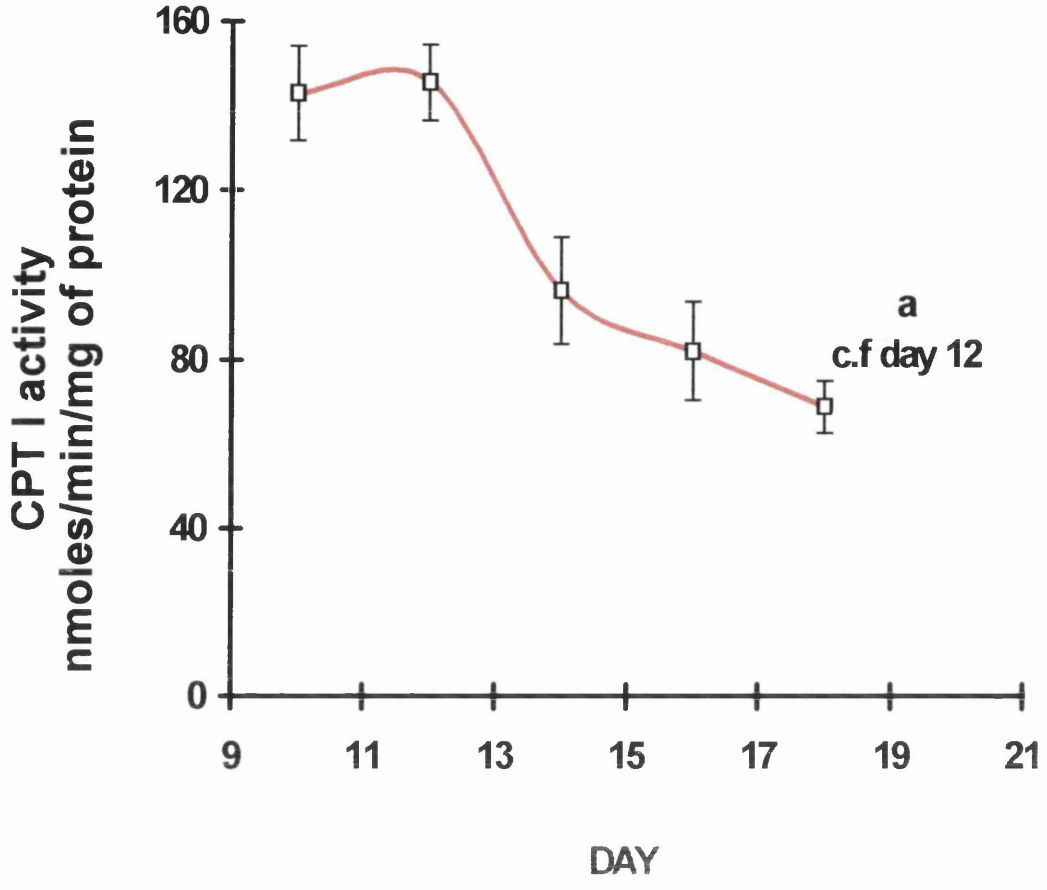


Figure 9: **CPT I activity in the yolk sac membrane mitochondria. Values are expressed as means ± S.E.M. of 4 replicates. Statistical analysis was carried out by comparing values of day 12 and day 18,** $^{a}P<0.001$.

Table 14: **Sensitivity of CPT I to malonyl-CoA inhibition (mM of malonyl CoA for 50% of inhibition). Values are means ± S.E.M. of at least 3 replicates. cf day 12 [a]P<0.001.**

SENSITIVITY OF CPT I TO MALONYL COA INHIBITION	
Days of development	**ID_{50} Malonyl CoA (mM)**
10	18.56 ± 3.05
12	26.67 ± 1.85
14	18.84 ± 0.51[a]
16	28.96 ± 6.51
18	24.79 ± 4.60

3.3.2. MGAT activity in yolk sac membrane and liver during embryonic development.

MGAT activity was present in both yolk sac membrane and liver. Results are presented on ***Figure 10*** and ***11***. The activities are expressed as nmoles/min/mg of microsomal protein. The MGAT activity found in the yolk sac membrane was very high compared to that found in the liver (at day 12: 285 ± 13.2 and 25 ± 4.34 nmols/min/mg of protein, respectively). In the yolk sac membrane, the MGAT activity remained constant at around 248 nmoles/min/mg of microsomal protein throughout development. However at hatching the MGAT activity fell significantly to 144 nmoles/min/mg of microsomal protein. In the liver, MGAT activity significantly increased to a peak on day 19 and decreased significantly at hatching.

3.3.3. GPAT activity in the yolk sac membrane and the liver during embryonic development.

Results are presented on ***Figure 12*** and ***Table 15*** and activities are expressed as nmoles/min/mg of either microsomal or mitochondrial protein. GPAT activity was associated with predominantly the microsomal fraction but also in the mitochondrial fraction in the yolk sac membrane. Microsomal GPAT was 10 fold greater than that found in the mitochondria. Microsomal GPAT activity peaked significantly on day 12 at 26.6 nmoles/min/mg of protein and then steadily declined to an activity of 6.6 nmoles/min/mg of protein at hatching. Similarly, GPAT activity was found in both microsomal and mitochondrial fractions in the liver. In comparison GPAT activity was very low compared to that found in the yolk sac membrane. A significant difference was found between microsomal GPAT activity on day 10 and that found on day 22. Mitochondrial activity remained low and did not change significantly during development.

3.3.4 DGAT activity in the yolk sac membrane and the liver during embryonic development.

DGAT activity (nmols/ min/ mg of microsomal protein) was found in both yolk sac membrane and liver tissues (***Table 16*** and ***Figure 15***). In the yolk sac membrane, DGAT activity did not significantly change during embryonic development remaining at around 30 nmoles/min/mg of protein. However in the liver, DGAT activity was lower than found in the yolk sac membrane. Moreover an increase in DGAT activity was noted as development proceeded between day 14 and day 17, 9.55 ± 2.55 and 22.4 ± 1.45 nmoles/min/mg of microsomal protein respectively.

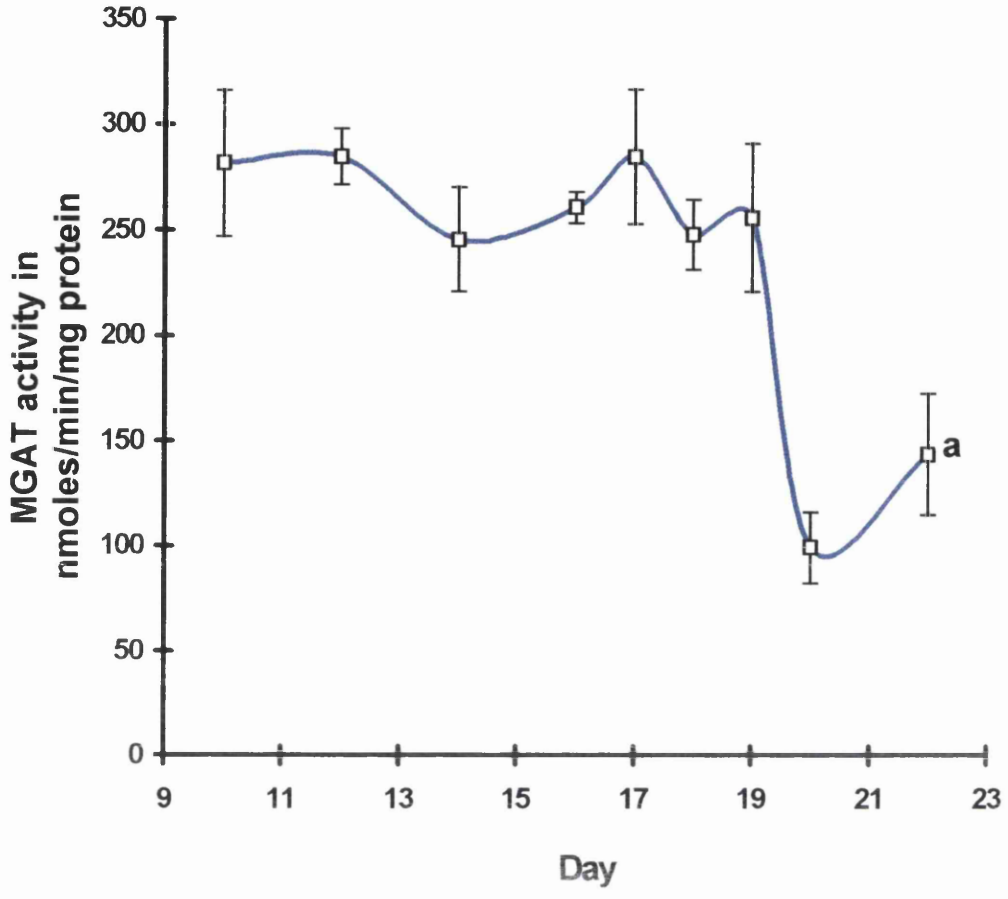


Figure 10: **MGAT activity in the yolk sac membrane microsomes. Results are means of 4 replicates ± S.E.M. Statistical analysis was carried out by comparing day 12 and day 22. [a]P<0.001**

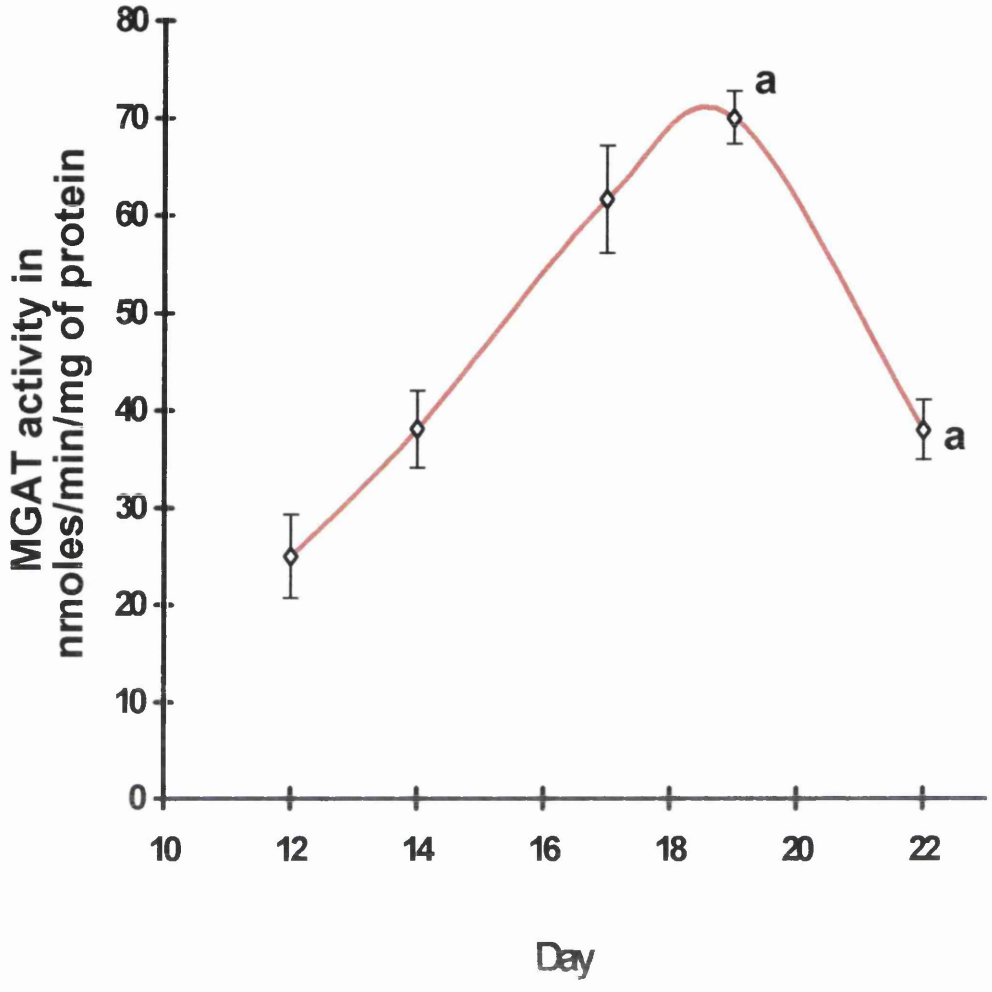


Figure 11: **MGAT activity in the liver during embryonic development. Results are means of 4 replicates ± S.E.M. Statistical analysis was carried out by comparing values between day 12 to day 19 and day 19 to day 22.** $^{a}P<0.001$.

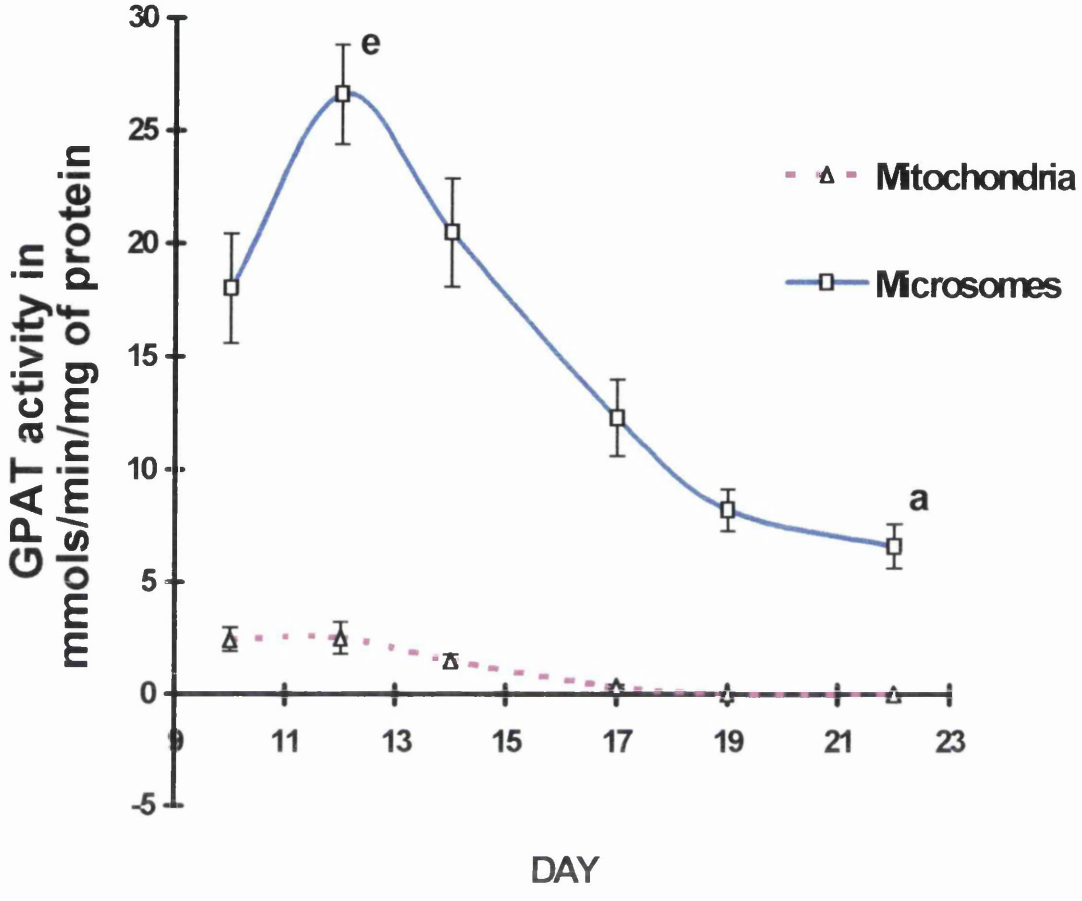


Figure 12: **GPAT activity in the yolk sac membrane during embryonic development. Results are means ± S.E.M. of at least 4 replicates. Statistical analysis was carrying out by comparing day 10 to day 12 and day 12 to day 22. $^{e}P<0.05$, $^{a}P<0.001$.**

Table 15: **GPAT activity in the liver during embryonic development. Results are means ± S.E.M. of at least 4 replicates. Statistical analysis was performed, no significant differences were found.**

Day of development	10	12	14	17	19	22
GPAT activity						
nmoles/min/mg of protein						
Microsomes	2.5 ± 0.3	2.6 ± 0.1	3.0 ± 0.3	3.1 + 0.4	2.5 ± 0.3	3.6 ± 0.3
Mitochondria	1.4 ± 0.3	0.5 ± 0.1	0.4 ± 0.0	1.1 ± 0.1	0.6 ± 0.0	1.2 ± 0.1

Table 16: **DGAT activity in the yolk sac membrane during embryonic development. Results are means of 4 replicates ± S.E.M. Statistical analysis was performed, no significant differences were found.**

Day of development	10	12	14	17	19	22
DGAT activity						
(nmoles/min/mg of protein)						
Yolk	27.3 ± 4.1	33.5 ± 8.5	36.1 ± 4.4	25.7 ± 8.6	25.7 ± 7.8	28.8 ±
sac membrane						

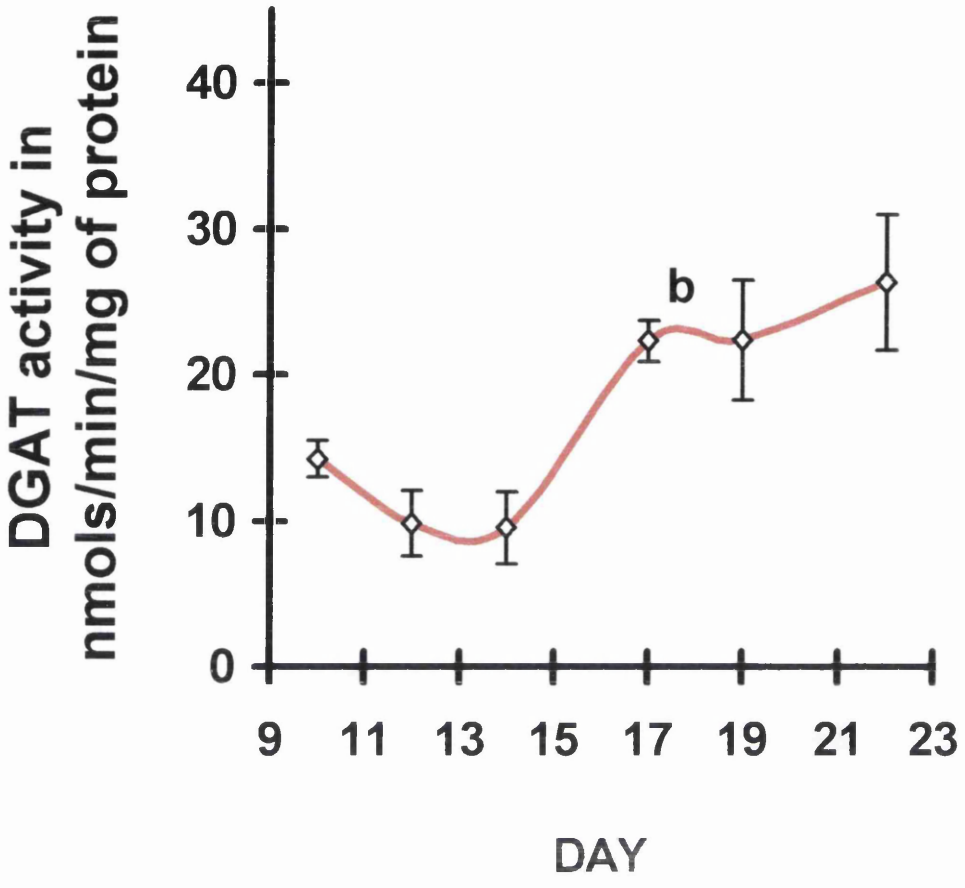


Figure 13: **DGAT activity in the liver during embryonic development. Results are means of 4 replicates ± S.E.M.. Statistical analysis was carried out. $^{b}P<0.005$ cf day 14**

3.4. FATTY ACID COMPOSITION OF PLASMA LIPOPROTEINS FROM DEVELOPING CHICK EMBRYO.

Analysis of lipid class and lipid composition of blood lipoprotein species very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), high density lipoprotein (HDL) were performed on day 14 and one day post hatch (day 22). Most of the plasma lipid is associated with the VLDL fraction on both days 14 and 22 (***Table 17***). In contrast, the IDL fraction accounts for only a small percentage of plasma lipid. On day 14 the LDL fraction is the second major contributor of plasma lipid, whereas the HDL fraction contributes only 8.5 %. In contrast, on day 22 the lipid associated with the LDL is reduced and a dramatic increase in the lipid contribution by HDL is noted.

Analysis of the lipid composition of day 14 lipoproteins shows a pattern of changes characteristic of lipoprotein metabolism. Such that the percentage of TG declines in accordance with LPL action, and there is a reciprocal increase in the percentage of PL. Interestingly the percentage of CE increased almost 5 fold from VLDL to HDL.

The lipid profile of day 22 lipoproteins was slightly different from that found on day 14. Namely, the percentage of TG in the VLDL fraction was considerably lower, whereas the percentage of cholesteryl ester is 6 fold higher. The reciprocal changes in TG and PL are again noted, reflecting the action of LPL. The subtle differences suggest that the lipoproteins have a different origin. The day 14 lipoproteins possibly originated from the yolk sac membrane and the day 22 lipoproteins possibly originated from the liver.

Table 18 and ***19*** details the fatty acid composition of each lipid moiety in the different lipoproteins. In general there were no major differences in the fatty acid lipid composition on day 14 and day 22. Moreover, the fatty acid composition of the TG fraction was found to be similar in all lipoproteins, indicating a non-specific action of LPL. However, there is some evidence to suggest that LPL is less selective towards

TG containing DHA, since a progressive enrichment of this fatty acid was observed from VLDL through to HDL. DHA also made a significant contribution to the free fatty acid fraction. However, since this fraction is a minor part of the total lipid the absolute amounts of DHA would be necessarily small.

Table 17: **Composition of lipoprotein (% w/w of lipid class) in embryonic blood at day 14 and one day post hatch (day 22).**

Days of development	14				22			
	VLDL	**IDL**	**LDL**	**HDL**	**VLDL**	**IDL**	**LDL**	**HDL**
Lipoprotein fraction (%, w/w of total lipid)	**49**	**7.5**	**35**	**8.5**	**45**	**4**	**28**	**23**
Lipid class (%, w/w of total lipid)								
Phospholipid	16.6	23	22.5	35.4	13.9	17.8	23.2	38.2
Free fatty acid	0.71	3.1	0.69	2.55	0.6	6.8	0.9	2.3
Triacylglycerol	71.1	48.6	31.5	21.6	31.8	13.4	7.9	6.2
Cholesteryl ester	6.62	15.6	33.5	32.5	43.8	53.3	53.6	40.1
Free cholesterol	4.96	9.63	11.9	7.96	9.8	8.8	14.4	13.1

Table 18:

Fatty acid composition at day 14 (% w/w of total lipid) of lipid class in lipoprotein species: VLDL, (very low density lipoprotein), IDL (intermediate density lipoprotein), LDL (low density lipoprotein), HDL (high density lipoprotein). Analysis expressed results of plasma pooled from 20 embryos.

	VLDL				IDL				LDL				HDL			
Lipid class	**PL**	**FFA**	**TG**	**CE**	**PL**	**FFA**	**TG**	**CE**	**PL**	**FFA**	**TG**	**CE**	**PL**	**FFA**	**TG**	**CE**
Total lipid (mg)	**2.06**	**0.09**	**8.84**	**0.82**	**0.47**	**0.06**	**0.99**	**0.32**	**2.15**	**0.07**	**3.01**	**3.21**	**0.80**	**0.06**	**0.49**	**0.73**
Fatty Acid (% w/w of total lipid)																
Palmitic acid	30.2	25.9	28.4	6.5	31.2	22.7	26.4	7.3	32.9	30.9	25.2	12.4	31.6	34.5	28.4	17.0
Palmitoleic acid	0.7	2.0	2.8	2.4	0.7	1.5	2.4	1.8	0.7	1.1	1.5	2.1	0.7	1.6	1.5	2.2
Stearic acid	17.5	13.0	7.0	3.8	16.7	13.5	8.4	3.9	16.0	19.5	9.3	3.6	16.1	13.4	10.1	3.3
Oleic acid	12.7	26.1	33.4	62.2	14.7	25.2	32.6	60.2	14.0	21.4	31.2	51.4	13.1	24.9	30.6	36.7
Linoleic acid	21.5	9.0	13.2	15.3	21.3	10.3	10.9	17.9	21.3	5.7	9.4	22.4	19.1	7.0	8.6	31.5
Linolenic acid	0.0	0.6	0.6	0.7	0.0	0.6	0.4	0.6	0.0	0.0	0.3	0.5	0.0	0.3	0.3	0.3
Arachidonic acid	11.8	2.0	2.0	2.2	10.3	2.7	3.1	2.3	10.4	2.4	4.5	3.0	11.5	1.8	3.8	3.6
Eicosapentaenoic acid	0.3	0.5	0.3	0.8	0.3	1.3	0.5	0.7	0.3	0.4	0.8	0.9	0.3	0.2	0.7	0.7
Docosapentaenoic acid	0.1	1.1	0.9	0.3	0.0	1.4	1.0	0.1	0.2	0.7	1.1	0.2	0.1	0.6	1.0	0.1
Docosahexaenoic acid	2.2	9.6	6.9	2.3	1.5	13.7	9.9	1.7	1.5	7.8	12.6	1.9	2.0	6.1	10.8	1.7

Table 19:

Fatty acid composition at day 22 (% w/w of total lipid) of lipid class in lipoprotein species: VLDL, (very low density lipoprotein), IDL (intermediate density lipoprotein), LDL (low density lipoprotein), HDL (high density lipoprotein). Analysis expressed results of plasma pooled from 20 embryos.

	VLDL				IDL				LDL				HDL			
Lipid class	**PL**	**FFA**	**TG**	**CE**	**PL**	**FFA**	**TG**	**CE**	**PL**	**FFA**	**TG**	**CE**	**PL**	**FFA**	**TG**	**CE**
Total lipid (mg)	**2.56**	**0.11**	**5.87**	**8.08**	**0.27**	**0.10**	**0.20**	**0.80**	**2.82**	**0.12**	**0.96**	**6.51**	**3.79**	**0.23**	**0.61**	**3.98**
Fatty Acid (% w/w of total lipid)																
Palmitic acid	27.1	32.0	29.4	7.0	27.5	28.9	28.2	8.4	28.1	31.3	25.6	10.6	25.0	21.7	26.3	12.0
Palmitoleic acid	0.9	1.6	2.4	2.2	0.8	2.1	1.7	2.1	0.9	1.0	1.2	2.1	0.8	1.0	1.2	2.0
Stearic acid	20.3	17.0	8.4	4.1	19.6	9.4	11.5	4.3	19.8	21.9	10.2	3.7	19.9	18.0	9.9	2.9
Oleic acid	15.5	24.6	37.4	61.9	15.6	27.9	38.5	53.1	15.3	23.0	31.7	45.5	14.5	33.9	31.0	38.1
Linoleic acid	20.0	9.1	12.8	18.2	19.3	13.7	9.9	22.8	20.0	8.8	10.9	28.2	19.3	8.9	10.9	34.9
Linolenic acid	0.2	0.5	0.5	0.6	0.2	0.5	0.4	0.6	0.1	0.2	0.3	0.6	0.1	0.3	0.3	0.7
Arachidonic acid	9.7	2.0	1.4	2.5	9.1	2.3	1.4	3.1	9.7	1.4	3.7	4.0	11.6	2.3	3.8	3.8
Eicosapentaenoic acid	0.3	0.7	0.2	0.6	0.3	0.5	0.1	0.7	0.3	0.5	0.6	1.1	0.4	0.3	0.6	0.8
Docosapentaenoic acid	0.3	0.7	0.6	0.1	0.4	1.1	0.3	0.2	0.2	0.5	2.6	0.3	0.6	0.6	2.6	0.2
Docosahexaenoic acid	2.7	4.2	1.9	1.2	2.2	4.6	1.3	1.3	2.6	3.9	8.1	1.7	3.0	4.0	8.2	1.5

DISCUSSION

INTRODUCTION

An inadequate supply of PUFAs may result in irreversible impairment of brain and retinal function. A recent major conference on the role of fatty acids in human health (Fourth International Congress on Essential Fatty acids and eicosanoids, Edinburgh, Scotland, July 1997) implicated PUFA as an important factor in a number of illnesses such as alcoholism, schizophrenia, diabetes, ischaemic heart, disease and cancer. The formation of neural tissues depends on a continuous supply of essential fatty acids, n-3 and n-6 (linolenic acid and linoleic acid based respectively) at a critical period of development. In mammalian species during development, adequate amounts of these essential fatty acids cross the placenta to meet the demands of the foetus. In the avian it is very different, once the egg has been laid the embryo has to rely on pre-packaged nutrients to meet its development needs. Moreover, delivery of essential fatty acids such as DHA to the developing embryo must be co-ordinated to meet the demands of various tissues (Noble, R.C. & Cocchi, M. 1989a).

The aim of this study was to examine the metabolic processes underlying the fatty acid compositional changes occurring during avian embryo development (Maldjian, A. 1996). The study encompassed three distinct stages of PUFA transfer namely (1) uptake of fatty acids from the yolk by the yolk sac membrane, (2) analysis of the enzymatic machinery in fatty acid remodelling between different lipid moieties, and (3) delivery of fatty acids to the developing embryo.

4.1. UPTAKE OF POLYUNSATURATED FATTY ACID FROM THE YOLK.

The transfer of fatty acids from the yolk to the yolk sac membrane occurs by non specific phagocytosis. Yolk lipids are trapped by the apical microvilli and they are then enclosed within apical vesicles and appear within the yolk sac membrane (Noble, R.C. & Cocchi, M. 1990). As discussed previously DHA uptake from the yolk does

not follow the general pattern of other fatty acids (Noble, R.C. & Cocchi, M. 1990; Maldjian, A. *et al* 1995). Analysis of the yolk has also shown that there is a significant disappearance of DHA from the PE fraction during development and that this appears to be specific for DHA (Noble, R.C. & Moore, J.H. 1967b). This study lends support to these earlier findings. Analysis of the yolk lipid composition shows that the percentage of DHA associated with the free fatty acid fraction significantly increased and there is an associated decrease in the DHA associated with the PL fraction. As development proceeds there is a steady loss of DHA from the FFA fraction and this correlates with the uptake of DHA by the yolk sac membrane (Maldjian, A. 1996).

4.1.1 Phospholipase A_2.

As C_{20}- C_{22} fatty acids are esterified at the second position of the glycerol backbone, it has been proposed therefore that the decrease in the percentage of DHA associated with the phospholipid fraction and its appearance in the free fatty acid fraction may be the result of PLA_2 action. In support of this concept, PLA_2 activity was found in the yolk at day 10, although no activity was measured on day 0. This increase in enzyme activity in the yolk during development at a time when the percentage of DHA in the free fatty acid fraction increase's, supports a role of PLA_2 in the uptake of DHA. DHA has been shown to be preferentially released as compared to other C_{22} fatty acids and mono- and di-unsaturated C_{18} fatty acids, from sheep erythrocyte phospholipids (Van Den Bosch, H. 1980). Detailed analysis of yolk PL has shown that it is the PE species that is preferentially hydrolysed. Although no substrate preference has been documented for PLA_2 it is perhaps noteworthy that the PLA_2 activity reported here was carried out using a probe esterified to PE. Therefore yolk PLA_2 will hydrolyse PE, however no conclusions can be drawn with respect to its selectivity without analysing its activity against a PC substrate.

PLA_2 has been shown to be secreted by exocrine glands such as the pancreas, or be membrane bound as is found in sheep erythrocytes (Van Den Bosch, H. 1990; Dennis,

E.A. 1983). Therefore the yolk sac membrane was analysed for PLA_2 activity as a probable source of the yolk enzyme. Analysis of the PLA_2 activity in the yolk sac membrane was shown to be similar to that found in the yolk although the level of activity found on day 10 was considerably lower. PLA_2 activity in the yolk sac membrane increased to a plateau level at day 14. The PLA_2 activity in the yolk was constant between day 10 and day 16. On the basis of these results it is proposed that the yolk sac membrane may secrete PLA_2 into the yolk at around day 10. However, in order to substantiate this a more detailed analysis of the PLA_2 species in the yolk and the yolk sac membrane is required. For instance, purification of the enzymes would enable a more detailed comparison.

In this study it was found that PLA_2 was Ca^{2+} dependant. For PLA_2 to have a physiological role in the mobilisation of essential fatty acids there therefore must be an adequate Ca^{2+} concentration. It has been shown that the yolk Ca^{2+} concentration increases significantly at around day 10 (Johnston & Camar 1955), coincident with the reported increases in PLA_2 activity. At this time it can be concluded that PLA_2 may play an important role in the uptake of essential fatty acid from the yolk by the yolk sac membrane.

4.2. METABOLISM OF POLYUNSATURATED FATTY ACID IN THE YOLK SAC MEMBRANE.

Fatty acids required for the development of the embryo are supplied by the yolk. The yolk provides synthetic maternal and a source of energy for the developing embryo. The total energy requirements for embryogenesis are derived from the oxidation of fatty acids. It has been found that some essential PUFA, namely DHA and AA, appear to be preserved and are not oxidised. A high percentage of the initial yolk content of AA and DHA was recovered in the newly hatched chick, compared to the major C_{16} and C_{18} fatty acids (Maldjian, A. 1996). Alternatively, *de novo* synthesis of these fatty acids is possible due to the existence of a desaturation/elongation system. *De novo* synthesis of DHA is unlikely however due to the low level of the substrate, linolenic acid. However *de novo* synthesis of AA may occur due to the presence of linoleic acid, the substrate. Within the yolk sac membrane it has been found that PUFAs are remodelled between lipid moieties, for example DHA is found associated with the PL fraction in the yolk but associated with the TG in the plasma. All these metabolic changes suggest a major role of the yolk sac membrane in the successful co-ordination of fatty acid transfer to the developing embryo. One aim of this study was to investigate how PUFAs are transferred to the embryo without being oxidised for energy. The existence and activity of rate limiting enzymes of the oxidative and esterification pathways were investigated to determine the possible metabolic fate of PUFAs and their possible regulation during development.

4.2.1. Oxidation pathway

The rate of β-oxidation was determined by measuring the activity of the rate limiting enzyme carnitine palmitoyltransferase I (CPT I). The yolk sac membrane expressed considerably high activity of CPT I compared to that found in other animals tissues, for example, liver CPT I activity in the starved refed rat was 44 nmoles/min/mg of protein (Granthan, B.D. & Zammit, V.A. 1986), and in the rat heart CPT I activity

was 35 nmoles/min/mg of protein. The metabolic processes taking place within the yolk sac membrane will place a high demand on energy provision therefore it is not surprising that CPT I activity is high to insure an adequate supply of energy. CPT I activity declines from day 12 which presumably parallels the yolk sac membranes decreasing energy demands. Despite this high oxidative activity in the yolk sac membrane, major PUFAs are protected from oxidation. It may be that the substrate specificity of yolk sac membrane CPT I allows selective retention of PUFAs. In rat heart CPT I was shown to prefer palmitoyl-CoA compared to docosahexaenoyl-CoA (Nada, A.M., Abdel-Aleem, S. & Schulz, H. 1995). Moreover in the rat liver, the rate of oxidation of C_{20} fatty acid was lower than with other unsaturated fatty acid such as oleic acid, linoleic acid and α-linoleic acid. These differences were explained by the selective incorporation of C_{20} into membrane phospholipids (Leyton, J., Drury, P.J. & Crawford, M.A. 1987). Inhibition by malonyl-CoA is known to be a mechanism by which mitochondrial oxidation of long chain fatty acids can be regulated (Schulz, H. 1991). Yolk sac membrane CPT I was shown to be more sensitive to malonyl-CoA at day 14. This change takes place exactly at the time when CPT I activity decrease from 145 to 96 nmol/min/mg of mitochondrial protein. This feature may have a major role on PUFA protection against oxidation, especially since the rate of DHA uptake from the yolk is maximal at around this time.

4.2.2. Esterification pathways.

Fatty acid esterification is possible via acylation of glycerol 3-phosphate or acylation of monoacylglycerol phosphate. Each pathway however ultimately results in the formation of diacylglycerol which can then be acylated by DGAT to form triacylglycerol or diverted to phospholipid formation. The rate limiting enzymes for both the glycerol 3-phosphate and monoglycerol phosphate pathways were found in the yolk sac membrane, therefore fatty acid esterification by either route is metabolically feasible. Moreover the presence of DGAT activity in the yolk sac membranes completes the TG synthetic pathway. Yolk sac membrane DGAT activity showed no fluctuation during development, the activity of this tissue to synthesise

triacylglycerol from diacylglycerol does not appear to be regulated at least at the enzyme level during development. The activity of yolk sac membrane DGAT must be sufficient to allow adequate acylation of diacylglycerol to supply the need for TG for lipoprotein synthesis.

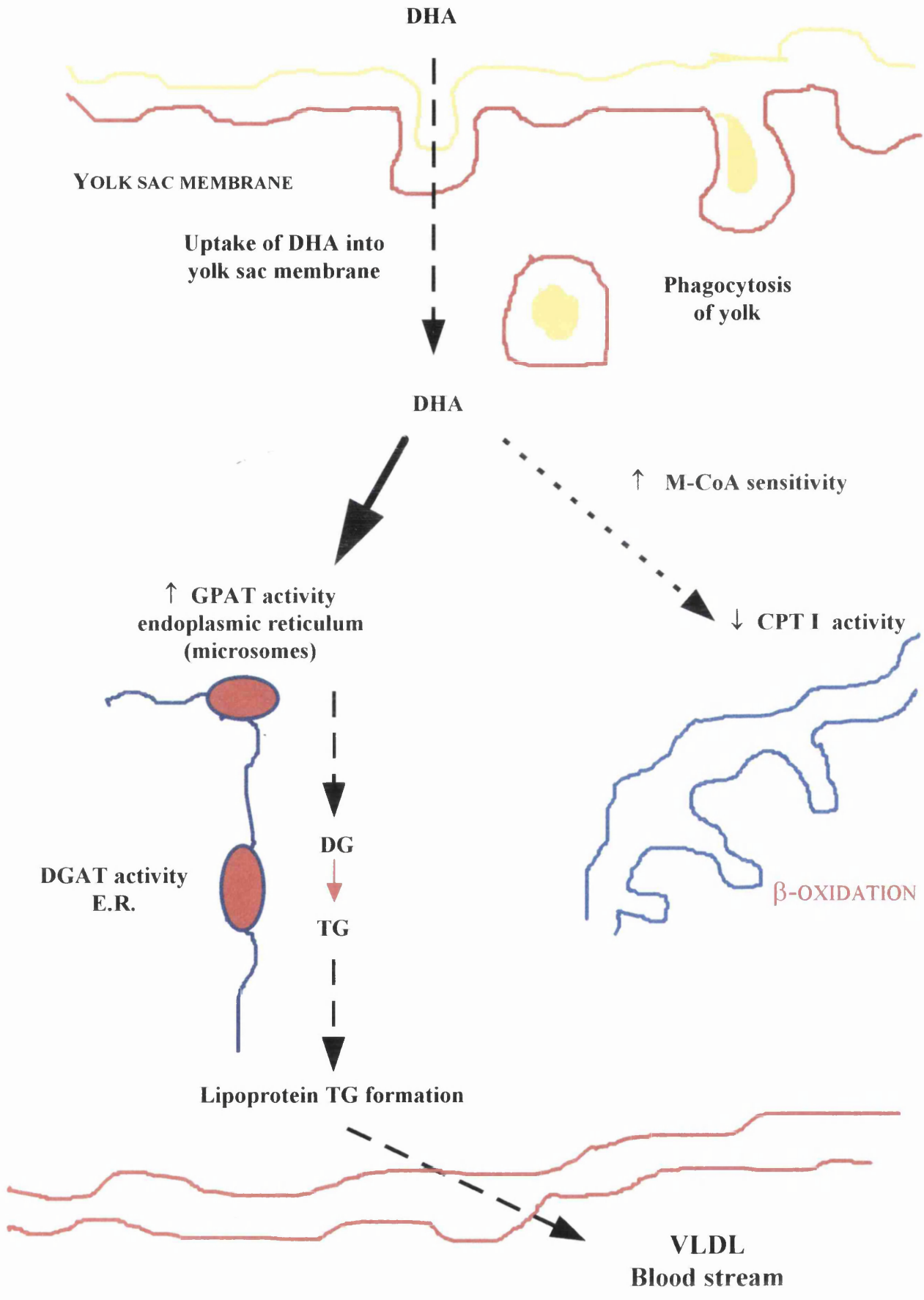
GPAT and MGAT were present at all stages of development in the yolk sac membrane. The MGAT activity showed no fluctuation during development and was at least 10 fold greater than that found for GPAT activity, suggesting that in this tissue the MGAT athway may be of greater importance for glycerolipid synthesis that the GPAT pathway. However, it is noteworthy that the MGAT pathway does not appear to be regulated at least at the enzyme level. As suggested previously, the MGAT pathway may be a way of selectively retaining essential fatty acids (Tian Xia, *et al* 1993). MGAT used monoacylglycerol as its substrate with the fatty acid esterified at the 2 position. DHA is present in the second position of PE. If the MGAT pathway is the mechanism whereby DHA is selectively preserved then the PE containing DHA must first be acted on by a PLA_1 and then presumably a phospholipase C, to provide a suitable substrate. The yolk sac membrane PLA_2 suggests an alternative mechanism. The activity of yolk sac membrane GPAT was found to be regulated during development with a peak at day 12. Therefore this may be an important route for PUFA reesterification if the PUFA is in the form of a free fatty acid, as suggested in the case of DHA. Maximum rates of DHA uptake from the yolk occurred at around day 12, if DHA is taken up as a free fatty acid, the GPAT pathway may be the preferred esterification route. Moreover at this time CPT I activity was found to decrease which may contribute to free fatty acids being directed towards esterification and away from oxidation. However, as discussed earlier no information was obtained with respect to substrate specificity of either CPT I or GPAT and this would obviously dictate the fate of certain fatty acids.

Diagram 5:

Suggested mechanism by which DHA present in the yolk is transferred into triacylglycerol in the yolk sac membrane. Docosahexaenoic acid (DHA); malonyl-CoA (M-CoA); carnitine palmitoyltransferase I (CPT I); glycerol acyltransferase (GPAT); diacylglycerol acyltransferase (DGAT); free fatty acid (FFA); phospholipase A_2 (PLA_2).

YOLK

Phospholipase A_2: action; release of DHA as FFA.



4.3. DELIVERY OF POLYUNSATURATED FATTY ACID TO THE TISSUES.

Clearly one of the functions of the yolk sac membrane is to supply the embryo with adequate levels of C_{20} and C_{22} polyunsaturated fatty acids, since deficiencies in the supply of DHA during the relevant period of development are associated with irreversible impairment of neural development (Neuringer, M., Anderson, G.J. & Connor, W.E. 1988). With respect to the supply of DHA, the transfer of this fatty acid is possible by the simultaneous action of PLA_2 in the yolk and an esterification system able to divert essential fatty acid from oxidation. The delivery of PUFAs to the embryo was studied by carrying out a lipoprotein analysis at day 14 and day 22.

4.3.1. Adipose tissue and lipoprotein lipase activity.

A constant provision of DHA is essential for the normal development of the brain, the results of a detailed study (Lin, D.S., Connor, W.E. & Anderson, G.J. 1991) have indicated that the levels of DHA do not respond proportionally to major changes in the yolk content of this fatty acid. These authors suggested that the brain PL may have an optimal fatty acid composition that is compatible with normal functional development. Furthermore, it was suggested that to maintain this optimal composition, the brain may possess the capability to regulate its incorporation of PUFAs in response to changes in provision from the yolk. But having established that lipoprotein lipase activity is not expressed by the brain of the chick embryo, PUFA delivery must occur via an alternative mechanism and if such mechanism is mediated at the level of PUFA transport from the yolk to the brain, the adipose tissue seems to have an important role. High lipoprotein lipase activity is expressed by adipose tissue and the fatty acids released by this enzyme are taken up by the adipose tissue for storage (Speake, B.K. *et al* 1993). In accordance with LPL action the percentage of TG in the metabolised lipoproteins declines with a reciprocal increase in the percentage of PL. However an increased percentage of DHA associated with TG

within the resulting lipoprotein particles was found. This is in accordance to the study of Melin *et al* (1991) who showed TG species containing C_{20}-C_{22} PUFA exhibited a degree of resistance to LPL action. However, the high LPL activity in the adipose tissue resulted in some incorporation of DHA and AA (Speake, B.K. *et al* 1991). In contrast to other major fatty acids present, *in vitro* studies have shown that DHA and AA are selectively mobilised from adipose tissue at the end of embryonic development (Speake, B.K. *et al* 1997). Most of the fatty acids have been transferred from the yolk complex by this time, importantly the transfer of DHA is complete by day 19. The functional significance of this selective mobilisation and release of DHA from adipose tissue could be to constantly support the embryonic development of neural tissues (Noble, R.C. & Speake, B.K. 1997). The PUFA stored within adipose tissue may represent a reserve which allows a steady supply of essential PUFA's to the developing tissues at critical development points. It has been suggested that free fatty acid bound to albumin (or α-fetoprotein) is a preferred source of PUFA's for the brain (Anderson, G.J. *et al* 1988). Therefore release of DHA from adipose tissue as a free fatty acid could be a possible mechanism for delivery to neural tissues. Alternatively, free fatty acid released by the adipose tissue may be taken up by the liver which would then co-ordinate the supply to the developing tissues.

4.3.2. Role of the liver

Lipoprotein analysis showed that following the action of LPL the resulting lipoproteins were enriched in DHA within the TG faction (***Table 18***). This DHA could then be released by further lipase action, although not LPL, or the lipoprotein may be removed by the liver. However, our results do not support further hydrolysis since there was a progressive enrichment in the percentage of DHA associated with the TG in the metabolised lipoproteins. Previously it has been shown that liver DHA is initially associated with TG fraction at around day 14 (Maldjian, A. 1996) which suggests that the DHA enriched TG is cleared by the liver. As development proceeded this DHA was found to be gradually transferred from TG to PL (Nobel, R.C. & Cocchi, M. 1990). As discussed earlier the MGAT pathway has been

suggested to be responsible for protecting PUFAs from oxidation. The increase in liver MGAT activity up to day 19 of development suggests a possible role for this enzyme in the DHA remodelling between the lipid moieties. Moreover the low GPAT activity compared to the MGAT activity (at day 19, 2.5 ± 0.3 and 70 ± 2.7 nmoles/ min/ mg of microsomal protein respectively) suggests that this acylation pathway is less important. However the substrate specificity of each enzyme is not known, which would of course have important ramifications.

The hepatic accumulation of CE during embryonic development derives from the uptake of cholesterol rich lipoproteins secreted from the yolk sac membrane as well as the inefficiency of the embryonic liver in disposing of this excess material (Noble, R.C. & Cocchi, M. 1990; Speake, B.K. *et al.* 1993). A proportion of the CE may also be hydrolysed to free cholesterol through lysosomal fusion. Free cholesterol can then be incorporated into the liver cell membrane, or excreted as bile. Alternatively, the cholesterol can be esterified back to CE by the action of ACAT enzyme. Tarugi *et al* (1994) showed that the depletion of hepatic CE was coincident with an increased in hepatic synthesis of apoB and apoA-I and suggested that the liver syntheses and presumably secretes lipoproteins enriched in CE. The significant increase in hepatic DGAT activity from day 12 to day 19 (25 ± 4.3 and 70 ± 2.7 nmoles/min/mg of microsomal protein respectively) supports this view, demonstrating that the liver has the capacity for TG synthesis necessary for lipoprotein assembly. Moreover, analysis of plasma lipoproteins at day 22 showed that the percentage of CE associated with the VLDL fraction was considerably higher than that found on day 14. Secretion of lipoproteins from the liver with DHA associated with the PL fraction may be a possible route of DHA delivery to the brain (Scott, B.K. & Bazan, N.G. 1989). Analysis of lipoprotein at day 22 showed that DHA was ended associated with the PL fraction. Alternatively, it has been proposed that lyso PC is the preferred substrate for brain uptake of DHA (Thies, F. *et al.* 1994). Further work is however necessary to clarify this possibility.

4.3.3. Delivery to the brain

The phospholipids of the brain and retina of the embryo are especially enriched in DHA. In this system, DHA delivery to the brain could be mediated via the adipose tissue regulating delivery as the free fatty acid, alternatively the supply of DHA to the brain may be controlled by the liver. DHA within the liver as been shown to be remodelled from TG to PL and this DHA could then be secreted associated with VLDL particles or secreted as lysoPC. ***(See Diagram 6)***

Clearly more study is needed to identify the origin of the isolated lipoproteins at each day of development, which could have come from either the liver or the yolk sac membrane. The mechanism by which DHA is remodelled between lipid moieties requires further detailed analysis. The incorporation of radioactively labelled fatty acids in the yolk at early stage seems to be a next step on polyunsaturated fatty acid investigation.