FATTY ACID SYNTHESIS IN THE DEVELOPING BRAIN

A Thesis Submitted for the Degree of

Doctor of Philosophy

by

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DECLARATION:

The Work contained in this Thesis is the result of experiments carried out by Richard Charles Cantrill.

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i. ABBREVIATIONS:

All chemical symbols have their usual meanings.

ATP	adenosine triphosphate
CoA	coenzyme A
CoASH	free coenzyme A
$NADPH_2$	reduced nicotinamide-adenine dinucleotide phosphate
$NADH_2$	reduced nicotinamide-adenine dinucleotide
NADP	nicotinamide-adenine dinucleotide phosphate
Tris	Tris (hydroxymethyl) methylamine
EDTA	ethanolaminediaminetetraacetic acid
TCA	trichloracetic acid
DNA	deoxyribosenucleic acid

Ci	Curie
nm	nanometer
M	molar
mM	millimolar
µM	micromolar
mol	mole
nmol	nanomole

SUMMARY

ii. The study of biochemical aspects of brain metabolism is very complex because brain tissue is composed of different cell types. The two major cell types are neurones and glia. These two cell types have differing roles but are very closely associated with normal brain development and function. Oligodendroglia are responsible for the synthesis and maintenance of the myelin membrane. In the developing brain, the myelin membrane is formed during a very short period of time.

The aims of this study are (i) to investigate age-dependent changes in the activity of the lipogenic enzymes, fatty acid synthetase and palmitoyl-CoA synthetase which are intimately involved in the synthesis and activation of fatty acids for the formation of phospholipids for incorporation into the myelin membrane; (ii) to determine the sub-cellular location of these enzymes; (iii) to investigate the effect of thyroid hormone on the activity of these enzymes and (iv) to determine the route of fatty acid incorporation into myelin lipids. The results presented indicate (i) that the activities of fatty acid synthetase and palmitoyl-CoA synthetase increase during the period of myelination; (ii) fatty acid synthetase is found predominantly in the cytoplasmic fraction from brain homogenates, whereas palmitoyl-CoA synthetase activity is present in all membrane fractions, although activity measured in myelin may be due to contamination; (iii) the administration of thyroid hormone has no effect on palmitoyl-CoA synthetase, whereas its specific antagonist, propylthiouracil, causes a decrease in enzyme activity in the mitochondria-enriched fraction; (iv) the accumulation of radioactivity in the myelin fraction from labelled fatty acid involves all subcellular organelles including the cytoplasm and no precursor-product relationship is apparent. The results will be considered in the light of data available for lipogenic and other enzymes involved in lipid synthesis.

1. INTRODUCTION:

In this study, the activities of two lipogenic enzymes will be considered. One, fatty acid synthetase, is found in the cytoplasmic compartment of the cell, while the other, palmitoyl-CoA synthetase, is membrane bound. In order to understand their separate and sequential roles in the synthesis and turnover of brain lipids, it is important to consider firstly the gross morphological and chemical changes that take place in the brain during development and secondly the changes in composition and enzyme content of the various subcellular fractions.

The oligodendroglial cells are responsible for the formation and maintenance of the myelin sheath around the nerve axon and in the following introduction the synthesis and turnover of myelin lipids will be considered.

The results of both *in vitro* and *in vivo* investigations will be discussed in order to illuminate the key roles which the two above enzymes must play. The effect of diet and hormones will also be considered since both of these are central in the formation of a normal functional brain. However, no attempt will be made to discuss either the accumulation of protein during development or the role of neurotransmitters since these topics are beyond the scope of this thesis.

1.1 MORPHOLOGICAL CHANGES IN THE WHOLE BRAIN

Growth and development of the brain occurs in two phases (1,2). The initial phase, which occurs in the rat and rabbit during late pregnancy and between 15 and 20 weeks gestation in man, is characterised by neuronal multiplication. The second phase is generally referred to as the "brain growth spurt". This period lasts about three weeks in the rat, beginning at birth. In man it occupies the period from 25 weeks gestation until the third or fourth postnatal year. The rabbit undergoes its "brain growth spurt" from about ten days prior to birth to about thirty days afterwards. Figure 1.1 shows the velocity curves for brain growth in various species in relation to birth (1). The first half of this growth spurt is associated with the proliferation of glial cells and the second half with myelin synthesis, which is considered to be most active between 5 and 20 days after birth in the rabbit. This interval has been called the "period of active myelination" or simply "myelination".

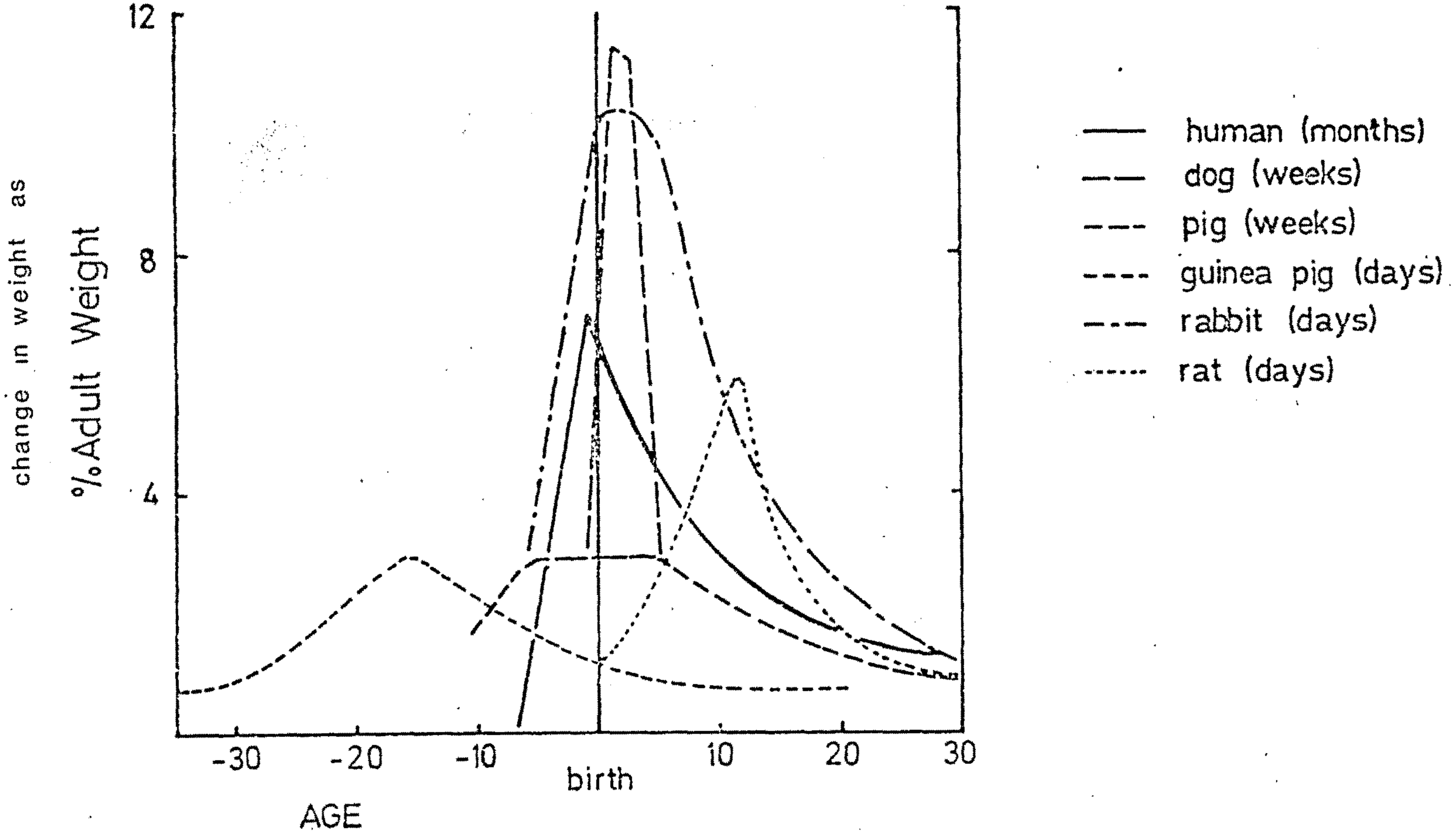
During this period, the weight of the brain increases rapidly and a number of other development processes including myelination, proceed at a very high velocity. This period has been identified as a period of vulnerability where it is only necessary to retard growth to cause distortions of pattern as well as quantitative deficits which are detectable in the adult brain.

The timing of the "brain growth spurt" in relation to birth makes it possible to divide animals into "prenatal", "perinatal" and "postnatal" brain developers (3). It was shown by Harel et al

FIGURE 1.1

Velocity curves for brain growth in various species. Changes in weight as % adult value against time. Arbitrary values for age are given and the time periods are shown in the key. Taken from refs.3 and 4.

Figure 1.1 Velocity curves for brain growth in various species



(4) that the rabbit is a "perinatal" developer undergoing its period of fastest growth during the birth period. Other species whose "brain growth spurt" occurs during the perinatal period are the dog, pig, and human (3). The guinea-pig (5) and the rhesus monkey (3) are examples of animals which show prenatal brain development, while the rat and mouse show postnatal brain development. The use of the rabbit as an experimental animal offers a convenient means of studying factors, involved in brain development during birth and early life, which may be directly applicable to human brain growth.

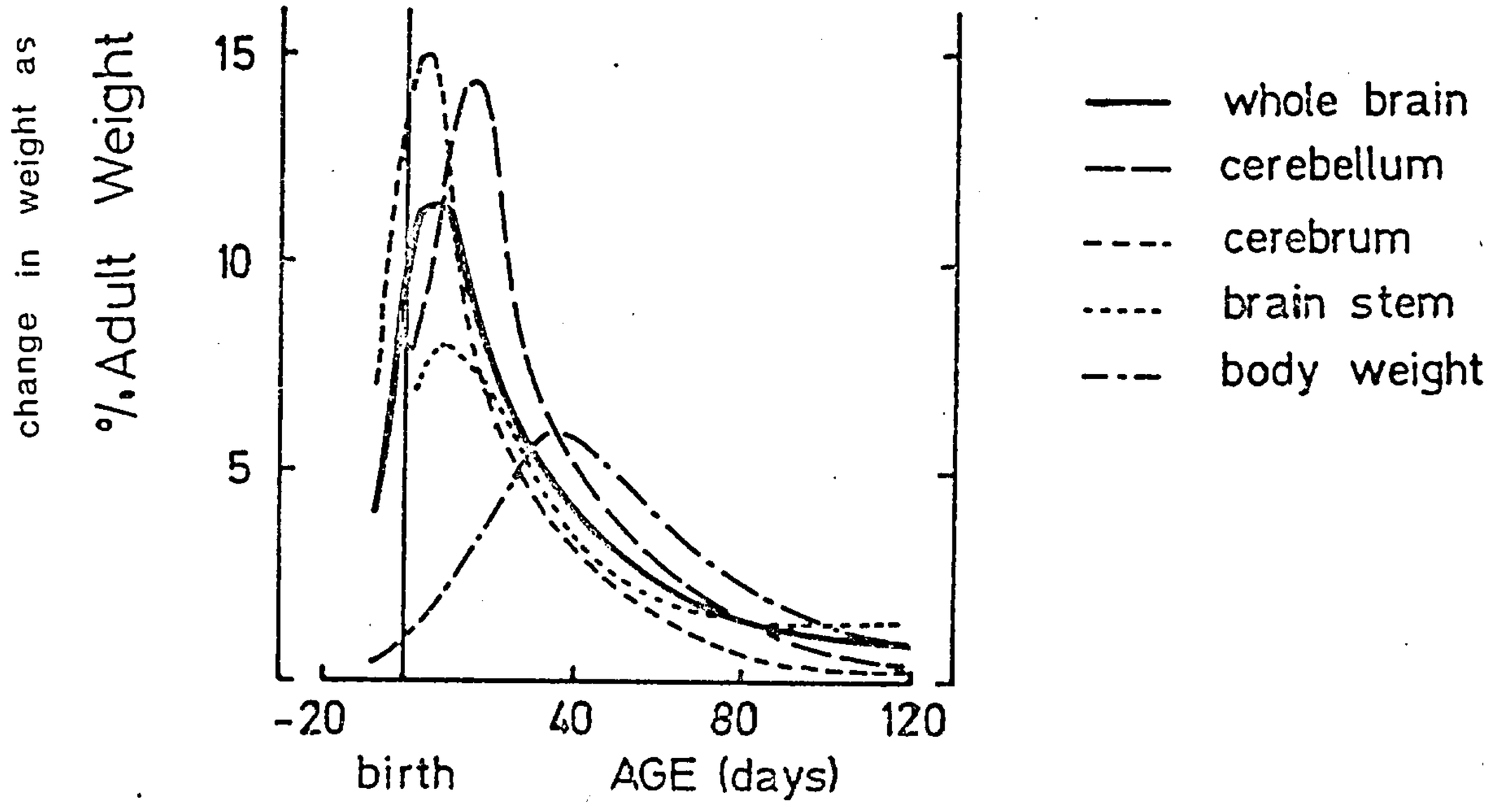
In common with other mammals the "brain growth spurt" in the rabbit precedes that for body growth. The brain/body weight ratio has its peak prior to birth when the brain is immature and has a relatively faster rate of growth than the body.(4) Figure 1.2 shows the rate of weight increase of the brain and the body together with the velocity curves for three distinct regions of the brain. The cerebrum shows the earliest peak velocity at five days of age. The brain stem shows its peak velocity around ten days of age but this is much lower than either that of the cerebrum or cerebellum. The peak velocity of weight increase in the cerebellum occurs at fifteen days and is only very slightly lower than that of the cerebrum.

During the last third of the gestational period of the rabbit, the primitive cerebral cortex increases in size 100 times and continues to increase rapidly after birth, as myelination proceeds.

FIGURE 1.2

Growth curves for different regions of the rabbit brain.
Changes in weight as % adult value against age in days.
The different regions examined are shown in the key.
Taken from ref. 4.

Figure 1.2 Growth curves for different regions of rabbit brain.



1.1.1 Myelin Formation:

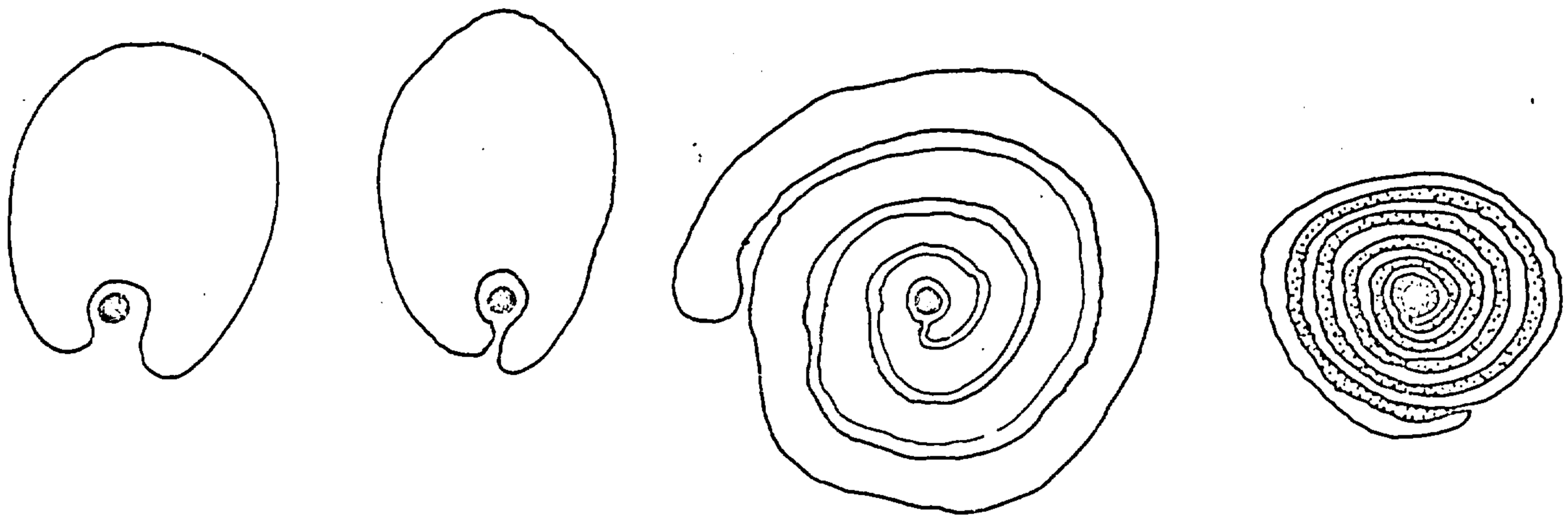
From electron microscope studies it has been shown that myelin is formed around nerve axons by processes derived from oligodendroglial cells in the central nervous system and Schwann cells in the peripheral nervous system. Figure 1.3 is a diagrammatic representation of the oligodendroglial cell cytoplasmic membrane progressively forming the multilamellae sheath around the axon. As the number of windings increases so compaction takes place and the myelin spiral takes on the appearance of mature myelin.

In the central nervous system a single oligodendroglial cell may provide processes for the sheaths of more than one adjacent axon, whereas in peripheral nervous tissue more than one Schwann cell may be required to form the sheath between two nodes, and the thickness of the membrane varies along the length of the axon. Figure 1.4 represents a transverse section of the myelinated axon, the upper part represents a nodal region in the central nervous system. In the peripheral nervous system the Schwann cell provides both the inner and outer collar of cytoplasm around the compact myelin. The outer collar is extended into the nodal region as a series of interdigitating processes. Terminating loops of compact myelin come into close contact with the axolemma in the region of the node apparently providing some barrier for movement of materials into or out of periaxonal space. The Schwann cell is covered with a basement membrane. In the central nervous system the myelin ends in terminal loops near to the node and there are periodic thickenings of the axolemma where the glial membrane is applied in the paranodal region. These may serve as diffusion barriers and thus confine the material in the periaxonal space.

FIGURE 1.3

The progressive envelopment of a nerve axon by glial cell processes to form myelin.
Stages 1 and 2: the gradual envelopment of the axon by the glial cell.
Stage 3: formation of loose myelin
Stage 4: compaction. Taken from ref. 4.

Figure 1.3 Formation of myelin.

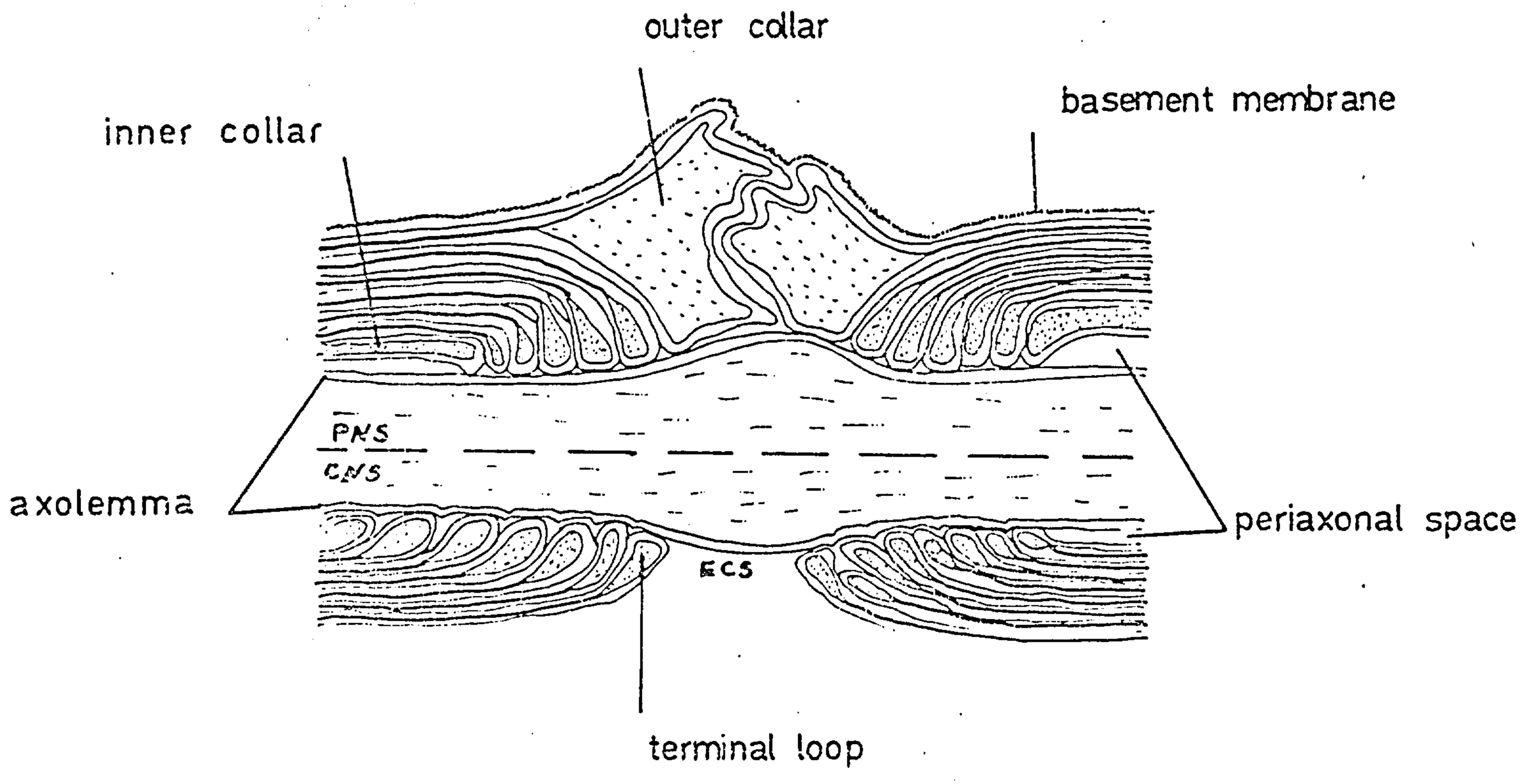


1. 2. 3. 4.

FIGURE 1.4

Comparison of the nodal region of axons in the central nervous system (CNS) and peripheral nervous system (PNS).
ECS: extracellular space.

Figure 1.4 Comparison of nodal regions of axons in the CNS and PNS.



At many nodes in the central nervous system there is considerable extracellular space (6).

If the myelin membrane is unwound hypothetically, as in figure 1.5, there is a continuous circuit of cytoplasm around the periphery of the membrane and also through the centre that is connected directly to the oligodendroglial cell body. It is possible that lipid and protein moieties are transported though the channels of oligodendroglial cell cytoplasm (shown in figure 1.5) and membrane components are replaced in this way (7).

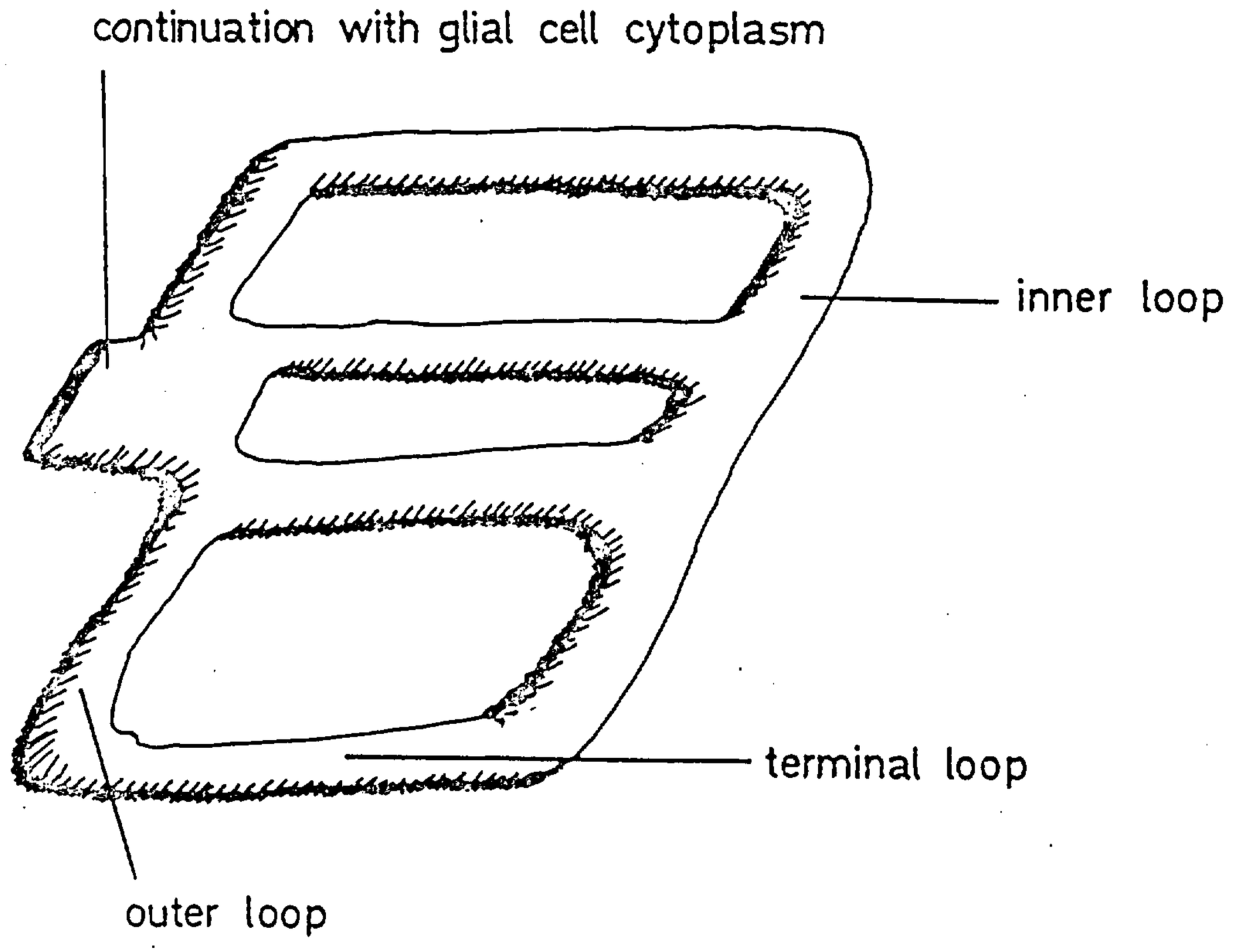
The amount of myelin surrounding an axon can be correlated with the rate of conduction of an action potential in the developing chick sciatic nerve (8, 9) although the unmyelinated axons are not without electrical activity. The intimate relationship between myelin and nerve conduction velocity has also been demonstrated in experimentally induced focal demyelination (10).

Since K^+ and Na^+ ions do not move across the ensheathed part of the axonal membrane, the myelin sheath allows increased conduction velocity along the axon by making the local bielectric current generated at a node of Ranvier, act ahead at the next node and so provide a limited access for current flow. Bunge (6) described the myelinated axon as the "super highway" of the nervous system. This high velocity phenomenon is that of the sheath as a whole and must depend on the characteristics of the axolemma as well as the structural morphology and chemical composition of the myelin surrounding it.

FIGURE 1.5

Hypothetical structure of the unwound myelin sheath.
This figure is a diagramatic representation of one of the several areas of myelin sheath from a single glial cell unwound to show the loops of cytoplasm that are present.

Figure 1.5 Hypothetical structure of an unwound myelin sheath.



1.1.2 Myelin membrane stability:

Myelin stability, in part, may be caused by the lipid of the membranes which make up the sheath. The amount of lipid found in different types of membrane varies greatly. Myelin has the highest lipid content (80% dry weight) erythrocytes an intermediate content (40 - 50%) and mitochondria the lowest (28%) (11). Of the lipid molecules in myelin, cholesterol and sphingolipids comprise 66% of the total. These two lipid classes account for less than 20% of the total mitochondrial membrane lipid, the rest being made up by glycerophosphatides.

The fatty acid composition of myelin may also contribute to its stability. The myelin membrane is particularly rich in saturated medium and long chain fatty acids, 70% and 25% respectively, but low in polyunsaturated fatty acids (5%) (11). By comparison, the erythrocyte membrane is rich in medium chain length (65%) and poly-unsaturated (30%) fatty acids, whereas rat liver mitochondrial membrane lipids contain 60% polyunsaturated fatty acids (11). The effect of the increased amount of polyunsaturated fatty acid is to increase membrane permeability and decrease its stability.

O'Brien (12)postulated that the stability of myelin membranes was caused by the interaction of the non-polar fatty acid chains of the lipid moeities in the bilayer. The central region in each membrane is only lightly stained with osmium tetroxide in electron microscope studies, indicating an area 51 angstroms wide which is hydrophobic in nature.

There is only room for two lipid molecules packed tail-to-tail in this region, since the average length of lipid molecules, from phosphate group to hydrocarbon tail, is 26 - 28 angstroms. Using the models of Vandenheuval, O'Brien suggested that the lipid molecules in myelin are more tightly packed together than in other membranes and the high degree of stability is due to the presence of fatty acids with greater chain lengths than those found in other membranes (12).

1.2 CHANGES IN THE LIPID COMPOSITION OF WHOLE BRAIN

Large amounts of biochemical data has been accumulated concerning the growth and development of the central nervous system in many species: pig (13) guinea-pig (5,14), rat (3, 15, 16, 17), dog (3), and chick embryo (18), with less information existing about humans (3,15,19,20, 21). The development of the central nervous system in the rabbit has not been investigated as thoroughly as in the rat and other species, although lipid and protein changes during maturation have been reported (22, 23, 24). During the development of the central nervous system there is a progressive accumulation of a relatively large amount of lipid compared with other tissues (25,26). Cholesterol and sphingolipids have been shown to be the major constituents of total cerebral lipids and these are present mainly in the isolated myelin membrane.

In the developing rabbit brain there is a continuous increase in both wet and dry weight up to adult age (Fig. 1.6)(24). Lipid is deposited up to adult age but accumulates maximally between 3 and 20 days. Although the amount of protein (mg/brain) increases thoughout development, it accounts for a decreasing proportion of the dry weight of the brain (64% in the neonate to 44% in the mature brain). The 3-fold increase in DNA during development, in fact, represents a sharp decrease on a dry weight basis and may reflect major increases in cell size during this period (24).

The highest rate of accumulation of lipid corresponds with the most active period of myelination.

FIGURE 1.6

Relative changes in composition of rabbit brain during development.
Changes in wet weight, DNA, protein and lipid content are plotted against age in days. The values prior to birth are days gestation. Drawn from values presented in ref. 24.

Figure 1.6 Changes in the composition of rabbit brain.

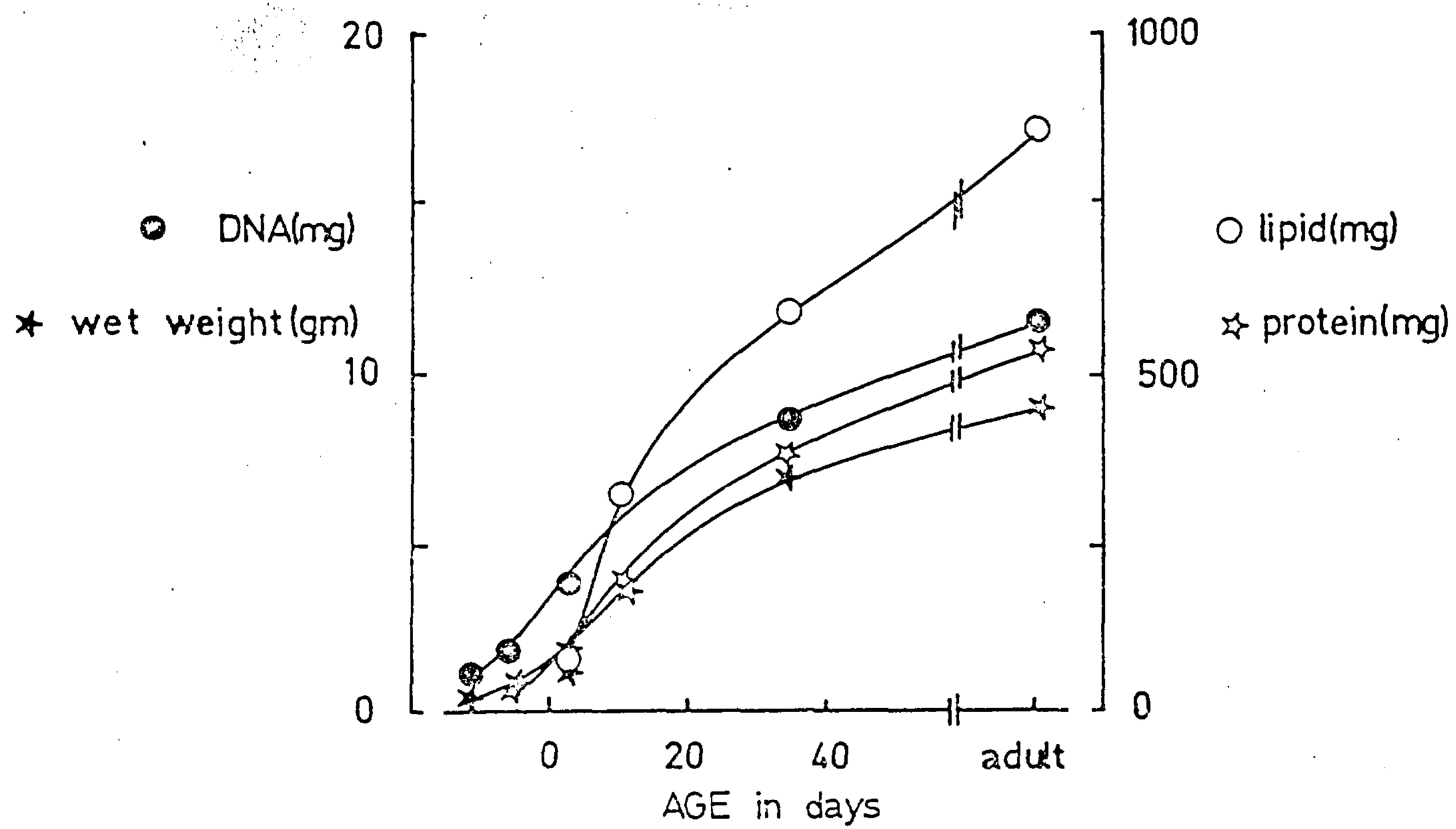


Table 1a (59) shows that increasing amounts of various lipids are deposited in the brain during development. There is a striking increase in cholesterol content of rat brain during the period between 3 and 20 days post partum, and a much more marked accumulation of phospholipids occur during the same time. Dalal and Einstein (22) showed a large increase in the cholesterol content of the developing rabbit brain, and a similar change in the cholesterol, cerebroside and sulphatide can be seen in the results of Radin (28) for whole rat brain. The appearance of cerebroside is delayed in the rat brain and at 20 days of age its rate of deposition is comparable with that of the other lipids (27). Similar results are seen in the developing rabbit brain (see table 1.1b)(24).

In the developing rat brain, dramatic increases in certain phospholipids are seen. Figure1.7shows the levels of different phospholipids (μmol/brain) in the rat brain expressed as a percentage of the adult value.

Both ethanolamine plasmalogen and triphosphoinositide are important components of myelin, although they are found in other membranes and they show a larger increase during the period of most active myelin deposition than phosphatidylethanolamine and phosphatidyl-choline which are more general components of cell membranes. Sphingomyelin is also closely associated with myelination.

TABLE 1.1a

Lipid composition of the developing rat brain.
The values shown are % adult value (μmol/brain)
for total lipid, cholesterol, cerebroside and total phospholipid
at each age in days. Taken from ref. 27.

TABLE 1.1a

LIPID COMPOSITION OF DEVELOPING RAT BRAIN

AGE: (Days)	3	7	10	12	14	16	20	30	36	50	Adult
Total lipid	4	8	21	27	31	33	38	51	67	93	100
Cholesterol	3	4	17	27	27	28	32	53	69	91	100
Cerebroside	0	0	1	1	2	6	12	36	54	70	100
Total phospholipid	6	14	28	34	42	44	54	55	68	100	100

THE RESULTS ARE EXPRESSED AS % ADULT VALUE. TAKEN FROM REF. 59

TABLE 1.1b

Lipid composition of the developing rabbit brain.
The values shown are % adult value (mg/brain) for total lipid, cholesterol, cerebroside and phospholipid at each age in days.
Taken from ref. 24.

TABLE 1.1b

LIPID COMPOSITION OF DEVELOPING RABBIT BRAIN

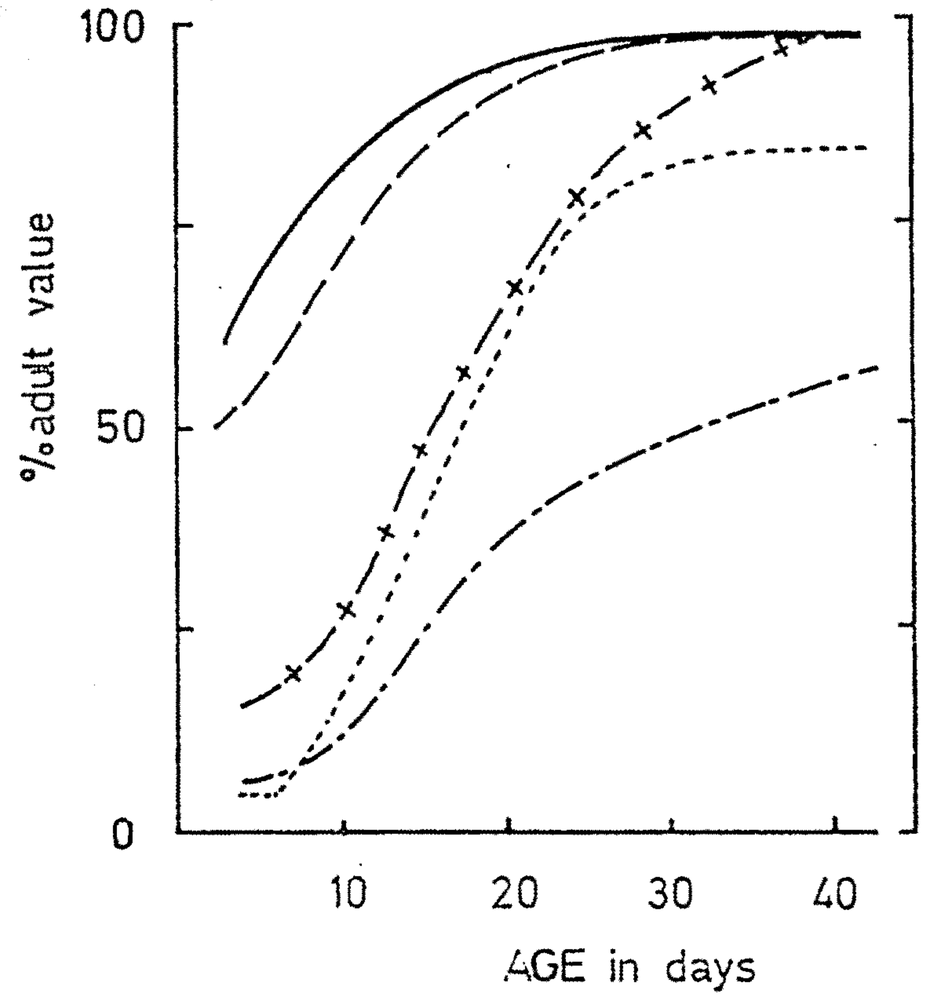
AGE: (Days)	3	11	45	200
Total lipid	9	34	65	100
Cholesterol	4	23	62	100
Cerebroside	3	20	54	100
Phospholipid	15	50	73	100

THE RESULTS ARE EXPRESSED AS % ADULT VALUE. TAKEN FROM REF. 19

FIGURE 1.7

The levels of some phospholipids in the developing rat brain shown as % adult value (μmol/brain) against age in days. The phospholipids shown are: ——— phosphatidylcholine, —·—·— phosphatidylethanolamine, — + — ethanolamine plasmalogen, - - - - - - sphingomyelin and —·—·— triphosphoinositide. Taken from ref. 27.

Figure 1.7 The levels of some phospholipids in rat brain.



If the concentration of phospholipid (mmol /100gm fresh tissue) from rat brain is plotted against $\log_{10}$ of the age, as suggested by Rouser and Yamamoto (29), an increase in concentration occurs up to 100 days, since this form of data expression condenses the time scale. The increase in phospholipid concentration (mmol) and concomitant increase in the size of the rat brain during maturity probably indicates that myelin formation continues well into adult life.

During the rapid accumulation of lipid in the myelinating brain, the composition of the phospholipid fraction varies. Table 1.2a.(24) shows the phospholipid composition of the developing rabbit brain. Phosphatidylethanolamine and sphingomyelin account for an increasing proportion of the phospholipid while the proportion of phosphatidylcholine falls. Phosphatidylserine, phosphatidylinositol and phosphatidic acid show only a slight decrease in proportion with age. In the rat brain (Table 1.2b)(27) the proportion of phosphatidylserine and phosphatidylinositol does not fall as in the rabbit brain, but phosphatidylethanolamine and sphingomyelin increase while phosphatidylcholine decreases in proportion. The differences in the trends shown by each animal may be due to differences between the species. In addition to changes in the relative proportions of each phospholipid, an alteration in the fatty acid composition of the phospholipids also occurs. In the developing rat brain the proportion of $C_{18:0}$ and $C_{18:1}$ fatty acids in phospatidylcholine increases at the expense of $C_{16:0}$ and $C_{16:1}$ (30) and this is probably true for most glycerophosphatides (31).

TABLE 1.2a

Phospholipid composition of the developing rabbit brain.

The amount of each phospholipid present at each age is expressed as % total phospholipid at that age. Taken from ref. 24.

TABLE 1.2a.

PHOSPHOLIPID COMPOSITION OF THE RABBIT BRAIN

AGE: (Days)	3	11	45	200
Phosphatidyl choline	39	37	27	23
Sphingomyelin	5	10	13	17
Phosphatidyl ethanolamine	25	32	41	47
Phosphatidyl serine	9	6	7	5
Phosphatidyl inositol	9	6	6	4
Phosphatidic acid	5	2	3	2

THE RESULTS ARE EXPRESSED AT % TOTAL AT EACH AGE. TAKEN FROM REF. 19

TABLE 1.2b

Phospholipid composition of the developing rat brain.
The amount of each phospholipid present at each age is expressed as % total phospholipid at that age. Taken from ref. 27.

TABLE 1.2b.

PHOSPHOLIPID COMPOSITION OF THE RAT BRAIN.

AGE: (Days)	3	7	10	20	36	50	Adult
Phosphatidyl choline	42	37	38	28	35	38	36
Sphingomyelin	2	2	2	3	6	5	6
Phosphatidyl ethanolamine	29	31	33	33	40	39	41
Phosphatidyl serine	10	8	10	10	15	-	12
Phosphatidyl inositol	4	4	4	4	6	-	5

THE RESULTS ARE EXPRESSED AT % TOTAL AT EACH AGE. TAKEN FROM REF. 59

The increase in $C_{18:0}$ and $C_{18:1}$ is due to the high level of these two fatty acids in phosphatidylcholine in myelin which makes an increasing contribution to the total phosphatidylcholine of the brain with increasing age (32). Changes in fatty acid composition of the developing rabbit brain are also seen (24). Rabbit brain phosphatidylcholine shows a decrease in the amount of $C_{16:1}$ and $C_{18:0}$ and an increase in longer chainlength mono-unsaturated fatty acids especially $C_{18:1}$.

The fatty acid pattern of sphingomyelin varies only slightly during maturation. The predominant species are $C_{18:0}$ and $C_{24:1}$ and an increasing proporation of very long-chain fatty acids are esterified at the expense of the medium chainlength fatty acids. Cerebrosides also show similar changes (24).

Dhopeshwarker (33) also noted a decrease in the palmitate/stearate ratio and a corresponding rise in the oleate/stearate ratio in older rat brains. In the human brain, similar changes in the fatty acid composition have also been shown (34, 35).

1.3 THE PREPARATION OF SUBCELLULAR FRACTIONS FROM BRAIN TISSUES

In order to resolve the problems of relating the gross changes in the lipid composition of the brain to structure and enzyme content it has been necessary to characterise the chemical composition and enzyme content of the sub-cellular organelles.

The first systematic use of sedimentation characteristics in a gravitational field to separate subcellular fractions was in 1934 when Bensley and Hoerr (36) isolated mitochondria. But subcellular fractionation techniques were not applied to brain tissue until 1952.

Electron microscopic examination of the mitochondrial fraction of brain tissue, revealed the existence of sealed off nerve endings in this preparation (37, 38). These were separated by density gradient centrifugation and named synaptosomes (39). The early work of Whittaker et al (39, 40) and De Robertis et al (41) has been developed (42, 43) to improve the separation of subcellular organelles from different parts of the nervous system. Methods have also been developed for specific nervous tissue components such as microtubules, myelin fragments and postsynaptic membranes, as well as for isolation of nuclei, mitochondria and lysosomes.

The methods of tissue disruption are more important when fractionating nervous tissue than when disrupting other tissues. The formation of synaptosomes depends upon the method of tissue disruption used.

Synaptosomes are formed when weak shear forces are used, such as in a teflon-glass homogeniser. More severe methods of homogenisation can be used when synaptosomes are not being investigated, but the more violent the techniques used, the greater the tissue disruption and the potential loss of other organelles and ultrasonication causes the formation of small non-specific membrane vesicles (44).

Differential centrifugation of nervous tissue is based on schemes originally devised for the liver, but it yields fractions that are far more heterogenous because nervous tissue is composed of a variety of cell types and membrane structures formed from cell processes. Further purification of each fraction has been achieved by both manipulating the centrifugation conditions, to cause enrichment of a particular organelle, and by density gradient centrifugation. By the latter method, subcellular particles are separated according to their bouyant density (see Table 1.3). Sucrose gradients are most frequently used, although caesium chloride (45) and Ficoll (a high molecular weight dextran with low osmotic effect) gradients have also been used (46 - 50). Subcellular fractions are characterised by a variety of criteria. These are :

(i) Marker enzyme content

(ii) Protein and lipid composition.

(iii) An even homogeneous field when viewed under the electron microscope.

When all subcellular fractions of a particular tissue are required it is usual to sacrifice absolute purity and use methods which yield each fraction.

TABLE 1.3

The size and bouyant densities of many subcellular particles from brain tissue.
For each organelle its size range in μ and the molarity of sucrose having the same density are given. Taken from ref. 49.

TABLE 1.3

SIZE AND BOUYANT DENSITY IN SUCROSE OF SUBCELLULAR ORGANELLES

Organelle	Size in μ	Molarity of Sucrose Having same Density
Nuclei (neuronal)	7000 - 10000	2.0 - 2.2
Nuclei (glial)	4000 - 6000	2.3 - 2.6
Myelin	1000 - 3000	0.6 - 0.7
Synaptosomes	400 - 1000	1.0 - 1.2
Mitochondria	300 - 700	1.4
Lysosomes	200 - 500	1.7
Membranes Fragments	400 - 1000	0.7 - 1.1
Ribosome Particles	10 - 30	1.6
Microtubules	50 - 200	1.4
Synaptic Vesicles (small agranular)	30 - 80	0.4 - 0.5
Synaptic Vesicles	30 - 80	0.5 - 0.6

TAKEN FROM REFERENCE 42.

1.3.1 Preparation of Myelin:

Most methods of myelin preparation rely on two properties of the myelin membrane, (i) the large vesicle size, and (ii) the low density. Homogenisation of brain tissue in sucrose solution, causes the myelin to swell and peel off the axons and reform in spherical vesicles which are in the size range of nuclei and mitochondria, and therefore sediment with these two fractions during differential centrifugation. Myelin also forms multilamella structures which will form single membrane vesicles after a hypotonic shock. Myelin vesicles have the lowest density because of their high lipid to protein ratio and so density gradient procedures were developed to separate myelin from nuclei, mitochondria and synaptosomes. Methods used for the preparation of pure myelin fall into two groups, depending on whether they are designed to isolate myelin solely or whether myelin is obtained as only one fraction in a preparation scheme designed to isolate all brain subfractions. Although the former methods may give rise to a low yield and a sample of myelin that is not representative of myelin in vivo, and the latter methods might give a sample that is of lower purity, it has been shown that the composition of myelin prepared by these methods is not significantly different (51).

1.3.2 The Enzyme content of Myelin:

The criteria for myelin purity are complex since it has little enzyme activity although some lipid synthetic enzymes could be expected.

Under an electron microscope it should give a uniform field of large vesicles but there are then difficulties in identifying any small vesicles that may be present. Purified myelin is 90% soluble in chloroform:methanol (2:1 v/v). It should be free from the plasma membrane enzymes acetylcholine esterase (EC 3.1.1.7) and 5'-nucleotidase (EC3.1.3.5.). These two enzymes have been frequently reported in myelin preparations. There should be minimal levels of Na^+/K^+ ATPase (EC3.6.1.3) succinic dehydrogenase (EC 1.3.99.1) from mitochondria, and microsomal NADPH cytochrome c reductase (EC 1.6.2.4)and nucleic acids from endoplasmic reticulum and cell nuclei. Purified myelin is associated with 2', 3'-cyclic nucleotide-3-phosphohydrolase (52) although it has no apparent metabolic function because of its high K_m for the substrate. The enzyme may be associated with either the axolemma or myelin as it is not possible to separate them. The absence of measurable activity of the enzyme in the unmyelinated brain and its presence in the plasma membrane of glial cells, suggests that 2', 3'-cyclic nucleotide-3-phosphohydrolase is associated with myelin. Two other enzymes have also been ascribed to myelin (i) leucine amino peptidase (53) (ii) cholesterol ester hydrolase (54).

1.3.3. <u>The Protein Content of Myelin</u>:

The electrophoretic pattern of myelin proteins isolated from the central nervous system is simple compared with other membrane fractions (55). There are two major proteins, the proteolipid protein and the basic protein, which are not found in the neuronal axolemma, and also a fraction called the "Wolfgram Protein" which may be heterongeneous.

The high degree of solubility of myelin in chloroform:methanol (2:1 v/v) is to a large extent accounted for by the presence of the proteolipid protein complex which is soluble in lipid solvents and accounts for 60% of the proteins in myelin (56). The proteolipid protein is poor in acidic and basic amino acids and rich in the less polar amino acids. This could explain its association with lipid and the solubility of the complex in lipid solvents. It may have a structural function since proteolipid proteins isolated from the myelin of peripheral nerves and other membranes have a similar amino acid composition (57).

The basic protein has been extensively studied since it exhibits encephalitogenic activity. Bovine brain contains 10 mg. basic protein/g wet weight (58), and it accounts for 30% of protein in human white matter. It is of interest because it produces allergic encephalomyelitis when injected into experimental animals. It is highly basic and exists as an extended polypeptide chain with an isoelectric point greater than 12. The bovine brain species has a molecular weight of 18,000. Palmer and Dawson (59, 60) found that basic protein could form a complex with triphosphoinositide of 0.145μmol TPI/mg protein which was comparable with the ratio of TPI to basic protein in the brain.

1.4. CHANGES IN THE LIPID COMPOSITION OF MYELIN AND OTHER SUBCELLULAR FRACTIONS.

1.4.1 Changes in the lipid composition of myelin:

Myelin accounts for 50% of the dry weight of bovine brain white matter and 65% of the total lipid. Lipid accounts for 70% of the dry weight of myelin from both the central nervous system and the peripheral nervous system (61). Myelin from the peripheral nervous tissue contains a higher proportion of sphingomyelin and a lower concentration of cerebroside than myelin from the central nervous system. Triphosphoinositide was thought to have been exclusive to myelin (62) though more recent work by Hawthorne indicates the presence of a small non-myelin pool (63). In 1965 Horrocks (64) reported that the composition of mouse brain myelin changes during development and later it was shown by several investigators that the relative proportions of galactolipids, cholesterol and ethanolamine plasmalogens increase and the relative proportions of choline phosphoglycerides decreases during maturation in the mouse brain (165), the rat (27, 66-68), the rabbit (22) and in man (55,69). In the rat and mouse the increase in the relative proportions of galactolipids and cholesterol are nearly equal on a molar basis to the decrease in phospholipid (70).

Cuzner and Davison (27,67) described an even more pronounced increase in these lipids, although Eng and Noble (71) showed no appreciable change in the cholesterol content of myelin from the developing rat brain. The cholesterol and galactolipid content of myelin from the developing human brain does not appear to vary with age (55,72, 73, 74).

1.4.2. "Pro-myelin" and formation of the myelin membrane:

It has been suggested that some of the variations in myelin lipid composition are due to the presence of a "pro-myelin" fraction.

The preparation of myelin from the brains of animals up to 25 days of age, creates special problems for the interpretation of results. Myelination proper, does not begin in the rat until about 10 days of age, at which time the first rings of myelin can be seen around the axon in electron micrographs. It is not until this time that myelin can be isolated. The material isolated using the normal method of myelin preparation was found to have a lipid composition that was different from that of the adult rat (see Table 1.4). Myelin prepared from the young brain has a higher proportion of phospholipid and a lower proportion of sterol and galactolipid than the adult brain. The composition of myelin from young brains resembles that of endoplasmic reticulum and plasma membrane rather than mature myelin. Davison and co-workers (75, 76) proposed that the myelin prepared from young brains (early myelin) was a mixture of mature myelin and a "pro-myelin" fraction derived from the oligodendroglial cell plasma membrane which is involved in the formation of myelin. The "pro-myelin" fraction has also been called the myelin-like fraction due to its co-sedimentation with mature myelin in the preparation procedure. The "pro-myelin" material has been prepared (77) and shown to be poor in cerebrosides and to contain a higher proportion of shorter chain fatty acids in the phosphoglycerides than mature myelin. Further studies by Agrawal et al (78) showed that the material resembled myelin in enzyme activity but differed in that the basic protein was absent.

TABLE 1.4

Changes in the lipid composition of rat brain myelin are shown. The figures for lipid content are expressed as % dry weight and for desmosterol as % total sterol. All other values are expressed as wt % of total lipid. Age is expressed in days. Taken from ref. 66.

TABLE 1.4 RAT BRAIN MYELIN COMPOSITION DURING DEVELOPMENT

AGE: (Days)	15	20	30	60	144	190	425
Lipid % dry wt.	74.2	70.4	72.1	72.5	70.4	70.1	68.0
Sterol	25.1	25.7	25.7	25.9	27.8	27.9	27.4
Desmosterol	3.0	1.8	1.5	1.0	0.3	0.2	0.2
Galactolipid	21.4	23.6	26.2	30.5	33.3	30.7	31.7
Phospholipid	50.4	48.3	48.0	44.0	43.7	43.5	44.9
PE - Phosphatidylethanolamine	18.4	18.1	17.5	16.8	15.1	17.1	17.7
PC - Phosphatidylcholine	16.4	15.6	13.6	12.4	10.7	10.7	11.5
PS - Phosphatidylserine	7.0	6.9	7.4	6.5	6.7	7.1	7.6
PI - Phosphatidylinositol	1.4	1.4	1.0	1.3	1.2	1.1	1.2
Spingomyelin	3.4	3.5	3.3	3.4	3.2	3.1	3.1
Plasmalogen	14.1	13.1	13.7	14.4	13.9	13.7	14.3

"Early myelin" therefore consists of the "pro-myelin" and compact myelin fractions from the developing brain and the myelin-like fraction is probably a transition state between the oligodendroglial cell plasma membrane and compact myelin. The "pro-myelin" fraction may also contain some fragments of oligodendroglial cell plasma membranes. Rouser et al (72) comment that although it is acknowledged that lipid gain or loss during subcellular fraction-ation is possible, there has been little attempt to evaluate this. Values for myelin total lipid between 59 and 90% of the dry weight have been obtained using human brains, using the method of Norton (60). Selective loss of either lipid or protein during isolation could account for the range of values obtained.

The proposed existence of a myelin precursor is supported by the occurrence of the myelin specific constituents, basic protein and triphosphoinositide in rats only 1-2 days old. This is well before the period of maximum myelin deposition at between 10 to 20 days and before any myelin with the mature lipid composition can be found (79). Also at this early stage lipid droplets can be detected in the glial cells which may serve as a source of lipid material for myelin formation since they are observed less frequently as myelination proceeds (80). Additional evidence for a myelin precursor which may be glial cell plasma membrane or microsomes comes from the work of Mandel (81, 82) and Baumann et al (83) on myelin deficient mutants. In the Quaking mouse, a genetic mutant in which myelin is never properly formed and the number of glial cells in the brain appears normal, there is a qualititive defect.

This defect can be correlated with a loss of metabolic differentiation shown by the failure of long-chain fatty acids to appear in cerebrosides. The Jimpy mouse, a different genetic mutant, studied by Mandel, has a decreased number of oligodendroglial cells and this gives rise to a decreased amount of myelin. Since normal myelin formation is prevented, other membrane components such as proteins are degraded.

Mokrash (56) suggested that there is a 'threshold' compostion for precursor myelin, perhaps similar to that of plasma membrane with its large percentage of phosphatidylcholine, which confers on the membrane some degree of stability. From this composition the membrane attains the mature composition by replacement and exchange of lipid molecules and the addition of myelin-specific proteins which may be required to initiate a spontaneous compaction process (indicated diagrammatically in Figure 1.3)

1.4.3 Changes in the lipid composition of subcellular fractions other than Myelin:

Although the composition of mouse brain microsomes does not change appreciably with age (65) the molar ratio of galactolipid to total phospholipid increases with age in rat brain microsomes, nuclei and mitochondria (72). The phospholipid composition of chick brain subcellular fractions also changes during development. As seen in the developing rat and rabbit brain (24), the amount of sphingomyelin increases at the expense of phosphatidylcholine. Cholesterol and desmosterol can also replace each other in membranes (84) and cholestanol can replace cholesterol (85).

1.4.4. Changes in the fatty acid composition of major lipid classes:

The overall changes seen in the fatty acid composition of major lipid classes in the whole brain have been demonstrated in sub-cellular fractions from developing brain. Skrbic and Cumings (32) showed that in the rat brain the increase in $C_{18:0}$ and $C_{18:1}$ is due to the high level of these two fatty acids in phosphatidylcholine in myelin. There is a general tendency for the chain-lengths of the fatty acids of phosphatidylcholine in all brain subfractions to increase with age (32). The fatty acid composition of cerebrosides from subfractions of mouse brain has also been studied (86). 2-Hydroxy-fatty acids account for 80% of the myelin cerebrosides fatty acids and 55% in microsomal fractions. The majority of fatty acids in myelin are of chain lengths greater than C_{20} whereas, in microsomes fatty acids of chain length C_{16}-C_{20} predominate.

1.5 LIPID METABOLISM IN THE DEVELOPING BRAIN

Both *in vivo* and *in vitro* studies have been used in the elucidation of lipid metabolism in brain tissue. Many of the early hypotheses of brain lipid metabolism originated from the *in vivo* incorporation of radioactive precursors into brain tissue. The precursor was usually administered by intraperitoneal injection, or via the carotid artery. *In vitro* studies of lipid metabolism involve fractionating brain tissue homogenates by differential and density gradient centrifugation and assaying the resultant fractions for enzyme activity.

1.5.1 In Vivo Incorporation of Lipid Precursors:

The uptake of lipid precursors by the developing brain has been the subject of several reviews (87-89). Early experimenters (90-95) concluded that lipids could only be labelled during early life and in particular, during the period of myelin formation and the slow incorporation of precursors into the adult brain was due to the metabolic stability of myelin lipids and the blood-brain barrier.

More recently, Smith and Eng (96) supported the idea of a low rate of adult myelin lipid turnover by estimating the half-lives of the myelin phospholipids phosphatidylcholine, phosphatidylinositol and sphingomyelin to be 41 days, 18 days and 250 days respectively. However, following intraperitoneal injection of labelled acetate and glucose (97) the rate of turnover, as measured by loss of radioactivity, was the same in both adult and weanling rats.

The major criticism of experiments involving the uptake of labelled compounds from the circulation into the brain has been the concept of the blood-brain barrier.

In recent reviews, Dobbing (15, 98) and Dhopeshwarkar (87, 99) have rejected the idea of there being any physical restriction to lipogenic metabolites entering the brain. They argue that the entry of any component into the brain is governed by the metabolic properties and state of the tissue and not by any physical property of the capillaries. The metabolic properties envisaged are:1. the many types of uni- and bi-directional active transport mechanisms; 2. the varying metabolic activities of cellular components which determine the net flux and exchange of material between the blood and the brain; and 3. the size of the precursor pool in the tissue.

Results have been published (94, 100 - 104) involving the incorporation of radioactivity into the cerebral lipids of adult animals which cannot be explained in terms of myelin lipid stability. Short term experiments by Ansell and Dohmen (100) and Dawson and coworkers (101,104) showed that inositides and phosphatidic acid were rapidly labelled with ^{32}P-phosphate but other phospholipids were labelled more slowly. ^{14}C-cholesterol remained for as long in the mitochondrial membranes as in myelin (102,103) and Davison and Dobbing concluded (94) that the time taken for the transfer of circulating ^{32}P-phosphate to enter the brain was less than its subsequent transfer from the acid-soluble phosphate pool into brain phospholipid phosphate. Thus, a slow flux of label through the lipids of adult brain tissue could be indicative of a low rate of de novo synthesis and not a low rate of lipid turnover.

The use of intracerebral injection may overcome any selective uptake phenomena which may affect the incorporation of precursors into brain lipid following intraperitoneal and carotid injection. Ansell and Spanner (105,106)showed that ethanolamine plasmalogen and phosphatidylethanolamine were extensively labelled in all brain subcellular fractions following intracerebral injection of ^{14}C-ethanolamine. Similarly, Jungalwala and Dawson (107) using a variety of lipid precursors (^{32}P-phosphate, ^{14}C-glycerol and ^{14}C-ethanolamine), showed that there was considerable labelling of phospholipids in the microsomal and myelin fractions of adult rat brain. The specific activity of microsomal phospholipid was higher than that of myelin initially, but by 20 days after intracerebral injection, the reduced specific activities were the same. Also, following injection of 10 day old rats, some label remained in the adult brain. These results together with those of Ansell and Spanner (105)indicate that there are two pools of lipid in myelin, one rapidly exchanging and the other turning over slowly, which can be labelled in the developing animal.

It is probable, therefore, that the exchangeable pool of myelin lipids is larger and more accessible than had been originally thought. However, Davison and Gregson (53) cautioned that the metabolically active pool of cerebroside sulphate was only 0.2% of the total.

1.5.1.1 Effect of diet upon the fatty acid composition of cerebral lipids:

Although it appears that lipid exchange in brain tissue is a common phenomenon, the fatty acid patterns of lipids are only slightly affected by dietary constraints such as starvation or essential fatty acid deficiency.

Changes in the fatty acid pattern of lipids due to dietary effects, are reversed when the animal returns to a normal diet (108,109). Since only small changes in the fatty acid patterns could be detected, endogenous fatty acid synthesis or re-utilisation of pre-formed fatty acids must be major routes of fatty acid metabolism in the adult brain (110).

1.5.2 In Vitro Studies of Brain Lipid Metabolism:

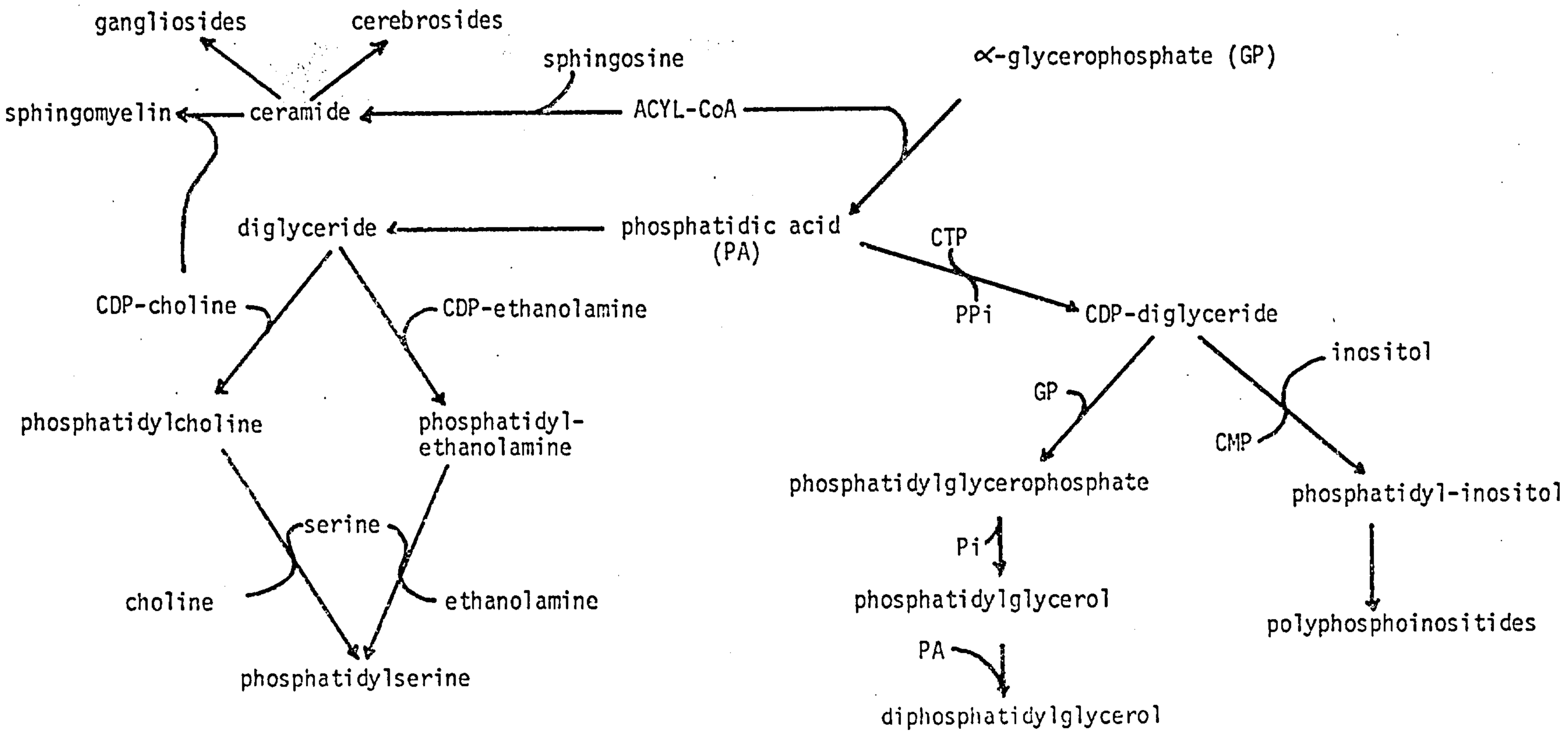
The in vitro activities of many brain enzymes and enzyme systems involved in lipid synthesis have been investigated. Cerebral lipid metabolism, which is of special importance because of the large amount of lipid found in the myelin sheath, is outlined in figure 1.8 . Investigators have used slices, homogenates and subcellular fractions in the elucidation of cerebral lipid metabolism.

Although the synthesis of phospholipids from phosphatidic acid in the brain is well documented, little attention has been paid to the de novo biosynthesis and activation of the fatty acids whose ultimate fate is incorporation into complex lipids. Brady (111) showed fatty acid synthetase activity in rat brain in 1960, but the first report of age dependant changes in the specific activity of the enzyme was not published until 1972 (112). Changes in the specific activity of rat brain acetyl CoA carboxylase during the period of myelination, were not reported until 1974 (113). Because of this paucity of knowledge, it was decided that changes in the activity of the enzymes, acetyl-CoA carboxylase, fatty acid synthetase and palmitoyl-CoA synthetase would be investigated, primarily, in the developing rabbit brain. A brief consideration of the modes of action of these enzymes follows in section 1.5.2.1.

FIGURE 1.8

A simplified view of the routes of phospholipid synthesis.

FIG. 1.8 A SIMPLIFIED VIEW OF PHOSPHOLIPID SYNTHESIS



Age dependant changes for the incorporation of fatty acid precursors into brain slices have been reported for both rat (114) and rabbit brain (115) and will be discussed in the light of results presented subsequently.

The exchange and turnover of myelin lipids is only possible if it is mediated by some form of transport mechanism, since myelin is devoid of lipogenic enzyme activity and the microsomes are considered to be the site of lipid synthesis. Dawson (116)postulated that newly synthesized phospholipids are transferred to other cellular membranes and organelles by a process of exchange diffusion involving soluble cytoplasmic lipoprotein by a similar mechanism to that described in hepatic tissue (117,118). Such protein factors, similar to those described in liver cell phospholipid transfer (119), have been described for the transport of cerebral lipids (120-123).

The catabolism of brain lipids is not clearly understood, although phospholipase A_1 activity has been found (124) and phospholipase A_2 activity has been recovered from acetone powder of brain tissue (125). Both a sphingomyelin specific phospholipase C (126) and a phosphatidyl-ethanolamine specific phospholipase C (127) have been isolated from rat brain tissue. Ansell and Spanner (128, 129) showed the presence of Mg^{2+} dependant plasmalogenase, hydrolysing the alkenyl ether linkage of ethanolamine plasmalogen, especially in the crude mitochondrial fraction of white matter. Enzymes of triphosphoinositide breakdown have been found by Dawson and Thompson and co-workers (130-133).
The catabolism of fatty acids in the brain is uncertain. Since brain mitochondria are capable of oxidising fatty acid at the same rate as liver mitochondria (134) but the rate of <u>in vivo</u> fatty acid oxidation, as measured by the oxidation of radioactive fatty acid to $^{14}CO_2$ is low.

Warshaw et al (135) showed that fatty acid oxidation was highest in foetal and early neonatal life. This is in agreement with the accepted theory that glucose is the major energy source of the adult brain with a respiratory quotient of almost 1.

1.5.2.1 In Vitro Fatty Acid Metabolism:

Much information about the uptake and incorporation of fatty acids and their precursors into brain lipids was obtained by studies in vivo. In Vitro experiments can be divided into two types : (i) Investigations into de novo synthesis and (ii) the further metabolism of preformed fatty acids.

(i) De novo synthesis of saturated fatty acid is catalysed by two enzyme systems which function sequentially in many biological systems. The enzymes are : 1. Acetyl-CoA carboxylase and 2. Fatty acid synthetase. This aspect of fatty acid metabolism has been reviewed thoroughly recently (136-138).

1. The first enzyme, acetyl-CoA carboxylase, has a biotin prosthetic group and the carboxylation reaction to form malonyl-CoA requires the formation of a carboxyl-biotin intermediate. The two step carboxylation reaction can be seen below

(i) ATP + HCO_3^- + biotin enzyme = CO_2^- - biotin-E + ADP + Pi

(ii) CO_2^- - biotin-E + CH_3CO-SCOA = $^-OOCCH_2CO$-SCOA and biotin-E

This enzyme has been purified from many sources but has not been investigated thoroughly in the developing mammalian brain.

Acetyl-CoA carboxylase is frequently considered to be the rate limiting enzyme of fatty acid biosynthesis. Initial observations of the activity of the enzyme in liver extracts showed that acetyl-CoA carboxylase had a much lower activity than fatty acid synthetase. But when the carboxylase was fully activated in the presence of citrate and assayed under optimum conditions, its activity is similar to that of fatty acid synthetase. Therefore the degree of enzyme activation may be important in the regulation of the in vivo rate of fatty acid synthesis.

The addition of citrate causes polymerisation of acetyl-CoA carboxylase from animal sources, but not in yeast or bacteria. The enzyme is competitively inhibited by palmitoyl-CoA *in vitro* with respect to citrate at physiological concentrations. The degree of this inhibition *in vivo* is hard to estimate, as the cytoplasmic levels of free palmitoyl-CoA are not known. The activity of the liver enzyme is depressed in starved rats, rats fed a high fat diet or made alloxan diabetic and is elevated in rats fed a fat-free diet. These changes of activity have been shown to be changes in enzymes content. The rat brain enzyme has not been investigated under different dietary conditions, although it would be reasonable to suggest that there may be a change in enzyme content during the period of maximum lipid deposition during myelination.

2. The second step in the synthesis of fatty acid is their formation from acetyl-CoA, malonyl-CoA and NADPH by the multi-enzyme complex fatty acid synthetase. The generalised equation for the synthesis of fatty acids is :

$$CH_3CO\text{-}SCOA + n\ HOOCCH_2\text{-}CO\text{-}SCOA + 2n\ NADPH + 2nH^+ \rightarrow CH_3(CH_2\text{-}CH_2)_n\text{-}CO\text{-}SCOA + nCO_2 + nH_2O + nCOASH + 2n\ NADP^+$$

For synthesis of palmitate from acetyl-CoA:

n = 7

The enzymes required for the synthesis of fatty acids from malonyl-CoA have been isolated from many unicellular organisms, plant and animal tissues. The multi-enzyme complex system was discovered by Lynen (139) in yeast, and subsequently comparable complexes have been isolated and purified from various avian and mammalian tissues (138).

The various intermediate reactions were elucidated using E.coli where the enzyme components exist as discrete units. Acyl-carrier protein (ACP) is a central component of all fatty acid synthetases, and contains a 4'-phosphopantetheine prosthetic group. The acyl intermediates in fatty acid synthesis are bound in thioester linkage to the SH group of the prosthetic group. The ACP from animal complexes has only been isolated as fragments and it appears that ACP is covalently attached to one of the two non-identical polypeptide chains that comprise the synthetases of the multi-enzyme complex type (140). Recent studies on mammary gland (141, 142) and rat and rabbit liver (143, 144) showed that fatty acid synthetase was more active with butyryl-CoA as primer than with acetyl-CoA.

Yeast fatty acid synthesis is terminated by the transfer of the acyl-chain from the SH - group of the 4' phosphopantetheine prosthetic group to CoASH. Termination of mammalian fatty acid synthesis in vitro results in the release of non-esterified fatty acids by an intrinsic mechanism. In E.coli a specific palmityl-ACP thioesterase has been isolated although its role in vivo is not clear since the ACP derivatives of the fatty acid products have been shown to be incorporated directly into phospholipids (137).

Fatty acid synthetase undergoes short and long-term control, and this enzyme may be important in the long-term regulation of fatty acid synthesis. The effects of hexose diphosphates (145, 146) and long-chain acyl-CoA derivatives on the enzyme do not appear to have a physiological role.

A regulatory role for the rapid turnover of the prosthetic group remains to be elucidated. Long term control of the content of fatty acid synthetase appears to be exerted by a variety of factors, nutritional, hormonal, genetic and developmental, although only developmental factors appear to affect fatty acid synthetase in the brain (145).

3. From in vitro studies of phospholipid metabolism, fatty acids are further metabolised in the presence of ATP and CoASH or as Acyl-CoA synthetase derivatives. Acyl-CoA synthetase has been investigated in sub-fractions from rat liver (147, 148) and the highest percentage of total activity was found in the microsomal fraction. Similar results were reported for the adult rat brain by Pande and Mead (149). Changes in the activity of this enzyme have not been investigated in the developing brain.

The formation of fatty acids with a chain length longer than C_{16} (the predominant product of brain fatty acid synthetase), is carried out by both mitochondrial and microsomal enzyme systems (150). The mitochondrial system elongates fatty acids by the addition of acetate units from acetyl-CoA whereas the microsomal system is malonyl-CoA dependant (151). Baumann et al have identified at least two different malonyl-CoA dependant elongation systems located in the microsomes from the brains of normal and 'quaking' mice. Ammonium sulphate precipitation was used to separate active microsomal elongation systems (152) but density gradient centrifugation has not been used.

1.6 Hormones and the Developing Brain:

Some behavioural changes affecting the fully mature brain are associated with hormonal imbalance. In these cases, normal adult behaviour can be restored by hormone therapy, but certain abnormalities are caused by lesions resulting from hormonal imbalance during certain periods in the development of the central nervous system and these are permanent. It was postulated by Pfeiffer (153) that the 'organization' (in terms of the formation of the fully developed functional brain) of the central nervous system is influenced by hormones acting at certain periods during the early development of the brain. Most investigations relate to the effect of adreno-cortical and thyroid hormones on the brain development of laboratory animals.

The postnatal growth of the brain in most animals is due partly to cell proliferation. Different areas of the brain increase in cell number (as measured by DNA content) to a differing degree and at different rates. The adult rat brain cerebrum contains 80% more cells than at birth and the increase in cell number is even greater in the cerebellum than in the cerebrum.

The timing of cell proliferation also varies from region to region in the brain. In the rat brain, the rate of cerebral cell acquisition is rapid in the first 15 days after birth whereas in the cerebellum there is a period of slow cell multiplication lasting a few days preceding extensive cell formation. Glial cell formation accounts for most of the postnatal increase in cell number (154).

Thyroid hormone is involved in the regulation of postnatal cell formation in the brain. Neonatal thyroidectomy causes a retardation in the rate of DNA increase, although normal cell numbers are attained after some delay (155, 156). Administration of triiodothyronine in early life also influences postnatal cell formation. This leads to a permanent reduction in cell numbers (157).

Triiodothyronine increases the rate at which enzymes involved in glucose metabolism and amino acid formation attain adult activity in developing rats, but these enzymes are depressed in thyroid deficient animals (158). The biochemical observations of brain maturation can be related to the morphological effect of thyroid hormone. Thyroid deficiency causes a reduction in neuronal cell-cell interactions (159) and a reduction in synaptosomal enzyme activity and glucose metabolism (156). There is significant reduction in the amount of myelin deposited in the brain of the thyroidectomised rat (160) although this may be due to a decrease in axonal density (161). The administration of thyroid hormone in early life only increases the rate of myelination in the initial stages. The effect of thyroxine on the activity of most enzymes associated with myelin formation has not been studied. Myelin formation in thyroidectomised rats is retarded and the peak incorporation of cerebrosides, sulphatides and cholesterol occurs between 22 and 30 days after birth, not 12 - 18 days as in normal rats (162).

Corticosteroid administration causes a marked inhibition of cell multiplication in the rat brain, although no significant change in cell size was noted (163). Balazs et al (164, 165) showed that various enzymes of nervous tissue have been induced prematurely by the administration of corticosteroids. Volpe and Kishimoto (112) noted that rat brain fatty acid synthetase activity was not affected by hydrocortisone or triiodothyronine administration, but the enzyme from C_6 glial cell culture was depressed by glucocorticoids (166). Rat brain glycerol-3-phosphate dehydrogenase activity is also affected by glucocorticoids. Hypophysectomy leads to a 40% decrease in enzyme activity (167).

Corticosteroid or thyroid hormone treatment in early life causes inhibition of postnatal cell development in the cerebrum by 30 - 40% but the appearance of substances (such as triphosphoinositides) used as indices of biochemical differentiation (165) was not affected. Thyroid deficiency, although it has no effect on cell formation in the cerebrum, produces retardation of enzyme activity. Since glial cell proliferation accounts for most of the increase in cell number in the cerebrum after birth, and cortisol and thyroid hormone cause a reduction in the number of glial cells in the brain, it remains to be seen if these hormones play a role in the control of lipogenic enzymes associated with myelin formation.

2. MATERIALS AND METHODS

2.1 Materials:

2.1.1 Chromatography:

Kieselgel H was obtained from E. Merck Laboratory Chemicals, Darmstadt, West Germany. Sephadex G25 was purchased from Pharmacia, Uppsala, Sweden. 10% Diethyleneglycol adipate - 3% phosphoric acid (DEGAP) precoated onto Chromasorb W was supplied by Jones Chromatography & Company, Newport, Mon.. Standard fatty acid methyl esters were obtained from Applied Science Laboratories, Pennsylvania, U.S.A. Lipid standards for thin-layer chromatography and fatty acid standards for gas liquid chromatography were obtained from P.L. Biochemicals, Milwaukee, U.S.A.

2.1.2 Radiochemicals:

$NaH^{14}CO_3$, (^{3}H-methyl) carnitine, (9,10-^{3}H) palmitic acid and (1,2(n)^{3}H) and (1.^{14}C) n-hexadecane were purchased from the Radiochemical centres, Amersham. Bucks.. (1-3^{14}C) malonyl-CoA and (2-^{14}C) malonyl-CoA, were supplied by New England Nuclear Chemicals, GMbH, Dreichenhain, West Germany.

2.1.3 Other Chemicals:

ATP, CoA, $NADH_2$, $NADPH_2$, NADP and oxaloacetic acid were obtained from Boehringer Mannheim, GMbH, Mannheim, West Germany. Dithiothreitol was purchased from Sigma Chemical Company, St. Louis, U.S.A. Crystalline bovine plasma albumin was supplied by Armour Pharmaceutical Company Limited, Eastbourne, Sussex. L-carnitine, thyroxine, propylthiouracil, heparin and the scintillation chemicals 2,5-diphenyloxazole (PPO) and 1,4, bis-2-(5-phenyloxazolyl) benzene (POPOP) were obtained from Koch-Light Laboratories Limited, Colnbrook, Bucks..

Basic scintillator was obtained from Intertechnique. All other chemicals, of A.R. purity, were obtained from either British Drug Houses, Poole, Dorset or Fisons Scientific Apparatus Limited, Loughborough, Leics.,

Air, nitrogen, hydrogen and 95% argon/5%CO_2 mixture were purchased from British Oxygen Company, and methane from British Oxygen Company or British Drug Houses.

2.1.4 Animals:

Rabbits were of the New Zealand White strain (albino) supplied by the Gadsby Animal Farm, Penistone, Yorks. Adult rabbits were of the female sex, while younger rabbits were of both sexes. Suckling rabbits were removed from the mother immediately before killing. Weaned rabbits were fed ad libitum on a diet of water and Coney Pellets (BOCM, Stoke Mandeville, Bucks.). The pellets had the following fatty acid composition (expressed as a percentage of the total fatty acids) palmitic, 13: palmitoleic, 0.5: Oleic, 27: linoleic, 7: arachidonic, 4.

Wistar rats from the University colony, were fed ad libitum on a diet of ratcake and water. The diet was Oxoid Modified Diet 86 obtained from H.C. Styles (Bewdley) Limited, Bewdley, Worcs. Adult rats were in the weight range of 150-200 gm. and females were used at all ages except in the thyroid hormone studies. Pregnant rats used in the thyroid hormone studies were kept in the Animal House for the duration of the study, and fed pasteurised diet from the above source.

These rats were supplied with the exact date of conception known. This was determined by the Animal House staff looking for vaginal 'plugs' which are released by the female after mating has taken place. The average size of the litters used was twelve. Bovine and calf liver used for the preparation of carnitine acyl transferase was obtained from the local abattoir and transported to the laboratory on ice.

2.2 METHODS

2.2.1 Preparation of Subfractions from Whole Brain:

Animals were killed by cervical dislocation. The whole brain was quickly removed, chilled and weighed. For ages up to fifteen days, six brains from littermates were homogenised together, but fewer brains from older animals were combined. Foetuses were delivered by caesarian section immediately after the death of the mother. Only foetuses from the last third of gestation were used.

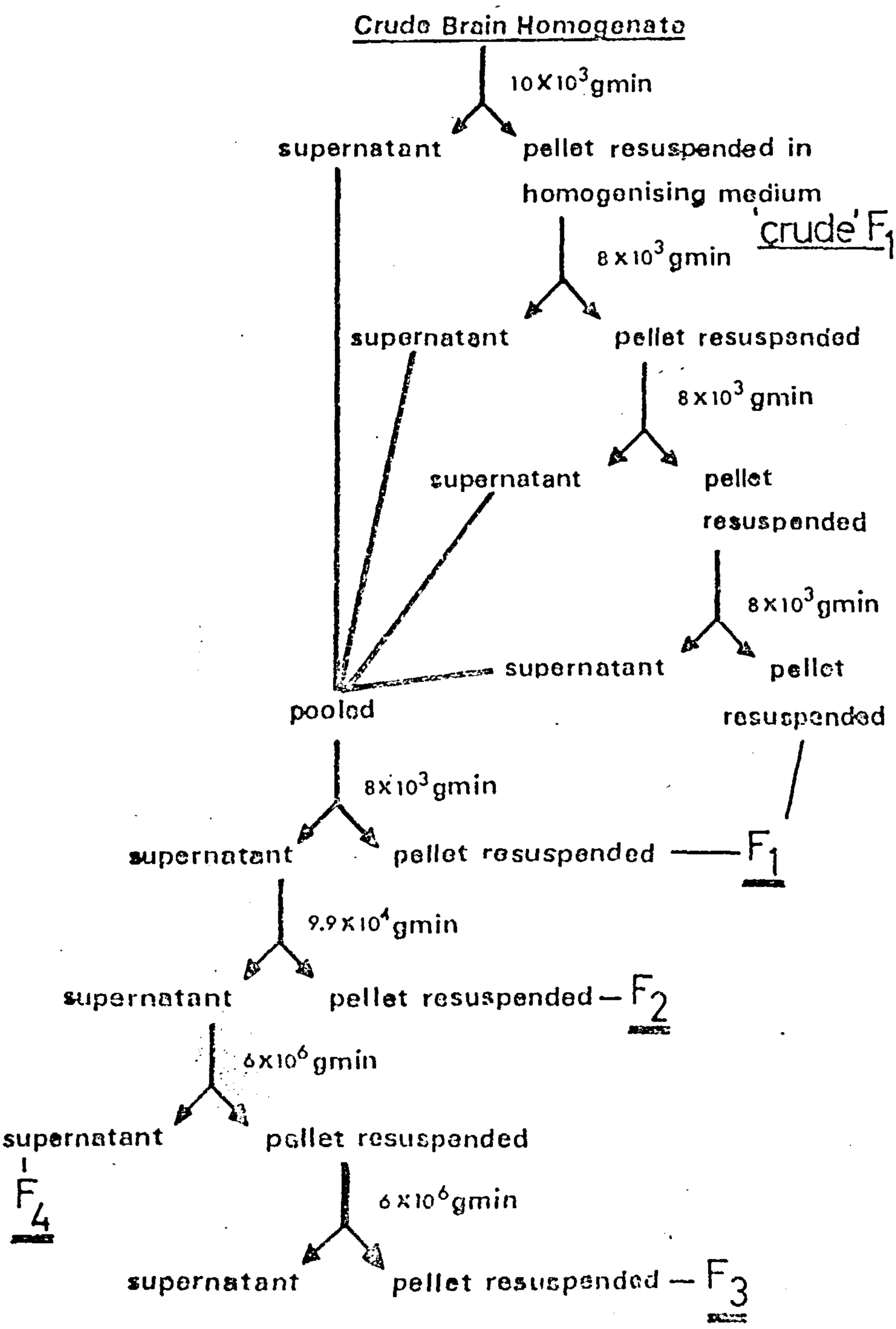
Whole brains were homogenised in ice-cold medium in the ratio of 1:2 (W:V) using a motor-driven Potter-Elvehjem homogeniser with a teflon pestle, clearance 0.1mm (15 passes). The homogenisation medium used contained 0.3M sucrose, 0.25M potassium phosphate and 10mM dithiothreitol at pH 6.8. Early experiments showed that the activity of palmitoyl-CoA synthetase was lower if the tissue was fractionated in sucrose alone. Dithiothreitol was added to prevent oxidation of the -SH groups of the proteins.

The scheme used for the subfractionation of brain tissue was a modification of that reported by Gray and Whittaker (38) outlined diagrammatically in Figure 2.1. The method used differed from the original in that the first pellet was resuspended in homogenisation medium and recentrifuged four times at 8000 gmin., as reported by Lapetina (168). This reduces any mitochondria or cytosol trapped in the pellet.

FIGURE 2.1

Flow diagram for the preparation of brain subfractions by differential centrifugation.
All values are given as gmin or g x minutes and the fractions prepared are labelled : 'crude' F_1, F_1, F_2, F_3 and F_4.

Figure 2.1 Centrifugation scheme for brain subfraction preparation.



All manipulations were carried out at $4^{o}C$ using ice baths and refrigerated centrifuges. All centrifugation steps involved in the preparation of the F_1 fraction (see Figure2.1) were carried out using the high-speed attachments of the MSE Mistral-6L. Fractions F_2, F_3 and F_4 were prepared by centrifugation in the 10 x 10 ml. and the 8 x 50 ml. rotors of the MSE Superspeed 50 or the 50 and 30 rotors of the Beckmann Spinco L-2 ultracentrifuges. There was no observable difference in the results obtained using either of these centrifuges and the use of either one depended upon availability. Subfractions were made up to known volumes with homogenisation medium and either assayed immediately or stored at $-20^{o}C$.

Fractions were designated as follows :

F_1 : plasma membrane, nuclei, and heavy myelin

F_2 : mitochondria, synaptosomes and light myelin

F_3 : microsomes

F_4 : soluble fraction

The contamination of the fractions by other subcellular particles will be discussed in the results.

Fractions F_1 and F_2 were further subfractionated by sucrose density gradient centrifugation (Section 2.2.2). The effect of (1) freezing and thawing, (2) sonication and (3) osmotic shock on these fractions has also been determined by density gradient centrifugation.

(1) Freezing and thawing was carried out in either test tubes or plastic vials. Up to 2 ml. of sample, containing 100 mg. protein, was first immersed in liquid nitrogen until frozen and then transferred to a water bath at 30^{o} until it thawed.

This was repeated four times before the sample was layered on a gradient.

(2) Sonication was carried out using an MSE sonication model 150 W. Full power was applied to the sample for a total of one minute in sessions of 15 seconds. The sample was sonicated standing in an ice bath to prevent overheating and subsequent protein denaturation.

(3) Samples were subjected to an osmotic shock by resuspending pelleted F_1 or F_2 material in ten volumes of distilled water. A motorised homogeniser was used to resuspend the pellets. The samples were then left on ice for one hour. After recentrifugation the samples were resuspended in homogenisation medium. As a control, aliquots of the same samples were subjected to the same treatment using homogenisation medium throughout.

2.2.2 Sucrose Density Centrifugation:

Sucrose density gradients were formed at room temperature using either a double-well perspex gradient mixer, with one well used as a mixing chamber, or a three-channel Pharmacia pump (Model P-3). Both methods gave good reproducible linear gradients, when checked using a refractometer, although the use of the mixer was later discontinued in favour of the pump since it was possible to make two gradients simultaneously. Gradients were formed with 0.5M sucrose containing 0.25M potassium phosphate pH6.8 as the starting solution and 1.5M sucrose containing 0.25M potassium phosphate pH 6.8 as the limiting solution.

1.0M sucrose/phosphate solution was also used as a limiting solution. Different sized gradients were formed in tubes for the Beckmann 50 rotor (8 ml) the Beckmann SW 41 (12 ml) and the MSE 3 x 25 ml (20 ml) swingout rotors, the figures in brackets refer to the size of gradient formed in each tube.

Gradients were prepared before the preparation of subcellular fractions F_1 and F_2, and left for two to three hours to equilibrate before use. Up to 0.5 ml of resuspended F_1 and F_2 containing up to 25 mg. protein, were loaded onto the 8 ml. and 12 ml. gradients and up to 1.0 ml. onto the 20 ml. gradient.

After centrifugation, bands were removed by three different methods :

(i) Bands were removed sequentially down the gradient by a manual process using a Pasteur pipette. The disadvantage of this method was that some material from each band was observed to have been left behind, and in cases where bands were very close together, cross-contamination could occur.

(ii) The LKB sucrose density gradient fractionator which pumps out the gradient from the bottom of the tube, was found to be unsatisfactory because passing the central stainless steel outflow pipe through the gradient caused some disturbance of the bands. Also thick bands tended not to pass through the pipe and adhered to the outer surface and tended occassionally to block the pipe.

(iii) The MSE unloader was found to be more satisfactory than the LKB model. Heavy 2.0M sucrose was pumped through a central pipe to the bottom of the centrifuge tube and this displaced the gradient, finally pushing it through the perspex cap and out through a plastic tube to the collecting tubes. Although the cap was made of perspex, it was not possible to see when each band reached the top of the centrifuge tube, passed through the cap and out to the collecting tubes, due to the optical properties of the machined perspex. In common with the previous method, material adhered to both the perspex cone and the delivery tube and it was found that large particles of agglutinated membrane appeared in fractions that were expected to be clear.

Fractions obtained from the gradient were analyzed, either straight from the gradient, or after dilution with distilled water, to give a final sucrose concentration of 0.3M, or dialysis against the homogenisation medium.

2.2.2.1 Development of Conditions for Continuous Sucrose Density Gradient Centrifugation:

Samples of freshly prepared F_1 fraction in 0.3M sucrose, were applied to continuous sucrose density gradients in the range 0.5M to 1.5M sucrose. Initially, these gradients were 8ml in volume and were run in the Beckmann 50 fixed angle rotor. The shortest centrifuge run was for one hour at 50,000g (3×10^6gmin) and this gave low resolution of three bands on the 8ml gradient.

The speed was increased to 63,000g for one hour (3.8×10^6gmin) which was used by Hemminki and Suovaniemi (169) to separate plasma membranes on discontinuous gradients. At this speed, four bands were observed. Increasing the running time only slightly increased the resolution and above two hours no difference was observed. Increasing the speed of the centrifuge did not improve the separation in the 8ml gradient (See Figure 2.2).

The diagramatic representation of the gradients in Fig. 2.2 shows a thick band (band two) tailing off on one side.

This was caused by using a fixed angle rotor. The centrifugal force is applied diagonally acrosss the tube and some of this fraction has remained adhered to the wall of the tube after completion of the centrifugation. In differential centrifugation, this phenomenon slightly disrupts the orderly sedimentation of particles and so could perturb a sucrose gradient in density gradient centrifugation. Because of this, the Beckmann SW41 swingout rotor was used. Using 12ml gradients, it was found that best resolution was obtained at 100,000g for ninety minutes (9×10^6gmin). The bands were broader than before because of the shallower gradient (see Figure 2.3a.). Figure 2.3b. shows the separation obtained on 20ml gradient at 100,000g for ninety minutes (9×10^6gmin). This gradient was used extensively in the analysis of fractions following intracerebral injection since it was possible to load more material onto the larger gradient and the bands were very sharply defined and so helped to reduce cross-contamination.

FIGURE 2.2

Diagrammatic representation of the results of fractionation of F_1 by sucrose density gradient centrifugation.
Centrifugation conditions are given in g x minutes from 50,000 x g for 60 minutes to 63,300 x g for 120 minutes. The three bands obtained retain the same identification throughout. The gradients were linear from 0.5M to 1.5M sucrose buffered with 0.25M potassium phosphate at pH 6.8. The tailing of band 2 is caused by the use of a fixed angle rotor.

Figure 2.2 Sucrose density gradient centrifugation.

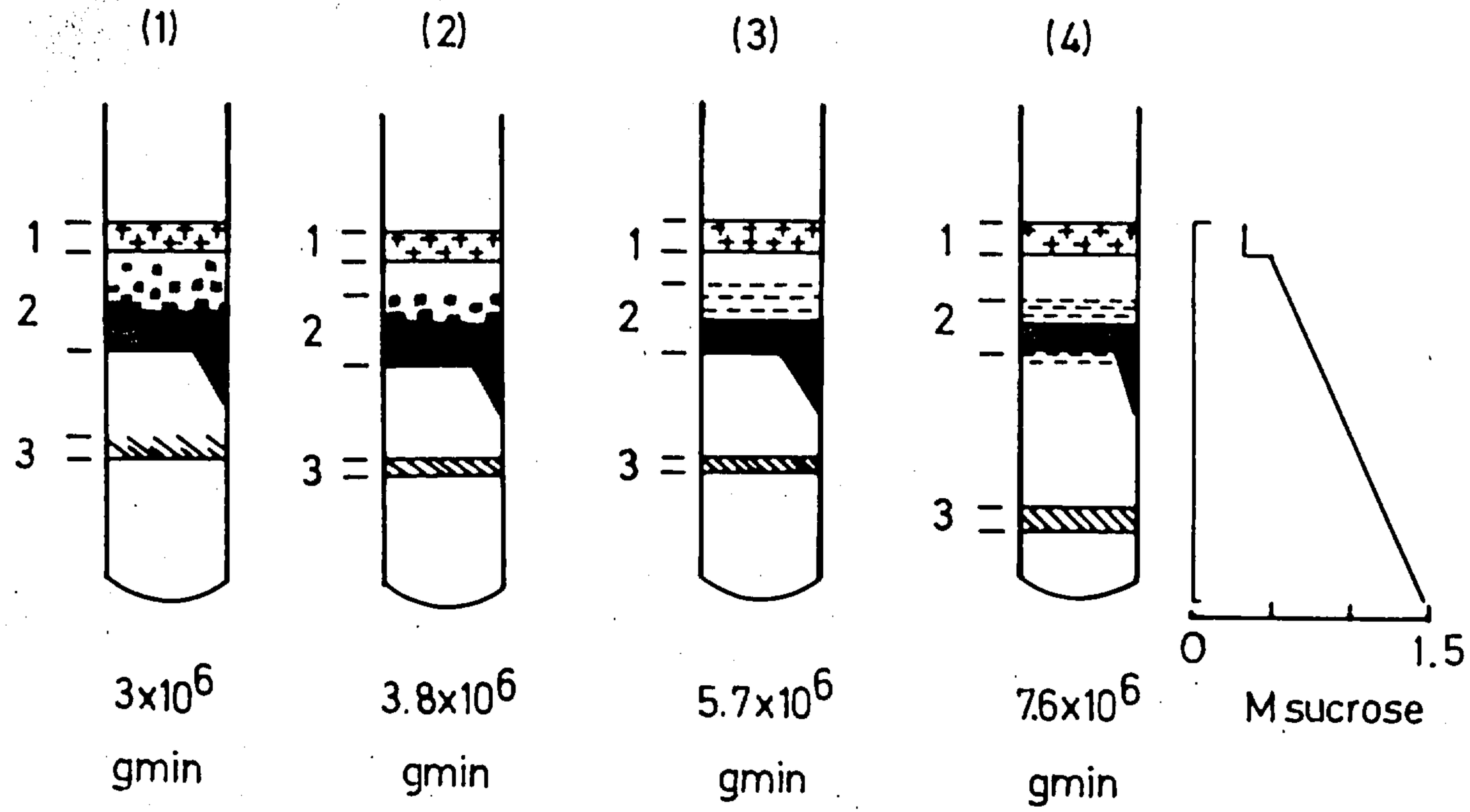
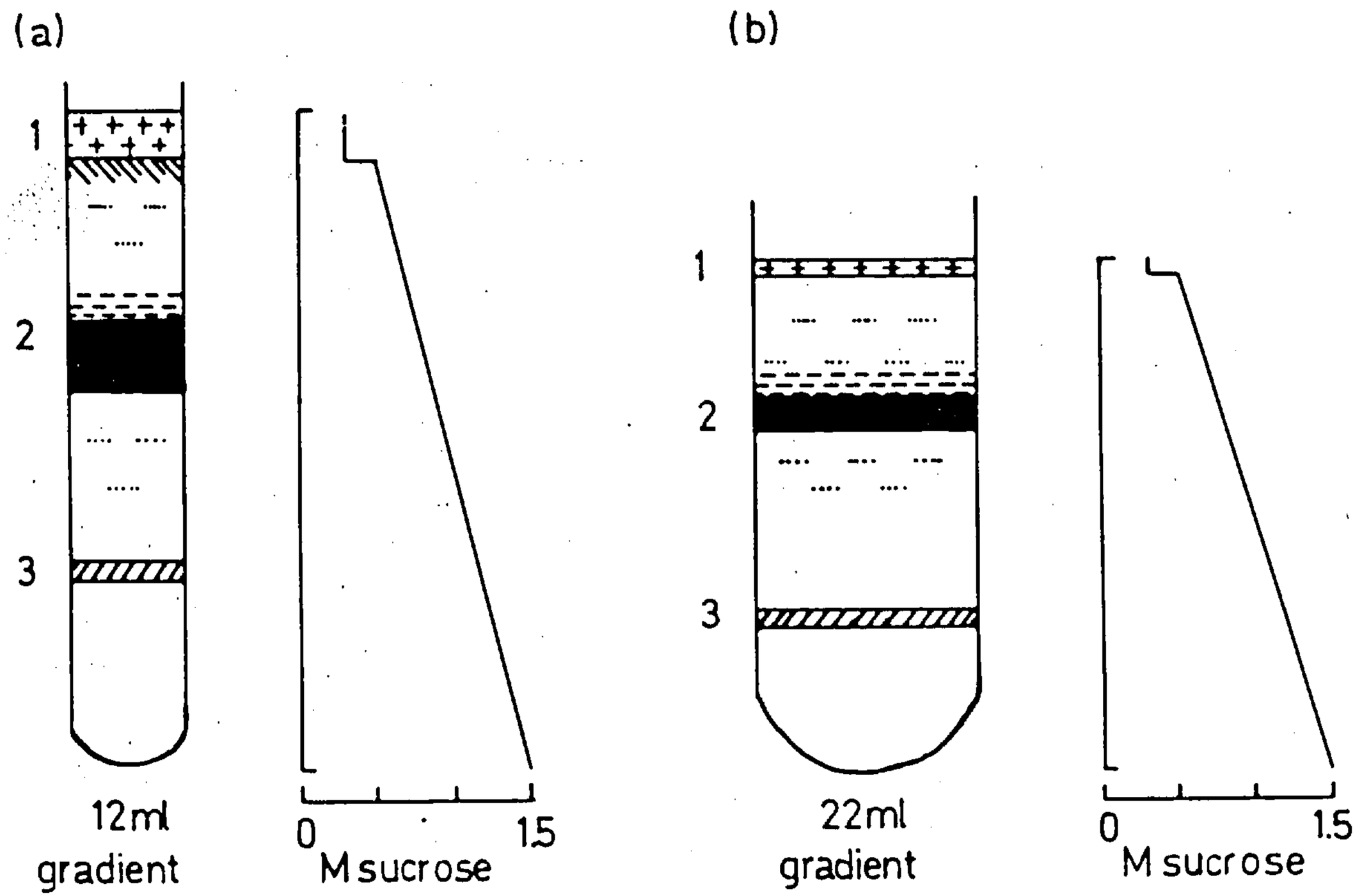


Figure 2.3 Sucrose density gradient centrifugation.



The material recovered from density gradients was designated according to its original source (eg: F_1) and the position of the band on the gradient (eg: 2) giving rise to fractions F_{12} etc.

2.2.3 PREPARATION OF ENZYMES

2.2.3.1 Preparation of carnitine palmitoyl transferase (EC. 2.3.1.2.)

Palmitoyl-CoA : carnitine palmitoyl transferase was prepared using the method of Norum (170). Bovine and calf liver were used in two separate preparations of this enzyme. The washed mitochondrial pellet was prepared and resuspended exactly as described by Norum and stored at -20°C. Small aliquots of this were taken and purified to yield a crude carnitine palmitoyl transferase fraction. The suspension of the freeze-dried extract obtained in the final stage of the preparation was spun in a bench top centrifuge and the transferase activity in this fraction was assayed using a radioactive carnitine exchange reaction (171). The equation for this reaction is as follows :

palmitoyl-carnitine + CoA + ^{3}H-carnitine $\rightleftharpoons$

palmitoyl-CoA + carnitine + ^{3}H-carnitine $\rightleftharpoons$

palmitoyl-^{3}H-carnitine + CoA + carnitine

The incubation mixture contained in a total of 1.0 ml :
0.5mM palmitoyl (L)-carnitine, 0.12mM CoASH, 0.2mM (^{3}H-Me) carnitine (1Ci/mole), 2mM dithiothreitol, 100mM Tris-HCl pH7.5, protein and water. All incubations were carried out in duplicate in a shaking water bath at 30°C. The reaction was started by the addition of protein and stopped with 0.1ml of concentrated HCl. Radioactive palmitoyl-carnitine was extracted with 1.0ml of n-butanol after the addition of 0.9ml distilled water. The butanol fraction was removed by Pasteur pipette and washed with 2ml butanol-saturated water. 0.2ml of this fraction was taken to dryness in a scintillation vial and the radioactivity determined. The enzyme preparation was used in the assay of palmitoyl-CoA synthetase.

2.2.4 PREPARATION OF SUBSTRATES

2.2.4.1 Preparation of defatted bovine serum albumin:

The solutions of defatted bovine serum albumin prepared (172) had protein concentrations of 70-90 mg/ml and were stored at -20°C and diluted as necessary.

2.2.4.2 Preparation of potassium palmitate solutions:

Equimolar amounts of palmitic acid and potassium hydroxide were used to give a final solution of 5mM potassium palmitate. The solution was stored at 4°C and warmed to 30°C before use.

2.2.4.3 Preparation of acetyl-CoA:

A modification (173) of the method of Ochoa (174) was used in the preparation of acetyl-CoA. The yield was determined by measuring the appearance of free-SH groups (see section 2.2.6.4) after hydrolysis of the thioacyl bond by 0.2M KOH. The yield (routinely greater than 80%) was calculated by subtracting the amount of unreacted COASH, determined by the free-SH concentrate before hydrolysis, from the total -SH concentration after hydrolysis.

2.2.4.4 Preparation of malonyl-CoA:

This was routinely prepared by the method of Trams and Brady (175) by firstly synthesising monothiophenylmalonate and then reacting this with CoASH. Care was taken to exclude traces of water by passing the nitrogen through a calcium chloride drying tube before entering the reaction vessel, and by only using freshly redistilled dimethyl formamide and thiophenol.

The yield of monothiophenylmalonate was estimated from its absorbance at 237nm in absolute ethanol. This intermediate was stored at $4^{o}C$ and found to be stable for several months. Malonyl-CoA was prepared by reacting excess (20 µmol) monothiophenylmalonate with 10 µmol CoASH in 0.1M bicarbonate solution at pH 8.3 at $4^{o}C$. A gentle steam of nitrogen was passed through the reaction mixture. Unreacted intermediate and thiophenol were removed by extraction with diethyl ether, and the yield (percentage bound to CoASH) was estimated as for acetyl-CoA.

It was found that malonyl-CoA was stable for several weeks but it was usually prepared just prior to use. Both acetyl- and malonyl-CoA were used without further purification.

2.2.4.5 Preparation of palmitoyl L-carnitine:

Palmitoyl chloride and free L-carnitine were used in the preparation of palmitoyl-carnitine according to the method of Bohmer and Bremer (176) Palmitoyl chloride was prepared by refluxing 500 mg palmitic acid with 15 ml thionyl chloride. Unreacted thionyl chloride was removed at room temperature under a gentle stream of dry nitrogen until constant weight was reached. Immediately on preparation, the acyl chloride was mixed with a solution of 300mg L-carnitine in 0.5 ml trifluoro-acetic acid in a test tube fitted with a calcium chloride drying tube and left in a water bath overnight at 40-$45^{o}C$. Isolation of palmitoyl carnitine was carried out as described (176).

2.2.5 ASSAY OF ENZYMES

One unit of enzyme activity is defined as the amount of enzyme required to convert 1 nmol of substrate to product per minute. All assays were carried out in duplicate at 30°C in a shaking water bath. Spectrophotometric assays were carried out at 30°C as described in section 2.2.5.4. All enzyme assays used were checked for linearity with respect to protein and time.

2.2.5.1. Fatty acid synthetase:

Fatty acid synthetase was assayed in subcellular fractions by the method of Volpe and Kishimoto (112). The final volume of each incubation was 0.5 ml and contained 40mM potassium phosphate pH 6.4, 130 µM NADPH, 50µM acetyl-CoA, 160µM (^{14}C) malonyl-CoA (either 2-^{14}C or 1,3-^{14}C, 0.5 Ci/mol), 40µM EDTA, 3mM dithiothreitol and up to 2mg protein. The reaction was initiated by the addition of the protein and stopped by the addition of 4 ml chloroform: methanol (2:1 v/v), after 5 and 10 minutes. Lipids were extracted using the method of Folch et al (177)(See methods section 2.2.7). A portion of the lipid extract was taken to dryness at room temperature in a scintillation vial and the radioactivity determined by liquid scintillation counting (see section 2.2.6.4). The radioactive products were analysed by thin-layer and radiogas - liquid chromatography.

2.2.5.2 Acetyl-CoA carboxylase (EC 6.4.1.2.):

This enzyme was assayed in the F_4 fraction of brain homogenates using the method of Gross and Warshaw (113) which was a modification of that of Greenspan and Lowenstein (178). This method was developed specifically for the assay of rat brain acetyl-CoA carboxylase.

The enzyme was preincubated at 30°C for 30 minutes in a shaking water bath in a medium (0.5 ml) containing 40mM Tris-HCl pH 7.5, 20mM $MgCl_2$, 8mM sodium citrate 4mM EDTA and 2 mg defatted bovine serum albumin, (using up to 2 mg brain supernatant protein). The assay was started by the addition of 0.5ml medium containing: 40mM Tris-HCl pH 7.5 20mM $MgCl_2$, 8mM sodium citrate 4mM ATP, 0.8mM acetyl-CoA and 160mM $NaHCO_3$, containing $NaH^{14}CO_3$ (specific activity 0.05Ci/mole). The complete mixture was then incubated for intervals up to 15 minutes at 30°C and terminated by the addition of 0.1 ml 10% tricholoracetic acid. The samples were then centrifuged in a bench-top centrifuge to sediment the protein. The supernatant was then removed and dried down in a scintillation vial under a stream of hot air to remove ^{14}C-bicarbonate. Radio activity was determined by liquid scintillation counting. See section 2.2.6.4. This method was used for both the "crude" supernatant fraction and that purified by filtration through a Sephadex G-25 column to remove low molecular weight compounds.

2.2.5.3 Palmitoyl-CoA synthetase (EC 6.2.1.3):

This enzyme was assayed by the method of Farstad et al.(179) and is based on the conversion of palmitoyl-CoA to palmitoyl-(^3H) carnitine. Incubations were carried out at 30°C in a 1 ml system containing: 100mM Tris-HCl-pH 7.5, 80μM CoA, 3.0mM dithiothreitol, 1.5mM ATP, 0.4mM potassium palmitate, 3mg defatted bovine serum albumin, 2mM $MgCl_2$, 5mM L-(^3H-Me) carnitine (0.1Ci/mole), excess palmitoyl-CoA carnitine palmitoyl transferase and protein from the full homogenate or subcellular fractions.

The reaction was started by the addition of ATP, and stopped with 0.1 ml concentrated HCl. Palmitoyl-(^{3}H) carnitine was extracted by either the method of Bremer (180) or Solberg (171). The former method required the addition of 0.9ml of distilled water and 1.0 ml n-butanol and thorough mixing before the upper phase, which contained radioactive palmitoyl carnitine, was removed and washed with butanol-saturated water. The latter method required the addition of 0.9 ml saturated ammonium sulphate solution and 1.0 ml iso-butanol saturated with ammonium sulphate. There was no observable difference in the results obtained by either method. Radio activity was determined by liquid scintillation counting after evaporation of 0.2 ml of the washed non-aqueous fraction.

2.2.5.4 Glucose-6-phosphate dehydrogenase (EC 1.1.49):

Glucose-6-phosphate dehydrogenase was assayed in subcellular fractions of the brain by the method of Baumann (181). The system contained in a final volume of 2.5ml: 0.15mM Tris-HCl pH 7.5, 10μM $MgCl_2$, 0.11mM NADP, 10μM glucose-6-phosphate, protein and water. The reduction of NADP was followed at 340nm at 30°C in either a Gilford Model 2410 or Pye SP1800 recording spectrophotometer. The reaction was started by the addition of glucose-6-phosphate after a steady background rate had been recorded.

2.2.5.5 Malate dehydrogenase: NAD Dependent (EC 1.1.37):

Malate dehydrogenase was assayed (182) using oxaloacetic acid and NADH. The reaction mixture contained in 1 ml: 200mM potassium phosphate pH 7.5, 1.5mM oxaloacetic acid 0.7mM NADH and protein from brain subcellular fractions. The reaction was started by the addition of oxaloacetic acid after a steady background rate had been recorded. The reaction was followed at 340nm using a recording spectrophotometer.

2.2.6. NON-ENZYMIC DETERMINATIONS

2.2.6.1 Determination of protein concentration:

Protein concentration was estimated in the crude homogenate and all subcellular fractions except the microsomal fraction using Biuret reagent (183). Protein, from a known volume of each fraction, was precipitated using 10% trichloroacetic acid (5% final conc.) and the samples were centrifuged. The supernatant was carefully decanted and the pellets were suspended in 1.0 ml distilled water and mixed with 2.0 ml Biuret reagent. The samples were incubated at 30°C for 30 minutes for full colour development. The optical densities of the samples were read at 555nm together with blanks containing no protein, in either Pye or Gilford spectrophotometers. The protein concentration was determined by reference to albumin standards in the range of 1-10mg protein/ml. Since this assay is not sensitive at low protein concentrations the Lowry-Folin method (184) was used to determine the protein concentration of the microsomal fractions, which contained in the order of 1mg/ml protein. This method also avoids the use of large amounts of protein in the assay, requiring only 25-300µg protein. The assay was carried out exactly as described using freshly made up solutions and the samples were read at 750nm together with albumin standards and protein-free blanks.

2.2.6.2 DNA estimation:

DNA concentration was estimated by the method of Burton (185). Samples of full homogenate were hydrolysed in 10% TCA and made up to 1.9ml with distilled water. 0.1 ml 70% perchloric acid was then added. After thorough mixing 2.0ml of diphenylamine-acetylaldehyde reagent was added, mixed, and the samples left for 18 hours at room temperature.

The absorbance of the samples, hydrolysed calf thymus DNA standards and DNA-free blanks was determined at 600nm.

2.2.6.3 Free thiol groups:

SH groups were determined using the Jocelyn method (186). 0.1 ml sample was added to 0.9 ml distilled water and mixed before the addition of 1.5 ml, 50mM potassium phosphate pH 6.8 and 0.5 ml disulphide reagent (0.4gm dithiobisnitrobenzoic acid (DTNB) in 100 ml 0.2M potassium phosphate pH 6.8). Samples and blanks were left for five minutes before the optical densities were read at 412nm. CoASH was used as standard in the range 1-10 μmole.

2.2.6.4 Determination of radioactivity:

Radioactive samples in chloroform or chloroform:methanol (2:1), from the assay of fatty acid synthetase and from the fractionation of brains removed from animals after intracerebral injection (see section 2.2.8), were taken to dryness in scintillation vials before the addition of scintillation fluid. These samples after drying down, were counted in 10 ml xylene scintillator containing 5.0 gm PPO and 0.5 gm POPOP per litre xylene. Samples containing water or n-butanol were counted in 10 ml Triton-toluene scintillator (1:2 v/v) containing 4.0 gm PPO and 0.2 gm POPOP in 1 litre.Butanol samples, after solvent evaporation, were not counted in xylene scintillator as they were found to contain a small quantity of water and this produced a cloudy emulsion which gave rise to variable counting efficiency.

All samples were counted in a Packard liquid scintillation spectrometer model 3385. The counts per minute (cpm) were converted to disintegrations per minute (dpm) by the application of quench curve correction obtained by the external standard ratio method. Quench curves were generated by the addition of 0-100μl chloroform to

standards containing 69 x 10^3dpm (^{3}H) or 30 x 10^3 dpm (^{14}C)-hexadacane. The data was fed into a Wang desk top computer model 700, and the generated curves were stored on tape cassettes and then used to convert cpm to dpm, by reference to the external standard ratio . The programme for the Wang computer for single-label counting was written by Dr. T. Hoy. Data was obtained from the counter as punched tape and was processed by the computer using a punched tape reader.

2.2.6.5 Determination of radioactivity in the carboxyl carbon:

The method of Brady et al was used in this determination (187). Extracted fatty acids were decarboxylated via the Schmidt degradation using concentrated H_2SO_4 and sodium azide. The labelled carbon dioxide liberated was trapped in basic scintillator Intertechnique.

2.2.7 LIPID EXTRACTION AND ANALYSIS

2.2.7.1 Folch extraction procedure:

The Folch method (177) of extraction was used to extract all radioactive lipids, both from the assay of fatty acid synthetase, and from brain subfractions obtained after intracerebral injection. All samples were extracted with 2:1 chloroform:methanol (v:v) and the volumes were adjusted to give the Folch extraction ratio of chloroform: methanol: water 8:4:3. The upper phase was washed twice with synthetic lower phase and the combined lower phases were washed once with synthetic upper phase before being dried down under nitrogen or by rotary evaporation under partial vacuum. The lipid samples were then dissolved in a known volume of chloroform and aliquots were taken for analysis by thin-layer chromatography, and gas-liquid chromatography and for the determination of radioactivity.

2.2.7.2 Thin-layer chromatography:

Thin-layer chromatography was carried out on 20cm x 20cm glass plates spread with a layer of Kieselgel-H 0.6mm thick. The plates were activated for one hour at 100°C before use and were pre-run in petroleum ether:diethyl ether:acetic acid, in the ratio 75:25:1 before the application of samples (188). Samples were applied to the plate in a small volume of chloroform using capillary tubes. The position of each sample on the plate was determined using a Shandon spotting guide. Purified standards were also run with the samples in order to identify the separate spots. The standards for the identification of neutral lipids were cholesterol stearate, glycerol tripalmitate, palmitic acid, cholesterol, glycerol dipalmitate, glycerol monopalmitate and lecithin (phosphatidyl choline). Rf values were 1.0, 0.8, 0.55, 0.4, 0.42, 0.2, 0, respectively. The solvent used was petroleum ether: diethyl ether: acetic acid 75:25:1 (188).

Phospholipids were separated using a solvent containing chloroform: methanol: water in the ratio 65:25:4 (188). Phospholipid standards used were phosphatidylcholine, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, sphingomyelin and phosphatidic acid. Rf values were 0.6, 0.4, 0.3, 0.83, 0.29, and 0.88 respectively.

The separated lipids were visualised by brief exposure to iodine vapour. The radioactivity in each spot was determined using a Berthold thin-layer scanner. The areas under the peaks on the trace from the thin-layer scanner, were calculated and the relative percentage of radioactivity of each lipid class was calculated.

The method used to separate phospholipids depended upon the amount present in the sample. In a neutral lipid solvent, phospholipids remained at the origin and were eluted from silica gel, scraped from the origin of the chromatogram, with chloroform: methanol (1:1 v/v). The eluate was then concentrated and applied onto a new chromatogram. The recovery of phospholipid from silica-gel was 50-60%. If there was insufficient material to allow neutral and phospholipids to be run on separate chromatograms, the neutral lipid chromatogram was rerun in the more polar phospholipid solvent. In this solvent, the neutral lipids run with the solvent front and the formerly immobile phospholipids were partitioned. There was no interference from the neutral lipids and material was not lost in this method.

2.2.7.3 Gas-liquid radiochromatography:

The radioactive fatty acid composition of the products extracted from the assay of fatty acid synthetase was determined by gas - liquid radiochromatography. Samples were firstly saponified with 1M KOH at 100^{o}C for 10 minutes and then extracted with petroleum ether. Unesterified fatty acids in petroleum ether were then taken to dryness in screwtop vials and methylated using 0.2 ml boron trifluoride in methanol at 60^{o}C for 5 minutes (189). The samples were then cooled on ice and 0.5 ml distilled water was added and the methyl esters were extracted with pentane. The lower phase was extracted twice with 0.5ml pentane and the combined extracts were taken down to 10μl under nitrogen. The methyl esters were then injected onto a gas-liquid chromatograph.

To ensure the quantitative transfer of radioactive fatty acids and methyl esters a carrier containing equimolar quantities of standard fatty acids was added to the samples before saponification.

The carrier contained 10 μmole of the following fatty acids :

$C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, $C_{20:0}$, $C_{20:1}$, and $C_{22:0}$.

A standard mixture of methyl esters was also injected onto the GLC before running radioactive samples to check the separation of each fatty acid methyl ester and make it possible to identify the constituents of the samples by reference to retention times. The gas-liquid chromatograph used was a Pye 104 with a Panax radiogas detector unit, equipped with a gas proportional counter. The proportional counter measured the amount of $^{14}CO_2$ in the gas stream after combustion of the methyl esters in a combustion tube packed with copper oxide at 300°C. The system employed a stream splitter at the end of the GLC column before the H_2 flame ionisation detector with a ratio of 1:5, therefore 20% of the column effluent went to the mass detector and 80% of the column effluent passed through the combustion tube. The 48 inch column was packed with a stationary phase of 10% diethylene glycoladipate -3% phosphoric acid (DEGAP) on a support of chromasorb W. The gas phase was 95% argon/5% carbon dioxide at a flow-rate of 40ml/min. Samples were chromatographed at an oven temperature of 180°C. The amplified signals from the ionisation detector and the gas proportional counter were continuously monitored and superimposed using a two-pen recorder. The radioactivity in each sample was quantitated by using a scaler/timer with an attached printer. The radio-active peaks were identified by direct comparison with the mass trace and reference to the retention times obtained with the standard mixture of methyl esters.

2.2.8 INTRACEREBRAL INJECTION OF SODIUM (9,10-^{3}H) PALMITATE

The radioactive palmitic acid was converted to the sodium salt by the addition of an equimolar quantity of sodium hydroxide to give a final concentration of 20$_{\mu}$mole/ml and specific radioactivity of 500Ci/mole. In two separate experiments, two groups of rabbits (littermates) of 10 and 24 days of age were injected intracranially with 0.1ml of the palmitate solution at a point midway between the ears and to a depth of 5mm. The depth of injection was controlled by fixing a plastic sheath to the syringe body which covered the needle except for the final 5mm of its length, and therefore gave a reproducible depth of injection when the sheath was in contact with the top of the head. Palmitate solution was injected very slowly to prevent the animal going into convulsions. At various times after injection up to 24 hours, animals were sacrificed and the brains quickly excised and subfractionated according to the scheme outlined in section 2.2.1.

2.2.9 THYROID HORMONE STUDIES

Routinely, each litter used was divided into test and control pups. All pups were given intra-peritoneal injections daily from birth according to their body weight. At various ages up to 20 days pups were sacrificed and their brains quickly removed and stored at -20^{o}C. Blood samples were taken after decapitation and stored in heparinised tubes. The blood from control and test animals at each age were pooled separately. Total plasma concentration was assayed by Dr. I. Broadhead in the Pharmacology Department, University of Sheffield.

2.2.9.1 Hyperthyroid studies:

The test pups from each litter were given daily doses of thyroxine from birth. 0.01% thyroxine in physiological buffered saline (PBS) containing 0.02% sodium hydroxide, was administered in the ratio of 0.1mg/100gm body weight. Control pups were injected with PBS in the ratio of 1ml/100gm body weight.

2.2.9.2 Hypothyroid studies: Throughout the experiment pregnant rats and lactating mothers were provided with drinking water containing n-propylthiouracil (PTU)(0.02% in tap water containing 0.02% NaOH) from the 13th day of pregnancy. At birth, some pups from each litter were given intraperitoneal injections of PTU daily.(PTU$_b$)The solution used contained 0.5% PTU in PBS containing 0.02% NaOH and was injected into the pups in the ratio of 1ml/100gm body weight. The other pups were given PBS in the same volume/body weight ratio.(PTU$_a$)

3. RESULTS

3.1 DISTRIBUTION OF MARKER ENZYMES IN SUBCELLULAR FRACTIONS FROM BRAIN TISSUE.

In order to determine the subcellular location of the lipogenic enzymes investigated in this study, it was necessary to characterise the cellular components isolated in each of the fractions (F_1 - F_4) produced by the procedure outlined in section 2.2.1. Marker enzymes were chosen as a suitable method of identifying the constituents of the various fractions.

3.1.1 GLUCOSE-6-PHOSPHATE DEHYDROGENASE (EC 1.1.49)

The activity of the enzyme glucose-6-phosphate dehydrogenase was used as a marker of the soluble fraction. In measuring the activity of fatty acid synthetase in all subcellular fractions it was important to assess the amount of enzyme activity that was due to contamination by soluble proteins. The results are shown in table 3.1. Most of the activity of glucose-6-phosphate dehydrogenase was associated with the supernatant fraction F_4 (85%). This is to be expected since the location of this enzyme is the cell cytoplasm. The activity associated with the particulate fractions (F_1, F_2, F_3) may be due to cell cytoplasm both trapped in, and adhering to, membrane vesicles and organelles. Bourre et al have shown a similar distribution of glucose-6-phosphate dehydrogenase in their subfractionation of mouse brain (181).

TABLE 3.1

Distribution of marker enzymes in subcellular fractions of rabbit brain.

Glucose-6-phosphate dehydrogenase was assayed as outlined in the methods in the total homogenate and subcellular fractions. Results are expressed in μmol/minute per total fraction and % total activity and are mean ± S.E. of duplicate determinations of 3 separate samples.

Malate dehydrogenase was assayed in sonicated fractions and the results are expressed as for glucose-6-phosphate dehydrogenase.

(i) recovery of malate dehydrogenase activity from 'crude' F_1 fraction

(ii) recovery of malate dehydrogenase activity following washing 'crude' F_1 fraction to give F_1.

TABLE 3.1 DISTRIBUTION OF MARKER ENZYMES IN SUBCELLULAR FRACTIONS OF ADULT RABBIT BRAIN

Enzyme	Full homogenate	Crude F_1	F_1	F_2	F_3	F_4	% recovery
Glucose-6-phosphate dehydrogenase							
Total activity of fraction µmol/minute	± 1.72 ± 1.3	± 0.14 ± 0.023	-	± 0.08 ± 0.01	± 0.01 ± 0.005	± 1.46 ± 0.12	
% total activity	100	8.0	-	4.7	0.6	85.0	98.2
Malate dehydrogenase							
(i) Total activity of fraction µmol/minute	± 330 ± 23	± 106 ± 9.4	-	± 161 ± 12.3	± 3.0 ± 0.18	± 32 ± 0.9	
% total activity	100	32	-	49	1	10	92.0
(ii) Total activity of fraction µmol/minute	± 330 ± 23	-	± 17 ± 4.3	± 250 ± 31	± 4.1 ± 0.21	± 32 ± 0.6	
% total activity	100	-	5	79	1	10	95.0

3.1.2 MALATE DEHYDROGENASE: NAD DEPENDANT (EC 1.1.37)

The distribution of the activity of malate dehydrogenase was used to determine the localisation of mitochondria in the subcellular fractions obtained (table 3.1). Since the enzyme is found in the mitochondrial matrix, the activity measured in sonicated samples of the particulate fraction F_1 - F_3 (80 - 85%) is derived from whole mitochondria, whereas the activity in the cytoplasmic fraction F_4 (10%) is a measure of mitochondrial rupture during the fractionation procedure.

Initially, the resuspended pellet obtained from the first 10,000gmin. centrifugation was used as the crude F_1 fraction. This fraction was also used in the determination of the subcellular location of palmitoyl-CoA synthetase (section 3.2.3) but was found to contain high levels of mitochondrial malate dehydrogenase. Repeated resuspension and centrifugation of crude F_1 at 8,000gmin. gave rise to the F_1 fraction which shows a much reduced malate dehydrogenase activity (5%). The longer F_1 preparation procedure reduced the mitochondrial contamination, as measured by malate dehydrogenase activity from 32% of the total activity to 5%.

Fraction F_2, designated mitochondria-enriched fraction, in fact contains 79% of the total marker enzyme activity. It is clear from table 3.1 that the crude F_1 fraction contains an appreciable amount of mitochondrial material entrapped in it and that the washing procedure which gives rise to fraction F_1 liberated much of the mitochondrial contamination. The distribution of microsomal material throughout the subfractions will be discussed in section 3.2.3 when the subcellular location of palmitoyl-CoA synthetase is considered.

3.2 THE ACTIVITIES OF LIPOGENIC ENZYMES IN MAMMALIAN BRAIN TISSUE.

The activities of the enzymes involved in the synthesis and further metabolism of fatty acids considered in this study are (1) fatty acid synthetase (2) acetyl-CoA carboxylase and (3) palmitoyl-CoA synthetase. The results obtained from the assay of these enzymes in developing rat and rabbit brain will be presented in the following sections:

3.2.1 FATTY ACID SYNTHETASE.

3.2.1.1 Assay of Rabbit Brain Fatty Acid Synthetase.

Acetyl -CoA dependent incorporation of ^{14}C-malonyl-CoA into a chloroform:methanol (2:1 v.v) soluble product by the cytoplasmic fraction (F_4) from rabbit brain, 12 days post partum was linear with time for up to 10 minutes (see figure3.1). Figure 3.2 shows that the incorporation of ^{14}C-malonyl-CoA was linear up to 2.2mg protein from the F_4 fraction from a 12 day old rabbit.

Thin layer chromatography of the chloroform : methanol extract from the assay gave only one spot on the chromatogram. The spot, detected by visualisation with iodine vapour and radio-thin layer scanning, had an Rf value of 0.55 equivalent to free fatty acid in the neutral lipid solvent (petroleum ether:diethylether:acetic acid 75:25:1 v/v).

Gas-liquid radiochromatography of methyl esters of the assay product showed (table 3.2) that the major fatty acid synthesized was palmitic $C_{16:0}$ (79.8%) with smaller proportions of myristic $C_{14:0}$ (4.5%) and stearate $C_{18:0}$ (15.7%). The ratio of the radioactivity in the carboxyl carbon to the total radioactivity in the fatty acid chain was determined to distinguish between de novo synthesis and elongation.

FIGURE 3.1

The activity of fatty acid synthetase from adult rabbit brain. The activity is plotted as nmol ^{14}C-malonyl-CoA incorporated into non-polar material per mg protein from fraction F_4 in the standard assay against time in minutes. Results are mean ± S.E of duplicate determinations of three different animals.

Figure 3.1 The activity of fatty acid synthetase.

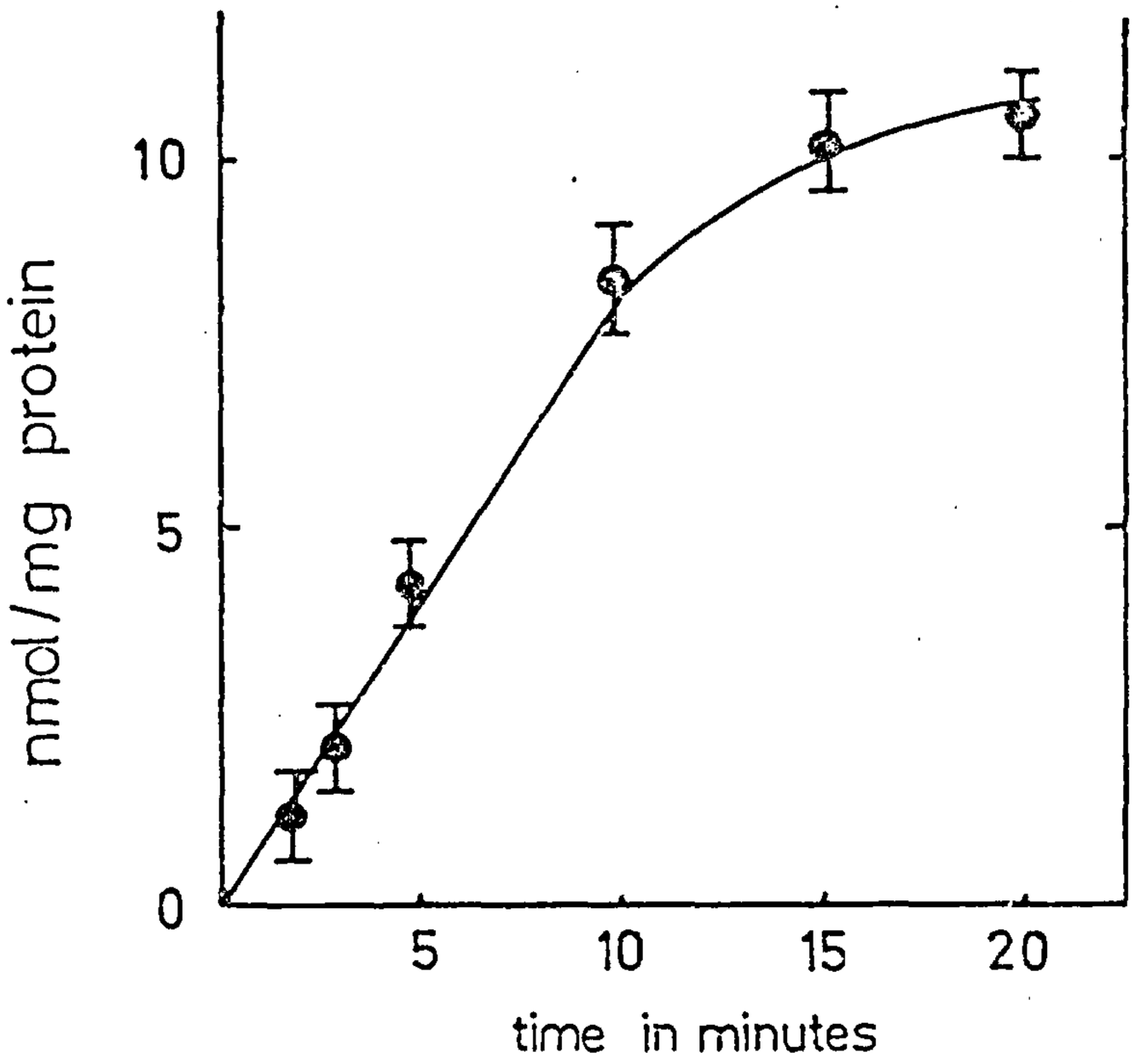


FIGURE 3.2

The activity of fatty acid synthetase from adult rabbit brain. The activity is plotted as nmol ^{14}C-malonyl-CoA incorporated into lipid material per minute in the standard assay against mg protein from fraction F_4. Results are mean $\pm$ S.E. of duplicate determinations of three separate brains.

Figure 3.2 The activity of fatty acid synthetase.

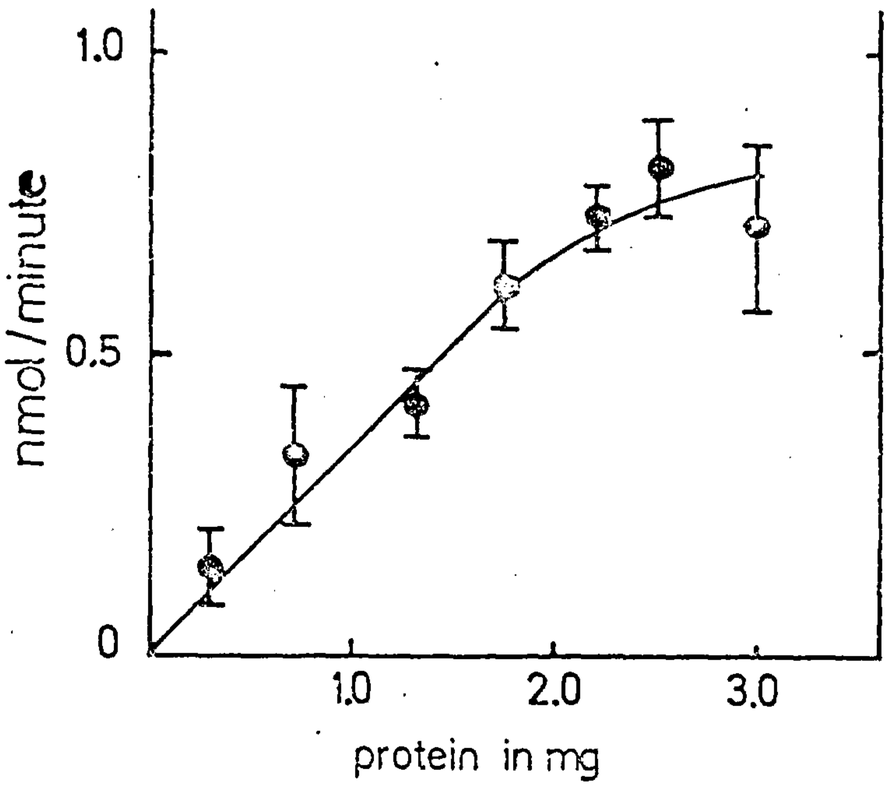


TABLE 3.2

The products of fatty acid synthetase.

Values are expressed as % radioactivity recovered in each fatty acid. Fatty acids were separated as their methylesters by gas-liquid chromatography and radioactivity was determined by on line radiogas proportional counting. The values shown are the mean % of up to six determinations and were the products of the experiments to determine age dependant changes in fatty acid synthetase activity shown in Figure 3.3.

TABLE 3.2 PRODUCTS OF FATTY ACID SYNTHETASE

Age	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$
27 days gestation	6.5	79.0	14.5
4 days post partum	3.0	86.0	11.0
12 days post partum	4.6	87.0	8.4
40 days post partum	2.0	76.0	22.0
Adult	6.6	71.0	22.4
Average	4.5	79.8	15.7

Expressed as % radioactivity recovered in each fatty acid.

The theoretical value of de novo synthesis of palmitic acid from ^{14}C labelled malonyl-CoA is 12%. The result obtained, 11 ± 2% (6 det.) is in good agreement with this value. Elongation of a precursor fatty acid molecule would give rise to a value larger than this depending upon the size of the fatty acid chain.

3.2.1.2 Subcellular localisation of Fatty Acid Synthetase:

Fatty acid synthetase from all mammalian tissues studied is a soluble protein complex. Rabbit brain tissue is no exception to this statement. Table 3.3 shows the distribution of fatty acid synthetase in rabbit brain subfractions. The majority of the enzyme activity recovered was detected (97%) in the F_4 fraction which contains the cytoplasmic enzymes. This is in agreement with other workers (68, 112). Aeberhard and Menkes (150) showed that de novo synthesis of palmitic acid takes place in particulate fractions in the absence of ATP, but the activity recovered from the soluble fraction was several fold higher than microsomal and mitochondrial enzyme activities. The high total activity of the crude homogenate of rabbit brain, may be due, in part, to the elongation of endogenous medium and long-chain fatty acids as well as de novo fatty acid synthesis. The synthetic activity seen in the absence of NADPH and acetyl-CoA may be due to the presence of these endogenous substrates in the crude homogenate fraction.

3.2.1.3 Changes in the Activity of Fatty Acid Synthetase during development:

Changes in the activity of rabbit brain cytoplasmic fatty acid synthetase are shown in figures 3.3 and 3.4.

Results are expressed as :

a. activity per mg protein in the soluble fraction (F_4)

TABLE 3.3.

Recovery of fatty acid synthetase from all fractions prepared from 10 day old rabbit brain.

Results are expressed as nmol malonyl-CoA incorporated into lipid per mg protein per minute and are mean $\pm$ S.E. of four separate determinations. Total activity is expressed as nmol malonyl-CoA incorporated per minute per fraction. The standard assay was used in all cases except where either $NADPH_2$ or acetyl-CoA were absent.

TABLE 3.3 BRAIN FATTY ACID SYNTHETASE - 10 DAY OLD RABBIT

Fraction	Malonyl-CoA incorporation nmol/mg/minute	Total activity per fraction (nmol incorp./min.)	% recovery
Crude homogenate	0.502	335	100
Without NADPH	0.075	-	-
Without Acetyl-CoA	0.037	-	-
Crude F_1	0.063	15	4.6
F_2	0.05	4	1.2
F_3	0.16	2	0.6
F_4	0.85	112	30

b. the total activity of the enzyme in the soluble fraction (F_4)

c. the activity per mg DNA

d. the activity per gram wet weight of tissue.

There was a steep rise in the specific activity of fatty acid synthetase (Figure 3.3a) during the last third of gestation to a peak at 5 days of age. The maximum specific activity at 5 days of age was 1.2 nmol malonyl-CoA incorporated/mg protein/minute. This was followed by a progressive decline to 35 days of age, where the specific activity was 0.43 nmol/mg/minute and not significantly different from the value obtained for the adult rabbit brain.

The total fatty acid synthetase activity of the whole brain (Figure 3.3b) reached a maximum at 15 days of age and then fell only slightly to the adult value of 48.6 nmol /whole brain/minute. When the activity of brain fatty acid synthetase is expressed per mg DNA (Figure 3.4a) a sharp rise is seen to a peak activity of 8.6 nmol malonyl-CoA incorporated/mg DNA/minute followed by a rapid decline to the adult value of 3.6 nmol malonyl-CoA incorporated/mg DNA/minute. The activity per gram wet weight of tissue was very similar to that seen per mg DNA although initially there was a fall in activity from 21 days gestation to 27 days gestation (Figure 3.4b).

The peak specific activity of fatty acid synthetase occurred at 5 days of age which is before the period of maximum myelin synthesis of 10 - 25 days after birth (4). This apparent early maximum may only be due to the use of protein concentration as a parameter for enzyme activity, since, during the early post-natal period, there is a rapid increase in cell number and, although the total amount

FIGURES 3.3 AND 3.4:

Acitivty of rabbit brain fatty acid synthetase.
The enzymes was assayed in the soluble F_4 fraction of developing rabbit brain using the standard assay. Duplicate incubations for 5 and 10 minutes were carried out. Activity was determined as nmol malonyl-CoA incorporated into lipid per minute and shown as 3.3(a) incorporation per mg protein per minute and 3.3(b) incorporation per brain per minute.
Figure 3.4(a) shows incorporation per mg DNA per minute and 3.4(b) incorporation per gm wet weight of tissue.
All values are the mean. All determinations and S.E. values are shown where three or more animals or groups of animals were used. Age is shown in days from 21 days gestation (dg) to adult.

Figure 3.3 Rabbit brain fatty acid synthetase.

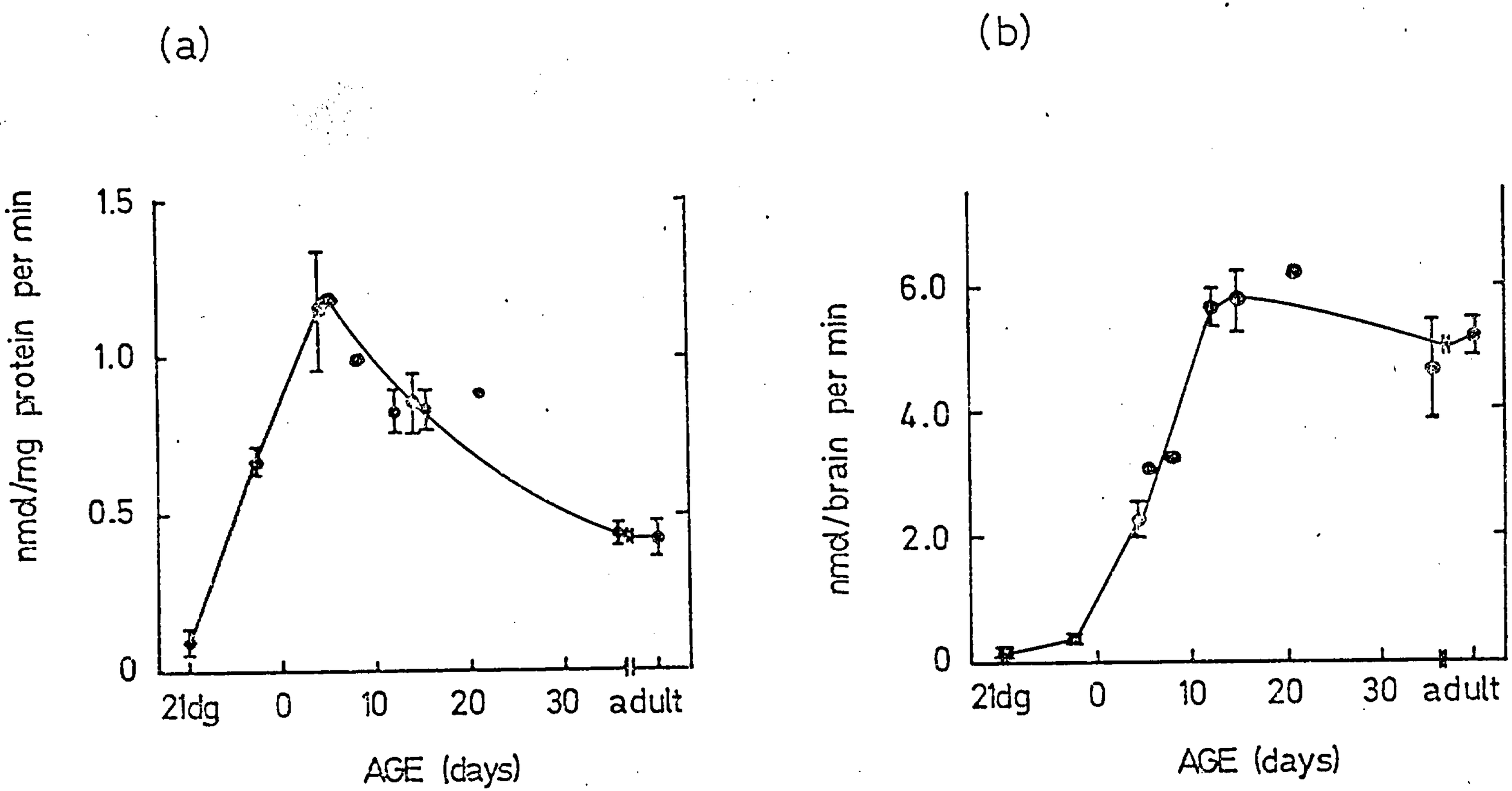
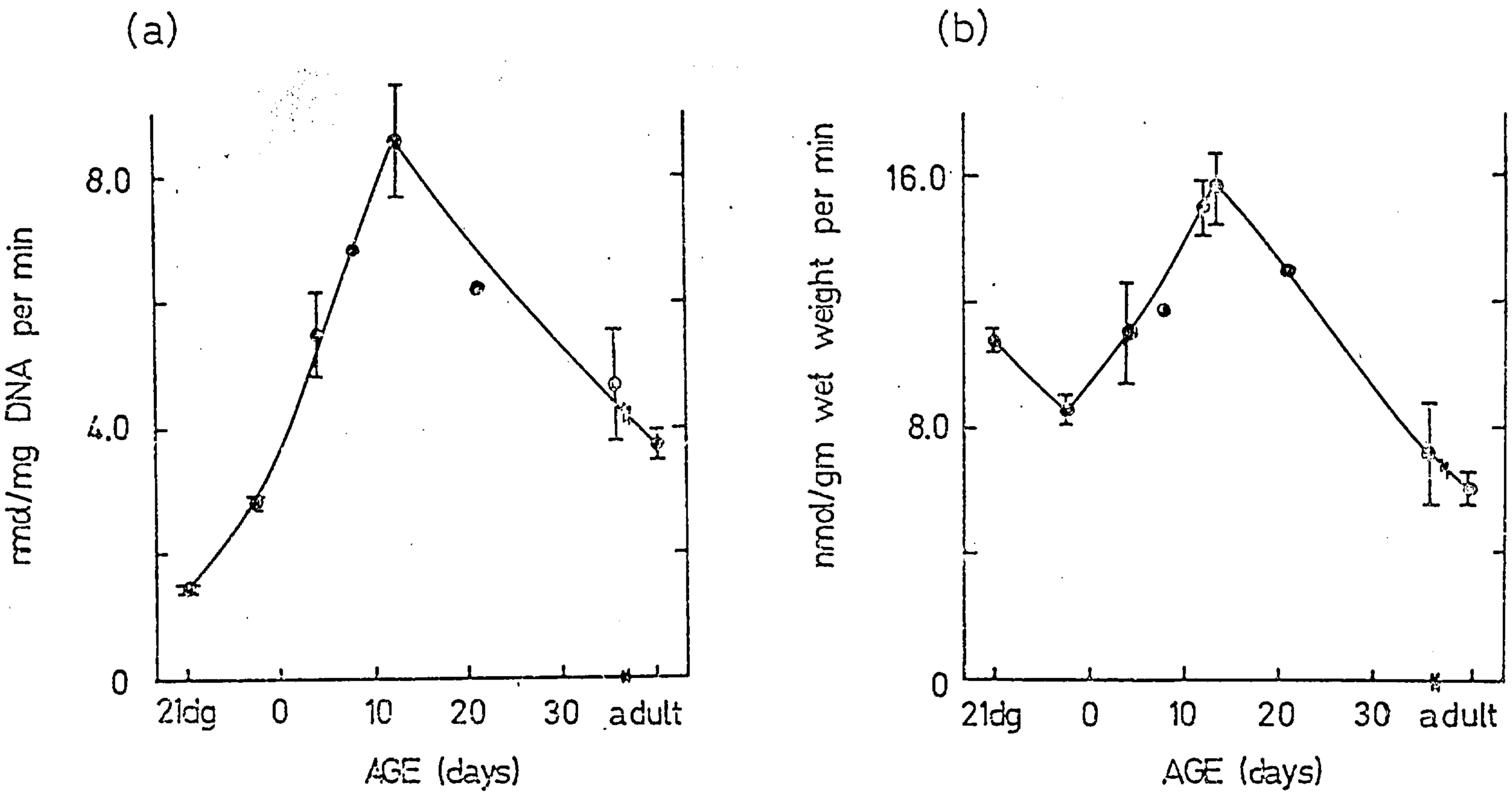


Figure 3.4 Rabbit brain fatty acid synthetase.



of fatty acid synthetase protein may be increasing during this period, this fact is hidden by a larger increase in the amount of other proteins. Expressing the results in terms of DNA may give a more realistic picture of the changes in enzyme activity taking place during cell multiplication. Because the DNA content of each cell is constant, changes in the total amount of DNA in brain reflect changes in cell number. Therefore, enzyme activity expressed per mg DNA will give an indication of cellular changes in enzyme activity. The activity of fatty acid synthetase, when expressed per DNA (Figure 3.4a) showed peak activity at 15 days postnatal which is during the period of maximum myelin formation.

Expressing the data as incorporation per brain also showed maximum enzyme activity at 15 days of age, but this was not followed by a steep decline in activity, showing that fatty acid synthetase was still very active in the adult animal. Results expressed per gram fresh tissue also indicated that maximum enzyme activity was at 15 days.

The development pattern of rat brain fatty acid synthetase was similar to that from rabbit brain (Figures 3.5, 3.6).
The maximum incorporation of malonyl-CoA per mg protein into fatty acid occured at 5 days of age whereas the data expressed per mg DNA, per gram fresh tissue and per whole brain, showed maximum activity at 13 days of age. Expressed per gram fresh tissue (Figure 3.6b) the activity of brain fatty acid synthetase in the newborn rat was the same as in the adult rat, although between 5 and 19 days of age the activity doubled to give a peak value of 24.3 nmol malonyl-CoA incorporated/gram fresh tissue/minute at 13 days after birth.

FIGURES 3.5 and 3.6

The activity of rat brain fatty acid synthetase.

The enzyme was assayed in the soluble F_4 fraction of developing rat brain using the standard assay. Incubations were carried out for 5 and 10 minutes in duplicate. Activity was determined as nmol malonyl-CoA incorporated into lipid per minute and expressed as 3.5 (a) per mg protein per minute 3.5 (b) per whole brain per minute, 3.6 (a) per mg DNA per minute and 3.6 (b) per gm wet weight of tissue. All values are the mean of all determinations and S.E. values are shown where three or more animals were used. Age is shown in days.

Figure 3.5 Rat brain fatty acid synthetase.

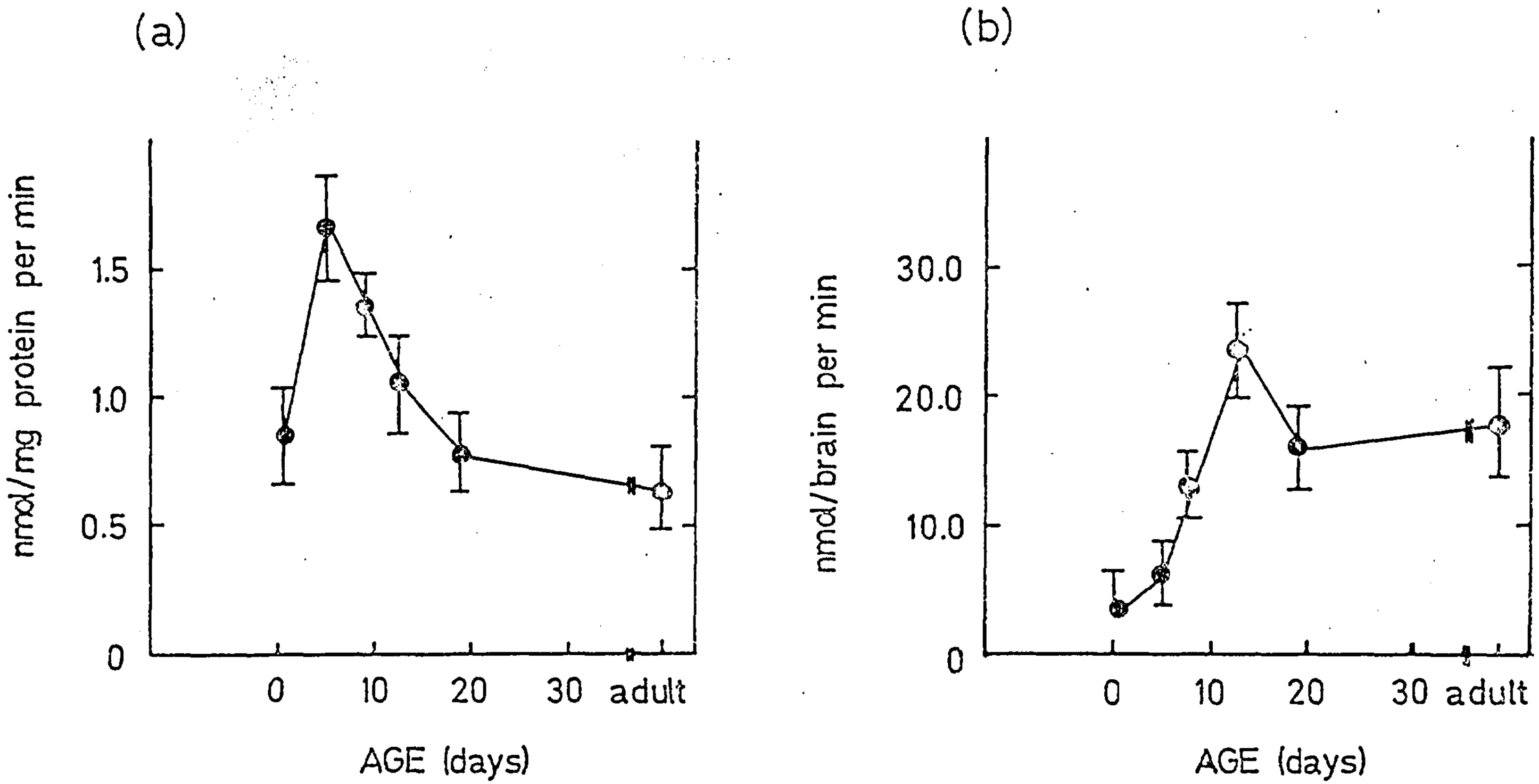
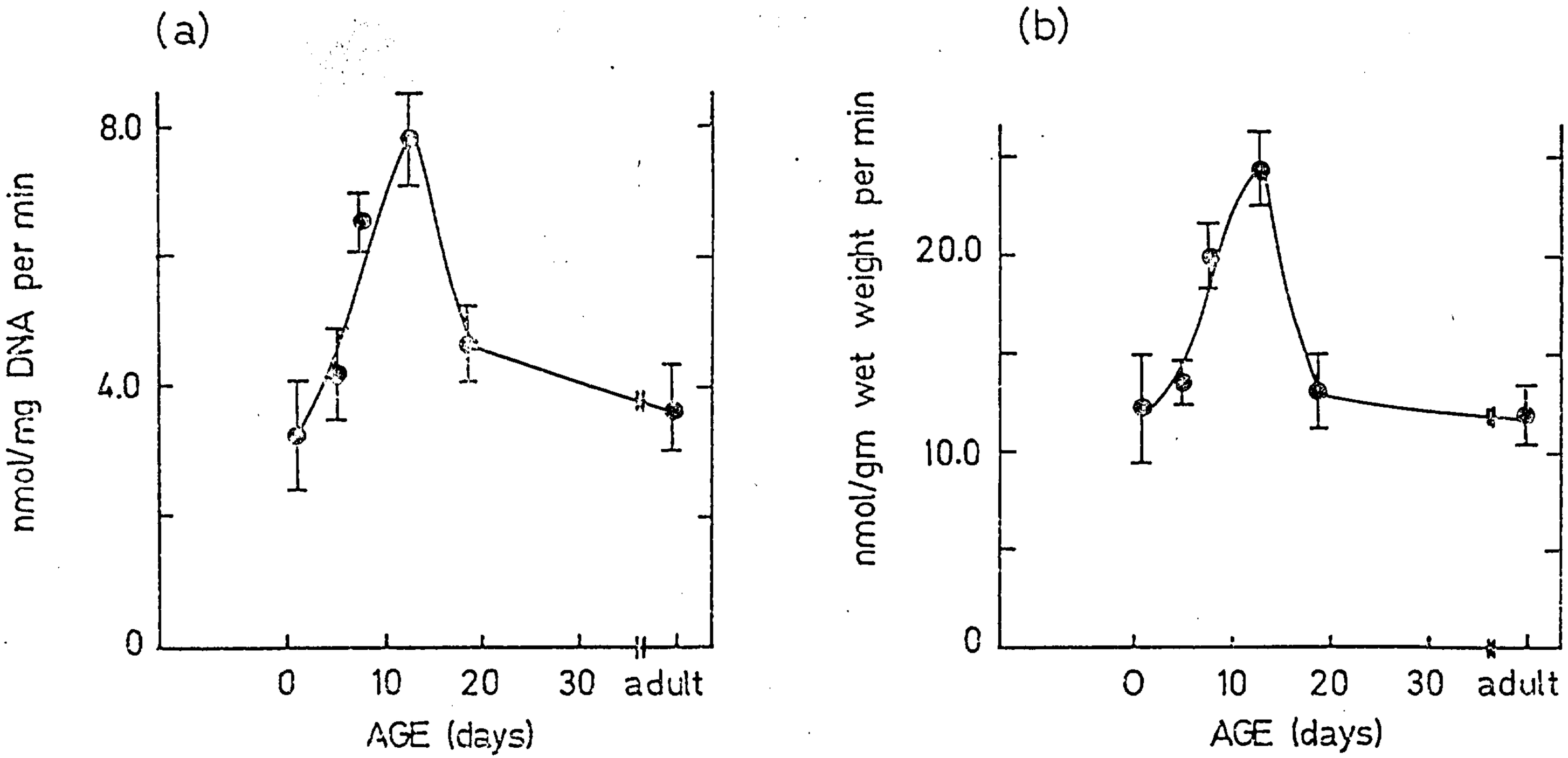


Figure 3.6 Rat brain fatty acid synthetase.



3.2.2 ACETYL-CoA CARBOXYLASE

Acetyl-CoA carboxylase activity detected in the developing rabbit brain was very low. See Table 3.4. Pre-incubation of the sample with citrate did not give any appreciable increase in activity. The rate of bicarbonate incorporation into acid soluble material in the absence of acetyl-CoA accounted for 60% of the total rate in the crude homogenate of 12 day rabbit brain and 51% in the crude homogenate of 18 day rabbit brain. In the F_4 fraction the rate of bicarbonate incorporation in the absence of acetyl-CoA was 57% of the activity measured using the full assay mixture in an 18 day rabbit brain. Although a G-25 sephadex column was used to separate protein from smaller molecular species in order to remove any inhibitors of acetyl-CoA carboxylase that may have been the cause of the low activities shown in Table 3.4 no change in activity was seen.

The enzyme was also assayed by measuring the incorporation of acetyl-CoA into lipid material. NADPH and ^{14}C-acetyl-CoA and unlabelled sodium bicarbonate were used in the standard acetyl-CoA carboxylase assay. The result obtained using a rat liver F_4 preparation was 1.03 nmol /mg protein/minute. This value agrees closely with the result of 1.4 nmol /mg protein/minute using the incorporation of $H^{14}CO_3^-$ into malonyl-CoA (178).

The rat brain enzyme has been investigated by Gross and Warshaw (113) who showed that its specific activity was 3.0 nmol /mg protein/minute in the supernatant fraction of foetal brain. The specific activity rose slightly to a maximum of 3.4 nmol /mg protein/minute at 14 days of age and then after an initial steep decline fell slowly to the adult value which was 55% of that obtained during the early postnatal period.

TABLE 3.4

Activity of Acetyl-CoA carboxylase.

Results are expressed as nmol HCO_3^- incorporated into acid soluble material per mg protein per minute. The standard assay was used in all cases except where specific omissions are recorded. Experiments with radiolabelled acetyl-CoA were carried out in the standard assay mixture plus $NADPH_2$ and activity was determined as radioactivity extracted in lipid per mg protein per minute. All values are the means of duplicate determinations carried out for 5 and 10 minutes.

TABLE 3.4

ACTIVITY OF ACETYL-CoA CARBOXYLASE

		Crude Homogenate	F_4
12 day old Rabbit Brain	Full assay mixture	0.16 ± 0.02	0.014 ± 0.002
	Without acetyl-CoA	0.096 ± 0.01	0.008 ± 0.001
	Without citrate	0.15 ± 0.03	0.013 ± 0.005
18 day old Rabbit Brain	Full assay mixture	0.049 ± 0.005	-
	Without Acetyl-CoA	0.025 ± 0.004	-
	^{14}C-acetyl-CoA	0.041 ± 0.008	-
Adult Rat Liver	^{14}C-acetyl-CoA	-	1.03 ± 0.09

Activity is expressed as nmol HCO_3^- incorporated into acid soluble material per mg protein per minute.

The activity of the rabbit brain enzyme, measured under the same conditions, was 100 fold lower.

The specific activity of rabbit brain acetyl-CoA carboxylase, in the crude homogenate was 70% lower at 18 days than at 12 days; a smaller fall in activity was shown by the rat brain enzyme (113). A decrease in the activity of both rat and rabbit brain fatty acid synthetase was also seen at this time.

Although variations in substrate concentration did not lead to a higher specific activity, rabbit brain acetyl-CoA carboxylase may differ from the rat brain enzyme in its requirements for activator and substrate molecules.

3.2.3 PALMITOYL-CoA SYNTHETASE

This enzyme was studied since it is intimately involved in the further metabolism of both the products of fatty acid synthetase and free fatty acids taken up by the brain, because only the CoA derivative of fatty acids are further metabolised. Palmitoyl-CoA synthetase was assayed in all subfractions using a linked assay involving acyl-CoA carnitine acyl transferase. Optimum conditions for the assay of acyl-CoA synthetase were determined before studying changes in palmitoyl-CoA synthetase activity during development.

3.2.3.1 Assay of Acyl-CoA carnitine acyl transferase from bovine and calf liver:

This assay is based upon the reversibility of the reaction

$$\text{palmitoyl-carnitine} + \text{CoA} \rightleftharpoons \text{palmitoyl-CoA} + \text{carnitine}$$

$$\text{palmitoyl-CoA} + {}^{3}\text{H-carnitine} \rightleftharpoons \text{palmitoyl} - {}^{3}\text{H carnitine} + \text{CoA}$$

By determining the amount of palmitoyl-^{3}H-carnitine formed per minute, a measure of the activity of the carnitine acyl transferase will be obtained. However, the amount of palmitoyl-^{3}H-carnitine formed per minute in this assay is not a direct measure of the ability of this enzyme to convert palmitoyl-CoA to palmitoyl-^{3}H-carnitine in the linked assay system. The amount of palmitoyl-CoA carnitine acyl transferase required to saturate the linked assay system was determined empirically with each carnitine acyl transferase preparation. (see ref 171)

The formation of palmitoyl-^{3}H-carnitine in the exchange reaction was found to be linear with time up to 45 minutes (Figure 3.7) and with protein up to 1.0mg of enzyme preparation. Routinely however, 0.2 - 0.5 mg of protein was sufficient for the acyl-CoA synthetase assay.

3.2.3.2 Assay of Palmitoyl-CoA Synthetase:

The linked assay with carnitine acyl transferase was found to be linear with up to 3.0 mg of protein from the crude homogenate of rabbit brain (Figure 3.8a). The formation of palmitoyl carnitine was linear for up to 12 minutes using 0.1 mg of protein from the F_3 subfraction of adult rat brain (Figure 3.8b). The conditions used for the assay of palmitoyl-CoA synthetase were constant for the assay of material from both rat and rabbit brain.

3.2.3.3 Subcellular location of Palmitoyl-CoA synthetase:

The subcellular location of this enzyme has been described by other workers as microsomal and mitochondrial.

FIGURE 3.7

The activity of carnitine acyl transferase prepared from calf and bovine liver.

(a) Time-dependant labelled acyl-carnitine formation was measured using the standard assay containing 0.25mg protein. Points shown are mean $\pm$ S.E. of determinations in duplicate over a period of 10 days.

(b) palmitoyl-carnitine formation was measured with increasing protein concentration in the standard assay system incubated for 10 minutes. All points shown are the mean $\pm$ S.E. of duplicate determinations of activity in three different enzyme preparations from bovine liver mitochondria.

Figure 3.7 The activity of carnitine acyl transferase.

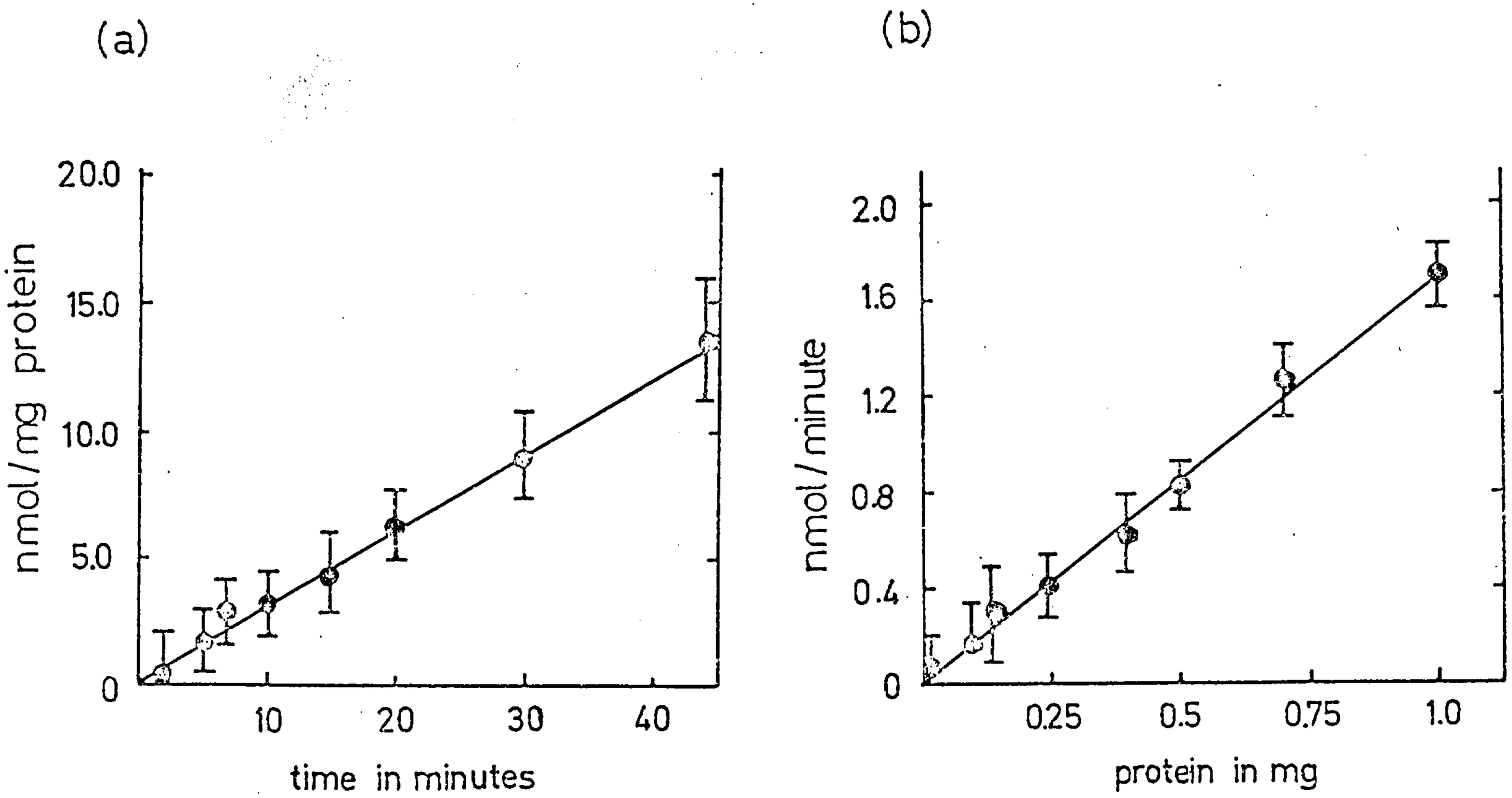
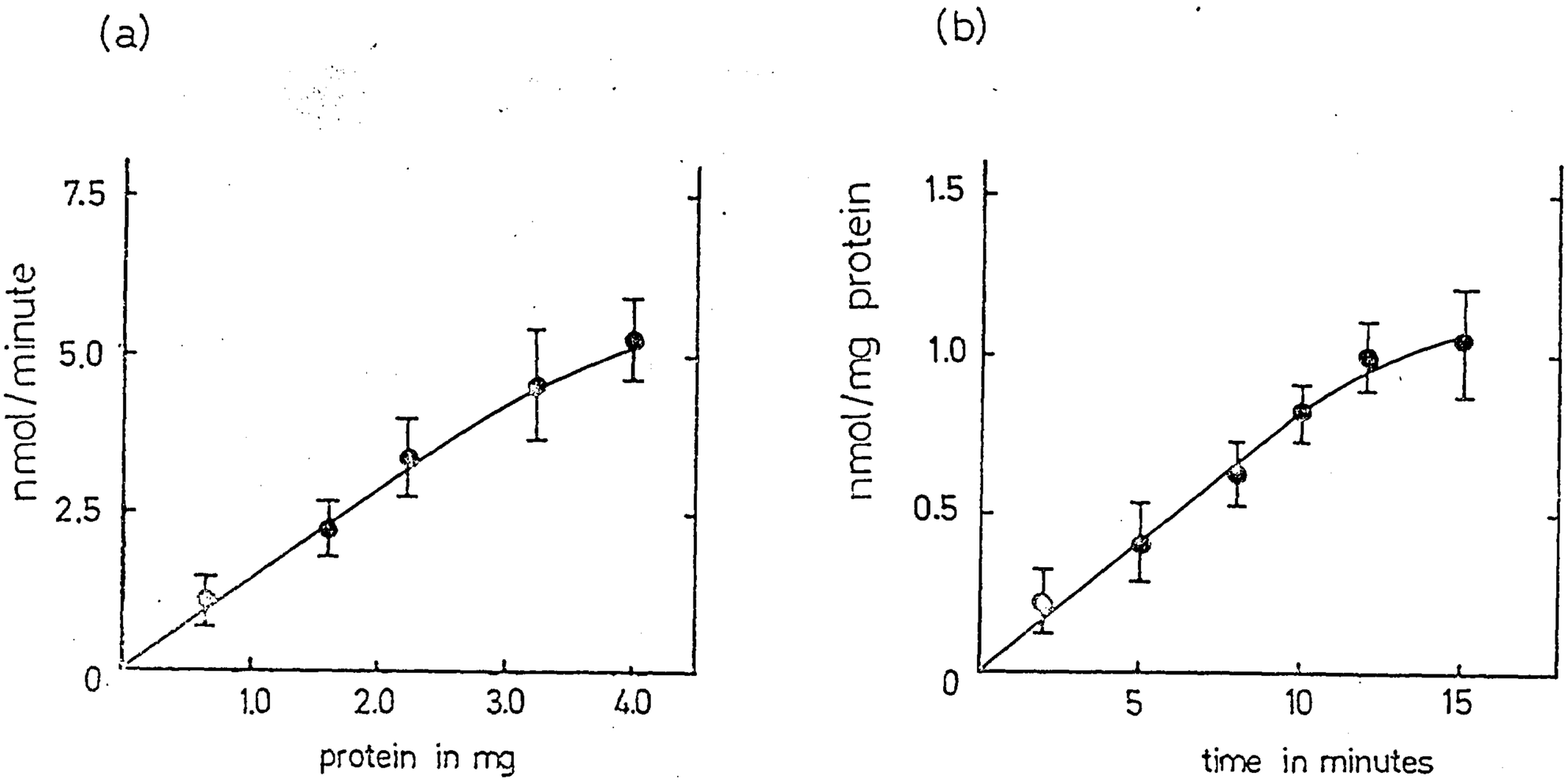


FIGURE 3.8

The activity of rabbit brain palmitoyl-CoA synthetase.
Palmitoyl-CoA synthetase activity was determined using the linked-enzyme assay as palmitoyl-carnitine extracted from the standard assay mixture. (a) Palmitoyl-CoA synthetase activity as a function of protein from the crude homogenate of 17 day old rabbit brains. All points are the mean $\pm$ S.E. of results obtained in assays for 5 and 10 minutes carried out on homogenates from different animals.
(b) The time-dependant formation of palmitoyl-carnitine was measured in duplicate assays containing 0.1mg of protein from the microsomal (F_3) fraction. All points are mean $\pm$ S.E. of six determinations.

Figure 3.8 Rabbit brain palmitoyl-CoA synthetase.



However, as seen in Table 3.5a, activity could be detected in all subfractions obtained from adult rat brain prepared according to the method outlined in section 2.2.1. The study of developmental changes in palmitoyl-CoA synthetase activity described in section 3.2.3.4 was carried out using a fractionation scheme which omitted washing the crude F_1 fraction. This method was modified in an attempt to decrease the large discrepency between the palmitoyl-CoA synthetase activity in the whole homogenate and that recovered in the mitochondrial and microsomal fractions. Table 3.5b shows the effect of washing the crude F_1 fraction according to the fractionation scheme. Although by this three step procedure the total amount of protein in the supernatants was only 17.6% of that present in the crude F_1 fraction, the acyl-CoA synthetase activity released was 47% of total activity of the crude F_1 fraction. This indicated that there was a large quantity of material trapped in the membranous F_1 fraction.

The recovery of palmitoyl-CoA synthetase activity from all the fractions(Table 3.5a) was 98%. The soluble fraction (F_4) and the membranous F_1 fraction, account for more than 40% of this activity. An attempt to identify the activity associated with fraction F_1 by continuous density gradient centrifugation will be discussed in section 3.2.3.5. Activity in the cytoplasmic fraction F_4 may be caused by small microsomal vesicles that were not sedimented in the preparation of the microsomal fraction (F_3). The fraction with the highest activity (37%) was fraction F_2 which also contained the highest activity of malate dehydrogenase (49% see Table 3.1) and was shown by Gray & Whittacker (38) to contain a high proportion of mitochondria. By contrast, this fraction only contained 12.5% of the total protein giving an enzyme activity: protein ratio of 0.5.

TABLE 3.5 a

The activity of palmitoyl-CoA synthetase in subcellular fractions of adult rat brain.

Results are mean ± S.E. of four separate fractionations. All activities (nmol palmitoyl-carnitine/fraction) are the result of duplicate determinations carried out for 5 and 10 minutes using the standard assay and then corrected to give total activity per fraction. Protein determinations were carried out in duplicate.

TABLE 3.5a

THE ACTIVITY OF PALMITOYL-CoA SYNTHETASE IN SUBCELLULAR FRACTIONS FROM ADULT RAT BRAIN

Fraction	Total activity (nmol/fraction)	% Activity	Total Protein (mg)	% recovery
Homogenate	108.8 ± 11.1	100	640 ± 52	100
F_1	34.0 ± 2.3	31	300 ± 27	47
F_2	40.0 ± 3.7	37	80 ± 6	13
F_3	16.4 ± 0.9	15	10 ± 1	2
F_4	14.8 ± 1.1	13	210 ± 18	33
Crude F_1	85.6 ± 9.4			

TABLE 3.5b

The effect of washing the crude F_1 fraction from adult rat brain.

Palmitoyl-CoA synthetase activity is expressed as nmol palmitoyl-carnitine formed per fraction per minute using the standard assay. All total activities are the result of duplicate determinations carried out for 5 and 10 minutes using the standard assay. Each value is the mean $\pm$ S.E. of four separate fractionations.

TABLE 3.5b

THE ACTIVITY OF PALMITOYL-CoA SYNTHETASE IN SUBCELLULAR FRACTIONS FROM ADULT RAT BRAIN :

THE EFFECT OF WASHING THE 'CRUDE' F_1 FRACTION

Fraction	Total activity (nmol/fraction)	% activity	Total Protein (mg)	% recovery
'Crude' F_1	85.6 ± 9.4	100	380 ± 35	100
8000g min supernatant 1	24.0 ± 2.1	28	40 ± 2.1	11
8000g min supernatant 2	12.8 ± 1.4	15	20 ± 1.0	5
8000g min supernatant 3	4.0 ± 0.6	4	7 ± 0.5	2
F_1 fraction	34.0 ± 2.3	40	300 ± 27	79

The microsomal fraction F_3 contains an even higher concentration of enzyme activity giving an activity : protein ratio of 1.6.

Changes in Palmitoyl-CoA synthetase activity during development.

The data presented in this section was obtained using fractions F_2 and F_3 prepared directly from the supernatant obtained in the preparation of the 'crude' F_1 fraction. Although the 'crude' F_1 fraction prepared from adult brain is heavily contaminated with mitochondrial and microsomal material (sections 3.2.3.3 and 3.2.3.5) this data was not corrected for contamination since animals of different ages were used.

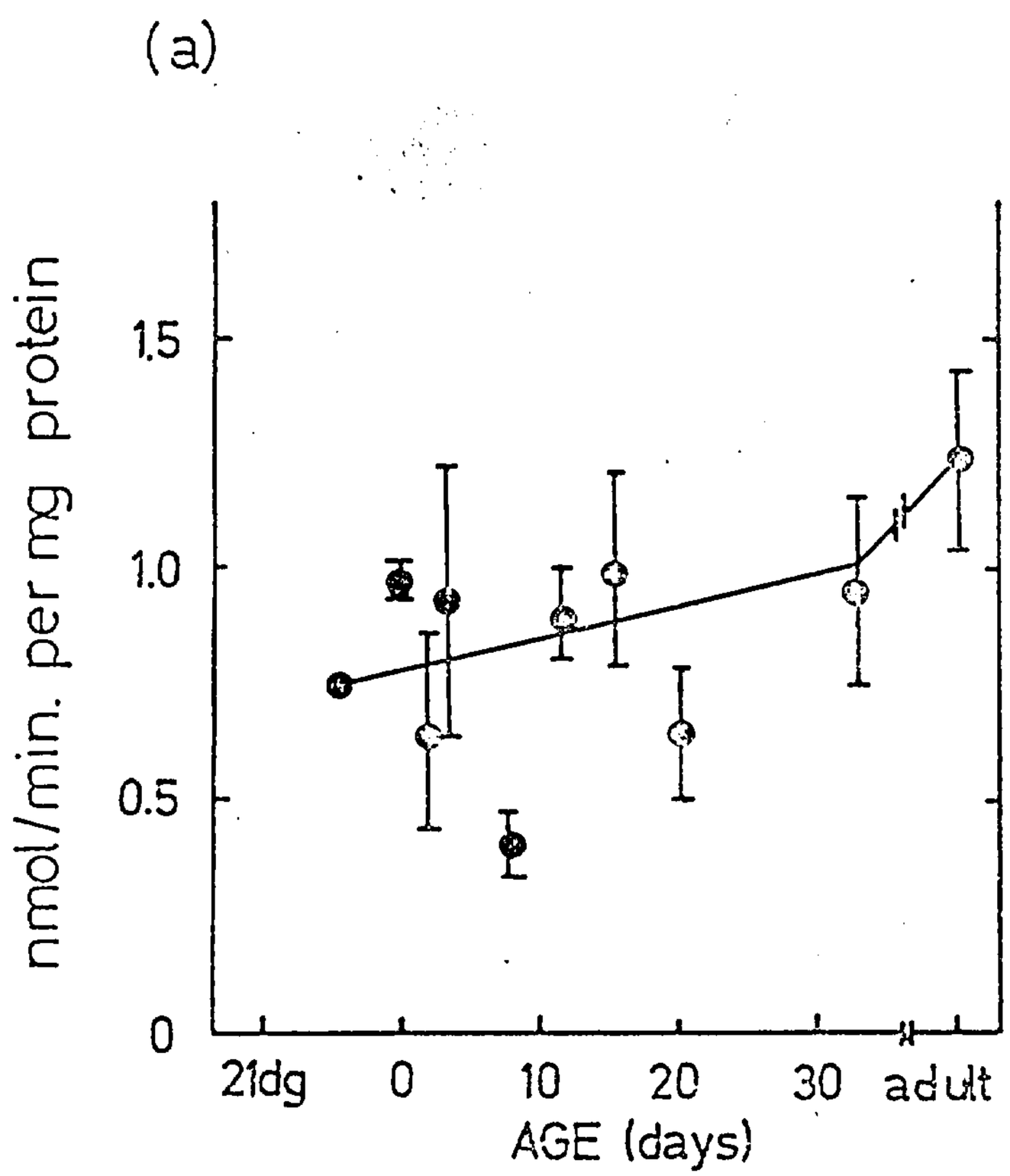
The specific activity of palmitoyl-CoA synthetase in the crude homogenate of rabbit brain was approximately 0.8 nmol/mg homogenate protein per minute throughout development. (Figure 3.9a) The highest specific activity of palmitoyl-CoA synthetase in the rabbit brain mitochondrial fraction F_2 (Figure 3.9b) was in the late foetal and early neonatal period. The specific activity declined to a value similar to that of the adult brain, 0.55 nmol/min per mg. protein, by twelve days of age. In contrast, the specific activity of the enzyme in the rabbit brain microsomal fraction (F_3) increased from the late foetal period to reach a maximum at 15-20 days of age. The maximum activity of 5 nmol/mg per minute was a three-fold increase from the activity at birth. From twenty days the specific activity of the enzyme declined to the adult value, which was about half the maximum specific activity (Figure 3.9b).

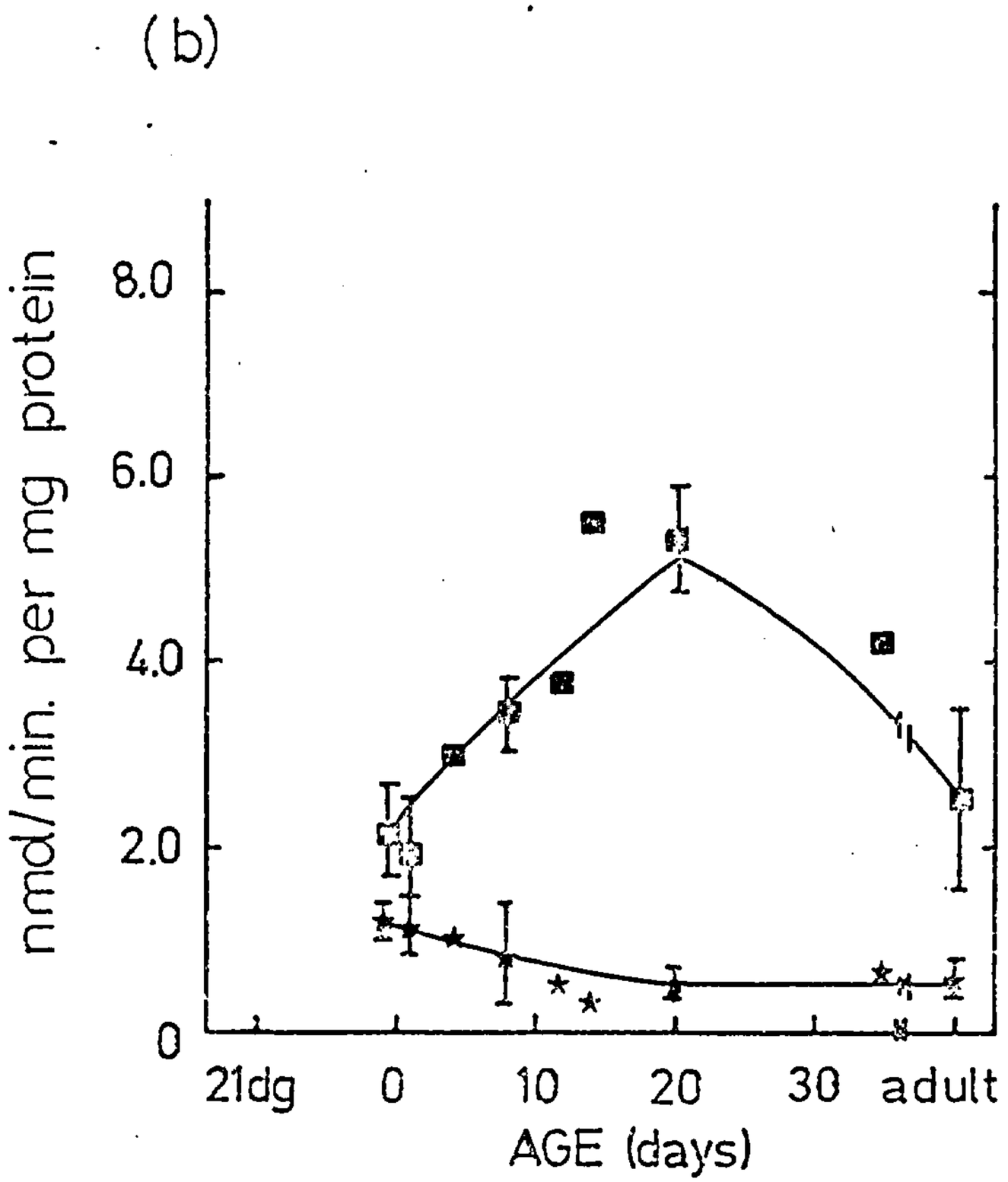
The specific activity of the microsomal enzyme was twice that of the mitochondrial enzyme at birth and ten times higher between 15 and 20 days of age.

FIGURE 3.9

Changes in the specific activity of palmitoyl-CoA synthetase in rabbit brain during development.
Activity is expressed as nmol palmitoyl-CoA synthesised/minute per mg protein in (a) the full homogenate (⊙——⊙) and (b) the microsomal F_3 (■——■) and mitochondria-enriched (F_2) fractions (✱——✱). Each point is the mean value $\pm$ S.E. from at least four groups of animals. The foetal age is shown as days gestation (dg).

Figure 3.9 Changes in rabbit brain palmitoyl-CoA synthetase activity.





These changes in specific activity were not seen in the cell-free homogenate results because of the large increase in brain total protein taking place during this period. The wide variation in enzyme activity may be due to a greater variation in the amount of protein used in assays of this fraction than in incubations containing microsomal or mitochondrial protein.

The whole brain activity of palmitoyl-CoA synthetase rose steeply from birth to adult values using the cell-free homogenate of rabbit brain (Figure 3.10). The mitochondrial (F_2) and microsomal (F_3) fractions obtained from the supernatant of the 'crude' F_1 fraction accounted for only 40% of the total whole brain activity at birth and 10% in the adult brain. Therefore 60-90% of the enzyme must be either trapped or located in the crude F_1 fraction. Since 80% of the total activity of the rat brain enzyme is trapped in the crude F_1 fraction (Table 3.5) it is not unreasonable to conclude that a similar amount must be trapped in the 'crude' F_1 fraction of rabbit brain.

In order to test this hypothesis, the 'crude' F_1 fraction was washed three times as outlined in section 2.2.1. The resultant fractions, F_1, F_2, and the washings, were further analyzed by rate-zonal density gradient centrifugation, and the subfractions obtained were assayed for palmitoyl-CoA synthetase activity.

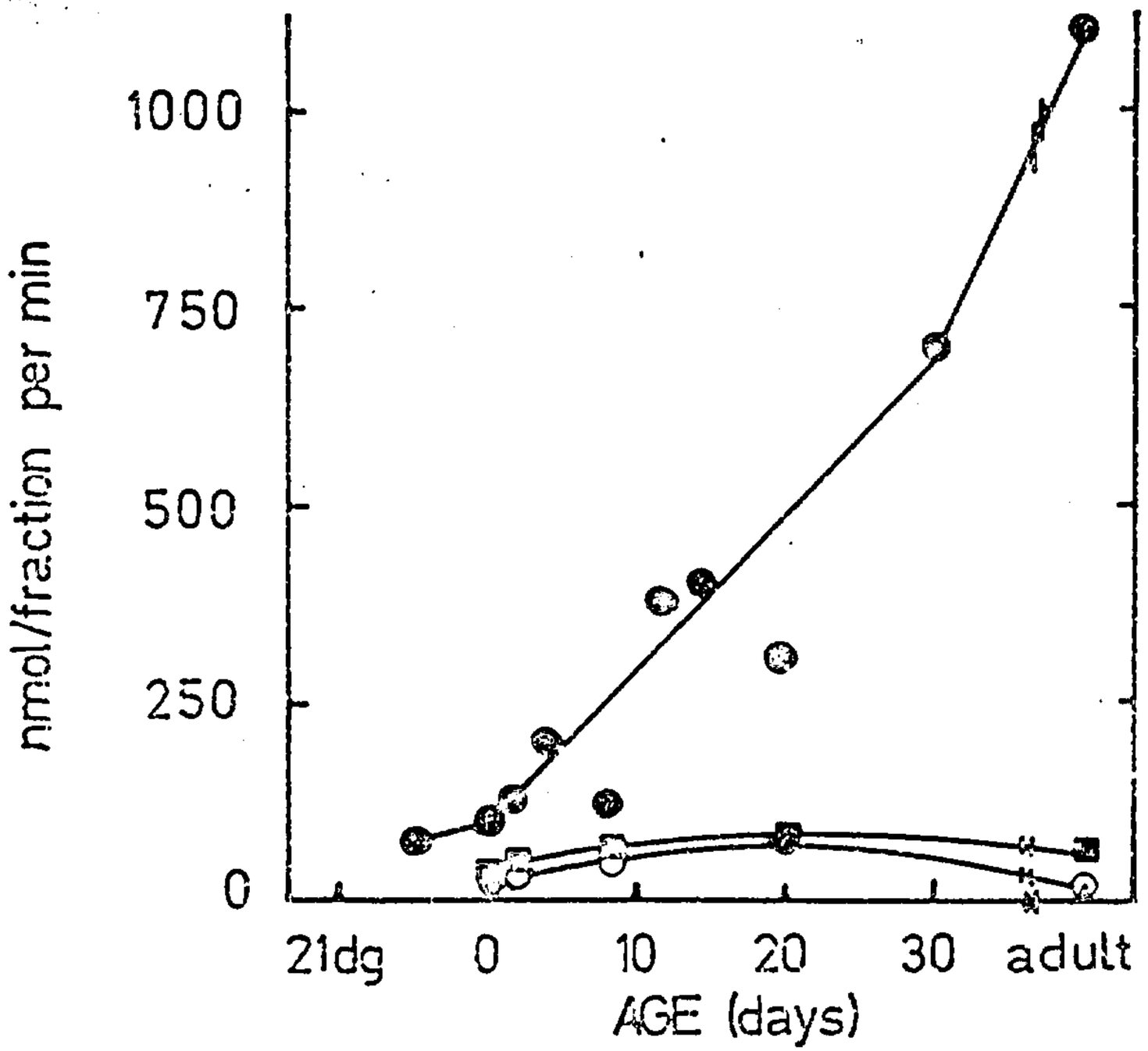
Distribution of Palmitoyl-CoA Synthetase in Fractions F_1 and F_2 in rat brain after Density Gradient Centrifugation.

Figure 3.11 is a visual representation of the pattern of bands obtained after sucrose density gradient centrifugation of the membrane fraction (F_1) the mitochondrial fraction (F_2) and the pooled supernatants (S)

FIGURE 3.10

Changes in the whole brain activity of palmitoyl-CoA synthetase in rabbit brain during development.
Activity is expressed as nmol palmitoyl-CoA synthesized/minute per total fraction for the full homogenate (○—○) the mitochondria-enriched fraction (F_2) (■—■) and the microsomal (F_3) fraction (○—○). Each point is calculated from the mean values presented in Figure 3.9.

Figure 3.10 Changes in rabbit brain palmitoyl-CoA synthetase activity.



obtained by washing the 'crude' F_1 fraction. In general, subjecting the fractions to hypotonic lysis caused a displacement of the bands down the gradient and a subsequent broadening. Palmitoyl-CoA synthetase activity was found in bands 1-3 on the gradients. The position of band 1 was equivalent to that occupied by washed material from the microsomal fraction (F_3). Purified myelin, prepared by the method of Autilio (43) gave a band in the region of 0.7-0.8 M sucrose which was in the same position as band 2. Material from band 3 (0.9 M sucrose) was found to be high in malate dehydrogenase activity and therefore of mitochondrial origin.

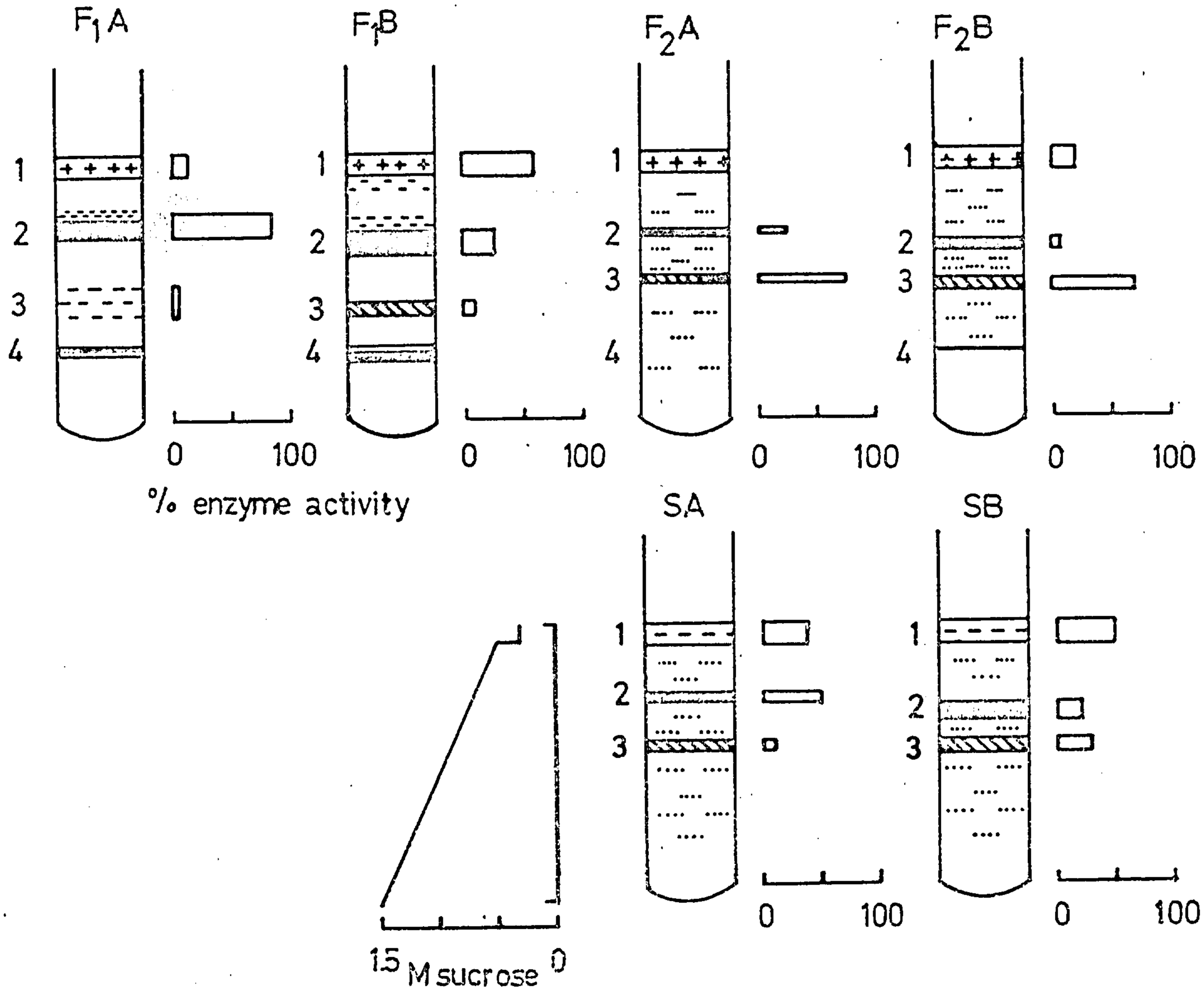
The effect of osmotic shock on all fractions applied to density gradients was to cause a lowering of the palmitoyl-CoA synthetase activity in band 2, the myelin fraction, and an increase in the enzyme activity found in bands 1 and 3. This indicates that mitochondrial and microsomal material was trapped in the myelin fraction (band 2). The origin of band 4 (1.1 M sucrose) obtained by gradient centrifugation of F_1 material after hypotonic lysis is unknown, but a similar band was obtained after freezing the crude membrane fraction (F_1) during the isolation of radio labelled material following intracranial injection of ^{3}H-palmitate. Since this band has no acyl-CoA synthetase activity, it probably is composed of large aggregations of myelin membrane.

From the above results it appears that the fraction F_1 contains an appreciable amount of mitochondrial and microsomal material which contributed up to 60% of the palmitoyl-CoA synthetase activity measured in that fraction (Figure 3.11).

FIGURE 3.11

Distribution of palmitoyl-CoA synthetase activity following density gradient centrifugation of fractions from adult rat brain. Fractions F_1 and F_2 were prepared as in section 2.2.1 and Fraction S were the pooled supernatant fractions from washing the 'crude' F_1 fraction. All fractions A were control and all fractions B were the result of hypotonic lysis. Gradients were formed in a linear fashion from 0.5M to 1.5M sucrose buffered at pH 6.8 with 0.25M potassium phosphate and centrifuged at 9×10^6 gmin. Palmitoyl-CoA synthetase was determined using the standard assay and the activities shown are typical for % distribution.

Figure 3.11 Palmitoyl-CoA synthetase activity on density gradients.



The recovery of palmitoyl-CoA synthetase activity from the gradients was between 40 and 50% (Table 3.6) whereas the recovery of protein was between 40 and 70%. The apparent low recovery of protein from gradients containing material from the F_1 fraction may be due to the presence of large quantities of lipid in this fraction. The increase in palmitoyl-CoA synthetase activity seen in Band 1 following hypotonic lysis of all three fractions may be derived from either microsomal material that was occlude in large membrane vesicles or mitochondrial membranes formed by intact mitochondria rupturing.

Fraction S, obtained by pooling the supernatants when washing the 'crude' F_1 fraction, had a similar band pattern to the mitochondria-enriched fraction (F_2) (Figure 3.11). There was some palmitoyl-CoA synthetase activity associated with the microsomal band (1) before osmotic shock and this could represent microsomal material washed from the 'crude' F_1 fraction. The enzyme activity in the myelin band (2) was decreased by osmotic shock and there was a concomitant increase in activity in both bands 1 and 3 indicating that mitochondrial and microsomal material was trapped in the myelin fraction.

The results presented in this section show that the brain fractions prepared as in section 2.2.1 were not homogeneous and there was a degree of contamination of each fraction by mitochondrial and microsomal material. From the data in Table 3.6 75% of the palmitoyl-CoA synthetase activity in the membrane fraction F_1 was associated with microsomal and mitochondrial material.

TABLE 3.6

Activity of palmitoyl-CoA synthetase in subfractions of adult rat brain obtained by density gradient centrifugation.
All values are mean $\pm$ S.E. of at least four separate determinations using duplicate 5 and 10 minute assays. The nomenclature for fractions and bands is the same as in Figure 3.11.

TABLE 3.6

ACTIVITY OF PALMITOYL-CoA SYNTHETASE IN SUBFRACTIONS OBTAINED BY DENSITY GRADIENT CENTRIFUGATION

Fraction	Amount Applied	Band 1	Band 2	Band 3	Amount Recovered	%
F_1 Control						
Enzyme activity (nmol palmitoyl-carnitine formed per minute)	5.6	0.18 ± 0.02	2.52 ± 0.12	0.04 ± 0.01	2.74 ± 0.3	47
Protein	28.0	0.02 ± 0.005	13.8 ± 0.95	0.08 ± 0.01	14.08 ± 1.2	50
F_1 (osmotic shock)						
Enzyme activity	5.6	1.21 ± 0.08	0.56 ± 0.04	0.60 ± 0.05	2.37 ± 0.21	42
Protein	28.0	0.80 ± 0.05	12.0 ± 0.81	1.15 ± 0.09	13.95 ± 0.9	50
F_2 Control						
Enzyme activity	16.07	-	2.13 ± 0.11	5.63 ± 0.43	7.73 ± 0.50	48
Protein	14.0	-	3.2 ± 0.24	6.31 ± 0.35	9.51 ± 0.62	68
F_2 (osmotic shock)						
Enzyme activity	16.07	0.62 ± 0.01	1.40 ± 0.09	5.41 ± 0.31	7.43 ± 0.35	46
Protein	14.0	0.30 ± 0.01	2.9 ± 0.14	6.29 ± 0.47	9.49 ± 0.71	68
S_1 Control						
Enzyme activity	6.1	0.93 ± 0.04	1.25 ± 0.09	0.50 ± 0.027	2.68 ± 0.11	45
Protein	7.0	0.6 ± 0.03	3.3 ± 0.21	1.0 ± 0.08	4.9 ± 0.20	70
S_1 (osmotic shock)						
Enzyme activity	6.1	1.14 ± 0.09	0.71 ± 0.05	1.00 ± 0.05	2.85 ± 0.14	45
Protein	7.0	0.7 ± 0.02	2.2 ± 0.14	1.9 ± 0.16	4.8 ± 0.25	70

Removal of band 2, rehomogenisation and density gradient fractionation only lead to a broadening of the myelin band, no change was seen in the distribution of enzyme activity in F_1 and F_2 The mitochondria-enriched fraction (F_2) however, only contained 8% microsomal contamination as measured by palmitoyl-CoA synthetase activity in band 1.

6. The Distribution of Palmitoyl-CoA Synthetase Activity in the Microsomal Membrane Fraction (F_3) of Mature Rabbit Brain.

Because the microsomal fraction contains membrane vesicles from a number of different cell types, sucrose density gradient fractionation was used to separate this material according to its buoyant density in order to determine the distribution of palmitoyl-CoA synthetase in this fraction. 0.5-1.0 mg of microsomal protein, prepared as outlined in section 2.2.1 were loaded onto 12 ml continuous sucrose density gradients. The sucrose gradient, buffered with 0.25 M phosphate pH 7.5, was 0.25-1.25 M. After centrifugation at 40,000xg for six hours each gradient was unloaded and each fraction was read at 260nm to give the protein distribution shown in Figure 3.12a.

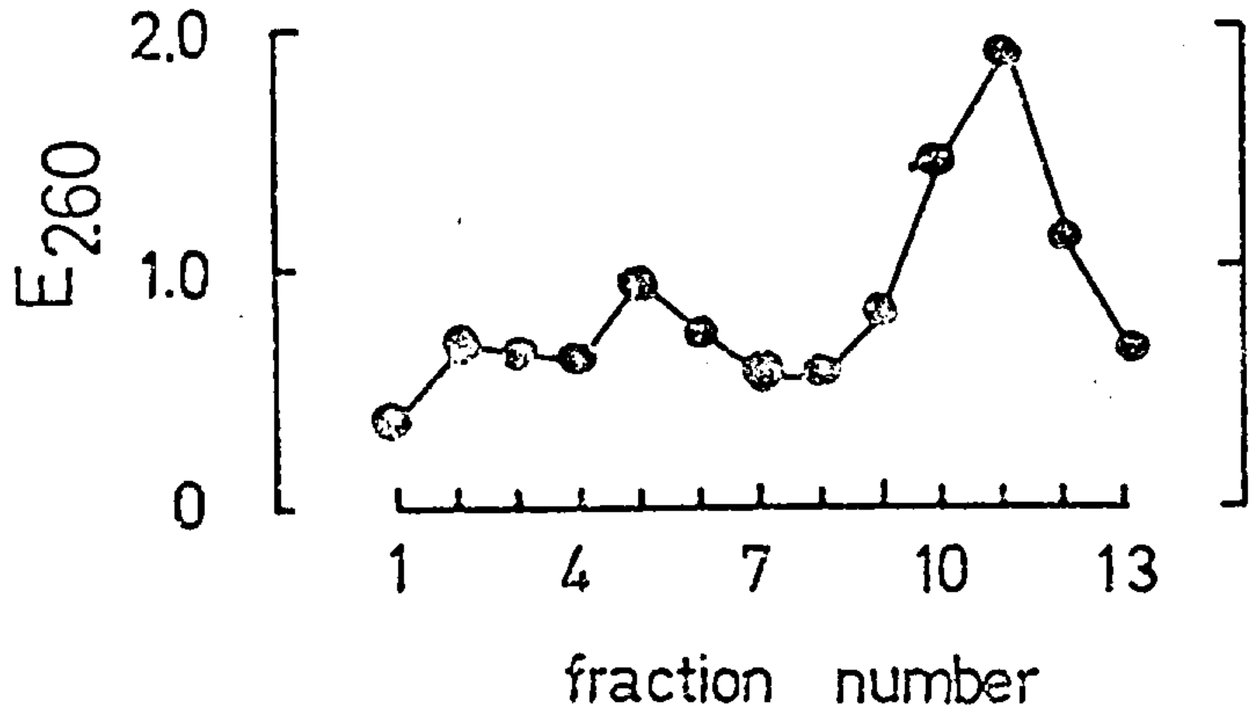
The distribution of palmitoyl-CoA synthetase activity is shown in Figure 3.12b. Two peaks of activity were detected in fractions 4-7 and 11-13 respectively. The relationship of this activity with the incorporation of malonyl-CoA into fatty acids in the microsomal fraction will be discussed in a later section.

FIGURE 3.12

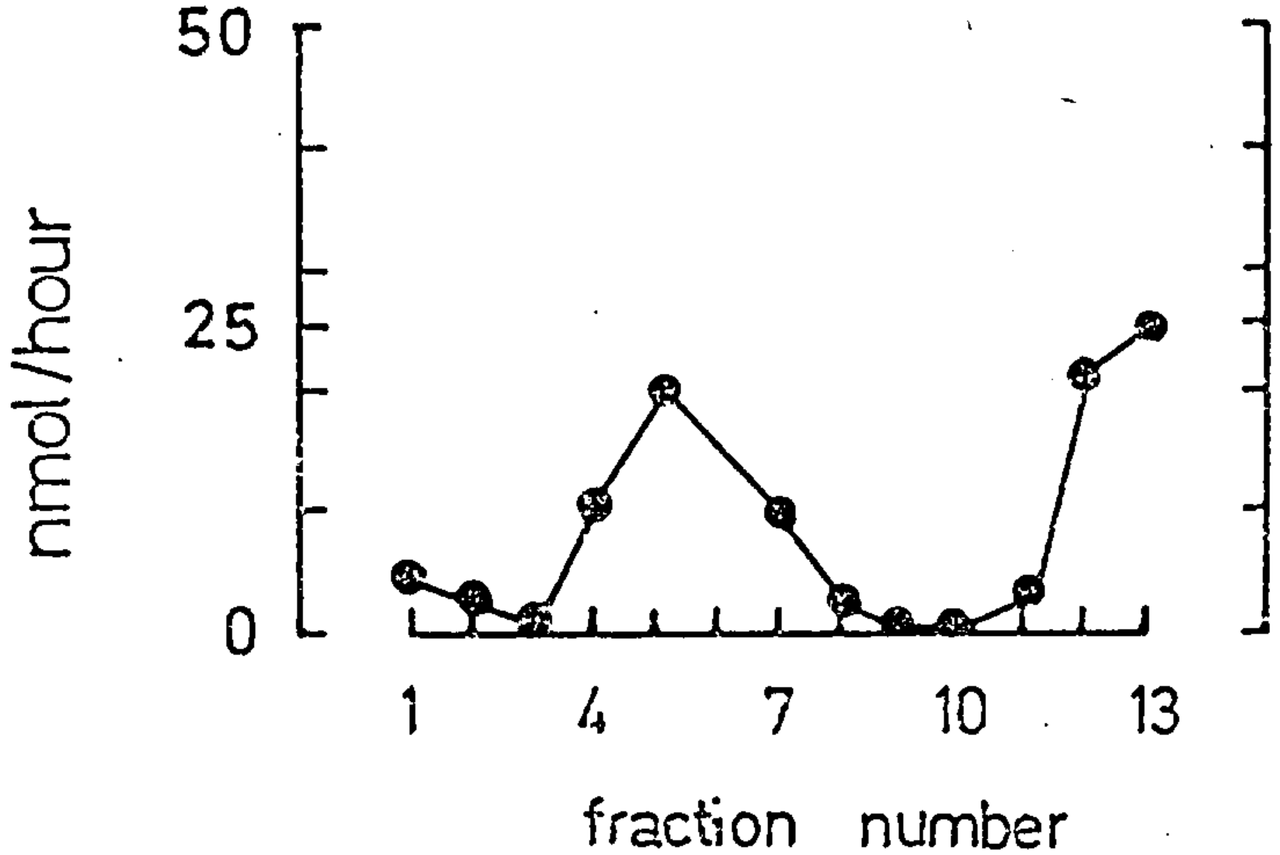
Sucrose density gradient centrifugation of washed microsomes from adult rabbit brain. 1ml fractions were collected by unloading from the bottom therefore fraction 1 is at the bottom of the gradient and fraction 13 is at the top. (a) protein content was measured as E 260 during unloading. (b) palmitoyl-CoA synthetase activity was determined in duplicate using the standard assay and is expressed as nmol palmitoyl-CoA synthesized/hour per fraction.

Figure 3.12 Gradient centrifugation of washed microsomes.

(a)



(b)



THE EFFECT OF THYROID HORMONE AND PROPYLTHIOURACIL ON THE DEVELOPING RAT BRAIN.

Thyroid hormone has been implicated in the normal maturation of the brain. Although it was shown to have no effect upon fatty acid synthetase (112) there were no data available for its effect on the palmitoyl-CoA synthetase systems in subfractions from the developing brain. Therefore the results presented in the following section are from an investigation into this area of potential hormonal control.

3.1. Thyroid Hormone, Propylthiouracil and Normal Brain Development.

Rats given thyroxine were morphologically and behaviourally more advanced than the littermate controls, whereas those given propylthiouracil were retarded with respect to the control rats. Eye-opening, hair formation and the unfolding of the pinna of the ear, were all advanced in the animals given thyroxine. These rats were also more physically active. Conversely, the rats given propylthiouracil were behaviourally and morphologically retarded by one or two days.

From four days of age, the weight of the brains from thyroxine-treated rats was consistently lower than the average weight of the brains of the saline-injected litter mate controls (Figure 3.13a). Although there was no difference between the weights of the brains of the rats given propylthiouracil via the maternal circulation while *in utero* (group PTUa) and the weight of the brains from rats receiving daily doses of propylthiouracil until they were sacrificed (group PTUb)(Figure 3.13b), these brains were also of lower weight than the saline-injected littermates.

FIGURE 3.13

The effect of thyroxine and propylthiouracil on the weight of the developing rat brain.
(a) the effect of thyroxine administration on brain weight increase. Values are for the average brain weight from a single litter.

■ thyroxine administration ● saline-injected controls. (b) the effect of maternally ingested propylthiouracil (PTUa) and daily injections of propylthiouracil (PTUb) on the increase in brain weight. All values are the mean for the litters

■ PTUa ● PTUb

N.B:

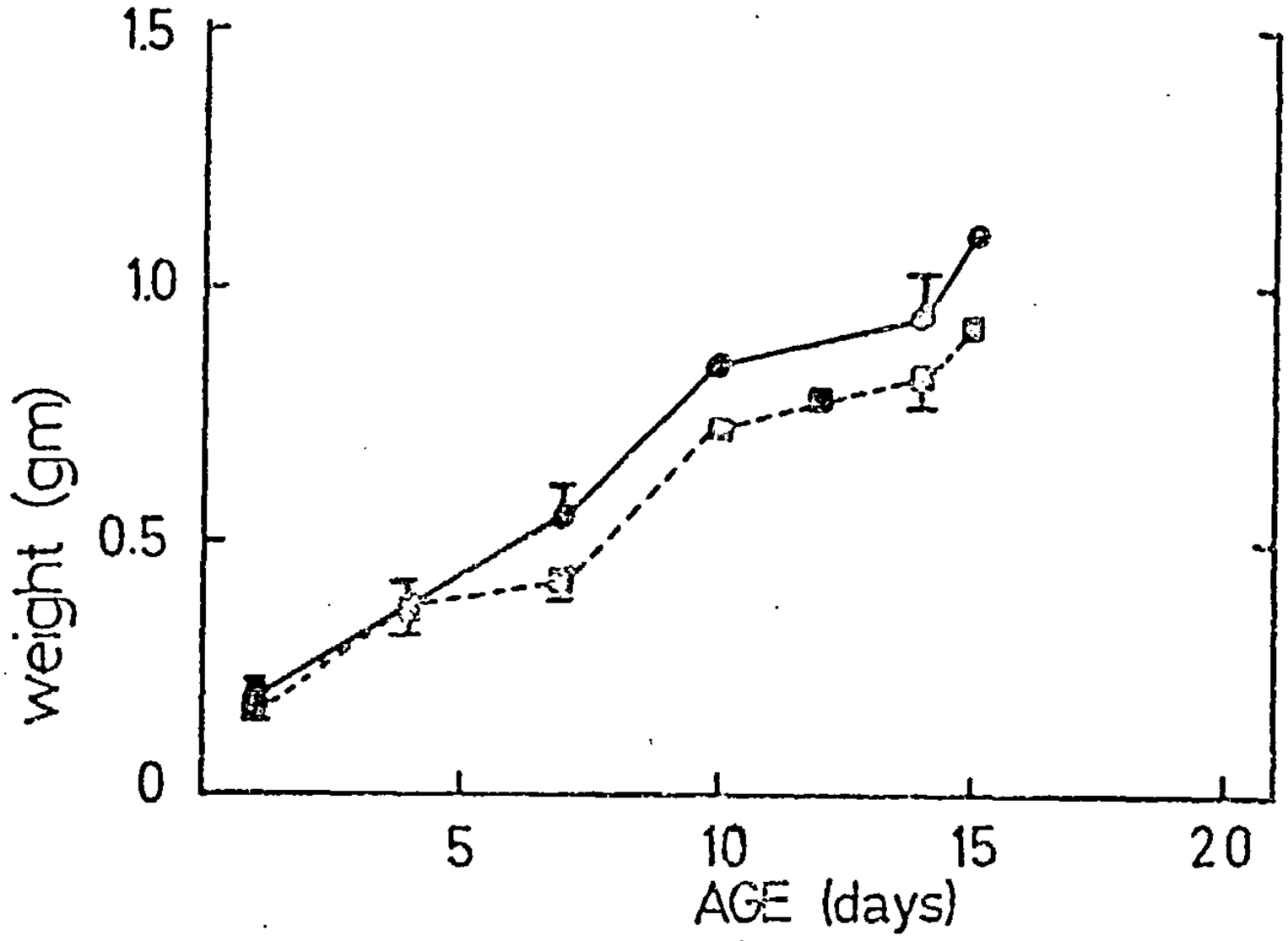
Figures 3.13 and 3.14, Tables 3.7 and 3.8:

In the study of the effect of hormones on the developing rat brain the values presented are the mean ± either difference from the mean or standard error depending on the number of litters used. Below are the numbers of litters used in this study for saline-injected controls, thyroxine treatment and propylthiouracil treatment.
(PTUa - maternally ingested propylthiouracil; PTUb - daily injection of propylthiouracil)

Age in days	Control	Thyroxine	PTUa	PTUb
1	2	2	-	-
4	4	4	1	1
7	2	2	1	1
8	-	-	1	1
9	-	-	1	1
10	1	1	1	1
13	-	-	2	2
14	3	3	-	-
15	1	1	3	3
16	-	-	2	2
18	-	-	3	3
20	-	-	1	1

Figure 3.13 The effect of thyroxine and propyl-thiouracil on brain weight.

(a)



(b)

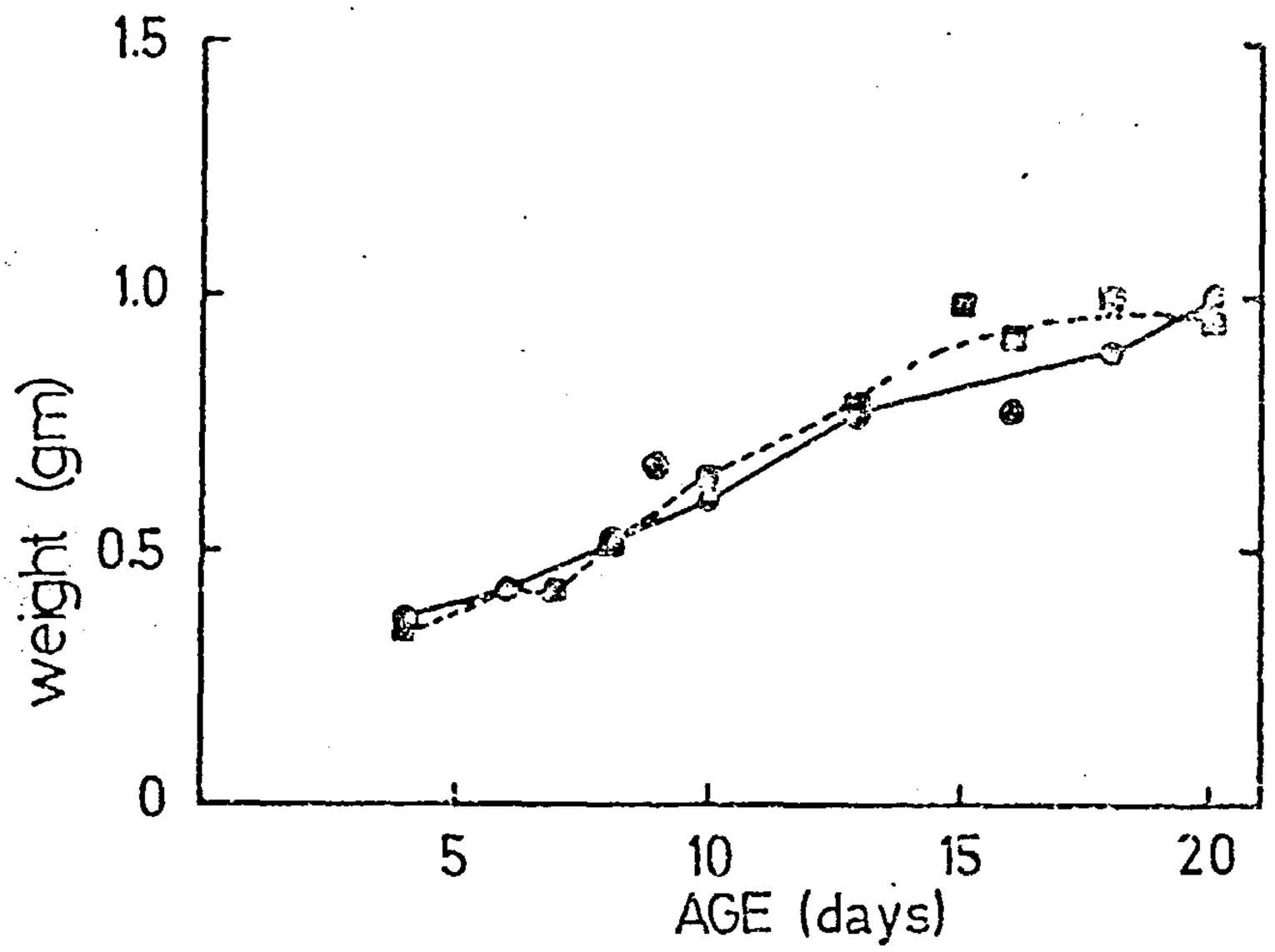


FIGURE 3.14

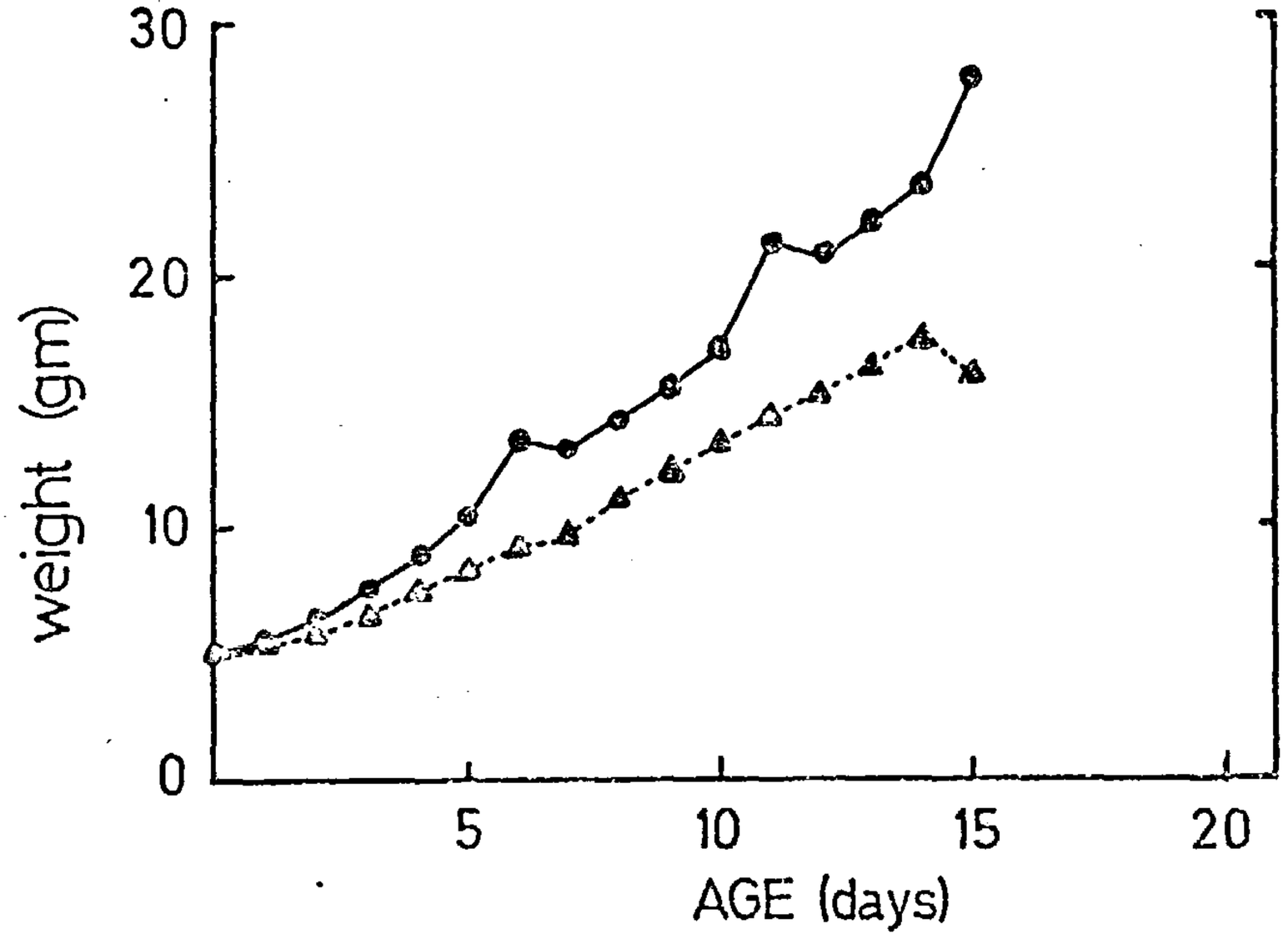
The effect of thyroxine and propylthiouracil on the body weight of the developing rat.

(a) the effect of thyroxine administration on body weight. Values are the mean of all the animals in two litters. △—△ thyroxine ●—● saline-injected controls.

(b) the effect of maternally ingested propylthiouracil (PTUa) and daily administration of propylthiouracil (PTUb) on body weight. Values are the mean of all the animals in the litters. △—△ PTUa ●—● PTUb.

Figure 3.14 The effect of thyroxine and propyl-thiouracil on body weight.

(a)



(b)

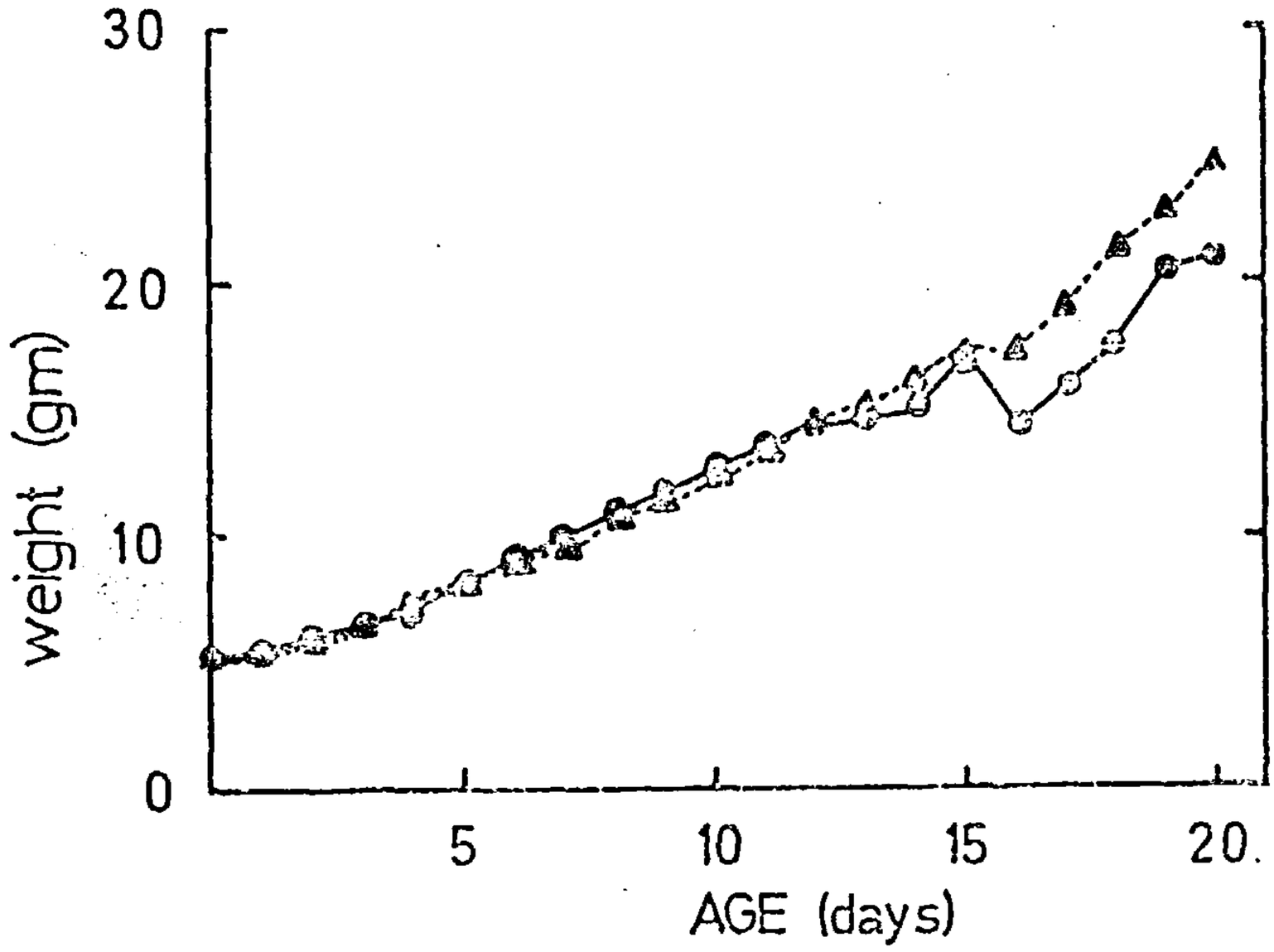


TABLE 3.7

The effect of hormone treatment on the level of rat brain DNA. Values are shown in mg DNA per brain for control rats, thyroxine-injected and those receiving propylthiouracil via the maternal circulation (PTUa) and receiving daily doses of propylthiouracil by injection (PTUb). All values are expressed as outlined for Fig. 3.13.

TABLE 3.7

THE EFFECT OF HORMONE TREATMENT ON THE LEVEL OF RAT BRAIN DNA (MG PER BRAIN)

Age in days	Control	Treatment		
		Thyroxine	PTU(a)	PTU(b)
1	1.06	0.97	-	-
4	1.48	1.50	0.76	0.40
6	-	-	0.25	1.27
7	1.80	1.13	0.80	1.32
8	-	-	0.91	1.34
9	-	-	1.55	2.10
10	2.85	3.0	2.92	3.00
13	-	-	3.00	2.28
14	3.11	3.59	-	-
15	3.16	3.66	3.87	-
16	-	-	-	1.75
18	-	-	2.52	1.41
20	-	-	2.61	1.54

TABLE 3.8

The effect of hormone treatment on plasma Iodine levels.

All values represent plasma iodine concentrations in pooled sera from each litter and are expressed as outlined for Figure 3.13.

TABLE 3.8

THE EFFECT OF HORMONE TREATMENT ON PLASMA IODINE LEVELS

Plasma Iodine (µg iodine per 100ml plasma)

	Treatment			
Age in days	Control	Thyroxine	PTU(a)	PTU(b)
1	8.85 ± 4.6	26.8 ± 4.3	-	-
4	9.56 ± 3.0	16.37 ± 0.7	-	1.17
6	-	-	-	1.43
7	6.40 ± 0.7	14.0	-	0.5
8	-	-	0.8	1.5
9	-	-	0.57	-
10	7.60	9.20	0.57	1.78
13	-	-	-	2.83 ± 0.9
14	4.00 ± 2.9	6.67 ± 2.5	-	-
15	7.50	12.0	1.89	2.32 ± 0.5
16	-	-	1.49 ± 0.2	2.89 ± 1.3
18	-	-	2.21 ± 0.7	3.89 ± 0.4
20	-	-	-	3.06

The weights of the brains from both the thyroxine-injected and propylthiouracil-injected groups of rats were very similar.

Similar results were obtained plotting body weight against age. The control rats had higher body weights than the littermate test animals. There was little difference in body weight between the thyroxine and propylthiouracil-injected rats (Figures 3.14a and 3.14b).

There was little difference seen in the DNA content of whole brains from test and control rats at all ages (Table 3.7). Since different regions of the brain increase in both weight and cell number at different rates during development, changes in whole brain DNA will only give an overall picture of brain cell multiplication.

The total plasma iodine concentration, shown in Table 3.8 in the rats given thyroxine was higher than that of the control group of all ages. In both groups of rats receiving propylthiouracil, the plasma iodine concentration was lower than the control value. The reason for the lower values from group PTUa compared with group PTUb is not clear.

Figures 3.15 and 3.16 show the amount of protein recovered from the cell-free homogenate (CFH) and the fractions F_1 - F_4 with increasing ages. In general, except in the cell-free homogenate, fractions obtained from rats given thyroxine had a higher protein content than control rats at all ages studied. The protein content of fractions F_1 - F_3 from both groups of rats given propylthiouracil (PTUa and PTUb) was lower than that of the control rats.

FIGURE 3.15

The effect of hormone treatment on the protein content of rat brain homogenates and subfractions.
Control (●–●) thyroxine-injected (o–o) propylthiouracil via maternal circulation (PTUa)(▣–▣) and propylthiouracil via daily injection (PTUb) (□–□). All values are expressed in mg per brain or mg per fraction and are the mean of duplicate determinations. (a) protein content of the full homogenate plotted against age in days. (b) protein content of the membrane fraction (F_1) plotted against age in days.

Figure 3.15 The effect of thyroxine on brain protein content.

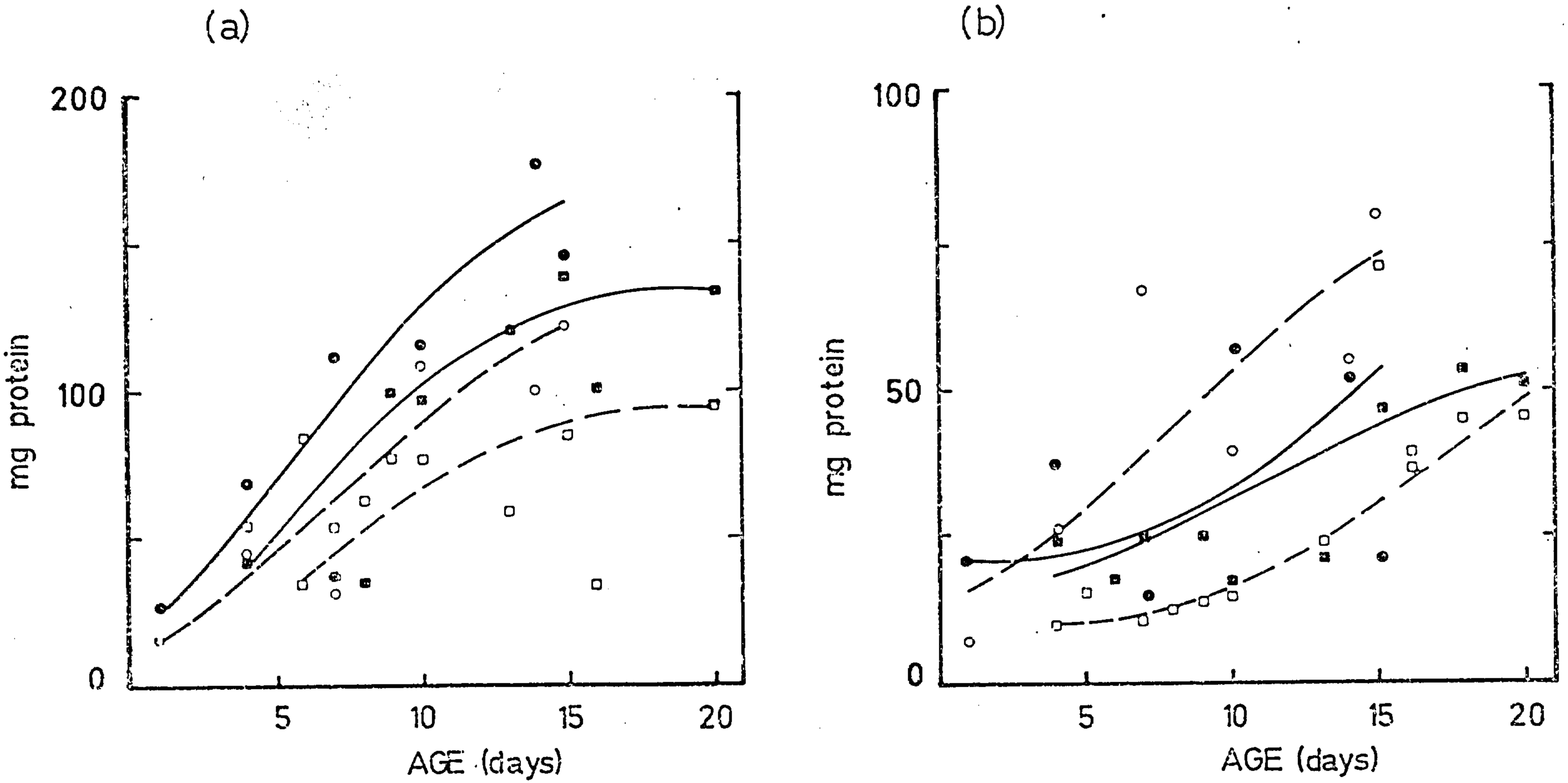


FIGURE 3.16

The effect of hormone treatment on the protein content of rat brain subfractions.
Control (●–○) thyroxine-injected (○–○) PTUa (▣–▣) and PTUb (□–□). All values are expressed in mg per fraction and are the mean of duplicate determinations.
(a) Protein content of the mitochondria-enriched fraction (F_2) plotted against age in days.
(b) protein content of the microsomal fraction (F_3) plotted against age in days.
(c) protein content of the soluble (F_4) fraction plotted against age in days.

Figure 3.16 The effect of thyroxine on brain protein content

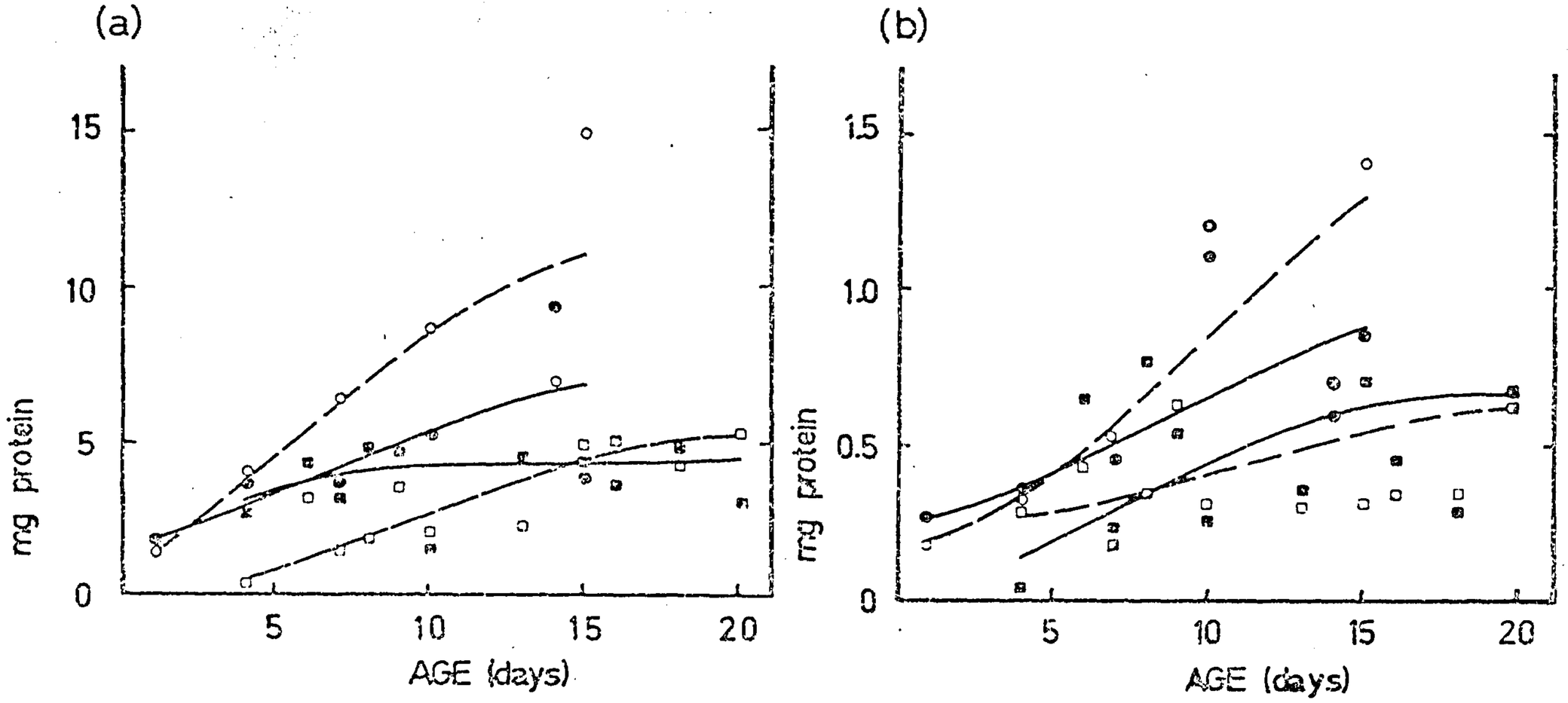
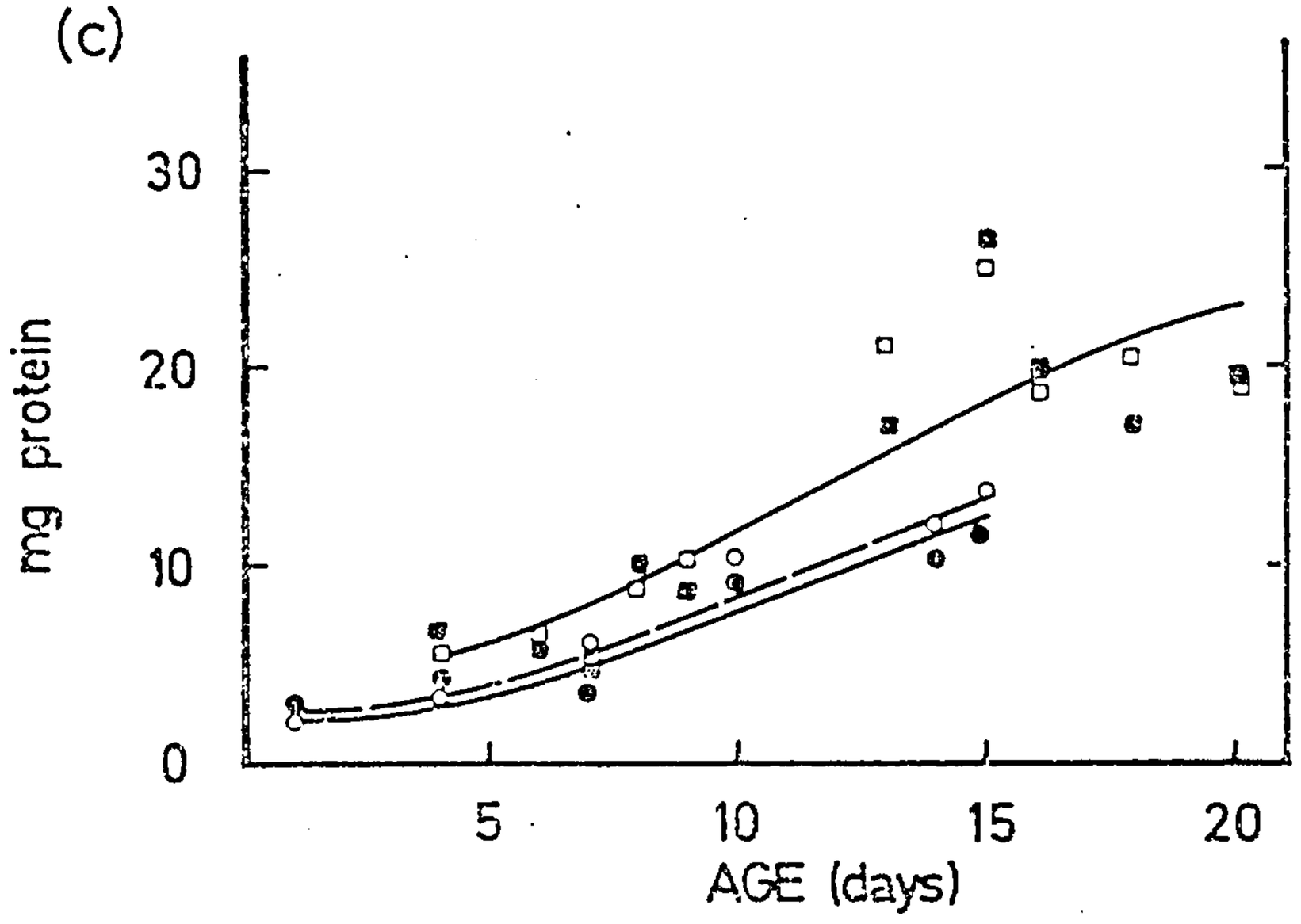


Figure 3.16 The effect of thyroxine on brain protein content



3.3.2. The Effect of Thyroxine and Propylthiouracil on the activity of Rat Brain Palmitoyl-CoA Synthetase.

Palmitoyl-CoA synthetase activity was assayed in the crude homogenate and the washed membrane (F_1) mitochondria-enriched (F_2) and the microsomal (F_3) fractions prepared as outlined in section 2.2.1.

Because of the small brain size, the brains from either the test or control pups from each litter were homogenised together to provide enough material for the enzyme assays. Where two or more litters were sacrificed at the same age, the enzyme activities were determined separately and the values obtained have been expressed as in the previous section. Age dependent changes in palmitoyl-CoA synthetase activity have been expressed in the following ways :

Specific activity (Fig. 3.17a-d)

palmitoyl-CoA synthetase activity/mg DNA (Fig. 3.18a-d)

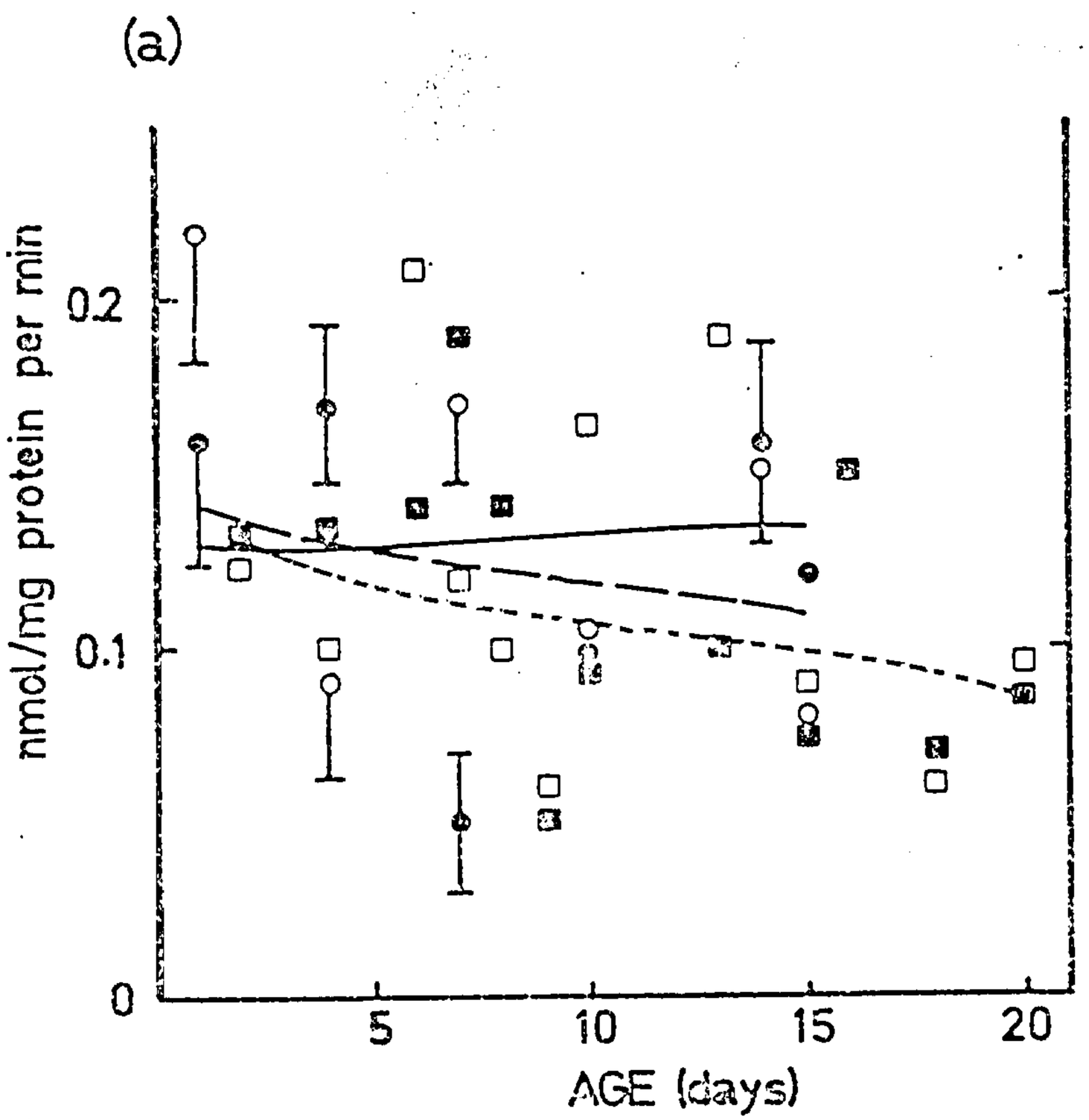
palmitoyl-CoA synthetase activity per brain. (Fig. 3.19a-d)

There was a slight decrease in palmitoyl-CoA synthetase activity/mg protein from 0.2 to 0.1 nmol/mg per minute in the cell-free homogenate with increasing age in all groups of rats (Figure 3.17a) but no similar trend was apparent in the enzyme activity recovered from the subcellular fractions. Only the mitochondria-enriched fraction (F_2) showed any clear difference in palmitoyl-CoA synthetase activity between control rats and those receiving hormonal treatment. The activity of the enzyme in fraction F_2 prepared from the brains of rats receiving propylthiouracil was 6-fold lower (0.5 nmol/mg per min) than the activity in the same fraction from control rats (3.0 nmol/mg per min) and only slightly lower than that recovered from rats receiving thyroxine (Fig. 3.17c).

FIGURE 3.17

Activity of palmitoyl-CoA synthetase in subfractions of brain tissue following hormone treatment.
Activity was measured as palmitoyl-CoA synthesized using the standard assay and all points are the mean of duplicate determinations carried out for 5 and 10 minutes. The activity of palmitoyl-CoA synthetase is expressed as nmol palmitoyl-CoA synthesized/mg protein per minute in (a) the full homogenate (b) the membrane fraction (F_1) (c) the mitochondria-enriched fraction (F_2) and (d) the microsomal fraction F_3. All values are expressed as outlined for Figure 3.13. Control (●—●), thyroxine (○--○) PTUa (··□··) and PTUb (··□···).

Figure 3.17 The effect of thyroxine on palmitoyl-CoA synthetase activity



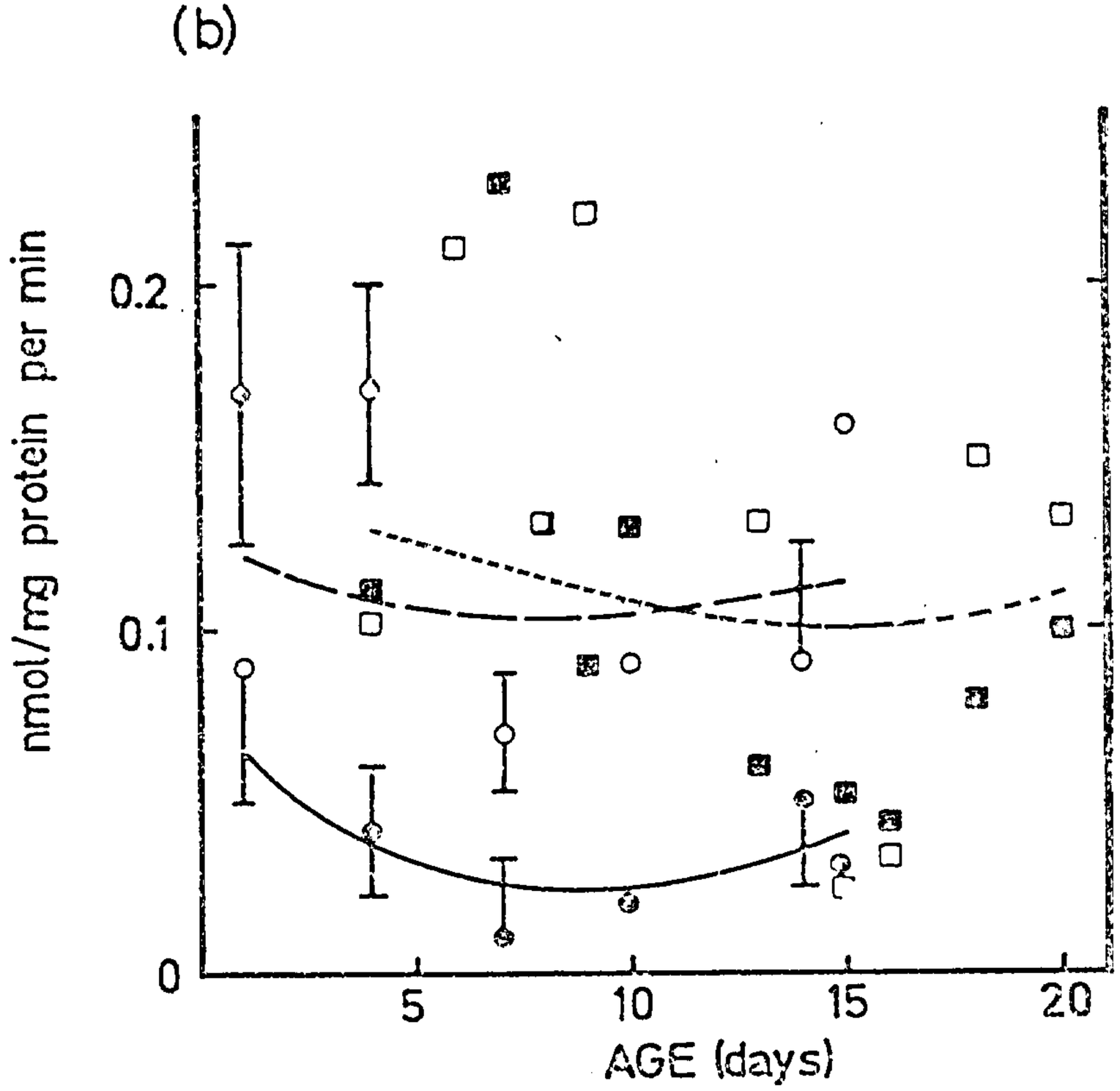
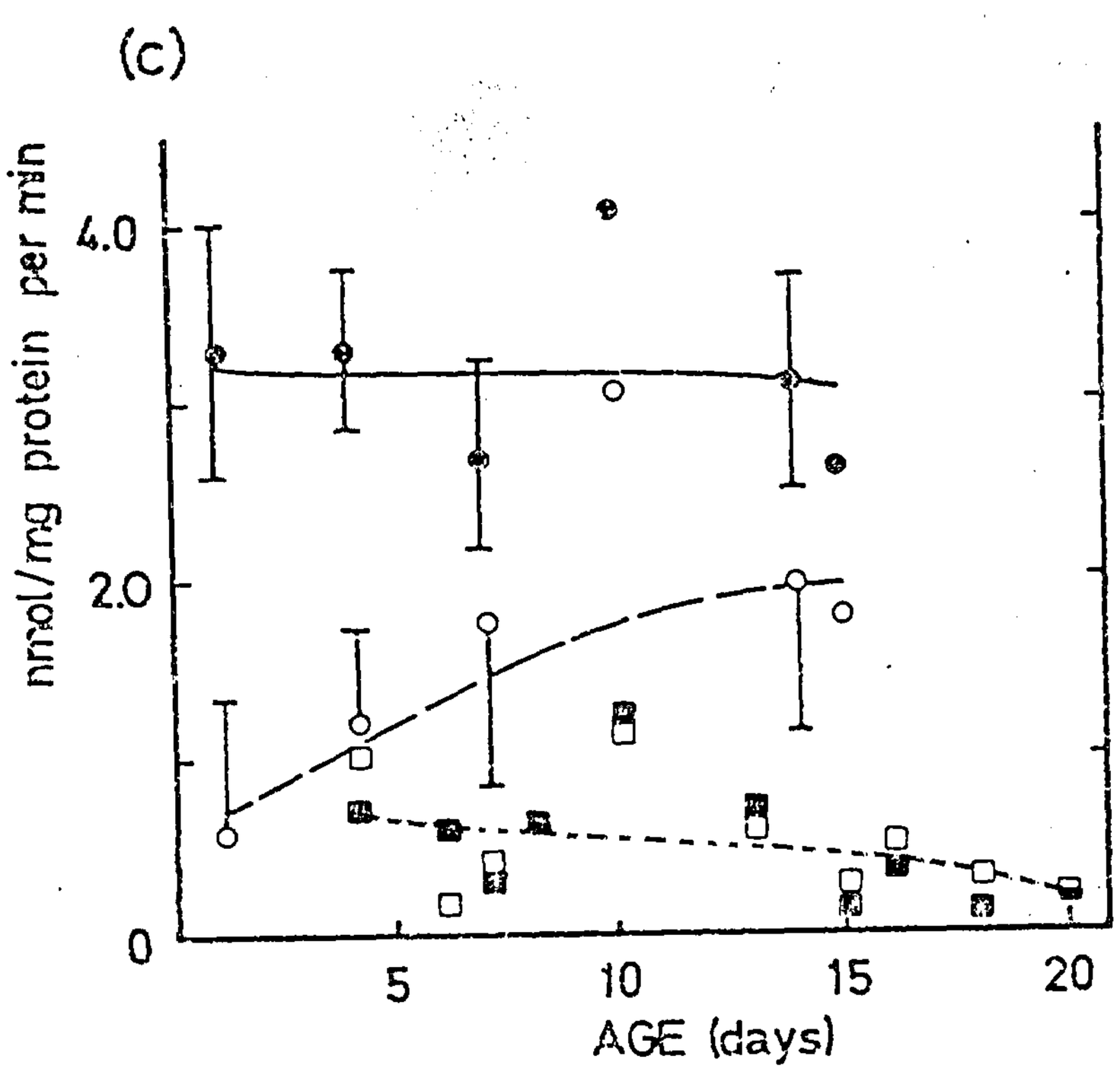


Figure 3.17 The effect of thyroxine on palmitoyl-CoA synthetase activity



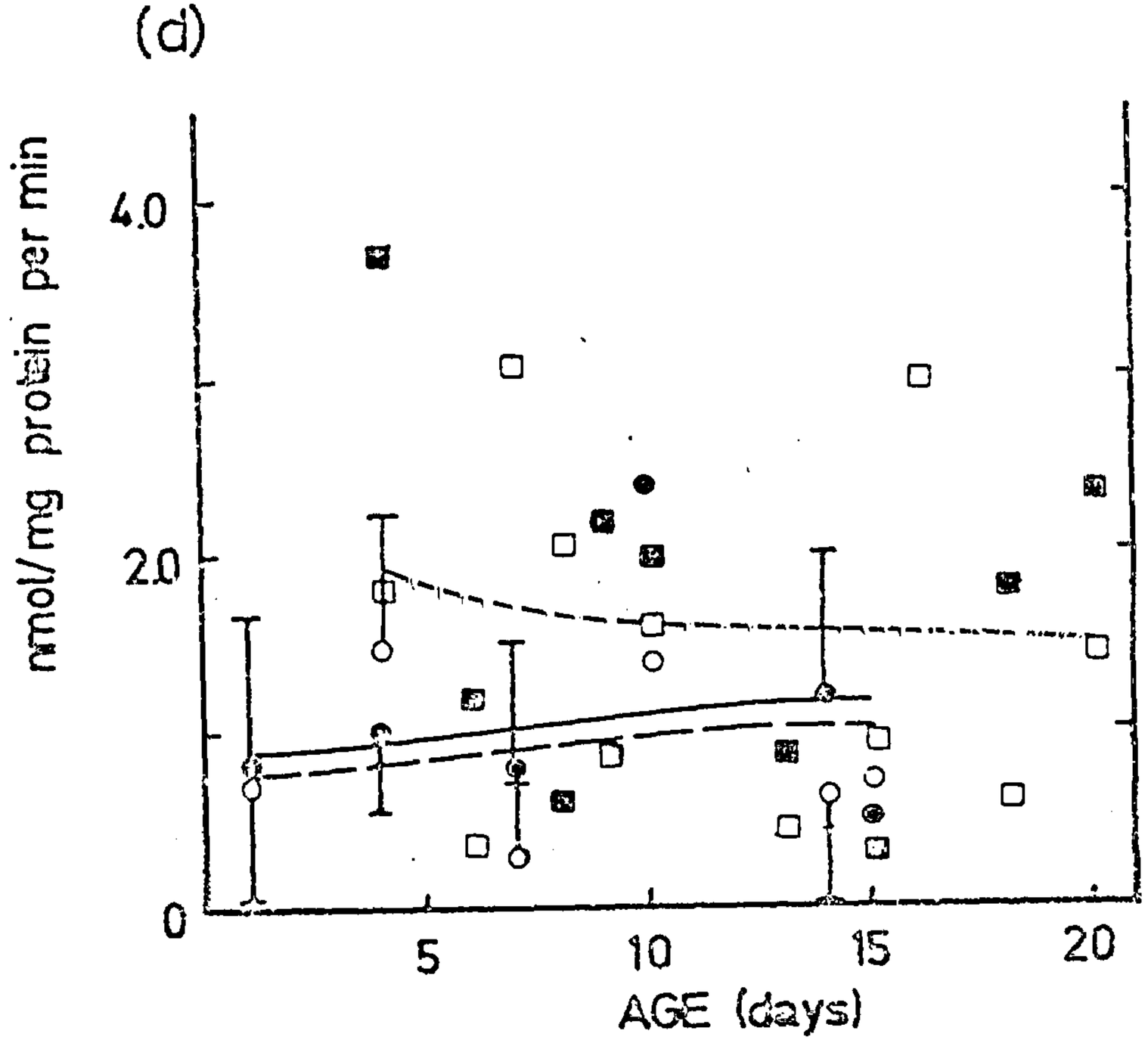


FIGURE 3.18

Activity of palmitoyl-CoA synthetase in subfractions of brain tissue following hormone treatment.

Activity was measured as palmitoyl-CoA synthesized using the standard assay and all points are the mean of duplicate determinations carried out for 5 and 10 minutes. The activity of palmitoyl-CoA synthetase is expressed as nmol palmitoyl-CoA synthesized/mg DNA per minute, in (a) the full homogenate (b) the membrane fraction (F_1) (c) the mitochondria-enriched fraction (F_2) and (d) the microsomal fraction F_3. All values are expressed as outlined for Figure 3.13. Control (●—●) thyroxine (○-○) PTUa (····▨··) and PTUb (····□··).

Figure 3.18 The effect of thyroxine on palmitoyl-CoA synthetase activity

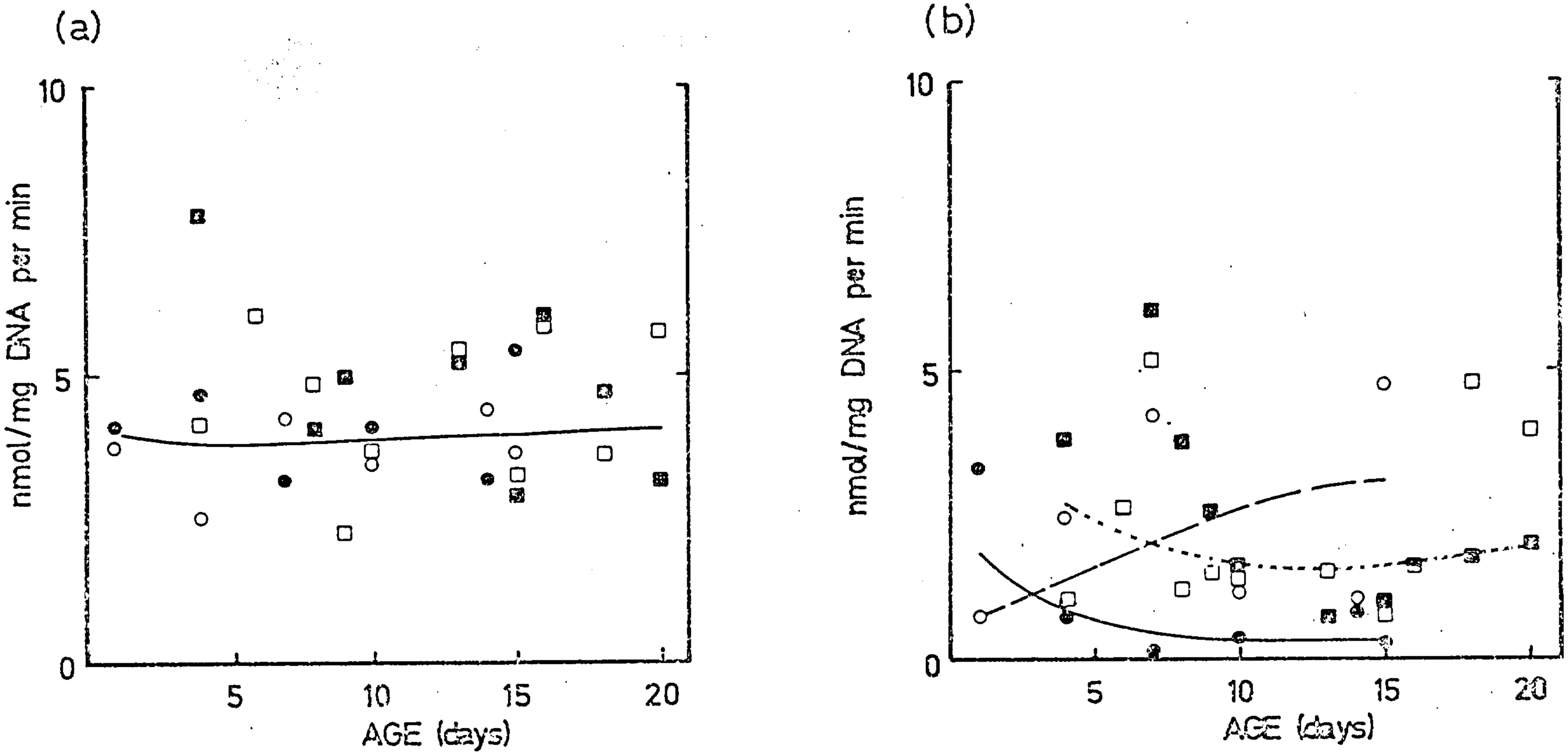
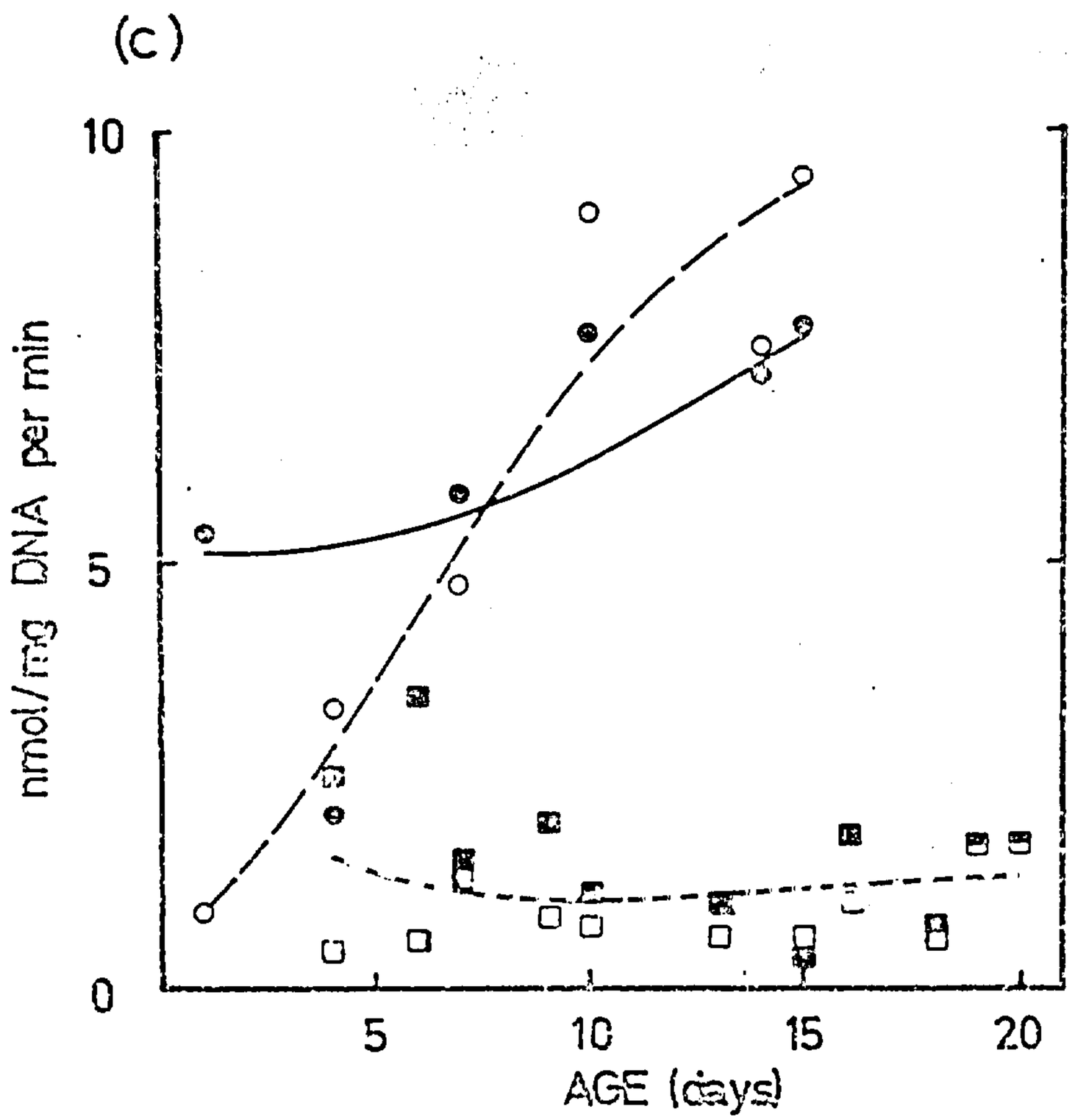
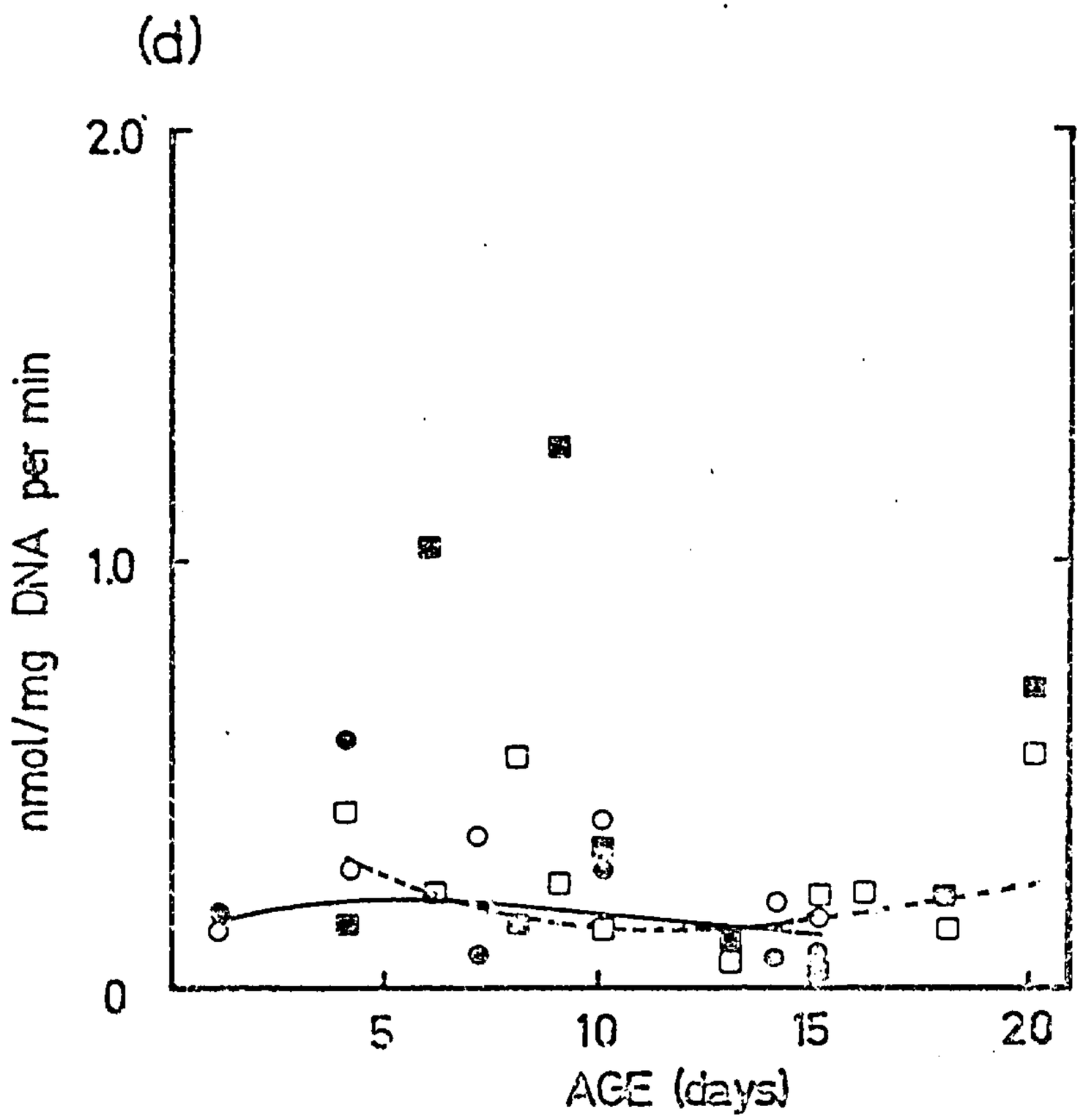


Figure 3.18 The effect of thyroxine on palmitoyl-CoA synthetase activity





There was no change with age in the homogenate activity of palmitoyl-CoA synthetase (4,0 nmol/mg DNA per min) when expressed per mg DNA (Figure 3.18a). Again, the only apparent differences caused by the various treatments were seen in the mitochondria-enriched fraction (F_2). The enzyme activity in this fraction from control and thyroxine treated rats rose steeply with age (1.0 - 9.0 nmol/mg DNA per minute) whereas there was little change in the activity measured in the propyl-thiouracil-treated rats (1.0nmol/mg DNA per minute) (Fig.318c).

Expressing the data in terms of total activity per fraction per brain (Figures 3.19a - d) the palmitoyl-CoA synthetase activity in the homogenate increased with increasing age (4.0 - 12.0 nmol/brain per min.). Thyroxine and propylthiouracil had no effect on the activity of the enzyme in the membrane (F_1) and microsomal (F_3) fractions, but again the F_2 fraction from the thyroxine-treated and control rats showed a steep increase in activity (2.0 - 28.0 nmol/brain per minute). The same fraction recovered from propylthiouracil-treated rats, showed no change and had a very low enzyme activity (2.0 nmol/brain per minute) (Figure 3.19c).

FIGURE 3.19

Activity of palmitoyl-CoA synthetase in subfractions of brain tissue following hormone treatment.

Activity was measured as palmitoyl-CoA synthesized using the standard assay and all points are the mean of duplicate determinations carried out for 5 and 10 minutes. The activity of palmitoyl-CoA synthetase is expressed as nmol palmitoyl-CoA synthesized/brain per minute, in (a) the full homogenate (b) the membrane fraction (F_1) (c) the mitochondria-enriched fraction (F_2) and (d) the microsomal fraction F_3. All values are expressed as outlined for Figure 3.13. Control (●—●) thyroxine (○—○) PTUa (···□···) and PTUb (···□···).

Figure3.19 The effect of thyroxine on palmitoyl-CoA synthetase activity

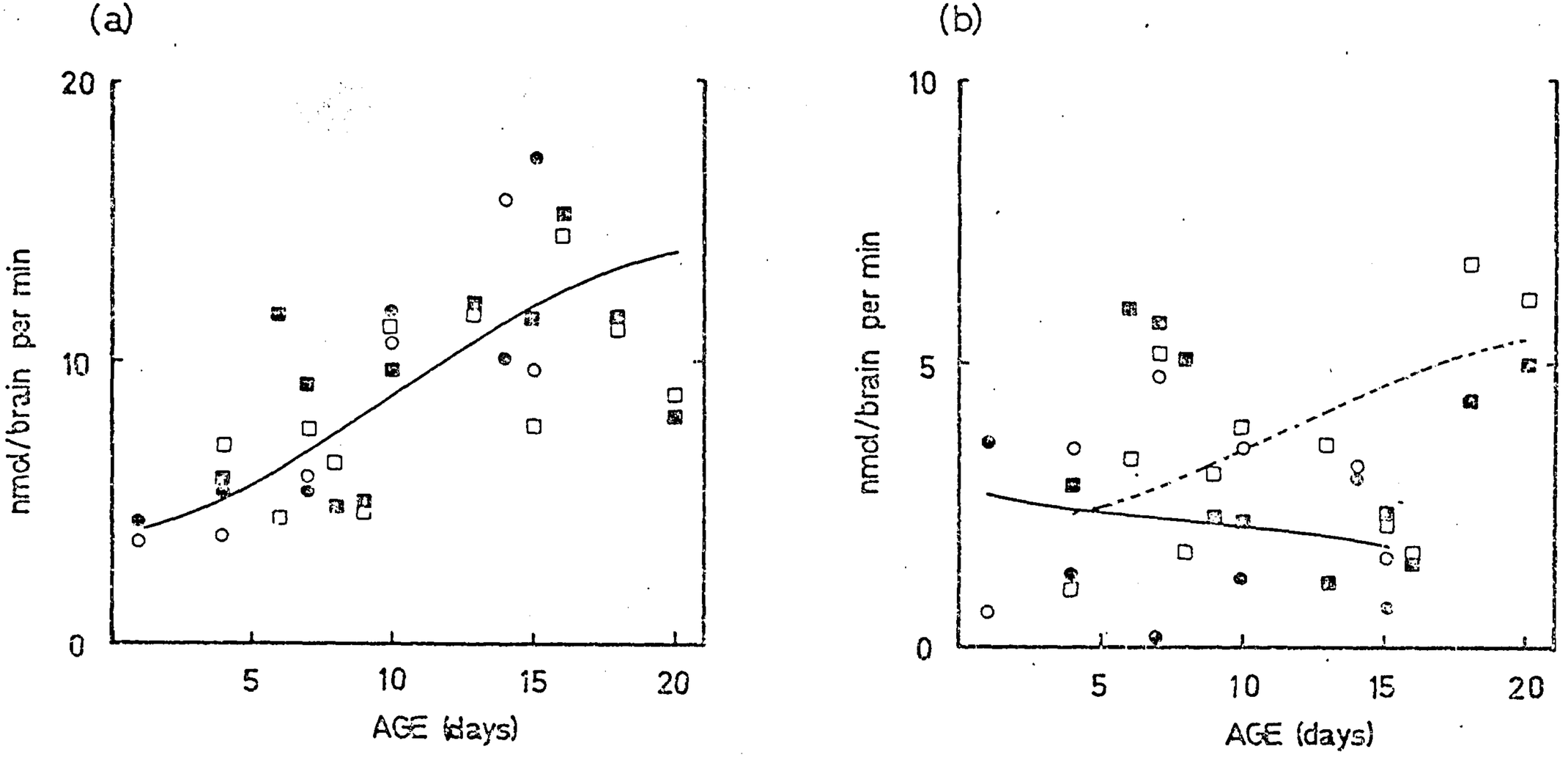
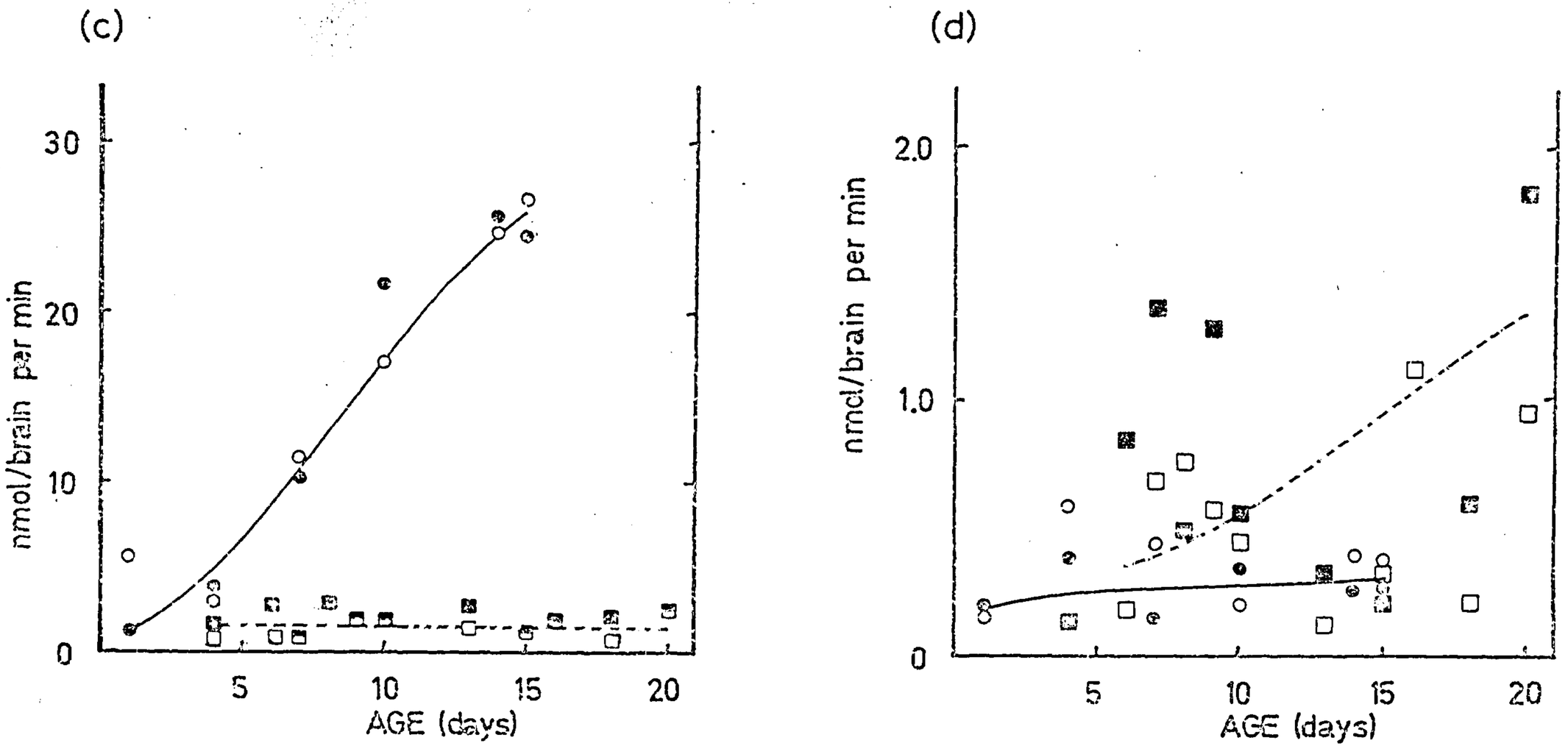


Figure3.19 The effect of thyroxine on palmitoyl-CoA synthetase activity



3.4 THE INCORPORATION OF SODIUM [^{3}H] PALMITATE INTO THE LIPIDS OF BRAIN SUBCELLULAR FRACTIONS FOLLOWING INTRACRANIAL INJECTION.

The incorporation of [^{3}H]-labelled palmitate into brain tissue was investigated in order to determine the inter-relationship between the various subcellular fractions and the role of fatty acids in complex lipid metabolism. The study was carried out over two different time periods; 10 day old rabbits were sacrificed within one hour of intracranial injection and 25 day old rabbits were sacrificed within 24 hours. These two time periods were chosen to measure the initial uptake of fatty acid moeities and their subsequent incorporation into complex phospholipids. 10 day old rabbits have just reached the period of maximum myelin formation, whereas at 25 days the rate of myelination is declining (4). Animals were sacrificed by cervical dislocation and the brains were fractionated as outlined in section 2.2.1.

3.4.1. The Incorporation of [^{3}H] palmitate into the lipids of subcellular fractions of rabbit brain:

Although the radioactive fatty acid was injected intracranially, the recovery of radioactive material was low. Direct solubilisation of the crude homogenate or extraction of lipid material prior to counting gave the same results. The mean % recovery of radioactivity from solubilised tissue was 10.2 $\pm$ 3.1% for the 20 animals used in this study.

Immediately following intracranial injection, 80% of the radioactivity recovered from the brain of 10 day old rabbits was found in the F_1 fraction. This fell to 70% within one hour (Table 3.9).

The radioactivity recovered from the mitochondria-enriched fraction F_2 rose slightly from 15% to 23% during the same period. Less than 15% of the recovered radioactivity was found in the microsomal (F_3) and soluble (F_4) fractions.

Incorporation of radioactivity into fractions F_1, F_3 and F_4 from 25 day old rabbits was similar over 24 hours to that seen during the first hour following injection, in 10 day old rabbits (Table 3.9).

TABLE 3.9

Incorporation of ^{3}H palmitate into lipid extracted from subfractions of young rabbit brain.

All values are expressed as % total amount of radioactivity recovered. Gradient subfractions are expressed as % total radioactivity in the original fraction. Experiment I was carried out over 60 minutes using 10 days old rabbits and Experiment II over 24 hours using 25 day old rabbits. All determinations were carried out on two animals in duplicate.

TABLE 3.9

INCORPORATION OF ^{3}H-PALMITATE INTO LIPID EXTRACTED FROM SUBFRACTIONS OF YOUNG RABBIT BRAIN.

Fraction	Experiment I Time in minutes						Experiment II Time in hours				
	0	1	3	10	30	60	0.25	1	5	16	24
F_1	82	75	94	83	56	70	70	47	75	73	67
F_{11}	48	79	74	19	20	20	16	24	8	12	27
F_{12}	8	11	20	7	5	15	26	16	6	8	27
F_{13}	6	5	3	13	22	22	48	51	79	37	19
F_{14}	38	5	3	61	53	42	10	9	7	43	27
F_2	15	23	4	13	29	23	27	30	17	16	12
F_{21}	21	20	24	22	32	23	22	26	24	25	21
F_{22}	72	66	69	67	59	66	64	67	68	65	64
F_{23}	7	14	7	11	9	11	14	7	8	10	15
F_3	2	1	1	2	6	5	1	7	3	5	7
F_4	1	1	1	2	9	2	2	16	5	6	14

The % radioactivity recovered from the mitochondria-enriched fraction (F_2) however, decreased from 27% to 12% during the period of study. It is apparent, that fractions F_1 and F_2 play a major role in the incorporation of fatty acids into cerebral lipids. The % radioactivity incorporated into the lipids of gradient sub-fractions obtained from fraction F_1 and F_2 varied greatly in both 10 and 25 day old rabbits. In general, only gradient fractions from fraction F_2 gave consistent results in both studies. Fraction F_{22} identified as myelin (section 3.2.3.5) at all times contained at least 50% of the radioactively-labelled lipid; (Table 3.9) the microsomal fraction F_{21} contained a large proportion of the balance in the F_2 fraction.

The radioactivity recovered in the lipids extracted from subfractions of F_1 was mainly in fractions F_{11} and F_{14} of 10 day old rabbits, but was recovered in greatest quantities from fractions F_{13} of 25 day old rabbits. Fractions F_{11}, F_{13} and F_{14} have been tentatively identified as microsomal, mitochondrial and possibly originating artefactually from myelin

The presence of fraction F_{14} in subfractions of F_1 makes interpretation difficult, because it is present to varying degrees in each sample and appears to increase with the time of storage at $-20^{o}C$. Assuming that fractions F_{11} and F_{21} are mainly microsomal, F_{22}, F_{12} and F_{14} are myelin, F_{13} and F_{23} mitochondrial in origin , it is possible to express the data given in Table 3.9 to give the percent incorporation of radioactivity into the lipids of each of the above subcellular organelles with time.

Radioactivity from microsomal material was taken as the sum of the radioactivity from fractions F_{11}, F_{21}, and F_3. The incorporation of radioactivity with time into organelles from 10 and 25 day old brains, is shown in Figs. 3.20 a and 3.20 b. In both cases there is a rise in the myelin content of radiolabelled lipid, but in the one hour study (Fig. 3.20 a) this increase is balanced by a fall in the microsomal radiolipid content; whereas over a 24 hour period the rise in myelin radioactivity is balanced by a fall in the mitochondrial radioactive lipid (Fig. 3.20 b). The differences in the distribution of radioactivity between the organelles from the two groups of animals one hour after intracerebral injection may be attributed both to age and differences in the composition of the subcellular fractions prepared.. The low percent radioactivity recovered from the soluble F_4 fraction may be due to the presence of soluble lipid-carrier proteins.

3.4.2. <u>The Incorporation of [^{3}H] palmitate into the phospholipid fraction of subcellular fractions of rabbit brain</u>:

Scanning thin layer chromatograms for radiolabelled lipids following separation of samples in petroleum ether: diethylether : acetic acid (75 : 25 : 1), it was apparent that only two radiolabelled spots were present. These were identified using authentic standards as free fatty acid and phospholipid.

In all fractions prepared the amount of label found in the phospholipid fraction was at least 25% of the total radioactivity recovered one hour after intracerebral injection and greater than 70% of the total after 24 hours (table 3.10) Since the amount of label found in the phospholipid fraction (expressed as a percentage of the total) was determined in subfractions of fractions F1 and F2 from 10 day old rabbits, it was possible to plot the changes in the percentage of the total radioactive lipid recovered as phospholipid in the various subcellular organelles (Fig.3.21)

FIGURE 3.20

The incorporation of ^{3}H-palmitate into the lipids of subcellular organelles of developing rat brain.

(a) Incorporation of label into subcellular organelles of 10 day old rabbit brain. Values plotted are % radioactivity recovered in each organelled against time in minutes. Myelin ●–○ Microsomes ○–○ mitochondria □–□ and soluble fraction ✳–✳.

(b) Incorporation of label into the lipids of subcellular organelles of 25 day old rabbits. Values are plotted as % radioactivity recovered in each organelle against time in hours. Myelin ●–○ microsomes ○–○ mitochondria □–□ and ✳–✳ soluble fraction. The experiments were carried out using two animals at each time and all values are the mean of several determinations carried out on the lipid extracts.

Figure 3.20 Incorporation of ^{3}H-palmitate into subcellular organelles

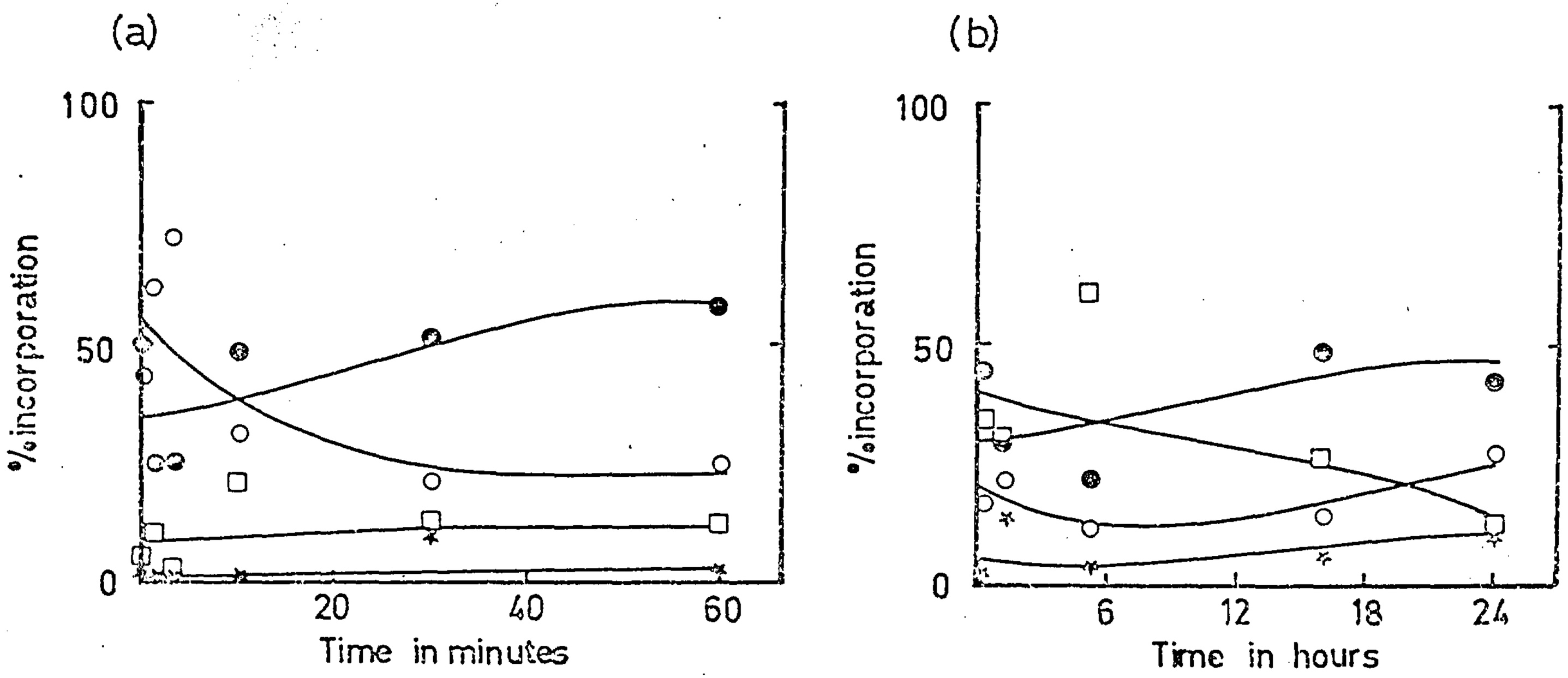


TABLE 3.10

Incorporation of ^{3}H-palmitate into phospholipid in subfractions from developing rabbit brain. Values are expressed as the amount of radioactivity recovered from the phospholipid component of the total lipid extract expressed as a % of the total radioactivity recovered. Experiment I was carried out on 10 day old rabbits and time is expressed in minutes. Experiment II was carried out on 25 day old rabbits and time is expressed in hours. Subfractions have the same designation as outlined in sections 2.2.1 and 2.2.2. All values are the mean of several determinations carried out on the lipid extracts from two animals at each time.

TABLE 3.10

INCORPORATION OF ^{3}H-PALMITATE INTO PHOSPHOLIPID IN SUBFRACTIONS FROM DEVELOPING RABBIT BRAIN

Fraction	Experiment I						Experiment II				
	Time in minutes						Time in Hours				
	0	1	3	10	30	60	0.25	1	5	16	24
F_1	20	11	35	30	80	45	21	37	80	85	95
F_{11}	9	8	10	23	99	75	-	-	-	-	-
F_{12}	32	15	12	28	99	38	-	-	-	-	-
F_{13}	25	8	50	13	25	25	-	-	-	-	-
F_{14}	6	13	70	36	13	30	-	-	-	-	-
F_2	12	-	14	19	39	74	79	76	72	96	99
F_{21}	30	-	14	21	70	99	-	-	-	-	-
F_{22}	50	-	99	23	45	99	-	-	-	-	-
F_{23}	99	-	11	36	20	99	-	-	-	-	-
F_3	45	50	60	45	50	-	83	94	74	97	97
F_4	-	-	-	-	-	-	50	49	72	76	72

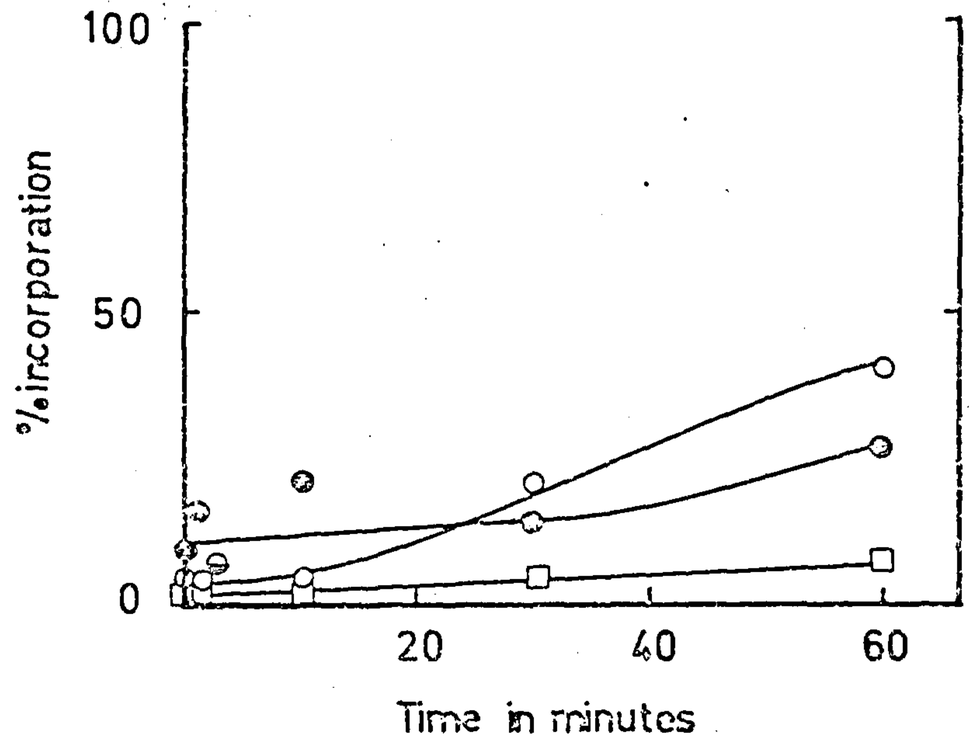
FIGURE 3.21

The incorporation of ^{3}H-palmitate into the phospholipids of subcellular organelles of 10 day old rabbit brain.

Values are expressed as the amount of radioactivity recovered from the phospholipids as a percentage of the total radioactivity recovered in the lipid extract of that subcellular organelle. All points represent the mean for two animals.

Myelin ●–● ($\bar{F}_{12} + \bar{F}_{14} + \bar{F}_{22}$) mitochondria □–□ ($\bar{F}_{13} + \bar{F}_{23}$) microsomes ○–○ ($\bar{F}_{11} + \bar{F}_{21} + \bar{F}_{3}$).

Figure 3 21 Incorporation of ^{3}H-palmitate into the phospholipids of subcellular organelles



The rise in radiolabelled phospholipid in the microsomes is interesting since it occurred while the total microsomal radioactive lipid content was falling. As indicated in the crude fraction data (Table 3.10) the % radio-phospholipid content of mitochondria and myelin also increased. The radiolabelled phospholipids from membrane (F_1), mitochondrial-enriched (F_2) microsomal (F_3) and soluble (F_4) fractions from the 24 hour study were identified by radio thin layer chromatography (Fig. 3.22 a,b,c, & d) and expressed as % total phospholipid. In fractions F_1 and F_2 (Fig.3.22a & b) which contain all the myelin and mitochondria, the predominant radiolabelled phospholipid is phosphatidylcholine, whereas the microsomal (F_3) and soluble (F_4) fractions (Fig.3.22c & d) contain predominantly phosphatidylethanolamine. The occurrence of a large proportion of phosphatidic acid, 10-15% in fraction F_1, F_2 and F_4 may indicate either a high level of turnover of labelled phospholipids or, as is more probable, a large degree of spontaneous breakdown of the more labile phospholipids. The incorporation of label into most phospholipids identified was detected in trace amounts in all fractions at different time intervals following intracerebral injection (Fig.3.22a,b,c, & d). Thus indicating that all phospholipid classes are involved in the uptake and further metabolism of fatty acids and to differing degrees in different organelles. The presence of labelled phospholipids in the soluble fraction (F_4) probably indicates the presence of lipid carrier-proteins in the brain cell cytoplasm.

FIGURE 3.22

The incorporation of ^{3}H-palmitate into various species of phospholipid in subcellular fractions of 25 day old rabbit brain. Values are expressed as % total radioactivity recovered from the phospholipid fraction. ■-·-·-■ phosphatidylserine and phospatidylinositol

□——□ sphingomyelin ▲—·—▲ phosphatidylcholine

△— —△ phosphatidylethanolamine ●- - -● phosphatidic acid

○····· ○ cardiolipin.

(a) changes in the distribution of radiolabelled phospholipids in the membrane fraction (F_1)

(b) changes in the distribution of radiolabelled phospholipids in the mitochondria-enriched fraction (F_2)

(c) changes in the distribution of radiolabelled phospholipids in the microsomal fraction (F_3)

(d) changes in the distribution of radiolabelled phospholipids in the soluble fraction (F_4).

Figure 3.22 Incorporation of ^{3}H-palmitate into phospholipid.

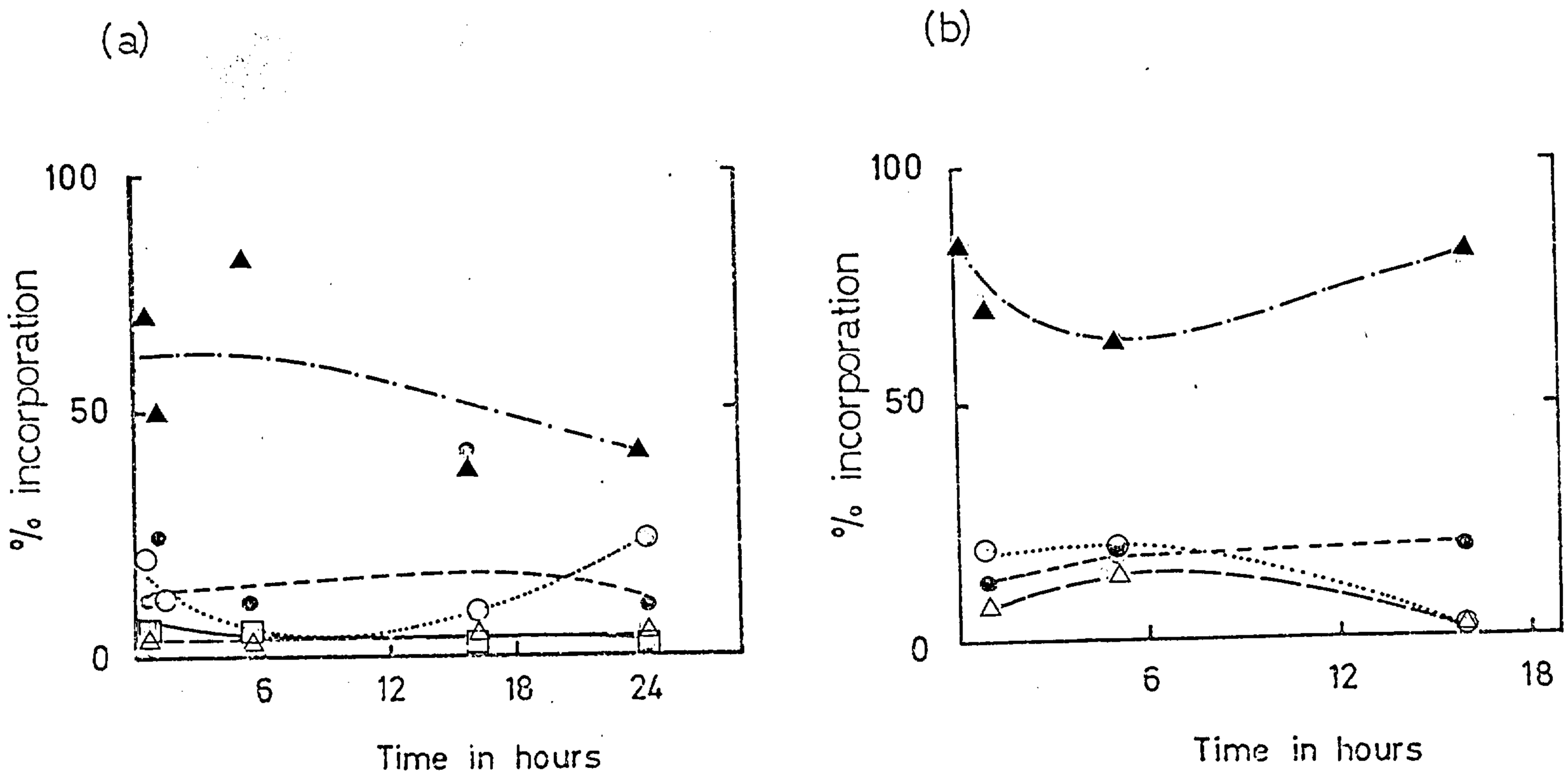
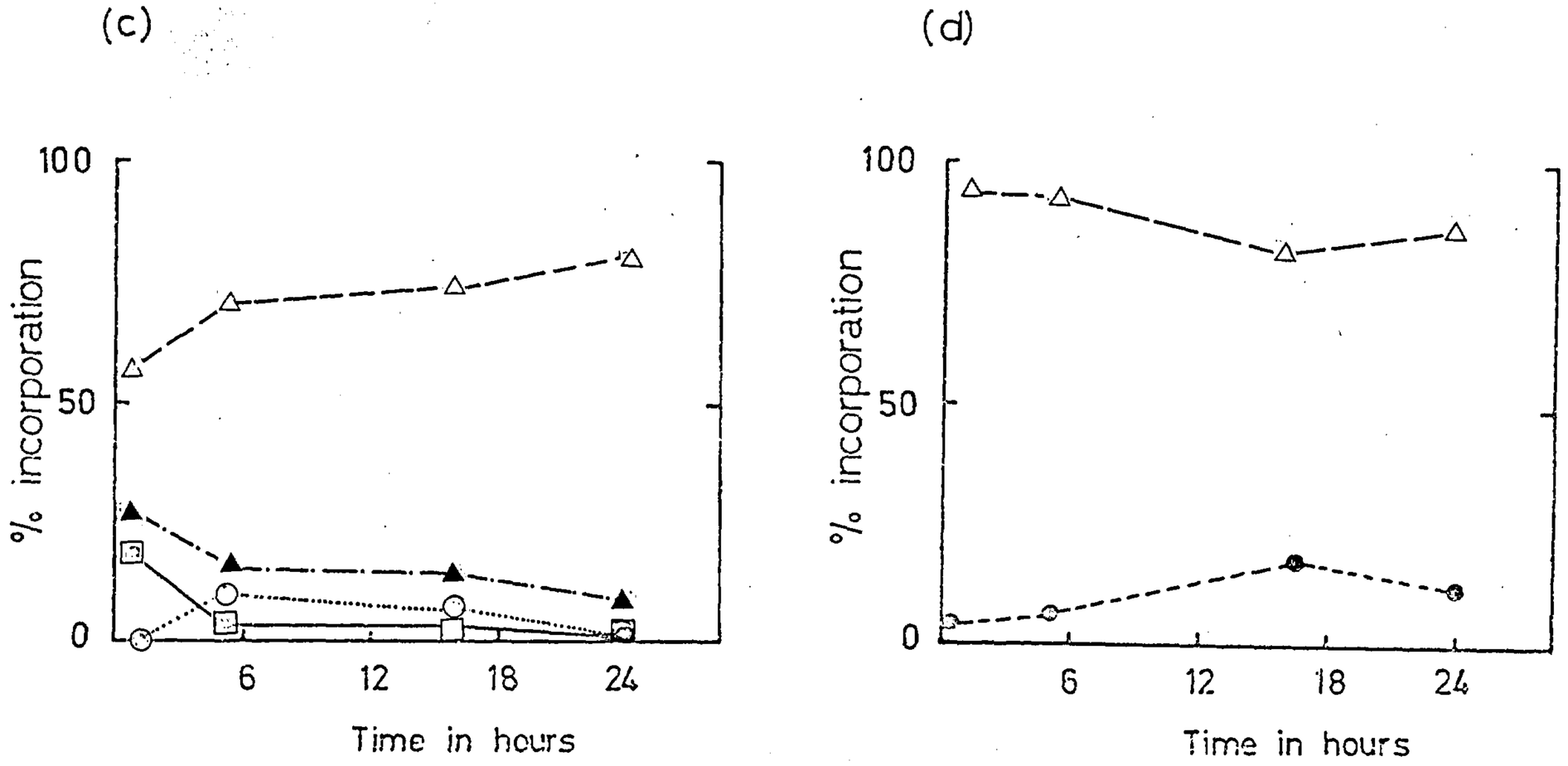


Figure 3.22 Incorporation of ^{3}H-palmitate into phospholipid.



4. DISCUSSION

Myelination is a period of brain development during which complex structural and functional changes take place. The synthesis of lipid material for the myelin membrane must therefore be under strict control. It has been shown in other studies (1, 2, 15, 153, 190) that slight disturbances during this period can lead to major changes in both structure and function although some of these are reversible.

It is important therefore, to consider the key role played by fatty acids in normal brain development and, in particular, changes in lipogenic enzyme activity during myelination. It is also necessary to determine the subcellular site or sites of fatty acid synthesis, activation, and incorporation into complex lipids, in order to understand the major routes of lipid biosynthesis and at which points malfunction may lead to quantitative and qualitative changes in myelin.

4.1 The incorporation of fatty acids into myelin lipids following intracerebral injection:

Intracerebral injection of labelled precursors has been used to circumvent any blood-brain barrier effect which may inhibit the uptake of metabolites by the brain (105), although Dhopeshwarkar showed cerebral uptake of acetate and palmitate within 15 seconds of carotid injection in the adult rat (99) and Davison and Dobbing (94) concluded, from their experiments, that the uptake of circulatory phosphate into the brain was faster than the incorporation of the phosphate into phospholipid.

In the present study, intracerebral injection was chosen as a means of introducing labelled fatty acid into the brain because:

1) two separate groups of rabbits of different ages were used and intracerebral injection of labelled fatty acid overcame any age-dependent changes in the selective uptake phenomena exhibited by brain tissue, and

2) since young animals were used, intracerebral injection of substrate avoided undertaking the technically more demanding technqiue of carotid injection.

It is apparent, that of the labelled lipid recovery from the brain, at least 95% of the material was located in the membrane fractions even immediately following intracerebral injection (Fig.3.20a) and of the subcellular organelles prepared, only myelin showed an increase in the % label recovered. Since the results in Figs.3.20 a and b and Fig. 3.21 are expressed in terms of % total radioactivity and no measure of the actual amount of radioactivity or the change in specific activity with time of the seperate lipid classes was made, the time-dependent accumulation of the label originating from [^{3}H] palmitate in the myelin fraction represents either a net gain in labelled material or a slower rate of loss of radioactivity than in other subcellular particles. That the total % recovery of injected radioactivity remained fairly constant during the 24 hour study supports net accumulation of radiolabelled lipid by the myelin fraction during this period.

The constant % recovery of labelled lipid from the soluble fraction over 24 hours and the mitochondria in the short-term study and microsomes in the long-term study may be indicative of either a fixed pool of labelled material or a dynamic situation in which the radiolabelled fatty acid is undergoing enzymatic modification while being translocated from one organelle to another. The changing composition of the F_2 and F_3 phospholipids (Figs.3.22b & c) over 24 hours suggests

that there is a dynamic flux of phospholipid through these fractions. In the soluble fraction, however, the appearance of only one major phospholipid (Fig.3.22 d) could indicate either a tissue pool or the presence of specific carrier lipoproteins which are involved in the transfer of phosphatidylethanolamine from the microsomes to other subcellular organelles.

The involvement of soluble phospholipid carrier proteins has been ably demonstrated in both brain (120, 123, 191, 192) and liver (116-119). Exchange of phosphatidylcholine between mitochondria and microsomes has been shown in both liver (119,118) and brain (191) but no evidence of in vitro exchange has been demonstrated between microsomes and myelin (192).

Brammer and Shaltawy (193) suggest that the observed lack of phosphatidylcholine exchange in myelin is due to the high negative charge of this group of phospholipids. The interaction of phosphatidylcholine with myelin proteins may also inhibit the exchange of this phospholipid since Braun and Radin (194) showed that sulphatide phosphatidylcholine and phosphatidylinositol could be bound to proteolipid protein by nonionic association and Palmer and Dawson (50, 60) showed that triphosphoinositides and phosphatidylcholine combine readily with myelin basic protein.

It is apparent that myelin is a very specialised membrane structure and the properties of the individual lipid and protein species give it its structural and functional properties.

Lipid compositional changes which take place during maturation may be the result of exchange with protein-carried phospholipids which could be present in the continuous circuits of cytoplasm which are present in both immature and mature myelin (7).

That there is no clear pattern of transfer of radioactivity between each class of subcellular particle and myelin indicates that the method of myelin formation during early postnatal development may be much more complex than envisaged by cytoplasmic phospholipid transfer and may firstly involve membrane extrusion and then modification by phospholipid exchange and protein replacement and addition.

4.2 Changes in Lipogenic enzyme activity during development:

The investigation of age-dependent changes in enzyme activity is of paramount importance in the understanding of myelin formation and subsequent maintenance of its composition. The enzymes investigated in this study, fatty acid synthetase and palmitoyl-CoA synthetase, obviously play a central role in the formation and activation of fatty acids for incorporation into the phospholipids of the myelin membrane. Since a large amount of lipid is incorporated into the developing myelin membrane during a very short time period, during most active myelination, changes in the activity of lipogenic enzymes would be expected.

4.2.1 Fatty acid synthetase:

The changes in the activity of the soluble fatty acid synthetase complex are very similar in both the developing rabbit and rat brain (Figs.3.3 - 3.6).However, the timing of the peak activity varied depending upon how the data was expressed. The peak specific activity (per mg protein) of the rabbit brain enzyme occurred at 4 days of age and of the rat brain enzyme occurred at 5 days. This is before the period of most active myelination and may account for the appearance of lipid droplets in the glial cells prior to myelin formation. However, the same data expressed in terms of mg DNA, whole brain or gm wet weight showed peak enzyme activity at 12 days of age which is during the period of most active myelination.

That rat brain fatty acid synthetase has peak activity during myelination is in direct conflict with the results of Volpe and Kishimoto (112).

From their results rat brain fatty acid synthetase activity (per gm wet weight) shows maximum activity during the neonatal period and there is a progressive fall to adult values. This is in contrast with the concept that most active myelination is associated with raised enzyme activity. However, this difference may be explained by the fact that their rats were of the Sprague-Dawley strain whereas the Sheffield University colony is Wistar strain.

Assuming that the brain weight of rats of both strains is comparable at all ages, then it is possible to express the results of Volpe and Kishimoto (112) in terms of fatty acid synthetase activity per brain (Fig.4.1 b). The brain weights shown in Fig.4.1 a were obtained from Wistar rats and were used in the conversion of activity /gm wet weight into activity/whole brain.

The shape of the curve is essentially that seen in Fig.3.5b except that peak activity appears to be at 18 days of age instead of 13 days and the values are consistently 4 fold higher.

Results expressed in terms of whole brain activities may, therefore, show changes in enzyme activity that are not apparent when the results are expressed per gm wet weight of tissue. The use of gm wet weight of tissue in expressing enzyme activity, introduces a further parameter into the calculations which, although negligible in the adult animal, is directly related to the change in brain weight with increasing age.

FIGURE 4.1

(a)

Increase in the brain weight of the developing Wistar rat. All values are the mean ± S.E.M. of all normal animals used and are plotted as gm wet weight against age.

(b)

The activity of fatty acid synthetase in the developing rat brain. The data of Volpe and Kishimoto (112) is re expressed in terms of nmol malonyl-CoA incorporated per brain per minute using the weights shown in Figure 4.1 (a)

(c)

The activity of acetoacetyl-CoA thiolase in the developing rat brain.
This figure is compled from the data of Buckley and Williamson (206) and re expressed in terms of nmol acetyl-CoA formed per brain per minute using the weights shown in Figure 4.1 (a).

Figure 4.1 Activity per brain: re-expression of data.

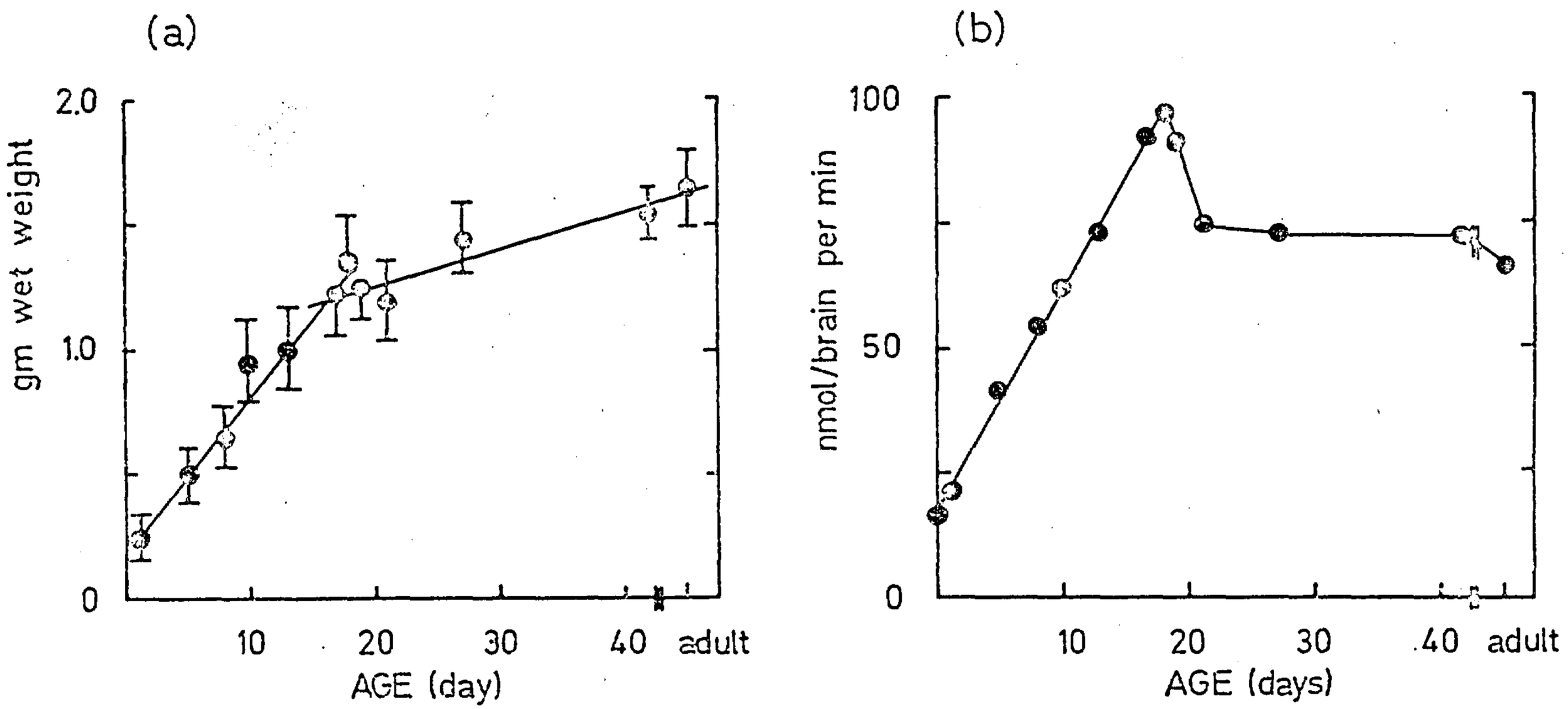
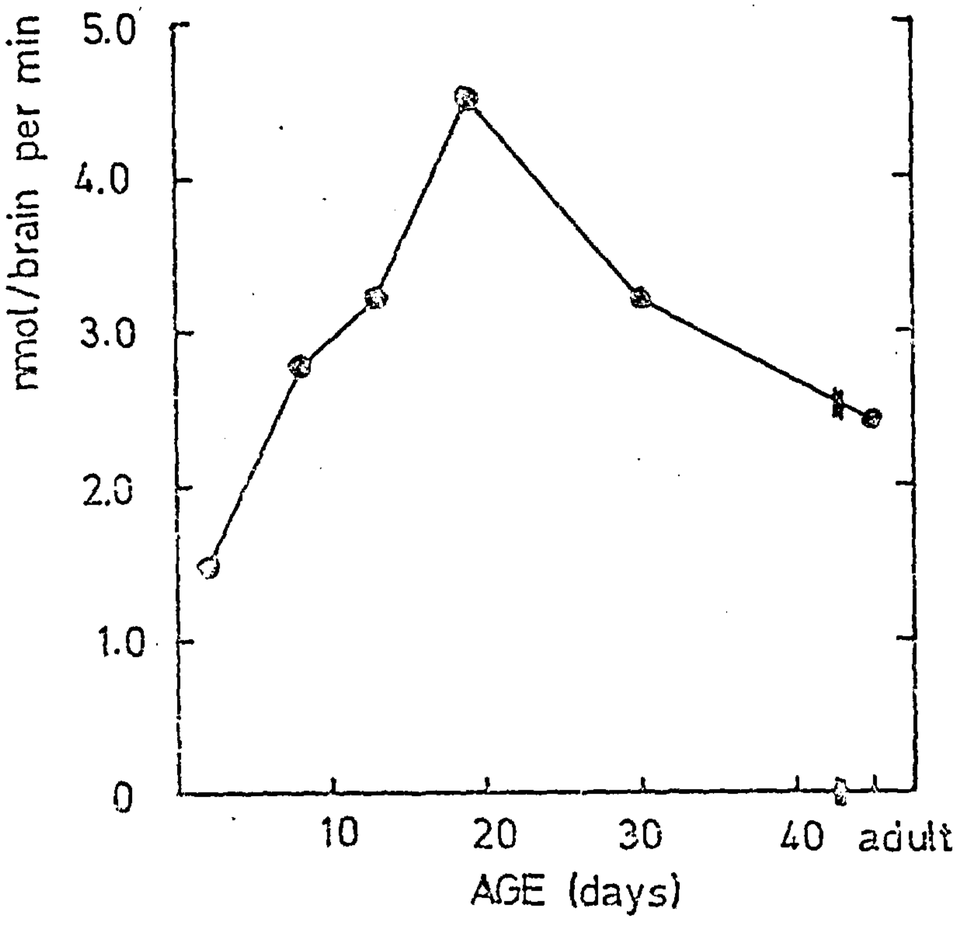


Figure 4.1 Activity per brain: re-expression of data.

(c)



Up to 13 days of age the rat brain weighs less than 1.0gm (Fig. 4.1a) whereas above this age the brain weighs more than 1.0gm and the rate of weight gain decreases. Volpe and Kishimoto (112) showed that the activity of rat brain fatty acid synthetase from animals fed a fat-free diet from birth to 65 days of age was 40% higher than the activity of the enzyme from the brain of rats fed a high-fat diet. This suggests that the uptake of circulatory fatty acids may be involved in the regulation of intracellular fatty acid synthesis in the normal developing brain.

The existence of fatty acid synthetase activity in the microsomal fraction of brain tissue has been discussed by several workers (152, 195, 196). Ammonium sulphate precipitates fatty acid synthetase from suspensions of both microsomes and soluble proteins between 30% and 50% saturation (195) and the enzyme can also be separated from crude microsomal material by sucrose density gradient centrifugation. The activity of the membrane-bound fatty acid synthesising system has been shown (196) to have a similar age-dependent activity profile as the soluble enzyme, indicating that the soluble enzyme could be trapped within membrane vesicles or physically associated with the membrane fraction. Therefore, it is possible for the fatty acid synthetase complex to be loosely attached to the microsomal membrane and liberated into the soluble fraction by homogenisation.

In an elegant study, Volpe et al (197) investigated the turnover of fatty acid synthetase using antibodies. The liver enzyme content varied depending upon the nutritional status of the animal, whereas the brain enzyme content did not change.

The changes in brain fatty acid synthetase activity (112) seen on different diets must, therefore, be due to changes in short-term control, possibly allosteric control by the cytoplasmic concentration of fatty acids or fatty acyl-CoA. The amounts of liver and brain enzyme are controlled by the rates of synthesis and degradation of the enzyme. In the brain, the half-life of the enzyme increases from 1.9 days in the young suckling rat to 6.4 days in the adult animal. The increase in brain activity during myelination may be due to an increase in the rate of enzyme synthesis since the rate of degradation is high, as measured by the half-life. Obviously, the turnover of the enzyme in the adult brain is maintained by a much lower rate of enzyme synthesis and a decrease in the rate of degradation. Volpe and Vagelos (198) also showed that the activity of brain fatty acid synthetase was not altered by the turnover of the 4-phosphopantetheine prosthetic group, although this moeity has a far higher turnover rate than the whole enzyme.

4.2.2 Palmitoyl-CoA-Synthetase

It is apparent from the results presented in section 3.2.3 that any discussion of the cellular role and activity of palmitoyl-CoA synthetase must, of necessity, include some comment on its location among the subcellular fractions and the composition of those fractions. Of the four fractions that were prepared by differential centrifugation (section 2.2.1) only fraction F_4 contains the soluble proteins from the cell cytoplasm but may also contain some very low density, small vesicles of unknown origin, which may be the cause of the palmitoyl-CoA synthetase activity in this fraction (table 3.5).

Fraction F_3 was designated the microsomal fraction. The composition of fractions F_1 and F_2 was shown by rate zonal centrifugation (Fig. 3.11) to be similar in the number of constituent bands but differing in the amounts of them. The three major bands appear to be microsomes, myelin and mitochondria (section 3.3.2.5). Thus, if enzyme activity is detected in either of these fractions (F_1 and F_2) its subcellular location must be ascertained.

The subcellular location of brain acyl-CoA synthetase was shown by Pande and Mead (149) to be microsomal and mitochondrial. Since they used a tissue fractionation procedure essentially the same as the one used in this study, the only fractions assayed for changes in palmitoyl-CoA synthetase activity in the developing rabbit brain (section 3.2.3.4) were the crude homogenate, the mitochondria-enriched fraction F_2 and the microsomal fraction F_3. However, it was apparent from the large discrepancy between the crude homogenate activity and that recovered from the fractions (Table 3.5)that a large proportion of the enzyme activity was lost during fractionation. Measurement of palmitoyl-CoA synthetase in all the subcellular fractions prepared from adult rat brain showed 96% recovery (table 3.5). However the 'crude' F_1 fraction contained 80% of the enzyme activity.

The discrepency between the activity in the homogenate and that recovered in the mitochondrial and microsomal fraction was also noted by Pande and Mead (149) who suggested a plasma membrane location for palmitoyl-CoA synthetase. Washing the 'crude' F_1 fraction and osmotic shock of the resultant F_1 fraction liberated large quantities of palmitoyl-CoA synthetase (Table 3.5) but a small residual amount of activity remained in the myelin band which could not be removed by further density gradient centrifugation of that fraction. (Table 3.6)

This residual activity may be associated with a membrane fraction which has a similar buoyant density to myelin, since purified myelin contains only limited enzymatic activity (52, 54, 199-201).

Both heart and skeletal muscle contain a species of palmitoyl-CoA synthetase which is sedimented in the slow nuclear fraction (148). This enzyme could be involved in the acylation of fatty acids diffusing through the plasma membrane in order to inhibit their leaving the cell. The presence of such an enzyme in brain tissue could play an important role in the control of uptake of circulatory fatty acids.

The role of palmitoyl-CoA synthetase in the microsomal fraction is obviously to convert fatty acids synthesised in the cytoplasm into CoA-derivatives for incorporation into phospholipids since this is the site of phospholipid synthesis and max enzyme activity occurs during myelination. The low level of fatty acid activation in the mitochondrial-enriched fraction (F_2) seen when the results were expressed per mg protein (Fig.3.9b)is in agreement with the theory that brain tissue mainly uses glucose as a source of energy and so, although capable of carrying out β-oxidation (134), this is not a major route of energy production in brain mitochondria. In contrast, the maximum whole brain activity of the enzyme in this fraction occurred during myelination. These results could be explained if fatty acids were being activated for elongation by the mitochondrial system prior to incorporation into newly synthesised membranes.

Much attention has been focussed on the microsomal fatty acid elongation system. Baumann et al (150-152) have identified two separate malonyl-CoA dependant elongation systems by ammonium sulphate precipitation. This method relies on the protein content of the

membranes comprising the microsomal fraction, whereas in the separation of palmitoyl-CoA synthetase activity in the microsomal fraction (F_3) by density gradient centrifugation (section 3.2.3.6), the inherent bouyant density of the membranes has been employed. Density gradient centrifugation has not been employed to differentiate between the elongation activity of heavy and light microsomal fractions or its spatial relationship with other lipogenic enzymes. The first peak of palmitoyl-CoA synthetase activity (fraction 4-7 Fig. 3.12b) corresponds to a region of palmitoyl-CoA dependent incorporation of malonyl-CoA into long-chain fatty acids (195). The second region (fractions 11-13) of activity corresponds to an area of palmitoyl-CoA independent malonyl-CoA incorporation into long-chain fatty acids. In these membranes the size of the endogenous fatty acid pool may be sufficient for elongation. However, the two regions may have distinct roles in the metabolism of fatty acids. The first (fractions 4-7) may be involved in the activation of <u>de novo</u> and exogenous fatty acids while the second region may be involved in the turnover of endogenous fatty acids and <u>de novo</u> synthesis.

The administration of propylthiouracil (0.01% w/v, in drinking water) to pregnant rats, after day 13 of gestation has been shown in other studies (202, 203) to cause hypothyroid progeny. In the present study, pregnant rats were given drinking water containing 0.02% propylthiouracil and the pups showed classical signs of hypothyroidism. Their behavioural and morphological development was retarded by one or two days. Although test rat pups were maintained hypothyroid by daily intraperitoneal injections of propylthiouracil, Clos et al (203) induced hypothyroidism in young rats solely by giving propylthiouracil to the mothers.

Since, in this study, no difference in brain and body weight (Fig.3.13 & 3.14) was detected between rats given propylthiouracil from birth (PTUa) and those deriving the inhibitor from the mother (PTUb) it can be concluded that the level of thyroid hormone in the young rat is either dependent upon the level of hormone in the maternal circulation and in the milk or that the potent inhibitor of thyroxine production, propylthiouracil, is secreted in the milk and controls the synthesis of thyroxine in the developing animal.

In the normal rat, plasma thyroxine levels increase gradually from birth to a peak value at approximately 16 days of age (202) and daily administration of propylthiouracil has been shown to depress this maximum (202). Plasma iodine concentrations (Table 3.8) show that there is a significant decrease in animals given propylthiouracil and thyroxine administration causes an increase. However, the plasma iodine concentration is a function of the dietary intake of iodine as well as the circulating levels of thyroxine.

Wysocki and Segal (202) showed that daily injections of 1.0µg tri-iodthyronine (T3) per gm body weight caused a 17% decrease in body weight and elevated the levels of UDP-galactose:sphingosine galactosyl-transferase and 2'3'nucleotide 3' phosphohydrolase in the rat spinal cord at 5 days. In the present study, there was also a 17% decrease in body weight after five days of thyroxine administration (control 10.5gm, test 8.7gm) together with an 11% decrease in brain weight. However, in this study, no change in the activity of palmitoyl-CoA synthetase was detected.

The only detectable change in palmitoyl-CoA synthetase activity was recorded in the mitochondrial-enriched F_2 fraction. In this fraction, the administration of propylthiouracil depressed the activity of the enzyme with respect to the control, whereas thyroxine had no effect.

Balazs et al (155) suggested that the species of mitochondria in nerve terminals were selectively affected in the brains of hypothyroid animals. This conclusion was reached following studies of the enzyme content of synaptosomal and free mitochondria. Although, in this study, no attempt was made to separate the two types of mitochondria, the suppression of palmitoyl-CoA synthetase activity could be explained by a decrease in the number of synaptosomal mitochondria.

Extending this premise, the high levels of fatty acid activation detectable in the F_2 fraction of the developing rabbit brain (Fig.3.10) may be due to the presence of palmitoyl-CoA synthetase in synaptosomal mitochondria. This species of mitochondria may utilise fatty acids as an energy source during suckling, although glucose is the major energy source of the mature brain.

Volpe and Kishimoto (112) showed that thyroxine, hydrocortisone and adrenalectomy had no effect on rat brain fatty acid synthetase, but hypothyroidism caused a decrease in the activity of the enzyme. The method of this control is unclear. Thus it appears that the hormonal control of brain fatty acid synthetase is different to that in the liver.

4.3 GENERAL DISCUSSION

In the development of the central nervous system, the period of maximum lipid accumulation correlates with active myelinogenesis (4, 27). Although there is considerable variation between mammalian species as to the timing of the period of most active myelination, in the rat, mouse and rabbit this occurs between 10 and 30 days after birth. Different regions of the brain commence myelin formation at different times, for example, myelin appears in the cerebellum before the cerebral cortex (204). Therefore in the developing brain there are temporal and anatomical differences in the deposition of cerebral lipids. The morphological differences probably reflect changes in the activity and control of those enzymes involved in myelin synthesis.

The use of whole brain homogenates, by definition, will only yield results that are the mean of all the regional activities of a particular enzyme. However, more material is available for separation into subcellular fractions so that fewer animals are required and the biological variation is minimised. Data for many enzymes of lipid and energy metabolismhave been collated and are presented in Fig.4.3 and Fig.4.4 . Before considering temporal changes in specific brain enzyme activity it is worth considering the results of experiments using brain slices.

Maximum synthesis of lipid from acetate occurs between 4 and 35 days in tissue slices (114 , 115 , 205). However, the synthesis of lipid from glucose (115 , 205) appears to precede that from acetate by several days and follows the increase in brain lipogenic enzyme activity (205).

FIGURE 4.2

Comparison of the activities of rabbit brain fatty acid synthetase and palmitoyl-CoA synthetase with the activities of other enzymes involved in fatty acid synthesis and energy metabolism in the brain at 20 days of age. The activity of fatty acid synthetase (number 8 in Figure 4.3) is shown in terms of nmol malonyl-CoA incorporated per mg protein per minute (- - - - -) and also nmol fatty acid synthesized per minute per mg protein (———). All data are converted to activity per mg protein per minute and the width of the bars represents the percentage homogenate protein. Three different ranges of activity are shown :

Key to enzyme number and source. For Figures 4.2 and 4.3

No.	Enzyme	Ref.
1	Acetyl-CoA carboxylase	113
2	Acetyl-CoA synthetase	213
3	Butyryl-CoA synthetase	213
4	Propionyl-CoA synthetase	213
5	Acetyl-CoA synthetase	208
6	Acetoacetyl-CoA synthetase	208
7	Acetoacetyl-CoA thiolase	208
8	Fatty acid synthetase (rabbit)	214
9	Fatty acid synthetase (mouse)	163
10	Fatty acid synthetase (rat)	112
11	Microsomal fatty acid synthesis (de novo)	196
12	Acyl-CoA synthetase	214
13	Fatty acid desaturase	209
14	Fatty acid desaturase	215
15	Fatty acid desaturase	216
16	Synthesis of long-chain fatty alcohols	217
17	Acyl-CoA hydrolase	211
18	Acyl-CoA elongation (C_{18}-C_{20})	163
19	Acyl-CoA elongation (C_{20} -)	218
20	Acyl-CoA elongation (lignoceric acid)	218

FIGURE 4.2 (Contd)

No.	Enzyme	Ref.
21	Acyl-CoA elongation (C_{16} - C_{18})	163
22	Acyl-CoA elongation (rat microsomes)	196
23	Hydroxylation of acyl groups	219
24	Squalene 2 (3) epoxide lanosterol cyclase	218
25	7 dehydrocholesterol reductase	220
26	Plasmalogen synthesis	210
27	UDP galactose : Diglyceride galactosyl transferase	221
28	UDP galactose : HFA ceramide transferase	221
29	UDP galactose : NFA ceramide transferase	221
30	Monogalactose diglyceride synthesis	222
31	Digalactose diglyceride synthesis	222
32	Sulphatide synthesis	223
33	Ceramide synthesis	211
34	Acylation of glycerol-3-phosphate	228
35	Glucose-6-phosphate dehydrogenase	224
36	Lactate dehydrogenase	224
37	Isocitrate dehydrogenase	225
38	Glycerol-3-phosphate dehydrogenase	226
39	Hexokinase	227
40	Aldolase	227
41	ATP citrate lyase	208
42	Pyruvate dehydrogenase	206

Figure 4.2 Comparison of the activities of lipogenic enzymes at 20 days

(nmol/mg protein per minute)

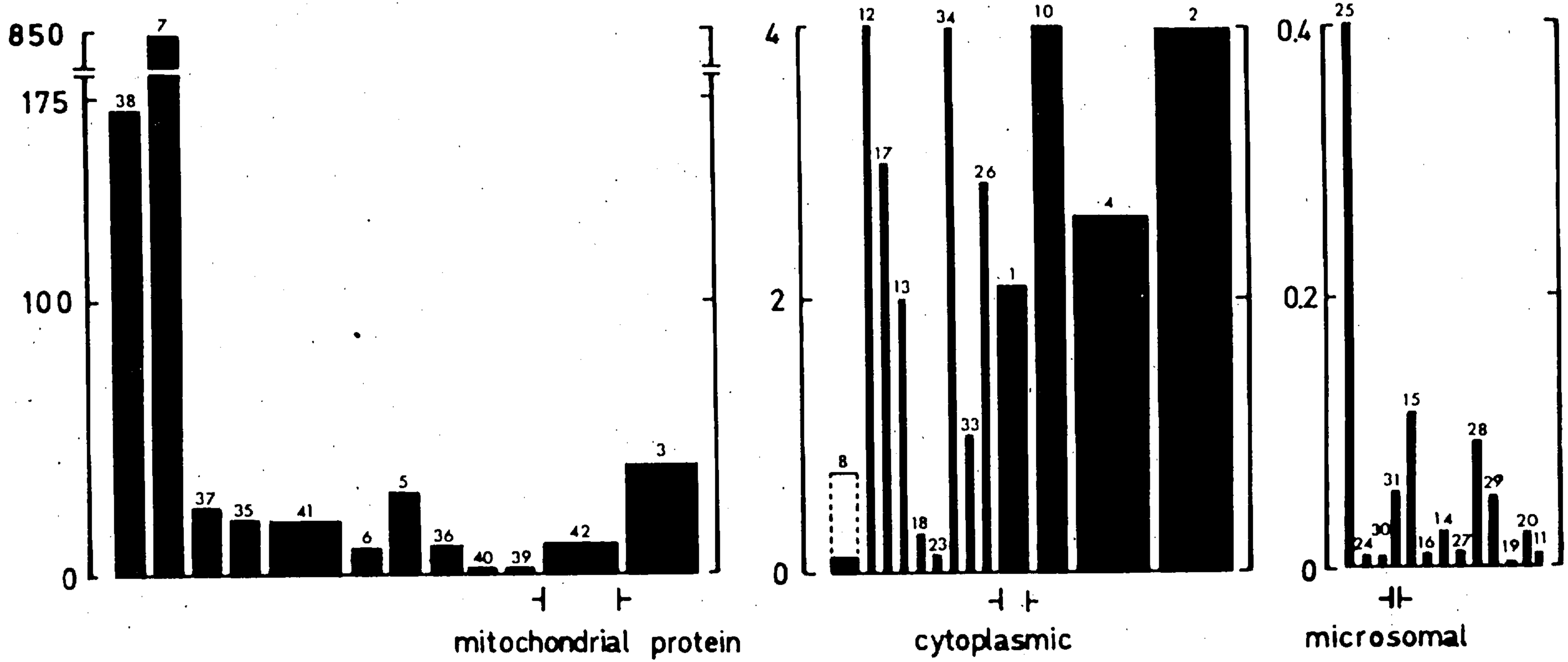
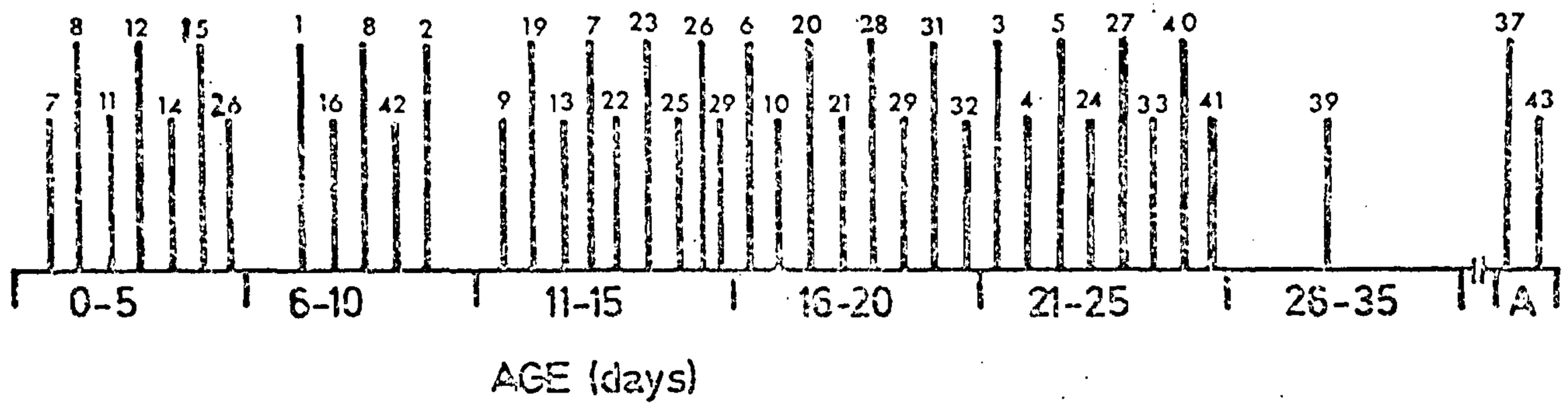


FIGURE 4.3

Peak activity of lipogenic and energy producing enzymes shown in days.

Enzymes are identified as in the key for Figure 4.2 and the time scale is in 5 day intervals from birth. All values are taken directly from the original data and no account is taken of the original mode of expression in compositing this figure.

Figure 4.3 Peak activity of lipogenic and energy-producing enzymes



Although the amount of acetyl-CoA formed from glucose may rise from birth to 20 days of age as a result of the increased activity of pyruvate dehydrogenase (206), Carey (115) showed that the amount of glucose converted to lipid in rabbit brain cortical slices decreased with age as the amount of glucose oxidised to CO_2 increased. Also, Patel and Owen (207) showed that maximum incorporation of ketone bodies into rat brain slices occurred 7 days after birth and the rate of lipid formation from this source was equal to the maximum rates of the constituent component enzymes as measured by Buckley and Williamson (208). It is apparent that the rate of ketone body metabolism in brain slices is the same as the rates of enzyme activity measured in tissue homogenates and subcellular fractions.

In Fig.4.3 the activities of rabbit brain fatty acid synthetase and palmitoyl-CoA synthetase are compared with the activities of various brain enzymes involved in lipogenesis. Values are expressed in terms of nmol/mg protein per minute at 20 days of age. Since, in the original papers, enzyme activity was expressed in several different ways, the conversion to activity per mg protein per minute obviously involved the application of correction factors. It was assumed, in the calculations, that the protein content of the different sub-cellular fractions was the same as determined in the present study. Furthermore, in compiling Figs. 4.2 and 4.3 , the various enzymes were grouped according to their functions. Enzymes 1-7 are involved in the generation of precursors for fatty acid synthesis. Enzymes 8-23 are involved in the synthesis, activation and further modification of fatty acids. Enzymes 24-34 are involved in cholesterol and phospholipid synthesis and enzymes 35-42 are involved in energy metabolism and the production of reduced NADP for lipid synthesis.

It is apparent that the cytoplasmic enzymes involved in glucose oxidation and $NADPH_2$ production have the highest activities and therefore, the generation of energy is not a rate limiting factor in the synthesis of cerebral lipids. The enzymes involved in the generation of precursors for fatty acid synthesis (No's 1-7 Fig. 4.2) also have high activities indicating that fatty acid synthesis may be regulated by acetyl-CoA carboxylase or fatty acid synthetase and not by the availability of suitable precursors. In the present study, the activities of rat and rabbit brain fatty acid synthetase are very similar at 20 days (0.75nmol/mg protein/min) and are considerably lower than those measured by Volpe and Kishimoto (112)(4.1 nmol/mg protein/min). Furthermore, the values available for the activity of acetyl-CoA carboxylase at this time are in the region of 2.1nmol/mg protein/min (113, 205); thus, acetyl-CoA carboxylase may not be the rate limiting enzyme of fatty acid synthesis since the difference in activity between the two enzymes is small. The microsomal enzymes, palmitoyl-CoA synthetase, fatty acid desaturase (209), glycerol-3-phosphate acyl transferase (210) sphingosine acyl transferase (211) and of plasmalogen synthesis (212) all have higher levels of enzyme activity than rabbit brain fatty acid synthetase. The other enzymes quoted in Fig. 4.2 all have lower activies than fatty acid synthetase and are involved in complex lipid formation. Therefore, the activity fatty acid synthetase must regulate , the flow of <u>de novo</u> synthesized fatty acids into the brain pool where they are modified and then incorporated into phospholipids. Furthermore, the total activity of endogenous palmitate synthesis is 100 times greater than that of the microsomal elongation system. In terms of acyl group formation and activation, the specific activity of the microsomal palmitoyl-CoA synthetase is 50 times higher than that of fatty acid synthetase.

Taking into account the relative distribution of protein between fractions F_3 and F_4, the whole brain activity of microsomal palmitoyl-CoA synthetase is eight times that of fatty acid synthetase. Since palmitoyl-CoA synthetase is also present in the mitochondria-enriched fraction (F_2) and membrane fraction (F_1) and only 15% of the whole brain activity is found in the microsomal fraction (Table 3.5) then there is at least a 50 fold excess of palmitoyl-CoA synthetase activity over that of fatty acid synthetase.

In the rabbit brain, the incorporation of fatty acids into cerebral lipids in vivo during the period of maximum lipid deposition is approximately 10mg/day (24). This value is at least three times higher than the amount of fatty acid that can be synthesised by fatty acid synthetase per 24 hours in the whole brain. It is obvious, therefore, that the developing brain is dependent upon exogenous fatty acid during the period of maximum myelination and palmitoyl-CoA synthetase is necessary to activate any fatty acid taken up from the circulation. Since it has been suggested that acyl-CoA synthetase may also have a plasma membrane location (149) it would be ideally situated to be intimately involved in the activation of extracerebral fatty acids and would stop a two-way diffusion of fatty acids across the plasma membrane by forming the CoA-derivative.

Fig.4.3 shows diagrammatically the day at which the enzymes considered in Fig. 4.2 show peak activity. The majority of the enzymes show peak activity between 10 and 25 days. Since no attempt was made to standardise the methods of data expression, it is possible that some of the enzymes would show peak activity at other times.

However, the effect of expressing the results of Volpe and Kishimoto (112) for rat brain fatty acid synthetase in terms of whole brain activity instead of gm wet weight was to move peak activity from birth to 13 days of age. Similarly, the results of Buckley and Williamson (209) for acetoacetyl-CoA thiolase (No.7 Fig. 4.2) when expressed in terms of whole brain activity (Fig. 4.1c) also show peak activity at 18 days of age. Thus, it is possible that if maximum whole brain activity were plotted against age then the number of enzymes showing peak activity during the period of most active myelination, between 10 and 25 days after birth, would be even greater.

The relationship of the enzymes involved in the catabolism of phospholipids is not well understood but they may be specifically involved in the maintainence of the composition of membrane lipids and the regulation of metabolic pools which must exist in brain tissue.

The role of palmitoyl-CoA synthetase in the mitochondria-enriched fraction is not clear since, although brain mitochondria are capable of oxidising fatty acid, the measured RQ for brain tissues is almost 1 indicating carbohydrate rather than lipid as the major energy source. However, acetyl-CoA dependent fatty acid elongation takes place in this fraction and palmitoyl-CoA synthetase could be involved in the activation of fatty acids prior to elongation by this system.

The synthesis and turnover of myelin lipids is a very complex process; however, the increase in activity of fatty acid synthetase and palmitoyl-CoA synthetase in the rat and rabbit brain, together with other lipogenic enzymes during myelination (Fig. 4.3) indicate that this process is under very strict control.

Hormones would be the obvious controlling factors, and especially thyroid hormones since the highest concentrations of thyroxine are found during the period of most active myelination. However, the administration of thyroxine does not increase the activity of fatty acid synthetase (112) or palmitoyl-CoA synthetase in the developing rat brain. Therefore, the role of this hormone in the control of fatty acid synthesis and activation of fatty acids, may not be direct but may be involved in the rates of turnover of these enzyme systems and thus control their activity by controlling the amounts present in the cell. The incorporation of labelled fatty acid into subcellular particles also does not appear simple since all fractions become labelled and only myelin lipids appeared to accumulate label. However, several different membrane particles may be involved in the formation of a single phospholipid molecule and the transfer of a labelled moeity by cytoplasmic carrier-molecules could be responsible for the general labelling of subcellular fractions resulting in no obvious time course of incorporation.

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