C H A P T E R I

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION AND LITERATURE REVIEW

Contd.....

.....

Tell me where is fancy bred,
or in the heart or in the head?
How begot, how nourished?

..... Merchant of Venice
III, ii, 64.

The search for the answers for the above questions asked by Shakespeare was "fancied" much earlier in the history of mankind. Brain and its function intrigued the probing mind of man for number of centuries. Since the pioneering attempts of Thudichisum, the father of neuro-chemistry, we have come a long way in unfolding the mystery of brain-composition and function. But this is not to say that we have come to an end of the search for complete understanding of this unique tissue, the Brain. Our knowledge of physiological, biochemical and electrical processes taking place in the brain is far from complete. The knowledge of exact mode of nourishment to the development and functioning of the central nervous system still remains partly incomplete. The relationship between chemical and electrical processes; their effect on the structure and function of the CNS and above all the behavioural correlates are some of the vexing themes of present day research programmes.

The effects of various stress conditions on the brain function have attracted good deal of attention from large number of researchers. Nutritional stress occupies a special position due to the vast multitude of children being subjected to early undernutrition and malnutrition associated with mental subnormality.

Estimates of the current magnitude of the world hunger problem depend upon the criteria used, and vary from the conservative WHO figure of 500 million persons moderately to severely malnourished (FAO/WHO, 1976) to a World Bank estimate (Reutlinger and Selowsky, 1976) of over a billion persons suffering under-or malnutrition to a degree influencing growth and development or health. In India alone, approximately 35 per cent of all children between 1 and 5 years of age are classified as moderately or severely undernourished. The ominous prospect of some 300 to 500 million malnourished children growing up to become brain-damaged adults, who in term, will parent another deprived, mentally subnormal generation is a real one in every respect.

There is no question that severe malnutrition during early life is associated with varying degrees of cerebral degeneration and mental subnormality. To what degree the cerebral inadequacy is causally related to protein energy malnutrition and its various associated disturbances

remains to be determined. However, a large body of accumulated evidence suggests that longer and more severe the malnutrition during the first two years of life, more serious the effects will be on subsequent intellectual development. Thus, the potential for reversibility of the defects is drastically curtailed if the malnutrition overlaps the brain growth phase in early life.

Since the adequate nutrition is recognized to be necessary for the structural development of the brain in early life, the past two decades have witnessed the conduct of several studies with experimental animals on the effects of undernutrition in early life. Most of these studies have been concerned with parameters which reflect structural development such as brain weight, DNA, RNA, protein and cholesterol. The studies concerning protein deficiency in early life are few in number; that too those with emphasis on the enzyme systems involved in the neuroregulators are still much limited and scattered. Same is true for the deficiencies of individual vitamins.

In the subsequent pages of this Chapter an attempt has been made to present, in brief, the data available on the morphological and biochemical development of the brain, its vulnerability to nutritional insults both in experimental animals and actual case studies of human population, the

detailed discussion of the individual components of the cholinergic system and its vulnerability to different stress conditions.

1. Morphological Development of the Brain :

The key role of the central nervous system in the orchestration of the growth, metabolism, regulation and function of the living organism is very well known. The anatomical and metabolic heterogenity of the brain is still not clearly understood. Another dimension to this heterogenity is the spatial heterogenity. The growth of different anatomical structures, the development of different metabolic patterns and the spacing of the behavioral patterns have been known to be sequential processes. The complexity of the relationship between the anatomical, metabolic and chemical compartments of the developing brain is often changing until the matured adult stage is reached. This change is a result of many growth processes reaching their optimum activity and falling away at different times during the development of the brain.

In view of the fact that, the brain plays such a vital role in the living system, it is not surprising that its development is initiated and to some extent completed earlier than that of many other organs (Winick et al.,1972).

The development of CNS commences with the formation of neural tube in the embryonic stage. This is followed by the development of the brain regions from the anterior part of the tube. The embryonic cells lining the lumen of the tube subsequently give rise to glia and neurons of the CNS (Altman, 1969).

The development of the brain is characterised in the initial phase by organogenesis and neural multiplication. This is followed by the "growth spurt" characterised by axonal and dendritic growth, glial multiplication and growth in size. This sequence can also be described briefly in the following way : The initial event is the cell division, i.e., hyperplasia; the next event is the cell enlargement in addition to cell division, i.e., hyperplasia and hypertrophy; the final event is the completion of cell division and only continuation of cell growth, hypertrophy, till the maturity of the brain is complete (Winick and Noble, 1966).

The different stages of the brain development have been arbitrarily defined by various workers (Flexner, 1955; Oja, 1966; and Davison and Dobbing, 1968). The stages based upon developmental progress, in a particular species, will avoid the overlapping of the arbitrary definitions of the different stages. The above mentioned morphological development can best be summarised on the stages suggested by

Davison and Dobbing (1968).

Stage I : Organogenesis and neuronal multiplication.

Stage II : The brain "growth spurt" including -

a) a maturation period of axonal and dendritic growth, glial multiplication and myelination,

b) a later, but overlapping period of growth in size.

Stage III : The mature adult stage.

Stage IV : The senile regression.

These stages occur at different chronological ages in different species. These stages, as they occur in man and rat, are outlined in Table 1.

The brain consists of many anatomical, morphological and functional compartments. The pattern of maturation and growth varies from region to region. The sequence of events taking place remains essentially same but the spatial heterogenity in the development of these regions differ. With this difference the complexity of defining the growth peaks or the optimal concentration of a particular parameter increases many-folds, particularly the values expressed on the basis of the whole brain estimates tend to confuse the true picture of the development of a particular region.

Table- 1 : Critical phases in the developing brain*

Developmental Features	Age at manifestation or completion	
	Man	Rat
Multiplication of neurons and astroglial cells	26th fetal week	Until birth
Multiplication of glial cells and micro-neurons.	26th fetal week to birth	Birth to 10th day of age
Out growth of dendrites and axons and synaptic formation	-	-
Continued synapto-genesis oligodendro-glial proliferation, onset of myelination	Birth to 6th month	11-21 days
Further myelination and development	After 6 months	After 21 days
Adult EEG patterns	10 years	14-16 days

* Caley and Maxwell (1968); Svennerholm (1974); Lindsley (1974).

The DNA concentration per diploid nuclei remains same in all cells including that of brain. The rate of proliferation of cells or the number of cells at a particular period during maturation of the brain can be quantified as per the concentration of DNA in a particular region or the whole brain. DNA per gram of wet weight of the region or the whole brain is a convenient index to express the cell population (cellularity). The cell size is indicated by the ratio of protein : DNA and RNA : DNA (Davison and Dobbing, 1968).

The DNA content of the brain increases to optimal level in about 3 weeks postnatally in rats (Winick, 1968) and one year in man (Altman, 1969). The completion of high rate of DNA synthesis takes place first in stem, followed by cerebellum and then in cortex (Fish and Winick, 1969). The peak levels of DNA in the cortex reach at 16 days in mice (Himwich, 1962), at 7 days in guinea pigs (Dobbing and Sands, 1970) and during 6 to 8 months in man (Winick et al., 1970). Two different peaks of DNA synthesis have been reported in rats (Brasel et al., 1970). The rate of DNA synthesis in different areas of the brain is different and this contributes much to the spatial heterogenity (Fish and Winick, 1969). Once the mitotic division of the brain cells is levelled off to a stable adult stage as indicated by insignificant rise in the DNA levels the neuronal multiplication is largely accomplished.

The sequence of developmental events is not so clearly marked out in the human brain. Neuronal multiplication is accomplished by 18 weeks according to Dobbing and Sands (1973) and by 30 weeks according to Robinson and Tizard (1966). Winick (1968) reported that the total cell number of the brain is accomplished by 8 or 12 months after birth. Mandel et al. (1964) place this period around 12 months. Moreover, Dobbing and Sands (1970) have shown that two peaks of DNA synthesis may occur normally in the human brain. The first peak is reached around 18 weeks of gestation and the second around birth. These two periods have been interpreted to correspond to the peak rate of neuronal division and the peak rate of glial multiplication. Subba Rao and Janardana Sarma (1972) have also reported the increased rate of DNA synthesis between 13 and 25 weeks of gestation, followed by lag phase between 22 and 34 weeks and then a sharp increase from 34 weeks onwards till birth. According to Dobbing and Sands (1973) the cell division in the cerebellum begins little late as compared to cerebrum and brain stem but finishes earlier. Adult cell number is achieved in the cerebellum around the age of 18 postnatal months, at a time when the forebrain and stem have only 60% of the same. The cell division in the cerebrum and cerebellum levels off between 12 and 15 months of age (Winick et al., 1970). In the brain stem, DNA synthesis continues at a slow but steady rate until at least upto 1 year of age.

As earlier mentioned, the different species are born at differing stages of maturity. It is not difficult to express this as different timing of the brain growth spurt in relation to birth. Thus, the rat (Dobbing and Sands, 1970) is characterized as a postnatal, the guinea-pig (Dobbing and Sands, 1970), a prenatal and the pig (Dickerson et al., 1967) as a perinatal brain developer. Earlier it was thought that human brain resembles pig in this respect based on the observations of Winick (1968). But the studies of Dobbing and Sands (1973) reveals that the 5/6th of the human brain growth spurt is postnatal and in this respect humans resembles rats much more closely.

The neuronal multiplication is followed by the increase in neuronal size which is associated with the growth of axons and dendrites and a tremendous increase in the complexity of the latter. Synaptic connections with the assymetrical membrane thickenings increase in number during the postnatal maturation of neocortex in the rat. These connections represent a "mature" form. After 10 days the synapses found are indistinguishable from those found in adult cortex. At the same time the dendritic cytoplasm is also well-developed, containing ribosomes, motochondria, the endoplasmic reticulum and a dense background matrix (Caley and Maxwell, 1971). In the human cerebral cortex dendritic growth is optimum from the later

fetal period to about one year of age and continues at slower rate for several years thereafter (Schade and Groenigen, 1961).

The characteristics that distinguish a mature neuron from other cells are its abilities to propogate an action potential and to make special contacts, through the synapses, with other neurons. The precise timings of the initiation of development of such properties in the neuronal membranes is not known. It may begin just after the mitotic division as in the case of motor neurons of chick spinal cord, in which axonal growth begins while the cell is migrating out of the germinal layer whereas in the retinal ganglion cells, this process does not take place until the neuron is placed in its adult position and has grown in size (Jacobson, 1970). The maturing neuron exhibits (a) decreasing numbers of microtubules and increasing number of microfilaments in the cytoplasm; (b) shift from multiple nucleoli to one large nucleolus, accompanied by an increase in nucleolar RNA; (c) decreasing amounts of free ribosomes and increasing amounts of rough endoplasmic reticulum and the smooth membrane bounded cisternae of the golgi apparatus; and (d) growth in size which is indicated by the increased ratio of protein : DNA (Jacobson, 1970; La Velle and La Velle, 1970; and Caley and Maxwell, 1968).

Axonal and dendritic growth is accompanied by rapid multiplication of oligodendritic cells. Macroglia, with neuroectodermal origin, initially proliferate in ventricular zone. After cessation of active cell division in this region glioblasts migrate into surrounding nervous tissue and continue to divide in situ (Jacobson, 1970). The appearance of astrocytes precedes that of oligodendrocytes. The appearance of oligodendrites and subsequently myelin proceeds in caudocranial direction. As the rate of cell proliferation deceases, RNA content and protein synthesis increase and lipid droplets appear in the oligodendroglia. The progressive deposition takes place which represents about 75% of the dry weight of myelin.

The composition of the myelin is characteristic. The largest lipid component is cholesterol but significant amounts of ethanolamine phosphotides and galactolipids are also present. The characteristic composition of myelin lipids shows a molar ratio of cholesterol : phospholipid : cerebrocide of 4:3:2 . In the rat brain, the rates of increase in the myelin lipids are maximum during the second and third postnatal week (Davison and Dobbing, 1968; Norton and Poduslo, 1973). But the increase in the concentration continues till adult age. In man, myelination progresses at a much faster rate from 7 months of gestation onwards and continues till 4-5 years of age according to Kokaady et al. (1971) and Dobbing and Sands (1973). In the earlier

studies this was believed to take place till a much later age of 10 years (Brante, 1949; Tingeyn, 1956; Cummings et al., 1958) or over adolescence (Davison and Dobbing, 1966).

The recognisable pattern of electrical activity emerges during the period of myelination. The evoked cortical responses can be very well correlated with the degree of maturation of the central nervous system. The immature cortical tissue elicites the evoked cortical response which is characterized by an initial negative deflection whereas the matured CNS elicites an initial positive response. According to Rosen (1971) the rats exhibit important change at 14 days of age, i.e., decreased latencies and the appearance of all components of the evoked response. In human brain similar observations have been reported at the post-conceptual age of 37 to 45 weeks which resemble the adult brain (Regan, 1972).

The regulation of overall growth of the organism is far from well understood. And the development of CNS in particular is believed to be regulated in more complex manner. During specific critical periods of the development, hormones may act directly on the CNS to produce permanent changes. A particular hormone may have different action at different times. Most hormones produce changes when administered in the prenatal or immediate postnatal period

(Balázs and Richter, 1973). These workers reported that the administration of testosterone to new born rats causes male sexual behaviour at maturity and the absence of the hormone causes female sexual behaviour. The action of this hormone is localized in the hypothalamus. The rats made hypothyroid at birth showed decreased RNA and protein content in cerebrum and cerebellum but the DNA content was not affected at 35 days of age suggesting reduction only in the cell size and not in the total number of cells (Balázs, 1971). The same studies also revealed that the administration of tri-iodothyronine at birth caused reduction in the number of cells in cerebellum and cerebrum. Thyroid hormone deficiency from birth has been shown to retard myelinization and the development of neuropil and decrease the respiratory activity of the brain (Hamburg, 1968). A number of other hormones have widespread effects on the developing nervous system. Corticosteroids appear to have selective effects on the timing of maturation of the brain, they reduce dendritic branching transiently and probably decrease the myelin formation (Howard and Benjamins, 1975). Most of these effects are mediated through cAMP. Some of the enzymes like cytoplasmic glycerol phosphate dehydrogenase are induced by glucocorticoids, both *in vivo* and *in vitro* tissue culture studies (De Vellis and Brooker, 1973).

In addition to the hormones, nerve growth factor (NGF) has been shown to have direct effect on the brain maturation. NGF is required for survival and maturation of peripheral, sensory and symphathetic neurons (Angeletti et al., 1973). It promotes regeneration of axons from lesioned adrenergic neurons (Bjerre et al., 1973).

2. Biochemical Development of the Brain :

The structural and functional changes taking place during the development of the brain are associated with several changes in chemical composition and metabolism. As earlier mentioned, the estimation of such substances as DNA, protein or cholesterol can provide invaluable index of morphological development. It is now possible to relate such information to the changing chemistry of the developing brain.

The main energy source of the brain is glucose. In the fetal brain, glucose is mainly catabolized through the glycolytic pathway to pyruvate or lactate with comparatively little energy production. As the brain develops, the oxygen consumption increases and this necessiates the complete oxidation of glucose to carbon dioxide and water. This change is very well reflected in the enzyme activities of the developing brain. Throughout the postnatal period there is relatively very little elevation in the activities

of the glycolytic enzymes in rat brain. During the first few days after birth there is a very little increase in the activities of the TCA cycle enzymes and after 10 days there is a steady rise in the activities of these enzymes reaching maximum at about 40 days in rats (Laatsch, 1962). An exceptional pattern is shown by isocitrate dehydrogenase, the activity of which is maximum at birth and decreases to about 30% of this activity in the adult (Robins and Lowe, 1961). This may be due to the fact that this enzyme utilizes NADP as coenzyme and the reduced form of this coenzyme is required for the synthetic requirements of the developing brain. In general, the activity of glycolytic enzymes undergoes much smaller change in the various cortical layers than those of the respiratory enzymes during the period of development. During the most rapid phase of lipid synthesis, glucose is metabolised in much greater proportions through the hexose-monophosphate shunt and there after a small proportion passes through this pathway which favours both nucleic acid and lipid synthesis. For the former it supplies ribose nucleus and for the latter it generates, as mentioned earlier, NADPH (Winick, 1970).

It is evident that the characteristic features of the carbohydrate metabolism of adult brain develop relatively slowly and are not present in the initial stages

of brain development. This change does not take place uniformly throughout the brain but commences at the spinal cord and eventually reaches the cerebral cortex. The relative importance of brain respiration for energy supply to the brain at different ages presumably determines relative vulnerability at different ages to anoxia. Thus, rats survive 50 minutes in nitrogen on their day of birth but only 3 or 4 minutes from 11 days onward (McIlwain, 1966). Narcotics such as pentobarbital, and ethanol exercise a larger percentage depression of respiration in the adult than the young rat brain (Himwich et al., 1941). A significant positive correlation has been found between the rate of respiration at different ages and sensitivity to barbiturates (Desbrats-Schonbaum and Birmingham, 1959). A similar correlation between age and the increased response, in the form of respiration, by brain tissue to electrical stimulation has been reported by Greengard and McIlwain (1955).

Another interesting feature of the immature fetal brain is the ability to utilise ketone bodies like β-hydroxybutyrate and acetoacetate (Smith et al.,1969 ; Toth and Quastel, 1970; Hawkins et al., 1971). Although the adult brain seems to retain the limited capacity to oxidise the ketone bodies, under the acute conditions of nonavailability of glucose for normal respiration, the

activity of the enzyme, β -hydroxybutyrate dehydrogenase, has been reported to decrease to 50% of the activity at birth (McIlwain, 1966; Cremer, 1971).

The composition of the fetal brain lipids is similar in many respects with that of many other tissues but as the brain maturation proceeds the composition distinctly changes. The relatively high content of glycosphingolipids, i.e., cerebrocides, sulphatides and gangliosides, is a characteristic feature of the matured nervous tissue. Gangliosides are predominantly located within neurones (Lodwen and Wolfe, 1964); whereas, the cerebrocides and sulphatides predominate in myelin. Another category of lipids is the phospholipids which are mainly located in the myelin and account for 20 to 25 % of the dry weight of mammalian brain. Phospholipids are the compounds in which phosphatidic acid is combined with a base and include the following kinds of lipids : lecithin (phosphatidylcholine), cephalin (phosphatidylethanolamine), lipositols (phosphatidylinositol), phosphatidylserine, plasmalogens and spingomyelin. Cholesterol is the only sterol present in appreciable amounts in the adult nervous system. In the earlier stages of development, however, demosterol and zymosterol have been reported (Holstein et al., 1966; Kritchevsky and Holmes, 1962). These appear to be the precursors of cholesterol. Although most

of the cholesterol in the nervous system is in free form, fatty acid esters of cholesterol are also present, especially in the infant brain in both rats (Adams and Davison, 1959) and man (Ramsey and Davison, 1974). Fatty acids are mainly present in the form of esters (Svennerholm, 1968) and only trace amounts of free acids are present.

In the rats, cholesterol concentration increases in both grey and white matter and in the cerebellum from early fetal life (Howard and Granoff, 1968). In man, cholesterol concentration of the whole brain is about 2.4% in fetuses of less than two months of postconceptual age and rises to 5.4 % at term, 6.6 % at nine months and 10.7 % in adult brain (Brante, 1949). This is in agreement with the changes observed in the total lipids in the brain. A very little change occurs during the first seven months of gestation in the concentration of total lipids in either grey or white matter. Thereafter, lipid deposition proceeds rapidly in the grey matter and adult concentrations are reached at about three months of age. In the white matter, which accounts for the most of the actual myelination, lipid deposition is less rapid but continues for a much longer period. Ninety per cent of adult values for total lipid are achieved by 2 years of age and adult values by ten years of age (Brante, 1949; Cummings et al., 1958; Tingeyn, 1956).

The concentration of cerebrocides also appears to increase during development although extensive data on the same are not available during development in white matter. All phospholipid fractions increase during development. Phosphotides show little increase after seven months of gestation. In contrast, cephalin concentration increases from 7 % at 4 months gestation to adult value of 12 % in grey matter and 17 % in white matter by 3 months. Conde et al. (1974) have found that the neutral glycosphingolipids, sulphatides and sphingomyelin to be present at 22 weeks and to show a marked increase after 32 weeks. In another studies ganglioside concentration was found to increase approximately 3-folds in the cerebral cortex from the 15th fetal week to the age of about 6 months (Vanier et al.,1971).

Although the chemical composition of the brain undergoes very little change after maturity this can not be considered as a total state of metabolic inertia as has been demonstrated by studies of the incorporation of labelled precursors such as glucose and amino acids (Lajatha et al., 1959; Berl et al., 1961). Since the lipids constitute a major component of the brain, it has led to several studies on the turnover rates of the same using the incorporation of precursors such as radioactive sulphatides (Davison and Greson, 1966), ^{14}C-acetate into fatty acids (Dhopeshwarkar et al., 1969) and ^{32}P into various phospholipids (Mandel and Nussbaum, 1966). Acetate - 15 seconds - Y cholest in Adult Rats (BBA 246, 41, (1971))

The protein content of the brain increases from 5% at birth to 10 % of the wet weight in the 30 day old rat. Thereafter there is an increase in absolute content of brain protein ending at about 50 days of age after which the brain weight alone increases slowly. There is some evidence to suggest that the chemical composition of the brain proteins changes during the development of the brain (Prensky and Mosher, 1967; Wender and Waligora,1964). The characteristic acidic protein of the brain appears at ten days of age and finally comprises of 0.5 to 1.0 % of the total soluble proteins of rat brain (Ramussen, 1966).

The brain has a high concentration of free amino acids such as glutamic acid, aspartic acid and glutamine. In the adult brain a significant proportion of the same is derived from glucose and this accounts, partly, for the considerably higher glucose utilization of the brain. In the fetal and neonatal brain the rapid flux of glucose to amino acids is not yet present. In the rats this appears about 10 to 21 days after birth when the cerebral cortex becomes functionally mature (Gaitonde and Richter, 1966) and adult levels of glutamic acid and glutamine are attained at 21 and 14 days of age respectively. Gamma amino butyric acid and aspartic acid reach adult levels at about 30 days (Agrawal et al., 1966; Bayer and McMurry, 1967).

Incorporation of labelled amino acids into brain proteins both *in vitro* and *in vivo* are faster in young animals than in adults (Gaitonde and Richter, 1956). The reduced incorporation of labelled amino acids into brain proteins with increasing age has been reported by Johnson and Luttges (1966). The, mean turnover rate of mixed brain proteins has a half life of about 20 days in adult animals as compared to the halflife of 3 days in very young animals (Gaitonde and Richter, 1956; Lajtha, 1964).

3. Vulnerability of the Developing Brain :

I) Animal Studies :

a) Undernutrition and Protein Deficiency :

The developing organism is vulnerable to various external stress conditions; the times at which it is more vulnerable depends upon the stage of maturation of the particular tissue which is the target of the external stress. This concept forms the basic foundation of modern subject of teratology. The precise timing of exposure of the organism to the particular stress correlates well with the key developmental events. The damage to the anatomical structure of a particular tissue could be instituted at a time when early stages of organogenesis are taking place. Such a damage taking place during this particular period

has been shown using various agents like viruses, vitamin imbalances, drugs and various forms of irradiations.

The developing brain is not an exception to the damage caused by various agents such as drugs, virus, toxins, nutritional imbalances and environmental deprivation. This period of vulnerability in the brain is placed at the time of fastest growth. In terms of whole brain this period coincides with that of "growth spurt" described earlier. The different regions and metabolic sequences of the brain are naturally likely to be affected differently at a particular stage of growth spurt because of the fact that each subcomponent of the brain has a different rate of growth in relation to overall rate of the brain growth timings. Hence each subcomponent of the brain may have its own particular timing during maturation at which it is likely to be more vulnerable to the stress.

Since the brain has already acquired most of its definitive shape before the growth spurt begins, it is to be expected that the stress or the insult at this later stage will not produce any gross anatomical deformity. The effects of the insult will be restriction of the growth and this would result in certain deficits in the brain. In other words, if this assumption has any validity the net result will be a smaller brain consisting of less than the

normal complement of anatomical and chemical constituents and perhaps even with some distortion of their normal pattern. The same may be said to be true for the behavioural development which would indicate deficits in the behavioural parameters with some variation in normal pattern.

Dr. Dobbing and his group who are the pioneers of such studies have aptly put the hypothesis of vulnerability in the following words : "In its simplest form the hypothesis (of vulnerability) states that if a developmental process be restricted by any agency at the time of its fastest rate, not only will this delay the process, but will restrict its ultimate extent, even when the restricting influence is removed and the fullest possible rehabilitation obtained. There are two important corollaries to the hypothesis. Firstly, the severity of the restriction required to produce a given ultimate deficit will be less, the nearer it is applied to the time of fastest growth. The same effects may be achieved later in development, but only much greater restriction over a much longer period of time. The second corollary is that such effects should not be achievable in the adult" (Dobbing, 1968a).

The development of the behaviour can not occur until the physical and neurological apparatus required for

the performance of behaviour is developed to some extent. And the parallels observed between the developments of the brain and behaviour in various studies, justify to some extent the correlation in these two systems. This is not to suggest that an ultimate evidence exists to draw a strong parallel between these two systems. The testing of the above hypothesis particularly in relation to the behavioural studies is far from complete.

It is interesting to note at this juncture that several different theoretical models can be constructed to account for the ultimate deficits as mentioned in the above hypothesis. All such models must assume a failure of 'catch-up' when rehabilitation is attempted. This is particularly true when one considers that many of the brain's biosynthetic mechanisms are only present at the time of the "brain growth-spurt".

Dobbing and Sands (1971) have put up two such models. According to them one possibility is that nutritional growth retardation results in a chronological delay of the growth-spurt complex, and it may be delayed beyohd the point when biosynthetic mechanisms have declined to insignificant proportions, resulting in an ultimate deficit. Alternatively the growth-spurt in nutritionally restricted animal may occur at a fixed chronological time but to a

lesser extent and would produce a quantitatively similar ultimate deficits. If the later model is true then there exists a "once-only" opportunity to construct a complete brain and if this opportunity is missed it is not possible to remedy the situation later. The authors have shown that the later model may truely reflect the manner in which the brain deficits are achieved due to growth retardation.

Contrary to the vulnerability of the developing brain, the adult brain seems to be quite immune to prolonged starvation or undernutrition or protein deficiency. The chemical composition of the brain is also virtually unaffected by severe starvation in the adult as suggested by the values for RNA, DNA, protein nitrogen and brain lipids (Lehr and Gayet, 1963; Lipton, 1966; Dobbing, 1968a). It is, of course, possible that compositional alterations could be detected in the different regions under the effects of the above stress conditions but the adult brain, on the whole, seems to be more stable particularly in comparison with the developing brain.

The fact that adequate nutrition is necessary for the structural development of the brain in early life has prompted many studies using the animal experimentation with a particular emphasis on the effects of protein deficiency and undernutrition during this period. A few attempts

have also been made to study the effects of vitamin deficiencies. Degenerative changes in the CNS have been found with a deficiency of vitamin A (Dam and Sondergaad, 1964), vitamin E (Gordon and Nitowsky, 1968), thiamine (Collins et al., 1970; Dreyfus, 1976b), and riboflavin (Zimmerman, 1943). Biochemical changes have been reported in the deficiencies of thiamine (Holowach et al., 1968; Collins et al., 1970; Gubler, 1976a), niacin (Garcia-Bunnel et al., 1962) and pyridoxine (Eiduson et al., 1964). These studies have been reviewed elsewhere (Coursin, 1968; Rajalakshmi and Ramakrishnan, 1972; Dreyfus, 1976a,b). The most extensively investigated parameters are DNA, RNA, and protein which reflect the structural development. The various lipids, particularly cholesterol and phospholipids have also been investigated because of their major role in myelination.

The studies on the effects of manipulating the plane of nutrition during the prenatal period are relatively few in number. Progeny born of rats fed a protein deficient diet during gestation had low birth weights and brain weights (Zamenhof et al., 1968; Shrader and Zeman, 1969; Zeman and Stanbrough, 1969; Envonwu and Glover, 1973; Siassi and Siassi, 1973; Coupin et al., 1977). Brain DNA content was reduced but concentration was not found to be affected suggesting a reduction in cell number (Zeman and Stanbrough,

1969; Zamenhof et al., 1971; Envonwu and Glover, 1973; Siassi and Siassi, 1973; Coupin et al., 1977). The similar deficits in body weight and brain weight have been reported in other species such as dog and pig (Stewart, 1968); rabbit (Schain and Watanabe, 1973) and human (Stoch and Smythe, 1963 and 1967). In contrast, progeny born of mothers fed a quantitatively restricted diet had significantly lower body weights but brain weight was not affected (Kumar and Sanger, 1970; Balázs and Patel, 1973).

Various techniques have been used to produce undernutrition in rats during the neonatal period. The most common technique is to manipulate litter size and consequently the supply of milk to the pups (Kennedy, 1957; Widdowson and McCance, 1960). Another method consists of restricting the food intake of the mother during lactation (Chow and Lee, 1964) or feeding the mother low protein diet (Barnes et al., 1966). Another technique is that of restricting the access of pups to the mother (Eayrs and Horn, 1955). In some investigations the maternal diets have been manipulated both during gestation and lactation (Simons et al., 1968). A combination of both approaches i.e., increase in litter size and the restriction of maternal diet, has also been used to produce a more severe degree of undernutrition (Guthrie and Brown, 1968). In all these cases the effect is

a decreased supply of milk but the protein content of the milk is not affected (a similar inference in undernourished lactating women have been drawn by Jelliffe, 1955). Consequently these techniques do not enable us to manipulate the protein concentration of the diet.

A more rigidly controlled technique for modifying the diet of the suckling rat was reported by Miller and Dymsza (1963). In this method newborn pups were fed using a specially designed needle. Czajka and Miller (1968) have achieved various degrees of undernutrition by hand-feeding the pups from birth with a milk formula the protein content of which was varied. They found weight gain during the neonatal period to depend on the protein content of the liquid formula as might be expected. A similar conclusion has been arrived at by computing the milk protein content of the different species and comparing the time taken for doubling the body weight (Jelliffe, 1955). However, this technique has not yet been extensively exploited for studies on the brain.

Neonatal undernutrition has been associated with a reduction in brain weight (Dobbing and Widdowson, 1965; Winick and Noble, 1966; Chase et al., 1969; Swaiman et al., 1971; Sobotka et al., 1974; Dyson and Jones, 1976). A decrease was found in the DNA content (Winick and Noble,1966;

Dobbing, 1968[a,b]; Guthrie and Brown, 1968; Swaiman et al.,1971; Envonwu and Glover, 1973), RNA content (Winick and Noble, 1966; Swaiman et al., 1971; Envonwu and Glover, 1973), cholesterol content (Dobbing, 1964; Benton et al., 1966; Geison, 1967; Dobbing, 1968[a,b]; Dickerson and Jarvis, 1970), protein content (Swaiman et al., 1971; Envonwu and Glover, 1973; Gambetti et al., 1972) but the concentrations were not affected (Guthrie and Brown, 1968; Chase et al., 1969; Swaiman et al., 1971; Envonwu and Glover, 1973; Sobotka et al., 1974). However, a reduction in the concentration of DNA, RNA and protein has been reported by Winick and Noble (1966).

Neonatal undernutrition has been reported to result in the reduction of concentration of brain lipids including cholesterol and phospholipids (Dobbing, 1968[a,b]; Geison and Waisman, 1970), gangliosides (Dickerson and Jarvis, 1970; Geison and Waisman, 1970), cerebrocides (Culley and Mertz, 1964; Benton et al., 1966; Geison, 1967; Geison and Waisman, 1970), proteolipids, sulphatides and plasmalogens (Geison, 1967).

The effects of dietary deprivation have been reported to vary from region to region, depending on their maturation period. The most extensively studied regions are the cerebrum, cerebellum and brain-stem. The cerebellum has

been reported to be most affected in rats by Culley and Lineberger (1968), Cheek et al. (1969), Fish and Winick (1969) and Chase et al. (1969) and in mice by Howard and Granoff (1968). The brain-stem has been reported to be the least affected (Fish and Winick, 1969). The studies of Patel et al. (1973) emphasize the timing of undernutrition imposed in relation to the maturity of the regions studied. Moreover, studies by these authors suggested a compensatory mechanism in undernourished rats i.e., a depression of mitotic activity and a reduction in the normal rate of cell loss. All these studies suggested a decrease in DNA and protein synthesis and reduction in RNA content. The reduction in protein synthesis is believed to be brought about by the effects of the same on RNA metabolism, as the same is found to be associated with an increase in alkaline ribonuclease and decrease in DNA polymerase activities (Winick et al., 1972).

The EEG pattern has been used as a determinant for assessing the functional activity of the brain. For the maintenance of the background activity, proper development of arborization of dendrites and synaptic contacts are very essential. Synaptic contacts increase in number during the early maturation of the brain. Although the periods of most rapid increase in numbers vary from region to region and even differ depending on the depth of the synaptic contacts

within any part of the cerebral cortex (Molliver and Loos, 1970), some general principles are applicable (Jones, 1976). It appears that within the rat brain there is gradual increase in synaptic numbers throughout the first three weeks of postnatal life with an acceleration taking place in the second week (Armstrong and Johnson, 1970; Bloom, 1972). It has been estimated that the total number of synapses may double each day between 4 and 11 days postnatal, and may increase 100 fold between 4 days and the adult in rat cerebrum.

Accompanying this increase in the number of synaptic contacts is a gradual alteration in the ultrastructural features of individual synapses. Here again, the sequence of events follows a different time course in different situations. Nevertheless three principal stages in synaptogenesis stand out as of general significance (Jones, 1975). In stage I only axodendritic synapses are recognizable, these synapses contain few vesicles and have undifferentiated or symmetrically thickened membranes. Stage II is characterized by an increase in numbers of synaptic vesicles, assymetrical membrane thickenings and the appearance of the first axosomatic synapses. Dendritic spines also make their appearance during this stage. These characteristics are further accentuated during stage III with a marked increase in the percentage of junctions displaying adult features.

As mentioned earlier, the period of active neuronal proliferation, which is primarily prenatal in rat, is followed by the "growth spurt". This would mean postnatal undernutrition may not severely affect cerebral neurons. Dobbing et al. (1971) reported that the postnatal undernutrition in the rat caused a reduction in neuronal density in those areas in which postnatal neurogenesis takes place. Cragg (1972) observed 33 % increase in neuronal density of cerebral cortex of postnatally undernourished rats suggesting the relative sparing of the neurons at the expense of neuropil. Salas et al. (1974) reported reduction in the number of dendritic processes in postnatal undernutrition. Cragg (1972) found 38 to 41 % deficits in the number of synaptic connections associated with neuronal layer of the cortex of undernourished rats. Undernutrition has been reported to affect the proper arborization of dendrites and also reduces the number of synapses (Eayrs and Horn, 1955; Bass et al., 1970; Cragg, 1972; Shoemaker, 1972; Gambetti et al., 1972a,b; Hogan et al., 1973). Gambetti et al.(1974) failed to find any effect on synaptogenesis.

This brings us to the next logical question : whether the nature of the synapse is affected ? The electron microscopic studies carried out by Jones and Dyson (1976) indicate that the thickness of the pre- and post-synaptic densities were affected in the 20 day old undernourished rat.

The deficits may be quite large, as evidenced by a 30 % deficit in the maximum presynaptic thickness. Further analysis of this presynaptic index demonstrates that the heights of the dense projections are significantly affected, being 24 % less in undernourished junctions.

Dense projections are thought to be intimately related to the synaptic vesicles of the presynaptic terminal (Gray, 1963; Gray and Willis, 1970; Jones and Bradford, 1971). If this is the case, the projections are probably involved in the accumulation and release of transmitter at the presynaptic membrane. A delay in their proper formation during brain development would be anticipated to delay the onset of neurological functions, a not unreasonable suggestion in view of the delay in the appearance of reflexes (Simson et al., 1968; Smart and Dobbing, 1971a,b) and of cortical electrical responses (Mourek et al., 1967; Salas and Cintra, 1973) reported in undernourished conditions. A corollary of this finding concerning the retarded development of dense projection is that the synaptic vesicles themselves may be another target of nutritional insults. At present, there is only very sketchy evidence on this, but what there is, suggests that number of vesicles per terminal unit erea is decreased while there is also a decrease in the intravesicular area of the junctions of twenty day undernourished rats (Dyson and Jones, 1976). These changes are thought to be

subsequently related to the mechanism of a particular neurotransmitter involved in the particular junction. The involvement of cholinergic system in these junctions has been very well suggested and extended to mean to reflect such changes through the biochemical parameters observed in undernourished brains, e.g. changes in the activity of AChE and ChAc as reported by Adlard and Dobbing (1971a,b, 1972a,b) and Gaetani et al. (1975). The vulnerability of cholinergic system to undernutrition and malnutrition is reviewed elsewhere in this Chapter.

Undernutrition during the neonatal period has also been found to result in the decreased activities of several brain enzymes, namely, succinic dehydrogenase, aldolase and acetylcholine esterase (Adlard and Dobbing, 1971a, 1971b). Similar observations were made with regard to AChE by Sereni et al. (1966) but Gambetti et al. (1972) found an increase in the activity of this enzyme. Cerebrocide sulfotransferase is also found to be affected in undernutrition (Chase et al., 1967).

The various deficits imposed during the prenatal and postnatal periods are not fully reversed by subsequent rehabilitation in the later life (Dobbing, 1968a; Guthrie and Brown, 1968; Geison and Weisman, 1970; Rajalakshmi et al. 1974a). On the other hand, the deficits imposed during post-

weaning undernutrition, have been fully reversed by the subsequent rehabilitation (Dobbing, 1968a).

Undernutrition during pre- and post-natal periods has been implicated to result into delayed neuromotor development (Chow and Lee, 1964; Smart and Dobbing, 1971a; Altman et al., 1971). The adequate nutrition just after birth seems to be more vital for this purpose (Smart and Dobbing, 1971b). However, Simpson et al.(1968) reported similar effect to last even in prenatally undernourished rats.

The maturation of evoked cortical response to visual or auditory stimuli were found to be delayed in neonatally undernourished rats. The electrophysiological studies suggest that experimental undernutrition in early life results in increased latencies of major components of the visual, auditory and somatosensory evoked potentials (Callison and Spencer, 1968; Mourek et al., 1967; Salas and Cintra, 1973). However, this effect does not persist into adulthood even if the undernutrition is continued after weaning (Bronzino et al., 1975; Forbes et al., 1975).

The degree of maternal and environmental stimulation may also influence neuromotor development in early life.

Maternal behaviour has been found to be altered with malnutrition (Frankova, 1972, 1974). Similarly the interaction between pups varies when they are reared in small or large litter size. Pups in large litter size are found to be more independent and more active (Frankova, 1972).

A slow and delayed development of exploratory activity(Frankova and Barnes, 1968a) and lower intensity of exploratory behaviour (Lat et al., 1961; Barnes et al., 1966; Barnes et al., 1968) have been found in rats subjected to neonatal undernutrition.

Although developmental retardation has been noted in early life in several studies, no permanent effects on complex learning were observed in undernourished and rehabilitated rats (D'Amato, 1960; Guthrie & Brown, 1968; Barnes et al., 1966; Frankova and Barnes, 1968b). However, some persisting effects such as hoarding behaviour, increased emotionality and decreased exploratory activity have been reported (Seitz, 1954; Novakova, 1966; Frankova and Barnes, 1968a; Chow et al., 1968).

Severe undernutrition or protein deficiency during the postweaning period preceded by neonatal undernutrition has been reported to impair psychological performance (Frankova and Barnes, 1968a). In this case the impairment

was much greater in case of protein deficient diet. Protein deficiency in the postweaning period has been found to impair performance in the visual discrimination and reversal learning and water maze performance in rats (Rajalakshmi et al., 1965; Rajalakshmi and Ramakrishnan, 1969a). In pigs it is found to impair performance on tasks such as electric shock avoidance (Barnes et al., 1968). Dogs subjected to protein deficiency showed lack of interest in the surroundings and were less playful and more aggressive even after rehabilitation (Platt and Stewart, 1968).

From the foregoing discussion it is evident that the developing brain is much more vulnerable to dietary insults. The dietary deprivation is most likely to result in the deficits in the brain constituents, impairment of brain function and abnormal behavioural pattern. The extrapolation of these studies to man poses a special problem due to differences in the ontogenic development of the brain and developmental profiles of behaviour in different species.

b) Thiamine Deficiency :

Thiamine deficiency has been associated with a triad of symptoms - anorexia and weight loss; cardiomegally and bradycardia; and neuromuscular disturbances (ataxia and convulsions).

Knowledge of the relation of thiamine to the oxidation of pyruvate in brain started with the finding of a fault in the disposal of lactate, especially associated with the lower parts of the pigeon brain (cf: Peters, 1936; 1940). From this it was found that there was a depression of oxygen uptake in the presence of lactate or glucose but not in the presence of succinate. This depression occurred when the acutely thiamine deficient pigeons exhibited signs of opisthotonus. The convulsions induced at the acute stage of deficiency could be cured with great rapidity by injection of thiamine solution into the subarachnoid space of the bird. Further studies revealed that in the terminal stages of thiamine deficiency there was an increased amount of pyruvate in the blood (Sherman and Elvehjem, 1936). Similar observations were made in beriberi patients as early as 1939 by Platt and Lu. The timely discovery of thiamine pyrophosphate (TPP) and its role as a coenzyme in the decarboxylation of α-keto acids like pyruvate (Lohmann, and Schuster, 1937) extended a much needed role for this vitamin. Ochoa and Peters (1938) reported the decrease in brain TPP levels of thiamine deficient pigeons. This observation has been extended to rats made thiamine-deficient by the vitamin analogue, pyrithiamine (Koedam, 1958). The convincing evidence of the involvement of TPP in pyruvate oxidase system of the pigeon brain was reported by Banga et al. (1939). Thiamine-dependent reactions of significance

to the nervous system are listed in Table-2 (Dakshinamurti, 1977).

Thiamine pyrophosphate (TPP) is one of the coenzymes associated with the pyruvate dehydrogenase multiple enzyme complex (PDH). The detailed sequence of different reactions taking place in the conversion of pyruvate to acetyl-CoA is shown in Figure-1.

Pyruvate occupies a pivotal position in the metabolic pathways; hence its oxidative decarboxylation is crucial to the cell. PDH complex is very well regulated by not only the products of reaction, acetyl coenzyme-A and NADH but also by an intricate phosphorylation-dephosphorylation sequence. Phosphorylation of the pyruvate dehydrogenase component of PDH complex is associated with a decrease in enzyme activity and dephosphorylation restores the activity. Such a sequence is known to occur in porcine brain (Siess and Weiland, 1972; Weiland et al., 1971). Interestingly, this complex is also regulated by TPP itself which inhibits phosphorylation of the pyruvate dehydrogenase by ATP and PDH-kinase (Reed et al., 1973).

The oxidative decarboxylation of α-ketoglutaric acid takes place in a manner similar to that of pyruvate.

Table - 2 : Thiamine-dependent Reactions.

+ Enzyme	Reaction catalyzed	Coenzymes
Pyruvate dehydrogenase complex	Pyruvate + CoA + NAD^+ ------ Acetyl CoA + CO_2 + NADH + H^+	TPP, lipoamide, FAD
∝-Ketoglutarate dehydrogenase complex	∝-Ketoglutarate + CoA + NAD^+ ----- Succinyl CoA + CO_2 + NADH + H^+	TPP, lipoamide, FAD
∝-Ketoacid decarboxylase (∝-ketoisocaproic, ∝-keto-methyl valeric, ∝-ketoisovaleric)	R.CO.COOH + CoA + NAD^+ --------- R.CO.S CoA + CO_2 + NADH + H^+ (isovaleryl CoA, ∝-methyl butyryl CoA, isobutyryl CoA)	TPP, lipoamide, FAD
Transketolase	Xylulose-5-phosphate + erythrose-4-phosphate ⟶ fructose-6-phosphate + glyceraldehyde-3-phosphate	TPP.

Adapted from Dakshinamurti (1977).

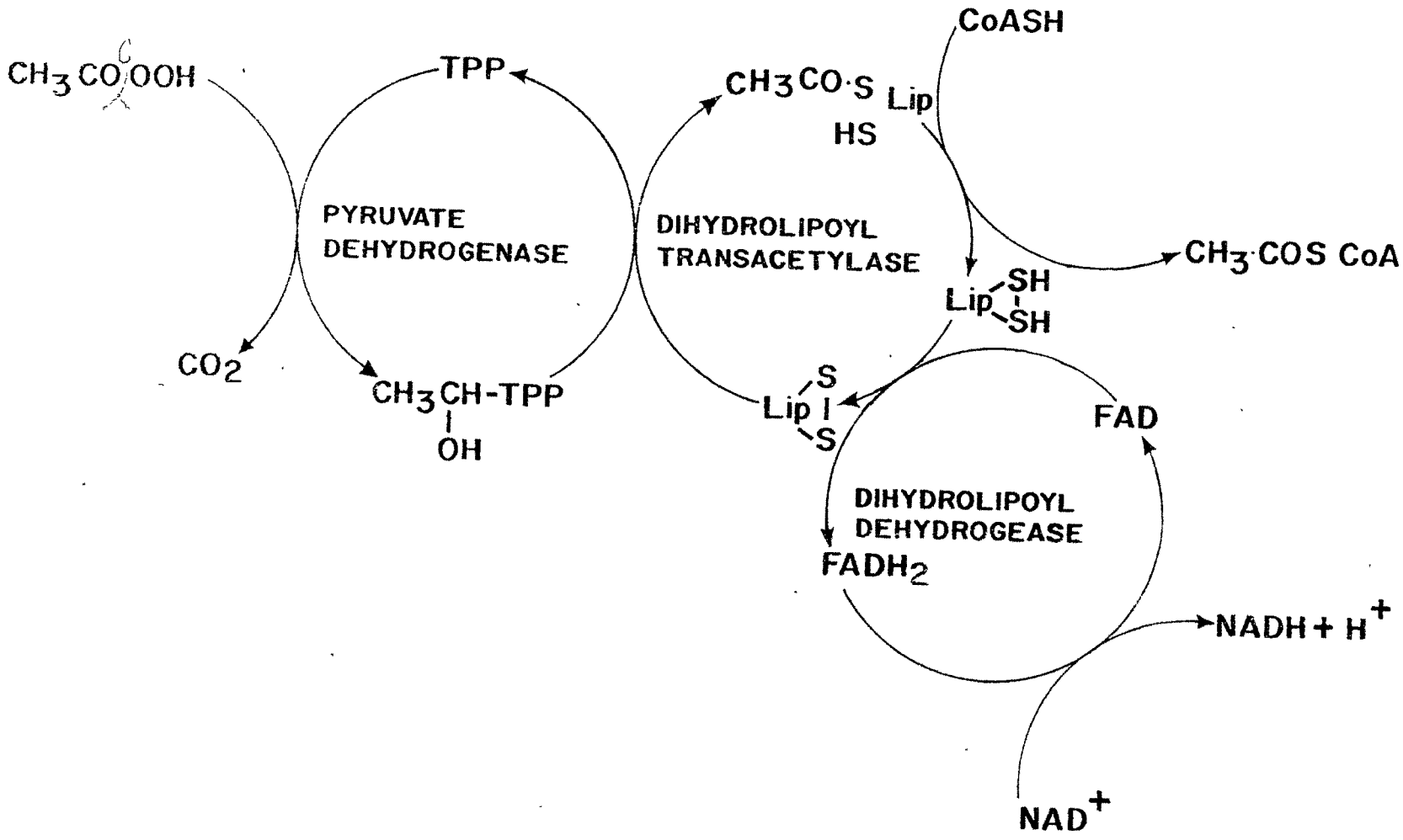


Fig. 1– Reaction sequence of decarboxylation of pyruvate and conversion to acetyl-CoA

The ∝-ketoglutarate dehydrogenase complex is analogous to that of PDH complex. This multienzyme complex consists of ∝-ketoglutarate dehydrogenase component, a transsuccinylase and a dihydrolipoyl dehydrogenase. TPP is the co-factor for the first component.

TPP is also involved as cofactor in the metabolism of branched chain amino acid (leucine, isoleucine and valine). These amino acids are transminated to the corresponding ∝-keto acids, which are then oxidatively decarboxylated and then metabolized further. TPP is involved at the stage of oxidative decarboxylation.

TPP is also involved as a cofactor in transketolase reaction in HMP pathways (Figure-2). It is highly bound to transketolase and its role in this reaction is same as in PDH reaction i.e., transfer of activated aldehyde unit.

In 1947, von Muralt proposed a dual role for thiamine in nervous tissue, one being the well known coenzyme function in intermediary metabolism, as discussed above, and the other possible role in nervous excitation. He has reiterated his original suggestion after the critical analysis of earlier work (von Muralt, 1962). Recently, considerable evidence has accumulated which demonstrates the

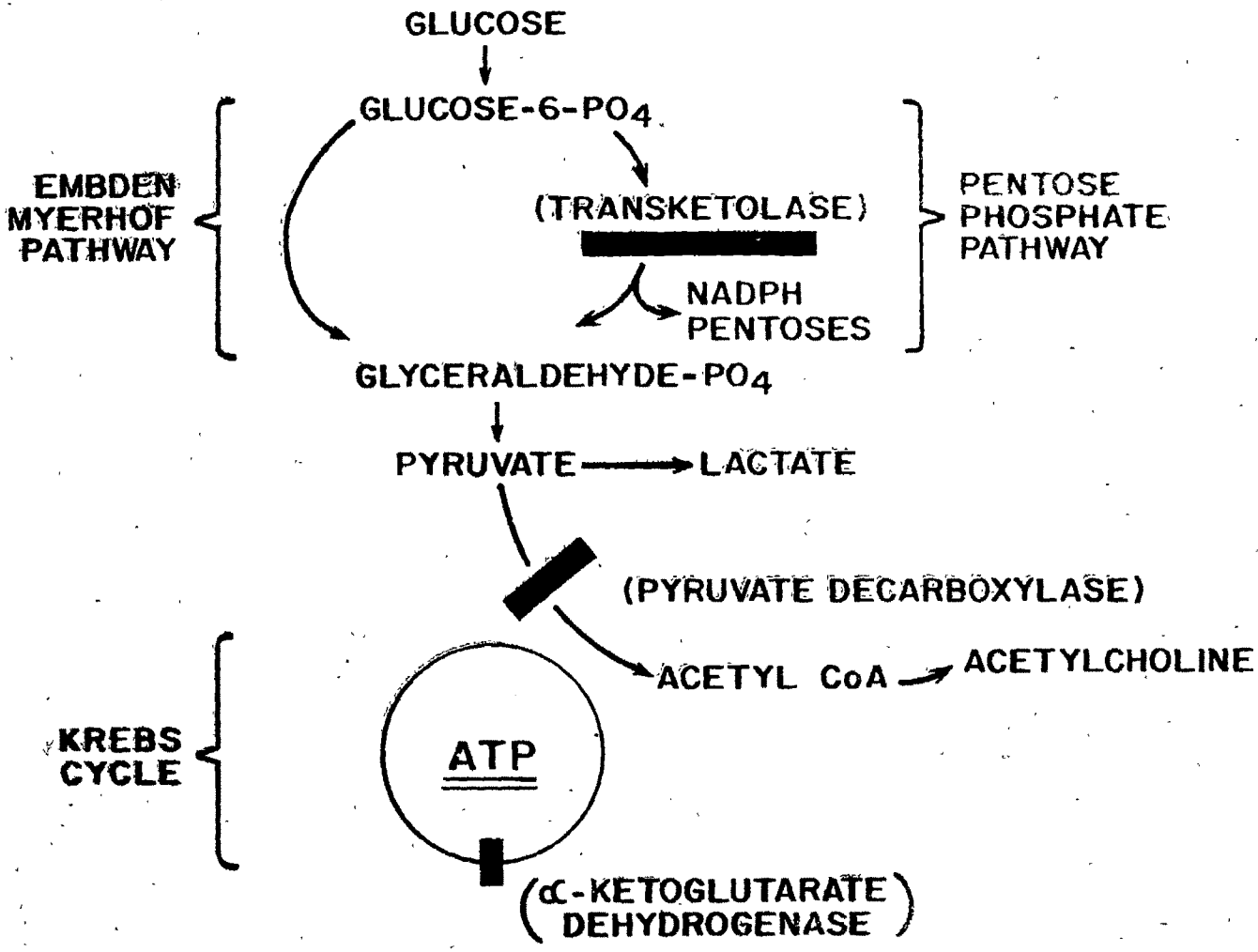


Fig 2 – Schematic diagram of the biochemical sites at which thiamine pyrophosphate acts as a coenzyme. These sites are indicated by the bold bars.

non-coenzymatic function of the vitamin. Such evidence includes - i) electrical stimulation of a variety of nervous tissue preparation result into the release of thiamine (Minz, 1938; Gurther, 1961; Cooper et al., 1971); ii) The electrical activity of nerve preparation is profoundly affected by antimetabolites of thiamine (Kunz, 1956; Petropulos, 1960; Armett and Cooper, 1965); iii) the addition of thiamine can restore the action potential of UV-irradiated nerve preparation (Eichenbaum and Copper, 1971); iv) the polyneuritis that is associated with a state of thiamine deficiency does not always correlate with an inhibition of enzymes in nervous tissues which require TPP as coenzyme (Lofland, et al., 1963; Koeppe et al., 1964; Dreyfus et al., 1965); v) thiamine triphosphate, a non-coenzymatic form of thiamine phosphate, is lacking in the brains of patients with subacute necrotizing encephalomyelopathy (SNE) (Fuente and Diaz-Cada-Vieco, 1954) and may be closely related to the etiology of this fatal disease (Cooper et al., 1969; Pincus et al., 1969; Murphy, 1973); Vi) the presence of active and highly specific TTPase in the brain (Barchi and Brown, 1972). The last observation has been taken to indicaate a role for TTP in the permeability of the excitable membrane to sodium ions during propogation of the nerve impulse.

In the acute stage of thiamine deficiency the following changes have been reported : edematous swelling

of astrocytic foot processes, increased permeability of vasculature and splitting of the basement membrane of capillaries in the species specific selected areas of the brain (Robertson et al., 1968; Watanabe, 1975). Blank et al. (1975) reported the splitting of myelin lamellae in the brain stem. The tissue changes in terminal thiamine deficiency gives an appearance of the brain having undergone pan-necrosis (Dreyfus, 1976b).

Earlier studies of the structural lesions in the CNS indicated involvement of the pons, medulla and cerebellum. Dreyfus and Victor (1961) showed that the lesion is highly localised with discrete symmetrical areas of pan-necrosis in the dorsal pontine tegmentum in the region of the lateral vestibular nucleus of Dieters. Ultrastructural changes in the brain-stem of thiamine deficient rats have been studied by number of workers. Collins (1967) reported that the glial cells are primarily involved. Robertson et al. (1968) also reported that the earliest lesions are associated with intracellular edema involving glial cells. Tellez and Terry (1968) have suggested that early changes occur in the axons and nerve endings. However, except in advanced deficiency neuronal cell bodies are not affected. Work of Pincus and Grove (1970) suggests that the parts of the nervous system which are affected earliest

and to the greatest degree tend to have the highest concentration of thiamine diphosphate and that progressive thiamine deficiency results in marked depletion of the diphosphate ester in the affected areas.

Dreyfus and Victor (1961) have reported the concentration of thiamine (total) in different areas of the brain of the control and thiamine-deficient rat. They found that the cerebellum had a higher thiamine content and the lateral pontine tegmentum, the site of profound histological changes, a slightly lower thiamine content than the rest of the brain. In depletion studies it was obvious that the brain thiamine was decreased to 50% of normal levels even before growth of the animals was retarded. Further depletion to 35 % of the normal levels was associated with unsteady gait and slow movements. Brain thiamine had to be drastically reduced (to 20 % of normal levels) before the well recognized disturbances in posture and equilibrium were seen. However, decreases in thiamine content of vulnerable areas of the brain as an antecedent of morphological changes could not be established.

Dreyfus (1965) and Dreyfus and Hauser (1965) studied the activities of TPP-dependent pyruvate dehydrogenase and transketolase in thiamine deficient rats. Although, the total brain PDH activity decreased by less than 20 % with

the progress of deficiency, the activity in the brain stem registered a significant reduction of 44 % at the extremely deficient state. During progressive thiamine depletion, transketolase activity of the cortex remained relatively constant, whereas that of the later pontine tegmentum decreased almost by 40 %. The decrease in brain thiamine had to be drastic before changes in transketolase activity could be observed. Dreyfus (1965) suggested that in rat brain transketolase rather than PDH is more susceptible to thiamine depletion. The studies of McCandless and Schenker (1968) confirmed the earlier findings of Dreyfus (1965). In these studies also it was apparent that brain thiamine levels had to decrease to approximately 20 % of normal before neurological symptoms appeared. Pincus and Grove (1970) reported a similar decrease of TPP in rat brain stem prior to manifestation of neurological symptoms. In thiamine-deficient symptomatic rats the pyruvate carboxylase activity of the cortex was not affected while that in cerebellum was reduced by 35.8 %; cortical lactate levels were not altered; whereas, the levels in cerebellum and brain stem increased by 32 % and 68.9 %, respectively. As reported earlier by Dreyfus (1965) there was a considerable decrease in brain stem transketolase activity, which did not respond to thiamine administration. The fact that the pyruvate carboxylase activity is reduced to 65 % of normal both in the cerebellum

and brain stem, along with the accumulation of lactate, suggests impairment in the utilization of pyruvate. Since the entry of acetyl CoA into TCA cycle is the main function of PDH complex one would expect this impairment to affect cerebral ATP levels. However, McCandless and Schenker (1968) did not find such a decrease in thiamine deficient rats. This would account for the equal reduction in rates of both synthesis and utilization. Since HMP pathway is substantially reduced through reduction in transketolase it could be well correlated with glutathione reduction in brain stem. Secondly, a substantial reduction in the neuronal activity of thiamine deficient rats would account for the greatly reduced expenditure resulting in unaltered ATP levels.

Holowach et al. (1968) observed the following changes in thiamine deficient rats (made deficient by dietary deprivation of the vitamin and also the administration of pyrithiamine): Increase in pyruvate levels, four-fold; ∝-ketoglutarate, seven fold; 6-phosphogluconate and xylulose-5-phosphate, 2.5 folds. ∝-KG dehydrogenase and transketolase activities were reduced to 50 % of normal.

All these changes observed in thiamine deficient animals give rise to one question : whether one or all these changes together could contribute to cause of subnormal CNS

function? The theories on the "primary biochemical lesion" have been very well reviewed (Dreyfus, 1976a,b; Gubler, 1976a,b).

Dakshinamurthi (1976) suggested the following possibilities :-

1) Since there is a considerable evidence to suggest a role for TTP in neural conduction, is it possible that the areas of the brain susceptible to thiamine deficiency are the ones which also use TTP primarily?

2) Is there a deficiency of acetyl CoA and consequently ACh too in the brain of deficient animals? It is interesting to note that Heinrich et al. (1973) observed reduced ACh levels in the brains of thiamine deficient rats which seems to reflect the decreased precursor pool of acetyl CoA.

The HMP pathway plays an important role in synthetic mechanisms by virtue of NADPH production for lipid synthesis and ribose production for nucleic acid synthesis. In adult brain this pathway probably plays a relatively minor role, assuming greater importance in the developing (Novello and McLean, 1968) and chronically depleted CNS. In severe thiamine deficiency, decreased NADPH production could impair fatty acid and nucleic acid synthesis and the

conversion of oxidised glutathione to its reduced form. The latter has been reported to be reduced in the brain of symptomatic thiamine-deficient animals (McCandless and Schenker, 1968). Reinauer and Hollman (1969) observed that the thiamine deficiency affects RNA synthesis and possibly, protein synthesis also.

Dreyfus (1976a) carried out extensive studies on the effects of thiamine deficiency, instituted on 14th day of gestation, in new born pups. Transketolase activity was reduced by 75 % at 25 days of age after birth. Brain DNA concentration was higher in deficient and pair-fed animals suggesting a smaller cell size. This was also reflected by lowered protein/DNA ratio and RNA levels per mg DNA. These pups exhibited characteristic neurological symptoms at this age. Whole brain ganglioside concentration was significantly greater in the thiamine deficient and pair-fed controls. Cerebroside and sphingolipid (minus ganglioside) concentrations in whole brain were markedly reduced in these two groups. Total phospholipid concentration and distribution remained unchanged. Somewhat similar results were observed when the lipid compositions of the brain stem, cerebellum and diencephalon were estimated.

Trostler et al. (1977) instituted the thiamine deficiency on 11th day of gestation and studied the effects

in the pups on day, 1, 14, 21 and 42 (23 days after rehabilation) postnatally. Body weights were reduced significantly at day 1, 14, 21 and 42 in thiamine deficient group and in pair-fed control group (except day 1). Total lipids per brain were affected from day 14 onwards, and cerebrosides and cholesterol from day 1 onwards. It is interesting to note that 23 days of rehabilitation failed to correct these deficits at day 42. Brain transketolase activity was reduced on day 1 onwards postnatally in thiamine-deficient pups. By 21 days of age thiamine deficient pups had only 34 and 25 %, respectively, of the transketolase activity of control and pair-fed groups. Pyruvate concentration was increased in the brains of thiamine deficient pups from the 7th day onwards reaching 3 to 4 times higher than in controls at the end of third week. Geel and Dreyfus (1975) studied brain lipid composition of offsprings of rats exposed to maternal and early postnatal thiamine deficienty. There was no change in brain lipid composition distinct from that of undernutrition brought about by anorexia. McCandless et al. (1976a) reported that the myelination proceeded at normal rate in the rats made thiamine deficient by injecting pyrithiamine during the critical period of myelogenesis.

Trostler and Sklan (1978) reported impairment, in thiamine deficient rat pups, in radioactive glucose incorporation into brain lipids and in acetyl CoA synthesis resulting

into altered lipogenesis. 36 % of uniformly labelled (^{14}C)-glucose is accounted for glutamate alone (Gaitonde et al., 1965) along with other amino acids which reflects the activity of HMP shunt. Gaitonde et al. (1974) reported reduced incorporation of ^{14}C glucose into amino acids of thiamine deficient rat brain. However, the activity of thiamine dependent enzymes was not affected. Similar findings were also reported by Gubler et al. (1974).

From the above cited studies it is apparent that immature animals are more vulnerable to dietary thiamine deficiency than are adults. Using sphingolipids and cerebroside levels as indices of the degree of the myelination, it appears that this process is equally impaired in thiamine deficiency and in the plain undernutrition represented by pair-fed control animals. Thiamine deficiency present during a period of rapid brain development does not seem to induce characteristic changes in brain lipids, which can be distinguished from the effects of simple undernutrition.

II) Human Studies :

a) Undernutrition and Protein Deficiency :

As mentioned earlier, a substantial section of the world's population is either undernourished or malnourished or both. Children in the postweaning period are the group

most vulnerable to malnutrition as the diets provided for them are often deficient in quantity and quality than adult diets while their nutritional requirements are greater in relation to body weight and energy intake. These children are usually adequately breast-fed and grow well for first four to six months, thereafter breast milk does not provide adequate protein (Scrimshaw and Behar, 1961; Jelliffe, 1955). Not only the inadequate diet but also unsanitary conditions result into frequent infections aggravating the condition.

The most obvious manifestation of early under-nutrition or malnutrition in these children is a reduction in body size associated with varying deficits in many other parameters. Although, weight is readily affected by short term nutritional insult, the height seems to be adversely affected only by long term severe insult. Head circumference is reportedly affected by inadequate nutrition in early life and is considered to be an index of brain size (Stoch and Smythe, 1963; O'Conell et al., 1965; Graham, 1968; Scrimshaw and Gordon, 1968; Monckeberg, 1968; Rabinow, 1972; Winick and Rosso, 1969; Engsner, 1974). The studies based on transillumination and echoencephalography suggest that the brains of malnourished children may be even smaller than is suggested by head circumference (Rozovski et al., 1971; Engsner and Vahlquist, 1975). Autopsy studies have confirmed the smaller brain size of malnourished children

(Udani, 1962; Brown, 1965; Rosso et al., 1970; Chase et al., 1972; Marcondes, et al., 1973). Since malnutrition has been found to be associated with both, a reduced brain size and mental retardation, it would be tempting to see an association between the two. A correlation coefficient of 0.6 between head size and (mental) development of malnourished children has been reported by Monckeberg (1975).

A reduction in brain size is associated with a deficit in cell number as measured by DNA (Winick and Rosso, 1969; Rosso et al., 1970; Chase et al., 1972; Ganguly et al., 1972; Subba Rao and Sarma, 1972). The achievement of a reasonable brain size in early life is important and this could account for the fact that brain growth is maintained even at the cost of other tissue growth with early malnutrition and that a certain ratio of brain size to body size is maintained in different animal species (Crile and Quiring, 1940). The significance of reduction of cell number should depend upon the type of cells affected leading to functional subnormality. One would expect that a uniform reduction in all cell types may not result into any subnormality. On the other hand, if only a particular cell type is affected this could very well lead to specific morphological, chemical and functional subnormality.

Since the brain development continues at different rates and in different ways in the late fetal period, infancy

and early childhood in man and during these periods the individual is highly vulnerable to nutritional deprivation a question arises as to the impact of early malnutrition on brain composition and function.

Severe protein energy malnutrition in children has been found to be associated with changes (besides on brain DNA) such as decrease in brain RNA, protein and total lipids (Winick and Rosso, 1969; Ganguly et al., 1972; Subba Rao and Sarma, 1972), cerebrocides and sulfatides (Fishman et al., 1969; Mokashi et al., 1972) and gangliosides (Mokashi et al., 1972). Bachawat (1972) reported deficits in the concentrations of glycolipids (cerebrocides plus sulfatides) and mucopolysaccharides in the whole brain and of gangliosides in the white matter in malnourished children.

Platt (1962) was the first one to point out the impact of severe malnutrition on CNS function. He suggested that the animal or child dying of severe protein malnutrition dies a 'central nervous death'. This conclusion was based on the observation of the cessation of the electrical activity of the brain before that of the heart. The histopathological changes observed by Platt et al.(1964) in piglets were independently observed in severely undernourished children by Udani (1962).

EEG abnormalities similar to those observed in malnourished animals have been reported in malnourished children (Engel, 1956; Nelson, 1959; Valenzula et al.,1959; De Silva, 1964; Botha-antocm and Harfouche, 1968; Taori et al., and Pereira, 1974; Coursin, 1974). Motor nerve conduction velocity is found to be affected more in kwashiorkar than in marasmus. Arakawa (1975) found the differences in the maturation of EEG patterns between normal and folate deficient animals. Rhodes and Snyder (1975) reported the aberrations of EEG patterns in malnourished children and the changes in the response of the same to light flashes. Similar abnormalities have been reported in congenital mental retardation, mongolism, hypopituitarism, and hypothyroidism and hypoxia in early life. Since some of these conditions are found to be associated with brain damage and mental retardation, a question arises about the significance of similar changes in malnourished children.

The psychological changes in the malnourished children were recognized long before the other changes, and more than two decades ago, Carothers (1953) stressed the mental arrest of malnourished children in his report to WHO. Extreme apathy is a common feature in children suffering from kwashiorkar (Platt, 1961). A kwashiorkaric child gives an appearance of total detachment with environment (Rajalakshmi and Ramakrishnan, 1972). A marasmic child is not so apathetic

and shows a fair interest in surroundings.

There is direct evidence that children experiencing early malnutrition perform less well on tests of learning and behaviour in direct relation to the degree of retardation in height and weight for age (Gomes et al., 1954; Ramos-Galván, 1964; Ramos-Galván et al., 1964). In these studies a high inverse correlation was evident between deficits in height and weight at various ages and performances on tests measuring, respectively, motor performance, adaptive behaviour, language skills and personal-social development. Dr. F. Monckeberg of Chile has reported a study with similar implications (1968). Champkam, et al. (1968) reported similar findings in India.

A number of studies have suggested the poor psychological status of the malnourished children (Stoch and Smythe, 1963; Cabak and Najdanvic, 1965; Stoch and Smythe, 1967; Monckeberg, 1968; Chase and Martin, 1970). Previously established reflexes are depressed or abolished and the elaboration of new conditioned reflexes are affected in protein deficient children (Brozek, 1962).

Persistant effects of early malnutrition on psychological performance are found in some studies (Cravioto, 1966; Hertzig et al., 1972). The studies of Cravioto

and Robles (1965) and Stoch and Smythe (1963, 1967) suggested a correlation between the age at which the children were hospitalized and intellectual impairment but such correlation was not found in the apparently more systematic studies of Hertzig et al. (1972).

Apart from nutritional status, factors such as paucity of the cultural environment, social and economic disadvantage, poor housing, poor maternal attention and child health are also bound to influence the intellectual development.

b) Thiamine Deficiency :

As mentioned earlier, the global picture of prevalence of beriberi has changed in recent time. But, still some pockets of beriberi are to be found in the Far East. Subclinical thiamine deficiency is presumed to be prevalent even in industralized nations.

Studies on thiamine deficiency in human with an emphasis on CNS function are very few. These studies are mostly correlated to clinical symptoms of thiamine deficient subjects. The typical neurological symptoms are ataxia, abnormal postures, vestibular disturbances, opthalmoplegia and acute confabulatory amnestic syndrome. In both animals and man asymmetrical, distal mixed sensory areflexic

neuropathy accompanied by autonomic disturbances is commonly encountered (Dreyfus, 1976b).

The involvement of thiamine, directly or indirectly in the CNS system in man has been suggested by various genetic diseases.

One of such diseases is "cerebeller ataxia" in which there is a defective pyruvate oxidation, specifically TPP involved step, has been reported by Lonsdale (1969), Lonsdale et al.(1969), Blass and Uhlendorf (1970) and Blass (1972). To explain the mode of action of PDH deficiency on CNS function numerous studies have been carried out using experimental animals. These studies suggest " a hypothesis that partial deficiencies of PDH may impair the flux of pyruvate to acetyl CoA in certain cells of the cerebellum without limiting flux in other parts of the brain" (Blass et al., 1976). Moreover, Balázs et al.(1970) suggested that pyruvate metabolism is particularly rapid in a nerve-ending compartment and Tuček and Cheně (1974) said that it is critical for the synthesis of ACh. The degree of inhibition of PDH by numerous inhibitors has been found to be correlated with decrease in ACh synthesis (Gibson et al., 1974; 1975).

Another interesting role of thiamine in the form of thiamine triphosphate (ThTP) has recently surfaced from the pioneering studies of Cooper and co-workers (1969). The syndrome known as subacute Necrotizing Encephalomelopathy (SNE) or Leigh's disease has been reported to be associated with failure to phosphorylate ThDP to ThTP in the brain. A presence of inhibitor, probably a lipid or proteolipid of the phosphoryl transferase has been detected in the urine of these patients (For review : Cooper and Pincus, 1976).

The studies described above suggest that thiamine plays a very vital role in the brain function either via its role of coenzyme involved in conversion of pyruvate to Acetyl-CoA or via its another form, ThTP. The former role and its effect on ACh synthesis, if any are part of lines of present study.

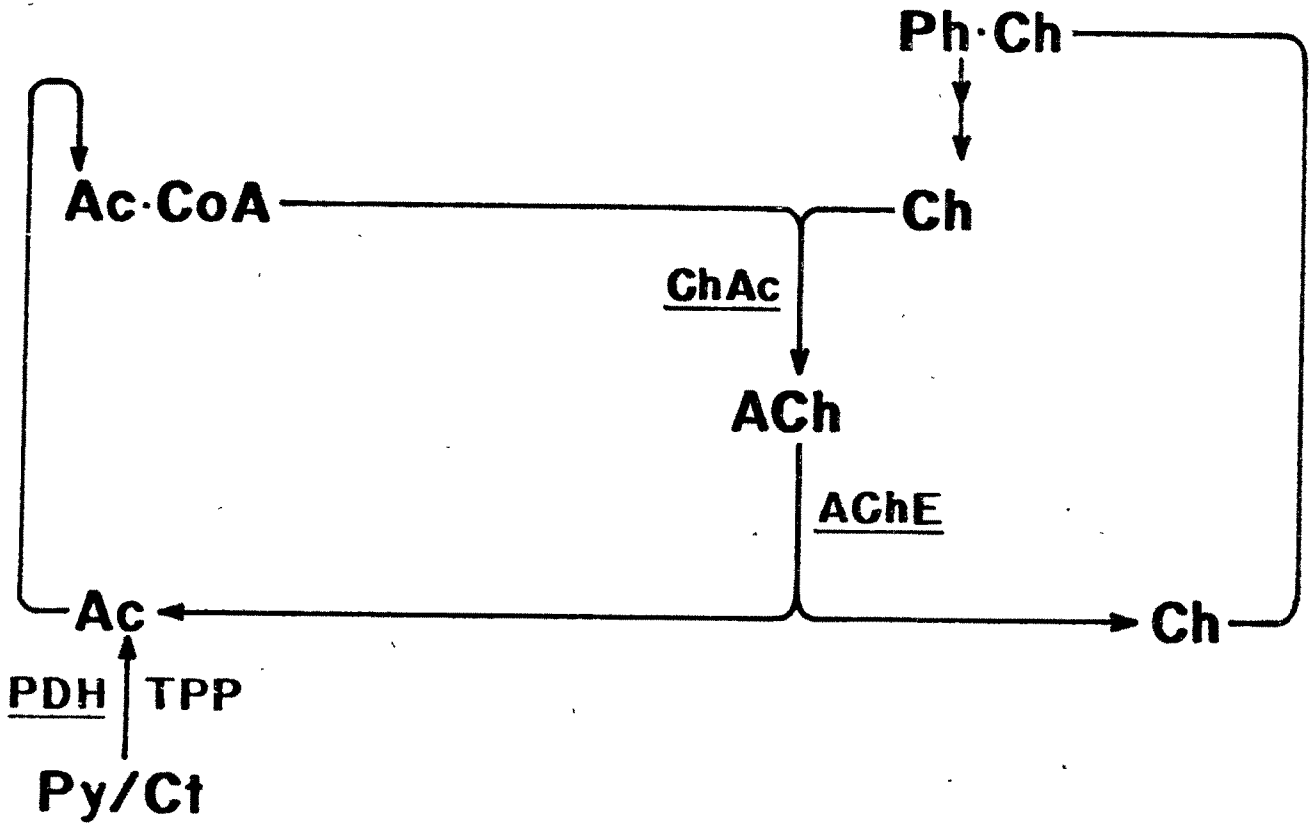
4. Cholinergic System :

The concept of a chemical substance being released at the nerve ending during nerve stimulation is of old date. This can be traced back to Du Bois Reymond who wrote in 1877:

" Of known natural processes that might pass on excitation, only two are, in my opinion, worth talking about : either there exists at the boundry of the contractile

Fig.3—

ACh METABOLISM IN CNS



1. Ac = Acetate
2. Ct = Citrate
3. Py = Pyruvate
4. Ph·Ch = Phosphatidyl choline
5. PDH = Pyruvate dehydrogenax complex
6. TPP = Thiamine pyro phosphate

substance a stimulatory secretion in the form of a thin layer of ammonia, lactic acid, or some other powerful stimulatory substance, or the phenomenon is electrical in nature".

It was not before the beginning of the 20th century that the idea of chemical transmission began to be seriously considered. Elliot (1904) made the bold statement that adrenaline might be the chemical stimulant liberated whenever the impulse arrives at the periphery. Hunt and Taveau (1906) demonstrated the biological activity of Acetylcholine suggesting it to be a likely candidate for chemical transmission in the parasympathetic system. Otto Loewi in 1921 showed for the first time that a substance was released from the isolated frog heart upon stimulation of its vagus nerves. This substance was named as "vagusstoff" by Loewi. In 1926 Loewi and Navratil further demonstrated that eserine from Calabar beans inhibited the enzyme breaking down the "vagusstoff". Feldberg and Krayer (1933) showed that the vagus stimulation to the dog's heart released a substance into the coronary blood which had the properties of acetylcholine (ACh).

The observation that some ACh releasing nerves ran into sympathetic nerves prompted Dale (1933) to introduce the terms cholinergic and adrenergic. Brown et.al., (1936)

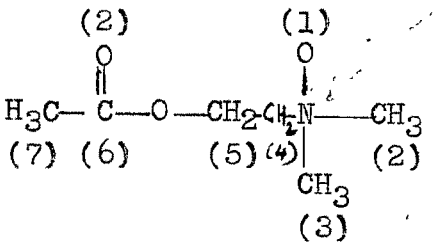
succeeded in showing that even small doses of ACh injected intra-arterially elicited a strong contraction of the cat gastronemius or tibialis anticus muscle. Thus, it seemed unavoidable to regard a release of ACh from the motor nerve endings as the cause of the muscle contraction. The demonstration of cholinesterase and the discovery of the synaptic vesicles through electron microscopy greatly enhanced, the progress in this direction. The brilliant findings of Castillo and Katz (1957) on the release of packets of ACh molecules and chemical studies on ACh synthesis by Nachmansohn et al. (1975) laid the basis for the better understanding of the cholinergic system.

A) Acetylcholine (ACh) :

ACh is one of several naturally occuring esters of choline. The other esters include propionyl choline, urocanylcholine, acrylcholine, seneciocholine, and gamma-aminobutyrylcholine. Of these, only ACh is found in substantial amount in the nervous tissue. Out of the rest of esters only GABACh has been reported to be present in the dog and pig brain (Hebb and Morris, 1969). The ubiquitious occurence of choline in the living organism makes it not unlikely that more of its esters may be discovered in future.

ACh itself is very widely distributed in the nature. Inspite of its well known function as a neuro-transmitter in the nervous system, it has been found in plants such as the Malayan jackfruit and the common stinging nettle, in organism without a nervous system, including two protoza and species of bacteria and in some non-nervous tissues of the multicellular animals (Hebb and Morris, 1969; Hebb, 1957 and Hebb, 1963).

Acetylcholine has the following chemical formula :



The only part of the ACh molecule which is susceptible to chemical attack, either in vivo or in vitro is the ester linkage. The hydrolysis of ACh at this site is accelerated by hydroxyl ions and to smaller extent by hydrions. ACh is much stable in solution at pH 4 and its half life is many hours at pH 7.4 and 37^{o} unless an esterase is present. At pH 12 or above it is destroyed within seconds.

Pauling and his colleagues (1974) have deduced its structure from crystallographic analysis. According to their studies though either end of the ACh ion (C_3-N-C_4-C_5 or

C_5-O_1-C_6-C_7) has all four atoms coplanar, rotation can occur around the bonds C_4 - C_5 and C_5 - O_1. Due to this double hinge, the two planes are twisted away from each other. The further displacement in the plane can occur when the ion reacts with a protein receptors. Pauling's group conclude that as the ACh ion attaches to a receptor site, it has about the same conformation in each case (i.e., nicotinic or muscarinic) but a different orientation. The methyl side of the ion has a high affinity for muscarinic receptors and the carbonyl group side has a high affinity for nicotinic receptors. The similar conclusions are drawn based on the results of quantum-chemical analyses (Green et al., 1974).

ACh is predominantly present in the cholinergic nerves, although substantial levels of the same are reported to be present in the placenta of higher animals (Morris, 1966) and corneal epithelium (Williams and Cooper, 1965). The levels of ACh in the whole brain are inversely proportional to the functional activity of the brain (Richter and Crossland, 1949). Apart from the variation due to the functional activity of the brain the reported values differ from investigator to investigator depending upon the method of sacrifice of the animals and also the method employed for the estimation of ACh. Since ACh is hydrolyzed in no time by acetylcholine esterase the value reported depends

mainly on the postmortem changes brought about by this enzyme. With latest innovations in techniques, the enzymes involved in ACh metabolism can be inactivated instantaneously using micro wave irradiation method (Guidotti et al., 1974; Stavinoha et al., 1973).

The values of ACh reported for adult rat whole brain, using conventional cervical dislocation method for killing the animal and using the tissue preparation for estimation, are as follows : ACh µgm per gram of brain - 1.27 (Richter and Crossland, 1949); 2.68 ± 0.02 (Crossland and Merrick, 1954); 3.45 ± 0.5 (Elliot et al., 1950); 5.0 ± 0.13 (Cheney, et al., 1969) and 1.36 ± 0.04 (Naik et al., 1970). The variation in these values range from 1.27 to 5.0 µgm per gram brain.

The above mentioned values were based on the estimation of ACh by the isolated tissue preparations like frog rectus abdominus, leech dorsal muscle or guinea pig ileum. Although these methods are very sensitive and can measure ACh upto nanogram quantities, the variation can set in due to difference in the skill of the experimenter and also the method of isolation of ACh from the brain in addition to the mode of killing the animal. Recently, various methods based on gas chromatography (Szilagyi et al., 1972; Kilbinger, 1973) and coupled enzymatic assay(Fegenson

and Saelenes, 1969; Reid et al., 1971; Goldberg and McCaman, 1973; Shea and Aprison, 1973) and fluorometric determination (Fellman, 1969; O'Neil and Sakaamto, 1970; and Browning, 1972) and pyrolisis with mass fragmentography (Polak, 1979) have been introduced. However, each of these methods is limited in some manner of other by one or more factors such as (a) the number of samples that can be assayed at a time, (b) the need for a specialized equipment, (c) the use of expensive labelled substrates (with a short ~~life~~ half life period in case of ^{32}P-ATP), (d) the use of columns for each sample to separate labelled compounds, (e) varying sensitivity or low sensitivity in the assay, etc. and thus may not be suitable for routine analysis or the analysis of large number of samples. The method of isolated tissue preparations, particularly frog rectus abdominus tissue, still remains a method of choice in the small laboratories due to its simplicity and inexpensive cost of analysing large number of samples.

Similarly, ACh content of the brain also depends upon the method of extraction employed to extract ACh from the brain. Generally following types of extractants are used for this purpose : hot hydrochloric acid, cold trichloroacetic acid, cold $HClO_4$ and acid-ethanol (MacIntosh and Perry, 1950; Crossland, 1961; Hebb and Silver, 1969). The most commonly used extractant is 10 % TCA as advocated

by MacIntosh and Perry (1950). According to Perry (1949) with 10% TCA losses upto 10% occur in the actual value. As an extractant TCA has some disadvantages, phospholipids are incompletely precipitated (McIlwin and Rodnight, 1962), TCA must be removed from the extract before assaying and it gives spurious results while chromatographing the separation of ACh (McLennan et al., 1963; Crossland (1961) has advocated extraction with acid-ethanol after the tissue has been frozen in liquid nitrogen but the freezing has been found to be cumbersome and disadvantageous (Hobbiger and Werner, 1948; Morris et al., 1965). Extraction with 15 % formic acid in acetone gave yields of 144 % higher than those obtained with acid ethanol (Toru and Aprison, 1966).

I) Synthesis of ACh :

a) Provision of Choline for ACh Synthesis :

Cholinergic neurons require exogenous supply of choline for the synthesis of ACh. Neurons, unlike liver cells, can not synthesise choline de novo and hence it has to depend on the free choline of the plasma. The concentration of choline in the plasma is homeostatically maintained at a constant level, 10 to 20 µM, by renal and other mechanisms. The small pool of extracellular choline undergoes constant turnover, exchanging with both intracellular free choline and

the much larger pool of choline covalently bound within tissue phospholipid. Choline taken up by the neurons is utilized not only for the synthesis of ACh but also for that of betaine, phosphorylcholine and phosphatidylcholine. There is no evidence to suggest that the nerve endings can make direct use of either endogenous or exogenous phospholipids as a source of choline for the synthesis of ACh. Although there is some evidence to suggest that lysophosphotidylcholine in the circulation can provide choline for ACh synthesis in brain (Illingworth and Portman, 1972), the bound choline only becomes available for uptake only after it has been extraneurally made free.

A decrease in the concentration of choline in the extracellular fluid of peripheral tissues has been shown to affect the rate of ACh synthesis. Isolated ganglia and muscle in choline free medium show gradual fall in choline concentration followed by decline in ACh synthesis with concomitant decrease in the rate of ACh release during nerve stimulation (Birks and MacIntosh, 1961; Potter, 1970). These studies confirm that there is little tissue synthesis of choline and the plasma has to supply most of the choline required for ACh synthesis. The plasma levels of choline have been shown to be remarkably kept constant in man regardless of food intake, exercise, sex and pregnancy (Bligh, 1952).

Concentrative uptake of choline has been shown for number of tissues. In the earlier studies mechanism of choline uptake by isolated nerve terminals (Marchbanks, 1968a; Potter, 1968; Kennedy, 1969), brain slices (Schuberth et al., 1966), squid axons (Hodgkin and Martin, 1965), erythrocytes (Martin, 1968) and kidney slices (Sung and Johnstone, 1965) appeared to be the same. In each case choline enters the cellular element by a saturable carrier mechanism and by passive diffusion. The uptake was found to be competitively inhibited by choline analogues like ACh and hemicholinium. The non-competitive inhibition was found with eserine. In these studies, the Michelis constant for the uptake of choline appeared to be in the range of 10 to 100 μM for most tissues including brain. This mechanism did not appear to be energy dependent or to require specific ions and was unaffected by oubain.

The amount of free choline in the brain and other tissues has been reported to be in the range of 50 to 500 μMoles per kg. (Potter, 1970a; Schuberth et al.,1970). Since all tissue extraction procedures release some lipid bound choline, these levels could be little higher and according to Potter (1972) an internal level of 20 to 200 μM of choline would be more accurate. These levels are much below the affinity constant of choline, acetyl-transferase for choline (750 μM in brain according to Glover and potter,

1971) suggesting that the enzyme is not saturated with the substrate under normal conditions. This definitely hampers the rate of ACh synthesis as the choline concentration of 20 to 200 μM is much below that of ACh concentration of 50 mM in nerve terminals (Potter, 1972). These observations and the fact that the choline acetyltransferase of nervous tissue has to depend for its supply of substrate on a carrier that could not be efficiently loaded at a level of 10 μM, which is the plasma concentration of free choline looked quite odd especially when the perfused ganglia were shown to maintain the ACh synthesis at this level (Birks and MacIntosh, 1961).

Several other studies indicating that when brain synaptosomes are incubated with low concentrations of choline a significant amount of ACh is formed, suggested that there might be more than one uptake system for choline in the brain (Green and Haubrich, 1971; Haga, 1971). This paradox was finally resolved by the demonstration of two kinetically distinct systems for transport of choline into synaptosomes of rat brain (Yamamura and Snyder, 1972). A high affinity system was observed to be highly dependent upon the presence of sodium and was associated with major proportion of ACh formation. By contrast low affinity system was much less dependent on sodium and resulted in little ACh formation. The further studies revealed that the high affinity uptake system had Km values of 1.4 μM for

corpus striatum and 3.1 μM for cerebral cortex; the values for low affinity system were 93 and 33 μM for these two regions respectively. In the cerebellum, choline was accumulated by low affinity system. The high affinity system was localized in the synaptosomes and represents a selective accumulation of choline by cholinergic neurons while the affinity uptake system has some less specific function (Yamamura and Snyder, 1973). Polak et al. (1977) reported that in the rat brain cortical slices all choline used for the synthesis of ACh is supplied by the high uptake system and this system is geared up to the rate of ACh synthesis. Murrin et al. (1977) demonstrated that in rat hippocampal synaptosomes, releasable ACh is derived from the sodium-dependent high uptake system. Kuhar and Murrin (1978) concluded from their studies that this system is a rate limiting, regulatory step for ACh synthesis.

When released ACh is hydrolyzed in the synaptic cleft, most of the choline produced is recovered by nerve terminals for ACh resynthesis. The studies on the isolated or stimulated ganglia and phrenic nerves indicate that 50 % of the choline released during the hydrolysis of ACh is recaptured by the nerve terminals (Collier and MacIntosh, 1969; Potter, 1970).

b) Provision of Acetyl CoA for the Synthesis of ACh:

One of the important problems in the biosynthesis of ACh in the brain is the origin of the acetyl group in the acetyl-CoA which is used for this purpose. Although a number of substrates have been shown to be able to serve as acetyl group donors for ACh synthesis *in vitro*, it is difficult to assess their relative importance for the biosynthesis of ACh *in vivo* (Hebb, 1972; Tuček, 1973). An additional dimension to this problem has been added by less known mode of transport of acetyl CoA or the acetate in any other form across the mitochondrial membrane to the cytoplasm. Another feature of interest is that of metabolic compartmentation for Kreb cycle intermediates and acetyl-CoA in the brain (Balázs and Cremer, 1973; Nakamura and Cheng, 1969; Nakamura *et al.*, 1970).

There has been a general agreement that the acetyl groups of ACh are derived from the intramitochondrial metabolism of glucose (Browning and Schulman, 1968; Sorbo, 1970; Tuček, 1970). *In vitro* experiments with brain tissue have shown that ACh can be synthesized with acetate, citrate, glucose, fructose, lactate, pyruvate, acetoacetate, acetylcarnitine, glutamate, N-acetylated amino acids, etc. (for review : Tuček, 1973). Although preferential utilization of acetate for ACh synthesis have been observed in other organs than mammalian brain (Fitzgerald and Cooper, 1967;

Fitzgerald, 1968; Cheng and Nakamura, 1970; Israël and Tuček, 1974), acetate seems to be relatively a poor source for ACh synthesis which takes place outside the mitochondria. This is because of the observations that acetate has a low metabolic flow in the extramitochondrial space and also the enzyme required for the incorporation of acetate into acetyl-CoA, acetyl-CoA synthetase, is predominantly located in the intramitochondrial space (Potter, 1972).

Tuček and Cheng (1974) carried out detailed studies on the incorporation of different labelled substrates injected intracisternally, into ACh in the rat brains. This comprehensive study revealed that the following order of decreasing contribution of different precursors to the ACh synthesis : lactate, pyruvate, glucose; acetoacetate; 3-hydroxybutyrate; acetate; citrate; Leucine; glutamate. Since both glucose and lactate will first be transformed to pyruvate before being metabolised to acetyl-CoA, pyruvate alone, according to these authors, seems to be the most important source of acetyl groups in the molecule of ACh. This finding is in agreement with previously reported greater incorporation of labelled pyruvate, as compared to acetate, citrate or glutamate into brain slices (Nakamura et al., 1970). The conclusion about the important role of pyruvate in the synthesis of ACh agrees well with the results of comparative measurements of the activities of choline

acetyltransferase, acetyl Co-A synthetase, ATP citrate lyase and pyruvate dehydrogenase complex in brain homogenates and nerve ending fractions (Tuček, 1967b; 1967c), and with previous studies demonstrating the ability of the brain to utilize pyruvate and glucose for the synthesis of ACh (Quastel et al., 1936; Mann et al., 1939; Sattin, 1966; Cheney et al., 1969; Itoh and Quastel, 1970; Sollenberg and Sorbo, 1970).

Since pyruvate is the major precursor for acetyl group of ACh, theoretically any disturbance in pyruvate dehydrogenase complex activity should result in the lower rate of ACh synthesis. Thiamine deficiency is known to result in decreased levels of pyruvate due to impaired activity of pyruvate dehydrogenase complex which requires thiamine pyrophosphate as a cofactor (Gubler, 1976). Some of the studies have revealed that ACh levels are significantly lowered in thiamine deficiency induced either by feeding thiamine deficient diet or feeding thiamine antagonists to rats, (Cheney et al., 1969; Heinrich, et al., 1973). Contrary to this finding, ACh levels were not found to be affected in thiamine deficient animal by Speeg et al. (1970).

The inner mitochondrial membrane is generally regarded as having very little permeability to acetyl-CoA (Lowenstein, 1968). Several ways have been suggested by

which the acetyl groups of the acetyl CoA generated in the mitochondrial matrix from pyruvate and fatty acids may become available for reactions occuring outside the mitochondria (Kornacker and Lowenstein, 1965; Srere, 1965; Greville, 1969; Sterling and O'Neill, 1978). The main possibilities are :

1. Acetyl-CoA is transformed to citrate intramitochondrially and then citrate diffuses to the cytoplasm and there it is subjected to the citrate cleavage reaction catalyzed by ATP citrate lyase with the formation of new acetyl-CoA ;

2. Citrate is further converted to alpha-keto glutarate and glutamate in the intramitochondrial space and then diffuse out and these compounds are converted back to citrate and acetyl-CoA extramitochondrially ;

3. Acetyl-CoA is converted to acetylcarnitine, the latter leaves the inner compartment of mitochondrion and is used for new synthesis of acetyl-CoA either in the outer compartment of mitochondrion or in the cytosol ;

4. Acetate is formed from acetyl-CoA in the mitochondrion, diffuses to the cytosol and is used for the synthesis of extramitochondrial acetyl-CoA ;

5. Acetyl-CoA leaves the mitochondria as such.

All these possibilities have found some experimental justification, but at the same time none of these modes of transport for intramitochondrial precursor of acetyl group of extramitochondrial acetyl-CoA have been conclusively proved. The problem of how mitochondrially generated acetyl-CoA can give rise to cytoplasmic acetyl-CoA has been a continuing puzzle.

B) Choline Acetyltransferase : (EC: 2.3.1.6)

Choline acetyltransferase, the enzyme responsible for synthesis of ACh from choline and acetyl-CoA, was recognized as a distinct enzyme by Nachmansohn and Machado in 1943. Since then many workers have tried unsuccessfully to obtain pure enzyme. Husain and Maunter (1973) have prepared an enzyme with a specific activity of 67 µmoles per minute per mg of protein, isolated from the squid ganglia, which with the fly brain is the richest known source (Smallman, 1956). In vertebrates, an enzyme with such a high activity has not yet been obtained. The highest specific activity, 2.4 µmole per minute per mg of protein was obtained by Morris (1966) starting from the immature human placenta, which is the richest ChAc source in vertebrates. Recently, Rossier (1976) has purified rat brain ChAc to the extent of a 30,00 fold with a specific activity of 20 µmoles per minute per mg of protein. This preparation was not found to be

homogenous on polyacrylamide gel electrophoresis. According to the author 1,50,000 fold purification will be required to obtain a homogenous preparation which would give approximately a specific activity of 100 μmoles per minute per mg protein.

ChAc, extracted from the nervous tissues of different species and partially purified, is a stable, relatively basic protein, the molecular weight of which is about 65,000 (Potter, 1970). There is some evidence that the native enzyme has a higher molecular weight and, during isolation, gives rise to two fragments that differ in size and activity. The positive charge on the molecule varies with species and several isoenzymes may be present in a single tissue (Fonnum, 1973). The composition of the ChAc molecule has not been very well studied, the basic amino acids are known to predominate, the imidazole ring is postulated to participate at the active site. This enzyme has been regarded as a sulphydryl enzyme, but whether the SH groups lie close to the active center has not yet been studied.

The substrate specificity of ChAc has been very well studied and found to be limited to mostly choline and choline esters and acetyl and acyl groups only. The ChAc activity is reportedly decreases as the number of methyl groups attached to choline N atom is reduced (Berman et al., 1953). However, Burgen et al. (1956) reported increase in

the activity to the extent of 40 % with the substitution of one of the methyl group by an ethyl group. These results could not be confirmed by others. Dauterman and Mehrota (1963) and later Hemsworth and Morris (1964) found that of the three analogues of choline, monoethyl choline was acetylated at 50 % of the rate obtained with choline, but the other two analogues failed to be acetylated. Since the analogues of choline do not compete effectively with choline for ChAc, they are unlikely to be utilized as substrates by ChAC (Hemsworth and Morris, 1964). The ChAc from mammalian brain has been reported to synthesize propionyl-Ch at 30 % rate that of choline (Berry and Whittaker, 1959). These workers also found additional choline acylases which can synthesise C_4-C_6 esters and palmitylcholine respectigely.

The ChAc-catalysed reaction is reversible with the large equilibrium constant toward ACh formation, the value of Keq at pH 7.0 and 37° being $5.1 \times 10^3 \pm 0.48 \times 10^3$ (Schuberth, 1966). Estimated Michaelis constants for choline and acetyl-CoA are 1 mM and 10 µM respectively. A number of quartenery ammonium compounds are reported to inhibit ChAc; tetramethylammonium chloride, at the concentration of 30 µmoles per ml inhibited the activity by 60 %; the tertiary analogue of neostigimine at 5 µmoles/ml inhibited the enzyme by 70 % (Reisberg, 1957). Both ACh and bromoacetyl-choline are found to inhibit the ACh synthesis without competing significantly

with either choline or acetyl-CoA (Glover, 1970). Potter (1972) has suggested that both rate of net synthesis of ACh and the level of free ACh in the cytosol depend upon the equilibrium position of ChAc with respect to its substrates and products.

The regional distribution of ChAc correlates well with that of ACh, their concentration from one part of the brain to another exhibit roughly similar variations. For all regions of gray matter that have been studied in the mammalian brain, ChAc and ACh are consistently found to be highest in the caudate or its associated nuclei and lowest in the cerebellar cortex (cf: Hebb and Morris, 1969). In the adult rabbit brain, ChAc has the following distribution as reported by McCaman and Aprison (1964) : cortex, 1.2; medulla, 1.32; thalamus, 1.38; superior calliculus, 1.47; and caudate nucleus, 5.77 μmples of ACh formed per g wet weight per hour.

Since subcellular fractionation studies have revealed that ACh is stored and released from several different pools or metabolic compartments within the nerve terminals, the studies on metabolic compartmentation of ChAc has acquired a special importance. This problem has been complicated by two main factors; high affinity of the enzyme to the membrane due to its strong positive charge and the differences in molecular

forms of ChAc in the different species. A high degree of controversy exists in the literature regarding the ultrastructural localization of this enzyme (De Robertis et al., 1963; Whittaker et al., 1964; McMann et al., 1965; Tuček, 1966; Fonnum, 1966; 1967; and 1973).

The available evidence suggests that ChAc is present in all parts of neurons that are cholinergic and that it may be one of the proteins which is formed in the cell body and migrates, by some form of flow along the axon to the nerve endings. This hypothesis is yet to receive experimental testing and confirmation. However, 50-70 % of the enzyme and the same proportion of ACh has been found in the fraction of brain homogenate which contains most of the nerve endings (Hebb and Smallman, 1956; Hebb and Whittaker, 1958; Gray and Whittaker, 1962). To explain the formation of vesicular ACh, Fonnum (1973) has theoritically summarized the following mechanisms of the possible membrane-ChAc interactions :-

1. ChAc is evenly distributed within the cytoplasm of the nerve terminal and ACh enters the vesicle only by an uptake mechanism.

2. A part of the ChAc is attached to the outer surface of the vesicle thereby establishing a high local concentration of ACh around the vesicle, thus enhancing ACh uptake.

3. A part of the ChAc is bound to the small membrane structures that may in some way be involved in the storage of ACh within vesicles.

4. ChAc is bound to the presynaptic axon membrane and is thus able to acetylate any choline entering the synaptosome from the synaptic cleft.

Until the problem of the subcellular localization of ChAc is not resolved the exact localization of ACh synthesis in the neuronal cell body will remain unsolved. Although the synaptic vesicles are capable of assimilating choline and converting the same into ACh (Marchbanks, 1968[a,b]; 1969), the vesicles are also capable of taking up preformed ACh in the suspending fluids (Guth, 1969). These findings suggest that neuronal cell body may synthesize ACh in more than one compartment.

The *in vitro* and *in vivo* studies suggest that synaptosomal ACh is localized in two compartments - Vesicular and cytoplasmic. The incorporation of labelled choline, in both the studies, result into poor synthesis in the former compartment (Whittaker *et al.*, 1964; Marchbanks, 1969; Richter and Marchbanks, 1971[a,b]). The high molecular weight fraction of synaptosomal ACh pool has been shown to be active one and is credited with functional importance (Aquilonius *et al.*, 1973).

The rates of ACh synthesis in the different areas of the brain are in agreement with the distribution of ChAc in these areas. McIllwin and Bachlelard (1971) have compiled the rates of ACh synthesis in the following areas : rate of ACh synthesis (μmoles/g fresh tissue/hr) cerebral cortex, 1.3 - 3.7, cerebellar cortex, 0.09; caudate nucleus, 13.0; thalamus, 3.1 and hypothalamus, 2.0. The whole brain tissue is estimated to have a rate of 3.0 μmoles/g/hr and may utilize 0.3 to 1 % of metabolically derived energy for this purpose.

The regulation mechanism for ACh synthesis is not yet clear, but it may be presumably dependent upon the concentration of ACh, choline and acetyl-CoA at the site of synthesis particularly in the manner of substrate-product ratios for ChAc. Recently is has been reported that the rate of ACh synthesis is proportional to plasma concentration of choline, acetyl-COA, rate of choline uptake by high affinity system and is regulated by the latter (Cohen and Wurtman, 1976; Lefresne et al., 1978; Jope and Jenden, 1977; Kuhar and Murrin, 1978).

C) Storage of Acetylcholine :

a) Site of Storage : As mentioned earlier, ACh is mainly present in the cholinergic nerves with the possible exception of the placenta of higher primates (Morris, 1960)

and corneal epithelium (Williams and Cooper, 1965). ACh is presumed to be present in association with ChAc in the cell bodies and dendrites of vertebrate neurons, but its concentration and exact location is still not clear. The percentage of total ACh present in the neuronal endings vary with the length of the axons. The proportion of total ACh present in the nerve endings of the cerebral cortex is about two-third, it is higher in the caudate nucleus and lower in the spinal cord according to Whittaker (1965). The estimated vesicular content of ACh is more than 80 % of the total in the nerves (Israel et al., 1968; Israel and Gautron, 1969). According to Barker et al. (1970) there would be 2,000 ACh molecules in a synaptic vesicle which would make the ACh concentration in the vesicle core about 0.2 M if all the ACh was in the free ionic form. It is interesting to note that Torpedo vesicle has a concentration of 0.065 M ACh which is equal to plasma electrolytes concentration (Marchbanks, 1973).

Whenever a peripheral tissue supplied by cholinergic axons is treated with a suitable anticholinesterase, its ACh content rises during an hour or so to about twice its initial value, even in the absence of nerve impulse (Potter, 1970; Collier and Katz, 1970). The ACh formed in this manner is called as a surplus ACh, it is intracellular and presynaptic

in location. According to Collier and Katz (1970) this type of ACh in ganglia and muscles can not be released by nerve impulses. However, it can be released by K^+ and by nicotinic agonists including ACh itself. These agents in the normal course do not release ACh from synaptic vesicles. This phenomenon is so far restricted to peripheral synapsis and the evidence that it occurs at central cholinergic synapses is lacking.

b) Synaptic Vesicles : Homogenization of brain tissue results in the formation of synaptosomes. This formation takes place by shearing of nerve terminals from their axonal connections and surrounding glial elements, these terminals are resistant to mechanical stress, the detached terminals are capable of resealing to form independent nerve ending particles i.e., synaptosomes. The synaptosomes consist of presynaptic membranes, synaptic vesicles and postsynaptic membrane fragments along with other macromolecules present in the nerve endings. Although the synaptosomes are the artefacts of the methodology of subcellular fractionation, they can be regarded as miniature, nonnucleated neurons for studying the various phenomena taking place in the synaptic endings and the junctions. The synaptosome is an excellent source for isolating the synaptic vesicles which are present in the presynaptic membranes.

In electron micrograph from intact cerebral cortex, the vesicles are seen in the nerve terminals as small bodies, spherical or oval in profile and about 0.05 micron in diameter, bounded by membrane of thickness of 40-50° A. The vesicles have been reported to be seen in the axoplasm either in isolated forms or in an orderly fashion along the neurofilaments giving rise to the speculation that the vesicles may be synthesised elsewhere in the cell body and transported to the nerve terminals. The synaptic vesicles can be separated from the synaptosomes using differential centrifugation. Such a preparation of synaptic vesicles from the rat brain was found to contain 45% of its dry weight as lipids with a high proportion of gangliosides (Burton and Gibbons, 1964). According to Eichberg, Whittaker and Dawson (1964), the composition of lipid components in the vesicles is as follows : phosphatidylethanolamine 15 %, phosphatidyleserine 11 %, phosphatidylinositol 5% and phosphatidylcholine 41 % of total lipids. The purified vesicles additionally contain one enzyme - Mg^{2+} activated ATPase which may be involved in the release of neurotransmitters (Poisner, 1970).

c) ACh compartmentation in the neuronal cells :

The problem of ACh compartmentation is two-fold - one is that of morphologic compartment and other is

the biochemical or metabolic compartment. While the former is somewhat easier to locate with the help of sophisticated techniques like electron microscopy which has better resolution to distinguish between the ultrastructural components of the nerve cell, the latter poses a special problem as no histochemical method exists which can pinpoint the metabolic pools of ACh with different biochemical movements in the cell body.

The morphological compartmentation of ACh can be said to be that of axonal or cytoplasmic ACh in the elongated axons and the other one being in the synaptic vesicles or at the nerve terminals. As earlier mentioned, cytoplasmic ACh (free ACh) fraction constitutes about 20 % of total ACh in the cholinergic neuron. The concentration of axonal ACh is about 0.3 mM. The rest of the 80 % ACh is believed to be present in the nerve endings. When the nerve terminal fraction was osmotically ruptured, it was found that some 50-60 % of the ACh in the preparation survived and could be recovered in the fractions containing synaptic vesicles (Whittaker et al., 1964). This particular fraction of the ACh is termed as "stable-bound ACh" and the rest of ACh in the nerve endings as "labile-bound ACh". Assuming, on the basis of the frequency of cholinoceptive cells in the cerebral cortex, that 14 % of the vesicles are cholinergic, Whittaker and Sheridan (1965) found about 2300 molecules of ACh in each vesicle from the

cerebral cortex giving the concentration of 0.2 M inside the vesicle provided all the ACh is present in the free form. As earlier mentioned the vesicular ACh has been found to be present in two pools - one that is large pool of low specific activity still bound in the vesicles and a small pool with a high specific radioactivity for ACh from the incorporation of labelled choline (Marchbanks and Israel, 1973). The problem of surplus ACh has already been discussed in the context of ChAc compartmentation.

B) Release of Acetylcholine :

a) Quantal Release of ACh :

The early work of Loewi, Dale, Feldberg and their collaborators which first indicated the occurence of cholinergic transmission at various sites has been extensively confirmed and amplified by subsequent investigators. The sequence of events which is now generally accepted as taking place during the passage of an impulse across a cholinergic synapse, such as those of the autonomic ganglia, is as follows (Grundfest, 1957) : With the arrival of the Nerve Action Potential (NAP) at the terminals of the preganglionic axon, the transmitter, ACh, is liberated from an intra-axonal storage site; it diffuses across the narrow synaptic cleft and combines with receptor groups on the ganglionic cell

membrane, causing the development of a localised non-propogated depolarisation known as the postsynaptic potential (PSP), the latter initiates electrogenically a NAP, which is propogated along the postganglionic fibre; the polarised state of the postsynaptic membrane is restored with the rapid destruction of the synaptic transmitter by the enzyme, acetylcholinesterase (AChE).

With the advent of microelectrodes, it became more obvious that the synapses were not very silent even when the nerve impulse is absent. Instead, there were spontaneous and random discharges of the post-synaptic membrane about once a second, amounting to a depolarization of 0.5 mV and lasting about 20 msec. These miniature end plate potentials (mepps) were shown to be due to ACh and were blocked by curare and potentiated by AChE inhibitors. Mepps can not be attributed to single or few molecules of ACh since the effect of applying graded doses of ACh at endplates is graded levels of depolarization. Thus, each mepps must be the result of the arrival of many molecules of ACh at post-synaptic membrane. These molecules must have been released at the same time from the nerve terminals as a burst or'quantum' package (cf: Potter, 1973).

The release of quantum depends upon the presence of calcium ions in the synaptic cleft at the moment of

depolarization of the nerve terminal membrane and on the ability of Ca^{2+} to move down a concentration of electrical gradient into the nerve terminal. The number of quanta released generally varies in a graded way with the degree of depolarization of the presynaptic membrane and as a power function of the extracellular calcium concentration, e.g. $(Ca^{2+})^4$ at endplates in the frog sartorius muscle (Dodge and Rahaminoff, 1967). Release is facilitated for short periods after one or more depolarizations of the nerve membrane apparently because Ca^{+2} ion accumulates within the terminals. Sodium ions compete with calcium ions on the outside and probably on the inside, of the presynaptic membrane. Release does not depend upon the entry of Na^+ during depolarization, which can be blocked by terodotoxin or upon the egress of K^+, which can be blocked with tetraethylammonium ions (Potter, 1973).

There is some variation in the rate of release of ACh depending upon the total store of the same in the neurons. Birks and MacIntosh (1961) found that the ganglia when stimulated with hemicholnium-3 (HC-3) released more slowly till the rate became proportional to the remaining ACh. Jones and Kwanbunbmpen (1970) demonstrated the delayed release of ACh from phrenic nerve terminals treated with HC-3. These results suggest that the part of total ACh is more rapidly released than the rest. This decreased

order is in agreement with the another finding which suggests that released ACh is mainly derived from the recently synthesised ACh pool (Potter, 1970).

b) Possible release mechanisms :

Number of possible mechanisms to explain the release phenomenon of ACh in the presynaptic regions have been put forward by various workers. All most all of these theories are lacking sound experimental evidence to substantiate the actual mechanism in vivo .

1. One of the classical postulated mechanism is that of calcium activated pores on the presynaptic membranes. It suggests that ACh is released in bursts by outward diffusion through calcium-activated pores in the presynaptic membrane. Such a mechanism seems to be unlikely because it would mainly depend upon the higher concentration of ACh in the nerve endings, whereas the ACh release is known to take place even if its concentration goes down and it does not increase with surplus ACh. Secondly, one would expect such a release to be dependent on the membrane potential changes requiring special channels instead of the pores exposed by smaller hydrated ions like potassium.

2. Another postulate is that of calcium-activated carrier molecules. This mechanism seems to be unlikely

because of the fact that it would require full saturation of the carrier molecule with ACh in the axonal cytoplasm and any fall in ACh concentration would decrease the quantal size; such a thing does not happen in experimental findings; such a carrier would appear to operate independent of changes in the membrane potential and this would be incomprehensive. Additionally, this hypothesis does not explain the evolution of synaptic vesicles. The known rates of carrier mediated reactions are less than 1 ion/msec/site and this would fall short of the rate of 10^3 ions/msec observed in the release of ACh.

3. The hypothesis that ACh release is regenerative i.e., that released ACh, besides acting post-synaptically, also acts presynaptically to release more ACh, has not withstood critical testing (MacIntosh and Collier, 1976). Although, axonal and pre-synaptic ACh receptors exist and have nicotinic properties, but they play very little part in the normal transmission and moreover, activation of presynaptic receptors appears to reduce and not increase ACh release (MacIntosh, 1976).

4. Since caffeine and dibutyryl cyclic AMP are found to increase the nerve evoked ACh release at the neuromuscular junction, the cyclic nucleotides have been

postulated to be involved in the ACh release mechanism. Although the numerous examples of mutual feedback between Ca transport and nucleotide-cyclase systems make such a mechanism plausible there is no convincing evidence so far to indicate the existence of such a mechanism in in vivo condition.

5. Stimulation of sympathetic or parasympathetic nerves is associated with release of prostaglandins, mainly PGE_2 and PGE_2 alpha. Hedquist (1973) has suggested that the involvement of prostaglandin in the negative feedback control of norepinephrine release. However, such a role is not involved in cholinergic system.

6. At present the best and most credible hypothesis seems to be that of vesicular release of ACh. The vesicles have all the components of ACh synthesising system. This hypothesis visualizes the following sequence : Nerve stimulation results into action potential which depolarises the presynaptic membrane through the influx of Ca^{+2} ions; the adjacent vesicles bind to Ca^{+2} ions and fuse with them, this stimulates the transient rupture of the membranes alongwith emptying of vesicular contents into the cleft. This is called as exocytosis. Ca^{+2} activated ATPase may trigger the withdrawal of the vesicles into cytoplasm following the removal of Ca^{+2} ions (Hammerschlag and Roberts, 1976).

However, this hypothesis will hold ground provided the descripancy of number of ACh molecules present in the vesicles and those present in the quanta agree with each other.

c) Action of Acetylcholine :

The bulk of the evidence now available indicates that ACh exerts its effect on the postsynaptic membrane by increasing the permeability of this membrane (Edelman, 1961). Several mechanisms have been proposed to account for this effect. One of the theories involves the reversible interaction of ACh with a constituent of the receptor membrane producing conformational changes which results in the increased permeability. Nachmansohn (1955) suggests that ACh on release may react with a protein moiety on the post-synaptic membrane. These receptors are now purified and found to be specific proteins; these receptors are strongly bound to the membrane; they not only combine reversibly with ACh but irreversibly with certain toxins like alkylating agents similar to ACh and venom toxins like alpha-bungarotoxin of the krait (Mac-Intosh, 1976).

The propogation of the post-synaptic potential is believed to take place through the mechanism which is

not very clear at this stage. There is a strong possibility that cyclic nucleotides may be involved actively in the propogation of PSP (Greengard, 1976).

d) Removal of Acetylcholine :

Acetylcholine is rapidly hydrolysed by two distinct classes of esterases known as pseudo- and true-cholinesterase (Mendel et al., 1943). Although the former category is found in all tissues including the central nervous tissue it may not contribute significantly to the hydrolysis of endogenous ACh released at the presynaptic membrane because it is not found significantly at the synapses. It seems possible that it may have more general metabolic role in the CNS. Its involvement in the ACh hydrolysis at muscarinic receptors has been suggested (Hebb and Silver, 1969).

A number of possible roles for pseudocholine-esterases have been mooted and for each there is some evidence but for none is convincing and unequivocal. Functions proposed for the enzyme outside the nervous system include its involvement in assimilation of food (Gerebtzoff, 1959), in the destruction of any butyrylcholine which might be produced in fatty acid metabolism (Zeller and Bissegger, 1943), or plasma choline levels (Funnel and Oliver, 1965),

in the metabolism of lipids (Ballantyne, 1967), in the regulation of tissue growth (Ballantyne and Burwell, 1965) and in membrane permeability with a particular emphasis on ionic movements. There is an equal scope for speculation about the function of pseudocholinesterase in the nervous system. The predominant site of its activity is in the white matter where the enzyme is mainly associated with glia although in some species it is also found in certain neurons. Earlier, there was a general belief that the enzyme was involved in the maintenance of myelin (Bloch, 1941) and subsequently this belief was proved to be quite erronous. Desmedt and La Grutta (1955) advocated its involvement in the neuronal function but this did not find much needed experimental support (Silver, 1974).

e) Acetylcholinesterase : (EC : 3.1.1.7)

The natural substrate for AChE is assumed to be ACh which is the predominant ester of choline found in the tissues. AChE preparations show maximum activity towards ACh but in some species it is found to have equal affinity towards other esters like propionylcholine (Banister et al., 1953). This enzyme has a low activity towards butyrylcholine as compared to ACh, but in certain species including rat AChE splits butyrylcholine to a significant extent (Bayliss and Todrick, 1956). Since the part of the

active site of AChE is negatively charged which enables the enzyme to accept cationic substrates like esters of choline and thicholine the enzyme is also capable of hydrolysing uncharged substrates like the carbon analogue of ACh, 3,3-dimethylbutyl acetate in which the quarternary nitrogen is replaced by an uncharged carbon atom. Adams and Whittaker (1948) and Adams (1949), using human erythrocytes AChE, showed that with aliphatic esters, as with choline esters, the nature of the acyl group determines the rate of hydrolysis. The rate of hydrolysis decreased in the following order :-acetate : propionate: formates: butyrates.

Cholinesterases, in general are known to be inhibited by physostigmine and organophosphorus compounds. A large number of anticholinesterase compounds have been found out; their mechanisms of action has been worked out; the significance of their inhibition towards the evaluation of role of AChE in the ACh hydrolysis has been a central theme of many a researches. Many of these compounds have been commonly used as experimental tools in the physiological, biochemical or histochemical investigation of cholinergic mechanisms.

The normal reaction between a cholinesterase and its substrate involves the attachment of the substrate to

the enzyme with the production of an acylated form of the enzyme which undergoes a rapid hydrolysis to yield reusable enzyme. Anticholinesterases interfere with this orderly sequence, the point of attack depending on the nature of the inhibitor. They may block a) the anionic site, b) the anionic and esteratic site, or c) the esteratic site, but in almost all cases the reaction is to some extent similar to that between substrate and enzyme. Some inhibitors are almost equally active against AChE and BuChE, but others have a preferential action on one enzyme or the other. None of the inhibitors is absolutely specific to one type of cholinesterase. The differential action is one of degree only and is reflected in the different concentrations at which the inhibitor affects the two types of enzyme. The effects of an inhibitor in <u>in vivo</u> and <u>in vitro</u> conditions differ depending upon the detoxification it undergoes in the liver and also the degree of its penetration into the site where ACh is stored.

To mention few of these compounds the following examples are cited :-

Inhibitors binding to the anionic site	:	Decamethonium, tetraethylammonium, tetraphenylammonium, edrophonium.
Inhibitors binding both the sites	:	Physostigmine, echothiopate, etc.
Inhibitor binding to esteratic site	:	Paraoxan, DFP, etc.

Substances which activate =AChE are less numerous and include cadmium, magnesium, lithium, potassium and sodium. Calcium which is an activator of pseudocholinesterase has a differential action on AChE of different species; it has a very little effect on horse erythrocytes (Cier et al., 1970) but activates human erythrocytes (Heller and Hanahan, 1972) and electric eel electroplaque AChE (Gridelet et al., 1970).

An important characteristic of AChE is its susceptibility to inhibition when substrate is present in excess. This particular property of the enzyme distinguish it from pseudocholinesterase. The V_{max} and K_m values of electrical eel AChE for ACh chloride 545 μmoles/hr/mg protein and 1.4×10^{-4} M at pH 7.6 (Ecobichon and Israel, 1967). Bovine brain AChE preparation has two major forms with the molecular weights of 430,000 and 291,000; highest specific activity of the order of 13,500 μmoles/hr/mg protein and K_m of 0.87×10^{-4} and 1.4×10^{-4} M at pH 7.8 respectively (Chan, et al., 1972). The rat brain AChE has been found to have two active forms with the molecular weights of 115,000 and 435,000; during the maturation of the brain the proportion of the latter form increased from 50 % at birth to 90 % in adult rat (Rieger and Vigny, 1976). Depending upon the method of solubilization and the separation, different investigators have reported different multiple

forms for rat brain AChE. Crone (1971) reported two forms with molecular weights of 100,000 and over 200,000; Wehthold et al. (1974) reported four different forms of AChE with molecular weight ranging from 150,000 to 650,000.

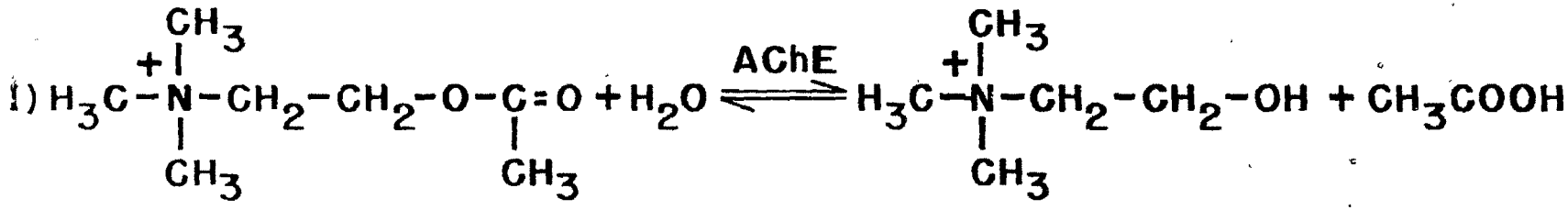
AChE is a membrane bound enzyme and it can be made free with the use of detergent or polar organic solvent. As mentioned above the enzyme can be broken down to number of subunits but it is not clear what these represent in terms of enzyme function. Leuzinger et al. (1969, 1971a, 1971b) suggested that electric eel AChE was a dimer with two non-identical protomers; each subunit possesses two active sites; there are two theories put forward by these workers :- i) each active centre would be built up from the alpha chain which provides the serine for the esteratic site while the beta-chain provides the anionic site; ii) only the alpha contributes to the active centre providing both the sites and beta-chain was of unknown function but could represent ACh receptor. Kremzner and Wilson (1964) are of the opinion that AChE has four active sites. A suggestion that electric eel AChE contains not 2 or 4 active site but 6 has come from Millar and Grafius, (1970). According to Bajgar and Zizkovsky (1971) rat brain has two forms of isozymes for AChE and one for BuChE with difference in Km values and pH optima as well as electrophoretic mobilities.

As shown in Figure No.4, AChE brings out the hydrolysis of ACh. To understand the intermediate steps in the reaction it is necessary to known some thing of the nature of the active centre of the enzyme. According to Wilson (1951) the active centre of the AChE has to subsites a negatively charged anionic site and an esteric site containing both the acidic (electrophilic) and a basic (nucleophilic) group (respectively A and B). At the simplest level, ~~the level~~ the events occuring in the hydrolysis may be described as follows :

1. The quarternary nitrogen of ACh is bound by Coulombic and Van der Waals' forces to the negatively charged anionic site and so oriented that the carbonyl group is presented to the esteratic site and an enzyme substrate complex is formed ;

2. Electron shifts occur at the esteratic site with the result that choline is liberated leaving an acyl-enzyme;

3. The acetylated enzyme is hydrolysed very rapidly yielding acetic acid and resulting in the restoration of the esteratic site to its inactive state.



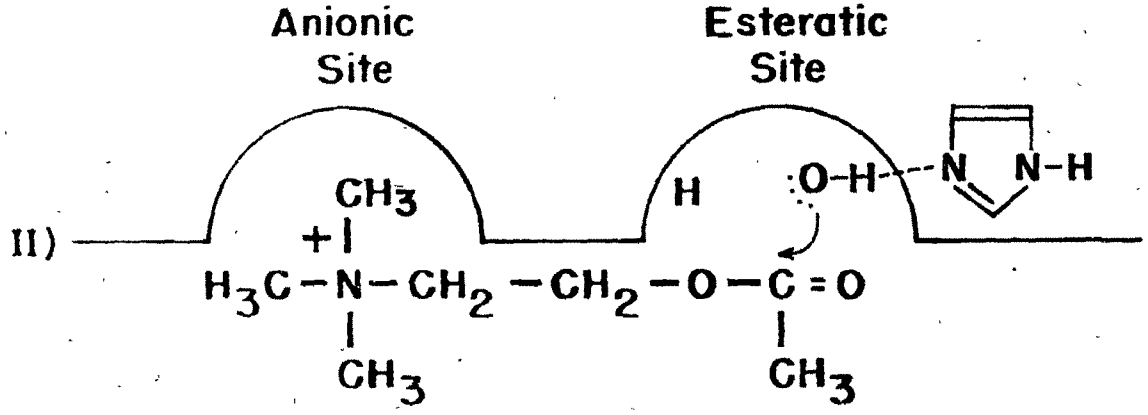


Fig. no. 4 –

I Hydrolysis of ACh by AChE.

II Schematic presentation of interaction between groups of AChE and substrate (from Wilson, 1967).

The regional distribution of AChE in the central nervous system has been a theme of many of the studies using either histochemical or biochemical technique. AChE distribution has been extensively studied in birds, fish, invertebrates and vertebrates (Silver, 1975). Table-3 gives some idea regarding the AChE distribution in the different areas of the rat brain. In general, the distribution of AChE is coextensive with the distribution of ChAc, but there is an exception. In almost all mammals (but not the rat) the cerebellar cortex has a very high concentration of AChE; in man it is only exceeded by the basal ganglia and substantia nigra. Contrary to this ChAc concentration in the cerebellum is very low. This is one of the reasons for thinking that AChE has some more function in the brain other than its role in ACh metabolism (Hebb and Morris, 1969).

Nathan and Aprison, (1955) demonstrated by subcellular fractionation that 90 % of the AChE in rabbit caudate nucleus was present in the cytoplasmic particulate fraction, the higher proportion being present in the mitochondria and microsomes. These findings were broadly confirmed by the subsequent studies (Aldridge and Johnson, 1959; Toschi, 1959). The electron microscopy examination revealed that AChE is confined to the membrane component (Hanzon and Toschi, 1959). De Robertis et al. (1962)

Table- 3 : Comparison of ChAc and AChE activity in certain areas of rat brain (Goldberg & McCaman, 1967).

Tissue	Choline acetylase*	Acetylcholin-esterase ‡
Mammilary body	52.5 ± 4.93 (5)	3498 ± 638 (4)
Caudate nucleus (Head)	22.0 ± 3.24 (7)	3866 ± 479 (5)
Dorsal grey composed of Gracilis, Cuneatus and Spinal 5th nuclei	12.7 ± 0.21 (5)	1760 ± 75 (3)
Olfactory bulb (Undissected)	11.7 ± 1.20 (8)	1693 ± 316 (4)
Thalamic nuclei (Caudal portion)	10.6 ± 0.98 (10)	2010 ± 258 (4)
Hippocampus	9.88 ± 1.15 (5)	2692 ± 303 (4)
Thalamic nuclei (Rostral portion)	4.60 ± 0.89 (9)	1037 ± 144 (4)

* Expressed as μmoles of acetylcholine synthesised/g dry wt/hr. ± Standard Error (number of sections).

‡ Expressed as μmoles of acetylthiocholine hydrolysed/g dry wt/hr. ± Standard Error (number of sections).

fractionated whole rat brain and demonstrated that the highest proportion of AChE is restricted to synaptosomes. The question of the precise location of AChE either near the vesicles, on the inside or outside of the presynaptic membrane, or in the cleft or the postsynaptic membrane is yet to be resolved in face of the vast mass of conflicting data. Koelle ~~et al~~. (1963) classified the AChE into two forms : one is the "functional AChE" which is present on the presynaptic membrane and facing outside and the second form is the "reserve AChE " present in the presynaptic membrane but facing inside the nerve terminal.

AChE seems to be synthesised in the axoplasm and then transported by not so well known mechanism to the nerve terminals. The precise location of the synthesis, regulation of the synthesis and the mode of transport are as yet far from clear understanding. Yaksh et al. (1975) reported recovery half ~~the~~ life times of average 3.6 days for AChE in the cat brain.

5) Turnover of Acetylcholine :

The rate of formation and the rate of disappearance of the radioactive ACh formed in the brain from intravenously injected labelled choline, have been used to determine the turnover rate of ACh in the brain. By

measuring both the initial rate of formation of labelled ACh and the isotope dilution of the labelled choline in the brain the turnover rate was estimated at 50 nmoles/g brain/min. in mouse (Schuberth et al., 1970). However, the subsequent work of the same authors suggest this rate to be about 20 nmoles/g brain/min. (Sparf, 1973). The ACh turnover rates in the different regions of the rat brain have been reported by various workers; Racagani et al. (1974) reported the following values :-

Region	ACh turnover rate (μ-mol/g/hr)
Striatum	1.3 ± 0.063
Hippocampus	0.52 ± 0.034
Occipital cortex	0.20 ± 0.010
Limbic cortex	0.20 ± 0.035
Brain stem	0.092 ± 0.0074

In general, the turnover rate of 5 % per minute in the resting state of the brain in situ have been reported (Sparf, 1973). The synaptic activity is believed to accelerate the synthesis and release of ACh with a concomitant increase in the ACh turnover rate; the increase in the rate of the order of 7-10 % has been reported in the stimulated ganglia (Birks and MacIntosh, 1961).

The methodology to study the turnover rates of different neurotransmitters has opened a new avenue to study the effects of drug action and different stress conditions on the central nervous system.

6) Central Cholinergic System and Behaviour :

With the accumulation of vast data on the cholinergic systems, it is not surprising that attempts have been made to correlate the central cholinergic mechanism with behaviour. Because of numerous difficulties it has not been possible to identify the particular cholinergic system in the CNS with specific behaviour.

The striking symptoms exhibited by human beings subjected to overdose of cholinergic antagonist or anti-cholinesterase agents are tempting to convince that cholinergic systems are involved in a number of vital functions and in behaviour. In general, ACh and other cholinomimetic agents stimulate the cerebral cortex (and hippocampus) and cholinolytic agents depress it. Additionally the inhibitory action of ACh on a system of intra-cortical neurons has been reported (Phillis and York, 1968).

Several reviews have described the possible functional roles of central cholinergic systems in some

details using the data obtained in histochemical, biochemical, phramacological and behavioural studies (DeFeudis, 1974); Several major functional roles for central cholinergic systems have been suggested. The cholinergic elements appear to be involved in the function of non-specific, reticulocortical pathways which are responsible for cortical arousal and hence for effective cortical function and behaviour. Cholinergic system has been implicated to be involved in behavioural regulation (Reeves, 1966; Russell, 1969; Fibiger et al. , 1970), in motor reflexes and postural mechanisms since the direct injection of cholinergic agents into extrapyramidal system cause tremors, catalepsy and "circling" behaviour (Mennear, 1965 ; Connor et al., 1966; Costall et al., 1972), in the central control of motor integration and coordination since cholinergic system innervates cerebellum, in the central autonomic mechanisms, in homeostatic functions and motivated behaviour. Further studies have shown the possibility of the involvement of cholinergic mechanisms in emotionality, learning and memory and mental functions (DeFeudis, 1974). Some of the roles in which cholinergic system may be involved based on experimental observations are listed in Table- 4.

Table- 4 : Some effects of intracerebrally administered cholinergic agents on physiology and behaviour *

AGENT	Site of injection (or implantation)	Species	Effect	Reference
ACh	H	Cat, Dog	Effects on blood pressure, heart rate and respiration	Dikshit (1934); Chand et al. (1937; 1938).
	V	Human	Thermoregulatory response	Henderson & Wilson (1936)
	Hip	Cat	Emotional-affective responses, attack	MacLean & Delgado (1953)
	LH	Rat	Polydipsia in sated subjects	Grossman (1950)
	S, PO, Cau.	Cat	Rage reaction	Hernandez-Peon & Chavez-Ibana (1963)
	Am	Cat	Aggressiveness, seizures, behavioral inhibition, sleep	Allikmets et al. (1969).
	PO/AH	Rat	Immediate decrease in body and hypothalamic temperature	Kirkpatrik & Lomax (1970); Grawshow (1973).

Continued....

Table- 4 (Continued)

AGENT	Site of injection (or implantation).	Species	Effect	Reference
Carbachol	LH	Rat	Polydipsia in sated subjects	Grossman (1960)
	DPH	Rabbit	Impaired aressive conditioned response	Kalyuzhnji (1962).
	LH	Rat	Convulsions (high dose)	Miller et al. (1964)
	LH,MRF	Cat	Contralateral circling, Increased heart rate.	Meyers (1965)
	MRF	Rat	Impaired acquisition of conditioned reflex	Grossman & Grossman (1966)
	MRF	Rat	Impaired behaviour in high shock situation	Grossman (1968)
	V	Rat	Elicited changes in blood pressure.	Brezenoff & Jenden (1969; 1970).
DFP	SON	Dog	Increased spontaneous uterine activity.	Abrahams & Pickford (1956).
	Hip	Cat	Localized seizures	Baker & Bendict (1967)
	Cau	Cat	Elicited test tremor	Lalley et al.(1970)

Contd...

Table-4 (Continued..)

AGENT	Site of injection (or implantation)	Species	Effect	Reference
Eserine	SON	Dog	Increased spontaneous uterine activity	Abrahams & Pickford (1956)
	Am	Rat	Produced deficit in acquisition of aversive conditioned reflex	Belluzzi (1972)
Atropine	VMH	Rat	Impaired appetitive conditioned reflex	Grossman (1966)
	MiTN,MTN	Rat	Suppressed attack & killing	Bandler (1971)
	Cau-Put, GP	Rat	Elicited contralateral circling	Costall et al.(1972)
Scopolamine	HIP	Cat	Antagonized localised seizure activity	Baker & Benedict (1968, 1970)
D-Tubocurarine	V	Cat	Tremor like responses, restlessness, motor seizures	Feldberg & Fleischauer (1965).
	AH	Cat	"Fear and Escape" reaction	Decsi & Varszgi (1969)
Oxotremorine	Cau, GP	Rat	Elicited rest tremors	Blazevic et al.(1965); Cox & Potkonjk (1965).
3-Hemicholinium	GP	Rat	Prevented against rest tremor	Ruzadik (1965).

* Based on De Feudis (1974)

7) Vulnerability of Cholinergic System :

A) Acetylcholine : Crossland (1951) observed that the brain ACh level in rats increase with the age. In one day old rat the concentration (µg/g brain) was 0.75 ± 0.02, in 21 days old it increased to 1.30 ± 0.08 and at more than 100 days old it registered a further increase upto 2.70 ± 0.21. Naik et al. (1970) reported increase in the brain ACh levels of the rats of increasing body weights e.g., rats weighing 40 and 200 g had the ACh levels of 0.43 ± 0.017 and 1.42 ± 0.023 µg/g respectively. Since the body weight increases with the age, this observation indirectly indicates and parallel increase in the ACh levels with increasing age. It is interesting to note that the consistent increase in the ACh level continues well beyond the lactation period (i.e., 21 days); this is suggestive of the increase beyond the completion of brain maturation.

It is interesting to note that brain ACh levels like other neurotransmitters are subject to diurnal variation. Friedman and Walker (1972) reported the variation in the ACh concentration of mid-brain and caudate nucleus of the rats; the lowest and highest concentration of ACh was observed at 12 O'clock noon and midnight respectively.

Diurnal variations in cerebral ACh content have been correlated with changes in the toxicities of cholinergic and anticholinergic agents (Friedman and Walker, 1972).

Naik et al. (1970) reported the increase in the brain ACh concentration of rats subjected to fasting, at the beginning of the fast the concentration was 1.35 ± 0.017 µg/g brain which increased to 2.4 ± 0.098 at the end of 24 hours of fasting. The effects of fasting or starvation on the brain metabolites are different from that of complete starvation. It is in this light the above results need to be interpreted.

Richter and Crossland (1949), suggested that the brain ACh concentration may be inversely proportional to the functional activity of the brain. In other words, the ACh level should be maximal in the conditions of reduced brain activity as in anaesthesia and sleep, while it may show a temporary fall in the state of increased neuronal activity as in the case of convulsions, etc. These workers based this suggestion on the observation that the ACh levels were increased in the state of anaesthesia induced by sodium pentabarbitone and sleep and the levels were decreased in the excited state induced either by electrical stimulation or emotional excitement caused by constant tipping sideways in the beaker (Table-5). One of the most

Table - 5 : Effects of different stress conditions on brain ACh levels.

Condition	Animal	Time after stress		ACh (µg/g brain) Control	ACh (µg/g brain) Experimental	REFERENCE
Spinning	Rat	2 hrs		1.35± 0.017	0.6± 0.03	Naik et al.(1970)
		4 hrs		1.35± 0.017	0.62± 0.02	"
Electrical stimulation	Rat	-		1.27	0.55	Richter & Crossland (1949).
Excitation	Rat	-		1.27	0.82	"
Convulsions (due to electric shock)	Rat	-		1.27	0.52	"
Disturbance (excitation)	Rat	-		3.9	2.6	Elliot et al. (1950)
Sleep deprivation (paradoxical)	Rat	96 hrs	T*	20.4± 0.7	18.0± 1.9	Tsuchiya et al. (1969)
			D*	25.3± 3.6	28.9± 4.4	
			M*	19.3± 0.09	20.0± 3.1	

The values have been cited from the references shown in last column.

* T = Tenecephalon; D = Diencephalon; M = Mesencephalon (ACh values are expressed as mµ moles/g region ± S.E.).

interesting observations was made in this study - the ACh levels decrease following the electrical shock, but the convulsions occur before the fall in the ACh level and for the next convulsions to occur the ACh levels have to come upto normal levels and then fall down.

Following these studies, number of workers have reported the effects of various drugs on the ACh levels in the whole brain or different ultrastructures of the brain. The most of the CNS depressants like reserpine, barbitone, nembutal, chloralose, urethane and many others are reported to elevate the brain ACh levels as summarized in Table-6 (Elliott et al., 1950; Richter and Crossland, 1949; Crossland and merrick, 1954; Naik et al., 1970; Mann and Hebb, 1977). Besides these drugs many others like narcotic analgesics such as morphine, the ganglionic blocking agents such as hexamethonium-3 and other CNS depressants are known to increase the ACh levels. On the other hand, the drugs like atropine, scopolamine, angiotensin, pentylenetetrazol, nicotine, amphetamines, picrotoxin and the psychomimetic agents like Ditran (1-ethylpiperid-3-yl phenylcyclopentaneglycolate) are reported to increase the ACh release from cortex suggestive of decreased ACh levels - Table-7 (DeFeudis, 1974). Khanna and Madan (1975) reported that the ethanol intake decreased the levels of free and total ACh in the rat brain. The maternal consump-

Table- 6 : Effects of CNS stimulants on Brain ACh levels.

Drugs	Animal	Time	ACh (ug/g brain)		Reference
			Control	Experimental	
Pentylenetetrazole (75 mg/kg, i.p.)	Rats	12 min.	2.86± 0.16	2.21± 0.24	Giarman & Pepea (1962)
3,5-Dimethylbutyl-ethyl barbiturate (20 mg/kg, i.p.)	Rats	10 min.	2.86± 0.16	2.26± 0.32	"
L.S .D. (0.2 mg/kg, i.p.)	Rats	2 hrs	2.86± 0.16	2.91± 0.22	"
Norbornane (20 mg/kg, i.p.)	Rats	2 hrs	1.35± 0.017	0.94± 0.05	Naik & Sheth (1970)
d-Amphetamine-SO_4 (15 mg/kg,i.p.)	Rats	1.5 hrs	1.35± 0.017	0.93± 0.004	"
Atropine-SO_4	Rats	30 mins	1.35± 0.017	0.82± 0.034	"
Pethidine-HCl (24 mg/kg, i.p.)	Rats	30 mins	1.35± 0.017	0.84± 0.032	"

These values have been cited from the references shown in the last column.

The list of drugs, studied, is not complete.

Table - 7 : Effects of CNS depressants on brain ACh levels.

D r u g	Animal.	Time of sacrifice after injection of drugs (sec/min)	ACh (ug/g of brain)		REFERENCE
			Control	Experimental	
1. Reserpine (5 mg/kg)	Rats	360 sec.	1.36± 0.04	1.95 ± 0.033	Naik et al.(1970)
2. Chloropromazine (20 mg/kg)	Rats	150 sec.	1.36± 0.04	1.89 ± 0.033	"
3. Pentobarbital (23 mg/kg)	Rats	45 sec.	1.36± 0.04	1.90 ± 0.017	"
4. Pentobarbitone	Rats	-	2.68± 0.02	3.47 ± 0.30	Crossland and Merrick (1954)
5. Ether	Rats	-	2.68± 0.02	3.2 ± 0.10	"
6. Nembutal (3.9 mg/rat)	Rats	30-40 min.	3.45± 0.50	4.9 ± 6.2	Elliott et al. (1950)
7. Chlorasole	Rats	30-40 min.	3.45± 0.50	5.8 ±	"
8. Chlordiazepoxide (25 mg/kg)	Rats	120 min.	1.35± 0.017	1.58 ± 0.05	Naik & Sheth (1970)
9. Meprobromate (100 mg/kg)	Rats	120 min.	1.35± 0.017	1.38 ± 0.029	"
10. Hydroxydione	Rats	45 min.	2.76± 0.30	4.23 ± 0.26	Giarman & repeu, (1962).

These values have been cited from the References shown in the last column.

The list of the drugs, studied, is not complete.

tion of ethanol has been reported to lower the brain ACh levels in the progeny (Moss et al., 1967; Rawat, 1977). The various other conditions resulting in "excitation" are reported to decrease the brain ACh levels. Richter and Crossland (1949) reported the decreased levels with excitation due to tipping the rats in the beaker. Convulsions and electrical stimulation due to light shock. The stress caused by spinning has been reported to decrease the levels (Naik et al., 1970). The acetylcholine concentration in rat telencephelone was reduced by 24 hours sleep deprivation (Tsuchiya, et al., 1969).

From the foregoing discussion it is obvious that the brain ACh levels are indeed inversely proportional to the functional activity of the brain. The change in the ACh level may be temporary, lasting till the effect of particular stress condition is over. It is apparent from the studies of Richter and Crossland (1949) that the electrical stimulation decreases the brain ACh level, but it is regained to normal level within minutes. Similarly, the effects due to a particular drug may last till the optimal concentration of the drug is in circulation (or in the brain). The question that may be asked at this stage: Whether such a change can occur in the case of dietary deprivation or malnutrition? If yes, then how far this change will last? Before the question can be answered the

discussion on the effects of malnutrition on the brain ACh levels needs to be reviewed.

Ladinsky et al. (1972) reported the effects of hypothyroidism on brain ACh levels in rats. At day 1 post-partum, brain ACh was 73 % of the adult value. Propyl-thiouracil induced hypothyroidism at 30 days resulted in significantly decreased ACh levels. This is agreement with earlier reported adverse effects of hypothyroidism on cerebral development and myelin formation (Eayrs, 1960; Balázs et al., 1969).

Since the brain does not have the enzymatic capacity for synthesizing choline (Ansell and Spanner, 1975) it has to ultimately derive choline from circulation (Schuberth and Jenden, 1975). Secondly, the choline transport into the brain is reportedly not saturated even at very high serum choline concentration (Freeman et al.,1975). This would mean that higher concentrations of serum choline should lead to increased synthesis of ACh in the brain or choline deficiency should lead to the decreased brain ACh levels. When rats were injected with choline chloride, serum and brain choline concentrations increased rapidly and were followed 40 minutes later by a significant rise in brain ACh (Cohen and Wurtman, 1975). Brain ACh concentrations have also been shown to increase after infusion of

choline intravenously (Haubrich et al., 1975 ; Racagani et al., 1975). The higher dietary level of choline was found to result in higher brain ACh level by Cohen and Wartman (1975). The rats given 129 mg choline per day (control - 23 mg/day) showed 45 % greater ACh levels in the caudate nucleus (Cohen and Wurtman, 1976). This observation opens a new line of therapeutic treatment for disease states thought to involve insufficient transmission across the cholinergic synapse. Davis et al. (1975) reported a patient with tardive dyskinesia who responded favourably to large oral doses of choline. Moreover, Morley et al. (1977) reported that the studies based on choline deficient and choline supplemented rats reveal that alterations in the ACh levels are positively related to the ACh receptor concentration in the brain. Nagler et al. (1968) showed a reduction of ACh levels in brain, kidney, and intestine of rats deprived of choline for six days after weaning. However, Haubrich et al. (1970) failed to find such reduction in 21 day old rats raised from birth on a choline deficient diet.

There has been a suggestion for some time that encephalopathy due to thiamine deficiency may involve an impairment of cholinergic system. The basis for this suggestion has been the decreased TPP levels in the brain, leading to its decreased availability which might interfere

with ACh synthesis by virtue of diminished production of Acetyl CoA or by impaired ATP synthesis resulting from decreased activity of TPP dependent PDH or a disruption of Kreb's cycle. Studies attempting to evaluate the direct effects of thiamine deficiency on brain ACh levels have rendered mixed results. Several groups have reported lower brain ACh levels as a result of experimental thiamine depletion while others have described normal ACh levels. Some of these studies are summarised in Table- 8.

One of the important problems in the biosynthesis of ACh in the brain is the origin of the acetyl group in the acetyl CoA which is used for this purpose. A number of substrates have been shown to be able to serve as acetyl donors for ACh synthesis *in vitro* (Hebb, 1972; Tuček, 1973). The problem acquires a special dimension when one considers the presence of distinct metabolic compartmentations for Kreb's cycle intermediates and in particular acetyl-CoA (Balázs and Cremer, 1973; Nakamura *et al.*, 1970). Recently, Tuček and Cheng (1974) have reported the incorporation of label from number of radioactive precursors into brain ACh *in vivo*. In their study, the contribution of pyruvate, lactate and glucose to the biosynthesis of ACh was considerably high. The highest specific activity of the acetyl group of ACh was observed 4 minutes after the injection of (2-^{14}C)pyruvate. From

Table- 8 : Studies on Thiamine Deficiency on Brain ACh levels.

S t u d y	Species	ACh levels	Type of Assay
Lissak et al.(1943)	Rat	Decreased	-
Gubler (1968)	Rat	Decreased	Bioassay
Cheney et al. (1969)	Rat	Decreased	Bioassay
Heinrich et al. (1973)	Rat	Decreased	Enzymatic
Hosein et al. (1966)	Rat & Pigeon	No change	Bioassay
Stern & Igic (1970)	Pigeon	No change	Bioassay
Speeg et al. (1970)	Rat	No change	Fluorometric
Reynolds & Blass (1975)	Rat	No change	G.L.C.
Vorhees et al., (1977)	Rat	No change	G.L.C.

these studies it was concluded that pyruvate appeared to be the most important precursor of the acetyl group of ACh.

Heinrich et al. (1973) have carried out detailed studies on thiamine deficiencies in rats. It was observed that the concentrations of acetyl-CoA and acetyl-choline were significantly reduced by 42 % and 35 % respectively in thiamine deficient animals. But none of the enzymes studied, AChE, ChAc, transketolase, PDH, were affected by thiamine deficiency. The point to note is the drastic reduction in a product concentration (i.e., ACh) without any apparent effect on the synthesising or breaking enzymes involved. The reduction in the ACh concentration is, partly, attributed to the reduction of precursor availability (i.e., of acetyl-CoA).

Cheney et al. (1969) reported the results of their studies on thiamine deficiency and rat brain ACh. The body weights of rats receiving thiamine deficient diet and also pyrithiamine treated group were increasing till the end of 10 days thereafter there was a constant fall in the body weight gain. The oxythiamine treated rats started losing weight from 3rd day onwards. In the former two groups the symptoms of neuropathy like convulsive seizure were noticed but the oxythiamine treated group did

not show these symptoms. In all three groups, there was a significant reduction in brain ACh concentration. The *in vivo* incorporation of the label form (3-^{14}C)-pyruvate into brain ACh was increased by treatment with pyrithiamine-thiamine. Although treatment with oxythiamine-thiamine resulted in an increase, and thiamine deficiency resulted in a decrease, in the quantity of labelled ACh obtained from (3-^{14}C)-pyruvate, neither change was statistically significant. The similar deficits in brain ACh levels in all three groups inspite of the lack of parallelism between the neurological symptoms seem to perclude the possibility that these changes play any major causative role in the development of the neurological symptoms associated with thiamine deficiency and particularly, with pyrithiamine treatment.

Recently, Vorhees *et al.* (1977) reported that the ACh levels in the cortex, corpus striatum, midbrain, diencephalon and brain-stem of thiamine deficient rats were comparable to those in the control groups. However, ACh utilization was slightly to moderately (10 - 41 %) decreased in cortex, midbrain, diencephalon and brain-stem. These workers have used for the first time, for this kind of studies, the precise techniques for killing the rats by head focused microwave irradiation and the measurement of ACh by gas-liquid-chromatography. This rules out the

earlier doubts expressed regarding the reliability and reproducibility of decapitation method for killing and bioassay for the ACh estimation. The results are in agreement with the recently published works of Stern and Igic (1970), Speeg et al. (1970), Reynolds and Blass (1975). However, the differences in the utilization of ACh in different brain regions of thiamine deficient rats need satisfactory explanation.

From the foregoing discussion it can be concluded that :

1. Brain ACh levels increase with age.

2. ACh level is inversely proportional to functional activity of the brain.

3. There are number of stress conditions affecting the ACh levels with or without changing the activities of the enzymes involved.

4. Hypothyroidism, a condition somewhat similar to undernutrition, results in the decreased levels of ACh in the brain.

5. Choline deficiency or choline administration seems to affect the ACh levels in the brain.

6. There is a considerable controversy regarding the effects of thiamine deficiency on brain ACh levels, a substantial evidence for and against the possible adverse effect of thiamine defieiency on brain ACh level exists.

B) Turnover of ACh : As mentioned earlier, the concentration of ACh in the brain has long been known to be inversely proportional to the functional activity of the brain. Until recently it was not possible to estimate the actual kinetics of ACh metabolism in vivo . With the recent developments in analytical methods, particularly the techniques of radioactive tracers, it has now become feasible to assess the kinetics. The rates of incorporation of labelled Ch into brain ACh can be measured and the dynamic rates of ACh turnover in vivo can be calculated. These approaches to study the kinetics of a neurotransmitter have opened new avenues to assess the effects of those conditions, known for long time, which affect the CNS eventually resulting in altered neurological manifestations.

Acute or chronic administration of ethanol has been known to reduce the levels of acetyl-CoA and ACh in mice; this was reported to be associated with decreased rates of ACh turnover suggesting increased degradation ACh (Rawat, 1974). The administration of morphine and other opiates

has been reported to decrease ACh turnover rates in cortex and hippocampus but not in striatum suggesting the differential action of the opiates in these regions (Zsilla et al., 1976). Further work suggests that activation of opiate receptors in n.accumbens through the inhibition of ACh turnover triggers a series of events that cause analgesia and/or catatoxia (Zsilla et al., 1977).

Intraventricularly injected beta-endorphin was found to induce analgesia and catalepsy associated with dose dependent decrease in ACh turnover rates in the cortex, hippocampus, n.accumbens and globus pallidus but not in n.candatus (Moroni et al., 1978). However, the effects of intraventricularly injected morphine were although found to be similar, it was 100 times less active. It is interesting to note that none of these drugs affected the levels of ACh or Ch in the brain regions.

Atropine administration has long been known to decrease the brain ACh levels (Holmstedt et al., 1963; Consolo et al., 1972) and increase the cortical release of ACh in the presence of AChE inhibitors (Dudar and Szerb, 1969). The studies of Lundholm and Sparf (1975) suggest that atropine increases the rate of ACh turnover. However, this effect is mainly restricted to already synthesised ACh (and not to newly synthesised ACh). Oxotremorine administra-

tion has been reported to diminish the rates of ACh turnover to 25 % of normal rate (Schuberth et al., 1969). Antipsychotic drugs like clozapine and haloperidol have been reported to reverse the decrease in striatal ACh turnover rate elicited by apomorphine (Racagani et al., 1976).

From the foregoing discussion it is evident that the kinetic measurements of synthesis, release, removal and destruction of neuroregulators can be studied through the measurement of turnover rates of the regulators. These studies only can provide a satisfactory basis for the precise definition and measurements of drug effects. However, this technique has not been utilized for studying the effects of malnutrition on the developing brain. Dreyfus (1976a) has suggested the usefulness of such studies in thiamine deficient animals. It would throw more light on the precise effects of malnutrition on the brain processes.

C) Acetylcholinesterase (AChE) and Choline acetylase (ChAc) : AChE is mainly localized in the synaptosomes and hence the measurement of its activity can provide a reasonably good quantitative index of synaptic connections. This measurement is considered more important since the methods, at present, for ACh estimations are not very accurate

and suitable for large number of samples. ChAc is somewhat uniformly distributed through out the neuronal body although there is some evidence to suggest its higher concentration in the synaptosomes. Hence, AChE rather than ChAc could serve as a better criterian for the quantification of synaptic connections.

McCaman and Aprison (1964) showed that both AChE and ChAc increased markedly in many areas of the rabbit brain between 3 and 20 days of age, a period of active brain growth and mental development. AChE was found to increase four-fold in the rat cerebral cortex (Hamburg and Flexner, 1957) and ChAc was found similarly to increase in the rat caudate nucleus between 1 and 20 days of age (McGeer et al., 1971). From the data of Adlard and Dobbing (1972[a,b]), AChE per g region seems to be increased from birth to 21 days of age by 11 % in forebrain, 2.8 % in cerebellum, 7 % in brain stem and 7.8 % in olfactory lobes. Ladinsky et al. (1972) reported a concomitant and parallel increase of ACh and ChAc in developing rat brain. The developmental profiles of these two enzymes seem to be parallel to that of many other enzymes in the developing brain as reported earlier in this Section. Moreover, it is apparent that these developmental profiles coincide with the gradual increase in synaptic numbers observed throughout the first

3 postnatal weeks (Armstrong and Johnson, 1970; Bloom, 1972). During this period only, not only the structural and chemical maturation of the brain is rapidly completed but also the definite neuromotor and behavioral correlates are being established. As mentioned earlier this is the most critical period in the brain maturation at which the animal is most vulnerable to the nutritional insults.

Sereni et al. (1966) undernourished the pups by manipulating the litter size (control, 4 and undernourished, 16) and studied the effects on the brain composition at different ages. The body weights and brain weights in undernourished rats were significantly reduced throughout the course of study. The activity of AChE was significantly reduced till the end of 14th day but thereafter the difference between the two groups narrowed down. And at 35 day AChE activity in the undernourished group was higher than that of control. By contrast, the activity of succinic acid dehydrogenase was not influenced by undernutrition. The characteristic effect of undernutrition on the AChE developmental profile was the maturational delay which eventually equalled at a later age.

Im et al. (1971a) reared the female rats on 25 % protein diet during gestation and further divided into control

(25 % protein) and low protein diet (12 % protein) till weaning. A 9 day undernourished rats had an increased activity of AChE per g. brain. At 21 day the total AChE activity was reduced due to microcephaly but the activity per g. brain remained same as that of control. This is in contrast to the earlier findings of Sereni et al. (1966). After 3 weeks the undernourished rats were weaned on 3 % protein diet and showed lowered total activity and increased activity per g. brain at 7 weeks suggesting that the structures rich in AChE are perhaps, "spared" from the effects of undernutrition. This is evident from the catch up of total activity to normal levels associated with persistent increased activity per g. brain at the end of 31 weeks of rehabilitation after earlier undernourished period of seven weeks. The rehabilitation "corrects" the total activity perhaps by virtue of increase in the brain weight. Interestingly this long lasting increases in brain AChE parallel the long term increases in the emotional reactivity observed in adult rats given identical nutritional treatment (Levitsky et al., 1970). Moreover, the long term effects of early handling give similar results in that both emotional reactivity (Denenberg, 1964) and subcortical cholinesterase (Tapp & Markowitz, 1965) decrease. The piglets previously undernourished showed elevated AChE activity which persisted even after rehabilitation of four months (Im et al., 1971b). Moreover, these piglets and also the rats reared in similar

manner exhibited abnormalities in various behavioral tests.

Adlard and Dobbing (1972) studied the effects of undernutrition, by restricting the food intake by 50 % of the control rats, from 7th day of gestation till the end of lactation with a standard litter size. At the end of 21 days, postnatally, the undernourished rats had body weight and brain weight deficits of 64 % and 20 % respectively. The deficits in the different brain regions were - forebrain, 18 %; Cerebellum, 29 %; brainstem, 18 % and olfactory lobes, 20 %. The total AChE activity in crude mitochondrial fraction showed deficits of 31, 38, 24 and 38 % in these regions while AChE in crude mitochondrial fraction activity per g of region had deficits of 16, 11, 8 and 22 % respectively. Of the other enzymes studied, fumarate hydratase and 5'-nucleotidase showed significant ~~deficits~~ elevation in the whole brain of undernourished rats at 21 days. AChE activity per g region was significantly lowered in three regions except cerebellum. This could be due to the fact that in all other regions this activity increases substantially (6-10 folds) in the first 21 days, and hence it is vulnerable to undernutrition. It is interesting to note that the neonatal undernutrition in rats results in reduced formation of synapses of somatosensory cortex (Bass et al., 1970) and hypothyroidism reduces the activity of glutamate

decarboxylase, an enzyme located in the nerve endings but not in other enzymes located in the cytoplasm (Balázs et al., 1968). These observations if correlated with chronological maturation of the brain and the observed effect of undernutrition of brain AChE activity consolidates the basis for identifying the AChE activity in crude mitochondrial fraction as a marker for cholinergic synapses.

In the earlier studies, Adlard and Dobbing (1971a) observed the deficits in AChE activity (in the crude homogenate) per g of region in forebrain, brain-stem and olfactory lobes (but not in the cerebellum) of undernourished rats at the end of 21 days. At 12 weeks of age undernourished animals weighed 26 % of controls but had significantly higher enzyme activities per g wet weight in the forebrains and brain stem. The total enzyme activity per whole region was not, however, raised although it had considerably increased inspite of continuing nutritional retardation of growth. This suggests that possibly the ultimate total number of synapses approaches a normal level although the tissue weight is much reduced. The rehabilitation instituted at day 21 resulted in the elevated AChE activity (14%) at 16 weeks of age inspite of the deficits of 28 and 10 % in body and brain weights (Adlard and Dobbing, 1971b). Whereas, the activities of SDH and aldolase returned to normal after rehabilitation.

The effect on AChE activity seems to be a permanent feature in the adult suggestive of, as mentioned earlier, relative sparing of regions rich in the enzyme. The elevation in the AChE activity per g of the region in undernourished animals is same as that of rehabilated animals except in the latter group the total activity tends to come back to normal (Adlard and Dobbing, 1972b).

Gambetti et al. (1972)[a,b] studied the effects of prenatal and postnatal undernutrition by feeding low protein diet (8 %) from 13-16th day of gestation in female rats. The body weight was diminished by 57% and 72 % for 12 and 24 days old rats respectively. At the same ages the weight of the cerebral cortex was diminished by 18 % and 20 % respectively. There was a significant reduction in protein content but not the concentration of the cerebral cortex of undernourished rats at both 12 and 24 days. Total AChE per cortex was significantly reduced (-20 %) at 12 but not at 24 days. Total ChAc activity was not significantly changed at either ages. In synaptosomal fractions, the recovered activities of both AChE and ChAc as well as the protein content were significantly increased (32-42 %) in the 24 days old undernourished rats while the specific activities of these enzymes remained unchanged. These results are indicative of the relative sparing of synapses over other structure during the critical undernutrition.

Eckhert et al. (1976a,b) reported the effects of undernutrition during different stages of development on the activities of AChE and ChAc in the three regions of the brain. The changes in the activities of these two enzymes were differential in the regions studied and also differed as per the institution of undernutrition at a particular time during maturation of the brain.

Bajgar et al. (1972) reported the increased AChE activity in crude mitochondrial and microsomal fractions of the brains of pups (16-25 days old) of adrenalectomized mothers. These changes were associated with decreased levels of the evoked aggression. AChE activity has been reported to be affected by prenatal X-irradiation (Maletta et al., 1967), by dark rearing and visual stimulation (Sinha and Rose, 1976) ChAc activity is also affected by complete light deprivation (Maletta and Timiras, 1968) and by immobilization of rat (Gottesfeld, et al., 1978). Increased ChAc activity in the temperal lobe has been associated with high learning ability of a mice strain C-57 by Mendel et al. (1974). AChE activity has been shown to be affected by combination of dietary manipulation and change in the environment (Im et al., 1976ø). The super high level of dietary protein (45 %) has been reported to affect the developmental profiles of AChE in rat brain in the manner similar to that of undernourished animal except

for the lesser degree of severity (Coupain et al., 1977; Tyzbir et al., 1977).

The studies of the alterations in the levels of ACh, AChE and ChAc under various experimental manipulations are very important to assess the vulnerability of synapses, particularly the cholinergic ones. Although large number of studies have been carried out on these lines the correlation of the observed alterations with that of changes in the synaptic structure remains to be explored although the later changes have been very well documented (Jones, 1976; Jones and Dyson, 1976). Similarly, the neuromotor development and behavioural tests need to be correlated with the observed alterations in the components of the cholinergic system. The studies so far carried out reiterate the need for more intensive animal studies to elaborate the models of undernutrition, malnutrition and catch-up vis-a-vis the vulnerability of developing brain as correlated with functional, biochemical and behavioral correlates.

7) AIMS AND OBJECTIVES

From the foregoing discussion it is apparent that inspite of large number of studies carried out so far on the effects of undernutrition and malnutrition on the cholinergic system of the developing brain so many questions remain unanswered. A systematic study to assess the effects of nutritional inadequacies on individual components of the cholinergic system as a whole under same set of conditions is much desired to answer certain specific questions.

In the present study the attempt has been made to study the effects of undernutrition and thiamine deficiency during prenatal, postnatal and postweaning period on the cholinergic system in the rat brain. For better understanding various parameters such as ACh levels, activities of AChE and ChAc, DNA and protein content of the brain, besides body weight and brain weight have been studied. In case of thiamine deficiency, brain thiamine levels and moisture content have been ascertained. Additionally, in order to throw more light on ACh turnover rates, the incorporation of (^{14}C)-acetate and (^{3}H)-choline has been attempted. It is hoped that the present work may serve to answer, at least partly, the following questions :

1) What are the effects of undernutrition and thiamine deficiency, individually, during pre- or/and post-natal period(s) on each component of the cholinergic system?

2) Is there any correlation between ACh levels, activities of the two enzymes and the incorporation of the label form either from acetate or choline into brain ACh under similar dietary deprivation ?

3) If at all the cholinergic system is affected, what is the degree of various deficits in the brain ?

4) Whether dietary rehabilitation can reverse some of these deficits ?