BRAIN ACETYLCHOLINE AND DOPAMINE IN SOME ABNORMAL FUNCTIONAL STATES

A Thesis

Presented to the

School of Graduate Studies

University of Ottawa

by

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in partial fulfilment of requirments

for the degree of

Master of Science

September 1974

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ACKNOWLEDGEMENTS

I wish to express my deep appreciation and gratitude to Professor Pavel D. Hrdina for his guidance and constant encouragement throughout the course of this investigation. I am grateful to Professor George M. Ling, Chairman of the Department of Pharmacology for his valuable advice and kind interest in this study. I also wish to thank Mrs. Denyse Marion, Mr. Clay Bagwell and Herman Van den Bergen for their expert technical advice and help, and Mrs. LeeAnn Cardinal-Perrier and Jeanine D'Ahoust for their generous help in typing the manuscript. The financial support of the Ontario Mental Health Foundation during my graduate training is gratefully acknowledged.

The kind assistance and constant encouragement of my wife Maureen were invaluable to the progress of my studies.

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INTRODUCTION

Our knowledge about neurotransmitters in the brain has evolved from the discovery of the compounds per se, through the elucidation of their biosynthetic and degradative pathways, and of the regulation of these processes. The transmitter theory for the central nervous system implies that neurons in this complex organization make do with relatively few neurohormones to accomplish their ends. Among the putative central neurotransmitters norepinephrine, serotonin and more recently acetylcholine and dopamine received most attention. The relationship of these brain biogenic amines to altered states of behaviour, mental diseases and their role in the action of psychotropic drugs has been the subject of vast number of reports. Sophisticated methods have been developed to study the levels, metabolism and the turnover of these biogenic compounds in variety of experimental conditions. Drugs which directly or indirectly affect the central nervous system have often been used as tools in an attempt to correlate the functional changes with neurochemical alterations in whole brain or discrete regions of the central nervous system. The investigation of the pharmacological interactions of antidepressant drugs with central adrenergic mechanisms and with the metabolism of brain norepinephrine and serotonin have provided the basis for the initial formulation of hypotheses on the key role of these amines in the etiology of affective disorders (Schildkraut, 1973). Deficiency of dopamine in basal ganglia of Parkinsonian patients was found to be a major factor in the pathology of this disease state (Hornykiewicz, 1966).

Studies on the hormonal regulation of the developing brain have shown that, in particular the thyroid hormone exerts a marked influence on the developmental pattern of various neurochemical components of the brain (Schwark et al., 1972). Attempts have also been made to correlate the neurochemical alterations resulting from the lack of thyroid function with the behavioural changes and other symptoms seen during experimental cretenism (Myant, 1971).

Although it has been suggested that brain biogenic amines may play a role in sleep mechanisms, their exact relationship to various stages of sleep has not yet been elucidated. Whereas serotonin and norepinephrine has been implicated in slow-wave and REM sleep, respectively, alterations in brain acetylcholine have been described during REM sleep deprivation in experimental animals (Bowers et al., 1966).

The aim of the present study was to investigate the changes in brain acetylcholine and dopamine during altered states of behaviour and after administration of some selected psychotropic drugs. In particular, the ontogenic development of brain dopamine, acetylcholine and acetylcholine esterase was studied in normal rats and compared to that during experimental cretenism. Neurochemical consequences of sleep deprivation were investigated by measuring dopamine and acetylcholine levels in the striatum of rats subacutely or chronically deprived of REM sleep. Finally, the effect of tricyclic antidepressants upon the striatal dopamine was examined and compared to that exerted by an antiparkinsonian drug, benztropine.

LITERATURE REVIEW

1. BRAIN ACETYLCHOLINE

A. Distribution, Levels and Metabolism of Brain Acetylcholine

a. Introduction

The development of new techniques for subcellular fractionation of the nervous tissue, new chemical methods for identification and quantitive measurement of brain acetylcholine and the use of tracer methods have resulted in a recent outburst of information on cholinergic transmission and the processes determining the function of the putative central neurotransmitter, acetylcholine (ACh). Drugs which directly or indirectly affect the central cholinergic mechanisms have often been used as tools in attempt to correlate the functional changes with biochemical alterations in the acetylcholine systems. Although the function of acetylcholine as a neurotransmitter at peripheral cholinergic synapses is now firmly established, there is still no definite answer to the question of whether or not acetylcholine serves as a transmitter within the brain. The evidence relevant to this problem was recently reviewed by Hebb (Hebb, 1970).

b. Distribution of Acetylcholine in the Brain

i.) Steady-State Levels in the Whole Brain and Discrete Areas.

Values reported for the concentration of ACh in rat brain range from 8.4 to 27.6 nmol/g wet wt. depending upon methods of sacrifice, extraction and estimation used. Among various brain areas, the highest concentration of ACh is found in ~~the~~ striatum (43-45 nmol/g) (Sattin, 1966; Schmidt et al., 1972; Hrdina et al., 1971a) and the

lowest in cerebellum (2-4 nmol/g) (Takahashi and Aprison 1964; Fink, 1968).

ii) Change in Steady-state Level of ACh. Ach content of the brain varies inversely with the degree of functional activity of the brain, being higher than normal during sleep or under anaesthesia (Richter and Crossland 1949, Crossland 1953, and Takahashi et al., 1961). The concentration of ACh also exhibits circadian rhythmicity (Hanin et al., 1970a,b, Friedman and Walker, 1969).

Drugs or altered physiological state could affect the synthesis, release, uptake or destruction of ACh. The change in the steady-state level of ACh would be dependent on the extent to which the various components of the ACh cycle (Fig. 1) have been altered.

c. Methods for Measuring Brain ACh Content.

A number of bioassays and chemical methods are available for measuring ACh.

i) Bioassays. Frog rectus abdominis muscle (Chang and Gaddum, 1933), clam heart (Welsh, 1943), dorsal wall of the leech, the cat's blood pressure (MacIntosh and Perry 1950) and the guinea pig's ileum (Blaber and Cuthbert 1961, Beani and Bianchi 1963) have been most frequently used as test objects. Bioassays are simpler in procedure compared to chemical methods, extremely sensitive (sometime responding to subpicomole concentrations) at the expense of being less specific and suffering from changes in sensitivity. The two objections led to search for chemical methods.

ii) Chemical methods. Gas chromatographic (Jenden et al., 1968;

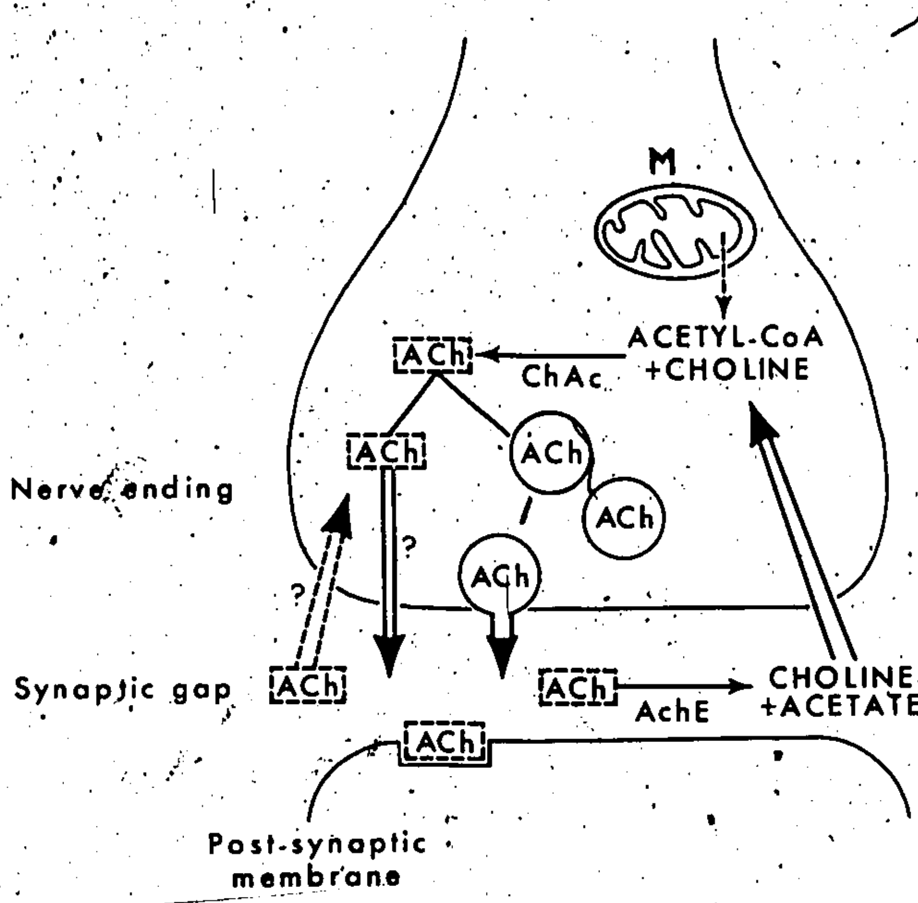

Fig. 1: Acetylcholine cycle at cholinergic nerve terminals.

(ACh) - vesicular pool of ACh

[ACh] - cytoplasmic pool of ACh

M - mitochondria

Szilagyi et al., 1968), fluorometric (Fellman, 1969) and radio-enzymic (Goldberg and McCaman, 1973) determinations of ACh have received most attention among physico-chemical methods. Besides the advantage of specificity and reproducibility, some chemical methods also approach the sensitivity of biological methods. Furthermore, in chemical assays more samples can be processed simultaneously.

d. Metabolism of Brain Acetylcholine.

i) Synthesis. Acetylcholine is synthesized from choline and acetyl-coenzyme A(acetyl-CoA) in a reaction catalysed by choline acetylase. The K_m of choline and acetyl-CoA under physiological condition is 750 μM and 10 μM, respectively (Potter, 1972).

Source of Acetyl-Coenzyme A: The rat brain contains 28 units/g of coenzyme A (CoA) (Kaplan and Lipmann 1948) and 5 nmoles/g of acetyl-CoA (Schuberth et al., 1966a). The latter is synthesized from glucose via pyruvate (Quastel et al., 1936) in the mitochondria of the brain cells. Mitochondrial membrane is impermeable to acetyl-CoA (Tucek, 1967) and choline acetylase is located in the cytoplasm (Fonnum, 1967). Most probably, acetyl-CoA is converted to citrate which crosses to the cytoplasm and is converted back to acetyl-CoA by the cytoplasmic enzyme ATP-citrate lyase (Sollenberg and Sorbo, 1970). Acetylcarnitine has also been postulated as a translocating intermediary (Potter, 1972).

Source of Choline: Choline for ACh synthesis might come from extracerebral supply (via blood) or from hydrolysed ACh at the synaptic cleft. Free choline content of the brain has been found to vary from 39.9 to 274 nmoles/g (Schuberth et al., 1970) according to species and

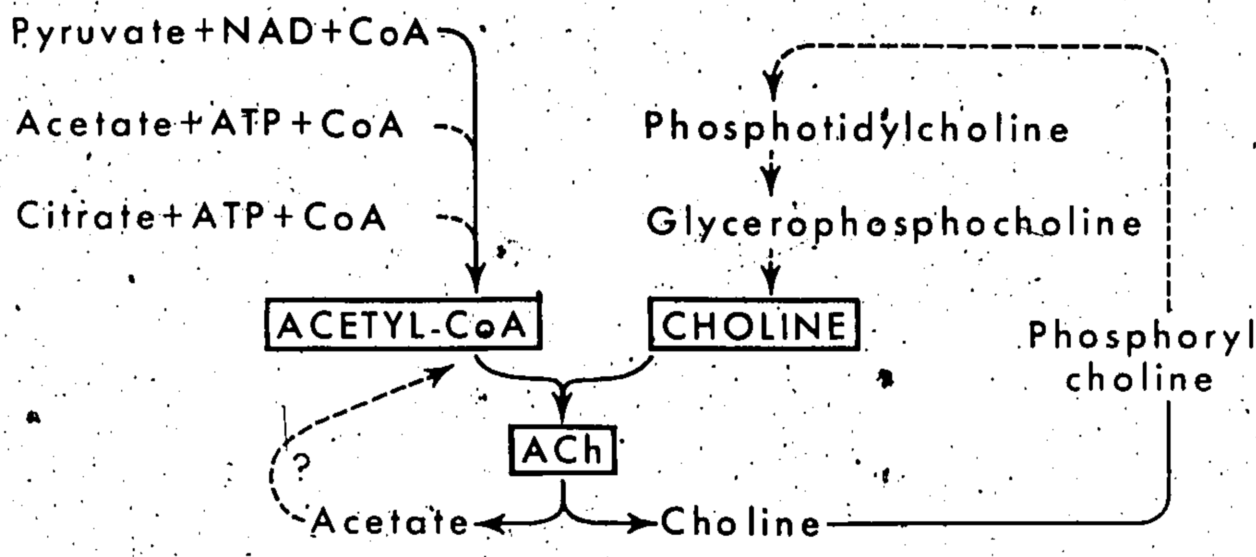

Fig. 2: Major pathways in the metabolism of acetylcholine.

methods used. Since choline does not cross easily the blood brain barrier it has been suggested (Ansell et al., 1970) that a phosphocholine compound may cross instead and then be partially degraded to yield free choline for ACh synthesis. Choline obtained from ACh hydrolysis is taken up by active process which requires oxygen and glucose utilization and can be blocked by dinitrophenol (Schuberth et al., 1966b).

Choline Acetylase: Choline acetylase (ChAc) is present in the soluble fraction of nerve endings (Potter et al., 1968; Tucek 1966) and has a molecular weight of 65,000 (Potter, 1972).

Regulation of Acetylcholine Synthesis: Level of ACh is believed to be controlled by a feed back mechanism: higher concentration inhibiting (Giarman and Pepeu, 1964) and lower levels (e.g. due to potassium induced release) enhancing its own synthesis (Sharkawi and Schulman, 1969a).

ii) Storage and Release.

Subcellular Distribution: Our present understanding of subcellular distribution of ACh in the brain comes mostly from the work of Whittaker and associates (Gray and Whittaker, 1962; Whittaker, 1969; Barker et al., 1970). 'Free' ACh is thought to be located in the axoplasm, 'labile bound' portion in the synaptosomal sap and 'stable bound' fraction is associated with vesicles within the synaptosome. Dynamic equilibrium is believed to exist between different components.

Release of ACh. Ach is known to be released from brain tissue 'in vivo' (Chakrin et al., 1968, Szerb 1963, Mitchell 1963; Beleslin et al., 1965) or 'in vitro' (Bowers 1967, Polak and Meews 1966). Electrical stimulation (Mitchell 1963, Szerb, 1967, Collier and Mitchell

1966) increased level of potassium (Mann et al., 1938, Sharkawi and Schulman, 1969b) and certain drugs enhance the rate of release The mechanism of ACh release from nerve endings within the central nervous system is however still obscure.

iii) Physiological Disposition.

Enzymic Destruction: The major means of disposing of released ACh within the CNS is its hydrolysis by acetylcholine esterase (AChE), which is associated with both pre- and post-synaptic membranes (Lewis and Shute 1964).

Reuptake: In the presence of an AChE inhibitor, slices of rat brain are capable of accumulating labelled ACh from the medium against concentration gradient (Liang and Quastel, 1969a, Hrdina and Ling 1973a, Schuberth and Sundwall, 1968). The physiological significance of the uptake transport system for ACh at the brain cell membrane is, at present unclear.

e. Ontogenic Development of Brain Acetylcholine

All the components of the cholinergic system in the brain were found to increase gradually during the postnatal life (Hamburgh and Flexner, 1957; McGeer et al., 1971; Ladinsky et al., 1972). The activity of the synthesising enzyme, choline acetylase in the whole brain or caudate nucleus of rats increases sharply during the first 20 days after birth (McGeer et al., 1971, Ladinsky et al., 1972). While acetylcholine concentration rises gradually, the content of choline showed a decrease in the brain of rats during the period of the fast growth (Ladinsky et al., 1972). During the period of active myelination (17 days), the uptake of

radioactive choline by rat brain synaptosomes was shown to be twice as high as by synaptosomes from brains of adult rats (Abdel-Latif and Smith, 1971). The developmental changes in the concentration of synaptosomal acetylcholine appear to parallel the increases in the activity of total and synaptosomal acetylcholine esterase. This is in contrast with the abrupt rise in the activity of Na^+-K^+-ATPase, which correlates well with the appearance of electrical activity in the rat brain (Abdel-Latif et al., 1967, 1970).

B. Effects of Centrally Acting Drugs on the Levels and the Metabolism of Brain Acetylcholine.

Drugs which affect brain ACh may do so by interfering with one or more of the following processes; 1) Synthesis of ACh by affecting the synthesizing enzyme ChAc or the availability of the precursors, choline and acetyl-CoA; 2) Storage of ACh by altering the pattern of subcellular distribution; 3) Release of ACh; 4) Physiological disposition of ACh by affecting AChE or reuptake. In addition, drugs can act on the site of action ACh and thus affect either the functioning or the metabolism of ACh.

a) Effects on Steady-State Levels in Whole Brain or Some Discrete Areas:

CNS depressant drugs such as pentobarbital (Giarman and Pepeu, 1962), thiopental (Beani et al., 1969), chloralose (Giarman and Pepeu, 1962), diazepam (Consolo et al., 1972), morphine (Giarman and Pepeu, 1962; Merali et al., 1974), nalorphine and cyclazocine (Howes et al., 1969) all increase the total brain ACh levels. None of these drugs alter the

reuptake of choline (Hemsworth et al., 1971).

Reserpine, unlike other CNS depressants decrease brain ACh content; this effect may be secondary to the catecholamines and serotonin-depleting action of this drug (Beani et al., 1966).

Convulsing or tremorogenic agents e.g. pentylenetetrazol (Slater 1971), p,p'-DDT and alpha-chlordane (Hrdina et al., 1971b; Hrdina et al., 1973) decrease ACh levels in the cerebral cortex or the striatum.

All cholinergic agonists tested enhance the concentration of total brain ACh (Cox and Potkonjak 1969, Bartollini et al., 1970, Haubrich and Reid 1972), while acute administration of anticholinergic drugs (e.g. atropine, scopolamine and benactyzine) results in a reduction of ACh levels in the whole brain (Giarman and Pepeu, 1964; Giarman and Pepeu, 1962), cortex and basal ganglia (Hrdina, 1974; Giarman and Pepeu, 1962, Fink, 1968).

b) <u>Effects on Synthesis.</u>

i. <u>Inhibition of Choline Acetylase</u>. Krell and Goldberg (1973) reported that two styrylpyridine analogues, NVP (4-[1-naphthylvinyl]-pyridine) and CS (3-chloro-4-stilbazole) produce a dose-dependent inhibition of ChAc in the mouse brain. However, these compounds do not lower ACh in the brain of quiescent animals suggesting that the concentration of ChAc is not a rate limiting factor in ACh synthesis.

ii. <u>Inhibition of Choline Uptake</u>. Intraventricularly injected hemicholinium-3 and triethylcholine (Slater, 1968) reduce the amount of brain ACh by blocking choline uptake. Decamethonium, hexamethonium, tetramethylammonium, d-tubocurarine and physostigmine were all found to

inhibit the uptake of choline by isolated synaptosomes (Hemsworth et al., 1971).

c) Effects on Subcellular Distribution.

Crossland and Slater (Crossland and Slater, 1968) found that anaesthetics (ether, urethane, chloralose and pentobarbital) increased the 'free' (extracted with eserinised saline) and 'bound' (extracted with acid ethanol) fractions of total brain ACh to the same relative extent. The 'free' part corresponds to the cytoplasmic and the bound to the synaptosomal fraction of Whittaker. Tremorine and eserine increase 'free' and 'bound' ACh, while atropine (Crossland and Slater, 1968), desmethylimipramine and reserpine (Hrdina and Ling 1973b) alter the ratio of 'free' and 'bound' ACh.

Beani et al., (Beani et al., 1969) further subdivided the bound ACh into the 'labile' and 'stable' bound fractions corresponding, according to Whittaker's scheme, to ACh in synaptosomal sap and vesicles, respectively. They found that anaesthesia (thiopental, gamma-hydroxybutyrate) increased all but the 'stable bound' fraction. Administration of eserine produced greater increase in 'free' that in 'bound' ACh fraction. Convulsive doses of pentylenetetrazole (80 mg/kg) resulted in marked decrease in the concentration of both 'labile' and 'stable' bound ACh without altering the 'free' ACh fraction. These findings support the view that the three subcellular ACh fractions may have different physiological significance.

d) Effects on Release.

Development of the 'collecting cup' technique (MacIntosh and Oborin,

1953; Mitchell, 1963) has greatly facilitated the studies on the effect of different drugs upon ACh release from the exposed cerebral cortex. CNS depressants such as cyclopropane, morphine, pentazocine, ether etc. were shown to suppress (Beani et al., 1968; Mitchell, 1963; Jhamandas et al., 1970) whereas CNS stimulants such as strychnine (Beleslin et al., 1965), D-amphetamine (Hemsworth and Neal, 1968) and picrotoxin (Szerb et al., 1970) etc. enhanced the release of ACh from the cerebral cortex. Δ9-THC was reported to reduce cortical ACh release (Domino and Bartolini, 1972) and to elevate total brain ACh levels (Domino, 1972), resembling in this respect the CNS depressants.

Morphine, meperidine and methadone have been shown to decrease the 'in vivo' release of ACh from the cortex and other brain areas in several species (Domino et al., 1973; Beleslin and Polak, 1965; Jhamandas et al., 1971). This effect can be fully reversed by narcotic antagonists (e.g. naloxone) (Domino et al., 1973; Jhamandas et al., 1971). It has also been demonstrated that tolerance develops to the anti-release effect of morphine (Jhamandas, 1973) as well as to the morphine-induced increases in total brain ACh (Large and Milton, 1970). Domino and Wilson (1973) showed that morphine and related narcotic analgesics prevent the hemicholinium-3-induced ACh depletion and that both a mixed antagonist like nalorphine and a pure antagonist like naloxone can abolish this anti-depletion effect of morphine. The above mentioned as well as other related evidence (Crossland, 1970, Merali et al., 1974) suggest that the hyperactivity seen during morphine withdrawal syndrome might, at least in part, be due to excessive release of ACh in brain. Support for this suggestion is gained from the reports that the abstinence syndrome in

man or animal can be attenuated by administration of atropine (Crossland, 1970).

Antimuscarinic drugs such as atropine and scopolamine were found to enhance the cortical release of ACh (Szerb et al., 1970; Bartollini and Pepeu, 1967). The increase in ACh release by amphetamine and DL-DOPA is probably mediated through the release of norepinephrine and formation of dopamine, respectively; both of these amines may in turn stimulate a corticopetal cholinergic pathway (Beani and Bianchi, 1970; Nistri et al., 1972).

e) Effects on Physiological Disposition.

i) Inhibition of Acetylcholine Esterase. Parenteral administration of AChE inhibitors such as eserine (Howes et al., 1969), TEPP (Giarman and Pepeu, 1962), DFP (Michaelis et al., 1954) and Di-Syston (Stavinoha et al., 1969) produces an increase of total brain ACh levels. Holmstedt and coworkers (1967) found a dose-dependent relationship between the increases of brain ACh and AChE inhibition. The CNS effects of anti-AChE agents like organophosphorous compounds can be blocked by atropine (Schaumann, 1960).

ii) Effects on ACh Uptake. Several drugs (e.g. hemicholinium-3, tetraethylammonium, tetramethylammonium and d-tubocurarine) have been found to inhibit the active uptake of choline in the brain. These compounds along with local anaesthetics such as cocaine, procaine and lidocaine (Liang and Quastel 1969b), autonomic drugs like succinylcholine, eserine, pilocarpine, nicotine, atropine and oxotremorine (Schuberth and Sundwall, 1967), the narcotic analgesic morphine (Schuberth and Sundwall, 1967) and the tricyclic antidepressant desmethylimipramine

(Hrdina and Ling, 1973a) were shown to inhibit the 'in vitro' uptake of ACh in cerebro-cortical slices in relatively low concentrations ($K_i = 10^{-5}$ M - 10^{-6} M). However, pentobarbital, amobarbital, chloral hydrate, reserpine, caffeine, sodium salicylate, pentylenetetrazole, epinephrine and norepinephrine failed to affect ACh uptake even in high concentrations (10^{-3} M) Liang and Quastel, 1969b; Schuberth and Sundwall, 1967).

f). <u>Alterations in ACh turnover</u>.

The concentration of choline in lumbar spinal fluid was recently used as an index of brain ACh release and turnover. The amount of choline in the cerebro-spinal fluid (CSF) was found to increase in patients during amphetamine intoxication and to return to normal values after detoxification (Aquilonius et al., 1970). This clinical finding is in agreement with the observation that amphetamine greatly enhances the release of ACh from exposed cerebral cortex of experimental animals (Dudar and Szerb 1969; Hemsworth and Neal, 1968). Among drugs which either decrease ACh turnover (oxotremorine, nembutal) or increase ACh release (atropine, amphetamine), only oxotremorine was found to produce a marked change (decrease) of choline concentration in the CSF from dog's lateral ventricles (Aquilonius et al., 1970). Although these experiments do not offer a clear-cut indication as to whether or not the choline in CSF is directly related to brain ACh, future studies along this line may bring valuable information concerning the nature of this choline pool.

2. BRAIN DOPAMINE

A. Distribution, Levels and Metabolism

a) Introduction

The notion that dopamine (DA) might have a role other than just as a precursor of norepinephrine was first clearly expressed by Blaschko in 1957 (Blaschko, 1957). In 1962, Hornykiewicz and his group in Austria demonstrated a deficiency of dopamine in the brain of Parkinsonian patients (Hornykiewicz, 1966). Using fluorescent microscopic technique it was found that the dopamine containing cell bodies of nerve terminals in the striatum are located in substantia nigra, namely in its pars compacta; lesions produced in substantia nigra of rats resulted in significant loss of DA in the ipsilateral striatum (Anden et al., 1964).

b) Distribution of Dopamine in the Brain

The highest concentration of dopamine in the rat brain is found in the caudate area (3.8 μg/g) and the lowest one in the cerebellum. The midbrain contains about 0.42 μg/g. Similar pattern of distribution is found in different animal species (Bertler and Rosengren, 1959). In man, the highest concentrations of DA are present in the putamen (8.25 μg/g) and caudate nucleus (5.74 μg/g) (Hornykiewicz, 1966).

c) Methods for Measuring Brain DA Content

Chemical methods of DA estimation are preferred to biological assays because of higher sensitivity of the former ones (Laverty and Sharman, 1965). Basically, there are two chemical methods available, both dependent on the formation of a fluorophor from dopamine. In the first method,

catecholamines are condensed with ethylenediamine to produce the fluorescent compound (Weil-Malherbe and Bone, 1957). The major criticism of this method lies in its lack of specificity. The second chemical method of estimation, widely used by various investigators involves conversion of dopamine into an indole derivative (by oxidation and treatment with alkali) which then shows a fluorescence in acid solution (Carlsson and Waldeck, 1958).

d) Metabolism of Brain Dopamine

i) Synthesis: Dopamine in brain is synthesised from L-tyrosine which is converted to L-dihydroxyphenylalanine (L-DOPA) by L-tyrosine hydroxylase. L-DOPA is converted to dopamine by L-DOPA decarboxylase (Hornykiewicz, 1966). Both tyrosine hydroxylase and DOPA-decarboxylase show the highest activity in the septum-caudate area (Porchen and Heller, 1972).

ii) Storage and Release: (a) Subcellular Distribution. The terminals of dopaminergic neurons form varicosities which show osmophilic densecore granules containing DA and ATP in a ratio of 4:1. The intragranular DA constitutes the storage pool which is in equilibrium with considerably smaller mobile pools within the granules and cytoplasm (Axelrod, 1963). (b) Release. Evidence of release of dopamine in the CNS has been obtained by push-pull cannula technique. Electrical stimulation of the nucleus centromedianus thalami in the cat was shown to increase the output of DA from the caudate nucleus and the stimulation of substantia nigra to enhance DA release from the putamen (McLennan, 1965).

iii) Physiological Disposition: (a) Enzymic Destruction. Released DA may be destroyed by two enzymes: monoamine oxidase (MAO) and catechole-o-methyl transferase (COMT). MAO acts upon intracellularly released DA, and COMT on the extracellularly released catecholamine (Kopin, 1968). Homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC) are the major end products of DA metabolism. (b) Reuptake. Evidence indicates that a specific active transport mechanism located in the presynaptic membrane exists in dopaminergic neurons and may be in part responsible for the removal of released DA from the synaptic cleft (Coyle and Snyder 1969).

e) Ontogenic Development of Brain Dopamine

The concentrations of dopamine and norepinephrine in the neonatal brain of several species are well below those seen in adult animals (Karki et al., 1962; Agarwal et al., 1966). The developmental pattern of enzymes involved in the biosynthesis (McGeer et al., 1967; Eberle and Eiduson, 1968) or degradation (Nachmias, 1960; Pryor, 1968) of these brain biogenic amines shows increases in various enzyme activities with age. The activities of DOPA decarboxylase, monoamine oxidase and catechol-o-methyl-transferase were found to increase at approximately same rate in different brain areas. In contrast, tyrosine hydroxylase activity as well as norepinephrine levels attain adult values much earlier in the brain stem than in the forebrain structures. In contrast to the sequential development of dopamine, tyrosine hydroxylase and DOPA decarboxylase in the mesencephalon, the developmental changes of this compound and enzyme in the septum-caudate area are concurrent. This can be

explained by the ingrowth of axons from the mesencephalon and the proliferation of biochemically intact dopaminergic nerve endings (Porcher and Heller, 1972).

B. Effects of Centrally-Acting Drugs on the Levels and Metabolism of Brain Dopamine.

The antiparkinsonian drug L-DOPA (Bartolini and Pletscher, 1968; Wurtman et al., 1970), monoamine oxidase inhibitors pargyline (Aghajanian and Roth, 1970) and tranylcypromine (Valzelli and Garrattini, 1968) all increase dopamine content of the brain. In contrast, the anticholinergic agent, atropine (O'Keefe et al., 1970; Perez-Cruet et al., 1971) as well as sympathomimetics such as amphetamine, mephentermine and norepinephrine failed to affect brain DA levels in rats (Breese et al., 1970).

Among other drugs, reserpine and α-methyl-DOPA were found to deplete brain DA stores (Anden et al., 1969). Phenothiazines (e.g. chlorpromazine) and butyrophenones (e.g. haloperidol) in low dose enhance but in high doses decrease DA levels in the rat striatum (O'Keefe et al., 1970). Marked reduction of brain DA has also been observed after administration of α-methyl m-tyrosine and α-methyl p-tyrosine (Anden et al., 1969; Spector et al., 1965). Gamma-butyrolactone was reported to increase the cerebral concentration of dopamine (Gessa et al., 1966); 1-hydroxy-3-aminopyrrolidione-2 (HA-966), a compound which chemically resembles the cyclic form of γ-aminobutyric acid was also found to enhance the DA content of the rat striatum (Bonta et al., 1971). Among narcotic analgesics, morphine has been reported to decrease for a short period of time the DA content in the whole brain of mice but to increase

striatal DA levels in the rat 1 hour after i.p. injection of the drug (Gauchy et al., 1973).

a) Effects on Synthesis

α-methyl-p-tyrosine inhibits the enzyme tyrosine hydroxylase and thus decreases the synthesis of DA (Koelle, 1968). Among other agents which inhibit tyrosine hydroxylase are: 6-hydroxydopamine (6-OH DA) (Breese and Traylor, 1970) and metaraminol (Breese et al., 1970).

On the other hand, benztropine, morphine (Gauchy et al., 1973) and oxotremorine increase the rate of DA synthesis by increasing the activity of tyrosine hydroxylase. Both DL-DOPA and L-DOPA enhance dopamine synthesis by increasing the availability of the substrate (Rinne et al., 1971).

b) Effects on Release.

Release of DA from the intragranular to the cytoplasmic pool is facilitated by guanethidine and blocked by bretylium and tranylcypromine (Koelle, 1968). Release from nerve endings has been found to be inhibited by the compound HA-966 (Hillen and Noah, 1971) and facilitated by amantadine (Stromberg et al., 1970).

c) Effects of Physiological Disposition

i) Effects on metabolic end products.

Alterations in the levels of HVA and DOPAC have been considered as indices of the turnover rate of brain dopamine. Among drugs which increase brain concentration of HVA in experimental animals are: oxotremorine and eserine (Perez-Cruet et al., 1971). On the other hand

atropine has been reported to decreased brain levels of HVA (O'Keefe et al., 1970). Levels of DOPAC were found to be enhanced by the compound HA-966 (Kopin, 1968), and reduced after administration of reserpine (Anden et al., 1963).

ii) Effects on Uptake:

'In vitro' studies using striatal synaptosomes from rat brain have revealed that antiparkinsonian agents, anti-histamines, tricyclic antidepressants and phenothiazines, reversibly inhibit the uptake of dopamine, but show marked differences as to their affinity for the uptake process (Horn et al., 1971). Certain structural features seem to underlie the relative selectivity toward dopamine neurons. For instance, replacement of alkylamino side chain by a tropine ring system enhances affinity for the dopamine uptake process. The effect of antiparkinsonian drugs on the uptake of striatal dopamine may play a role in the clinical effect of these agents by increasing the availability of this amine at central synaptic sites. (Snyder et al., 1970).

3. BRAIN BIOGENIC AMINES AND SLEEP

The intensive research of sleep processes during the last two decades has changed the classical neurophysiological concept of sleep as a passive resting state of the brain to that of a heterogeneous and rather complex succession of active phenomena (Jouvet, 1969). Sleep can be broadly classified into two states: The first one has been called slow wave (SWS). In this state the animal has a posture characteristic of sleep, the eyes are closed and the pupils are myotic. A degree of postural tonus always remains in some muscle groups of the body (including those of the neck). The electrical activity of the cortex is characterized by spindles and with high voltage slow waves. After some time slow wave sleep changes into rapid eye movement sleep (REMS) characterised by the loss of neck muscle tone, rapid movements of the eye (50 to 60 per minute in the cat) and fast waves with low voltage, characteristic of the waking electroencephalogram (EEG) recorded from the cortex.

A. Role of Catecholamines

As early as 1914, Bass induced "sleep" in dogs applying epinephrine under the dura or into the brain (Marinesco et al., 1929). Feldberg and associates (Feldberg, 1963) produced light anaesthesia or sleep by intra-

ventricular injection of norepinephrine (NE) in the cat. In one-month old chicks, who have no effective blood brain barrier (Waelsch, 1955), systemically administered NE and serotonin induced sleep-like patterns in EEG and behavior while dopamine (DA) induced arousal (Spooner and Winters, 1965). However, in adult animals all of these agents produced an arousal reaction.

Drugs which influence the levels and metabolism of brain catecholamines have been examined for their effects on the sleep pattern. In cats, dihydroxyphenylalanine (DOPA) given i.p. first produced wakefulness with low voltage fast EEG activity, followed by increase in SWS. This DOPA-induced state of wakefulness could be counteracted by administration of 5-hydroxytryptophan (Jouvet, 1967). In young chicks, both DOPA and DA induced alertness (Spooner and Winters, 1967). In rats, dihydroxyphenylserine (DOPS) increased SWS with no change in REMS. This increase was further enhanced by nialamide and α-n-propyl-3,4-dihydroxyphenylacetamide, an inhibitor of catechol-o-methyltransferase (Havlicek, 1967).

L-α-methyl-p-tyrosine (α-MT) which reduces brain NE level, was shown to significantly increase SWS and to suppress both REMS and wakefulness (Torda, 1968). Other studies in rats, however failed to show a clear effect of this compound on REMS (Branchey and Kisson, 1973). In cats, α-MT increased REMS, while in monkeys a suppresion of REMS and a proportional increase in SWS have been observed after administration of this agent (Weitzman et. al., 1969). In man, a small oral dose of α-MT was shown to produce an increase in REMS (Wyatt, 1970). Suppression of REMS was observed in cats following administra-

tion of disulfiram, α-methyl-m-tyrosine and α-methyl DOPA (Carlsson, 1965).

B. Cholinergic Mechanisms

Dikshit in 1934 produced a sleep-like state in cats by injecting a small dose of acetylcholine (ACh) into the lateral ventricle and the hypothalamus (Dikshit, 1934). Injection of ACh into various areas of the brain stem reticular formation (RF) between the caudal mesencephalon and the medulla in cats induced cortical synchronization and behavioral sleep, thus indicating the existence of a cholinergic mechanism in the RF area (Cordeau, et al., 1963). Extensive studies of Hernández-Péon and associates in cats demonstrated cholinoceptive hypnogenic areas in the lateral preoptic region (Hernández-Péon, 1962), the frontal cortex (Mazzuchelli-O'Flaherty, et al., 1967), the temporal lobe and the basal ganglia (Hernández-Péon, et al., 1967), midline and intralaminar nuclei of the thalamus, the anterior lingula of the cerebellum and the grey matter of the spinal cord in the lowest cervical region (Hernandez-Peon, 1965). But a state of alertness was elicited by local application of ACh in certain other areas of diencephalon and in the midbrain (Hernández-Péon, 1965). Injection of carbachol into the midbrain of cats (Baxter, 1969) produced REMS, the effect being antagonised by atropine (George, et al., 1964). Both pilocarpine and physostigmine were shown to be effective in inducing REMS in mesencephalic cats (Matsuzaki et al., 1968).

Among anticholinergic drugs, atropine was found to suppress REMS in both normal and pontine cats (Jouvet, 1962) and scopolamine to retard

the onset and to diminish the total amount of REMS in man (Sagales, et al., 1969).

Chlorpromazine, an antipsychotic drug and imipramine, an anti-depressant agent were both shown to suppres REMS in rats, rabbits and cats (Jouvet, 1961; Khazan, et al., 1967; Khazan and Sulman, 1966; Hishikawa, et al., 1965). Pretreatment with physostigmine or neostigmine effectively blocked the inhibitory effect of these psychotropic drugs on REMS (Khazan, et al., 1967).

Acetylcholine (ACh) content of the rat brain has been shown to increase during sleep and anaesthesia and to decrease during seizures (Richter and Crossland, 1949). Deprivation of REMS in the rat resulted in significant decrease of ACh in telencephalon (Bowers, et al., 1966). However, total sleep deprivation by maintaining the rats on a 24-hour avoidance schedule slightly increased the concentration of ACh in the same brain region (Tsuchiya, et al., 1969)..

Neurochemistry of sleep is a relatively recent field of inquiry. Although the studies of the chemical changes in the brain underlying various states of sleep and wakefulness have suggested the participation of some neurohormones such as acetylcholine, norepinephrine and serotonin in these processes, the understanding of their role and interactions requires further research.

II. EXPERIMENTAL PART

1. EFFECT OF TRICYCLIC ANTIDEPRESSANTS ON STRIATAL DOPAMINE

A. Introduction

It has been well established that imipramine-like antidepressant drugs inhibit the reuptake of neuronally released norepinephrine in the C.N.S. and thus increase the availability of this putative neurotransmitter within the synaptic cleft (Glowinski & Axelrod, 1964). The investigation of the pharmacological interactions of antidepressant drugs with central adrenergic mechanisms and with the metabolism of brain norepinephrine (Axelrod et al., 1961; Garattini et al., 1962; Glowinski & Axelrod, 1964) have provided the basis for the initial formulation of the "catecholamine hypothesis of affective disorders" (Schildkraut, 1965). Since then, however considerable amount of evidence has accumulated suggesting the involvement of not only norepinephrine, but also of other brain monoamines such as serotonin, acetylcholine and possibly also dopamine in the etiology of affective disorders and in the mechanisms of action of antidepressant drugs (e.g. Sjoerdsma et al., 1970; Sloane et al., 1966; Benesova and Nahunek, 1971; Kivalo et al., 1961; Schildkraut, 1973). Evidence indicates that imipramine-like antidepressants (e.g. desmethylimipramine) are capable of reducing the concentration of acetylcholine in discrete areas of the rat brain (Hrdina et al., 1971) as well as of inhibiting both the synthesis (Hrdina, 1974) and the uptake (Hrdina and Ling, 1973) of this neurohormone by isolated brain tissue. A recent clinical report of the alleviation of mania by physostigmine (Janowsky et al., 1972) further supports the view that alterations in brain acetylcholine system are also involved in certain affective disorders and in the mode of central action of tricyclic antidepressants.

An imbalance between the cholinergic and dopaminergic system whithin the striatum is thought to be an important feature in Parkinsonism (Connor, 1967; Duvoisin, 1967; Klawans, 1968). It is of interest that both imipramine and desmethylimipramine have been reported to have a beneficial effect on the Parkinsonian symptoms, alleviating not only the depression, but also the rigidity and akinesia (Strang, 1965; Laitinen, 1969). Since cholinergic dysfunction has been suggested as a link between depression and Parkinsonism (Mandel et al., 1962), the possibility exists that the effects of imipramine-like agents on the balance of acetylcholine and dopamine in the striatum might be a common basis of their beneficial effects in both depression and Parkinsonism. Although a short report by Alpers and Himwich (1971) indicates that imipramine can produce a moderate increase of dopamine levels in the whole brain of rats, detailed information on the effect of imipraminics on the concentration of dopamine in discrete brain areas is lacking. We therefore decided to examine the effects of imipramine, desmethylimipramine, amitriptyline and benztropine on the steady-state levels of striatal dopamine in rats.

B. Material and Methods

a) Animals

Sprague-Dawley male rats weighing 180 ± 10 g were used in these experiments.

b) Experimental Procedure

i) Time-Course Study: A dose of desmethylimiptamine (DMI, 10 mg/kg) previously shown to have a maximum effect on striatal ACh

(Hrdina et al., 1971) was administered to groups of rats and the animals were sacrificed 30, 60, 150 and 180 minutes later for the estimation of dopamine (DA).

ii) Dose-Response Study: Rats were given various doses of DMI (1.25, 2.5, 5, 10, 15 and 20 mg/kg) and the striatal levels of DA were estimated at the time interval (60 min.) at which maximum changes occured in the time-course study.

iii) Comparative Study: The effect of a selected dose of DMI (10 mg/kg) upon the concentration of DA in the striatum was compared with that of imipramine (IMI), amitriptyline (AMI), and of an anticholinergic drug used in parkinsonism, benztropine (BENZ).

Drugs were dissolved in physiological saline and injected intraperitoneally in a volume of 1.0 ml/rat. Control animals received an equal volume of the vehicle. The rats were sacrificed by a guillotine, their brains were rapidly removed and the striata dissected as described by Szabo (1972) and weighed on a torsion balance.

c) Biochemical Estimation of Dopamine.

Dopamine was extracted and isolated by a slightly modified method of Spano and Neff (1971).

Reagents:

1. 0.4 N perchloric acid containing 0.05% sodium metabisulfite
2. Alumina (WOELM, neutral grade 1)
3. Tris buffer, 0.5 M, pH 9.
4. EDTA reagent: 37.2 g of disodium ethylenediaminetetraacetate dihydrate was dissolved in 1 M sodium acetate and

the volume brought to 1 liter. pH was adjusted to 7 by adding 5 N NaOH.

5. Iodine solution: 1.27 g of iodine and 5 g of KI were dissolved in 100 ml of water.
6. Alkaline sulfite reagent: one ml of 25% sodium sulfite (anhydrous) was diluted in 4 ml of 5 N NaOH just prior to use.
7. Glacial acetic acid.
8. Stock solution of dopamine (in 0.01 N hydrochloric acid).

The tissue samples were homogenized in ice-cold 0.4 N perchloric acid containing 0.05% sodium metabisulfite. The homogenate was transferred to a beaker and brought to pH 7-7.2. 500 mg of neutral alumina was then added on which dopamine was adsorbed by gently spinning a bar magnet for five minutes. The supernatant solution was discarded, the alumina was washed twice with distilled water and transferred to a small glass column plugged at the bottom with glass wool. Dopamine was then eluted with 1.9 ml of 0.2 N acetic acid and the volume adjusted to 2.0 ml with water.

Dopamine was estimated by using the tri-hydroxy indole method of Chang (1964). One ml of the 0.2 N acetic acid eluent was brought to pH 6.0 with EDTA reagent. To form a fluorophor, 0.2 ml of iodine solution was added, followed in 3 minutes by 0.2 ml of alkaline sulfite reagent and 5 minutes later by 0.2 ml of glacial acetic acid. The final pH was adjusted to 4.4. The test tubes were then immersed into boiling water bath for 10 minutes. After they cooled to room

temperature, fluorescence was measured in an Aminco-Bowman spectrophotofluorometer by reading samples at 370 nm while activating at 320 nm. Dopamine content in tissue samples was calculated from the calibration curve of the internal standard. A portion of the cerebellum carried through the procedure served as a tissue blank. The recovery of dopamine under the above experimental conditions was about 60%.

During the work on the methodology of dopamine estimation, we have observed that the development of fluorophor depended on the final pH of the solution (after addition of all reagents) as well as on the time of heating. Furthermore, using an aqueous solution of iodine gave us a better reproducibility than the originally used alcoholic solution. Accordingly, we have modified the method of Spano and Neff (1971) in the following: a) An aqueous solution of $KI-I_2$ was used as oxidant; b) the oxidizing pH was set at 6.0; c) the acetic acid eluent was neutralized with EDTA; d) the final pH was set at 4.4; e) the immersion in boiling water was extended to 10 minutes.

Chemicals

All chemicals were of the purest grade available. Glass distilled water was used for preparing the solutions. Desmethylimipramine hydrochloride (the active ingredient of Pertoframe) and imipramine hydrochloric (the active ingredient of Tofranil) were kindly supplied by Geigy (Canada) Ltd., amitriptyline hydrochloride and benztropine mesylate by Merk, Sharp and Dohme of Canada Ltd. and dopamine hydrochloride was obtained from Nutritional Biochemicals Corporation.

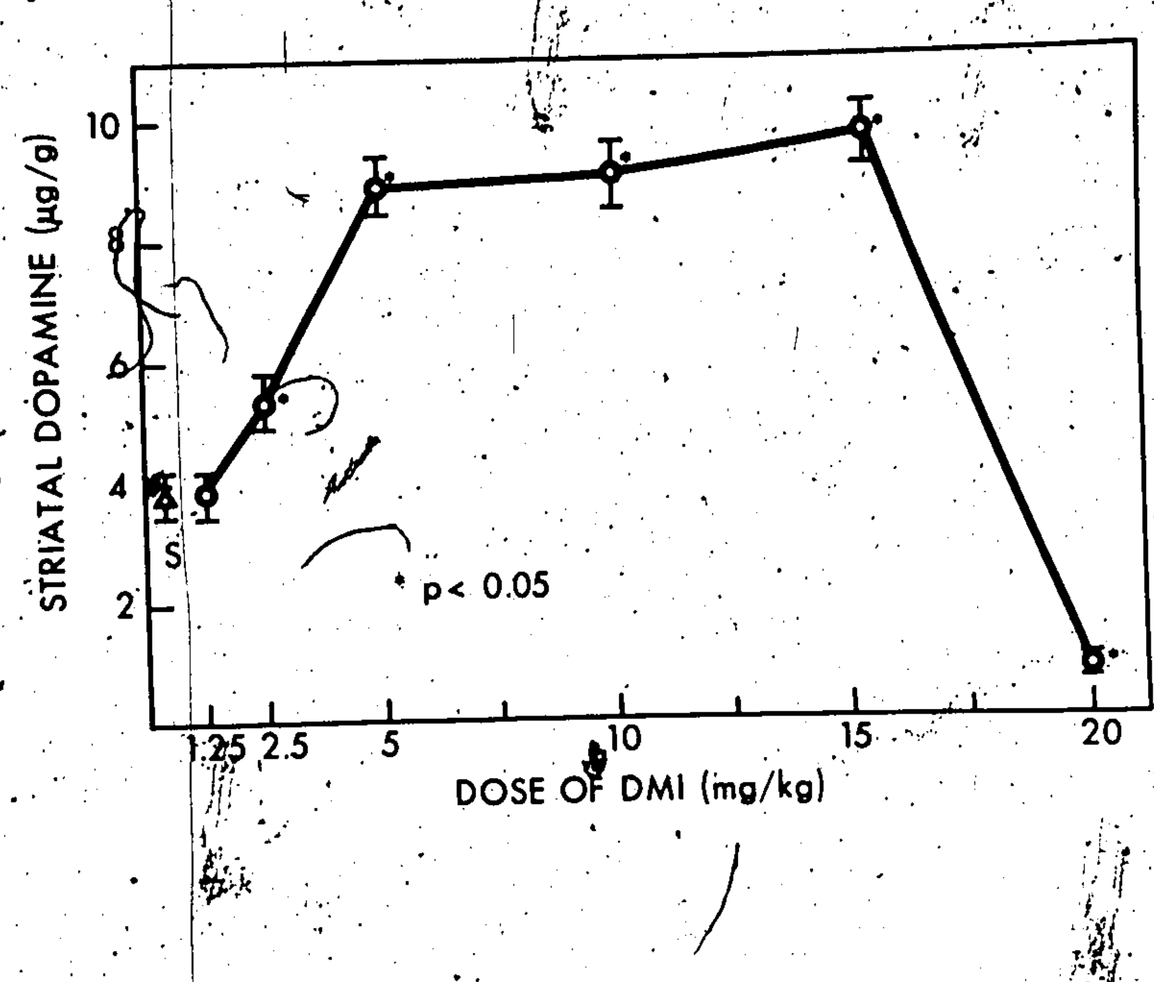

Fig. 3: Effect of varying doses of desmethylimipramine (DMI) on the striatal dopamine content in the rat. DMI was administered intraperitoneally and the animals were sacrificed one hour later. Each point represents the mean value from 6 animals; vertical bars indicate S.E.M. *Statistically significant difference when compared with the values from saline-treated controls (S).

Statistical Evaluation of Results

Mean values, standard errors of mean and the significance of differences between means were calculated by programmed statistical analysis using Student's t-test. The differences between means have been considered significant whenever the calculated p values were less than .05.

C. Results

a) Effect of Desmethylimipramine (DMI) on the Content of Dopamine in the Rat Striatum

The effect of varying doses of DMI (1.25, 2.5, 5, 10, 15 and 20 mg/kg) on the steady-state levels of dopamine in the rat striatum was investigated 60 minutes after the administration of the antidepressant drug. Data presented in Fig. 3 shows that whereas the lowest dose of DMI used (1.25 mg/kg) failed to produce any appreciable change in the concentration of striatal dopamine, administration of 2.5 mg/kg dose resulted in a significant enhancement of dopamine levels by 40% when compared to those seen in matched controls. When the dose of DMI was increased to 5 mg/kg, a large accumulation of striatal dopamine (130% above control values) was observed after the 60 minute period. Similar increases were noted with doses of 10 and 15 mg/kg of DMI. In contrast, the administration of 20 mg/kg dose of DMI resulted in marked drop of striatal dopamine content to 20% of that observed in control animals. (Fig. 3).

The time-course in changes of striatal dopamine levels produced by DMI (10 mg/kg) is presented in Fig. 4. Dopamine content was

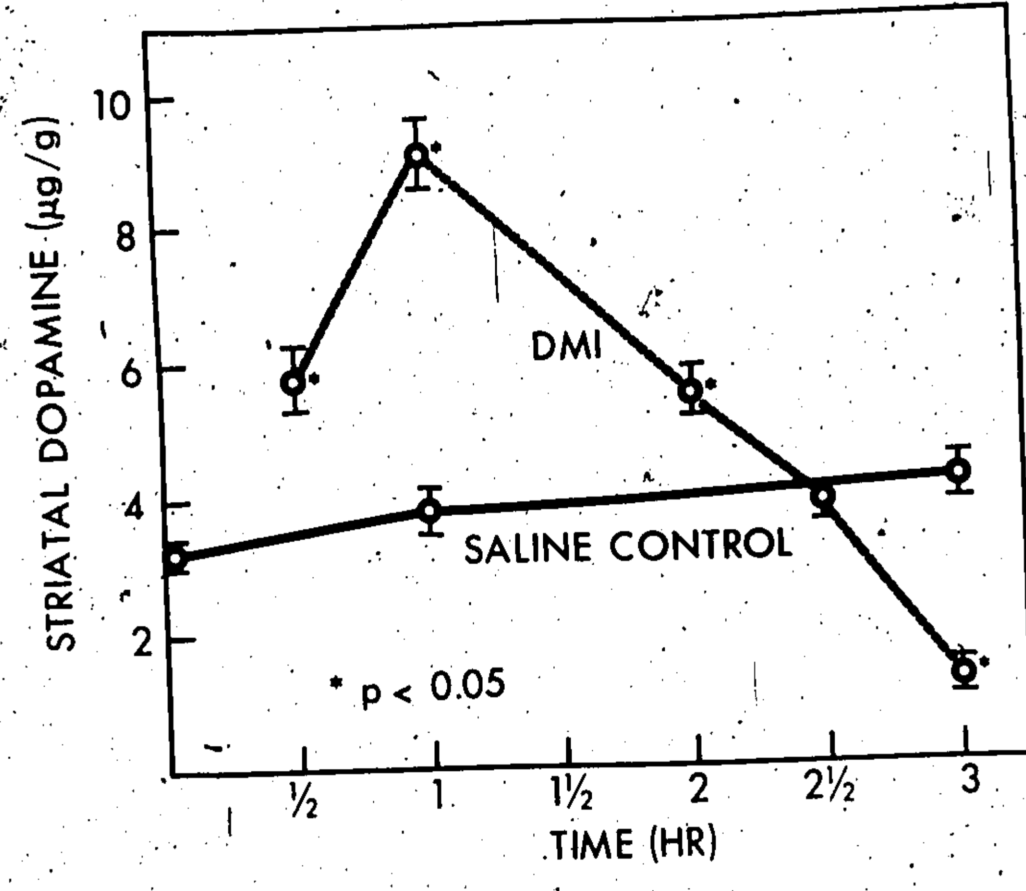

Fig. 4: Effect of desmethylimipramine (DMI, 10 mg/kg) on the striatal dopamine content in the rat at various time intervals after the injection. DMI was administered intraperitoneally. Each point represents the mean value from 6 animals; vertical bars indicate S.E.M.
*Statistically significant difference when compared with the values from saline-treated controls.

significantly enhanced already after 30 minutes and reached maximal values (140% above controls) 60 min. after the administration of the drug. Levels of striatal dopamine then gradually declined; they remained however significantly elevated at 120 min. and attained the range of control values at approximately 150 min. Three hours after the administration of DMI the dopamine content in the striatum was found to be markedly decreased to only about 21% of that observed in controls. It is of interest that the dopamine levels in the striatum of saline treated controls were higher but not significantly than those seen in non-treated animals.

b) Comparison of the Effects of Desmethylimipramine, Imipramine, Amitriptyline and Benztropine on Striatal Levels of Dopamine

Since desmethylimipramine (DMI) was capable of producing marked changes in the concentration of striatal dopamine, it was of interest to examine whether a similar effect can be induced by other antidepressant drugs of this class, such as imipramine and amitryptyline or by benztropine, an anticholinergic drug used in treatment of Parkinsonism.

Data presented in Fig. 5 show that the administration of both imipramine (IMI) and amitriptyline (AMI) in a dose of 10 mg/kg resulted after 60 min. in an enhancement of striatal dopamine levels similar to that seen after the same dose of DMI. In contrast, benztropine (BENZ) in a dose of 10 mg/kg produced a lesser, though still significant increase in striatal dopamine content.

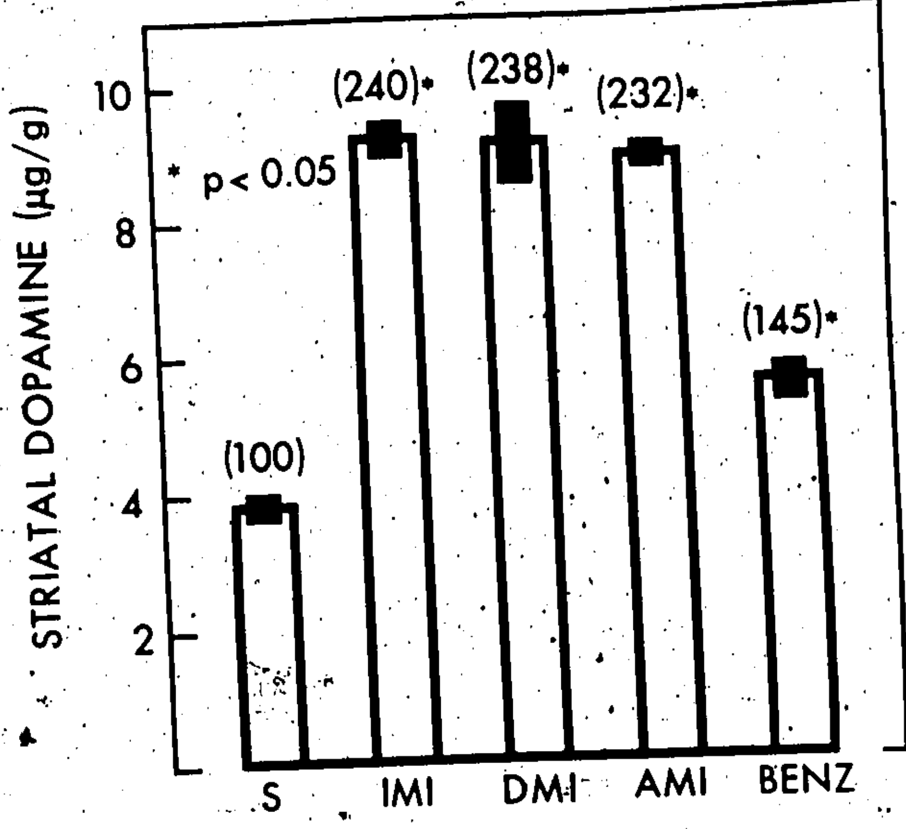

Fig. 5: Effect of imipramine (IMI), desmethylimipramine (DMI), amitriptyline (AMI) and benztropine (BENZ) on the striatal dopamine content in the rat. Drugs were administered i.p. in a dose of 10 mg/kg and the animals were sacrificed 1 hour later. Control rats were injected by saline. Dopamine concentration is expressed in micrograms per gram of fresh tissue. Columns represent mean values ± S.E.M. from six animals. Data are also expressed in percentages (in parentheses) taking the values of control animals 100%.
*Statistically significant difference when compared with the values of control rats ($p<0.05$).

D. DISCUSSION

The imipramine-like antidepressant drugs have not only provided invaluable help to the mentally ill but have served as chemical tools for studying the possible biochemical mechanisms underlying the disease state. The tricyclic antidepressants have also been of some help in alleviating the symptoms of Parkinson's disease (Laitinen, 1969). There are numerous experimental data to indicate that the imipramine-like compounds interfere with the metabolism of brain norepinephrine and serotonin (see Schildkraut, 1973). Recently, evidence has been presented that some tricyclic antidepressants, namely desmethylimipramine are also capable of reducing the concentration of acetylcholine (ACh) in discrete areas of the rat brain (Hrdina et al., 1971) as well as of inhibiting both the synthesis (Hrdina, 1974) and the uptake (Hrdina and Ling, 1973) of this neurohormone by isolated brain tissue. Since a balance between striatal ACh and dopamine (DA) is considered to be important for the maintenance of undisturbed functional activity in this important brain region, it was of interest to investigate whether the imipramine-like agents would exert an effect on the concentration of striatal dopamine.

Our results demonstrate that the tricyclic antidepressants tested (imipramine, desmethylimipramine and amitriptyline) as well as the anticholinergic agent benztropine are able to significantly increase the concentration of DA in the rat striatum. This effect was clearly dose-dependent as analysed with desmethylimipramine (DMI). While the lowest dose of the drug (1.25 mg/kg) was ineffective, increasing the dose to 2.5 or 5 mg/kg produced dose-dependent enhancement in striatal DA concentration. It appears, that there is a ceiling for DA levels

in the striatum, since increasing the dose to 10 or 15 mg/kg did not result in further increases of DA concentration. It is of interest that the highest dose of DMI used (20 mg/kg) produced an opposite effect, a reduction in DA levels below those seen in control animals. The time-course analysis revealed that the maximum increases in striatal DA concentration occur 60 min. after DMI administration. Thereafter the DA levels decline and after 3 hours even drop below the control values.

Our findings of increased striatal DA levels after treatment with tricyclic antidepressants are in agreement with the observation of Alpers and Himwich (1972) who found moderate increase of DA concentration in brains of imipramine-treated rats. In this connection, it should be noted that sometimes even marked changes in regional levels of brain biogenic substances can be masked when measured in the whole brain (Hrdina et al., 1971). The increase in striatal DA content could be due to 1) increased synthesis; 2) decreased breakdown and/or 3) decreased uptake of the neurotransmitter. There is experimental evidence to indicate that tricyclic antidepressants as well as benztropine significantly inhibit DA uptake by striatal tissue (Horn et al., 1971) and that benztropine in addition activates DA synthesis in the caudate nucleus (Cheramy et al., 1973). The possibility thus exists that increases in striatal DA seen in our experiments may have been due to uptake inhibition. The second phase of DMI effect, a suppression of DA levels seen also with the highest dose of DMI used (20 mg/kg) cannot be readily explained. One possibility is that the marked accumulation of DA might have triggered a negative feed-back mechanism.

resulting in suppression of DA synthesis and eventually of its concentration within the striatal tissue. Similar phenomena has been reported for regulation of brain acetylcholine synthesis by Sharkawi and Shulman (1969).

The ability of tricyclic antidepressants to increase DA concentration in the striatum may be relevant to the reported beneficial effect of these compounds in alleviation of parkinsonian symptoms which are believed to result from a decreased dopaminergic and enhanced cholinergic tone in this brain region.

It is also possible that DMI not only increases DA synthesis but increases its utilisation which is reflected in the depletion of DA level after 3 hours of administration of DMI (10 mg/kg). The therapeutic effect of DMI in Parkinsonism might also be due to the increased utilisation of DA.

2. ONTOGENIC DEVELOPMENT OF DOPAMINE ACETYLCHOLINE AND ACETYLCHOLINE ESTERASE IN THE BRAIN OF NORMAL AND HYPOTHYROID RATS.

A. INTRODUCTION

Available evidence indicates that the ontogenic changes in the chemical composition of the mammalian brain parallel the process of maturation, which in turn is deeply influenced by various hormones. Among them the thyroid hormone plays an important role (Myant, 1971). Neonatal thyroidectomy results in marked neurochemical changes in the developing brain (Valcana and Timiras, 1969; Pasquini et al., 1967; Schwark et al., 1972) which are associated with altered functional activity and may be related to an imbalance in putative neurotransmitter substances.

Information on the developmental changes in various brain biogenic amines and on the effect of neonatal thyroidectomy upon these parameters is rather limited. We therefore decided to study the ontogenic changes in the concentration of two putative neurotransmitters, dopamine (DA) and acetylcholine (ACh) and in the activity of acetylcholine esterase (AChE) in the brain of normal and hypothyroid rats. The question of whether the observed changes in the brain of hypothyroid animals could be reversed by specific treatment with the thyroid hormone, was also investigated.

B. MATERIAL AND METHODS

a) Animals

Newborn Sprague-Dawley rats were used in these experiments.

b) Experimental Procedure

i) Neonatal Thyroidectomy

Hypothyroidism was induced by a single injection of 200 μCi of I^{131} on the day of birth according to the method of Goldberg and Chaikoff (1949). Thyroidectomized animals and their control litter-mates were sacrificed on the 1st, 7th, 15th, 30th, 60th or 70th day by decapitation or using the 'near-freezing' technique of Takahashi and Aprison (1964).

ii) Administration of L-triiodothyronine to Thyroidectomized Rats

In separate experiments, 30 days old rats made hypothyroid in the way described above received daily injections of L-triiothyronine (T3, 250 μg/100g/day) for 4 days and were sacrificed 24 hrs after the last injection.

iii) Sample Preparation

After decapitation, the brains of experimental animals were rapidly removed and weighed. One half of the cerebrum was used for the determination of ACh and AChE, the other half for estimation of dopamine (DA). A portion of cerebellar tissue was used for tissue blank in DA determinations.

C. BIOCHEMICAL ESTIMATION

i) Acetylcholine Assay

Acetylcholine was estimated by using the pyrolysis-gas chromatography method described by Szilagyi et al. (1968). The method is based on gas chromatography of the volatile tertiary amines obtained by demethylation of quarternary halides at high temperature.

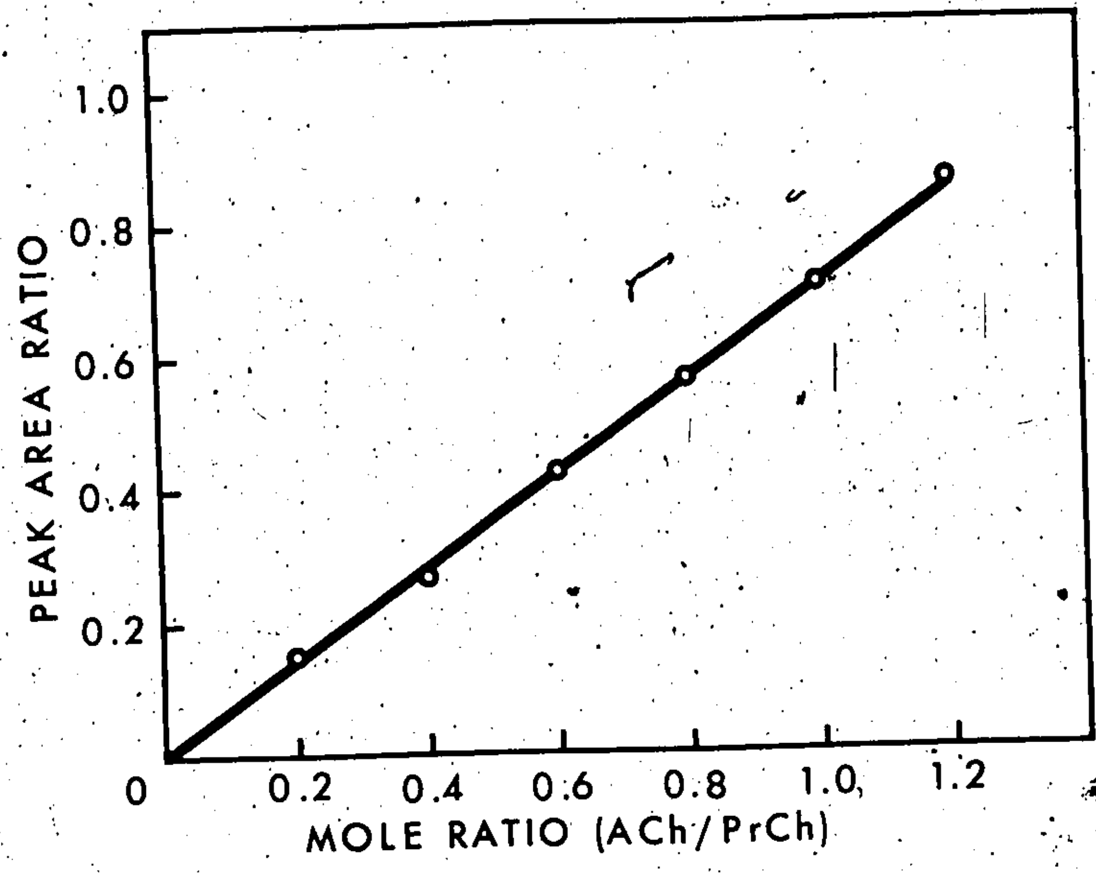

Fig. 6: An example of a calibration curve for acetylcholine determination by using pyrolysis-gas liquid chromatography. The peak area ratio of dimethylaminoethyl acetate and dimethylaminoethyl propionate is plotted against the initial molal ratio of acetylcholine and propionylcholine. Varying quantities of acetylcholine iodide were added to a constant amount of propionylcholine iodide (10 nmoles) in acetonitrile and carried through the procedure (see text for details).

The brain tissue was homogenized in acetonitrile containing 2% trichloacetic acid (3.0 ml/g of tissue), to which 10 nmoles of propionylcholine iodide was added as internal standard. After centrifugation, an equal volume of water was added to the supernatant and extracted twice with equal volumes of diethyl ether. The ether layers were removed and residual ether was blown off by a stream of nitrogen. The volume was made up to 10 ml with water and 20 µg of tetramethylammonium iodide was added as coprecipitant. The quarternary compounds were precipitated by addition of 0.3 ml iodide-iodine solution (2 g of KI and 1.8 g of I_2 in 10 ml of water). The tubes were put on ice for approximately 30 min. and then centrifuged. The precipitate was redissolved in acetonitrile and placed on the platinum ribbon in a Nuclear-Chicago pyrolyzer with block temperature of 165°C. After evaporation of the solvent, the solid material on the ribbon was pyrolysed for 10 seconds in 'analyze' position. The volatile materials from the pyrolysis were swept onto an aluminum column (8 feet by ¼ inch o.d.) packed with 20% Carbowax 6000 on Chromosorb W (HMDS), 60 to 80 mesh, previously conditioned at 180°C for 24 hrs in a stream of nitrogen. A Hewlet-Packard Model 402 gas chromatograph with a digital integrator was used for analysis. The temperature of the column was 135°C, of the flame ionization detector 230°C. The flow of nitrogen was 80 ml/min, of air 1 liter/min and of hydrogen 40 ml/min. Standard solution of ACh and propionylcholine were assayed simultaneously with the tissue samples to obtain a calibration curve. The amount of ACh present in the sample was calculated from the peak ratio of the demethylation products. An example of a calibration curve for ACh with propionylcholine (PrCh) as internal

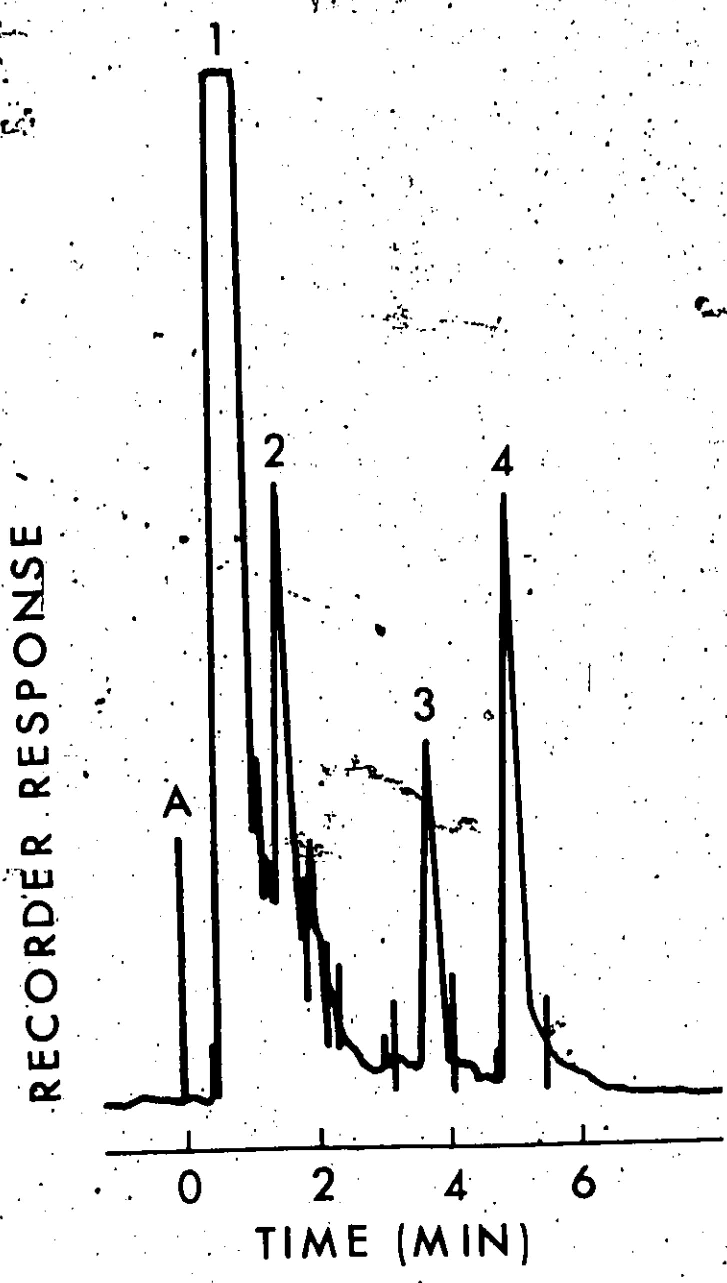

Fig. 7: Chromatogram of rat brain extract using 10 nmoles of propionyl-choline as an internal standard. "A" indicates moment of pyrolysis. Numbered peaks are: 1-trimethylamine; 2-methyliodide; 3-dimethylaminoethyl acetate; and 4-dimethylaminoethyl propionate. See text for details of the assay.

standard and a chromatogram of brain tissue sample are given in Fig. 6 and 7, respectively. In our hands, the method proved to be satisfactorily reproducible and yielded values for rat brain ACh concentrations which compare well with the figures in recent literature.

ii) Determination of Acetylcholine Esterase

The activity of acetylcholine esterase was determined colorimetrically according to the method of Ellman et al. (1961). The principle of this method is the measurement of the rate of production of thiocholine as acethylthiocholine (used as substrate) is hydrolysed by cholinesterase. This is accomplished by the continuous reaction of the thiol with dithiobisnitrobenzoate ion to produce the yellow anion of 5-thionitrobenzoic acid. The development of yellow colour formation is measured at 412 mμ in a photometer.

Samples of brain tissue, 40-60 mg, were homogenized in 0.1 M sodium phosphate buffer (pH 8.0) to produce a tissue concentration of 10 mg/ml). A 0.4 ml aliquot of the homogenate was added to a standard cuvette containing 2.6 ml of 0.1 M phosphate buffer (pH 8.0), followed by 100 μl of the dithiobisnitrobenzoic acid (DTNB, 0.1 M in phosphate buffer of pH 7) reagent. After allowing the mixture to equilibriate for five minutes, 20 μl of acetylthiocholine (AcSCh, 0.075 M) was added and the content of the cuvette mixed thoroughly. Readings were taken at 412 mμ in a Unicam 500 photometer every 30 seconds for 3 minutes and plotted on a graph paper. Activity of acetylcholine esterase was calculated from the slope of the line using a programmed statistical analysis and was expressed as moles of substrate hydrolysed per min. per gram of tissue x 10^{-6}. Appropriate blanks containing 2.6 ml of buffer and 0.4

ml of homogenate were run simultaneously.

Concentration of brain dopamine was estimated as described in Section II, 1,B,c.

All reagents were of the purest grade available. I^{131} (Atomic Energy of Canada, Ottawa) was injected intraperitoneally, L-triiodothyronine (T_3, Sigma) was dissolved in 0.02 mM NaOH and administered subcutaneously. Acetylcholine iodide and proprionylcholine iodide were obtained from Nutritional Biochemical Corp., and dithiobisnitrobenzoic acid (DTNB) from Aldrich Chemical Co.

The results were subjected to the same statistical analysis as described in Section II, 1,B.

C. RESULTS

a) Effect of Neonatal Thyroidectomy on Body and Brain Growth

As expected, the thyroidectomized (Tx) rats by about the fifteenth day displayed typical signs of experimental cretinism. These included markedly retarded growth, eyelid dysjunction, elevation of pinnae and snout elongation. In addition, hypothyroid rats appeared listless and relatively immobile. Their hair was dry and coarse and some animals exhibited a bilaterally symmetrical alopecia of the abdominal and flank regions. The mortality rate in the thyroidectomized rats was about 30%.

Fig. 8 shows the increases in body weight between day 1 and 70 of postnatal life in normal and Tx rats. Whereas there was no difference in body weight of control and experimental animals of the 7th day after birth, the body weight of hypothyroid animals was markedly lower and represented only 58, 33 and 25% of that in controls at the 15th, 30th

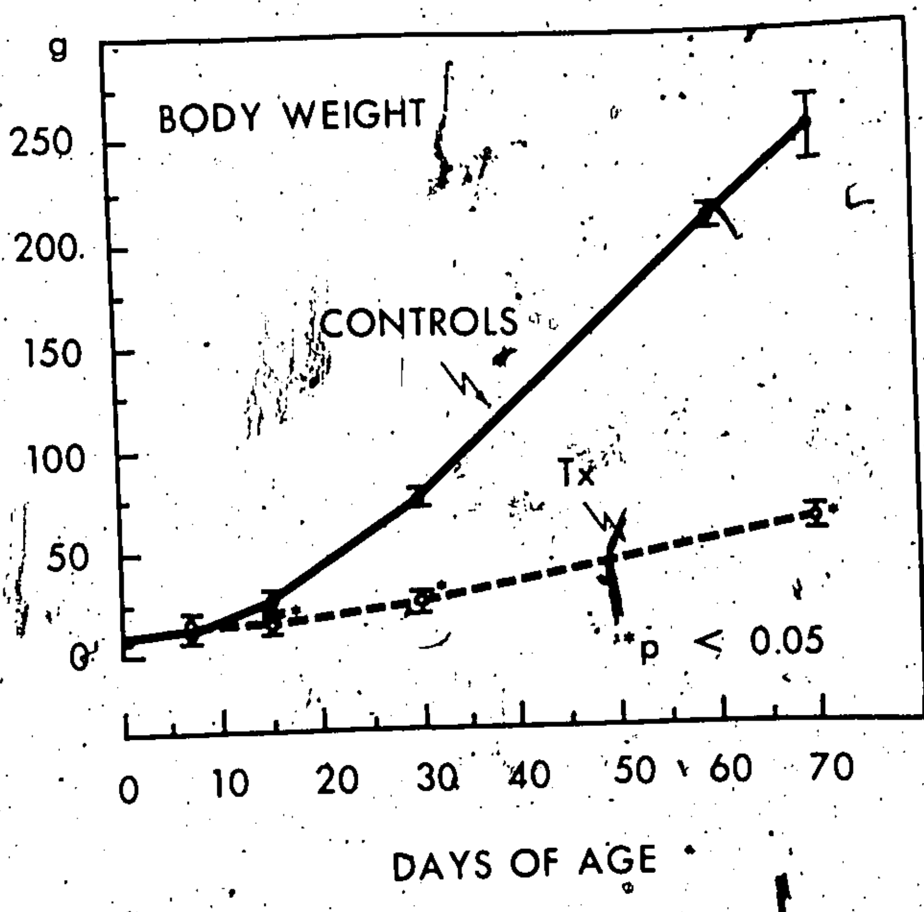

Fig. 8: Effect of neonatal thyroidectomy on the body growth of rats. Weight curve of normal male Sprague-Dawley rats compared to their litter-mates made hypothyroid by an i.p. injection of 200 µCi of I^{131} at the day of birth. Each point represents the mean body weight (g) of six animals ± S.E.M.

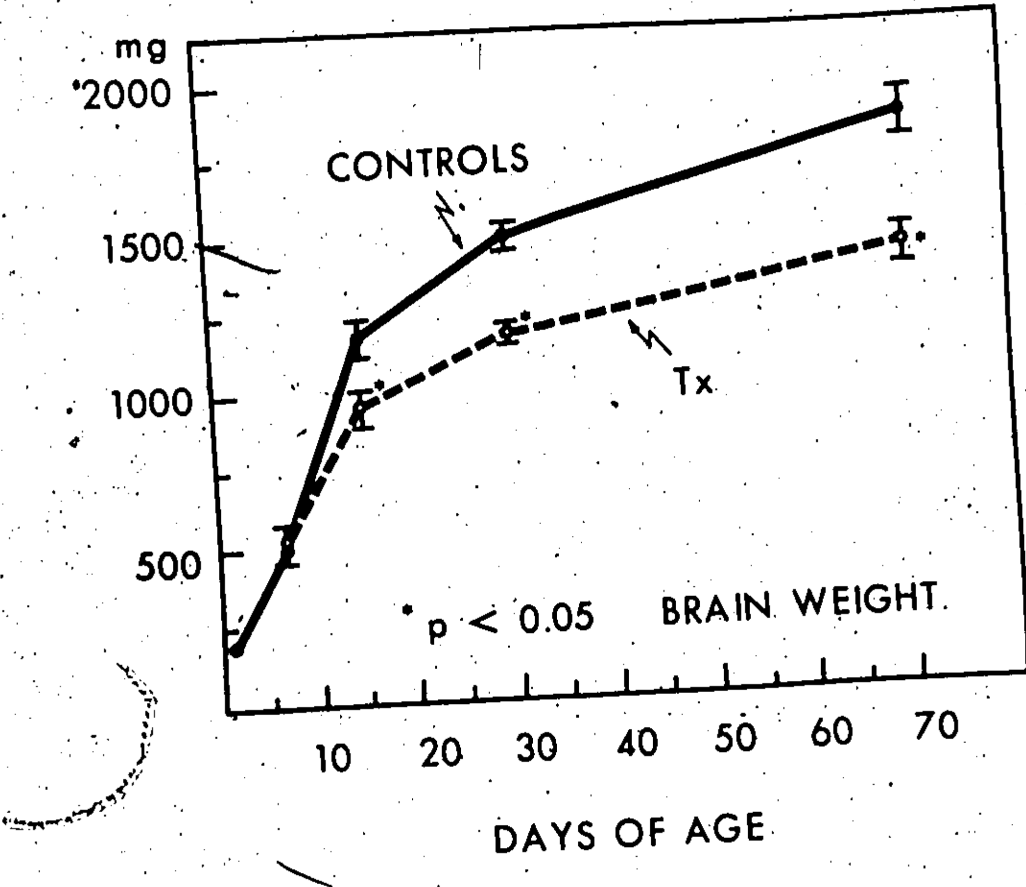

Fig. 9: Effect of neonatal thyroidectomy on the development of the rat brain. Brain weights (mg wet wt.) of normal Sprague-Dawley rats at various stages of postnatal life are compared to those of animals made hypothyroid by injecting 200 μCi of I^{131} intraperitoneally at day 1 of age. Each point represents means value ± S.E.M. from six rats.

and 70th day, respectively.

Developmental changes in brain weight are shown in Fig. 9. Brains of control as well as of Tx rats grew fastest in the period between the 1st and the 30th day. The developmental pattern of increases in brain weight of Tx animals was significantly lower, attaining only about 80% of the control values.

b) Effect of Neonatal Thyroidectomy on the Development Pattern of Brain Dopamine, Acetylcholine and Acetylcholine Esterase.

A comparison of the ontogenic changes in the concentration of dopamine (DA) in brains of control and hypothyroid rats is presented in Fig. 10. Dopamine content in the brains of control animals increased sharply up to the 30th day, when it almost reached the adult values. Neonatal thyroidectomy markedly retarded the observed developmental changes in brain DA, which in 30 days hypothyroid rats was only 46% of the control values and then increased slowly to attain 78% of controls at day 70.

Fig. 11 shows the changes in the concentration of acetylcholine (ACh, nmoles/g of wet tissue) in brains of control and Tx rats during post-natal life. In control rats, ACh levels were low at early stages of development and increases gradually to reach adult values of 20.6 nmol/g at the 70th day. Concentrations of brain ACh in Tx rats were consistently above those seen in controls, being significantly higher (by 49 and 64%) at the 15th and 30th day, respectively.

The activity of AChE in the developing brain of normal and hypothyroid rats is reported in Fig. 12. In control animals, the AChE activity increased sharply (5 times) between the 1st and the 30th day of

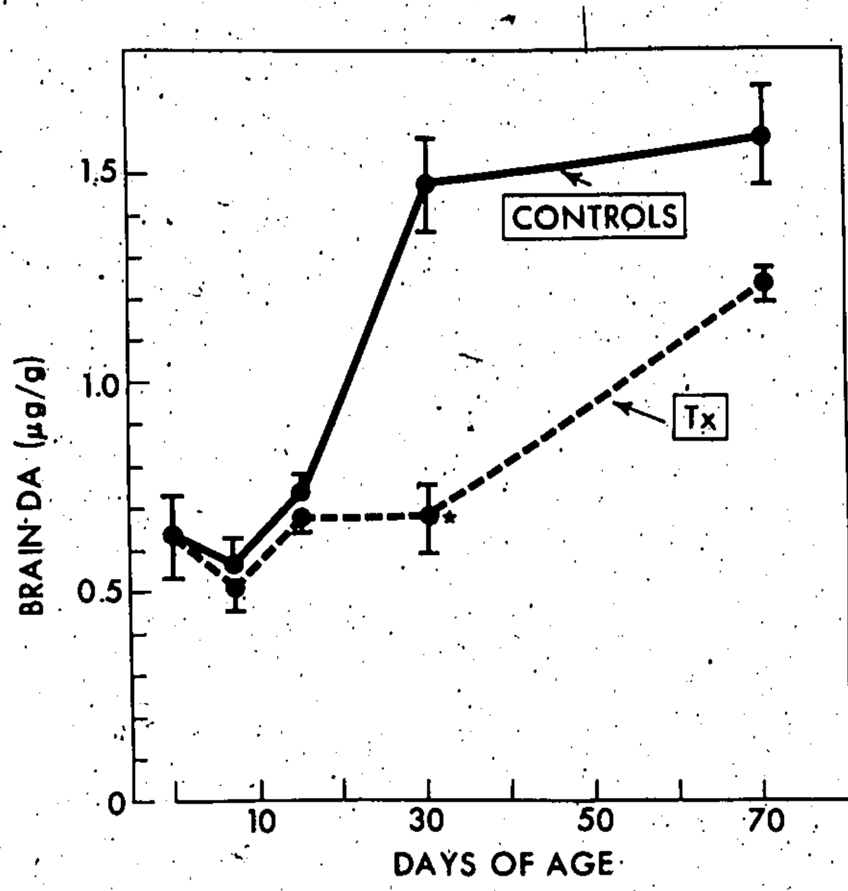

Fig. 10: Effect of neonatal thyroidectomy on developmental changes in brain dopamine. Brain dopamine concentration (μg/g wet tissue) of normal Sprague-Dawley rats at various stages of ontogenesis is compared to that of their litter-mates made hypothyroid by an i.p. injection of 200 μCi of I^{131} on the day of birth. Each point represents mean value ± S.E.M. from six rats.

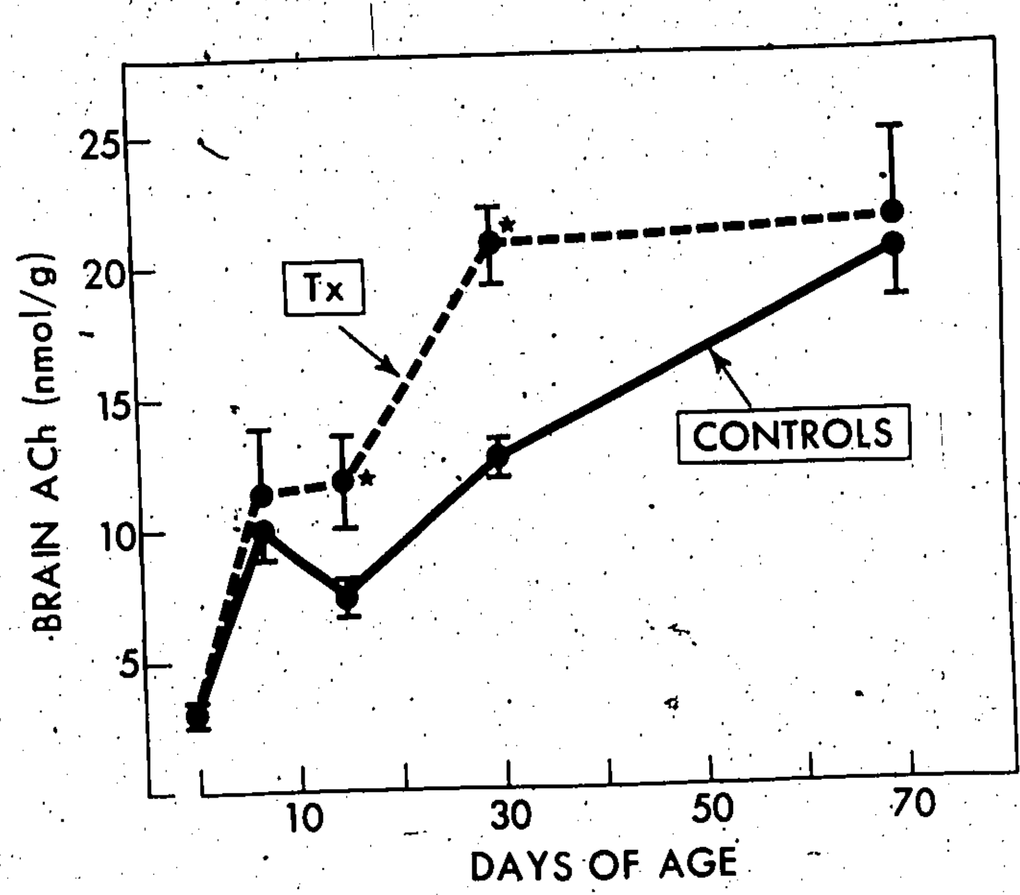

Fig. 11: Effect of neonatal thyroidectomy on developmental changes in brain acetylcholine. Concentration of brain acetylcholine (ACh, nmoles/g) in normal Sprague-Dawley rats at various stages of postnatal life is compared to that of their litter-mates made hypothyroid by an i.p. injection of 200 μCi of I^{131} on the day of birth. Each point represents mean values ± S.E.M. from six rats.

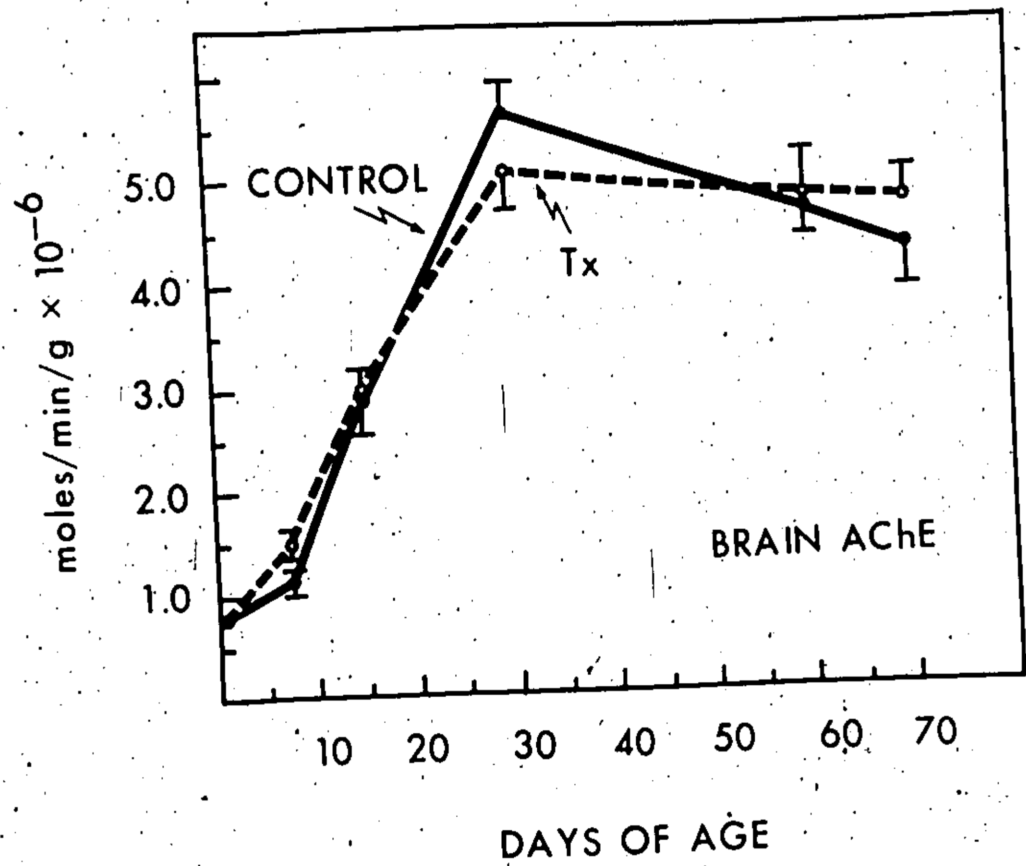

Fig. 12: Effect of neonatal thyroidectomy on developmental changes in brain acetylcholine esterase (AChE). Activity of AChE (expressed as micromoles of substrate hydrolysed per minute per g of tissue x 10^{-6}) in brains of normal Sprague-Dawley rats at various stages of ontogenesis is compared to that in the brains of their litter-mates made hypothyroid by an i.p. injection of 200 µCi of I^{131} at day 1 of age. Each point represents mean value ± S.E.M. from six rats.

postnatal life and slightly declined thereafter. Neonatal thyroidectomy failed to significantly alter the brain AChE activity, although somewhat lower values (by 10%) were noted in hypothyroid rats on the 30th day of postnatal life (Fig. 12).

c) Effect of L-triiodothyronin upon the Alterations in Brain Dopamine and Acetylcholine Produced by Neonatal Thyroidectomy.

Treatment of 30 days old neonatally thyroidectomized rats with L-triiodothyronin (T3) for a period of 4 days led to significant increase in the levels of brain dopamine which was virtually restored to values seen in normal rats of the same age (Fig. 13). The increase in level of ACh seen in brains of hypothyroid rats was also abolished by T3 treatment. No significant effect of T3 treatment was observed on the activity of AChE in the brain of neonatally thyroidectomized rats (Fig. 13).

D. DISCUSSION

Our data confirm and extend earlier findings (Hamburg and Flexner, 1957; MacCaman and Aprison, 1964; Valcana and Timiras, 1969; Abdel-Latif et al., 1970; Schwark et al., 1972) that the most pronounced changes in the biochemical maturation of the CNS occur in the period when the brain grows fastest and demonstrate that the development of experimental cretinism is associated with profound alterations in the concentration of brain dopamine (DA) and acetylcholine (ACh).

Sharp increase in brain DA from low values at early stages of post-natal development to almost adult values at 30th day is in agreement with earlier findings of low dopamine levels in the neonatal brain of several species and with the recent report by Porcher and Heller (1972)

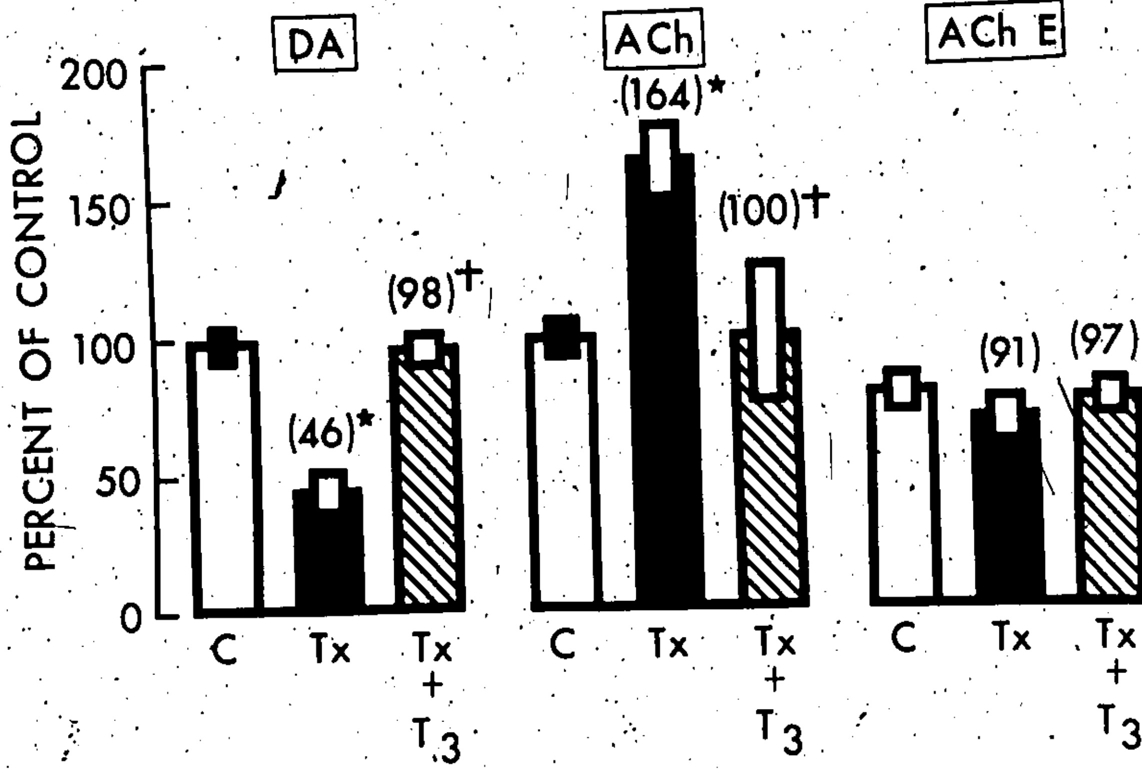

Fig. 13: Effect of L-triidothyronine treatment on dopamine (DA) and acetylcholine (ACh) content and acetylcholine esterase (AChE) activity in brain of neonatally thyroidectomised (Tx) rats. Animals were thyroidectomised by an i.p. injection of 200 μCi of I^{131} at day 1 of age. Groups of 30 days old Tx rats received daily injections of L-triiodothyronine (T_3, 250 μg/100g) for 4 days ($Tx+T_3$) and were sacrificed 24 hrs after the last injection along with non treated hypothyroid animals (Tx) and controls (C). Concentrations of brain DA, and ACh and the activity of AChE are expressed in percentages taking the values of control animals of the same age as 100%. Columns represent mean values ± S.E.M. from 5-6 animals in a group.

*Statistically significant difference when compared with the values of control rats ($p<0.05$).

+Statistically significant difference when compared with the values obtained from neonatally thyroidectomized rats not treated with T_3 ($p\leq0.05$).

who also observed a 3 fold rise of DA in rat caudate between the 8th and the 31st day of age.

Developmental pattern similar to that found for whole brain ACh in our experiment was reported by Abdel-Latif et al. (1970) for the appearance of ACh in rat brain synaptosomes. Low brain ACh levels at early stages of postnatal life correspond well with earlier finding of Crossland (1951) as well as with the low activity of the synthetizing enzyme, ChAc at the same age period, observed by Ladinsky et al. (1972). These authors, however, found the ACh content of the rat brain at day 1 to be already 73% of adult values and to attain nearly adult values at 30th day. The transient drop in brain ACh concentration observed in our experiments at the 15th day of postnatal life may result from increased utilization of this neurohormone due to development of the cholinergic receptor system and/or to the sharp increase in AChE activity during this time period. It is of interest that the activity of AChE attained its maximum at the 30th day and declined thereafter. Similar observation was made by Abdel-Latif et al. (1970) on the developmental pattern of AChE in rat brain synaptosomes.

Neonatal thyroidectomy not only impaired the normal growth of the body and of the brain but also markedly influenced the developmental pattern of two putative neurotransmitters studied - dopamine and acetylcholine. The concentration of dopamine in 30 days old hypothyroid rats was only 46% of the control values. In contrast, brain ACh levels in thyroidectomized rats were at all times higher than those seen in control animals. This was surprising and the reason for this finding is not immediately apparent. Accumulation of ACh in brain of hypothy-

roid animals is not likely to be due to its decreased hydrolysis, since activity of AChE at early developmental stages was found to be unaltered by thyroidectomy and was only moderately depressed at the 30th day. Considering that the activity of the synthetizing enzyme, ChAc is, according to Valcana (1971) and Ladinsky et al.,(1972)also slightly depressed in hypothyroidism, high levels of ACh might be due to a decrease in utilization of this neurohormone in the brain of hypothyroid animals. It is known that ACh levels vary inversely with the degree of functional activity of the brain and are higher than normal during sleep, under anesthesia and in certain states of behavioral depression (Richter and Crossland, 1949; Crossland and Slater, 1968). According to Eayers (1961) and Myant (1971), hypothyroid rats show marked impairment in various functional and behavioral tests. It is therefore conceivable that the functional hypoactivity in the brain of hypothyroid rats may result in observed accumulation of ACh. This in turn, by a negative feed-back mechanism may contribute to the suppression of the activity of the synthesizing enzyme. The most marked decrease of ChAc activity in the brain of hypothyroid rats (Ladinsky et al., 1972) occurs in fact during the same time period (from 15th day on) as the accumulation of brain ACh.

Ladinsky et al. (1972) found no changes in brain ACh levels of rats made hypothyroid by propylthiouracyl treatment until the 30th day when a decrease by 27% was noted. However, they observed an increased concentration of choline in the brain of hypothyroid rats between the 5th and 20th day of the postnatal life. The differences between our present findings and those reported by Ladinsky et al. (1972) cannot

be readily resolved. They may be due to different methods used to induce hypothyroidism and/or techniques of ACh estimation.

Evidence for the specificity of changes in the concentration of dopamine and acetylcholine in the brain of hypothyroid rats was provided by experiments in which we found that treatment of thyroidectomized rats with T3 virtually restored the levels of the neurotransmitters to values seen in control rats of the same age. Specific involvement of the thyroid hormone has also been demonstrated in the control of glycolytic enzymes (Schwark et al., 1972) as well as in the metabolism of serotonin and norepinephrine (Rastogi and Singhal, 1974; Hrdina et al., 1974) in the developing rat brain.

3. EFFECTS OF SUBACUTE AND CHRONIC REM AND TOTAL SLEEP DEPRIVATION ON BODY WEIGHT, BEHAVIOUR AND ON LEVELS OF STRIATAL DOPAMINE AND ACETYLCHOLINE.

A. INTRODUCTION

In experimental animals, the behavioural effect of REM sleep deprivation is characterized by increased responsiveness to external stimuli, irritability and agressivess (Jouvet, 1967). In this condition, an enhanced turnover of brain norepinephrine and significant decrease in brain ACh levels without concomitant alterations in levels of norepinephrine and serotonin have been reported (Bowers et al., 1966; Mark et al., 1969; Schildkraut et al., 1972). Since data on neuro-chemical changes in discrete brain areas associated with prolonged REM sleep deprivation is lacking, we decided to investigate the alterations in the levels of acetylcholine and dopamine in the striatum of rats chronically deprived of REM-sleep and compare them with those seen after subacute REM sleep deprivation.

B. MATERIAL AND METHODS

a) Animals

Male Sprague-Dawley rats, weighing 160-200 g were used in this study.

b) Experimental Procedure

Deprivation of REM sleep was achieved by using the procedure described by Ling and Usher, (1969). Experimental animals were isolated on small plastic islands, 7 cm in diameter, completely surrounded by water (Fig. 14). The rats were permited non-REM sleep, but were unable to obtain REM sleep since the ensuing relaxation of the muscles of the

Fig. 14: Experimental procedure for REM sleep deprivation in rats. The experimental animal is isolated on a small (7 cm in diameter) plastic island completely surrounded by water. The animal has free access to food and water.

neck would cause their heads to drop into the water. Other animals were placed on larger (12 cm in diameter) islands also completely surrounded by water. Under this circumstance the rats were allowed to rest and obtain both non-REM and REM sleep (Ling and Usher, 1969). The experimental animals had free access to food and water. Control rats were placed in individual cages kept in the same room. The weight of animals was recorded daily. Some rats were implanted with electrodes into the dorsal hippocampus, the cortex, the external margin of the eye and in the muscles of the back of the neck in order to permit electroencephalographic (EEG), electrooculographic (EOG) and electromyographic (EMG) recordings during the above experimental procedure.

Two separate experiments were carried out. In one, the rats were isolated on small islands for 96 hours (subacute REM sleep deprivation), in the other, for 10 days (chronic REM sleep deprivation).

Upon the termination of the experiments the rats were immediately sacrificed by using 'near freezing' technique of Takahasi and Aprison (1964). After decapitation, the brains were rapidly removed, the striata dissected and weighed on a torsion balance. The locomotor activities of REM sleep-deprived as well as of control rats were measured daily by using a selective activity meter (Columbus Instruments, Ohio).

Brain dopamine was measured by a slightly modified method of Spano and Neff (1971) as described in section II, 1, B, c. Acetylcholine was estimated by using pyrolysis - gas chromatography as described in section II, 2, B, c.

A

B

C

Fig. 15c Simultaneous recordings of eye movements (EOG, top tracing), electrical activity (EEG) from the cortex (second tracing from the top) and hippocampus (second tracing from the bottom) and from the neck muscles (EMG, bottom tracing) in a rat during wakefulness (A), slow-wave sleep (B) and REM sleep (C). Middle tracing: time in seconds.

Table 1

Analysis of sleep pattern during a typical chronic REM sleep deprivation experiment in the rat.

Parameters measured		Base-line day 3	Exp. day 1	2	3	4	7	8	9	10	11	After exp. day 1
TWT/TRT *	(%)	35.6	100	62.5	80.8	98.4	96.1	93.3	98.6	100	100	52.6
TST/TRT	(%)	64.4	0	34.8	19.2	1.6	3.9	6.7	1.4	0	0	47.4
SWS/TRT	(%)	55.7	0	34.8	19.2	1.6	3.9	6.7	1.4	0	0	26.2
REM/TRT	(%)	8.7	0	0	0	0	0	0	0	0	0	21.2
SWS/TST	(%)	86.5	0	100	100	100	100	100	100	0	0	55.3
REM/TST	(%)	13.5	0	0	0	0	0	0	0	0	0	44.7
No. of REM periods		17	0	0	0	0	0	0	0	0	0	14
Average Duration of REM period (min)		1.56	0	0	0	0	0	0	0	0	0	2.71

*TWT, total waking time; TRT, total recording time; TST, total sleep time; SWS, slow wave sleep; REM, REM sleep.

All chemicals were of the purest grade available and glass distilled water was used for preparing the solutions.

The results were subjected to the same statistical analysis as described in section II, 1, B.

C. RESULTS

a) Effects of Subacute REM Sleep Deprivation

It was first confirmed from EEG, EOG and EMG recordings that by isolation on small islands the experimental animals were indeed deprived of REM sleep. A typical example of such recordings during the states of wakefulness, slow-wave sleep and REM sleep are shown in Fig. 15. Before the start of the deprivation experiment (on the last day of baseline recording), the implanted rats had on the average 15 periods of REM sleep (of average duration of 1.8 min.) which accounted for 13.7% of total sleep time during five hours of recording. The deprivation procedure did not permit any periods of REM sleep (see Table 1) although the animals were able to obtain numerous brief periods of slow-wave sleep. Immediately after the termination of the chronic REM sleep deprivation procedure the recording has revealed a marked rebound increase in REM sleep to 44.7% of the total sleep time (Table 1).

i) Body Weight and Gross Behaviour

As seen in Fig. 16, the body weight of REM sleep-deprived rats decreased during the first 48 hours; between 48-72 hours the animals seemed to learn to cope with the stressful situation and their body weight relatively increased during this period of time and remained essentially the same till the end of the experiment. Rats placed on the large islands did not show much of the change in their body weights when

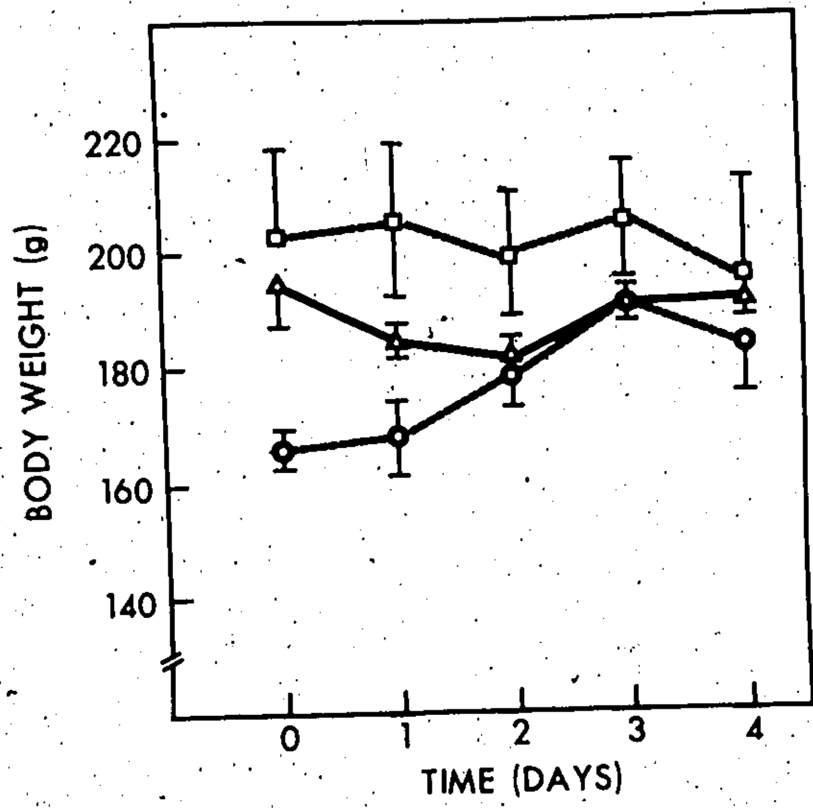

Fig. 16: Effect of four days of REM-sleep deprivation on the body weight of rats. Δ-Δ, mean values from REM sleep-deprived animals (n=6); □---□, mean body weight of rats placed on large (12 cm) islands (n=4); o-o, mean values from control rats kept individually in separate cages (n=6). Vertical bars indicate S.E.M.

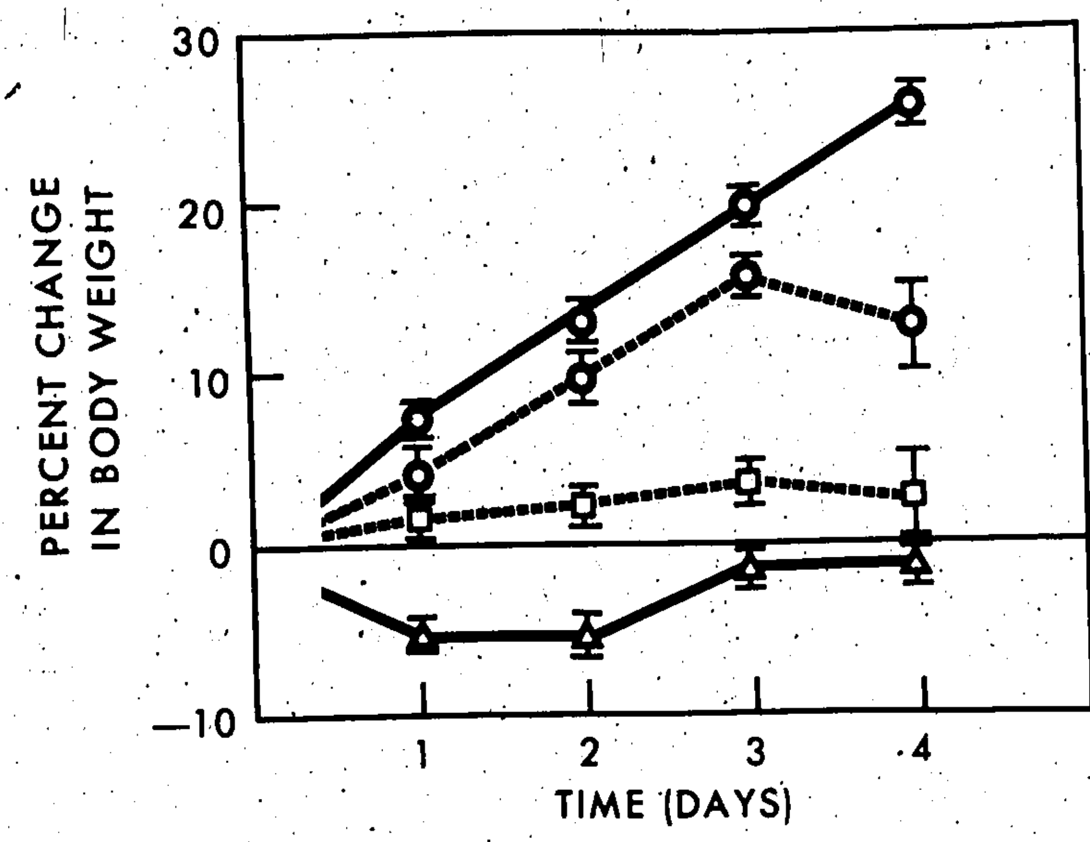

Fig. 17: Effect of four days of REM sleep deprivation on the percentage change in body weight of rats, taking the weight at 0 day as 100 per cent. Each point represents mean value of at least four animals; vertical bars indicate S.E.M. Δ-Δ, REM sleep-deprived rats, □---□ rats placed on large (12 cm) islands; o---o, control animals kept individually in separate cages; o——o, control rats caged in groups.

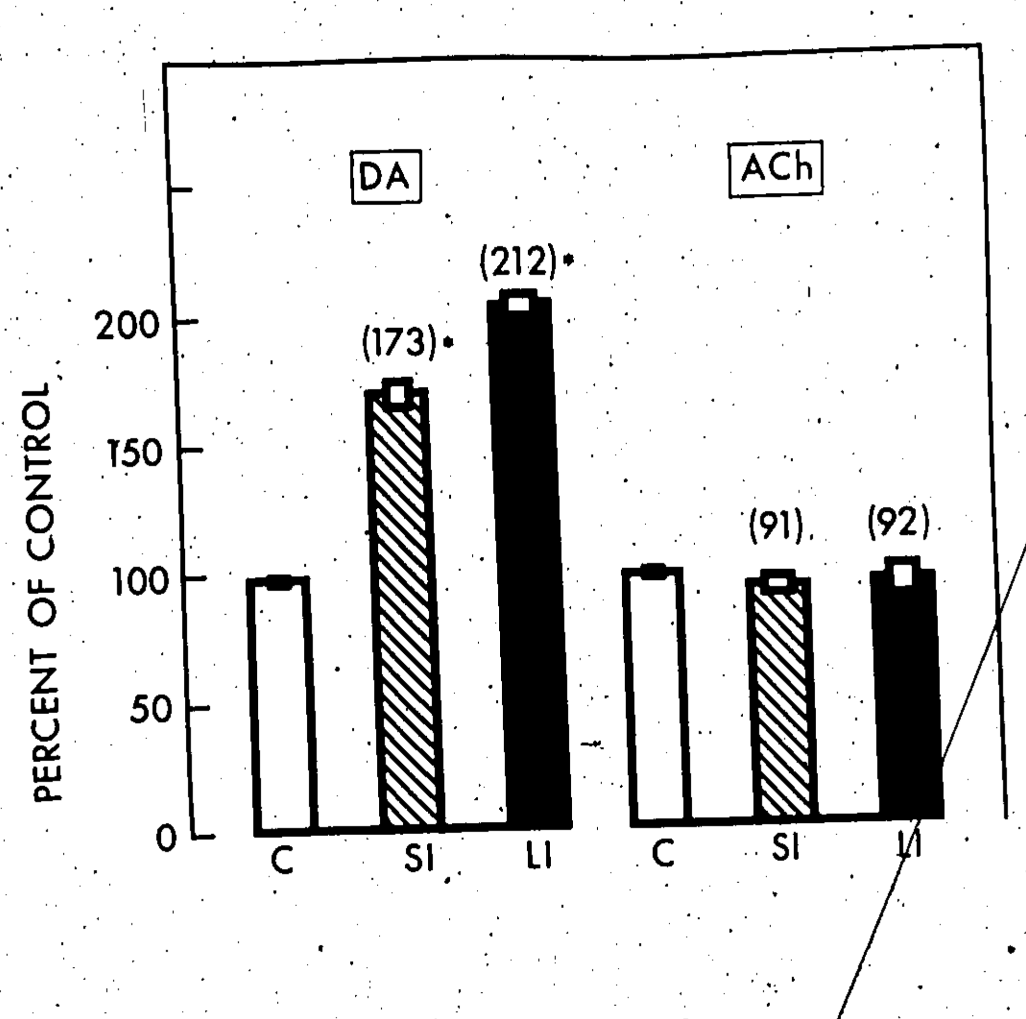

Fig. 18: Effect of subacute (4 days) REM sleep deprivation on the concentration of dopamine and acetylcholine in the rat striatum. Columns represent mean values ± S.E.M. of at least four animals in each group. Data are expressed in percentages taking the values of control animals as 100%. C, controls; SI, rats placed on small (7 cm in diameter) islands (REM sleep-deprived); LI, animals placed on large (12 cm in diameter) islands.

compared to those at the beginning of the experiment. The body weight of control rats, kept individually in separate cages increased linearly till 72 hours but between 72 and 96 hours showed a drop similar to that seen in animals kept on large islands.

The differences between the control and experimental groups become more apparent when the changes are expressed as percentages of the initial body weight (Fig. 17). Whereas there is an almost linear weight increment in control rats, animals isolated on large islands failed to gain any weight and the REM sleep-deprived rats (on small islands) even lost some (more than 5%) of their initial body weight during the first two days and failed to recover it during the remainder of the experiment.

Upon the termination of the subacute REM sleep deprivation procedure the rats were agitated, aggressive and showed increased irritability in response to external stimuli.

ii) Striatal Dopamine and Acetylcholine

Concentration of striatal dopamine was found to be markedly increased not only in the group of REM sleep-deprived animals but also in rats isolated on larger (12 cm) islands. As shown in Fig. 18, the increases represented 73 and 112%, respectively when compared to the values noted in control animals (3.2 ± 0.2 μg/g).

In contrast, levels of acetylcholine in the striatum of both experimental groups were slightly reduced to 91 and 92%, respectively when compared with controls (44.4 ± 1.9 nmol/g).

b) Effects of Chronic REM Sleep Deprivation

i) Body Weight and Gross Behaviour

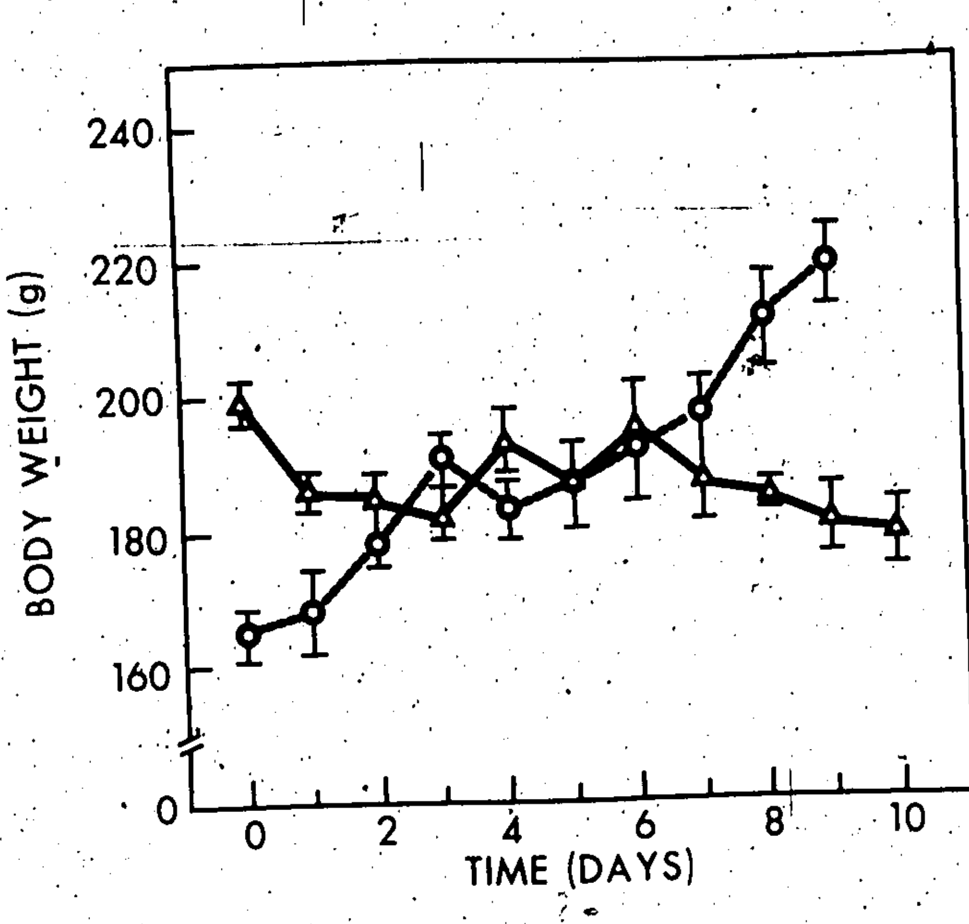

Fig. 19: Effect of chronic (10 days) REM sleep deprivation on the body weight of rats. Each point represents mean value of six rats; vertical lines indicate S.E.M. Δ——Δ, rats placed on small islands (REM sleep-deprived); o---o, controls.

Changes in the body weight of rats placed for 10 days on small islands (REM sleep-deprived) in comparison with those in control animals are presented in Fig. 19. Control rats in separate cages showed normal increases in body weight whereas the REM sleep-deprived animals displayed a two-phase decrease in their weight which at the 10th day was actually below that at the beginning of the experiment. Percentage changes in body weight (taking 0 day weight as 100 percent) in REM sleep-deprived rats and control animals kept in separate cages are shown in Fig. 20.

After prolonged REM sleep deprivation (for 10 days) the experimental animals appeared apathetic, exhausted and responded less to external stimuli.

ii) Locomotor Activity

The locomotor activity of control as well as of experimental (REM sleep-deprived) animals was measured daily at the same hour to avoid possible changes due to the circadian rhytm. As shown in Fig. 21, the locomotor activity of the control group has, after an initial fall during the first two days, stabilized around the values of approximately 200 counts per 5 min. In contrast, the locomotor activity of rats isolated on small islands (REM sleep-deprived) did not show an initial drop and was from the second day on consistently higher (by about 50%) than that of control animals.

iii) Striatal Dopamine and Acetylcholine

Levels of dopamine and acetylcholine in the striatum of rats chronically deprived of REM sleep in comparison with those of control

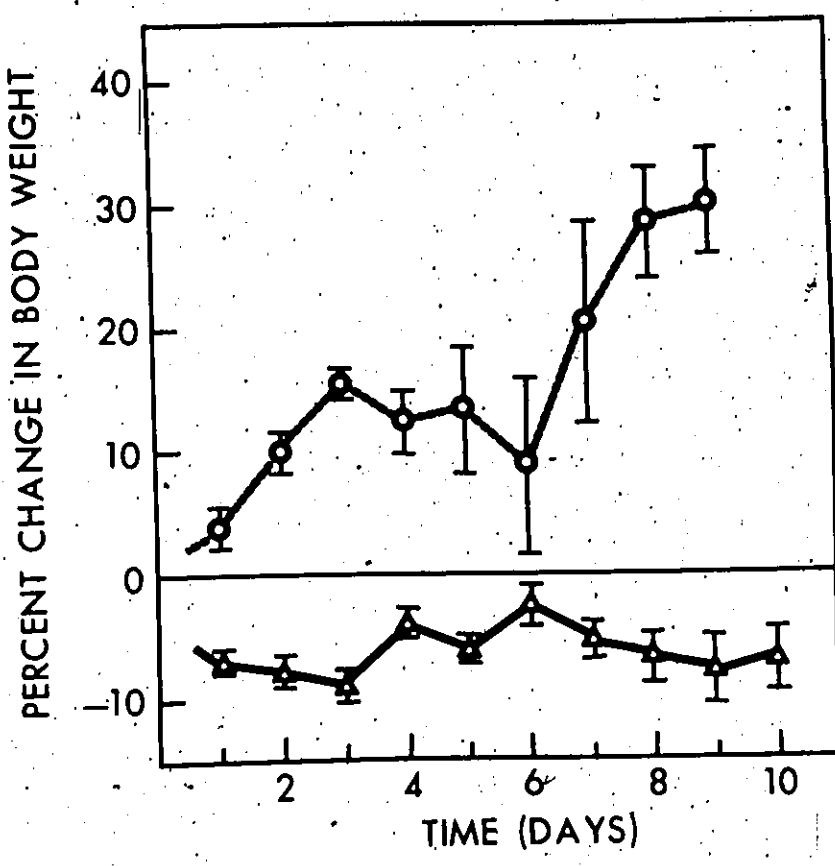

Fig. 20: Effect of chronic (10 days) REM sleep deprivation on the body weight of rats. Data are expressed as percentages taking the weight at day 0 as 100%. Δ——Δ, REM sleep-deprived rats; o---o, controls.

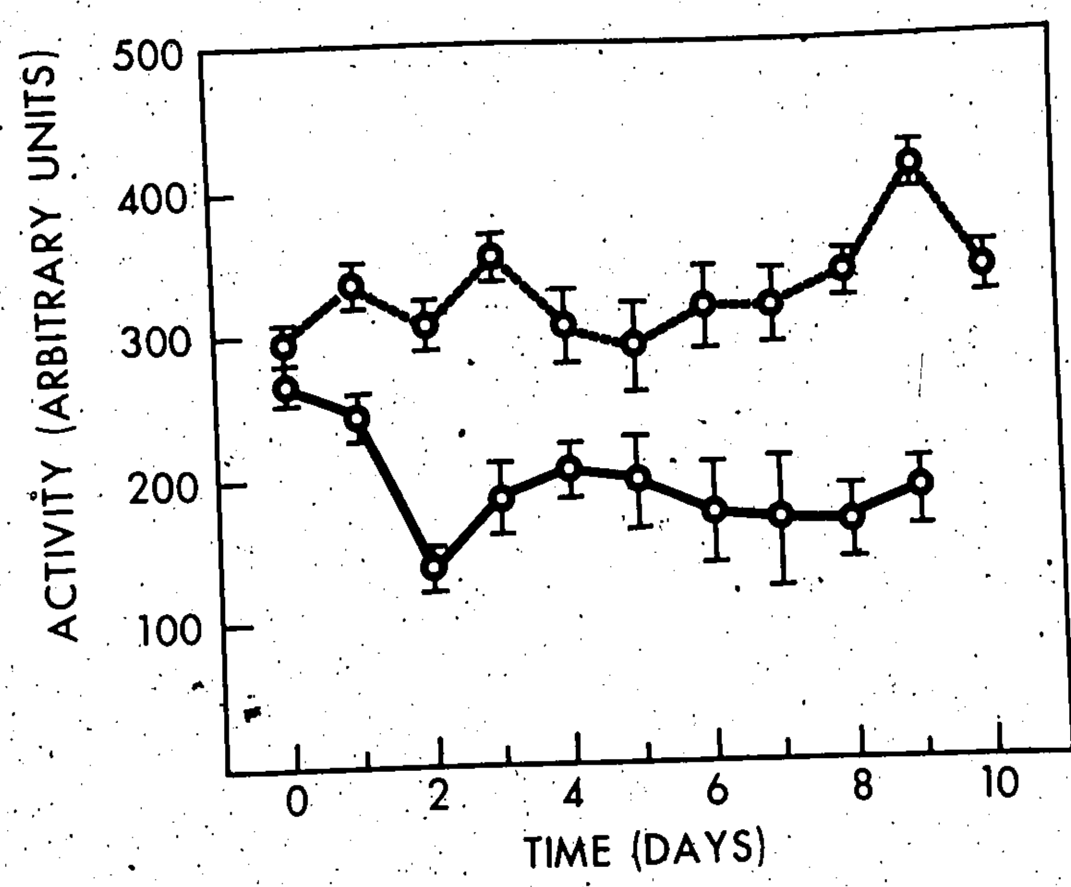

Fig. 21: Effect of chronic (10 days) REM sleep deprivation on the locomotor activity of rats. Each point represents mean value of six animals, vertical bars indicate S.E.M. o---o, rats placed on small islands (REM sleep-deprived); o——o, control rats. Locomotor activity is expressed as number of counts per 5 min.

animals are shown in Fig. 22. Similar to subacute REM sleep deprivation, the concentration of striatal dopamine in rats chronically deprived of REM sleep was markedly increased, attaining a mean value which was 133% higher than that found in the control group (3.2 ± 0.2 μg/g).

In contrast, alterations in the amount of striatal acetylcholine were opposite to those noticed after the subacute REM sleep deprivation. Fig. 22 shows that acetylcholine levels in the striatum of rats isolated on small islands for 10 days (chronically deprived of REM sleep) were significantly ($p<0.001$) higher (56.0 ± 2.5 nmol/g) than those in the control group (44.4 ± 1.9 nmol/g).

D. DISCUSSION

Our data indicate that REM and total sleep deprivation in rats results in marked alterations of both cholinergic and dopaminergic mechanisms in the striatum. Concentration of striatal dopamine was significantly increased after subacute and chronic REM sleep deprivation by 73 and 133%, respectively when compared to controls. Levels of acetylcholine in the striatum were significantly enhanced (by 28%) after chronic, but moderately decreased after subacute deprivation of REM sleep. Behavioural differences also seem to exist between the subacute and chronic REM sleep deprivation. Whereas upon the termination of the subacute experiment the experimental animals were agitated and aggressive, after prolonged deprivation (for 10 days) the rats appeared apathetic, exhausted and responded less to extern[illegible] [illegible] subacute and chronic deprivation procedure [illegible] loss in comparison with control rats.

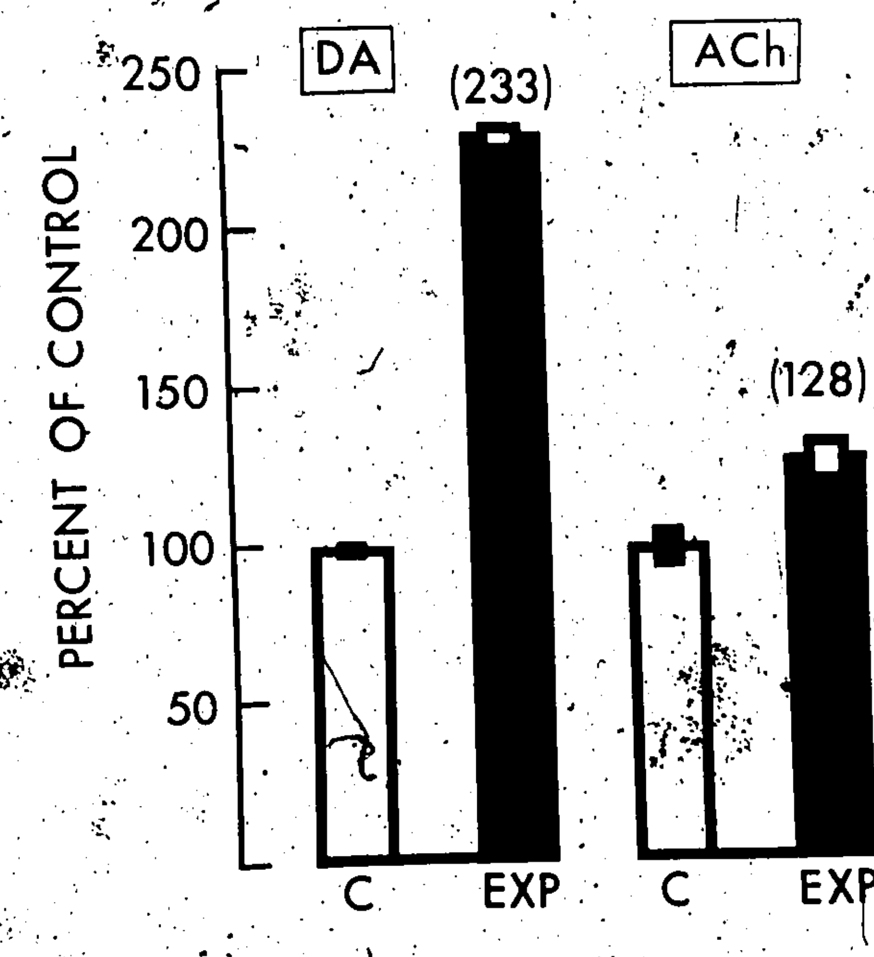

Fig. 22: Effect of chronic (10 days) REM sleep deprivation on the concentrations of dopamine and acetylcholine in the rat striatum. Columns represent mean values ± S.E.M. of at least six animals in each group. Data are expressed in percentages taking the values of control animals as 100%. C, controls; EXP, REM sleep-deprived rats.

Although it has been suggested that brain biogenic amines may play a role in sleep mechanisms, their exact relationship to both slow-wave and REM sleep remains to be clarified. Whereas serotonin is believed to be responsible for producing slow-wave sleep, norepinephrine has been implicated in the occurance of REM sleep periods (Jones et al., 1969; Torda, 1967). Furthermore, accumulation of brain acetylcholine during sleep has been reported by Richter and Crossland (1949).

One of the approaches to elucidating the possible role of biogenic amines in various stages of sleep has been the investigation of neurochemical consequences of prolonged total and REM sleep deprivation. REM sleep deprivation in rats was found to result in increased synthesis and utilization (Schildkraut et al., 1972) as well as enhanced turnover (Pujol et al., 1967; Mark et al., 1969) of brain norepinephrine. Whereas Schildkraut et al. (1972) reported that endogenous levels of norepinephrine in the whole brain of rats remain unchanged after 72 hours of REM sleep deprivation, Tsuchiya et al. (1969) found marked decreased in the concentration of this amine in the diencephalon and mesencephalon of rats deprived of REM sleep for 96 hours.

Bowers et al. (1966) reported that rats deprived of REM sleep (and partially of slow-wave sleep) show a significant fall (by 35%) in the amount of acetylcholine in telencephalon without any concomitant changes in diencephalon and brain stem. Reduction of acetylcholine levels in the striatum of rats deprived of REM sleep for 96 hours found in our experiments was similar to that reported by Tsuchiya et al. (1969) for telencephalon, a brain area including cortex, caudate and hippocampus. It is of interest that the latter authors found an opposite change,

i.e. an increase of acetylcholine levels in the telencephalon of rats after 24 hours of total sleep deprivation.

In all of the above studies the experimental animals were deprived of REM sleep for a maximum of 96 hours. We have extended the deprivation procedure to 10 days. It was shown in preliminary experiments that sleep deprivation beyond this time period results in death of experimental animals. Similar observation was made by Morden et al. (1967). Alterations in striatal acetylcholine found in our experiments after chronic REM sleep deprivation were opposite to those observed after the 96 hr deprivation procedure. It has been reported (Jasper and Tessier, 1971) that during REM sleep the cortical release of acetylcholine (which is suppressed during the slow-wave sleep) rises to waking levels. The possibility thus exists that chronic REM sleep deprivation may result in reduction of acetylcholine release and lead to accumulation of this neurohormone in brain tissue. Another possible explanation may be derived from observation of Tsuchiya et al. (1969) that in rats deprived of total sleep the telencephalic levels of acetylcholine were increased. The deprivation of REM sleep in last days of our chronic deprivation experiment might have been accompanied by total sleep deprivation as well, as it was in the typical experiment analyzed in Table 1.

Little attention has been paid to the possible role of brain dopamine in sleep mechanisms. The administration of the precursor of dopamine, L-DOPA was reported to supress REM sleep (Spitzer et al., 1971; Post et al., 1971). Our findings of increased dopamine concentration in the striatum of rats deprived of REM sleep for either 4 or 10 days is not at variance with the above observations. However, similar increases

of striatal dopamine were noted in rats placed on large (12 cm) islands; in this condition the animals may also be partially deprived of their REM (Schildkraut et al., 1972) and/or slow-wave (Mendelson et al., 1973) sleep. For this reason, the large island cannot be considered an appropriate control in REM sleep deprivation experiments. Placing of rats in water for one hour/day has recently been recommended as a better control insofar as in this circumstance the REM and slow-wave sleep times were found to be similar to those in baseline animals (Mendelson et al., 1973).

This, of course raises the question of whether the biochemical changes observed after sleep deprivation procedures can be considered as a specific consequences of REM and/or total sleep deprivation or result rather from concomitant stress factors. Opinions on this important question are divided. Some workers (Mark et al., 1969; Schildkraut et al., 1972) suggested that the increase in norepinephrine turnover as well as in the weight of adrenal glands may be entirely a result of the experimental stressful situation, since these changes were similar in rats kept on small islands (completely REM sleep-deprived) and on large platforms. Our findings of increased striatal dopamine content in both experimental groups would concur with the above suggestion. On the other hand, Bowers et al. (1966) and Tsuchiya et al. (1969) felt that the alterations in brain acetylcholine observed in REM sleep-deprived rats are an indication of metabolic changes due to REM deprivation and cannot be considered a nonspecific response associated with stress factors, since other stressful situations (food deprivation, restrain) did not lead to changes in telencephalic acetylcholine values.

Methods have yet to be devised to elucidate whether changes in the levels and metabolism of various brain biogenic amines are a specific consequence of REM and total sleep deprivation or reflect a response to a general stressful situation.

We are aware of the fact that the controls used by us in the sleep deprivation experiment may not be adequate. Animals in individual cages do not suffer from the stress factors which the experimental animals are subjected to besides REM deprivation. Large island animals on the other hand are also deprived of a part of REMS sleep. We used large island animals for comparison in lack of any better model of control available at the time we did the experiment.

The behavioural effects of subacute REM sleep deprivation (increased irritability, responsiveness to external stimuli, aggressiveness and failure to gain weight) noted in our experiments are in agreement with observations of other workers (Albert et al., 1970; Bowers et al., 1966). Apathy, lack of interest in the environment and diminished responsiveness to external stimuli observed at the end of chronic deprivation procedure can be ascribed to extreme exhaustion and fatigue of experimental animals. Results of the objective measurement of changes in locomotor activity as a function of REM sleep deprivation confirmed the earlier findings of Albert et al. (1970) in that the REM sleep-deprived animals display a substantially higher short-term activity than the control rats. To which extent are the observed behavioural changes related to the alterations in the levels and metabolism of brain biogenic amines found after REM sleep deprivation remains to be clarified.

III SUMMARY AND CONCLUSIONS

The levels and metabolism of acetylcholine and dopamine in whole brain or discrete cerebral areas such as striatum were investigaged in altered functional states of the central nervous system due to neonatally induced hypothyroidism and REM sleep deprivation as well as after administration of drugs used in treatment of mental depression and Parkinsonism.

The influence of neonatal thyroidectomy (Tx) on developmental changes in dopamine (AD), acetylcholine (ACh) and acetylcholine esterase (AChE) was studied in the whole brain of rats. In control animals, brain levels of ACh gradually increased and attained adult values at the 70th day. In contrast AChE activity showed a rapid increase between 7th and 30th day. Levels of DA were low during the early postnatal life but markedly increased to reach adult values of 1.47 µg/g at 30th day after which no further enhancement was noted. Neonatal Tx interfered with the normal growth of the animals, decreased brain weights and markedly influenced the developmental pattern of both brain DA and ACh. The concentration of DA in 30 days old hypothyroid rats was 46% of the control values. In contrast, brain ACh levels in Tx rats were consistently above those seen in controls, being significantly higher (by 49 and 64%) at 15 and 30 days, respectively. Activity of AChE in brains of hypothyroid animals was not significantly different from those in controls. Treatment of Tx rats with the thyroid hormone (T3) virtually restored the levels of DA and ACh to values seen in control animals. The question as to what extent these alterations in brain amines of Tx animals are associated with functional changes seen during hypothyroidism and experimental cretinisms

needs further clarification.

Changes in the concentration of striatal DA and ACh in rats chronically (10 days) deprived of REM sleep were compared with those obtained after subacute (4 days) deprivation. Animals placed on small (7 cm diameter) islands surrounded by water were completely deprived of REM sleep but able to obtain some slow-wave sleep. Concentration of striatal DA was significantly increased after subacute and chronic REM sleep deprivation by 73 and 133%, respectively when compared to controls. Levels of ACh in the striatum were significantly enhanced (by 28%) after chronic, but moderately decreased (by about 10%) after subacute deprivation of REM sleep. The short-term locomotor activity was significantly higher in REM sleep-deprived animals. Upon termination of the subacute deprivation procedure the rats were agitated and aggressive; in contrast, after prolonged REM sleep deprivation they appeared apathetic, exhausted and responded less to external stimuli. Our data indicate that REM and total sleep deprivation result in marked alterations of both cholinergic and dopaminergic mechanisms in the rat striatum. In addition, behavioural as well as neurochemical differences appear to exist between the subacute and chronic REM sleep deprivation.

Tricyclic antidepressants (imipramine, desmethylimipramine and amitriptyline) as well as an anticholinergic drug benztropine, used in treatment of Parkinsonism significantly increased the steady-state levels of dopamine in the rat striatum. Dose-response and time-course studies have shown that desmethylimipramine has a biphasic effect on the striatal DA concentration. These experiments have revealed an additional feature

in the central action of imipramine-like drugs which may be related to the reported beneficial effect of these compounds in the treatment of Parkinsonism.

The results presented in this dissertation suggest that changes in brain acetylcholine and dopamine play an important role in altered functional states of the central nervous system, such as in experimental hypothyroidism and during REM sleep deprivation as well as in the central action of imipramine-like antidepressants.

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