STUDIES ON THE EFFECTS OF LIGHT DEPRIVATION ON THE FORMATION OF ADENOSINE 3', 5'-CYCLIC MONOPHOSPHATE

by

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B.Sc., University of British Columbia, 1973

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in

THE DEPARTMENT OF BIOCHEMISTRY
FACULTY OF MEDICINE

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THE UNIVERSITY OF BRITISH COLUMBIA
March, 1976

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Morphological, electrophysiological and biochemical changes have been shown to occur in the retina, lateral geniculate nucleus, and visual cortex of light deprived animals. We attempted to determine whether the dark-rearing of rats from birth to 15, 30 and 60 days of age alters the ability of noradrenaline (NA) 30 μM, potassium chloride (KCl) 50 μM, adenosine 30 μM and combinations of NA and KCl with adenosine to stimulate the in vitro formation of cyclic AMP (cAMP) in visual cortical slices and, as an internal control, in frontal cortical slices. At 15 and 30 days of age there was an 11% and 21% reduction, respectively, compared to normally reared controls, in the stimulation of cAMP formation in a 5 minute incubation with NA in both frontal and visual cortical slices. After 60 days of dark-rearing, however, this was reversed in that the NA stimulation of cAMP formation was 23% and 35% higher than controls in frontal and visual cortical slices. In frontal cortical slices of rats dark-reared for 15 and 30 days there was a significant reduction in the stimulation of cAMP formation in a 20 minute incubation with NA. No differences were observed between 30 day old experimental and control animals in studies of the accumulation of cAMP in frontal and visual cortical slices incubated for various times with KCl. The stimulation of cAMP formation induced by KCl and adenosine in a 5 minute incubation was 57% and 39% higher, respectively, in frontal cortical slices of 60 day old experimental animals than controls while the response in visual cortical slices was unaffected. The differences found between 60 day old experimental and control animals were abolished in both visual and frontal cortical slices when adenosine was used in combination with NA or KCl. Studies of the
accumulation of cAMP in slices incubated for various times with NA revealed that the effect observed in the visual cortex after 30 days of light deprivation was due to a decrease in the maximum level of cAMP reached within a 20 minute incubation period, whereas in the frontal cortex the maximum level attained within a 20 minute incubation period was unaffected.

These results are discussed in terms of our present knowledge concerning supersensitivity and plasticity in the central nervous system and the role of cAMP in nerve.
ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Dr. S.C. Sung for allowing me the opportunity to work with him and for unfailing confidence in my abilities. I would like to thank W. Popow who spent innumerable hours providing valuable advice and encouragement when they were needed most. Thanks are also given to Drs. H.C. Fibiger and V.K. Singh for showing interest, giving encouragement and offering helpful suggestions during the course of this work. I would also like to offer my deepest appreciation to Drs. E. McGeer and V.K. Singh who took time out from their busy schedules for the critical reading of this thesis.
DEDICATION

I would like to dedicate this thesis to my wife, Kristy, who has taken me for better or for worse.
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INTRODUCTION

I. Sensory Deprivation as a Tool to Investigate Central Nervous System Development and Function.

Since the middle of this century there has been an enormous effort made to determine the extent to which the development and functioning of the central nervous system (CNS) may be affected by the environment. The term plasticity has been used to describe the ability of the CNS to respond and adapt at different structural levels to various types of insults. Without environmental demands for their use some CNS organizations are lost, their structural substrates diminished, and the brain chemistry altered. When certain sensory requirements are imposed, exaggerated neural growth patterns and restructuring of function beyond the ordinary occur. Compensation of structural and functional capacities result from a shift of environmental demands away from one sensory modality and towards others.

Although this information suggests that the CNS is highly plastic, it has been difficult to draw conclusions pertaining to the cellular mechanisms whereby plasticity is achieved and the impact that plastic changes have on function at various levels of structural organization. For example, if use promotes growth and function of the nervous system and disuse retards development or even induces atrophy, when and how are such effects occurring? Furthermore, the effects of learning, although certainly less pronounced than those of sensory deprivation, probably involve similar features. Therefore, can a thorough understanding of findings obtained from investigations involving sensory deprivation lead to the delineation of the morphological and chemical substrates of learning? It is only
through an interdisciplinary approach involving physiology, anatomy, and biochemistry that we can begin first to draw correlations and finally to answer these questions.

The environmental perturbations most used to investigate CNS plasticity have been sensory deprivation and controlled stimulation. Sensory deprivation refers to the reduction of the total number of stimuli delivered to sensory structures such as the motor, auditory and visual systems. More generalized approaches include impoverished and enriched environments as well as social isolation. Studies involving deprivation of the visual system of animals have been by far the most numerous. The main advantages of this system are as follows: a) The sensory input, which enters mainly via the optic fibers, can be easily modified and quantified. b) It is a system with minimal convergence of afferent fibers from other brain centers, which is very important because the non-visual afferents might reduce the effects of altered visual input. c) The visual influx is lateralized because of almost complete crossing of the optic fibers in the chiasma. From this point of view albino rats have been shown to be especially suitable (1,2).

A variety of procedures have been used to alter the total amount of input stimuli to the visual system. These include supplying an excess of light stimuli, fitting animals with translucent occluders, limiting the amount of incident light totally or to a few hours per day, and limiting the light to a particular frequency band. Most of the present discussion will be limited to investigations involving the total exclusion of light from experimental animals. The methods whereby this is achieved include rearing the animals in total darkness, enucleation of the eye or suturing the lids over the eye.
There are few studies that show little or no effect on visual struc-
tures following enucleation. Some of the findings obtained by this method
are important and are therefore included in this discussion even though it
may be argued that the technique involves deafferentation to produce the
desired deprivation and thus introduces the complicating factor of
anterograde transneuronal degeneration. The techniques of eye lid-suturing
and dark-rearing animals have advantages and disadvantages. Although
dark-rearing is relatively easy to accomplish and is readily reversible,
it may induce compensatory activation of other sense modalities (3).
Alternatively, light deprivation by lid-suturing can be done unilaterally
thus providing an internal control but it is uncertain to what extent
light can penetrate the eye lid.

Many review articles have appeared in the last decade regarding the
effects of total visual deprivation on various brain centers. Review
articles by Riesen (4-6) include neurochemical, neurophysiological and
morphological correlates of sensory deprivation and concentrate on the
requirement of adequate stimulation for growth of neuronal structures and
maturation of function. Mendelson and Ervin (7) and Globus (8,9) focused
their attention on the relation of post-synaptic structures to presynaptic
function and integrity. Scheibel and Scheibel (10) summarized the rela-
tion of the dendritic spine to presynaptic integrity and function.
Kreech et al. (11) and Rosenzweig (12) reviewed the anatomical findings in
the cortex of animals which have undergone different environmental experi-
ences. Extensive reviews by Fifkova (13), Cragg (14) and Raisman and
Mathews (15) have appeared on the morphological effects of sensory depriva-
tion. Rose et al. (16) have discussed the above findings with regard to
the implications they have on the processes that may be involved in learn-
ing. The biochemical correlates of sensory deprivation have been reviewed by Bondy and Margolis (17), and more recently by Walker et al. (18).

In the discussion to follow an attempt is made to integrate the key findings on the effects of sensory deprivation. The developmental aspects as well as the metabolism and function of cAMP in brain are also discussed. The adaptive nature of nerve is discussed with the intent of establishing a connection between the effects of sensory deprivation and the role of cAMP in the CNS. Finally, with reference to the foregoing discussion, the purpose and goal of the present investigation are delineated.

II. Anatomy of the Visual System.

The effects of light deprivation on the visual system have been investigated at four anatomical levels in the hierarchy of sensory information processing. These are the retina, superior colliculus, lateral geniculate nucleus (LGN) and the visual cortex. The relationship of these structures to each other is shown diagramatically in Fig. 1. Although the ganglion cells of the retina send their axons via the optic tract to a number of brain regions, the primary site of termination of these fibers in mammals is the LGN. In non-mammalian species the primary site of termination is the superior colliculus or as more generally referred to in lower vertebrates, the optic tectum. Neurons in the LGN which have received input from ganglion cells send their axons via the visual radiation to the visual cortex. Since the first synaptic site from fibers of the retina is the LGN, changes in the LGN as a consequence of any type of insult on the retina are called primary changes, while changes in the visual cortex being two synapses away from the primary sense organ are secondary effects. Tertiary changes are those found three or more syn-
FIG. 1  A schematic representation of the major visual pathways in the rat brain. The ganglion cells of the retina send their axons via the optic nerve and optic tract to the lateral geniculate nucleus and superior colliculus. The lateral geniculate neurons send their axons via the optic radiation to the visual cortex.
apses away from the initial site of insult.

In the rat unlike higher mammals only about 10% of the optic fiber are uncrossed (19). Anatomical (2), electrophysiological (1) and behavioral (20) studies have shown that in the albino rat this percentage is even less. As has been mentioned earlier this paucity of uncrossed fibers is one of the advantages of employing the albino rat in studies of the effects of visual deprivation. Investigations involving the effects of a host of visual deprivation conditions on a variety of animals have been conducted in each of the main visual centers described above. The following discussion will deal mainly with those changes which have been shown to occur in the visual cortex.

III. Effects of Sensory Deprivation of the Visual System.

a) Cytological.

The evidence accumulated to date strongly suggests that transneuronal degeneration is not halted at the first post-synaptic element, but that damage in the main afferent supply sets up a progressive involvement of successive neuronal links on the sensory neural chain. Although this is true of the trans-synaptic effects of visual deprivation in the optic system the most obvious changes occur in the retina, optic nerve, and subcortical centers and are more difficult to demonstrate in the visual cortex. The effects of visual deprivation are more pronounced the higher the animal's position in the phylogenetic scale and adult mammals are much less affected than young animals.

The dark rearing of mice from birth to various times up to 4 months causes a reduction in the thickness of the visual cortex (21,22). At 30 days of age there occurred in the visual cortex of these animals a reduc-
tion in the nuclear volume in glia and neurons, as well as a reduction in the quantity of cytoplasmic material. After prolonged dark rearing the percent difference in these parameters between control and experimental animals was shown to fall (22). The gradual recovery observed in some structures of dark-reared animals has been referred to as normalization (3). The nuclear volume of cells and the quantity of internuclear material was also found to be decreased in the auditory cortex of experimental animals at 2 months but increased above controls at 4 months (3). In the rat visual cortex, monocular deprivation for 30, 60 and 90 days caused a decrease in the tissue volume together with an increase in the cell density of various cortical layers (23,24). Thus, after 3 months there was an 8% decrease in the thickness of layers II to IV with a concomitant 11% increase in the cell density of layers III and IV (25).

There have been few cytological changes found in the visual cortex of rabbit, cat, dog, monkey and chimpanzee after light deprivation although other visual centers have been shown to be affected. Monocular and binocular eye-lid suturing of the kitten, for example, results in a decrease in cell, nuclei, and nucleolar volume in the LGN by as much as 35% (26-28). Visual deprivation of the rabbit results in a 74% decrease in the dry mass of the retinal ganglion cells (29). Dark-rearing of the chimpanzee for 6 months results in a 90% decrease in the number of ganglion cells in the retina (4).

b) Morphological.

Morphological changes in the visual cortex of animals dark-reared from birth have been repeatedly reported. Most of the studies have concentrated on quantitative and qualitative changes in dendrites, synaptic spines and synaptic sites. A reduction of dendritic length and branching
of stellate neurons was reported in the visual cortex of cats raised in the dark from birth to 100 days (30). In the visual cortex of the rabbit a greater variance of dendritic length has been shown to occur as a result of dark-rearing (31,32).

Employing the techniques of monocular lid-suturing and dark-rearing mice and rats, the deprived and non-deprived visual cortecies have been compared with respect to the number of spines on the apical dendrites of layer V pyramidal cells. Working with dark-reared mice, Valverde (33) concluded that certain spines will not develop without the input of light to the visual system. He has shown (33-36) a reduction in the number of spines on these dendrites and has described the distribution of spines on the dendrites as a function of distance from the soma. Although there is a reduction in the total number of spines in dark-reared animals, the shape of the curve of their distribution in relation to distance from the soma remains the same. In the rat the mean quantitative deficit in spines for the entire measured length of the apical dendrite was 17% and 28% after lid-suturing for 10 and 60 days, respectively (13,37). In the rabbit there have been reports of abnormally formed spines under conditions of light deprivation rather than a decrease in their numbers (31,32).

The electron microscope has been employed for studying the effects of light deprivation on cellular ultrastructure since the impregnation technique used in the above studies visualizes only a portion of the synaptic population. Cragg (38) using rats which had been deprived of light for 3 weeks found an increase in synaptic size in the superficial layers of the cortex but a decrease in deeper layers. Unilateral lid-suturing of rats reduced the number of synaptic sites by 20%, the upper layers of the cortex being most affected (39). The mean size of the axosomatic synaptic
contacts of the visual cortex supplied by the deprived eye was smaller by 23% through all layers studied when compared to the cortex supplied by the non-deprived eye (40). Axosomatic synapses having round vesicles showed a 35% and 29% reduction in layers II and IV, respectively, while synapses with flat vesicles were decreased by 16% and 14% in layers II and IV respectively (40).

Vrensen and Groot (41,42) studied the effects of dark-rearing and monocular lid-suturing on the synaptic terminals in the visual cortex of the rabbit and the recovery from these treatments after exposure of experimental animals to normal lighting. Dark-rearing for 7 months did not affect the number of synaptic contacts, their surface area, or their mean length. However, there was a 40% decrease in the number of synaptic vesicles in the visual cortex of dark-reared animals compared to controls, while no such difference was found in the motor cortex. This decrease was found to persist after raising experimental animals under normal conditions for 1 year. After eye-lid suturing the decrease in synaptic vesicles in the visual cortex was only 16% and there occurred, in the motor area of the deprived cortex, an increase in the density of synapses.

It is noteworthy that the changes in the visual cortex after enucleation or lesions at various points of the visual system bear marked similarities with those observed after light deprivation. Thus, a reduction in the number of spines of layer V pyramidal cells of the visual cortex has been reported after neo-natal enucleation of the mouse (34,43) and rabbit (32). The diminution in number of the dendritic spines in the mouse has been found to be larger at 24 days than 48 days of age indicating some degree of normalization possibly due to the involvement of compensatory mechanisms.
These findings suggest that the morphological integrity of the visual cortex is dependent on the structural integrity of the afferent systems as well as on its functional integrity.

c) Electrophysiological.

The information available indicates that sensory deprivation does affect the general electrical activity of the cortex although these effects are not well understood due to difficulties encountered in interpretation. Visual evoked potentials (VEP) and the ability of the cortex to follow various frequencies of light stimulation has been studied in normal rats and rats reared in the dark for up to 45 days of age (44). Differences in the latency of the VEP was small and disappeared by 20 days. The ability of the cortex to respond to high-flash frequencies was maximal by 30 days and 45 days in normally reared and dark-reared animals respectively. Monocular deprivation of rats for 70 to 170 days from birth was found (45) to cause a 27% diminution of the electrical activity and a 41% decrease in the amplitude of the VEP in the deprived compared to the non-deprived visual cortex. Although the overall conclusion of the latter study was that visual deprivation suppresses cortical electrical activity, it was found that the visual cortex as well as non-primary visual afferent cortical areas of some animals produced a greater response in the evoked potential in the deprived rather than the non-deprived cortex. Similarly, in the visual cortex of dark-reared rabbits (46) there was a longer latency and lower amplitude of the VEP compared to controls whereas sound and somesthetetic stimulation produced higher responses in all areas of the cortex of dark-reared animals. It has been suggested that the hypersensitivity observed to visual, auditory, and somesthetetic stimulation in other than specific projection areas of the cortex may be due to non-specific
systems at the sub-cortical or brain stem level which have become super-sensitive as a result of visual deprivation.

Electrophysiological recording from single cells of the visual cortex is another method that has been used to assess the effects of dark-rearing on the electrical activity of neural structures. This approach has allowed, to some degree, the determination of the functional changes that result from deprivation of sensory experience. In this regard recording from single cells is advantageous over biochemical and morphological studies since the latter allow correlations with function only to the extent that the consequences of the observed changes can be assessed from the known functions of the parameters affected.

There are neurons in the visual cortex that respond selectively to the orientation of an object as well as the direction of movement. Also, there are binocularly sensitive cells which respond only when stimulation is provided to both eyes. It appears that these cells have a dramatic dependence upon early usage for the maintenance of and the further development of specificity of responsiveness. The effect of unilateral eye closure in the kitten is to reduce drastically the number of cells in the visual cortex that remain responsive to stimulation of the occluded eye (26,47,48). Binocular eye closure results in the loss of cells responsive to stimulation of both eyes and a reduction in orientation selectivity (49). Some cells also become less selective in their definition of the orientation of moving edges (50) and stereoscopic cells show no peak responsiveness (51). If only certain orientations are available to the developing visual system, the cells that are later found to respond are restricted to those having sensitivities within a range of that orientation. Exposure of kittens to spots of light in a visual environment with-
out straight lines (52) results in many cells that are optimally stimulated by moving spots. The exposure of adult cats to vertical strips has been shown (53) to decrease the number of neurons sensitive to orientations around the vertical relative to those sensitive to horizontal orientations. This indicates that plasticity of functional properties of the cortical neuronal network still exists in adult animals.

The close correlation with function afforded by electrophysiological studies of single cells enables the determination of the degree of plasticity inherent in at least the visual cortex if not the brain. This is important because from the point of view of sensory deprivation experiments and the interpretations thereof, it must be established whether the changes taking place are indeed due to plastic and adaptive mechanisms intrinsic to the capabilities of the CNS or whether they are due to degenerative processes resulting from the subjection of animals to non-physiological conditions. The evidence to date is that at least a part of the structural basis for visual function is laid down at birth; but it still remains to be determined to what extent it is then modified and refined by experience.

A further method employed to study the electrical effects of sensory deprivation is to create surgical lesions at critical locations in a sensory system. Although lesions in the visual system are a form of sensory deprivation, this procedure may involve physiological and biochemical processes other than those occurring during lid-suturing or dark-rearing. Nevertheless, some of the findings obtained from these studies are relevant to the present discussion since the basic mechanisms responsible for altered brain function during these two experimental paradigms need not be mutually exclusive and may even be inseparable.

Lesions of the visual system may be produced at the level of the eye
(enucleation), lateral geniculate nucleus, or the cortex. Fentress and Doty (54) using chronically implanted electrodes in the cat and monkey to stimulate the optic tract and optic radiation showed that the electrical responsiveness of the visual cortex increases several fold after enucleation. Lateral geniculate lesions in the cat resulted in increased excitability to electrical stimulation of the visual cortex and a reduction in the electrical threshold for producing afterdischarges (55).

The isolated cerebral cortex, a preparation which involves undercutting the cortex but leaving the superficial blood supply intact, has been shown to become more susceptible to agents eliciting epileptiform activity and to electrical stimulation (56). It was further observed that this increased susceptibility could be prevented from occurring by "exercising" the disused neural elements through electrical stimulation (57). In the isolated cerebral cortex of kittens there is extensive collateral growth from injured axons (58). In the isolated cortex of cats there is increased capacity to bind $^{14}$C-D-tubocurarine, decreased acetylcholinesterase activity, decreased acetylcholine content and a reduction in the number of dendritic spines (59-61). All these changes could be prevented by electrically stimulating the isolated cortical slabs and it is this finding that brings the above studies into the realm of the present discussion. It shows that the changes resulting from isolation of the cortex may not be due to degenerative processes but rather to disuse. These observations support the contention that adaptive mechanisms are present and operative in the central nervous system.

d) Biological.

Biochemical investigations have not reached the degree of sophistication that the other disciplines have with respect to establishing the
effects of light deprivation on the visual system. One of the reasons for this is that biochemistry being at a more fundamental level of structural and functional organization, is inherently more complex. Also, the cellular complexity and morphological heterogeneity of brain results in difficulties in interpretation of biochemical data. Furthermore, the quantities of tissue involved in structures of the visual system are very small thus precluding some types of investigations due to limitations in available biochemical techniques. Despite these drawbacks, biochemical studies have been conducted and the information obtained, although sparse, does contribute to our understanding of the biochemical basis of neural function.

The emphasis with regard to the biochemical effects of environmental manipulation has been on protein and ribonucleic acid (RNA) metabolism since these are the processes that would be expected to lead to relatively permanent alterations in function.

The literature concerning RNA metabolism is fragmented and confusing. The polysomes in the cortex of rats kept in darkness for 3 days and subsequently exposed to light for 15 minutes were characterized by electron microscopy and sucrose-density gradients. The polysomes in the visual cortex and other cortical areas increased in number in experimental animals compared to controls. Moreover, the protein synthesizing capacity of ribosomes isolated from the cortex of dark-treated animals was increased (62). Consistent with this finding is the demonstration (63) that 22 day old dark-reared rats exposed to light for 2 hours have a higher incorporation rate of $^{14}$C-orotic acid into RNA of the visual cortex than do dark-reared controls. However, in the latter study only the visual cortex was affected. Unlike the frontal cortex the RNA content of the visual cortex of exposed animals was lower than unexposed dark-reared animals. De Bold
et al. (64) found no effects of darkness and other lighting conditions on the total amount of cortical RNA, but did obtain evidence suggesting an influence on the species of RNA produced in the visual cortex. In experiments on imprinting in newly hatched chicks in which one eye of the chicks was covered it was shown that the incorporation of $^3$H-uracil into RNA and the activity of RNA polymerase were 15% and 34% lower, respectively, in the forebrain connected with the covered eye as compared to the forebrain of the uncovered eye (65,66). Similar results were obtained when the incorporation of $^3$H-lysine into protein was measured (67).

Rose and his co-workers have conducted a number of studies in which they investigated the effects of dark-rearing and subsequent exposure to light on protein synthesis in the visual cortex of rats. Rats that had been dark-reared for 7 weeks followed by exposure to laboratory illumination for various times after they had been injected with $^3$H-lysine showed a transient increase in the incorporation of the radioactive amino acid into protein (68). The question of the biochemical specificity of these changes in protein synthesis was then investigated by differentially labelling the proteins of the visual cortex of experimental and control animals with carbon-14 and tritium labelled amino acids and fractionating the soluble and particulate proteins on polyacrylamide gels (69,70). It was found that 2 out of 21 soluble protein bands and 7 out of 20 particulate protein bands exhibited high differential incorporation rates between control (dark-reared) and experimental (light-exposed) animals. This suggests that certain protein fractions are disproportionately affected by visual stimulation after dark rearing.

A problem with the approach of fractionating proteins by gel electrophoresis is the difficulty of ascribing functional roles to those proteins
which are specifically affected. For this reason an alternate method of fractionation was adopted by these workers which involved the separation of the visual cortex into two cellular components: the neuronal and neuropil (glia, dendrites, axons) fractions. It was found that the elevation in incorporation of $^3$H-lysine into protein which occurs during first exposure to light, takes place amongst the neuronal proteins (71). In all areas of the cortex of normally reared animals the neuronal to neuropil incorporation ratio for short labelling times was 1.6 but after 4 hours decreased to 0.5 (72). Although this was the ratio obtained for the motor cortex of dark-reared animals, the ratio for the visual cortex even at short incorporation time intervals was 0.7 and increased to normal values only if the animal was exposed to light. From these studies it was suggested that the synthesis of rapidly labelled, rapidly transported particulate neuronal proteins is suppressed in the visual cortex but not the motor cortex of dark-reared rats (72,73).

The effect of unilateral eye-lid suturing on $\text{Na}^+$, $\text{K}^+$ activated ATPase and $\text{Na}^+$ and $\text{K}^+$ content has been studied in the optic tectum of the adult pigeon (74). There occurred a transient increase in this enzyme activity between 4 and 8 weeks of visual deprivation and this was accompanied by an increase in $\text{Na}^+$ content and a decrease in $\text{K}^+$ content. These changes in enzyme activity and $\text{Na}^+$ and $\text{K}^+$ ion content were correlated to the characteristic sensitivity of the brain protein synthesizing system to the ionic environment and it was suggested that the transient changes were evidence of functional adaptation.

The two neurotransmitter metabolizing enzymes choline acetyltransferase and acetylcholinesterase were studied in the visual centers of 21 day old dark-reared rats (75,76). There were no changes in these enzyme
activities in the visual cortex although changes in other optic centers (eg. LGN) were found.

In the visual cortex of dark-reared rats it has been found that the amino acid levels especially that of glutamate were generally elevated compared to normally reared controls (77). The fact that glutamate was higher by 25% in experimental animals is particularly interesting since it is suspected that glutamate may function as a neurotransmitter.

The biochemical changes observed in the visual cortex as a result of the perturbation of visual input must certainly form the basis of the morphological and electrophysiological changes that have been observed. It is clear that more detailed work needs to be done to determine what the biochemical signal is for a change to take place, what cascade of events this signal induces, and what the building blocks are that give rise to altered neural function. It is partly toward these problems that the present investigation is directed.

IV. cAMP and the Nervous System.

a) The Metabolism and Function of cAMP in Brain.

Adenosine 3', 5'-cyclic monophosphate is now recognized as an intracellular messenger mediating the actions of a variety of hormones in specific target tissues. As a result of work in the last decade, the involvement of cAMP in neurobiological events is also gaining acceptance. What follows is a discussion of the features of the metabolism and possible roles of cAMP in nerve tissue insofar as they are pertinent to the present investigation.

Adenylate cyclase, the enzyme responsible for the synthesis of cAMP from its substrate ATP, has been shown to be present in higher activity in
the CNS than in any other mammalian tissue (78). Subcellular fractionation studies revealed that the enzyme is localized to the plasma membrane and in particular to those fractions containing nerve endings and synaptic complexes (79,80). The enzyme responsible for the degradation of cAMP is nucleotide 3', 5'-cyclic phosphodiesterase which, like adenylate cyclase, is present in higher activity in the CNS than in any other mammalian tissue (81). Subcellular distribution and cytological localization studies have shown that this enzyme resides almost exclusively at the postsynaptic nerve ending and more precisely at the post-synaptic membrane (82-84). Both adenylate cyclase and phosphodiesterase are concentrated in those fractions containing the greatest quantity of the known neurotransmitters (85) as well as cAMP-dependent enzymes such as cAMP-dependent protein kinase (86), the protein substrates for protein kinase (87), phosphoprotein phosphatase (88), and N-acetyltransferase (89). The presence and strategic location of this enzymatic machinery, henceforth referred to as the cAMP system, indicates that cAMP may serve an important function in the CNS and that this function may be related to the process of synaptic transmission.

Further studies have led to the idea that cAMP may function as a mediator of the neurohormones involved in synaptic transmission. This concept has developed from the demonstration that a variety of putative neurotransmitter substances stimulate the formation of cAMP, (although only slightly in brain homogenates, do so profoundly in brain slices). Some of the substances that have been found to increase the content of cAMP in brain slices include noradrenaline (NA) (2,90,91), histamine (1, 90,91), serotonin (92), dopamine (93) and adenosine (94,95). The ability of some of these agents to elevate cAMP content in brain slices is greater
in some animals than others as in the case of serotonin in the rabbit, and is greater in some brain regions than others as in the case of dopamine in the caudate nucleus. This finding is consistent with the heterogeneity of the distribution of neurotransmitters in the CNS.

Nervous tissue functions by integrating information through excitation and inhibition. Thus it is noteworthy that perturbation of the electrical processes of nerve also affects cAMP levels. It has been shown that a variety of agents such as $K^+$, ouabain, batrachotoxin, and veratridine which are known to cause membrane depolarization also cause profound stimulation of cAMP formation in brain slices (96-98). Moreover, it has been demonstrated that application of electrical pulses to brain slices causes large increases in their cAMP content (99). An interesting finding has been that when adenosine or a depolarizing agent is incubated together with some of the biogenic amines (histamine, serotonin, NA), the stimulation of the formation of cAMP in brain slices is much more than additive. Since the mode of action of depolarizing agents and adenosine with regard to their ability to stimulate cAMP formation in brain slices is not known, the significance of the synergistic effects between these agents and the amines is not clear.

The strongest support for the involvement of neurohormone-sensitive and more specifically catecholamine-sensitive adenylate cyclases in synaptic transmission comes from the work of Greengard and his colleagues (100-102) on the sympathetic ganglion and Bloom and his associates (103-105) on the cerebellum. In the isolated superior cervical sympathetic ganglion of the rabbit electrical stimulation causes an acetylcholine mediated depolarization of ganglionic neurons. This excitation is followed by a slow and long lasting hyperpolarization of ganglionic neurons which
is thought to be mediated by dopamine. The inhibitory effect of dopamine is thought to be mediated by a dopamine-sensitive adenylate cyclase for the following reasons: (a) Stimulation of the ganglion increases cAMP levels. (b) cAMP can mimic the hyperpolarizing effect of dopamine when applied to ganglionic neurons. (c) Phosphodiesterase inhibitors potentiate both the hyperpolarization and the increase in cAMP levels induced by electrical stimulation and also potentiate the hyperpolarization induced by exogenous dopamine.

In the cerebellum Purkinje cells receive an inhibitory input from a diffuse system of NA-containing nerve terminals. The iontophoretic application of cAMP onto the surface of Purkinje cells was found to mimic the inhibitory actions of NE on the discharge rates of these neurons. The inhibitory effects of both NA and cAMP were potentiated by phosphodiesterase inhibitors. Furthermore, intracellular recordings from Purkinje cells showed that both NA and cAMP caused a hyperpolarization of the neurons similar to that produced by stimulating the NA pathway innervating these cells. Using an immunocytochemical method for detecting cAMP, it was shown that application of NA or stimulation of the NA pathway to these cells caused a large increase in the proportion of Purkinje cells that reacted positively.

After its production in nerve, little is known concerning the subsequent biochemical events that cAMP may participate in or concerning the possible mechanisms by which changes in cAMP concentrations would influence electrical events at the synaptic membrane. Once these processes are delineated it will become evident whether cAMP is, in fact, an intracellular messenger for some neurotransmitters. The study of the target enzymes of cAMP and their actions may provide the support for the involve-
ment of cAMP in mediating the fluctuations in the ionic environment of the membrane and provide some clues as to the mechanism whereby this is achieved. It has been suggested, for example, that the ion permeability characteristics of the membrane may be modified by the phosphorylation of specific synaptic membrane proteins by cAMP-dependent protein kinase (100). Initial conditions would be restored through the hydrolysis of cAMP by phosphodiesterase and dephosphorylation of the membrane protein by phosphoprotein phosphatase.

The participation of cAMP in metabolic events other than those at the synapse have not been systematically investigated. Therefore, the problem of relating and connecting the events which intervene between neuronal stimulation and the general metabolic responses which are known to occur in nerve under these conditions remain unsolved insofar as the involvement of cAMP is concerned. It is believed, however, that the synthesis and catabolism of glycogen in nerve might be affected by hormones in a manner analogous to that in other tissues, with cAMP as a mediator. cAMP has also been implicated in the process of axonal elongation since it is known that axonal elongation is directly dependent on the assembly of microtubules and that the cyclic nucleotide can stimulate this assembly process (106).

b) Development of the cAMP System in Brain.

Few investigations have been conducted on the development of the cAMP system in animals and as a result even fewer studies have been conducted where normal development has been perturbed and the effects correlated with the possible functions of this system. It is unlikely that the paucity of information in this area is due to the idea that such investigations might be fruitless since these studies will surely be of enormous
value in finally determining the functional role of cAMP in brain. Instead, it may be due to the diversion of efforts towards understanding the enzyme systems and neurohormones involved in the cAMP system. Apart from adding to our understanding of the function of cAMP in brain, developmental studies are important because cAMP may play an integral part in the development and maturation of the nervous system. Moreover, the participation of cAMP in certain aspects of ontogenesis of brain may form the basis for the function of cAMP once development is complete. Thus, the processes that cAMP may regulate might overlap the characteristic processes of development and differentiation (107). If cAMP is involved in synaptic transmission a corollary of the above hypothesis is that synaptic transmission itself may be involved in the development of the nervous system.

Support for this hypothesis is available from work which has been conducted with liver. In this organ, adrenaline and glucagon, both of which stimulate adenylate cyclase, stimulate the induction of several hepatic enzymes when injected into the fetus in utero (108,109) or when applied to fetal rat liver cells (110). Exogenously applied cAMP, in addition to mimicking the actions of these hormones, has been shown to enhance transcription and stimulate RNA polymerase in isolated liver nuclei (111).

In the rat brain the development of many enzyme systems occurs during the later half of the second post-natal week (112). Further indirect support for the above hypothesis is available from the observation that this increase in enzyme activity is preceded by an increase in brain noradrenaline and an increase in adenylate cyclase activity (113). Moreover, the maximal activity of the effector end of the cAMP system, that of
cAMP stimulated cAMP-dependent protein kinase, is fully developed in the newborn rat brain (114-116).

Neonatal thyroidectomy of rats has been shown to impair drastically brain development and to lead to anatomical, behavioral and enzymatic dysfunction in the mature rat. By this treatment an attempt was made at reducing the ability of brain to generate cAMP or to respond to it, thereby providing a means to study the possible role of cAMP in development (116). Although thyroidectomy at birth caused a 16% reduction in brain weight and a 70% reduction in body weight by 40 days of age, it had no effect on either whole brain or cortical activity of phosphodiesterase, adenylate cyclase, and the ability of NA to stimulate the production of cAMP in brain slices.

It has been found that in undernourished rats there is a 25% reduction in brain NA and dopamine and an increase in tyrosine hydroxylase as compared to adequately fed rats (117). This prompted an investigation to determine the effects of malnutrition on the cAMP system. It was found that in undernourished neonatal rats the capacity of the cerebral cortex to generate and metabolize cAMP, as shown by adenylate cyclase and phosphodiesterase activities, is insensitive to caloric restriction during early post-natal life (118).

Studies employing thyroidectomy or malnutrition appear to cast doubt on the involvement of cAMP, neurotransmitters, or synaptic transmission in brain ontogenesis. However, the above findings are completely consistent with the view pointed out earlier which was that the involvement of these processes in neural development may exist only at metabolic and morphologic levels that in the fully differentiated state succumb to the control of cAMP.
A further developmental aspect of the cAMP system in brain is the age dependency of the ability of various neurotransmitters to stimulate cAMP synthesis in brain slices. Thus, an important finding which has not been stressed in the literature is that the stimulation of cAMP formation by some neurotransmitters increases to a maximum at an early age and thereafter declines to a value observed in the adult. In rabbit cortical slices, for example, the histamine-induced formation of cAMP is highest at 8 days postpartum and lower at birth and in the adult by 75% and 37%, respectively (119). A similar diminution in the ability of NA to stimulate cAMP formation occurs by 25 days of age in the rabbit and is most pronounced in the frontal cortex and hypothalamus where the decrease from peak stimulation is 88% and 93% respectively (120). In rat whole brain slices, peak responsiveness to NA occurs at 16 days of age and decreases to 50% of this value by 25 days (113,116). Unlike rabbit, rat cortical slices did not show a decline in responsiveness to NA with age (121). This could be due to species differences but is probably due to differences in brain regions since in the rabbit frontal cortex was studied whereas in studies with rat either whole brain or whole cortex was employed.

An interpretation of the increased sensitivity of the cAMP system to neurotransmitters in the developing brain is that this may be one of the mechanisms whereby cAMP could influence morphogenesis. More stringent investigations are required to determine the validity of this concept.


It has been known for some time that excitable tissues including all types of muscle, the pineal gland, exocrine organs, and the central nervous system, can exhibit variable sensitivity to neurohormones and chemical
agents (122-124). For example, deprivation of nervous influence by various methods which bring about disuse causes effector organs to become more excitable while continuous excitation causes them to become less sensitive. Thus, excitable cells seem to have a feedback system that allows them to compensate for chronic changes in the level of stimulus they receive, becoming more sensitive when the stimulus is low and less sensitive when the stimulus is high. The terms supersensitivity and subsensitivity have been used to describe these phenomena.

The biochemical basis for alterations in sensitivity is not known and, with respect to the CNS, difficult to investigate. Therefore, the autonomic neuroeffector junction and the skeletal neuromuscular junction have been frequently used as models of CNS synapses. Some of the principles that have emerged from investigations in these systems are as follows: Supersensitivity in some effector organs is non-specific, for example, the smooth muscle of the nictitating membrane of the cat which is normally innervated by adrenergic fibers becomes sensitized after prolonged disuse to adrenomimetics, cholinomimetics, serotonin, and potassium ions (125). Supersensitivity in this system is slow to develop (requiring several weeks), is reversible (sensitivity reverting to normal when input is restored (126)), and is produced by withdrawal of excitatory influence only and not by pharmacological blockade or denervation of inhibitory input (127). To what extent these findings can be extrapolated to the CNS remains to be established. At present, however, they are useful as a point of departure.

From studies of peripheral systems one of the hypothesis put forward for the generation of supersensitivity is the proliferation of new receptors for neurotransmitters. Although not entirely satisfactory, this
explanation is supported by the finding that in normally innervated striatal muscle acetylcholine (ACh) sensitivity resides, and depolarization can be elicited, only within a few hundred microns from the neuromuscular junction. After denervation, however, the entire muscle becomes sensitized to ACh (128). Fetal muscle fibers are also sensitive along their entire length, the ACh-sensitive area shrinking to the end-plate region only after a functional myoneural connection is established (129). Important in its vindication of the peripheral system models of CNS function is the recent demonstration that at least some neurons undergo similar changes. After denervation of the parasympathetic ganglion cells of the frog heart, it was shown that the ACh sensitivity spread from their normally confined subsynaptic zones to the entire surface of the neuron (130, 131).

A further mechanism that has been proposed for the generation of supersensitivity in muscle is an increase in the efficacy of coupling between excitation and contraction (54). The analogous process in nerve would be increased coupling between post-synaptic transmitter-receptor interaction and the subsequent electrical activity of the neuronal membrane. The cAMP system in nerve lends itself well to acting as the mediator for this coupling. This is supported by the recent demonstration that treatments which alter the level of activity of nervous tissue and thus produce states of supersensitivity also alter the efficacy of neurotransmitter stimulation of cAMP formation.

The initial studies suggesting a possible involvement of adenylate cyclase and cAMP in the mechanisms of denervation supersensitivity were performed by Weiss and Costa (132) and Weiss (133). Ablation of the superior cervical ganglion of the rat causes denervation of the pineal and increased catecholamine-stimulated adenylate cyclase activity in vitro.
Subsequently, it was shown that in the cortex the cAMP formation induced by NA was augmented (134,135) after treatments with reserpine or 6-hydroxydopamine, both of which reduce the level of exposure of post-synaptic structures to NA by affecting NA containing terminals. That this phenomenon is not unique to NA was demonstrated by the finding that there is increased dopamine-induced cAMP formation in homogenates of caudate nucleus after radiofrequency of 6-hydroxydopamine lesions of the substantia nigra (136). These procedures result in the degeneration of dopamine containing terminals in the caudate nucleus thus inducing a state of disuse of post-synaptic structures by reducing the exposure of these structures to dopamine.

The above findings suggest that the cAMP system may be intimately associated with the mechanisms that form the basis for the plasticity and adaptability demonstrated by the CNS. It must be pointed out, however, that with regard to supersensitivity it is not known whether the participation of the cAMP system is the basis for or a by-product of this phenomenon and this will not be clear until a better understanding is achieved about the role of cAMP in nerve.

VI. The Present Investigation.

In the foregoing discussion evidence was presented that treatments, physiological or chemical, which precipitate disuse of certain neural structures result in an increased excitability of these structures and, where examined, the sensitivity of the cAMP system to stimulation by neurotransmitters has been shown to be altered. Data have been given which demonstrate that light deprivation of animals induces a variety of changes in regions of the brain subservient to this sense modality. It
was further pointed out that lesions of the anatomical pathway of the visual system result in changes similar in some respects to those caused by light deprivation. These changes include morphological agenesis and/or atrophy and electrophysiological supersensitivity. These findings support the contention that light deprivation of animals induces in the visual cortex a similar type of disuse of neural elements as surgical and chemical lesions have been shown to do in other neural systems. The involvement of the cAMP system in synaptic transmission has been discussed. Information was also presented that light deprivation of animals affects those morphologic structures of the visual cortex that the cAMP system is intimately associated with. It may be argued, therefore, that since the activity of the cAMP system in nerve has been shown to be altered as a consequence of disuse or diminution of function, it may also be affected in the visual cortex of light-deprived animals. The purpose of the present investigation is to test this hypothesis.

The experimental approach employed to accomplish this was to measure the ability of various agents to stimulate the formation of cAMP in visual and frontal cortical slices of dark-reared and normally-reared rats at various post-natal ages. The agents selected were NA, K⁺, and adenosine, and combinations of NA and K⁺ with adenosine. As mentioned earlier, in some instances a degree of non-specificity of excitation by a variety of agents develops in supersensitive tissue. Although the disuse of neurons imposed by visual deprivation and altered excitability may involve primarily neurotransmitters associated with the processing of visual input (and the biochemical identity of these is unknown) this may generalize to other substances such as those employed in this study.

The present investigation represents an initial attempt to determine
whether the cAMP system with all its ramifications can be used as a tool to study what influences environment and experience may have on brain development and subsequent function.
MATERIALS AND METHODS

I. Chemicals.

The sources of the chemicals used in the assay of cAMP were as follows: beef heart cAMP-dependent protein kinase and non-radioactive cAMP were obtained from the Sigma Chemical Company; bovine serum albumin (BSA) was obtained from Calbiochem; hydroxylapatite (Bio-gel HTP) was purchased from Bio-Rad Laboratories; and tritiated cAMP (37.7 Ci/m mole) was purchased from New England Nuclear.

The sources of the chemicals to which brain slices were exposed were as follows: 1-noradrenaline (1-arterenol) was obtained from the Sigma Chemical Company; adenosine (grade A) was a product of Calbiochem; and potassium chloride (reagent grade) was purchased from the Fisher Chemical Company. For liquid scintillation counting Triton X-100 was obtained from the Sigma Chemical Company. Unless otherwise noted, all other chemicals were obtained from either the Fisher Chemical Company or Mallinckrodt.

II. Maintenance and Treatment of Animals.

Albino rats of the Wistar strain, obtained from the vivarium of the University of British Columbia, were employed throughout this study. Animals were subjected to two different environmental conditions during weaning and subsequent maturation. One of these conditions involved groups of normally reared or control animals, while the other involved the rearing of animals in complete darkness. For the most part, control rats of appropriate ages and sex were obtained from the vivarium on the day they were to be used in an experiment. However, light deprived animals were raised and maintained by us in animal facilities in our laboratory.
Since the environment played an important role in this study it was necessary to maintain a group of normally reared rats in the laboratory facilities under our care until it could be established that these animals were not different from normally raised animals of the vivarium with regard to the biochemical systems under investigation. Initially this was achieved by transporting from the vivarium pregnant rats that were due to give birth within 3 to 5 days. This procedure, however, led to a high mortality rate among the litters and resulted in the death of some of the mothers. It was found that by transferring the rats from the vivarium roughly 3 days after the females had given birth, the mortality rate could be reduced to virtually zero.

Control rats raised in this lab were subject to similar conditions as those in the vivarium. This included a light-dark cycle of 12 hours, an equal density of rats per cage and food (Purina rat chow) and water ad libitum. The separation of litters from mothers was at 21 days of age and the young males were separated from the females after about 30 days of age.

Light deprivation of animals was achieved by placing 3 day old litters together with the mothers into a light sealed wooden box. This dark-box was constructed to accommodate 8 such groups of animals and ventilated sufficiently to maintain the temperature within the box, when filled to capacity, equal to that of the surrounding animal room in which the box was kept. These animals were exposed to a 15 watt red safety light for a maximum of about 3 minutes each day. During this time interval the animals were fed and the cleaning of the cages was accomplished.

Due to the limited facilities for raising animals in the dark it was necessary to include both males and females in all experiments in order to
obtain sufficient data. Consequently, all data were scrutinized for possible sex differences.

The light-deprived or experimental animals and the control animals were sacrificed by cervical dislocation. To avoid exposure of the experimental animals to the fluorescent light of the laboratory and to allow the same treatment for both the control as well as the experimental rats, the sacrificing of all animals was carried out under the illumination of a red safety light.

III. Techniques Involved in the Treatment of Brain Tissue.

a) Preparation of Kreb-Ringer Bicarbonate Buffer.

Kreb-Ringer Bicarbonate buffer (KR-buffer) was used for the rinsing of brains during dissection and in all the incubations of brain slices. The buffer was prepared (137) by bubbling a mixed gas (95% O₂ - 5% CO₂) through a 25 mM solution of sodium bicarbonate for 40 minutes. The following ions, at the final concentrations in the buffer, were then added from 10 times concentrated stock solutions: 118 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 2 mM KH₂PO₄, 2 mM MgSO₄, and 0.02 mM EDTA. Glucose was added to a final concentration of 12 mM. This stock buffer solution was gassed as above for an additional 10 minutes before use, and then for the remainder of the experiment, throughout which it was kept on ice.

b) Tissue Preparation.

A modified procedure of the method originally described by Kakiuchi and Rall (1) was used for the preparation of brain slices. Intact brain, immediately after removal from animals, was rinsed with ice-cold KR-buffer and placed on wet filter paper mounted on a glass Petri dish which was in
contact with ice. The area of the cortex described by Adams and Forrester (138) as that receiving the input from primary visual afferents was then dissected from both the left and right side of the brain. The area taken as frontal cortex was the left and right side of the most anterior pole of the forebrain. With this procedure four intact slabs of brain tissue were obtained from each animal.

After careful removal of the white matter from these slabs the tissue was weighed. The yield of tissue from each side of the visual cortex from animals 30 days and older was usually between 40 and 50 mg. The weight of the samples from the frontal cortex was of the same magnitude. From animals 15 days old and younger, the yield of cortical tissue from each side of the brain was usually less than 25 mg. The individual slabs of tissue were sliced using a McIlwain tissue chopper (Brinkman Instrument Company) with the blade adjustment set for a thickness of 0.3 mm. The dimensions of each tissue slice was about 3 x 1.5 x 0.3 mm. The entire dissection and chopping procedure required about 4 minutes, throughout which time the tissue was constantly rinsed with cold KR-buffer.

c) Incubation Procedures.

After the completion of slicing, each sliced slab of tissue was transferred to a 25 ml Ehrlemmyer flask containing 3.0 ml of KR-buffer. The flasks containing tissue and buffer were kept in ice until the beginning of the incubation. The flasks were topped with air tight rubber stoppers and transferred to a 37° water bath oscillating at 60 - 70 cycles/min. This marked the beginning of two preincubation steps at the end of which the tissue was exposed to various incubation conditions. The first preincubation was for a period of 30 minutes during the first 10 minutes of which the flask was flushed through the rubber stopper with
95% O₂-5% CO₂. Then the buffer was aspirated followed by the addition of 3 ml of fresh buffer to the flask, which was again gassed for the first 10 minutes of a 15 minute preincubation.

A total of 45 minutes for the preincubation is required to attain a constant level of cAMP in the cortical slices. During this time the cAMP content in the tissue is falling, due to metabolism by phosphodiesterase, from an initially high level which is known to be produced in brain tissue at the time of sacrifice of the animal (139,140).

The zero time for the exposure of brain slices to agents was the 15th minute of the second preincubation. Once the chemical agents were added, the incubation lasted usually for 5 minutes except in the case of time course studies where the incubation continued up to a maximum of 20 minutes. Baseline levels of cAMP was that measured at time zero of the incubation.

In initial experiments, visual and frontal cortical tissue from both the left and right cerebral hemispheres were incubated separately under similar experimental conditions. It was thought that a more reliable value for the cAMP content would be obtained per animal by taking the average of the two values from separate determinations for each of the cortical areas studied. However, it soon became apparent that, irrespective of the agent the cortex of any particular animal was exposed to, the left and right sides were always very similar with regard to cAMP content. Thus it was evident that the variation with which we and other workers in the field are plagued regarding the degree of stimulation of cAMP production in brain slices by certain chemical agents, does not arise from technical differences such as the measurement of small quantities of cAMP but may be due rather to differences among animals.
As a result of this finding for rats 30 days and older, the tissue from the left and right sides, whether visual or frontal cortex, were always incubated separately and exposed to different agents. Alternatively, one side was exposed to an agent while the other side served as a non-exposed control thus affording a value of cAMP content referred to as the baseline level. For rats 15 days of age and younger this was not possible as the yield of cortical tissue from one side was insufficient for an incubation. Therefore, the tissue from both sides of either the visual or frontal cortex was pooled from animals of these ages.

IV. Isolation and Recovery of cAMP from Brain Slices.

At the end of the incubation the contents of the incubation flasks were transferred to glass tubes which were used for both centrifugation and homogenization. The tubes were centrifuged in the cold room for about 30 seconds at 1000 x g and the KR-buffer was decanted off in order to avoid interference from the ions in the subsequent assay of cAMP (141). The pelleted brain slices were homogenized with a Teflon pestle in 1.0 ml of 5% (w/v) trichloracetic acid (TCA) and the pestle was washed with 0.5 ml of TCA. The TCA homogenate was transferred to Sorvall centrifuge tubes and the homogenization tubes washed with an additional 0.5 ml of 5% TCA. The TCA homogenate, total volume 2.0 ml, was centrifuged at 10,000 x g for 10 minutes. The supernatant, containing the cAMP, was transferred to 30 ml test tubes. The TCA precipitate was washed in 0.5 ml of TCA by rehomogenization and the pestle again rinsed with 0.5 ml of 5% TCA. The homogenate was centrifuged as above and the supernatant was pooled with the previous TCA soluble material. The numerous washings of the pestle, homogenization tubes and TCA precipitate were included to maximize
the recovery of cAMP. The TCA precipitate was stored at -20° and assayed for the protein content at a later date. The TCA soluble fraction was extracted 4 times with 2 volumes of ether to remove the TCA. The TCA remaining after extraction, as determined by titration, was negligible. The TCA soluble fraction was then lyophilized and stored at -20° prior to the cAMP assay.

To determine the recovery of cAMP from brain slices the following procedure was employed: Before the homogenization of slices which had been incubated in the normal manner, a known amount of radioactive cAMP was added to the homogenization tubes and the samples were taken through the routine isolation procedure for cAMP. After reconstitution of the lyophilized TCA soluble material, an aliquot was taken for determination of radioactivity. The percent recovery was calculated from the amount of radioactive cAMP in the aliquot and the known amount added prior to homogenization.

V. The Measurement of cAMP Levels in Brain Tissue.

a) General Principle of the Assay of cAMP.

The method used for the assay of cAMP was a modification of the method of Brostrom and Kon (141) who used a modified procedure of that originally described by Gilman (142). The method can be described essentially as the competition between a fixed known amount of tritiated cAMP and unlabelled cAMP for cAMP-dependent protein kinase (PK), a protein which has both high affinity and high specificity for the binding of cAMP. The source of the competing unlabelled cAMP is either from stock solutions of known concentrations for the purpose of generating a standard curve or from a sample containing unknown quantities of cAMP.
The construction of a standard curve involves the incubation of a series of tubes containing PK with a constant amount of radioactive cAMP and increasing concentrations of unlabelled cAMP. The net result of this is to incubate PK with decreasing specific activities of radioactive cAMP. The amount of radioactivity associated with PK is then measured and when this is plotted on log-log axes against cAMP concentration, a straight line is obtained. For the determination of cAMP content from an experimental sample, an aliquot is used which will produce a specific activity of cAMP that is within the limits of the standard curve. The amount of cAMP that must have been present in the sample to create the resultant specific activity is then interpolated from the standard curve.

The binding of cAMP to PK can be increased by including protein kinase inhibitor in the assay (142). We have chosen to include BSA in the assay mixture since it has been shown (141) that a number of proteins, including BSA, are protein kinase inhibitors.

A variety of methods have been reported to achieve the separation of PK-bound cAMP from free cAMP. These include the binding of the PK-cAMP complex to nitrocellulose membrane filters (142) or to hydroxylapatite (141) which is added to the assay mixture in the form of a slurry, or the adsorption of free cAMP on charcoal (143). In the present investigation the hydroxylapatite procedure was employed essentially because it is a relatively less expensive method. Initially the hydroxylapatite-PK-cAMP complex was separated from free cAMP by centrifugation as suggested by Brostrom and Kon (141). Since this procedure resulted in inconsistencies, the hydroxylapatite-PK-cAMP complex was collected on Whatman No. 1 filter paper discs by means of filtration. This technique was relatively faster and gave highly reproducible results.
b) **Materials for the Assay of cAMP.**

Stock solutions of tritiated cAMP contained 50 mM sodium acetate buffer, pH 4.5, 5 mg/ml BSA, and sufficient radioactivity to give about 80,000 dpm per 0.1 ml. Stock solutions of PK were made by dissolving lyophilysed beef heart PK in distilled water producing a concentration of 150 μg per ml. Small aliquots of the $^3$H-cAMP solution and of the PK solution were stored at -20° to avoid excessive freezing and thawing. The binding activity of PK was stable for up to 2 months.

The amount of PK used in the assay can be varied inversely with the amount of radioactive cAMP used. Thus, for the purpose of scintillation counting, the desired quantity of radioactive cAMP bound to PK can be achieved either by varying the quantity of protein kinase or $^3$H-cAMP employed per assay. It is important, however, to use an amount of PK that will be saturated by the cAMP concentrations being measured.

Batches of the hydroxylapatite slurry were made by adding 12 ml of distilled water to 1.5 gm of hydroxylapatite and were stored at 4°. Filter paper discs of 2.8 cm in diameter were cut from sheets of Whatman No. 1 chromatography paper.

The concentration of the unlabelled cAMP solution used for the production of standard curves was checked by measuring the adsorbance at 256 nm using the molar extinction coefficient of 14,500 for cAMP at pH 2.0.

For the filtration procedure Millipore funnels (15 ml capacity) equipped with a 25 mm diameter base and fritted glass filter supports, were employed. A Duo-Seal rotary vacuum pump was used to provide the vacuum for filtration.
c) Details of the Procedure for the Assay of cAMP.

The lyophilized TCA soluble fraction was reconstituted with 2.0 ml of 50 mM sodium acetate buffer, pH 4.5, (NaAc buffer) and an aliquot of this was assayed for cAMP content. The volume of buffer added to the dried samples may vary since the standard curve for cAMP estimation may be constructed to encompass a wide range of cAMP concentrations. However, caution must be taken not to dilute the cAMP to excess relative to the concentrations of other adenine nucleotides. The reason for this is the finding that adenine nucleotides can interfere in the assay of cAMP by competing for binding to PK. A dilution of no greater than 30 of the original tissue is recommended by Gilman (142). In the present work, problems were not encountered unless the tissue dilution was greater than 70. Thus, the lyophilized TCA soluble fraction of a sample of brain tissue weighing about 0.05 g was not taken up in more than 2.0 ml of acetate buffer, thereby affording a tissue dilution of about 40.

The assay of cAMP was conducted in a total volume 0.2 ml. To a sample volume of 80 µl, was added 0.1 ml of radioactive cAMP solution containing usually between 3 - 4 pmoles of cAMP. The assay tubes were cooled to 4° and after the addition of 20 µl of PK, the tubes were shaken and incubated for 1 hour at 4°. To generate the standard curve the 80 µl of sample was replaced by increasing amounts of unlabelled cAMP ranging from about .7 to 45 pmoles and where required, the 0.2 ml incubation volume was achieved by the addition of NaAc buffer.

At the end of the 1 hour incubation, 0.2 ml of the hydroxylapatite slurry (precooled to 4°) was added to the assay tubes and the tubes were incubated for an additional period of 5 minutes at 4°. Following this, 1.0 ml of ice cold 10 mM potassium phosphate buffer, pH 6.0, (KP buffer)
was added and the assay tubes were maintained at $4^\circ$ for a minimum of 5 minutes. The contents of the tubes were then filtered through Whatman No. 1 filter paper discs using the apparatus described earlier. The hydroxylapatite-PK-cAMP complex is stable in KP-buffer at $4^\circ$ for up to 2.5 hours. It was necessary to establish this stability factor since the filtration of a set of samples usually required 1.5 - 2 hours. The assay tubes were rinsed 4 times with 1.0 ml of cold KR-buffer and the filter paper discs were further washed with an additional 5.0 ml of this buffer. The filter paper discs were then placed at the bottom of scintillation vials and prepared for counting. All assays of cAMP were conducted in triplicate.

d) **Radioactivity Analysis.**

The hydroxylapatite on the filter paper discs was dissolved by the addition of 1.0 ml of 1.5 N HCl to the scintillation vials in which the discs had been placed. After the addition of 11 ml of a toluene based scintillation fluid containing 30% (v/v) Triton X-100, 0.02% (w/v) 1, 4-bis(2-(5-phenyloxazolyl))-benzene (POPOP) and 0.3% (w/v) 2,5 diphenyloxazole (PPO), the samples were counted in a Nuclear Chicago liquid scintillation counter at about 36% efficiency.

e) **Protein Determination.**

The precipitate obtained after homogenization of brain slices in 5% TCA was dissolved in 0.1 N NaOH and assayed for protein content by the method of Lowry *et al.* (144). The cAMP content in brain slices was always expressed as pmoles per mg of protein so as to facilitate the comparison of the present data with those in the literature.
VI. Treatment of Data.

As mentioned earlier chemical agents which stimulate the production of cAMP in brain slices always do so with a large degree of variation among different animals. As a result, a sufficient number of animals have to be included in an experiment to enable the treatment of data by statistical means. The baseline cAMP levels were obtained from 4 animals whereas at least 6 animals were used in all other experiments to be described in this thesis. All data are presented as the mean value for a group of animals plus or minus the standard error of the mean (S.E.M.). The student t-test was used for the analysis of significance, and the differences were considered significant if $p < 0.05$. 
RESULTS


It is generally acknowledged that rearing animals in the dark may have a multiplicity of physiological and biochemical effects not only on the visual system but on various facets of the animals' biological processes. An attempt was made to exclude some of the possible factors other than light deprivation which may contribute to alterations in the function of the visual systems and indeed in other CNS structures. One of these factors is nutrition and is related to the effect of dark-rearing on the animals' body weight gain. Since infant rats were fed by the mothers for the first 21 days of life, the nutritional status in terms of body weight of mothers of dark-reared animals may also be an important factor. The body weight gain of dark-reared and control rats as well as the body weights of mothers of these two groups of animals up to the time of weaning is shown in Fig. 2. No significant differences existed in the body weights of normally reared rats and those reared in the dark from birth. Furthermore, there were no significant differences in the body weights of nursing mothers of dark-reared and control animals.

The procedure for the estimation of cAMP in brain slices has been discussed in detail in the materials and methods section. A typical standard curve for the cAMP assay is shown in Fig. 3. That the technique employed in this study to measure cAMP concentrations is valid and reflects the true cAMP content of brain tissue is assumed from the fact that protein kinase has both very high affinity and specificity for cAMP. Moreover, in Fig. 3 it is shown that when a sample of the TCA soluble fraction of brain tissue which contains cAMP is used in the assay at increasing concentra-
FIG. 2 The effect of light deprivation on the body weights of rats at various postnatal ages and their mothers during weaning. A. The body weights of rats in litters of control (——) and dark-reared (––––) groups were measured at the ages shown. B. The body weights of nursing mothers of control (——) and dark-reared (––––) rats were measured 5 days after they had given birth and at intervals up to the time of weaning. In the case of light-deprived animals, this operation was conducted with the aid of a safety light to avoid exposure of animals to the intensity of room lighting. Each point and vertical bar represents the mean and S.E.M., respectively, of 10–15 animals.
FIG. 3 A typical standard curve for the determination of the cAMP content of brain slices. The standard curve (○—○) is constructed by incubating protein kinase with a fixed concentration of $^3$H-cAMP and increasing concentrations of unlabeled cAMP. The total quantity of cAMP per incubation is plotted on the abscissa and the radioactive cAMP bound to protein kinase is plotted on the ordinate. The broken line (●—●) represents a separate assay where the source of the unlabeled cAMP added to the assay tubes was brain tissue. The cAMP content of this tissue was assayed in a previous determination. The degree to which the two lines do not overlap represents the error in the assay procedure of cAMP which in this case was about 7%.
tions rather than pure cAMP, thus simulating the procedure for the production of a standard curve, a straight line is generated that is parallel to the standard curve. Since the material in the sample produced a line parallel to that of the standard curve and since the affinity constant for PK of this material and cAMP is the same, as calculated from the two curves, it is concluded that the material in the sample competing with radioactive cAMP for the binding of protein kinase must indeed be cAMP originating from brain tissue.

The two parallel lines in Fig. 3 should overlap and the extent to which they do not represents the error in the assay of cAMP. This error ranges from 3 to 7%.

The recovery of cAMP from brain tissue was 89%. This value is higher than those reported by others. The reason for this may be due to the numerous washings employed in the present study during the isolation of cAMP.

The amount of visual and frontal cortical tissue employed per incubation varies, unavoidably, due to the nature of the dissection of brain tissue. Thus it was necessary to show that the baseline levels of cAMP in brain slices as well as the stimulation of cAMP formation by various agents is linear with the quantity of tissue employed per incubation. This would be an important factor if substances were released from brain slices during the incubation which could stimulate the accumulation of cAMP since the amount of substance released would be dependent on the amount of tissue incubated. Moreover, during the incubation of cortical slices with NA, the NA may be taken up by the tissue or inactivated by some means thus reducing the quantity available to stimulate cAMP formation. This process would also be dependent on the concentration of tissue employed
per incubation. The effect of tissue weight per incubation as represented by protein content on the baseline cAMP levels and the NA-induced accumulation of cAMP in brain slices is shown in Fig. 4. The baseline cAMP content and the NA-induced accumulation of cAMP was linear for tissue-protein content per incubation ranging from 0.5 to 7.5 mg. Typically, the amount of tissue protein per incubation was between 1.5 and 4.0 mg.

It was desirable to determine the concentrations of NA to which brain slices were to be exposed during incubations with this agent. For this purpose investigators have used a variety of concentrations (134, 137, 145) or have adopted the concentration of 100 µM (94, 146-148) as producing maximal effects. Employing rat cerebral cortical slices Perkins and Moore have shown (121, 149) that 30 µM NA induces maximal stimulation of cAMP formation. In Fig. 5 is shown the time course of the accumulation of cAMP in visual cortical slices incubated with 30 and 100 µM NA. There is no significant difference in the accumulation of cAMP elicited by the two concentrations of NA in 1 and 5 minute incubations. However, for longer incubation times the cAMP content of brain slices incubated with 30 and 100 µM NA is significantly different. The cAMP concentration in tissue incubated with 100 µM NA is maintained at higher levels than that in tissue incubated with 30 µM NA. This difference could be explained if in the presence of 100 µM NA the rate of stimulation of cAMP formation by NA keeps pace with the rate of degradation of cAMP by phosphodiesterase, whereas in the presence of 30 µM NA the rate of degradation prevails. An alternative explanation is that after an initial stimulation of cAMP formation by NA no further or continuous stimulation takes place and for an unknown reason the degradation of cAMP in tissue incubated with 30 µM is faster than that in tissue incubated with 100 µM NA. Although this
FIG. 4 The effect of tissue weight per incubation on the baseline cAMP levels and the NA-induced accumulation of cAMP in brain slices. A range of tissue weights from the cortex of 15 day old rats were incubated under standard incubation conditions. The baseline cAMP content (•—•) and the NA-induced accumulation of cAMP (○—○) were measured. NA was employed at a concentration of 3 μM and incubations were for a period of 5 min. Baseline values of cAMP are those described in materials and methods. The amount of tissue per incubation is represented on the abscissa in terms of protein content.
FIG. 5 Time course of the stimulation of cAMP formation by two concentrations of NA. Visual cortical slices from 15 day old control rats were incubated with 30 μM (○—○) and 100 μM (●—●) NA for the time periods indicated. The cAMP content of the slices is expressed in terms of picomoles/mg protein. The protein yield from the tissue in the incubations varied between 1.5 and 4 mg. The B on the abscissa refers to the baseline cAMP content of slices at zero time and prior to the addition of agents. The time course studies begin at the time agents are added which is at the end of the second preincubation step as described in materials and methods. Each point and vertical bar represents the mean and S.E.M., respectively, of 4-6 animals.
explanation appears at first sight less satisfactory, it is the preferred one since it has been shown that there is a refractoriness to repetitive stimulation of cAMP formation by such biogenic amines as NA (92,137,146). Since at short incubation times the maximal accumulation of cAMP elicited by the two concentrations of NA studied correspond, the concentration of NA chosen to be used in incubations of brain slices was 30 μM. The advantage of employing this concentration is that any differences existing between dark-reared and control rats may reside in the rate of cAMP catabolism, thus the lower concentration of NA would allow inferences to be made in this regard. Phosphodiesterase inhibitors were not used during incubation of brain slices for similar reasons.

II. The Effect of NA and K⁺ on the Rate of Accumulation of cAMP in Brain Slices.

The effect of dark-rearing animals for 15 days on the time course of the NA-induced accumulation of cAMP in visual and frontal cortical slices is shown in Fig. 6. Dark rearing for 15 days did not affect the baseline levels of cAMP in either visual or frontal cortical slices. Nor were there any differences in the baseline cAMP levels of visual compared to frontal cortical slices of control or dark-reared animals. In a 5 minute incubation with NA there was a significant reduction of 11% in the cAMP levels in visual cortical slices of dark-reared rats compared to controls. At other incubation times there was no significant difference between experimental and control animals in the NA-induced accumulation of cAMP in visual cortical slices. In frontal cortical slices of dark-reared rats, although there was a trend toward a reduction in the NA-induced accumulation of cAMP at all incubation times, the only significant
FIG. 6 Time course of the stimulation of cAMP formation by NA in visual and frontal cortical slices of 15 day old control and dark-reared rats. Visual (—) and frontal ( — — — ) cortical slices from control ( O ) and dark-reared ( ● ) rats were incubated with 30 µM NA for the time periods indicated. Each point and vertical bar represents the mean and S.E.M., respectively, of 6-15 animals.

* Values significantly different from the same brain region of controls (p < 0.05).
difference from controls occurred in a 20 minute incubation. These results suggest that in both visual and frontal cortical slices of dark-reared animals there is a reduction in the maximal stimulation of NA-induced cAMP formation. Furthermore, the diminution of cAMP levels in frontal cortical slices of dark-reared animals after 20 minutes of incubation with NA suggests that there is a greater rate of degradation of cAMP in this brain region of experimental animals than controls. This is not evident in visual cortical slices of dark-reared animals.

After 30 days of dark-rearing, the effects on the NA-induced accumulation of cAMP in visual cortical slices is qualitatively the same as at 15 days. However, as shown in Fig. 7, there is a greater reduction from control values (21%) in the stimulation of cAMP formation by NA in a 5 minute incubation and there is a significant reduction (20%) in the cAMP level in a 1 minute incubation. As in the case of 15 day old animals, dark-rearing for 30 days had no effect on the baseline cAMP levels in visual cortical slices or on the levels after 10 or 20 minute incubations with NA.

The effects of dark-rearing animals for 30 days on the NA-induced accumulation of cAMP in frontal cortical slices are somewhat more complex than the effects on visual cortical slices. As shown in Fig. 8 the baseline cAMP content of frontal cortical slices was significantly higher (21%) for dark-reared animals than controls while the NA-induced accumulation of cAMP was 25% and 21% lower than controls in 1 and 5 minute incubations, respectively. At 10 minutes of incubation with NA the cAMP levels in slices from experimental and control animals are equal and in a 20 minute incubation with NA this level is maintained in slices from control animals but there is a 13% decline in slices from experimental animals.
FIG. 7  Time course of the stimulation of cAMP formation by NA in visual cortical slices of 30 day old control and dark-reared rats. Brain slices from control (○—○) and dark-reared (●—●) rats were incubated with 30 μM NA for the time periods indicated. Each point and vertical bar represents the mean and S.E.M., respectively, of 4–7 animals.

* Values significantly different from controls (p < 0.05).
FIG. 8  Time course of the stimulation of cAMP formation by NA in frontal cortical slices of 30 day old control and dark-reared rats. Brain slices from control (○---○) and dark-reared ( ● --- ●) rats were incubated with 30 \( \mu \text{M} \) NA for the time periods indicated. Each point and vertical bar represents the mean and S.E.M., respectively, of 4-7 animals.

* Values significantly different from controls (\( p < 0.05 \)).
Dark-rearing for 30 days appears to reduce the ability of NA to promote the synthesis of cAMP in visual cortical slices. In frontal cortical slices, dark-rearing for this period does not affect the maximal accumulation of cAMP elicited by NA, but there is a reduction in the rate at which cAMP is accumulated in response to NA, as well as a reduction in the maintenance of the maximally stimulated levels of cAMP.

The addition of 50 mM KCl to the incubation medium causes a much larger stimulation of cAMP formation in brain slices than does NA. Shown in Fig. 9 is the time course of this stimulation in visual and frontal cortical slices of 30 day old control and dark-reared rats. Dark-rearing did not affect the ability of K\(^+\) to stimulate the formation of cAMP in slices from either brain region at any of the incubation times studied.

In control and experimental animals, the K\(^+\)-induced accumulation of cAMP is greater in visual than frontal cortical slices and there is a greater decrement in the cAMP levels in frontal than visual cortical slices at incubation times of 10 and 20 minutes. The complete reverse is true of the NA-induced accumulation of cAMP where (see Figs. 6, 7 and 8) the stimulation induced by NA is greater in frontal cortical slices and in normally reared animals the subsequent diminution of cAMP levels is greater in visual cortical slices. In dark-reared animals the NA-induced accumulation of cAMP in frontal cortical slices is more K\(^+\)-like in that the diminution of cAMP levels at longer incubation times is increased.

III. The Ontogenetic Development of Responsiveness of Brain Slices to NA and K\(^+\)

The capacity of NA and K\(^+\) to stimulate the formation of cAMP was studied in brain slices from rats of various ages. Incubations of 5
FIG. 9 Time course of the stimulation of cAMP formation by K⁺ in visual and frontal cortical slices of 30 day old control and dark-reared rats. Tissue slices of frontal (— — ) and visual (— — ) cortex from control ( O ) and dark-reared ( ● ) rats were incubated with 50 mM K⁺ for the time periods shown. Each point and vertical bar represents the mean and S.E.M., respectively, of 4-7 animals.
minutes were chosen since the time course studies indicated that in brain slices from normally reared animals the accumulation of cAMP in response to these agents was maximal or almost maximal at this time. As shown in Figs. 10 and 11 sensitivity to NA was present at 5 days of age in both visual frontal slices. In visual cortical slices the responsiveness to NA increases at 10 days of age and after 15 days remains relatively constant. In frontal cortical slices the responsiveness to NA undergoes a drastic increase at 10 days and subsequently decreases by 30 days to a constant value.

The baseline levels of cAMP in frontal and visual cortical slices gradually decrease with age from an average of 80 pmoles/mg protein at 5 days to about 50 pmoles/mg protein at 60 days. In instances where the stimulation of cAMP synthesis by various agents is low this change in baseline may be important with regard to the interpretation of results inasmuch as the elevated levels of cAMP caused by these agents is the sum of newly synthesized cAMP and baseline levels. This is particularly true in the visual cortex (Fig. 10) where the change in baseline levels of cAMP with age appear to parallel changes in the NA-induced accumulation of cAMP. Thus, the changes in the response of visual cortical slices to NA may merely reflect changes in baseline cAMP levels. For the most part, however, the changes in baseline levels are of little consequence.

As shown in Fig. 12, the capacity of K⁺ to stimulate the synthesis of cAMP in visual cortical slices increases enormously from 5 day to 15 days of age whereupon it decreases slightly at 30 and 60 days. This is in marked contrast to the K⁺ sensitivity changes observed in frontal cortical slices (Fig. 13). Although a similar increase in K⁺ responsiveness occurs up to 15 days there is subsequently a progressive decrease
FIG. 10 Ontogenetic development of responsiveness of visual cortex to NA. Visual cortical slices from control (○—○) and dark-reared (●—●) rats of various ages were exposed to 30 μM NA for 5 min. Tissue cAMP content in the absence of NA (control ○—○; dark-reared ●—●) represents baseline levels. Each point and vertical bar represents the mean and S.E.M., respectively, of 4-15 animals.

* Values significantly different from controls (p < 0.05).
FIG. 11 Ontogenetic development of responsiveness of frontal cortex to NA. Frontal cortical slices from control (○—○) and dark-reared (●—●) rats of various ages were exposed to 30 μM NA for 5 min. Tissue cAMP content in the absence of NA (control○—○; dark-reared ●—●) represents baseline levels. Each point and vertical bar represents the mean and S.E.M., respectively, of 4-15 animals.
* Values significantly different from controls (p < 0.05).
FIG. 12 Ontogenetic development of responsiveness of visual cortex to \( K^+ \). Visual cortical slices from control (\(\circ\)) and dark-reared (\(\bullet\)) rats of various ages were exposed to 50 \(\mu M\) KCl for of 5 min. Tissue cAMP content in the absence of KCl (control \(\circ\); dark-reared \(\bullet\)) represents baseline levels. Each point and vertical bar represents the mean and S.E.M., respectively, of 4-6 animals.
FIG. 13 Ontogenetic development of responsiveness of frontal cortex to K⁺. Frontal cortical slices from control (C——C) and dark-reared (•—•) rats of various ages were exposed to 50 mM KCl for 5 min. Tissue cAMP content in the absence of KCl (control C——C; dark-reared •—•) represents baseline levels. Each point and vertical bar represents the mean and S.E.M., respectively of 4-6 animals. ★ Values significantly different from controls (p < 0.05).
until at 60 days the cAMP levels in frontal cortical slices incubated in the presence of high K\(^+\) is equal to that observed at 5 days. The net stimulation of cAMP synthesis by K\(^+\) is still greater at 60 days due to a decreased baseline at this age.

The major difference between frontal and visual cortical slices with regard to the ability to respond to K\(^+\) and NA by augmenting cAMP levels is the transient nature of the response that occurs with age in frontal cortical slices but is less pronounced in visual cortical slices. This transient response to NA with age has been observed in slices of rat whole brain (113,116) as well as in slices of rabbit frontal cortex, hippocampus and hypothalamus (120). The transient response of frontal cortical slices to K\(^+\) has not been previously reported.

The effects of dark-rearing animals for 15 and 30 days on the NA- and K\(^+\)-induced accumulation of cAMP in slices has been discussed. It might be further pointed out that the responsiveness of brain slices from dark-reared animals of these ages, whether significantly different from controls or not, parallels the developmental profiles of controls. At 60 days of age there is a turn of events with regard to the effects of dark-rearing. Whereas, at the former ages, dark-rearing caused a decreased responsiveness to NA and had no effect on the responsiveness to K\(^+\), after 60 days of dark-rearing the NA-induced accumulation of cAMP was 23% and 35% higher than controls in frontal and visual cortical slices, respectively (see Figs. 10 and 11). The K\(^+\)-induced accumulation of cAMP at 60 days was 57% higher in frontal cortical slices of experimental animals compared to controls but although the responsiveness to K\(^+\) was higher in visual cortical slices of experimental animals by 13%, this was not significant (p 0.1).
IV. The Accumulation of cAMP in Brain Slices in Response to Adenosine and Combinations of Adenosine with NA and K⁺.

Visual and frontal cortical slices from 60 day old dark-reared and control rats were incubated for 5 minutes with adenosine and combinations of adenosine with NA or K⁺. The results are shown in Table I together with the previously discussed results of incubations of slices of 60 day old animals with NA and K⁺ at comparable incubation times. Although brain slices incubated in the presence of 30 μM adenosine contained levels of cAMP significantly greater than baseline quantities, these did not approach the levels reported by others (121,146,148). This could be due in part to the fact that these workers incubated brain slices for periods of 15 to 30 minutes with concentrations of adenosine ranging from 30 to 100 μM.

There was no significant difference between experimental and control animals in the adenosine-induced accumulation of cAMP in visual cortical slices. However, as in the case of NA and K⁺, adenosine caused a significantly greater accumulation of cAMP in frontal cortical slices of dark-reared animals than controls. Incubations of visual and frontal cortical slices in the presence of adenosine in combination with NA or K⁺ produced no differences between experimental and control animals in either brain region. Thus, the difference observed in frontal cortical slices in incubations with adenosine, NA or K⁺ alone was abolished when NA or K⁺ was combined with adenosine.

The synergism between adenosine and biogenic amines that has been observed by others (96,98) is also demonstrated in the present work. For example, the accumulation of cAMP elicited by adenosine in combination with NA was greater than the sum of that elicited by NA and adenosine alone. The combined effects of depolarizing agents such as K⁺ and adeno-
The stimulation of cAMP formation by adenosine and combinations of adenosine with NA and K⁺ in visual and frontal cortical slices of 60 days old control and dark-reared rats.

<table>
<thead>
<tr>
<th>Agent(s)</th>
<th>Visual cortex</th>
<th></th>
<th>Frontal cortex</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Dark-reared</td>
<td>Control</td>
<td>Dark-reared</td>
</tr>
<tr>
<td>Baseline</td>
<td>47.8 ± 4.5</td>
<td>50.5 ± 3.4</td>
<td>46.3 ± 2.2</td>
<td>49.4 ± 6.2</td>
</tr>
<tr>
<td>Adenosine</td>
<td>83.1 ± 8.0</td>
<td>99.0 ± 11</td>
<td>75.2 ± 8.1</td>
<td>104 ± 6.4*</td>
</tr>
<tr>
<td>NA</td>
<td>108 ± 9.1</td>
<td>133 ± 8.8*</td>
<td>125 ± 6.8</td>
<td>169 ± 8.4*</td>
</tr>
<tr>
<td>Adenosine + NA</td>
<td>235 ± 15</td>
<td>275 ± 27</td>
<td>293 ± 25</td>
<td>349 ± 22</td>
</tr>
<tr>
<td>K⁺</td>
<td>235 ± 21</td>
<td>268 ± 16</td>
<td>115 ± 8.7</td>
<td>180 ± 21*</td>
</tr>
<tr>
<td>Adenosine + K⁺</td>
<td>312 ± 24</td>
<td>344 ± 53</td>
<td>251 ± 18</td>
<td>261 ± 20</td>
</tr>
</tbody>
</table>

Tissue slices were incubated for 5 min. in the presence of the following agents: 30 µM adenosine; 30 µM NA; 50 mM KCl; 30 µM adenosine + 30 µM NA; 30 µM adenosine + 50 mM KCl. Values are expressed as the mean ± the S.E.M. of 4-7 animals.

* Significantly different from the control value of the same brain region.
sine have been reported not to be synergistic but only additive (90). This was found to be true when visual cortical slices were incubated with a combination of adenosine and K\(^+\). However, frontal cortical slices of experimental and control animals gave a differential response such that in the former there was a synergistic response while an additive response was obtained in slices from dark-reared animals.

It is difficult to explain this result since the observed synergism between K\(^+\) and adenosine in frontal cortical slices of control animals was entirely unexpected and is contrary to the results reported by others. It might be pointed out, however, that many investigators employ, in their studies, the entire cortex which precludes the observation of regional differences. That such differences exist is not without precedent since in the present study numerous differences regarding the time course and degree of cAMP accumulation in response to NA and K\(^+\) have been found between frontal and visual cortical slices. Assuming that the synergism observed with adenosine and K\(^+\) in frontal cortical slices is valid then a possible explanation of why this was not observed in these slices from dark-reared animals may be that in this brain region of experimental animals both adenosine and K\(^+\) alone produced an accumulation of cAMP greater than that observed in slices of control animals. Thus, whatever mechanism is operative in the process of synergism may have already been activated and utilized to produce the augmented responses to adenosine and K\(^+\) alone.

There exists in the literature a wide variation in the reported baseline values of cAMP in brain slices as well as in the accumulation of cAMP levels elicited by various agents. Numerous brain regions of a variety of animals such as mouse, rat, rabbit and guinea pig have been used
in the study of the cAMP system. Thus, some of the above variations may be explained and indeed expected from such a heterogeneous use of animals and brain regions. The problem, however, is not resolved by this explanation since discrepancies exist in the reported results of different workers using the same brain region of the same animal. For example, in rat cortical slices baseline values ranging from 12 to 100 pmoles/mg protein have been reported (134,146,149,150). The reported values of the NA-induced accumulation of cAMP in rat cortical slices incubated with 10 μM NA range from 31 to 400 pmoles/mg protein (134,149). Although some of these differences may result from the fact that a variety of incubation times have been employed in the study of the NA-induced accumulation of cAMP in brain slices, this reason is not entirely satisfactory since the maximal accumulation of cAMP in response to this agent has been reported to occur at a variety of incubation times ranging from 10 to 30 minutes (134,146,149).

In spite of the fact that the percent increase in cAMP levels elicited by NA varies from 200 to 700%, a fairly consistent finding is that the baseline levels of cAMP in cortical slices vary proportionally with the NA-induced accumulation of cAMP. For example, in the case of a low cAMP baseline the increase in cAMP levels in response to NA is generally proportional to that in cases where higher baselines are obtained.

In the present study the baseline levels of cAMP of between 45 and 65 pmoles/mg protein for 30 and 60 day old animals agree fairly well with the values reported by some other investigators (134,151). However, the accumulated levels of cAMP in brain slices incubated in the presence of NA were generally lower by 25 to 80% than those reported by others who
obtained baseline levels similar to that reported here. This may be due to slight differences in technique or to the fact that discrete brain regions were examined in the present study whereas others have pooled the entire cortex in their studies.

The discrepancies in cAMP levels reported in the literature must certainly result from the methodological difficulties inherent in investigations of such a complex tissue as the brain. If the study of the cAMP system and the interactions of various agents with this system is to continue using the techniques employed in the present investigation, it is clear that an in-depth analysis is required to determine and eliminate the factors involved in the technique that contribute to variations in results.
DISCUSSION

We have found that effects of dark-rearing on the cAMP system occur in both the visual and frontal cortex and that these effects are bimodal with age. Dark-rearing rats for 1 month or less caused primarily a diminution in the ability of NA to increase cAMP levels in brain slices from these animals, whereas after 2 months of dark-rearing the response to NA and $K^+$ was increased. The bimodal nature of the effects of light deprivation and the fact that frontal cortex which is not the primary site of termination of visual input was affected must be reconciled not only with the available data on the effects of light deprivation but with the emerging concepts regarding plasticity and recovery of function in the CNS. The constructs into which the findings of the present investigation must be placed are not firmly established. Because this allows a certain amount of malleability in the interpretation of results it is indicative that perhaps a great deal of conjecture is unwarranted. However, speculation is desirable to the extent that it may aid in the planning of further experiments.

The finding that dark-rearing affects both the visual and frontal cortex renders suspect the conclusion that these effects stem from the elimination of visual stimulation. For example, an etiology involving the humoral system would be more appropriate since this system would have access to many brain regions. However, the studies alluded to earlier regarding the effects of malnutrition and thyroidectomy on the cAMP system tend to rule out at least some extraneous possibilities other than light deprivation as the causative factors for the results obtained in the present study. The redeeming feature of these investigations is that
neither thyroidectomy nor malnutrition of rats caused changes in the cAMP system in the brains of these animals. Both of these experimental approaches undoubtedly lead to gross abnormalities in the endocrine systems of treated animals. Since these humoral imbalances did not result in alterations in the brain cAMP system, it can be assumed that this system would not be affected by the humoral changes (152,153) resulting from a less traumatic treatment of animals such as light deprivation.

Despite the tentative conclusion that environment is responsible for the modified function of the cAMP system which we have found in the cortex of dark-reared rats, the neural systems that form the basis of these modifications is still uncertain. For example, it is not clear whether these effects are mediated by reduced afferent electrical impulses to the visual cortex which then influence the activity of the frontal cortex through intracortical neuronal associations or whether dark-rearing affects subcortical structures which in turn modulate electrical activity and thus neurochemical processes in the cortex. That the exclusion of light stimulus to animals and the reduction of activity in the visual system that this affords contributes directly, although perhaps not solely to the changes observed in the visual cortex, is suggested by the numerous morphological and biochemical studies (see introduction) where specific effects of light deprivation on the visual cortex have been demonstrated. For example, the number of spines on the apical dendrites of layer V pyramidal neurons have been shown to be reduced specifically in the visual cortex and not the temporal cortex of light deprived mice (36). These are the structures which comprise synaptic contacts and to which the cAMP system has been localized. Thus, the diminished responsiveness of visual cortical slices of 15 and 30 day old dark-reared animals to NA may in
part reflect reduced numbers of those structural entities with which exogenously applied neurotransmitters can interact. Unfortunately, investigators studying the effects of dark-rearing on brain morphology have chosen as their controls either the motor or temporal cortex, or, in the case of monocular visual deprivation, the visual cortex of the unoccluded eye. Therefore, if dark-rearing induces similar morphological effects in frontal cortex as in visual cortex then the above explanation may apply to the findings obtained for frontal cortical slices of 30 day old dark-reared rats despite the fact that only the time course and not the maximal accumulation of cAMP elicited by NA was affected.

As discussed above, the effects of dark-rearing which we have observed may reside in the simultaneous reduction through decreased neural contacts of all those components subservient to the production of cAMP. However, the altered capacity of brain slices from dark-reared rats to respond to various agents by augmenting cAMP synthesis might alternatively be due specifically to key events in the series of interactions that take place while a neuron responds to a transmitter. The elucidation of the biochemical mechanisms that may be responsible for altered responsiveness must await further investigations. This task although not insuperable does pose some difficulties. The reason for this is the many parameters that could potentially give rise to the observed effects of light deprivation. These effects may be ascribed to a change in a single variable or may be the net outcome of several processes acting in unison or opposition. Moreover, the effects of dark-rearing may be brain region specific causing different sets of events in brain regions receiving afferent supply for vision (e.g. visual cortex) and areas not receiving visual input (e.g. frontal cortex).
Some of the factors that may contribute to the effects of dark-rearing include those components that are involved in promoting the synthesis and degradation of cAMP. Thus, diminished responsiveness of visual cortical slices to NA after 15 and 30 days of dark-rearing may be due to, (1) decreased efficacy of the NA-receptor interaction which might involve cooperative changes in the receptor, (2) reduced number of receptors for NA, (3) decreased adenylate cyclase activity, (4) decreased coupling between the NA receptor and adenylate cyclase, or (5) increased phosphodiesterase activity.

Methods are available to distinguish between at least two of these possibilities. Phosphodiesterase inhibitors such as theophylline, aminophylline or diazepam may be included in the incubation of brain slices to determine whether the effects of dark-rearing are primarily on the synthetic or degradative processes involved in the metabolism of cAMP. Alternatively, phosphodiesterase activity could be assayed (154,155) in homogenates of the brain areas in question. Insofar as adenylate cyclase is concerned, its catalytic component could be quantified without the interference of other components through the known stimulation of this activity by fluoride ion (133,156), thus affording a measure of the absolute amount of enzyme protein.

In the visual cortex the time course studies (Figs. 6 and 7) tend to exclude the possibility that increased phosphodiesterase activity is mediating the effects of dark-rearing since visual cortical slices incubated for 10 and 20 minutes with NA showed no differences in cAMP levels between control and experimental animals. However, in frontal cortical slices, although the same processes as mentioned above may be operative to produce the observed changes in responsiveness to NA after 15 and 30
days of dark-rearing, the participation of phosphodiesterase is more suspect than in visual cortical slices. For example, the time course studies (Figs. 6 and 8) indicate that in a 20 minute incubation of frontal cortical slices with NA there is greater catabolism of cAMP in experimental than control animals. If augmented phosphodiesterase activity in frontal cortical slices of experimental animals is responsible for the diminished cAMP levels observed in slices after longer incubation times, then it is reasonable to assume that the increased catabolic activity of this enzyme may in part have caused the changes observed in these slices at short incubation times. The qualitative differences observed regarding the effects of dark-rearing on frontal and visual cortex may then be explained by assuming a differential effect on the activity of phosphodiesterase in these brain areas. Thus, in frontal cortical slices the effect of dark-rearing for 30 days on the rate of accumulation rather than the maximal levels of cAMP elicited by NA may reflect changes in cAMP degradative capacity whereas in visual cortical slices the reduced maximal response to NA may involve other processes more directly related to visual deprivation such as the structural changes alluded to earlier.

The control of cAMP levels by phosphodiesterase may be very stringent such that any attempt to elevate these levels would be immediately countered by degradation. Thus, dark-rearing may have caused a situation where large fluctuations in cAMP levels are intolerable and the maintenance of steady state levels, achieved in part by phosphodiesterase, becomes important. That phosphodiesterase may play a vital role in the regulation of cAMP levels in the cell is borne out in studies by Cheung (157,158) and Thompson and Appleman (159). These workers have shown that the enzyme displays all the features important for a regulatory function.
such that it has high affinity for its substrate, it exhibits negative cooperativity, and its activity is regulated by a protein factor as well as Ca^{++} ions.

There were no differences between 30 day old experimental and control animals in the K^{+}-induced accumulation of cAMP in frontal or visual cortical slices (Fig. 9). This tends to discount phosphodiesterase activity as the factor that precipitates the changes observed in responsiveness to NA in brain slices of dark-reared animals. The reason for this is that altered phosphodiesterase as a result of dark-rearing would presumably be manifested regardless of the circumstances that led to elevated cAMP levels. There are several intervening variables, however, which make this line of reasoning more complex than it appears. First of all, the stimulation of cAMP formation in brain slices by K^{+} is much greater than that of NA. These levels of cAMP could be high enough to inactivate phosphodiesterase through the negative cooperativity which the enzyme exhibits and thus obliterate any differences in its activity between brain slices from control and experimental animals.

Secondly, the mechanism whereby K^{+} and indeed all depolarizing agents stimulate cAMP formation in brain slices is not known. That depolarizing agents do not exert their effects on cAMP levels because of the increased respiration and glycolysis in brain slices which they cause is indicated by the finding that the increase in cAMP levels in slices incubated with progressively increasing K^{+} concentrations roughly parallels the known effect of K^{+} concentrations on the electrogenic membrane potentials (160) rather than the effect of K^{+} on respiration and glycolysis (161). Furthermore, it has been shown that under conditions where malonate inhibits enhanced metabolic activity by more than 50% (162)
there was no reduction in the accumulation of cAMP evoked by the depolarizing agent veratridine (163). It is suspected that depolarizing agents induce the release of adenosine (98) which then stimulates cAMP formation through an adenosine receptor (96). The problem encountered here is that phosphodiesterase activity may not be as tightly coupled to the adenosine receptor as it is to the NA receptor or that this coupling may exhibit different characteristics. It has, in fact, been suggested that biogenic amines activate phosphodiesterase whereas adenosine reverses this activation (148).

Finally, the possibility cannot be excluded that K+, in addition to causing the release of adenosine, causes the release of biogenic amines from nerve terminals. In this event, the interpretation of the results obtained in incubations of brain slices with K+ would be very difficult in view of the antagonistic effect of adenosine and biogenic amines on phosphodiesterase activity and the synergism that these substances exhibit with regard to the promotion of cAMP accumulation.

If phosphodiesterase plays a greater part in frontal than visual cortical slices with regard to the observed differences between experimental and control animals, then some of the findings obtained in incubations of brain slices of 60 day old animals with K+ might be explained specifically in terms of the differential effect of adenosine on phosphodiesterase activity in these brain regions. For example, the study of K+-induced accumulation of cAMP in brain slices of 60 day old animals showed (Figs. 12 and 13) that cAMP accumulation was higher in frontal cortical slices of dark-reared animals than controls whereas there was no difference between the two groups in visual cortical slices. Thus, it is possible that the K+-induced release of adenosine and the subsequent
stimulation of cAMP formation and the simultaneous inactivation of phosphodiesterase by adenosine was greater in frontal than visual cortical slices of dark-reared rats. In support of this is the demonstration (Table I) that cAMP levels in frontal cortical slices incubated with adenosine were significantly greater in experimental than control animals whereas there was no difference between the two groups in visual cortical slices. This result would be expected if the $K^+$-induced accumulation of cAMP were mediated by adenosine. Furthermore, this finding suggests that the altered responsiveness to $K^+$ in brain slices of 60 day old dark-reared animals is not due to changes in mechanisms controlling release of adenosine from cells but rather to changes in events subsequent to release such as those described for altered responsiveness to NA.

The processes that may be involved in diminished responsiveness to NA in brain slices of dark-reared animals have been discussed. The accentuated responsiveness of brain slices of 60 day old dark-reared animals to NA and $K^+$ may be explained in terms of the two processes of normalization and supersensitivity acting in concert. Normalization refers to the ability of the CNS structures affected by light deprivation to recover partially from deficiencies in morphological (22,25,33) and electrophysiological (44) development after prolonged durations of dark-rearing. In view of the findings that: (1) the effects of light deprivation may be likened to deafferentation (32,34,43,59-61); (2) denervated neurons may acquire supersensitivity to the transmitters that normally impinge upon them (164-167); 3) supersensitivity may generalize to other transmitters as well as $K^+$ ion (125); and 4) supersensitivity may lead to altered responsiveness of the cAMP system (132-136), it is reasonable to assume that in the cortex of 60 day old dark-reared animals supersens-
itivity of the cAMP generating system may have developed to or generalized to NA and to the agents released from nerve cells during depolarization. This hypothesis could be tested by measuring the accumulation of cAMP in response to varying concentrations of NA or K⁺. If supersensitivity in the cortex of rats dark-reared for 60 days does occur, there will be observed a shift in the log dose-response curve from the right to the left.

The recent demonstration of axonal growth in the CNS of animals affords yet another mechanism through which prolonged exposure to complete darkness may cause increased responsiveness of cortical slices to NA and possibly K⁺. Furthermore, axonal growth may explain some findings of heightened electrophysiological activity (45,46) of brain areas other than visual cortex after visual deprivation as well as some reports of increased synaptic densities of cortical layers which do not involve specific afferent systems (42). Axonal growth takes two forms, direct and collateral. It has been found (168-170) that ascending noradrenergic fibers begin growing after interruption by electrolytic or surgical lesions and invade the area of damage. The phenomena of collateral sprouting in the CNS involves uninjured fibers that can form new collaterals which invade regions deprived of their normal afferent inflow by damage elsewhere (171-173). The new collaterals make synaptic contacts with denervated postsynaptic membranes. Although axonal growth has only been demonstrated in cases where lesions have been introduced in the CNS it may be reiterated that there are numerous similarities between the effects of lesions and visual deprivation on visual cortical morphology and electrophysiology. During dark-rearing there is reduced afferent inflow to the visual cortex and possibly to other cortical areas which may be influenced directly or
indirectly by light deprivation. Thus, a further feature that light deprivation may have in common with denervation or deafferentation is the increased invasion by noradrenergic fibers into those cortical areas affected by visual deprivation.

It has been suggested that dark-rearing results in hyperactivity of the anatomical pathways projecting from the brain stem to the cortex (46). One of these projections is the noradrenergic fiber system. Thus, increased growth of axons and collaterals as a consequence of dark-rearing may form the anatomical substrate for apparent cortical hyperactivity and may be the basis for the suspicions of investigators that dark-rearing may have an effect on subcortical and brain stem structures. Since the NA-fiber system emanating from the brain stem innervates the entire cortex and since dark-rearing may perturb whatever function this system might serve, then the effect of dark-rearing on both the visual and frontal cortex is explained. That increased responsiveness to NA of cortical slices occurred after 60 days of dark-rearing also has a ready explanation through axonal growth. Increased arborization of noradrenergic fibers would lead to a greater number of synapses responsive to NA and thus to a greater capacity for the production of cAMP. The hypothesis of increased axonal growth could be tested by examining the histofluorescent pattern in the cortex of dark-reared rats; this is the technique used to demonstrate axonal growth of noradrenergic fibers.

The developmental profiles of the responsiveness of rat visual and frontal cortical slices to NA were found to be different in that the capacity of NA to elicit the formation of cAMP in frontal cortical slices passes through a maximum at about 10 - 15 days of age and thereafter declines by 30 and 60 days to values similar to that observed in visual
cortical slices which have changed little through the ages 10 to 60 days. This may be due to intrinsic differences in the development of NA-sensitivity in different cortical regions. However, a more palatable explanation is offered by the demonstration that NA is capable of stimulating dopamine (DA)-sensitive adenylate cyclase, albeit at higher concentrations than DA. In caudate nucleus of rat it has been shown (174) that NA stimulates the maximal accumulation of cAMP as effectively as DA although the concentrations needed to produce half maximal stimulation of cAMP synthesis was 4 μM for DA and 28 μM for NA. That NA interacts specifically with the DA receptor is supported by the following: 1) the classical -adrenergic agonist L-isoproterenol did not stimulate cAMP formation (174); 2) the effect of combinations of dopamine and NA on adenylate cyclase did not exceed that observed with optimal concentrations of the individual stimulatory agents (174,175); and 3) the increase in adenylate cyclase activity caused by NA was reduced by the specific DA antagonist haloperidol. The ability of NA to stimulate DA receptors takes on greater significance with regard to the present investigation in view of recent demonstrations of the existence of dopamine nerve endings in rat frontal cortex (176), as well as dopamine-sensitive adenylate cyclase in the anterior limbic cortex of the primate (177), in rat cerebral cortex (178) and, specifically, in the limbic forebrain of rats (179). Thus, the differences in responsiveness to NA between visual and frontal cortex may be explainable in terms of specific developmental characteristics of the dopamine-receptor adenylate cyclase complex in frontal cortex.

If the above interpretation is valid then the differential response of frontal and visual cortical slices to NA after 30 days of dark-rearing may be explained by assuming a differential effect of light deprivation
on the NA and DA systems in the cortex. Moreover, the increase in the
capacity of frontal cortical slices to generate cAMP seen at 10 and 15
days in normal rat pups may be correlated with the development of be-
havioral arousal which also passes through a maximum of about 15 - 20
days (180,181). In support of this correlation is the finding that in
the cortex of rats DA levels peak at 16 days of age, plateau, and then
increase substantially from 30 day to adult (182). Furthermore, it has
been demonstrated that treatments of rat pups with 6-OHDA which reduced
DA but not NA levels in brain results in the development of increased
behavioral activity earlier and to a greater degree than untreated controls
(180). This may have resulted from a supersensitive state of the DA
systems. This correlation is in agreement with the earlier suggestion
that the cAMP system may play a role in the development of the nervous
system.

The hypothesis that in the present experiments NA was stimulating
DA receptors in the frontal cortex might be tested by employing approp­
riate DA and NA agonists and antagonists in incubations of frontal corti­
cal slices. That the developmental characteristics of the DA-adenylate
cyclase system in frontal cortex is peculiar in that it may undergo a
supersensitivity state at earlier ages may be tested by establishing log­
dose response curves for DA in frontal cortical slices of rats of
appropriate ages.
CONCLUSIONS

Investigations into the morphology, electrophysiology, and biochemistry of the brains of light deprived animals suggested to us that in the cortex of animals so treated there may be an alteration in the biochemical processes responsible for the metabolism of cAMP. We have shown that in the visual and frontal cortex of dark-reared rats changes in these processes do occur and that in some respects these changes are different in the visual than in the frontal cortex. To explain our observations on the effects of dark-rearing on the cAMP system, several possibilities have been offered. It is evident that our lack of knowledge about the role of cAMP in brain, and all the systems involved in that role, make it difficult to interpret results showing environmental effects on the cAMP system. The question arises, therefore, whether it is deemed worthwhile to continue on from these preliminary studies. We believe that the possible link between two monumental findings, one demonstrating a second messenger role of cAMP in cells and the other showing the strong propensity of the CNS to exhibit plasticity in structure and function, warrant further investigations of the kind undertaken here.
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