REGULATION OF CARBOHYDRATE METABOLISM IN THE CENTRAL NERVOUS SYSTEM

A Thesis

Presented to

The School of Graduate Studies

of

The University of Ottawa

by

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In partial fulfillment of requirements

for the degree of

Doctor of Philosophy

August, 1970

TO DONNA AND OUR CHILDREN

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to Dr. Radhey L. Singhal for his guidance and constant encouragement throughout the course of this investigation. I am also grateful to Dr. George M. Ling, Chairman, Department of Pharmacology for his advice and constructive criticism.

The author acknowledges the financial support of the Food and Drug Directorate, Department of National Health and Welfare during the course of his Ph.D. program.

Finally, I am grateful to Miss Diane Bell for her expert typing of this manuscript.

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LIST OF ABBREVIATIONS

HK; Hexokinase

PFK; Phosphofructokinase

G-3PDH; Glyceraldehyde 3-phosphate dehydrogenase

PK; Pyruvate kinase

G-6PDH; Glucose 6-phosphate dehydrogenase

6-PGDH; 6-Phosphogluconate dehydrogenase

α-GPDH; α-Glycerophosphate dehydrogenase

ATPase; Adenosine triphosphatase

NAD; Nicotinamide-adenine dinucleotide

NADH; Nicotinamide-adenine dinucleotide (reduced)

NADP; Nicotinamide-adenine dinucleotide phosphate

NADPH; Nicotinamide-adenine dinucleotide phosphate (reduced)

ADP; Adenosine diphosphate

ATP; Adenosine triphosphate

Cyclic AMP; Adenosine 3',5'-monophosphate

DNA; Deoxyribonucleic acid

GABA; Gamma-aminobutyric acid

L-Phe; L-Phenylalanine

PEP; Phosphoenolpyruvate

T_3; L-Triiodothyronine

p-CMB; p-Chloromercuribenzoate

EDTA; Ethylenediamine tetraacetate

Oct; Octanoate

CNS; Central nervous system

Thyx; Thyroidectomized

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I. INTRODUCTION

Despite extensive investigations, our knowledge of the molecular processes which subserve the functioning of the mammalian central nervous system is still limited. Study of the biochemical or metabolic regulations provides an important approach for the elucidation of mechanisms involved in the maintenance of dynamic homeostasis in nervous tissue. Indeed, Atkinson, in 1967, suggested that "a thorough knowledge of cellular metabolism, including metabolic regulatory mechanisms, will be a necessary prerequisite to understanding the specialized biochemistry underlying the specialized functions of cells in the nervous system" (1).

Nervous tissue is equipped with many metabolic processes for the biotransformation of cellular constituents. An especially important role is played by carbohydrate metabolism in this tissue since it provides energy and precursor materials required for the maintenance of normal cerebral activity. The aim of the present study is to investigate some regulatory mechanisms that are involved in the control of carbohydrate metabolism in the central nervous system.

Recent studies have indicated that there exists, in individual cells, a complex network which controls enzyme activity and ensures a proper co-ordination of the overall metabolism. The importance of these regulatory mechanisms for the economy of the cell was stressed by Monod who said that "no cell could function, grow and survive if it were not for the existence of this regulatory network" (2). In

the present study, an attempt has been made to investigate the control of several important carbohydrate-metabolizing enzymes in the brain in order to explore the regulatory mechanisms involved in glucose metabolism in the central nervous system.

At least two mechanisms may be responsible for alterations in the activity of an enzyme. The amount of an enzyme may be affected by changes in the rate of its synthesis and/or degradation. This constitutes a quantitative change in the activity of an enzyme (enzyme induction) and is due to effects exerted on the protein-synthesizing machinery of the cell. Alterations in enzyme activity in this manner represent chronic adaptation as they generally require prolonged periods of time. Hormones control enzyme activity through enzyme induction in a variety of mammalian tissues although relatively little information is available on the hormonal regulation of enzymes in the central nervous system. Hormones secreted by the thyroid gland are known to exert pronounced effects on brain maturation but the neurochemical mechanisms affected by thyroid hormones are largely unknown. An important phase of the present investigation concerns the examination of the regulatory influence of thyroid hormone on certain carbohydrate-metabolizing enzymes in developing nervous tissue.

A second mechanism whereby cellular enzyme activity is altered involves qualitative changes in the activity of an enzyme. A number of specific metabolites have been demonstrated to interact with the enzyme molecule and produce positive or negative modulation of enzyme activity. The control of an enzyme through this mechanism is acute

in nature since rapid changes are produced in its activity as a result of enzyme-modifier interactions. In the present study, an attempt has also been made to investigate the acute modulation of two important enzymes of carbohydrate metabolism in rat brain.

This dissertation is concerned primarily with an elucidation of the mechanisms involved in the regulation of certain glycolytic, pentose phosphate shunt and α-glycerophosphate cycle enzymes in the rat central nervous system. In particular, the control of these enzymes by thyroid hormone in the developing cerebral cortex and cerebellum as well as the modulation of brain pyruvate kinase and α-glycerophosphate dehydrogenase by certain specific metabolites was investigated.

II. LITERATURE REVIEW

(A) SOME IMPORTANT PATHWAYS OF CARBOHYDRATE METABOLISM IN NERVOUS TISSUE

The mammalian central nervous system is highly dependent upon an adequate supply of glucose for the maintenance of its normal function (3,4). Energy provided by carbohydrate metabolism is necessary for processes such as ion transport and acetylcholine synthesis which are involved in some forms of nerve transmission (3,5). The profound importance of carbohydrate metabolism in the central nervous system can be readily demonstrated when the brain is deprived of the normal supply of glucose. Hypoglycemia induced either by hepatectomy or insulin administration leads to signs of nervous system dysfunction such as convulsions and/or coma (6). These symptoms, as well as the electroencephalographic abnormalities associated with hypoglycemia, become readily reversible following the administration of glucose (7).

Glucose is believed to be the main carbohydrate source responsible for maintaining normal brain function. Although other sugars such as mannose and maltose have been found to support cerebral activity in hepatectomized animals, they are believed to be converted to glucose before reaching the brain (7). In contrast, fructose is incapable of supporting normal cerebral function in hepatectomized animals (7). It is of interest that since the brain has a relatively small reserve of glycogen, it must depend for its energy requirements on the supply of glucose brought to it by the bloodstream (8). In addition to its role as a major metabolic fuel in the nervous system, glucose also

serves as a carbon source for the biosynthesis of many substances (9,10). Metabolic pathways leading to the conversion of glucose into amino acids, lipids and complex carbohydrate-containing substances such as gangliosides are known to exist in the central nervous system (11-14). Thus, carbohydrate metabolism is of considerable importance to the maintenance of structural features of the nervous system in addition to its role in the generation of energy for functional processes.

Although the brain has evolved a high dependence upon carbohydrate metabolism for normal activity, the metabolic pathways responsible for the utilization of this substrate in the central nervous system are generally similar to those found in other mammalian tissues. The classical Embden-Meyerhof pathway of glycolysis as well as the tricarboxylic or citric acid cycle exist in the nervous system (5). Similarly, there is evidence for the synthesis and degradation of glycogen and for activity of the pentose phosphate pathway of glucose metabolism in the brain (4,15). The characteristic features of the nervous system may thus be related not so much to the existence of unique pathways of metabolism but rather to specialized mechanisms for the control of metabolic rates and to the relative importance of various pathways (5). In this context, it is of interest to note the studies of Long who compared the activity of hexokinase, the enzyme responsible for the phosphorylation of glucose, in various tissues of the rat. The relative activity of this enzyme was: brain, 1.00; heart, 0.53; spleen, 0.31; kidney, 0.29; skeletal muscle, 0.27;

pancreas, 0.21; lung, 0.16 and liver, 0.05, indicating that the nervous tissue may be more adequately equipped to carry out this step of glucose metabolism (16).

In the present investigation, attention has been directed to some aspects of the regulation of carbohydrate metabolism in the rat central nervous system. In particular, the activities of certain enzymes in the glycolytic pathway (hexokinase, phosphofructokinase, glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase) and pentose phosphate pathway (glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase) of glucose catabolism were examined. In addition, the activity of α-glycerophosphate dehydrogenase, an enzyme which is of importance in the synthesis of myelin lipids in nervous tissue, was investigated. A schematic representation of the various metabolic pathways is shown in Fig. 1.

(a) Glycolysis

One of the outstanding chapters in the development of biochemistry is that concerned with the elucidation of the sequence of events in the glycolytic metabolism of carbohydrates. The existence of the glycolytic or Embden-Meyerhof pathway of glucose metabolism in the mammalian central nervous system was already established by the mid 1930's. Mazza and Valeri (17), Euler et al (18), Meyerhof (19), Ochoa (20) and Geiger (21) were among the first to demonstrate the presence of various intermediary stages of glycolysis in the brain.

Under aerobic conditions, pyruvate formed by the glycolytic

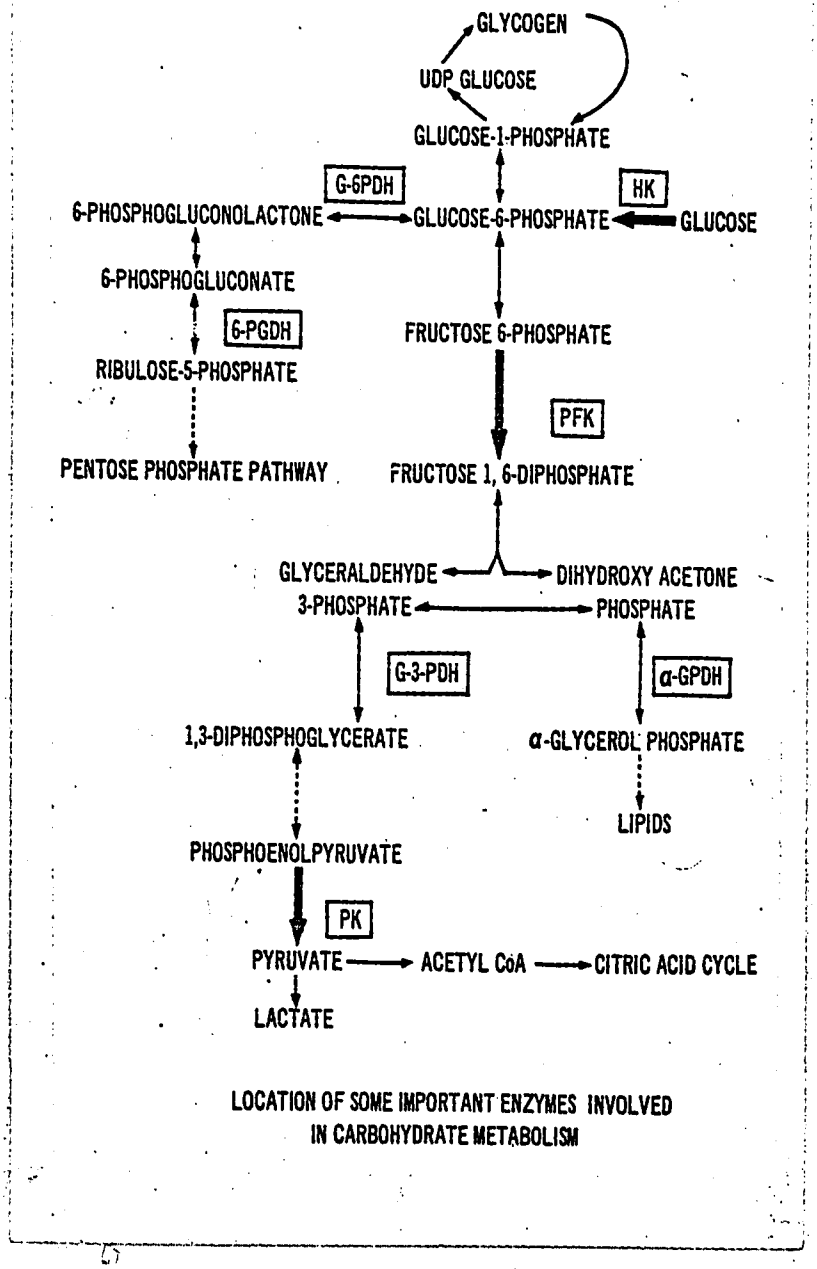

Fig. 1. Pathways of carbohydrate metabolism. The following abbreviations have been used: hexokinase, (HK); phosphofructokinase, (PFK); glyceraldehyde 3-phosphate dehydrogenase, (G-3PDH); pyruvate kinase, (PK); glucose 6-phosphate dehydrogenase, (G-6PDH); 6-phosphogluconate dehydrogenase, (6-PGDH); and α-glycerophosphate dehydrogenase, (α-GPDH).

breakdown of glucose is oxidized by the citric acid cycle whereas during anerobic conditions, pyruvate is reduced to lactate (Fig. 1). Normally, glycolysis and oxidation are consecutive reactions in nervous tissue, pyruvate formed during glycolysis being oxidized through the citric acid cycle (22). Glycolysis and oxidation differ markedly in the efficiency of energy yield; complete oxidation of 1 molecule of glucose to CO_2 and H_2O liberates 686 kcal of energy while the breakdown of glucose to lactate generates only 36 kcal (8). In terms of the yield of ATP, the conversion of glucose to lactate generates only 2 molecules of ATP per molecule of glucose whereas the complete oxidation of glucose results in 38 molecules of ATP (23).

The glycolytic rate is known to vary in different regions of the nervous system. Krebs and Rosenhagen demonstrated that white matter has a lower rate of glycolysis than grey matter (24). Chesler and Himwich found that the glycolytic activity in grey matter from different regions of the adult cat and dog brain decreases in the following order: caudate nucleus, cerebral cortex, thalamus, cerebellum, medulla oblongata and spinal cord (25).

Age is also known to influence the activity of the Embden-Meyerhof pathway in the brain. Tyler and van Harreveld demonstrated that the glycolytic rate was higher in the brain of 1-10 day old rats than in older animals (26). Mandel et al suggested that, in the rat brain, glucose oxidation increases from 36% in 3 day old animals to 56% in adults while at the same time glycolysis decreases from 45%

to 33% (27). Apparently this change does not take place uniformly throughout the nervous system but commences in the spinal cord and eventually reaches the cerebral cortex (23). The unique resistance of young animals to anoxia may be related to the high glycolytic rate in the immature nervous system (6). In contrast to adult animals, the high levels of anerobic glycolysis in the immature brain may provide adequate energy to sustain normal brain function under conditions of anoxia. Evidence for this was presented by Himwich et al who demonstrated that the tolerance of young animals to anoxia was markedly curtailed by pretreatment with inhibitors of glycolysis such as iodoacetate and fluoride (28).

The rate of glycolysis in nervous tissue is also affected by the functional state of the central nervous system. McIlwain demonstrated that glycolysis, as measured by lactate formation, was markedly increased in cerebral cortex slices following electrical stimulation (29). Sacktor et al found an increase in the glycolytic flux in mouse brain in situ after treatment with the convulsant drug, Indoklon (bis(2,2,2-trifluoroethyl)ether) (30). Treatment of animals with barbiturates also has been shown to enhance the rate of glycolysis in nervous tissue (31,32). In contrast, glycolysis in the brain is curtailed by treatment with iodoacetate, fluoride and glyceraldehyde, agents which inhibit specific points in the glycolytic scheme of reactions (33-35).

The presence of the enzymes involved in the Embden-Meyerhof pathway has been well documented for nervous tissue (36-38). The activity of the various glycolytic enzymes is known to be related to

factors such as age, species and the region of the brain (23,39,40). The nervous system may be somewhat unique in the subcellular localization of certain glycolytic enzymes. While the majority of the enzymes are localized within the cell sap or soluble fraction of the cell (41), Balazs and Lagnado found that approximately 10% of the glycolytic activity in the brain may be associated with a particulate fraction (42). Crane and Sols have shown that the activity of hexokinase is localized to a considerable extent in the particulate matter of the cerebral cortex (43). Furthermore, Johnson demonstrated that up to 75% of hexokinase activity in rat brain homogenates may be associated with the mitochondria (44).

Hexokinase, phosphofructokinase and pyruvate kinase are believed to be the three key enzymes in the glycolytic pathway since they play a rate-limiting role in determining the overall activity of this metabolic sequence in certain mammalian tissues (45). Lowry and Passonneau (37) and Lowry et al (46) examined glycolytic processes in the ischemic mouse brain and concluded that the steps involving hexokinase and phosphofructokinase were also rate-limiting in the regulation of cerebral glycolysis. Rolleston and Newsholme found that the steps catalyzed by hexokinase, phosphofructokinase and pyruvate kinase in guinea pig cerebral cortex slices were essentially unidirectional and suggested that glycolysis may be controlled at these points (47). More recently, Takagaki also presented evidence that pyruvate kinase plays a rate-limiting role in the process of glycolysis in

guinea pig brain (48). These studies indicate that, in accordance with other mammalian tissues (45), hexokinase, phosphofructokinase and pyruvate kinase may be of particular importance in the control of the glycolytic pathway in the central nervous system.

(b) Pentose Phosphate Pathway

Mammalian cells may channel glucose into the pentose phosphate pathway in addition to its metabolism through the Embden-Meyerhof pathway of glycolysis (Fig. 1). This metabolic sequence is of primary significance as a source of ribose and deoxyribose required for the synthesis of nucleic acids and nucleotide coenzymes (49). The pentose phosphate cycle also appears to have an important role in generating cellular reducing power, in the form of NADPH, for the synthesis of lipids, amino acids, steroids and purines (50,51).

The relative contribution of the pentose phosphate pathway to carbohydrate metabolism in the nervous system has not yet been firmly elucidated. Bloom suggested that the glycolytic pathway and citric acid cycle are the main mechanisms of glucose utilization in the brain and that the pentose cycle plays only a minor role (52). However, the various enzymes catalyzing the sequential reactions in the pentose phosphate cycle are known to be present in the brain (53-55), and DiPietro and Weinhouse have demonstrated the presence of most of the intermediates of this pathway in nervous tissue (15). Hostetler et al concluded that the maximum contribution of the pentose phosphate cycle to glucose metabolism in the monkey brain was no more than 5-8% (56).

In contrast, Moss has suggested that the pentose phosphate pathway might be the major route of glucose metabolism in the calf brain (57).

While no significant differences in the activity of this pathway were detected between various regions of the brain (56), age may have a marked influence on the activity of the pentose cycle in nervous tissue. Guerra et al found evidence for a substantial participation of the pentose phosphate cycle in fetal rat brain (58) and similar results were obtained by O'Neill and Duffy in neonatal canine brain (59). These workers suggested that the pentose phosphate cycle in the neonatal or fetal brain may contribute substantially to the biosynthetic activity in the nervous system at this stage of development (58,59).

A variety of pharmacological agents have been demonstrated to alter the activity of the pentose phosphate cycle in nervous tissue. Hoskin found that this pathway of glucose metabolism was activated in guinea pig cortex slices in vitro by arsenite and menadiol (60). Similar activation of the pentose pathway was found in rat brain after ethanol treatment (61). Takemori found that administration of morphine as well as several other central nervous system depressants produced an activation of glucose 6-phosphate dehydrogenase (the enzyme catalyzing the initial step of this pathway) in rat cerebral tissue (62). More recently, Herken et al demonstrated that the antimetabolite, 6-aminonicotinamide, markedly inhibits 6-phosphogluconate dehydrogenase in rat brain and related this inhibition to the neurotoxic effects of this compound (63).

(c) α-Glycerophosphate Dehydrogenase

It is known that certain intermediates of the glycolytic pathway can serve as precursors in the biosynthesis of various compounds in the cell (9). The cytoplasmic enzyme, α-glycerophosphate dehydrogenase, catalyses the conversion of dihydroxyacetone phosphate to α-glycerol phosphate which is required for lipid synthesis (Fig. 1). The work of Kennedy (64) and Kornberg and Pricer (65) led to the elucidation of this important pathway in mammalian tissues. In addition to its role in lipid biosynthesis, recent studies have indicated that α-glycerophosphate dehydrogenase may be involved in NAD-NADH interconversions by the "α-glycerophosphate cycle" (66,67).

Relatively few studies have been performed on the regulation of α-glycerophosphate dehydrogenase activity in nervous tissue. Laatsch demonstrated that the developmental changes in α-glycerophosphate dehydrogenase activity in rat brain closely paralleled the process of myelination and suggested that this enzyme may be of importance in myelin formation (68). It is of interest that the developmental pattern of glycerophosphatide formation in the whole rat brain also was similar to the ontogenic changes in the activity of this enzyme (69). Further evidence for the importance of α-glycerophosphate dehydrogenase in myelination was provided by DeVellis et al who demonstrated that the myelin deficiency observed in the nervous system of neonatal rats subjected to X-irradiation of the head was accompanied by a marked decrease in the activity of this enzyme (70).

(B) MATURATION OF THE NERVOUS SYSTEM

In accordance with other mammalian tissues, the central nervous system undergoes marked alterations in its structural, functional and chemical characteristics during development (6,71). McIlwain divided mammalian cerebral maturation into four periods (22). Phase 1 is characterized by extensive cellular proliferation throughout the nervous system. During this period, although the brain is only a fraction of the adult weight, it contains virtually the same number of cells as in the adult animal. In the rat, this period lasts until birth whereas in the guinea pig and human it occupies the first 3/4 of gestation. No significant signs of nerve impulse transmission are exhibited in the brain at this time (72). The second phase is characterized by growth in size of the brain cells. Nerve cells undergo marked outgrowth of axons and establish dendritic connections during this period. In the rat, phase 2 occupies the first 10 days after birth; in man it lasts until parturition. An outstanding feature of phase 3 involves the formation of myelin sheaths around axons. During this period, changes in electrical potential can be detected in the cerebral cortex and neuromuscular control begins (73). In man, this phase occupies the first few months after birth and in the rat, it occurs at about 10-20 days post-partum. In the final stage, myelination is still taking place but overall brain growth proceeds only very slowly to attain the adult size. This period is longer than the preceeding ones since it encompasses the remaining life span of the organism.

Davison and Dobbing have noted that the latter part of this phase may involve alterations associated with senile regression (74).

Elucidation of the changes occurring during maturation of the nervous system has involved the investigations of several disciplines. Anatomical studies have outlined morphological alterations, at both the gross and ultrastructural level, which accompany development of the central nervous system (75-77). Behavioural or psychological criteria which serve as milestones for evaluating the degree of maturation of the human nervous system have also been established (78,79), and more recently, electrophysiological investigations have demonstrated marked changes in bioelectrical activity of the brain during development (72,80,81).

Interest in the biochemical maturation of the nervous system was first expressed by Koch in 1913 (82). Studies in this field lagged for many years until they were revived by Flexner in the early 1940's (83). It has been demonstrated that marked alterations occur in the metabolism of carbohydrates, lipids, proteins and nucleic acids during the course of nervous tissue maturation (84-86). One of the more popular parameters that has been employed in studies on the biochemical maturation of the central nervous system involves the determination of enzyme activity in nervous tissue obtained from animals at various ages. Although this method suffers from the pitfall that the enzyme activity is usually measured under artificial conditions in vitro, it does yield information as to the presence or absence of a particular metabolic sequence in the tissue (87). Indeed, studies

of this nature have suggested that a temporal correlation often exists between the developmental changes in the activity of certain enzymes and the acquisition of various metabolic characteristics by the nervous system. For example, the enhancement of respiratory metabolic processes in the postnatal rat brain was paralleled by increases in the activities of cytochrome oxidase and succinic dehydrogenase (88,89). Laatsch demonstrated that the developmental increase in brain α-glycerophosphate dehydrogenase followed closely the course of myelination in rat brain (68); similar results were obtained with cholesterol esterase, another enzyme which may be involved in the process of myelinization (90). The ontogenic changes in chick brain ATPase, an enzyme involved in the regulation of ion fluxes and excitatory processes in nervous tissue (91), also have been shown to resemble closely the developmental pattern of electrical activity in this structure (92).

Although the majority of enzymes show marked increases during maturation of the nervous system, there are exceptions. Acid and alkaline phosphatase exhibited much higher activity in the fetal brain than in the adult (93,94). Similarly, glucose 6-phosphate dehydrogenase showed virtually no change in its activity in various layers of the rat cerebral cortex from birth to adulthood (95). In this context, Richter has suggested that maturation of the central nervous system involves the successive release of enzymes; those which are active in the embryo are mainly concerned with growth while enzymes concerned with functional activity develop later as the physiological function matures (87).

During the course of maturation, the central nervous system in all mammalian species traverses a period during which there is a rapid morphological and functional development of the brain and a more or less coincidental increase in chemical metabolism (84). The majority of brain enzymes also show marked alterations in activity at this time which Flexner has termed the "critical period" since it is of profound importance in the normal development of the nervous system (96). Wide variations in the time of occurrence of the critical period as well as in its duration have been observed among various species and between various regions of the brain. In the guinea pig cerebral cortex, the critical period occurs between 41-45 days of gestation (96) whereas in the rat it appears to begin at about 10 days post-partum (84).

Studies in recent years have indicated that exposure of the central nervous system to certain adverse influences during the critical period can markedly affect the subsequent development of this tissue. It is of interest that the presence of the same adverse factor after this period has elapsed produces far less effects on the organism (97). Winick (98) and Eichenwald and Fry (99) reviewed the influence of malnutrition on the physical and chemical growth and on the functional development of the brain. Both animal and human studies have suggested that there exists a critical period in brain development during which malnutrition may produce irreversible damage. It is believed that the earlier the malnutrition, the more severe and permanent are the effects produced. Additional factors such as neonatal X-irradiation have been shown to markedly alter the

maturation of the nervous system (100). Recently, Young called attention to our lack of knowledge in the area of developmental psychopharmacology which is concerned with the effect of drugs on the maturing nervous system (101).

A variety of hormones have also been demonstrated to affect the nervous system during critical periods and produce permanent changes in the subsequent psychophysiological processes of the organism (102). Administration of estrogens and androgens, respectively, to neonatal male and female rats resulted in abnormal sexual behaviour in the adult (103,104). Levine and Mullins presented evidence that exposure of neonatal animals to adrenocortical steroids exerts long-lasting effects on the nervous system (102). Finally, thyroid hormone is known to exert a profound influence on the development of the central nervous system.

(C) THYROID HORMONE AND CENTRAL NERVOUS SYSTEM MATURATION

A dual interrelationship is known to exist between the thyroid gland and central nervous system in the mammalian organism (105). First, there are factors concerned with the regulation of thyroid function by the nervous system. This relationship is mediated by the hypothalamus which controls the ability of the adenohypophysis to release thyrotrophic hormone which, in turn, stimulates the production of hormones by the thyroid gland (106). Secondly, thyroid hormones exert a marked influence on the central nervous system, the nature of which depends upon the age of the organism. Thus, in man, thyroid deficiency arising early in life has long been known to lead to cretinism which is characterized by

profound mental retardation (107). Although altered thyroid states in the adult human also lead to abnormalities of brain function, these effects are much less pronounced than those observed in the infant (108,109).

Extensive studies in experimental animals have demonstrated that both a deficiency and an excess of thyroid hormone during early life markedly influence the maturation of the central nervous system (110). The rat has proven to be a suitable subject for investigations of this nature for several reasons: (i) the rat is readily reared under laboratory conditions and produces litters of a size which permits the comparative studies of littermates; (ii) the rat can be readily thyroidectomized during infancy by chronic goitrogen administration (111), radio-iodine injection (112) or by surgical removal of the gland, all leading to a state of experimental cretinism (107), and (iii) the rat's nervous system is exceedingly immature at birth which makes investigations on the influence of altered thyroid states on maturational events more feasible (110).

Extensive alterations are known to result in the fine structure of the rat brain following neonatal thyroidectomy. Eayrs demonstrated a decrease in the size of nerve cells located in the sensorimotor cortex of cretinous rats (113). Impaired development of axons and dendrites has also been detected (113,114), and evidence suggests that interaction between these units in the cerebral cortex may be markedly reduced during cretinism (115). Neonatal thyroidectomy also produces extensive alterations in the histogenesis of the cerebellum as manifested by

delayed migration of cells from the superficial granular layer and decreased ramification of the Purkinje cells (116). Legrand demonstrated that thyroid hormone must act before the 10th postnatal day for normal histological maturation of the cerebral and cerebellar cortex, indicating that the early postnatal period constitutes a critical time for the influence of thyroid hormone on the central nervous system (117).

The maturational influence of thyroid hormone on the central nervous system was also demonstrated in terms of the ontogenesis of electrical activity in the cerebral cortex. Electroencephalograms obtained from neonatally thyroidectomized rats showed a reduced amplitude (118). It is of interest that thyroidectomy of mature animals resulted in no such alterations in the bioelectrical activity of the brain (119). Eayrs has suggested that electrocortical abnormalities observed in cretinous animals may be related to altered histogenesis and a consequent reduction in the liklihood of axodendritic interactions in the cerebral cortex (107).

Several investigators have demonstrated that the maturation of learning and behavioural responses is also impaired in rats made hypothyroid in early postnatal life. Eayrs and Lishman reported that the development of certain parameters of innately organized behaviour such as startle response to auditory stimuli and the capacity to land on the feet when dropped back downwards were grossly retarded by neonatal thyroidectomy (120). In addition, the capacity for adaptive behaviour, which depends upon the successful maturation of the cerebral cortex and is a measure of the learning ability (110), was markedly impaired

in rats subjected to experimental cretinism (120-122). The observation that these behavioural abnormalities do not arise when thyroid deficiency is produced in the mature animal further suggests the existence of an early critical period during which development of the central nervous system is readily affected by alterations in thyroid function (123). Interestingly, hyperthyroidism induced by thyroxine administration has been shown to enhance the ability of the new born rats to learn a conditioned avoidance response (124,125). However, Eayrs has pointed out that although innately organized responses exhibited an accelerated maturation when excess thyroid hormone was given to the neonate rat, the process of learning was subsequently impaired when the animal matured (126).

In recent years, a variety of investigations have demonstrated that extensive neurochemical alterations occur during experimental cretinism. Ramirez de Guglielmone and Gomez reported a transitory decrease in the concentration of glutamic acid, γ-aminobutyric acid (GABA) and glutamine and a permanent change in the levels of aspartic acid and taurine in the brain of neonatally thyroidectomized rats (127). A decreased incorporation of labelled leucine into protein has been shown in the cerebral cortex of rats made hypothyroid at 1 day of age (128). In addition, alterations have been found in nucleic acid metabolism (129-131) as well as in the level of several electrolytes in the central nervous system of cretinous rats (132). More recently, Walravens and Chase (133) and Balazs et al (134) have reported the occurrence of extensive changes in lipid metabolism and myelination

in the brain of neonatally thyroidectomized animals.

Many workers have also demonstrated alterations in enzyme activity in the nervous system of thyroid deficient animals. Hamburgh and Flexner found a decrease in the activity of succinic dehydrogenase and cholinesterase in the frontal cortex of cretinous rats (135). Geel and Timiras demonstrated that neonatal hypothyroidism produces a significant decrease in the level of acetylcholinesterase and cholinesterase in the cerebral cortex and in acetylcholinesterase activity in the hypothalamus (136). More recently, Garcia Argiz et al reported that thyroid deprivation from birth leads to an altered developmental pattern of glutamate decarboxylase, Mg^{+} and Na^{+}-K^{+} ATPase, and GABA transaminase in rat cerebral cortex and of GABA transaminase and Na^{+}-K^{+} ATPase in the cerebellum (137). The decreased level of several cerebro-cortical and cerebellar enzymes in neonatally thyroidectomized rats was successfully restored following administration of thyroid hormone (138).

The crucial effect of thyroid hormone on the maturation of the central nervous system was stressed by Levine and Mullins (102). They suggested that many of the defects observed in the nervous system following neonatal thyroidectomy could be partially or wholly corrected if thyroid hormone replacement was begun within a certain critical period which, in rats, is believed to extend up to 15 days after birth (102). However, once this critical period has passed, many of the effects of neonatal hypothyroidism become irreversible and cannot be corrected by thyroid hormone treatment during adulthood (139). A

similar situation is believed to exist for human cretinism since thyroid therapy must be initiated early in life or irreversible mental retardation ensues (140). Although these studies indicate that thyroid hormones exert a profound influence on the nervous system, particularly during its development, relatively little is known about the mechanisms which are responsible for eliciting these effects. During recent years, a variety of hypotheses have been advanced in order to explain the mechanism of action of thyroid, as well as several other hormones, on mammalian tissues.

(D) HORMONAL ACTION AND ENZYME REGULATION IN MAMMALIAN TISSUES

Hormones are generally thought to be chemical messengers, produced by special glands or cell groups and carried via the circulation to their target organs. However, there is little agreement on how the hormones produce their diverse effects in responding tissues or cells. Indeed, the multiplicity of hormonal effects and the wide variations in the chemical structure of hormones makes it quite conceivable that these biological substances may act by several different mechanisms (141).

Available evidence suggests that the action of certain hormones may be mediated by effects on the permeability of cell membranes. Hormones acting by this mechanism may cause alterations in intracellular substrate levels and thus exert a selective control on the metabolism of the cell (142-144). Many of the actions of insulin on carbohydrate distribution and utilization may be accounted for by the effect of

this hormone on permeability of the outer cell membrane (143). In addition to affecting the external cell membrane, certain hormones may alter transport processes of intracellular membranes; e.g., thyroid hormones are known to exert some of their physiological effects by influencing the permeability of mitochondrial membranes (145,146).

Hormones may also exert their effects by producing modifications in the activities of certain enzymes which govern the operation of vital metabolic processes. One mechanism whereby this effect may be produced is by hormonal interaction with the enzyme molecule and a resultant change in the catalytic activity of the enzyme. However, despite extensive investigations, the belief that hormones may cause a direct activation of enzymes by acting in this manner, analogous to certain vitamins which function as coenzymes (147), has not been substantiated (148).

Recently, the hypothesis that hormones may act through the agency of the cyclic nucleotide, adenosine 3',5'-monophosphate (cyclic AMP) has attracted considerable attention. According to this concept, certain hormones stimulate the activity of the enzyme adenyl cyclase in responsive cells, leading to an enhanced synthesis of cyclic AMP which then mediates the action of the given hormone (149). It is known, for example, that epinephrine- and glucagon-stimulated enhancement of hepatic glycogen metabolism occurs subsequent to an increase in the concentration of hepatic cyclic AMP. The cyclic nucleotide, thus generated, leads to an activation of phosphorylase, the enzyme responsible

for glycogen breakdown (149). Evidence for the involvement of the adenyl cyclase-cyclic AMP system in the action of a variety of other hormones and biogenic amines has been recently reviewed by Sutherland et al (150,151).

In addition to altering the catalytic properties of enzymes, hormones may affect enzyme activity in a quantitative manner by producing changes in the enzyme concentration. According to this hypothesis, hormones affect the rate of enzyme synthesis and degradation by acting on the protein-synthesizing apparatus of the cell. Increased enzyme activity as a result of this mechanism thus represents increased enzyme synthesis and is called enzyme "induction" (141). Several lines of evidence have been presented which support the suggestion that hormones induce quantitative changes in the amount of specific proteins or enzymes. Kidson and Kirby demonstrated that insulin, corticosteroids, androgens, estrogens, thyroxine and growth hormone all cause enhanced incorporation of labelled amino acids into protein (152). Stimulation of messenger-RNA production in the responsive cells of the target organ has also been shown for a variety of hormones. Williams-Ashman et al demonstrated that testosterone treatment enhances messenger-RNA synthesis in the prostate (153) and similar observations have also been made for estrogens (154) as well as growth hormone (155). The ability of inhibitors of RNA and protein synthesis, such as actinomycin D and cycloheximide, to block the hormone-induced increases in enzyme activity is an important criterion for establishing that the increased enzyme activity is the result of enzyme synthesis de novo (156).

Weber et al postulated that certain hormones may act on "functional genic units" to switch on the synthesis of groups of enzymes encoded by specific genes (157). Clever and Karlson demonstrated that the insect hormone, ecdysone, produces puffing at gene loci in salivary gland chromosomes and suggested that this hormone may regulate the expression of genetic material (158). Hormones also have been shown to interact with histones which are important components of the cell nucleus and are believed to be regulators of gene activity (159,160). More recently, Goldberg and Atchley demonstrated that hormones promote the separation of complementary strands of the DNA double helix which was considered as evidence for gene activation (161). While these studies suggest that hormones act on the gene loci, the precise mechanisms whereby this is accomplished have not yet been elucidated.

It is known that living organisms maintain dynamic homeostasis among various metabolic steps through the influence of certain specific metabolites which act as modifiers of enzyme action (2). In mammalian cells, hormones modify enzyme activity by promoting alterations in the concentration of the enzyme through an increase in the rate of its synthesis. The nutritional status of the organism and chemicals may also regulate the activity of an enzyme in this manner (162,163). This type of enzyme regulation, is generally of a chronic nature since prolonged periods of time are required for inducing de novo enzyme biosynthesis. The activity of an enzyme may also be modified acutely by interaction of the enzyme molecule with specific metabolites which produce rapid changes in the catalytic properties

of the enzyme. Hormones may also affect enzyme activity by this mechanism either directly or through the agency of an intracellular mediator such as cyclic AMP (141). The ability of endogenous and foreign metabolites to acutely modify enzyme activity in mammalian tissues has been reviewed by Atkinson (164) and by Stadtman (165). Monod et al presented a highly specific model for general interpretations of protein-substrate-modifier interactions and suggested that certain modulators affect allosteric sites of regulatory enzymes (166).

The basic concepts of acute enzyme modulation were established mainly by investigations with lower organisms (167,168). However, recent evidence indicates that the same mechanism of enzyme regulation may also be operating in the maintenance of dynamic balance among metabolic pathways in mammalian tissues. For example, the well known "Pasteur reaction" wherein anerobic glycolysis is inhibited during the generation of high energy yields, may be explained by the ability of ATP to inhibit the activity of a key glycolytic enzyme, phosphofructokinase (169). Similarly, the reciprocal relationship between carbohydrate and lipid consumption in mammalian tissues (170) has been explained by the ability of free fatty acids to act as a "metabolic directional switch" and inhibit the enzymes involved in the process of glycolysis (171). Weber demonstrated that L-phenylalanine, which accumulates in the tissues during phenylketonuria, produces a competitive inhibition of brain pyruvate kinase and suggested that inhibition of this important glycolytic enzyme may be involved in the pathogenesis of the defective brain development observed in phenylketonuric patients

(172). Thus, the acute modulation of enzyme activity by metabolites may be of importance in the production of abnormal metabolic states as well as maintaining dynamic balance among metabolic pathways under physiological conditions.

III. MATERIALS AND METHODS

(A) ANIMALS

Rats of the Sprague-Dawley strain, maintained on Master Laboratory chow and water ad libitum were used throughout this study. Pregnant animals were obtained from a local breeding colony and their litters, employed in certain phases of this investigation, were weaned at 22 days of age. No attempt was made to distinguish the sex of neonatal animals used in developmental studies.

(B) SAMPLE PREPARATION

(a) Preparation of the Tissue

Animals were killed by decapitation at the atlanto-occipital joint. The skin overlying the skull was cut in the midline and reflected laterally. One tip of a pair of scissors was introduced into the foramen magnum and a midline cut was made through the sagittal suture of the skull. Perpendicular cuts were then made through the coronal sutures and the bone flaps were reflected. The exposed brain was teased out of the cranial vault with the tip of a spatula, stripped of adherent meningeal tissue and blood vessels and weighed on a torsion balance (Federal Pacific Electric Co., Newark, N.J.). The whole cerebellum was excised by severing the three cerebellar peduncles. The remainder of the brain was then split into two halves and thin slices of the fronto-parietal region of the cerebral cortex were removed using a scalpel

blade. Grossly visible white matter was removed from the cerebrocortical slices using the tip of a scalpel blade.

Samples from several other regions of the central nervous system were obtained for studying the regional distribution of enzyme activity. Following removal of the cerebellum and a portion of the cerebral cortex, the remainder of the cerebral cortex was stripped from the brain using a scalpel blade and discarded. The exposed caudate nucleus-putamen was removed to represent the basal ganglia. The pituitary stalk was then located on the ventral surface of the brain and a small portion of tissue overlying this region was removed to represent the thalamus-hypothalamus. A cut was then made at the rostral aspect of the midbrain and a sample of the brain stem, including midbrain, pons and medulla oblongata was removed. The cervical spinal column of the decapitated torso was exposed by cutting the skin and dividing the dorsal neck muscles. The arches of the cervical vertebrae were split with a pair of scissors and the exposed cervical spinal cord was teased from the spinal column and stripped of adherent meningeal tissue.

All tissues were placed immediately in beakers immersed in crushed ice until used for the preparation of the homogenate. A diagrammatic representation of various regions of the central nervous system investigated is shown in Fig. 2.

(b) Homogenization and Centrifugation

The separately pooled tissues were finely minced with a pair of scissors. The tissue minces were weighed and 5% homogenates were

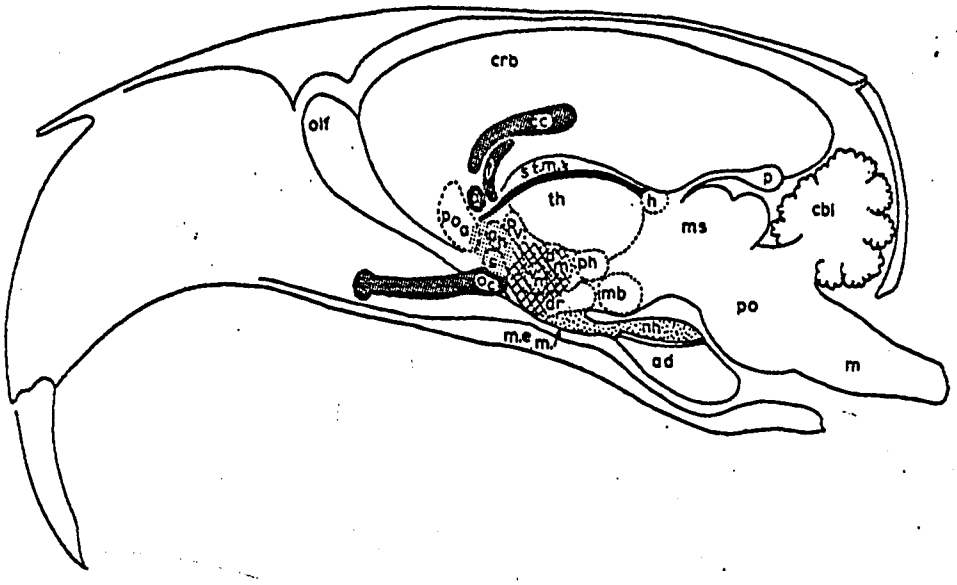

Fig. 2. A diagrammatic representation of the rat brain in situ showing the localization of some major structures. Areas investigated included: cerebral cortex, (crb); cerebellum, (cbl); brain stem including midbrain, (ms); pons, (po); and medulla oblongata (m); thalamus-hypothalamus, (th and the cross-hatched area). Two other areas, basal ganglia and cervical spinal cord which are not shown in this diagram, were also investigated.

prepared in isotonic KCl, pH 7.4. Homogenization was effected with a chilled Potter-Elvehjem homogenizer (Fisher Scientific Co., Fairlawn, N.J.) fitted with a Teflon pestle spinning at 700 r.p.m. for exactly 60 sec. After removal of an aliquot of the homogenate for DNA estimation, the homogenate was spun for 30 min at 100,000 X g at 5°C in a refrigerated I.E.C. model B-60 centrifuge (International Equipment Co., Needham Heights, Mass.). The supernatant fluids were decanted and stored in glass vials immersed in crushed ice throughout the course of the experiment.

(C) DNA ESTIMATION

DNA was used as a reference material for the expression of enzyme activity in certain phases of this investigation. The procedure used for DNA estimation was essentially that outlined by Burton (173).

(a) DNA Extraction

A 0.9 ml aliquot of the 5% homogenate was acidified with 0.1 ml 2.5 N perchloric acid, left on ice for 30 min and then centrifuged for 10 min in a refrigerated clinical centrifuge (International Equipment Co., Needham Heights, Mass.). The supernatant was discarded and the precipitate was resuspended in 0.5 N perchloric acid. The suspension was heated, with occasional stirring, at 70°C for 15 min and recentrifuged. This procedure was repeated twice using a total volume of 7.0 ml of 0.5 N perchloric acid. The DNA extract was stored on ice until used for the analysis.

(b) DNA Analysis

DNA was analyzed using the diphenylamine reagent which consisted of 1.5 g diphenylamine, 1.5 ml concentrated H_2SO_4 and 0.5 ml aqueous acetaldehyde per 100 ml glacial acetic acid. DNA was determined using 1.0 ml of the extract to which was added 2.0 ml of the diphenylamine reagent. Color was developed by incubating for 16-20 hr at 30°C along with a suitable blank and standard made from calf thymus DNA. The color was read at 600 mμ in a Unicam model SP 500 spectrophotometer (Unicam Instruments, Ltd., Cambridge, England) and total DNA content was calculated as mg/g wet weight tissue.

(D) ENZYME DETERMINATIONS

All enzyme activities were assayed in the 100,000 X g supernatant fluid. Enzyme assays were conducted at 340 mμ in a constant recording Unicam spectrophotometer model SP 800 (Unicam Instruments, Ltd., Cambridge, England) thermostated at 37°C. Preliminary experiments were performed with each enzyme to establish linearity with regard to time and amount of the tissue, and all enzymes were assayed under strictly linear kinetic conditions. Changes in optical density were recorded for a period of at least 5 min and the final pH and temperature were checked at the end of each assay. Enzyme activities were calculated as μmoles of substrate metabolized per hr per g of tissue at 37°C and expressed either per g of tissue or per mg DNA.

(a) Hexokinase

Hexokinase activity was measured according to the method of DiPietro and Weinhouse (174). The assay was based on the rate of formation of NADPH from NADP in a system coupled with glucose 6-phosphate dehydrogenase. The assay medium contained the following in a total volume of 2.5 ml at the designated final concentrations: glucose 6-phosphate dehydrogenase (1.43 µg of protein); glycyl-glycine buffer (pH 7.5), 50 mM; $MgCl_2$, 7.5 mM; ATP, 5 mM; NADP, 0.75 mM, cysteine, 2 mM and glucose, 0.5 mM. The reaction was initiated by the addition of supernatant fluids corresponding to 10 mg wet weight of the tissue and changes in absorbance were recorded against a blank which was devoid of glucose.

(b) Phosphofructokinase

This enzyme was assayed according to the procedure of Parmegianni et al based on the rate of disappearance of NADH in an assay system coupled with aldolase, α-glycerophosphate dehydrogenase and triose phosphate isomerase (175). The following components, at the designated final concentrations, were added to the reaction mixture (final volume, 3.0 ml) in the given sequence: glycyl-glycine buffer (pH 8.2), 50 mM; $MgCl_2$, 6 mM; EDTA, 1 mM; ATP, 1 mM; $(NH_4)_2SO_4$, 4 mM; 2-mercaptoethanol, 25 mM; NADH, 0.3 mM; bovine serum albumin, 0.6 mg; aldolase, 40 µg of protein; α-glycerophosphate dehydrogenase, 10 µg of protein; triose-phosphate isomerase, 4 µg of protein; D-fructose 6-phosphate, 1 mM. The reaction was started by the addition of the supernatant fluid

corresponding to 5 mg wet weight of nervous tissue and changes in optical density were recorded against a blank which contained no D-fructose 6-phosphate.

(c) Glyceraldehyde 3-Phosphate Dehydrogenase

The assay of this enzyme was based on the reduction of NAD to NADH in a system coupled with aldolase according to the procedure of Freedland (176). The reaction mixture (final volume, 3.0 ml) contained the following components added in the given order: glycine buffer (pH, 9.0), 27 mM; $MgSO_4$, 3.5 mM; Na_2HAsO_4, 17 mM; NAD, 4.2 mM; cysteine, 20 mM; aldolase, 50 µg of protein; supernatant fluid corresponding to 15 mg wet weight of the tissue. The reaction was started by adding D-fructose 1,6-diphosphate (36 mM) and changes in absorbance were monitored against a blank devoid of this substrate.

(d) Pyruvate Kinase

Pyruvate kinase activity was determined by measuring the disappearance of NADH in an assay system coupled with lactate dehydrogenase employing a modified procedure of Weber et al (177). The reaction mixture (final volume, 3.0 ml) contained the following components added in the given order: tris buffer (pH 7.4), 41.7 mM; $MgSO_4$, 6.25 mM; KCl, 25 mM; phosphoenolpyruvate, 5.3 mM; ADP, 1.3 mM; NADH, 0.22 mM; lactate dehydrogenase, 0.1 mg of protein. The reaction was initiated by addition of an appropriate dilution of the supernatant fluid and the changes in optical density were recorded against a blank which contained no phosphoenolpyruvate.

(e) Glucose 6-Phosphate Dehydrogenase and 6-Phosphogluconate Dehydrogenase

The activities of these pentose phosphate shunt enzymes were assayed according to the differential method of Glock and McLean (178) which is based on the reduction of NADP to NADPH. The assay medium contained the following in a total volume of 2.5 ml at the designated final concentrations: glycyl-glycine buffer (pH, 7.5), 50 mM; $MgCl_2$, 7.5 mM; D-glucose 6-phosphate, 2 mM; 6-phosphogluconic acid, 2 mM; NADP, 0.75 mM. In the presence of 6-phosphogluconic acid and glucose 6- phosphate as substrates, the activity measured represented the total activities of both enzymes. The activity of 6-phosphogluconate dehydrogenase was obtained using 6-phosphogluconic acid as the sole substrate. The activity of glucose 6-phosphate dehydrogenase was then calculated as the difference. The reaction was initiated by the addition of the supernatant fluid corresponding to 10 mg wet weight of the tissue and changes in absorbance were recorded against blanks devoid of the appropriate substrate.

(f) α-Glycerophosphate Dehydrogenase

α-Glycerophosphate dehydrogenase activity was assayed by measuring the rate of disappearance of NADH in an assay system coupled with aldolase according to the procedure of Freedland (176). The following components were added to the reaction mixture (final volume, 3.0 ml) in the given order of addition: tris buffer (pH, 7.4), 33.3 mM; NADH, 0.22 mM; aldolase, 50 µg of protein; supernatant fluid corresponding

to 15 mg wet weight of the tissue. The reaction was started by adding D-fructose 1,6-diphosphate (36 mM) and changes in optical density were recorded against a blank which contained no substrate.

(E) THYROIDECTOMY

Animals were thyroidectomized by treatment with ^{131}I according to the method of Goldberg and Chaikoff (112). The method is based on the ability of the thyroid gland to concentrate ^{131}I with a consequent radio-destruction of the thyroid tissue by β emissions. Litters of animals were injected intraperitoneally with various dosages of ^{131}I (obtained from Atomic Energy of Canada, Ltd., Ottawa). The radioisotope was administered via the muscles of the rump using a high gauge needle in order to prevent leakage from the site of injection. Littermate controls were injected with physiological saline. The mortality rate was extremely low and no special precautions were required to maintain these animals. The neck region was examined visually when the animals were killed in order to assess gross pathological changes. In addition, histological examination of the thyroid gland region was performed to estimate the degree of thyroid tissue destruction.

(F) CHEMICALS

All reagents were of the purest grade available and were dissolved in glass distilled water unless stated otherwise. Glucose 6-phosphate dehydrogenase, NAD and NADH were obtained from Boehringer Chemical Co. (London). α-Glycerophosphate dehydrogenase and DNA (from

calf thymus) were purchased from Calbiochem (Los Angeles). Various phenylalanine analogs (chloroacetyl-DL-phenylalanine, o-chloro-DL-phenylalanine and DL-p-chlorophenylalanine) as well as phenylalanine metabolites (phenylacetic and DL-β-phenyllactic acid) were obtained from Nutritional Biochemicals (Cleveland). ADP, ATP, L-alanine, aldolase, crystalline bovine serum albumin, p-chloromercuribenzoic acid, D-fructose 6-phosphate, D-fructose 1,6-diphosphate, D-glucose 6-phosphate, NADP, D-penicillamine hydrochloride, L-phenylalanine, phosphoenolpyruvic acid, 6-phosphogluconic acid, L-triiodothyronine and triose phosphate isomerase were purchased from Sigma Chemical Co. (St. Louis). Cycloheximide (Acti-dione) was obtained from Upjohn Co. (Kalamazoo, Mich.).

L-Triiodothyronine (T_3) was dissolved in 0.02 N NaOH (179) and administered subcutaneously. Cycloheximide was dissolved in physiological saline and given by the intraperitoneal route. Control animals were injected with the appropriate vehicle solution.

G. STATISTICAL ANALYSIS

Since it was necessary to pool tissues from a number of animals in order to obtain an adequate amount for sample preparation, studies on the regulation of carbohydrate-metabolizing enzymes by thyroid hormone were usually repeated 3 times. However, the variability within a given group was minor and it was felt that the means ± S.E. obtained from 3 determinations of enzyme activity were adequate to perform statistical analysis. The results were subjected to statistical evaluation using Student's "t-test" and significant differences between the means

(calculated as p values) are shown. No statistical significance is indicated when the p value was >0.05.

IV. RESULTS

(A) RELATIVE DISTRIBUTION OF ENZYMES AND DNA IN CERTAIN AREAS OF THE CENTRAL NERVOUS SYSTEM

The regional distribution of several carbohydrate-metabolizing enzymes in the central nervous system of adult female rats is shown in Table I. The activity of HK, PFK, G-3PDH and PK was lower in the spinal cord than in more rostral areas of the nervous system when expressed on a per g wet weight basis. With the exception of G-6PDH in the cerebellum, the two pentose phosphate pathway enzymes as well as the activity of α-GPDH was similar in all areas investigated. Table I also demonstrates that whereas the spinal cord contained somewhat less DNA, a higher concentration of the nucleic acid was found in the cerebellum. The data presented in Table II show the relative distribution of various enzymes in the central nervous system when expressed on a per mg DNA basis. The cerebellum was found to contain lower levels of all enzymes investigated when calculated per mg DNA which is due to the high levels of DNA encountered in this area of the brain.

(B) DEVELOPMENTAL CHANGES IN ENZYME ACTIVITIES AND DNA CONTENT IN THE CEREBRAL CORTEX AND CEREBELLUM

In order to investigate the biochemical changes accompanying development of the central nervous system, rats were killed at various ages after birth and DNA as well as various enzyme activities were determined in the cerebral cortex and cerebellum. The changes in DNA

TABLE I

DNA CONCENTRATION AND ENZYME ACTIVITIES IN SEVERAL REGIONS OF THE CENTRAL NERVOUS SYSTEM

Each value represents the mean ± S.E. based on three determinations of enzyme activity in tissues pooled from three adult female rats. Enzyme activities are expressed as μmoles of substrate metabolized/hr/g of tissue at 37°C. DNA content is calculated as mg/g of tissue.

REGION OF CNS	HK	PFK	G-3PDH	PK	α-GPDH	G-6PDH	6-PGDH	DNA
SPINAL CORD	80±15	307±15	143±5	8050±111	244±23	119±9	49.2±10.6	0.80±0.05
BRAIN STEM	126±18	500±90	221±13	8435±1780	317±9	150±16	58.0±6.4	1.02±0.10
THALAMUS-HYPOTHALAMUS	154±10	571±5	263±9	9582±704	248±1	122±17	54.7±9.0	1.52±0.14
BASAL GANGLIA	156±31	455±23	266±6	9033±1667	231±7	109±12	40.2±5.8	1.14±0.10
CEREBRAL CORTEX	178±11	615±43	301±8	9007±701	236±10	113±19	41.0±5.0	1.61±0.13
CEREBELLUM	149±24	647±58	347±25	10151±1631	273±27	205±21	55.5±11.9	6.12±0.51

TABLE II

DISTRIBUTION OF ENZYME ACTIVITIES IN SEVERAL REGIONS OF THE CENTRAL NERVOUS SYSTEM

Each value represents the mean ± S.E. based on three determinations of enzyme activity in tissues pooled from three adult female rats. Enzyme activities are expressed as μmoles of substrate metabolized/hr/g tissue at 37°C per mg DNA.

REGION OF CNS	HK	PFK	G-3PDH	PK	α-GPDH	G6-PDH	6-PGDH
SPINAL CORD	103±25	389±45	179±7	10115±1440	305±16	150±17	63.1±16.5
BRAIN STEM	126±21	490±55	220±18	8199±1188	317±29	150±24	58.4±9.9
THALAMUS-HYPOTHALAMUS	102±6	380±31	175±12	6387±665	166±16	80±6	35.7±3.2
BASAL GANGLIA	139±28	415±66	239±24	7984±1152	209±25	101±23	37.3±9.4
CEREBRAL CORTEX	111±9	383±11	185±15	5619±376	149±18	72±17	26.1±5.2
CEREBELLUM	25±5	109±17	58±8	1682±345	45±3	34±2	8.9±2.5

concentration during postnatal development of the cortex and cerebellum are shown in Fig. 3. A somewhat higher level of DNA was present in the cerebral cortex during the first few days of life and thereafter it remained relatively constant. In the cerebellum, DNA concentration increased between 1-20 days following birth, after which a gradual decline was observed in the level of this nucleic acid.

The ontogenic changes in the activity of four glycolytic enzymes are illustrated in Figs. 4-7. Marked increases in all enzyme activities, whether expressed per g of tissue or per mg DNA, were observed in the cerebral cortex during the first 20 days of life. The activity of G-3PDH and PK increased progressively after 20 days whereas constant levels of PFK were maintained after this age. A distinct decrease in HK activity was observable in the cortex after 60 days. Both G-3PDH and PK also showed marked increases in the cerebellum as the animals matured. Less pronounced increases in the activity of HK and relatively constant levels of PFK were observed in the cerebellum throughout the time course of this study.

Figs. 8 and 9 demonstrate the developmental pattern of the pentose phosphate shunt enzymes. With the exception of a decrease in G-6PDH activity (expressed per g of tissue) during the first 5 days after birth, only minor changes in these enzymes were detected in the cortex up to 60 days of age. The activity of cerebellar G-6PDH also showed a slight decrease during the first 5-10 days of life which was then followed by a progressive increase between 10-100 days. Slight changes in the activity of 6-PGDH were observed in the cerebellum

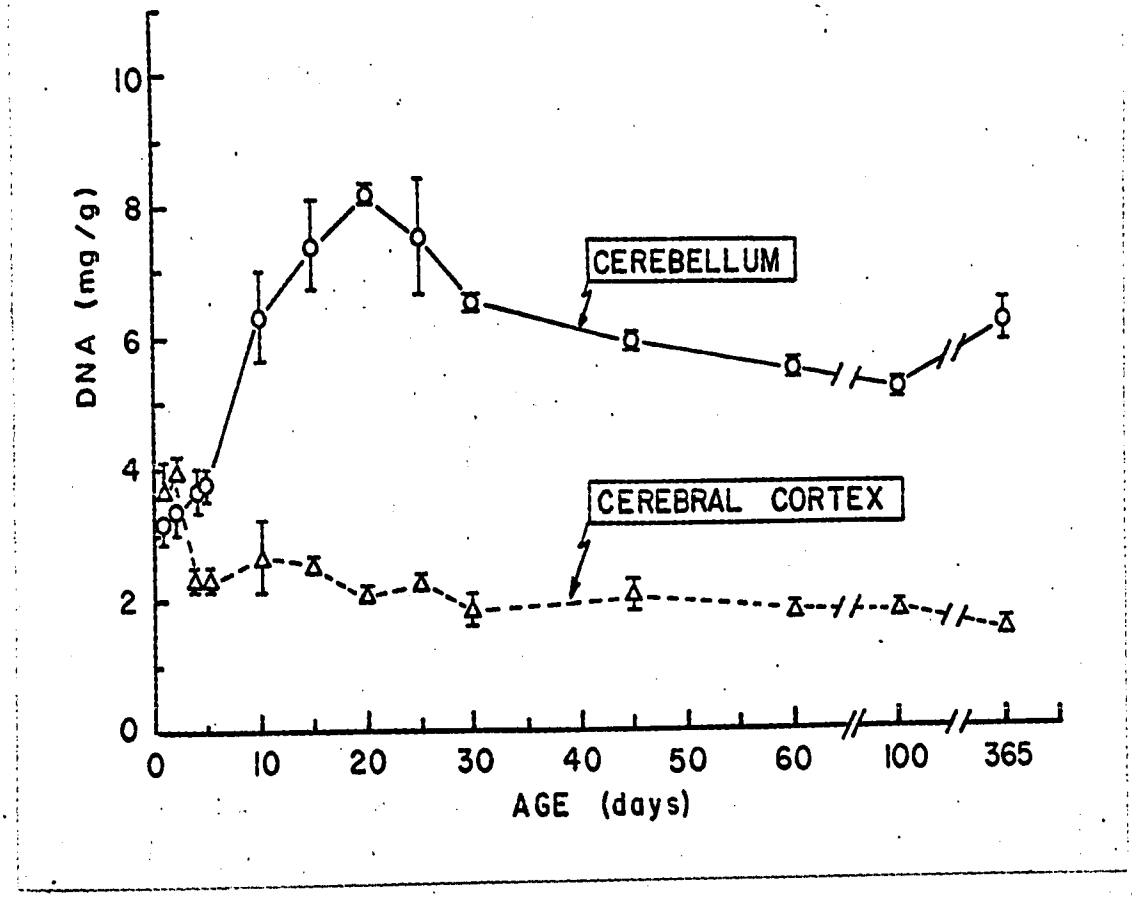

Fig. 3. Developmental changes in DNA content in rat cerebral cortex and cerebellum. Each point represents the mean ± S.E. of 3-4 determinations of DNA. Each assay was performed on tissues pooled from the entire litter in the case of young animals and from 2-3 rats in older age groups.

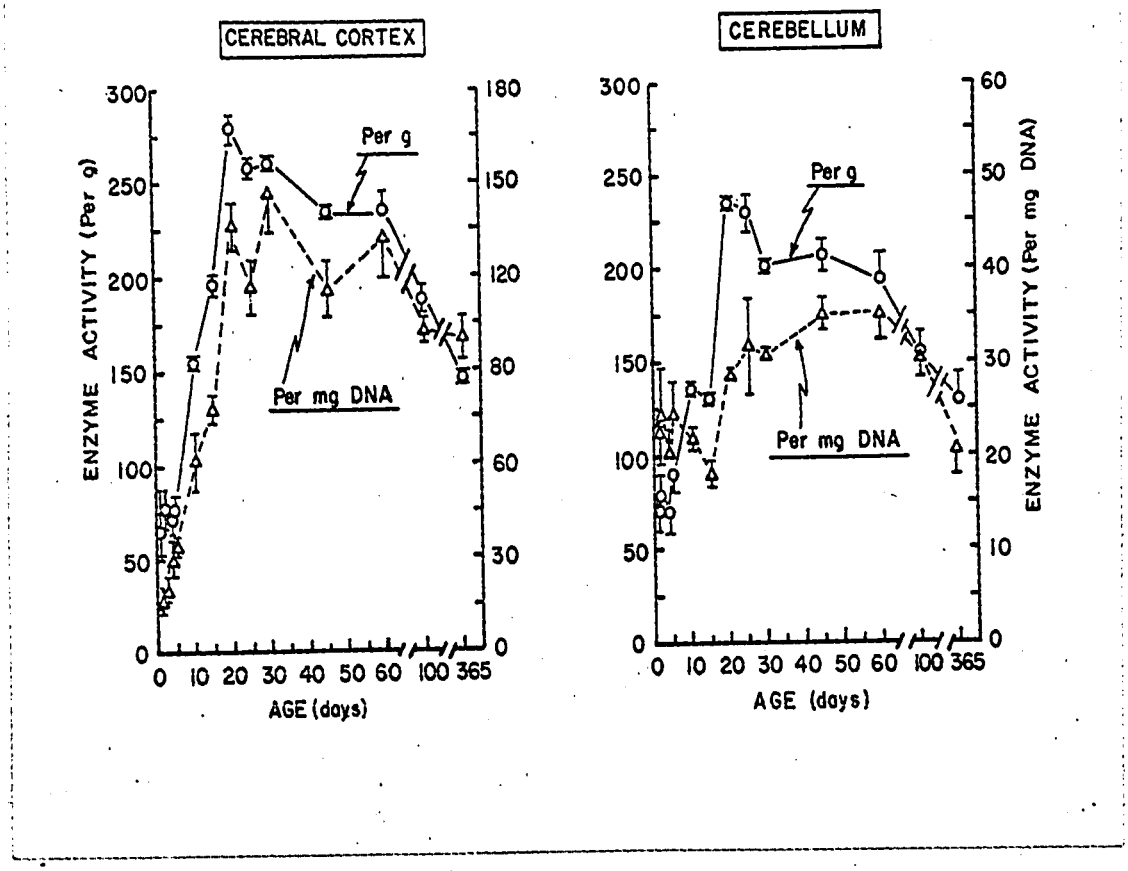

Fig. 4. Ontogeny of hexokinase activity in rat cerebral cortex and cerebellum. Each point represents the mean ± S.E. of 3-4 determinations of enzyme activity. Each assay was performed on tissues pooled from the entire litter in younger age groups and from 2-3 rats in older animals. Hexokinase activity is expressed as μmoles of substrate metabolized per hr per g of tissue or per mg DNA.

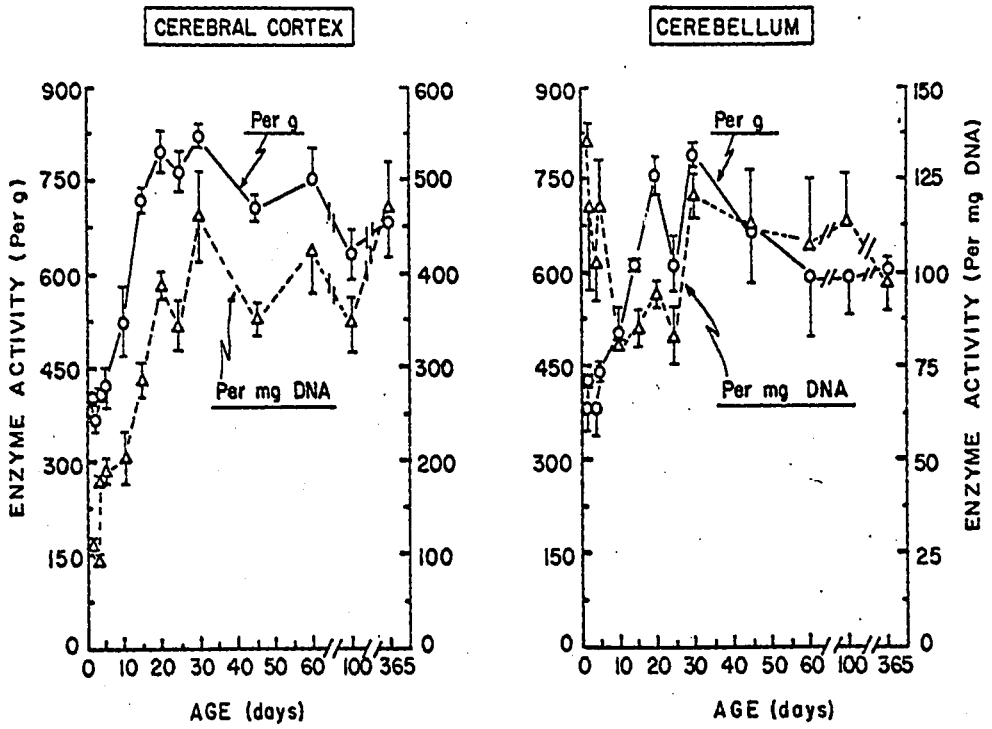

Fig. 5. Developmental changes in phosphofructokinase activity in rat cerebral cortex and cerebellum. Experimental conditions were the same as described in Fig. 4. Phosphofructokinase activity is expressed as μmoles of substrate metabolized per hr per g of tissue or per mg DNA.

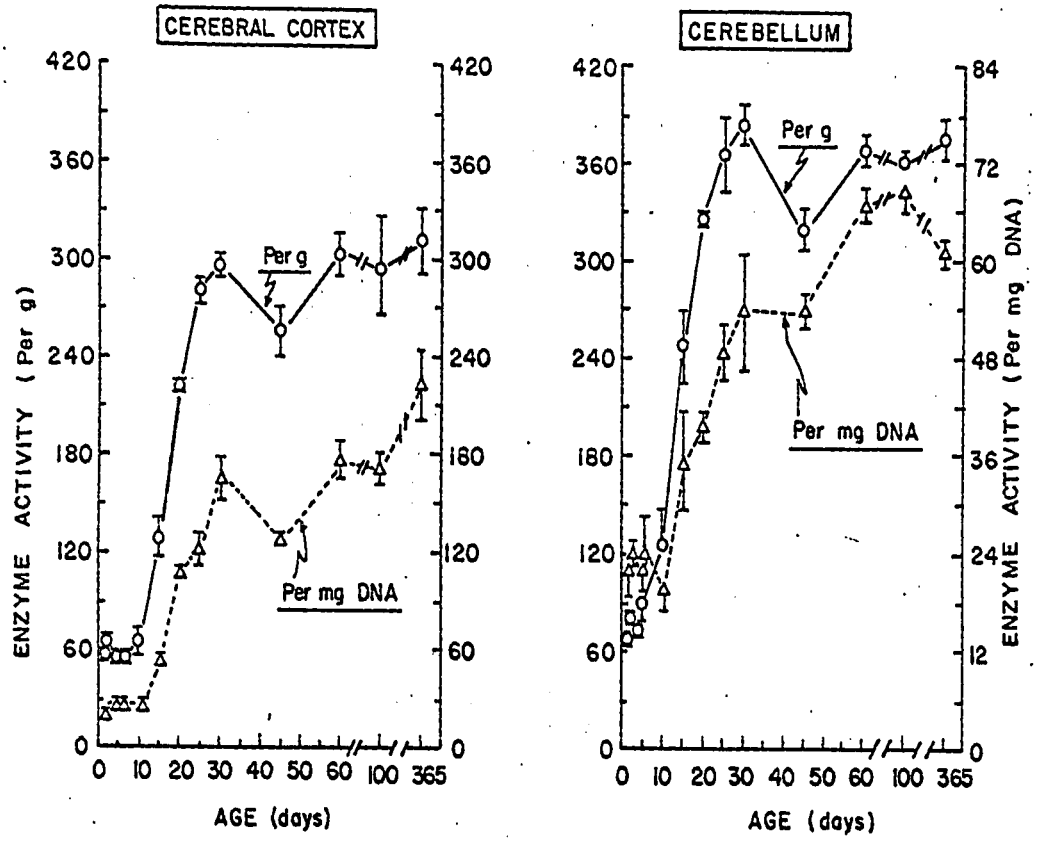

Fig. 6. Ontogeny of glyceraldehyde 3-phosphate dehydrogenase activity in rat cerebral cortex and cerebellum. Experimental conditions were the same as described in Fig. 4. Glyceraldehyde 3-phosphate dehydrogenase activity is expressed as μmoles of substrate metabolized per hr per g of tissue or per mg DNA.

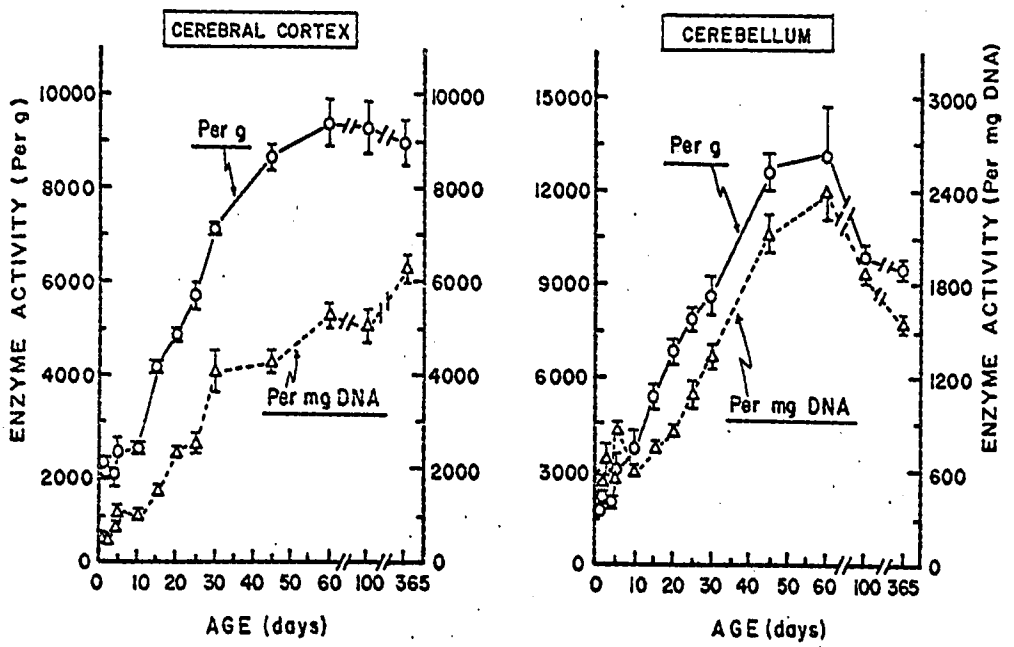

Fig. 7. Developmental changes in activity of pyruvate kinase in rat cerebral cortex and cerebellum. Experimental conditions were the same as described in Fig. 4. Pyruvate kinase activity is expressed as μmoles of substrate metabolized per hr per g of tissue or per mg DNA.

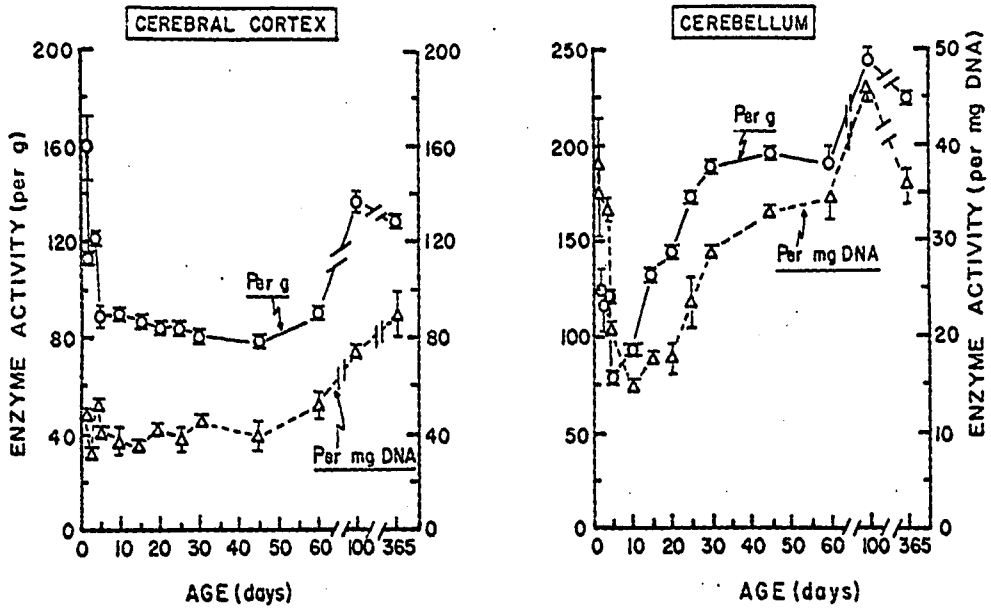

Fig. 8. Developmental pattern of glucose 6-phosphate dehydrogenase activity in rat cerebral cortex and cerebellum. Experimental conditions were the same as described in Fig. 4. Glucose 6-phosphate dehydrogenase activity is expressed as μmoles of substrate metabolized per hr per g of tissue or per mg DNA.

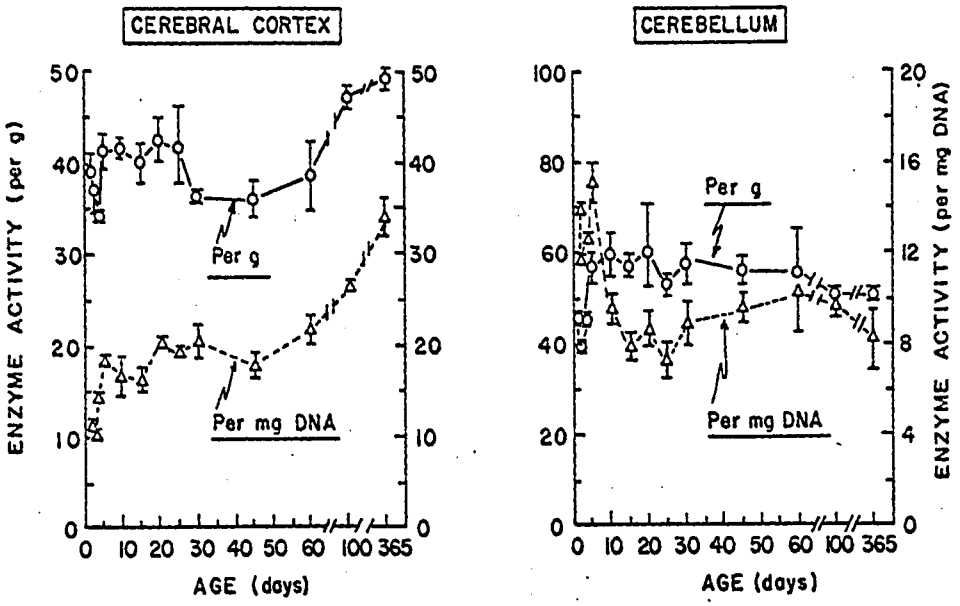

Fig. 9. Ontogeny of 6-phosphogluconate dehydrogenase activity in rat cerebral cortex and cerebellum. Experimental conditions were the same as described in Fig. 4. 6-Phosphogluconate dehydrogenase activity is expressed as μmoles of substrate metabolized per hr per g of tissue or per mg DNA.

between 1 day and 1 year of age.

Marked increases in the activity of α-GPDH in both the cerebral cortex and cerebellum were detected between 1-100 days (Fig. 10). The enzyme activity in the cerebellum decreased between 100 days and 1 year whereas relatively constant levels of α-GPDH were encountered during this period in the cerebral cortex.

The data demonstrate that most of the carbohydrate-metabolizing enzymes investigated show marked increases in the rat cerebral cortex and cerebellum during postnatal development. However, this was not a generalized phenomenon since some enzymes exhibited virtually no change in their activity from birth to adulthood.

(C) THYROID HORMONE CONTROL OF ENZYMES IN THE NERVOUS SYSTEM

The regulation of various carbohydrate-metabolizing enzymes in the central nervous system by thyroid hormone was investigated. First, the effects produced by thyroid hormone deficiency on the developmental changes in various enzymes were studied in both the cerebral cortex and cerebellum. Secondly, it was of interest to investigate what effects thyroid hormone could exert on these brain enzymes in normal as well as in hypothyroid rats. Finally, the nature of the thyroid hormone-induced increases in cerebro-cortical and cerebellar enzyme activities was investigated using a potent inhibitor of protein synthesis.

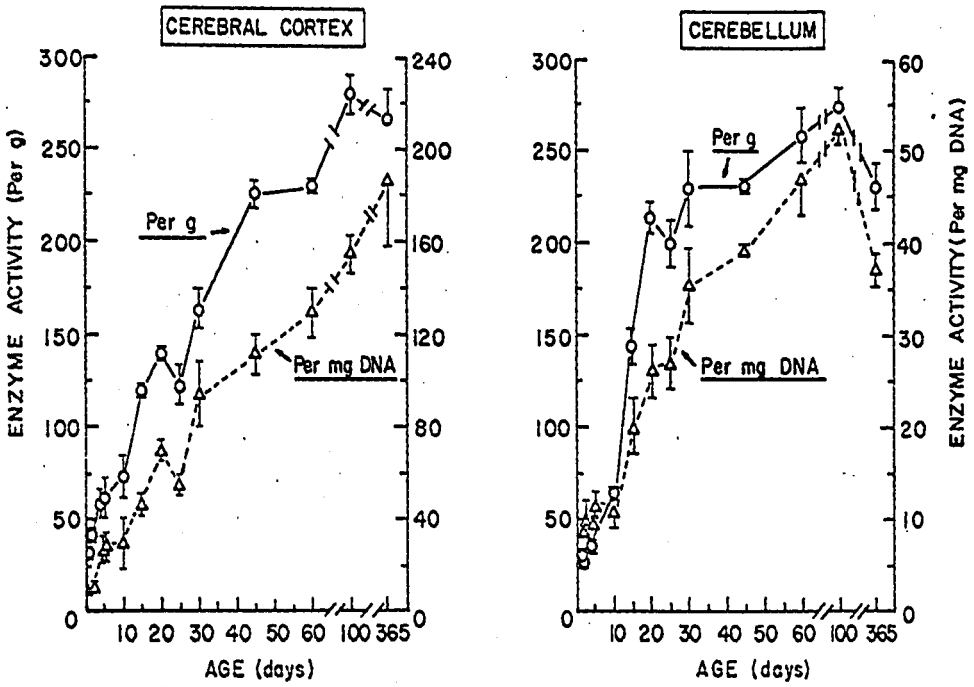

Fig. 10. Developmental changes in α-glycerophosphate dehydrogenase activity in rat cerebral cortex and cerebellum. Experimental conditions were the same as described in Fig. 4. Enzyme activity is expressed as μmoles of substrate metabolized per hr per g of tissue or per mg DNA.

(a) Influence of Thyroidectomy

(i) Effect of neonatal thyroidectomy on physical and behavioural development of young rats

Neonatal thyroidectomy, induced by a single injection of ^{131}I at 1 day of age, affected the physical maturation of young rats. In addition to producing a marked impairment of growth, neonatal thyroidectomy delayed the appearance of certain maturational changes such as eyelid dysjunction, elevation of the pinnae and snout elongation. The hair was dry and coarse and some animals exhibited a bilaterally symmetrical alopecia of the abdominal and flank regions. The demeanour of thyroidectomized animals also differed markedly from that of control littermates. The animals appeared listless and relatively immobile. There was a retardation in the development of the righting and placing reflexes in thyroidectomized rats. Some animals exhibited a peculiar high-stepping gait while others showed fine tremors of the head and limbs. A photograph comparing the appearance of a normal 45 day old rat with that of littermates treated with two different dosages of ^{131}I at 1 day of age is shown in Fig. 11. Fig. 12 is a photomicrograph demonstrating discrete destruction of the thyroid gland following treatment of a neonatal rat with 200 μc of ^{131}I.

(ii) Time course of various alterations observed following neonatal thyroidectomy

In order to investigate consequences of neonatal thyroidectomy on some parameters of maturation, groups of 1-day-old animals were

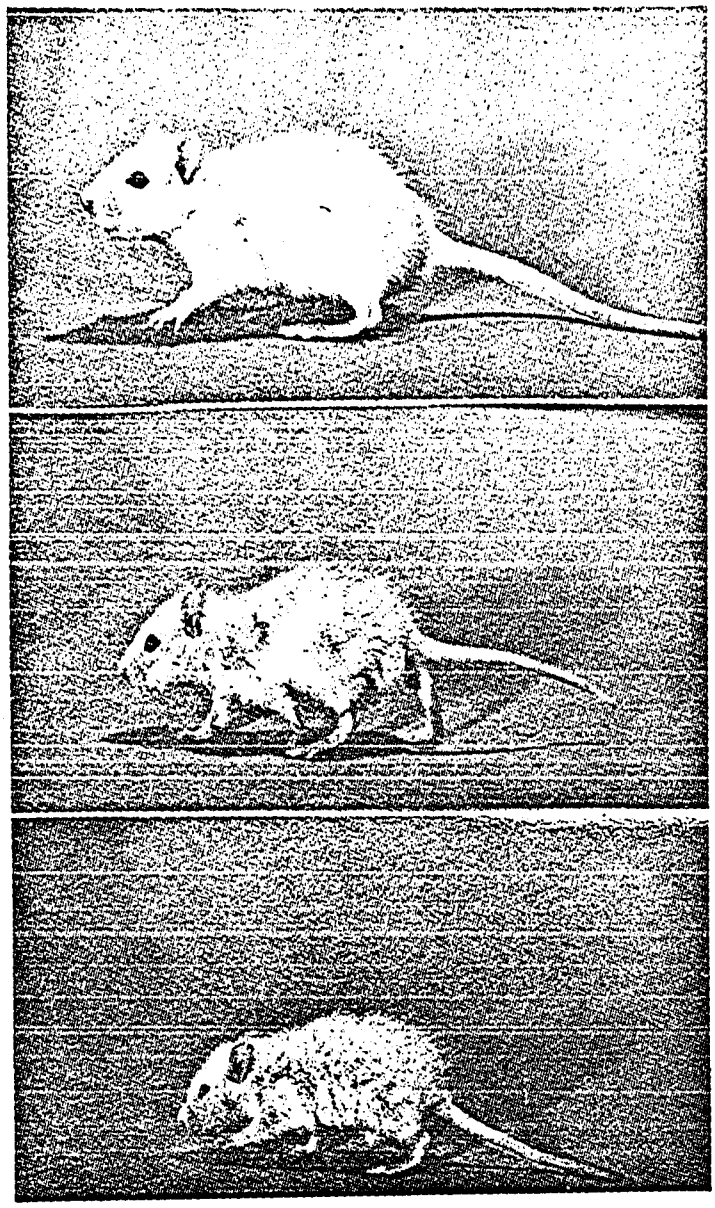

Fig. 11. Photograph comparing the appearance of a normal 45 day old rat (upper) with animals treated with ^{131}I at 1 day of age at a dose level of either 100 μc (middle) or 200 μc (lower) of the radioisotope.

Fig. 12. Photomicrograph showing a section of the thyroid region of a rat injected with 200 μc of ^{131}I at 1 day of age. Note the infiltration of connective tissue in between the remnants of the thyroid follicles (TF). The parathyroid (PT) seems to be relatively unaffected. (Hematoxylin-Phloxine B-Orange G; 10 μ; X 120). (Prepared through the courtesy of Dr. S. Jande, Department of Histology, University of Ottawa).

treated with 100 μc of ^{131}I and killed after various periods of time.

The influence of neonatal thyroidectomy on body and brain weight and DNA content in the cerebral cortex and cerebellum is shown in Fig. 13. Thyroidectomy markedly inhibited the normal increases in both body and brain weight. The inhibition was apparent at 20 days of age; neonatally thyroidectomized animals showed little or no increases in body and brain weight after this time. Fig. 13 also demonstrates that neonatal thyroidectomy produced a slight increase in DNA concentration in both the cerebral cortex and cerebellum.

The effect of neonatal thyroidectomy on the developmental changes in cerebro-cortical and cerebellar enzymes of carbohydrate metabolism is shown in Figs. 14-16. Activities of the 3 key glycolytic enzymes were significantly inhibited in both brain regions although this inhibition was comparatively less marked in the case of cerebro-cortical pyruvate kinase (Fig. 14). Marked inhibition of the developmental increases in α-GPDH activity was also observed in the cerebral cortex and cerebellum; at 60 days of age the activity of this enzyme was inhibited by more than 50% in both brain regions (Fig. 15). Fig. 15 also demonstrates that inhibition of G-3PDH in the cortex and cerebellum became apparent at 20 days of age. Although only slight decreases in the activities of the pentose phosphate shunt enzymes were observed in the cerebral cortex of thyroidectomized rats, pronounced inhibition of both enzymes was observed in the cerebellum (Fig. 16).

(iii) Influence of various dosages of ^{131}I

Since treatment of neonatal animals with 100 μc of ^{131}I

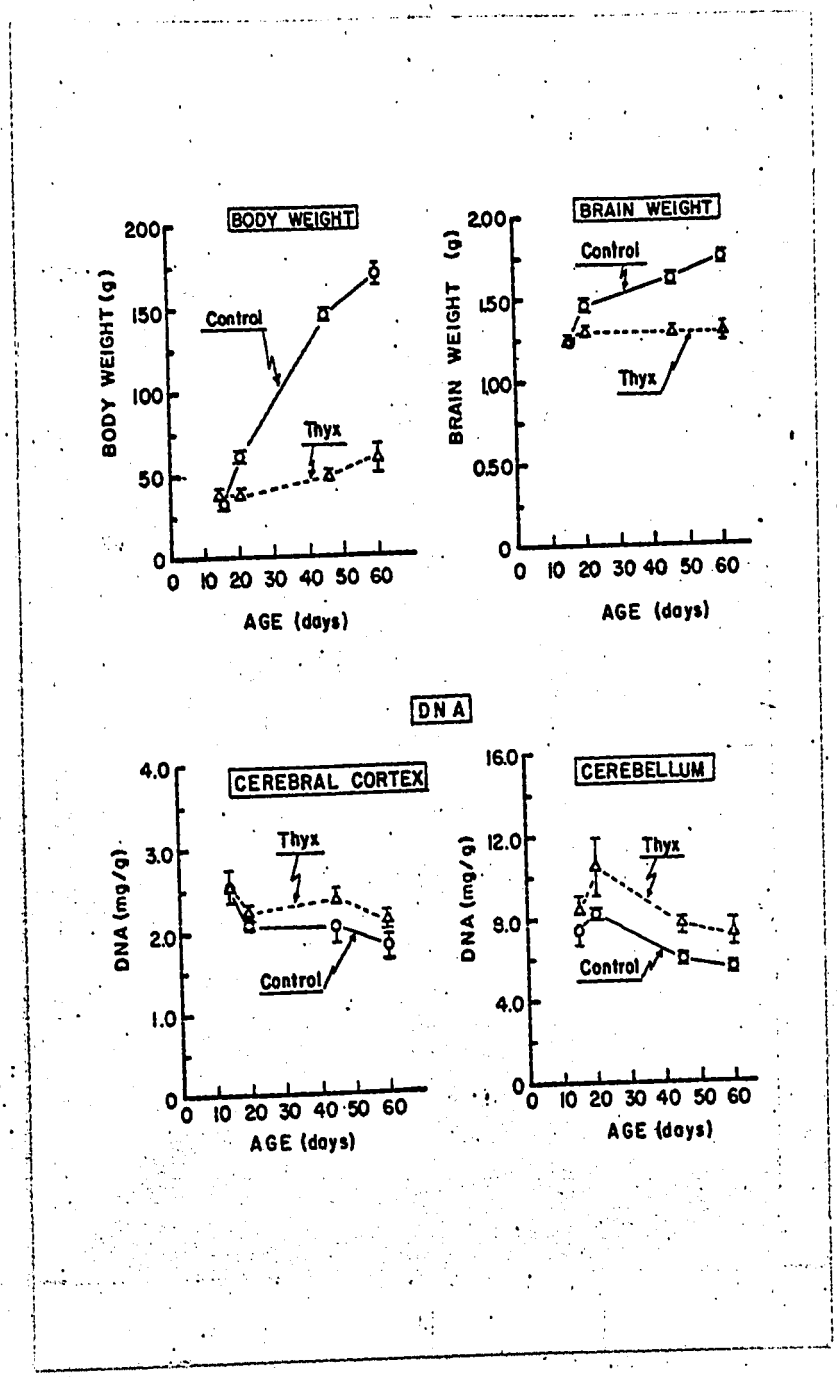

Fig. 13. Influence of neonatal thyroidectomy on body and brain weight and DNA concentration in cerebral cortex and cerebellum. Points represent means ± S.E. of three values in each group. One day old rats were treated intraperitoneally with 100 μc ^{131}I and killed after 15, 20, 45 or 60 days. THYX=thyroidectomized.

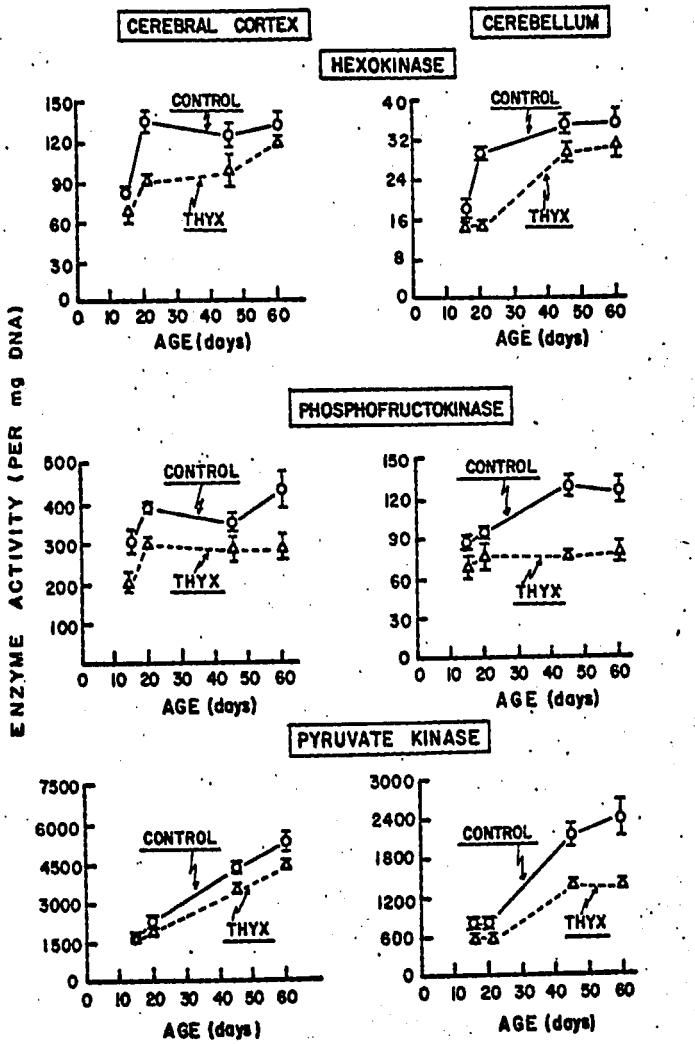

Fig. 14. Effect of neonatal thyroidectomy on the activities of key glycolytic enzymes in the cerebral cortex and cerebellum. Each point represents the mean ± S.E. based on three determinations of enzyme activity in tissues pooled from 2-3 rats. One day old animals were injected intraperitoneally with 100 μc ^{131}I and killed after 15, 20, 45 and 60 days. Enzyme activities are expressed per mg DNA. THYX= thyroidectomized.

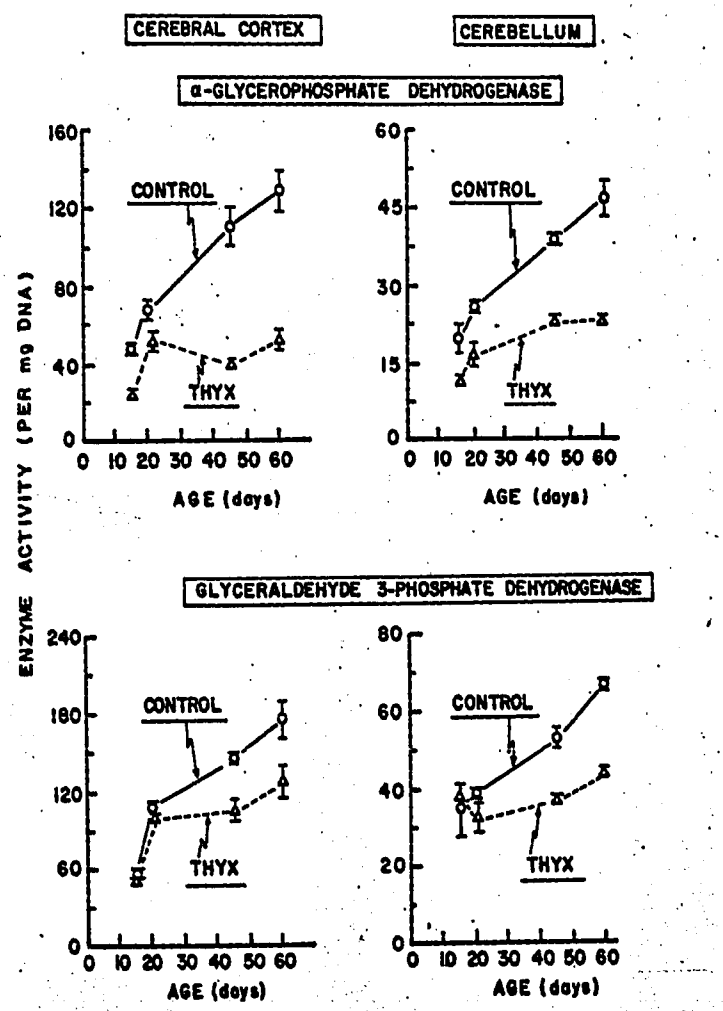

Fig. 15. Influence of neonatal thyroidectomy on α-glycerophosphate and glyceraldehyde 3-phosphate dehydrogenase activity in the cerebral cortex and cerebellum. Experimental conditions were the same as described in Fig. 14. Enzyme activities are expressed per mg DNA. THYX=thyroidectomized.

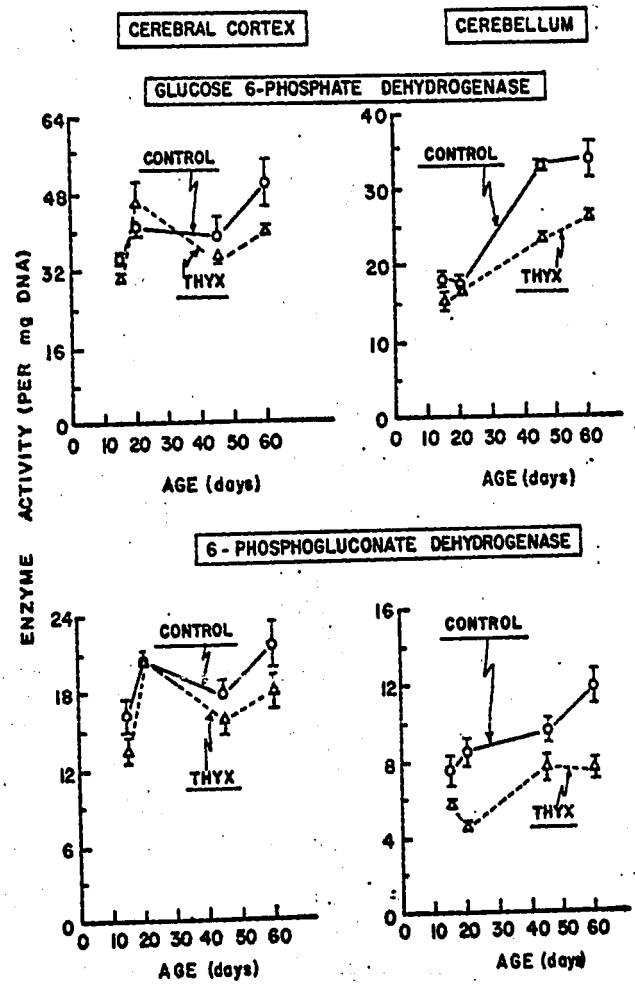

Fig. 16. Effect of neonatal thyroidectomy on the activities of pentose phosphate shunt enzymes in the cerebral cortex and cerebellum. Experimental conditions were the same as described in Fig. 14. Enzyme activities are expressed per mg DNA. THYX=thyroidectomized.

significantly affected the maturational changes in various brain enzymes, it was of interest to examine the effects of a higher and a lower dose of the radioisotope. Groups of 1 day old rats were therefore treated with 50, 100 or 200 μc of ^{131}I and killed after 30 days.

The results presented in Table III demonstrate that ^{131}I produced a dose-dependent inhibition of the increases in both body and brain weight. Conversely, a progressive increase in the DNA concentration of the cerebral cortex and cerebellum was observed as the dosage of the radioisotope was increased.

The effects of different dosages of ^{131}I on various carbohydrate-metabolizing enzymes in the cerebral cortex and cerebellum are shown in Tables IV and V. In all cases, the 50 μc dose of ^{131}I had little or no effect on the enzyme activity. Increasing the dosage of the radioisotope to 100 μc resulted in a significant inhibition of all 4 of the glycolytic enzymes in the cerebral cortex and of HK, PK, α-GPDH, G-6PDH and 6-PGDH in the cerebellum. Treatment of neonatal rats with 200 μc ^{131}I resulted in a marked inhibition of all enzymes investigated in the cerebral cortex and cerebellum. The results indicate that treatment of neonatal rats with ^{131}I produces a dose-dependent inhibition of carbohydrate-metabolizing enzymes in the developing brain.

(iv) Effect of delayed thyroidectomy

Since the above studies indicated that thyroidectomy produced by the administration of ^{131}I at 1 day of age interfered with the normal maturational events of the central nervous system, the influence

TABLE III

EFFECT OF VARYING DOSAGES OF ^{131}I ON BODY AND BRAIN WEIGHT AND DNA CONCENTRATION IN THE CEREBRAL CORTEX AND CEREBELLUM

Means ± S.E. represent three values in each group. Animals were treated with varying dosages of ^{131}I at 1 day of age and sacrificed after 30 days. Data are also given in percentages (in parentheses) taking the values of control animals as 100%.

DOSAGE OF ^{131}I (μc/rat)	BODY WT. (g)	BRAIN WT. (g)	DNA CONTENT (mg/g)	
			Cerebral cortex	Cerebellum
CONTROL	89±6 (100)	1.57±0.05 (100)	1.49±0.11 (100)	6.65±0.13 (100)
50	86±8 (97)	1.59±0.02 (101)	1.73±0.08 (116)	6.79±0.06 (102)
100	50±4 (56)*	1.41±0.03 (90)*	1.97±0.05 (132)	8.76±0.40 (132)*
200	32±6 (36)*	1.25±0.03 (80)*	2.19±0.03 (147)*	10.60±0.15 (159)*

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

TABLE IV

EFFECT OF VARYING DOSAGES OF ^{131}I ON ENZYME ACTIVITIES IN THE CEREBRAL CORTEX

Each value represents the mean ± S.E. based on three determinations of enzyme activity in tissues pooled from 1-3 rats. Animals were treated with varying dosages of ^{131}I at 1 day of age and killed after 30 days. Enzyme activities are expressed per mg DNA. Values in parentheses indicate the percentage of control which is taken as 100%.

DOSAGE OF ^{131}I (μc/rat)	HK	PFK	G-3PDH	PK	α-GPDH	G-6PDH	6-PGDH
CONTROL	186±11 (100)	426±32 (100)	214±8 (100)	4570±202 (100)	109±12 (100)	53.9±0.2 (100)	30.9±1.2 (100)
50	169±25 (91)	364±27 (85)	198±18 (93)	4279±218 (94)	97±8 (89)	46.0±3.5 (85)	32.3±1.2 (105)
100	113±8 (61)*	212±1 (50)*	144±10 (67)*	3522±37 (77)*	51±8 (47)	46.1±9.3 (86)	23.4±3.6 (76)
200	117±3 (63)*	148±22 (35)*	142±12 (66)*	2910±65 (64)*	43±5 (39)*	33.1±4.7 (61)*	21.3±1.0 (69)*

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

TABLE V

EFFECT OF VARYING DOSAGES OF ^{131}I ON ENZYME ACTIVITIES IN THE CEREBELLUM

Each value represents the mean ± S.E. based on three determinations of enzyme activity in tissues pooled from 1-3 rats. Animals were treated with varying dosages of ^{131}I at 1 day of age and killed after 30 days. Enzyme activities are expressed per mg DNA. Values in parentheses indicate the percentage of control which is taken as 100%.

DOSAGE OF ^{131}I (μc/rat)	HK	PFK	G-3PDH	PK	α-GPDH	G-6PDH	6-PGDH
CONTROL	34.7±1.4 (100)	109.5±15.0 (100)	63.4±3.4 (100)	1493±49 (100)	36.5±1.0 (100)	26.1±0.4 (100)	10.53±0.42 (100)
50	36.0±1.2 (104)	79.5±6.4 (73)	57.8±2.0 (91)	1397±32 (94)	32.9±1.0 (90)	25.9±0.4 (99)	10.06±0.59 (96)
100	21.3±2.5 (61)*	66.8±5.2 (61)	46.2±4.6 (73)	976±42 (65)*	23.0±1.5 (63)*	19.6±0.8 (75)*	6.89±0.58 (65)*
200	20.6±1.2 (59)*	46.0±6.4 (42)	37.3±2.1 (59)*	698±32 (47)*	15.8±2.5 (43)*	14.0±1.5 (54)*	5.33±0.52 (51)*

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

of delaying thyroidectomy for varying periods of time after birth was investigated. Groups of animals were treated with 200 μc of ^{131}I at various ages and killed after 30 days.

The data in Fig. 17 demonstrate that when ^{131}I was administered at 1 day of age, marked inhibition of the increases in body and brain weight was produced. However, when administration of the radioisotope was delayed for 5, 10 or 20 days after birth, progressively less interference with body and brain growth was observed. Fig. 17 also demonstrates that more marked increases in the DNA concentration in the cerebral cortex and cerebellum resulted when the animals were thyroidectomized in early neonatal life.

The effect of delayed thyroidectomy on enzyme activities in the cortex and cerebellum is demonstrated in Figs. 18-20. A similar pattern was observed for all enzymes investigated since the inhibition was more pronounced when ^{131}I was administered in early postnatal life. Progressively less inhibition of enzyme activity resulted when administration of the radioisotope was delayed for prolonged periods of time after birth. When ^{131}I was injected at 20 days of age, little or no inhibition of enzyme activity was observed in either brain region. These results indicate that the age at which hypothyroidism is induced is important in determining the degree of alteration in carbohydrate-metabolizing enzymes in the developing cerebral cortex and cerebellum.

(b) Effect of L-Triiodothyronine (T_3) in Normal Neonatal Animals

Since thyroid hormone deficiency resulted in marked impairment

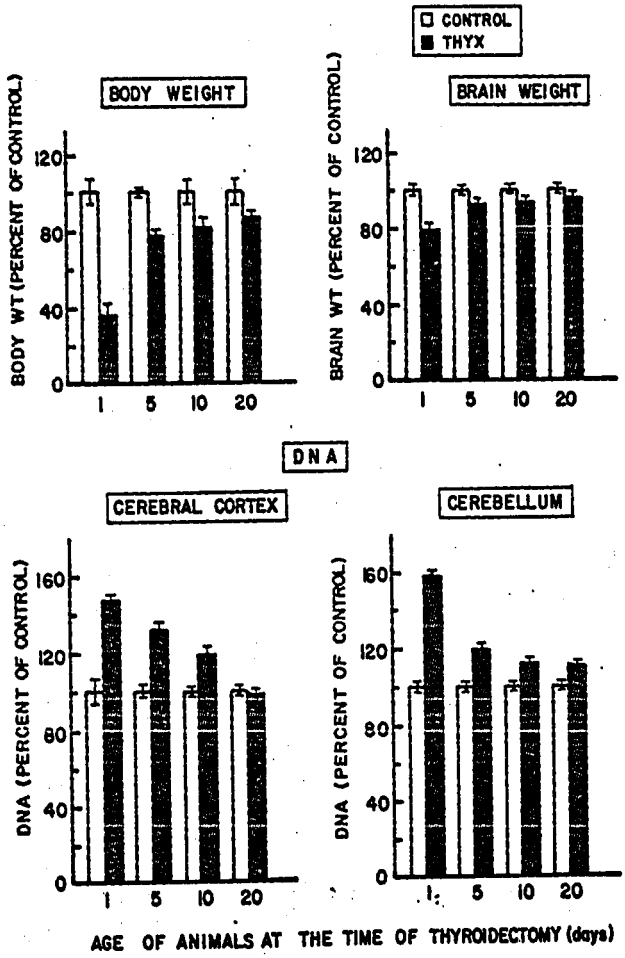

Fig. 17. Influence of delayed thyroidectomy on body and brain weight and DNA concentration in the cerebral cortex and cerebellum. Bars represent the means ± S.E. of three values in each group. Animals were injected intraperitoneally with 200 μc ^{131}I at 1, 5, 10 or 20 days of age and killed at 30 days. Data are given in percentages taking the values of control animals as 100%. THYX=thyroidectomized.

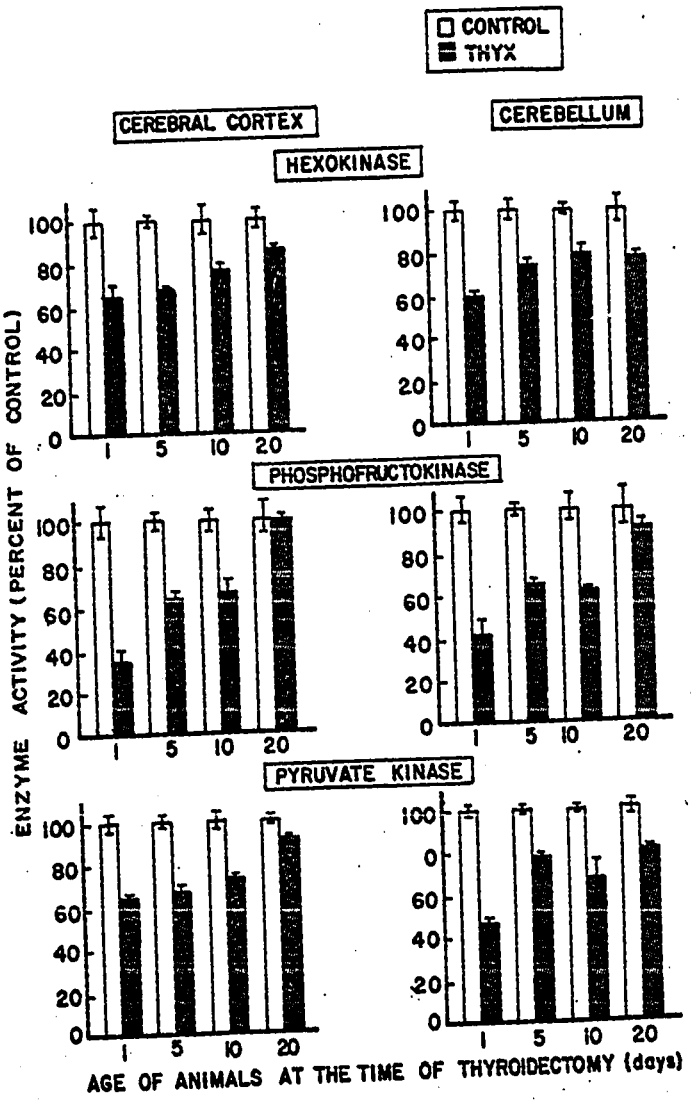

Fig. 18. Effect of delayed thyroidectomy on the activities of key glycolytic enzymes in the cerebral cortex and cerebellum. Bars represent the means ± S.E. of three determinations of enzyme activity. Each assay was performed using tissues pooled from 2-3 rats. Animals were injected intraperitoneally with 200 μc ^{131}I at 1, 5, 10 or 20 days of age and killed at 30 days. Enzyme activities were calculated per mg DNA and are given in percentages taking the values of control animals as 100%. THYX=thyroidectomized.

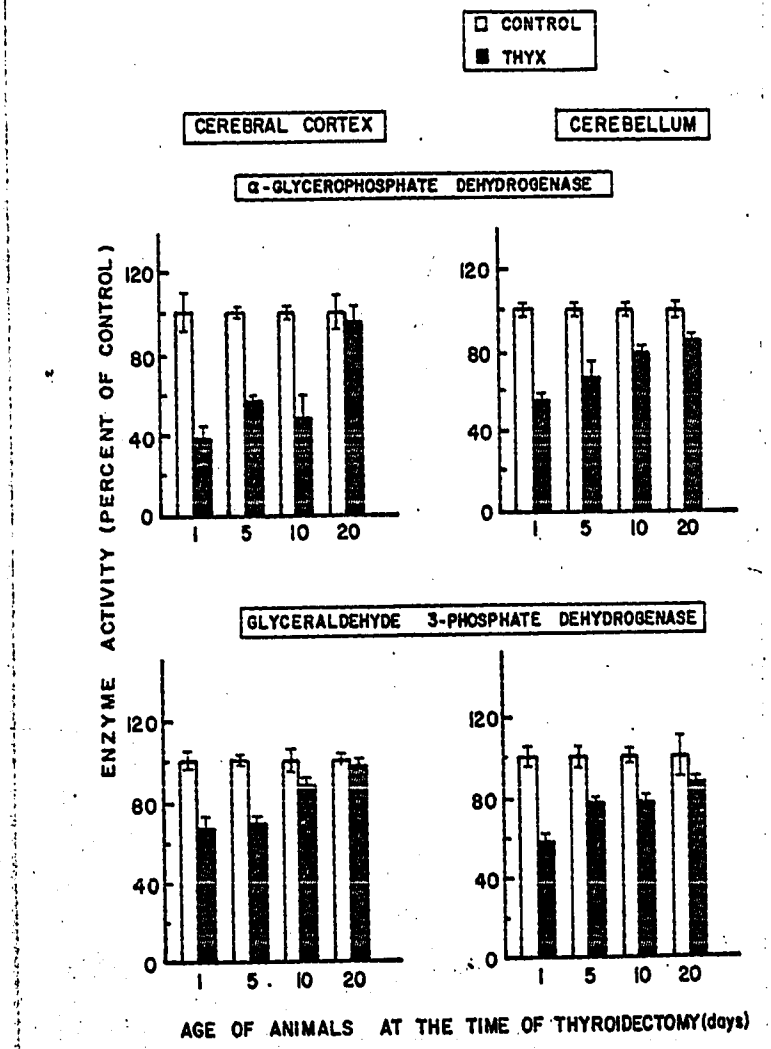

Fig. 19. Influence of delayed thyroidectomy on the activities of α-glycerophosphate and glyceraldehyde 3-phosphate dehydrogenase in the cerebral cortex and cerebellum. Experimental conditions were the same as described in Fig. 18. Enzyme activities were calculated per mg DNA and are expressed as percentage of the control which is taken as 100%. THYX=thyroidectomized.

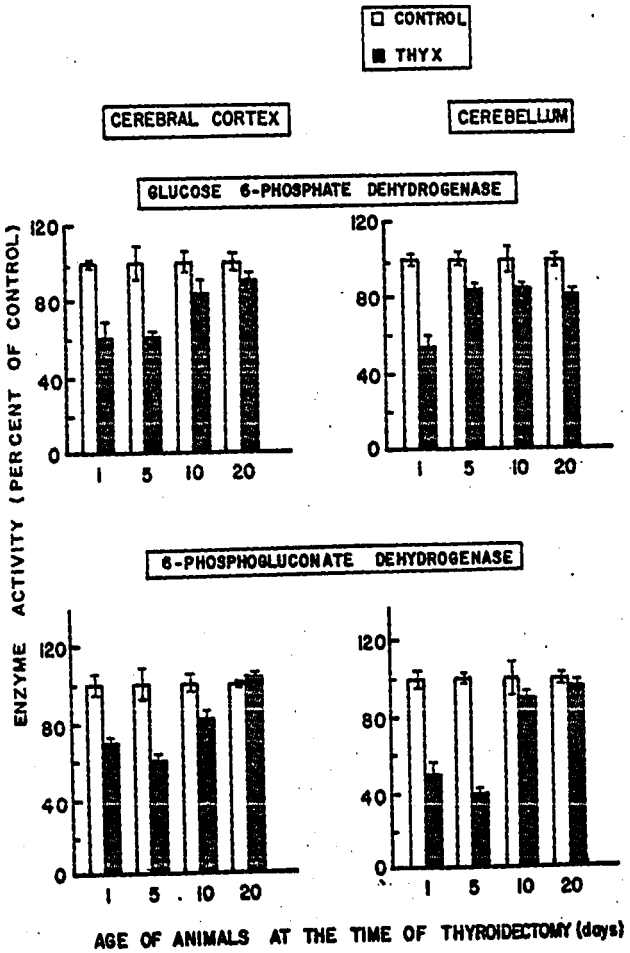

Fig. 20. Effect of delayed thyroidectomy on the activities of pentose phosphate pathway enzymes in the cerebral cortex and cerebellum. Experimental conditions were the same as described in Fig. 18. Enzyme activities were calculated per mg DNA and are expressed in percentages taking the values of control animals as 100%. THYX=thyroidectomized.

of certain maturational processes, it was of interest to examine the influence of thyroid hormone excess on normal neonatal animals. Groups of newborn rats were treated daily with L-triiodothyronine (T_3) at a dose of 25 µg/100 g/day for a period of 5, 10 or 15 days.

(i) Effects of physical and behavioural development

Administration of T_3 to normal neonatal animals caused an accelerated development of certain physical and behavioural characteristics which accompany maturation of young rats. Triiodothyronine treatment led to an increased rate of eye opening, pinnae elevation and snout elongation as compared to the littermate controls. After 10 days of treatment with T_3, the animals had the general appearance of a miniature adult. The acquisition of the placing and righting reflexes was accelerated. In addition, the animals developed co-ordinated movements and became mobile at an earlier age.

(ii) Influence on enzyme activity in the central nervous system

The results presented in Fig. 21 demonstrate that administration of T_3 to normal neonatal rats for 15 days produced no significant effect on the activities of the 3 key glycolytic enzymes in the cerebral cortex or cerebellum. In contrast, T_3 treatment resulted in a significant rise in α-GPDH activity in the cerebellum although no effect was observed on the cerebro-cortical enzyme (Fig. 22). Fig. 22 also demonstrates that T_3, when administered for 15 days, produced significant increases in G-3PDH activity in the cortex and cerebellum. Treatment

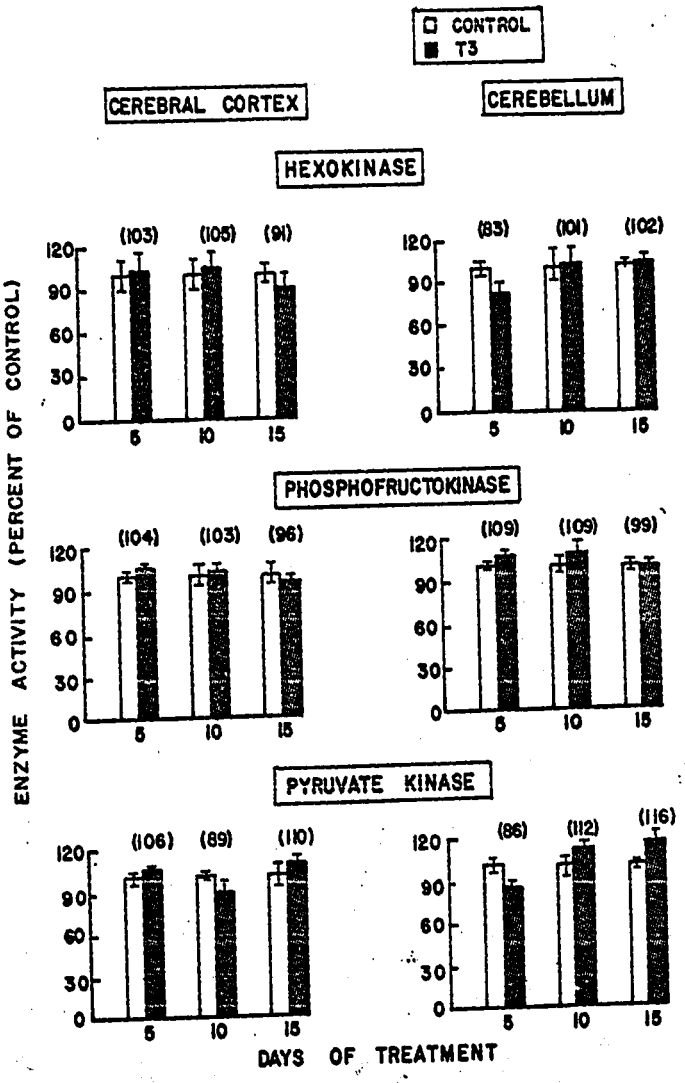

Fig. 21. Effect of T_3 treatment on activities of key glycolytic enzymes in the cerebral cortex and cerebellum of normal rats. Bars represent the means ± S.E. of three determinations of enzyme activity. Each assay was carried out in tissues pooled from 3-5 rats. Animals were given daily subcutaneous injections of T_3 (25 µg/100 g) for 5, 10 or 15 days starting on the day of birth and killed 24 hr after the last injection. Enzyme activities were calculated per mg DNA and are expressed in percentages taking the values of control animals as 100%. T_3=L-triiodothyronine.

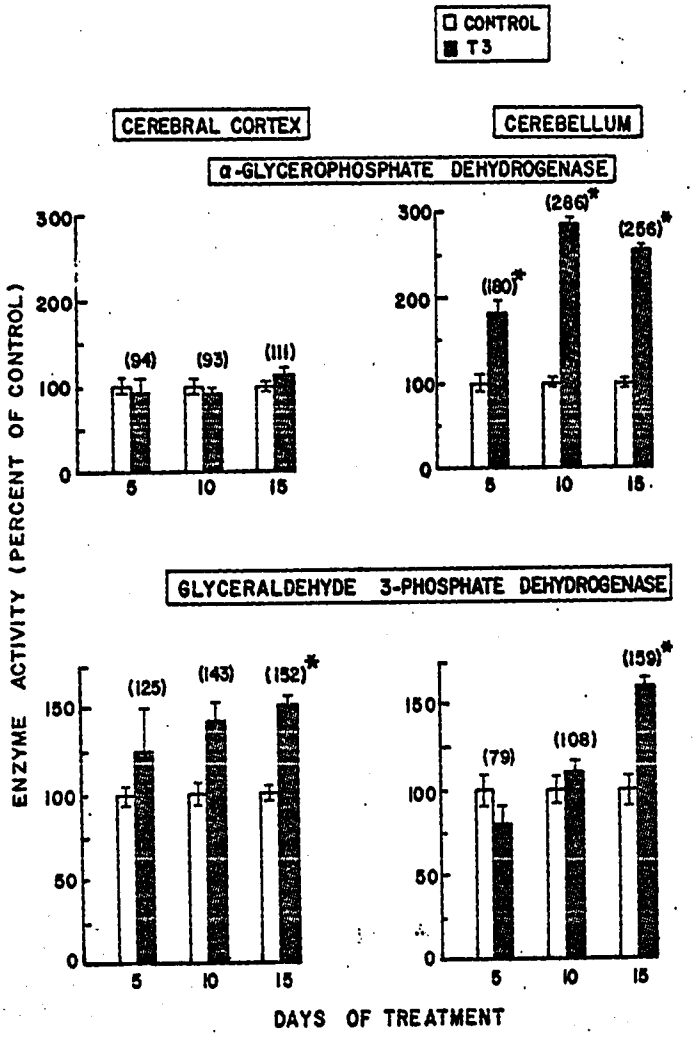

Fig. 22. Influence of T_3 treatment on the activities of α-glycerophosphate and glyceraldehyde 3-phosphate dehydrogenase in the cerebral cortex and cerebellum of normal animals. Experimental conditions were the same as described in Fig. 21. Enzyme activities were calculated per mg DNA and are expressed in percentages taking the values of control rats as 100%. The asterisks denote statistically significant alterations as compared with the values of control rats ($p = <0.05$). T_3=L-triiodothyronine.

with T_3 resulted in only slight changes in the activities of the pentose phosphate shunt enzymes in both regions of the central nervous system (Fig. 23). The data indicate that administration of thyroid hormone to normal neonatal animals exerts little or no effect on the activities of the majority of the enzymes investigated in the cerebral cortex and cerebellum.

(c) T_3 Treatment of Neonatally Thyroidectomized Animals

The influence of thyroid hormone on enzyme activity in the cerebral cortex and cerebellum was also investigated by examining the effects of T_3 administration to neonatally thyroidectomized rats.

(i) Time-course of T_3 effects

The results in Fig. 24 demonstrate the effects of T_3 treatment (10 µg/100 g/day) for various periods of time on body and brain weight in neonatally thyroidectomized animals. Both body and brain weight increased progressively as the duration of T_3 administration was increased. In contrast, the concentration of DNA in the cerebral cortex and cerebellum steadily decreased with the duration of hormone treatment.

Figs. 25-27 demonstrate that administration of T_3 to neonatally thyroidectomized rats produced significant increases in the activities of various carbohydrate-metabolizing enzymes in the cerebral cortex and cerebellum. Whereas the enzyme activities increased only slightly after 10 days of T_3 treatment, administration of the hormone for 25 days resulted in significant increases in all enzymes investigated in

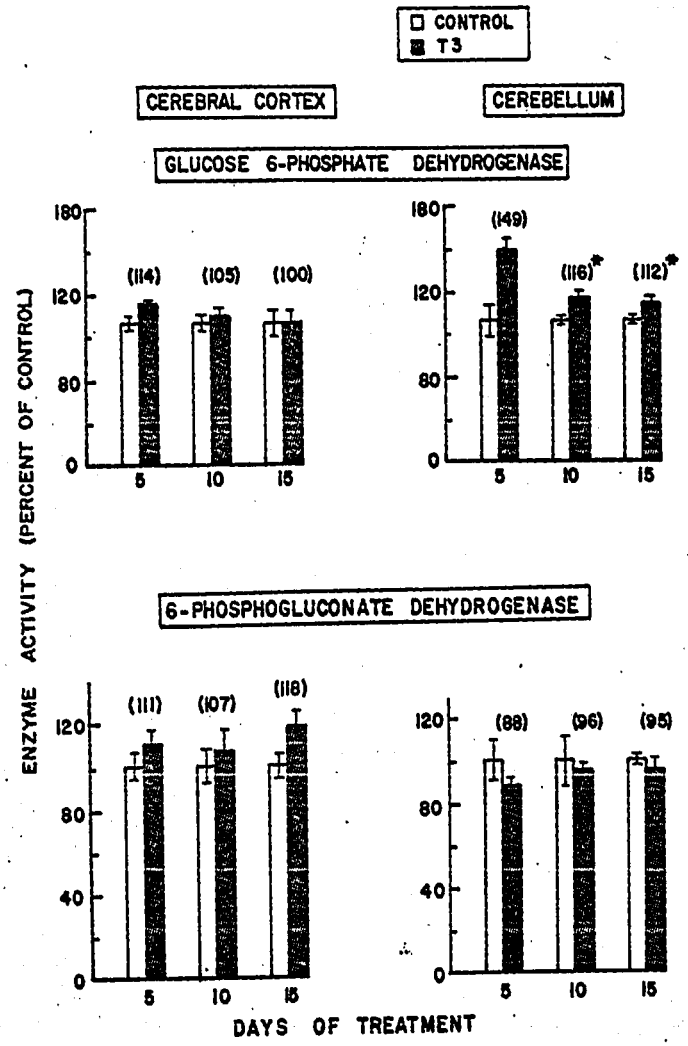

Fig. 23. Influence of T_3 treatment on activities of pentose phosphate shunt enzymes in the cerebral cortex and cerebellum of normal animals. Experimental conditions were the same as described in Fig. 21. Enzyme activities were calculated per mg DNA and expressed in percentages taking the values of control rats as 100%. Asterisks denote statistically significant differences from the values of control animals ($p = <0.05$). T_3=L-triiodothyronine.

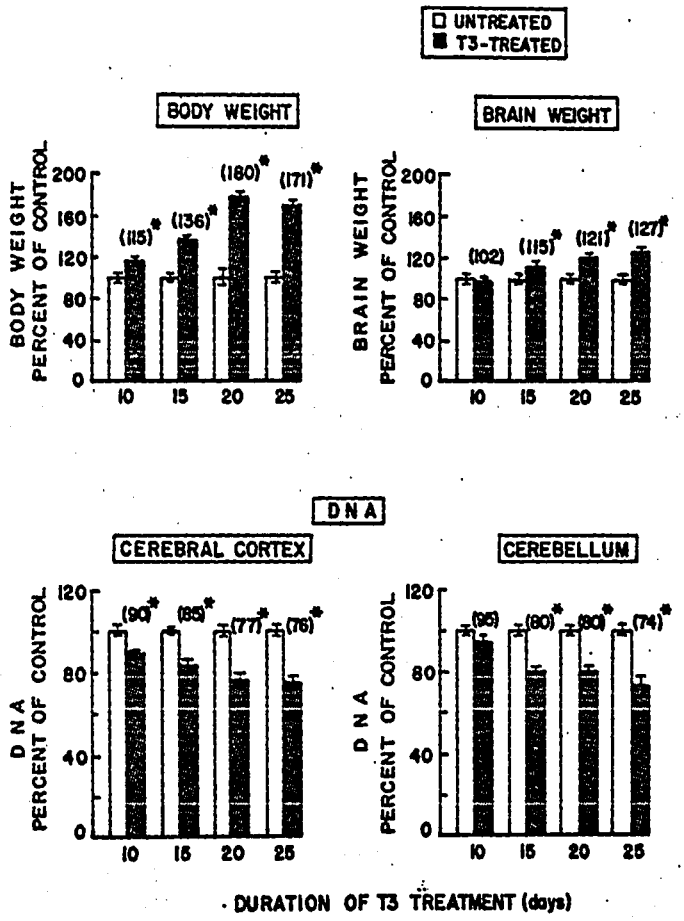

Fig. 24. Influence of T_3 treatment on body and brain weight and DNA concentration in the cerebral cortex and cerebellum of neonatally thyroidectomized rats. Bars represent the means ± S.E. of three values in each group. Thyroidectomized rats were treated daily with T_3 (10 µg/100 g) for 10, 15, 20 or 25 days and killed 24 hr after the last injection of the hormone. Data are expressed in percentages taking the values of control animals as 100%. The asterisks denote statistically significant differences when compared with the control values ($p = <0.05$). T_3=L-triiodothyronine.

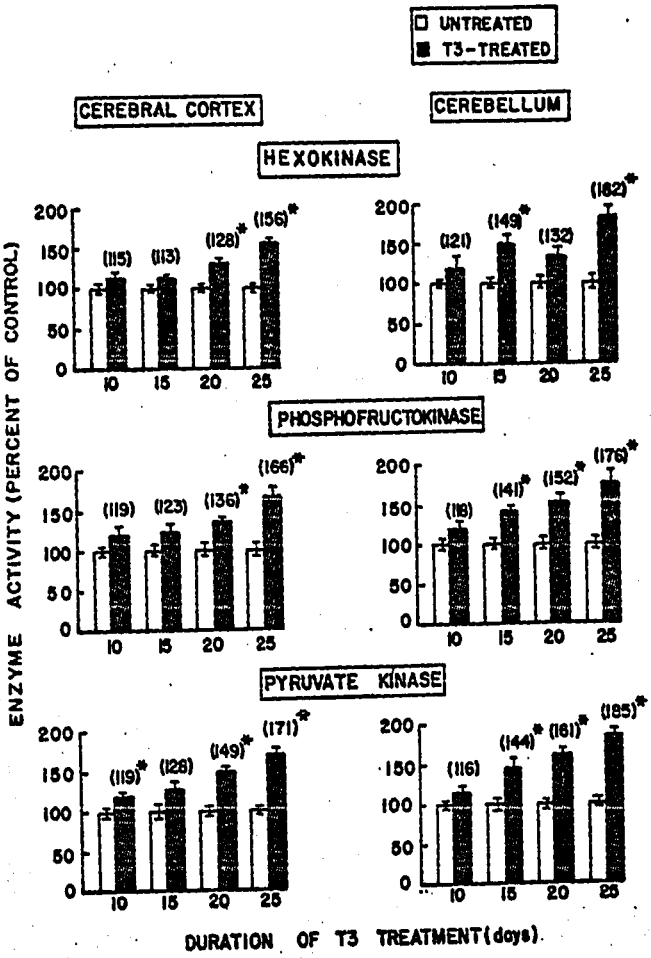

Fig. 25. Effect of T_3 treatment on the three key glycolytic enzymes in the cerebral cortex and cerebellum of neonatally thyroidectomized animals. Bars represent the means ± S.E. of three determinations of enzyme activity in tissues pooled from 2-3 rats. Thyroidectomized rats were given daily injections of T_3 (10 µg/100 g) for 10, 15, 20 or 25 days and killed 24 hr after the last injection. Enzyme activities were calculated per mg DNA and are given in percentages taking the values of control animals as 100%. The asterisks denote statistically significant alterations when compared with the values of control rats ($p = <0.05$). T_3=L-triiodothyronine.

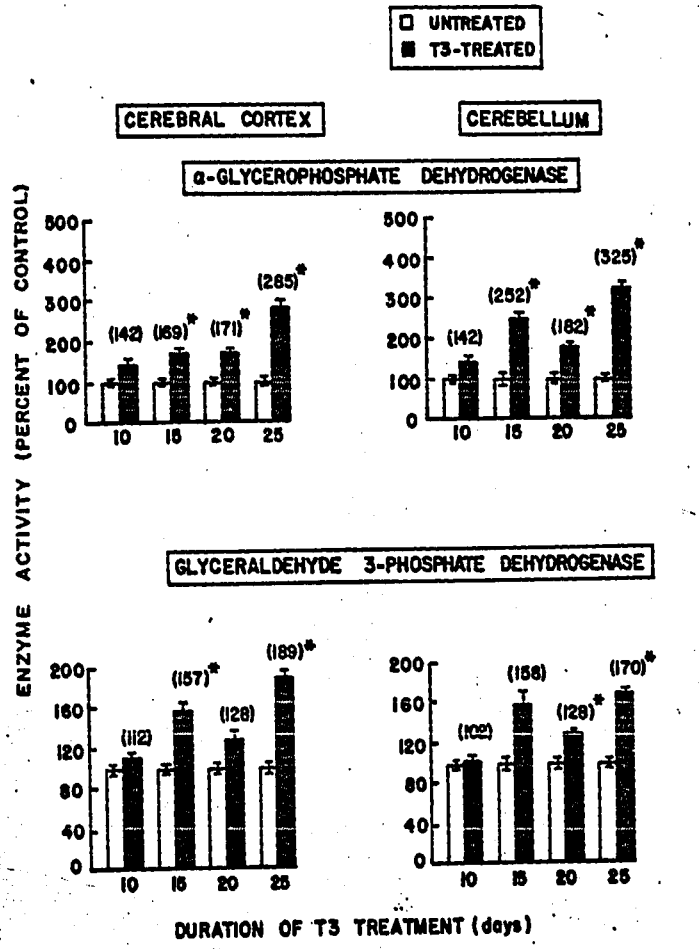

Fig. 26. Influence of T_3 treatment on the activities of α-glycerophosphate and glyceraldehyde 3-phosphate dehydrogenase in the cerebral cortex and cerebellum of neonatally thyroidectomized rats. Experimental conditions were the same as described in Fig. 25. Enzyme activities were calculated per mg DNA and are expressed as percentage of control which is taken as 100%. The asterisks denote statistically significant differences compared with the values of control animals ($p = <0.05$). T_3=L-triiodothyronine.

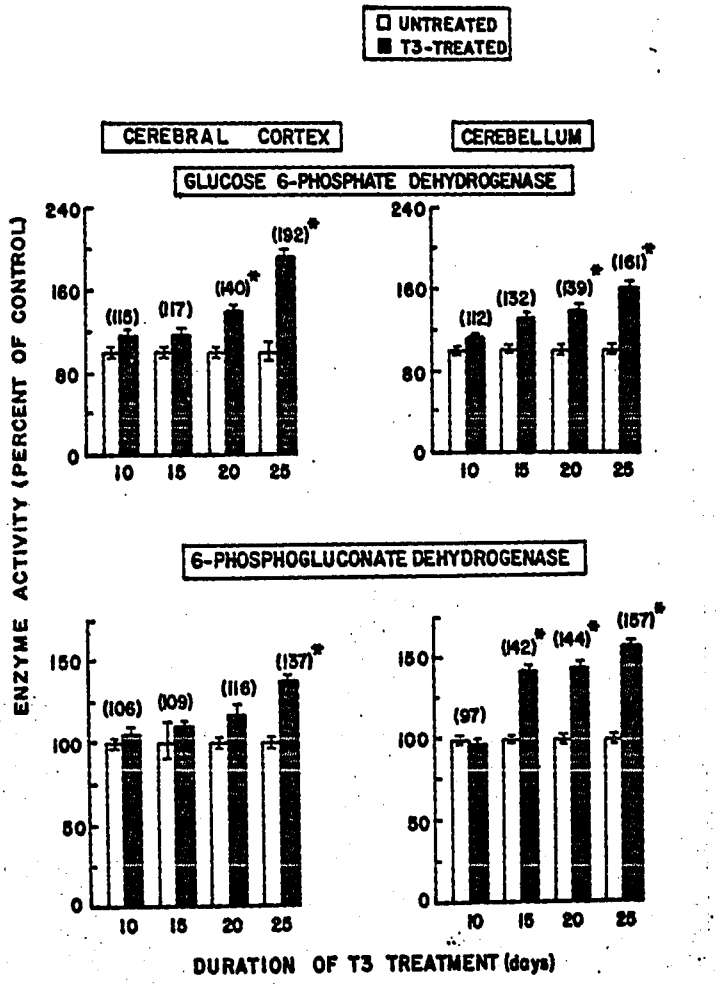

Fig. 27. Effect of T_3 treatment on the activities of pentose phosphate pathway enzymes in the cerebral cortex and cerebellum of neonatally thyroidectomized rats. Experimental conditions were the same as described in Fig. 25. Enzyme activities were calculated per mg DNA and are expressed as percentages of control values which are taken as 100%. The asterisks denote statistically significant alterations when compared with the values of control animals ($p = <0.05$). T_3=L-triiodothyronine.

the cerebral cortex and cerebellum. The two pentose phosphate pathway enzymes exhibited similar increases in the cerebellum. In contrast, a more pronounced increase in the activity of G-6PDH in cerebral cortex was obtained than that observed for 6-PGDH (Fig. 27).

(ii) Dose response studies with T_3

Since prolonged treatment of neonatally thyroidectomized animals with T_3 led to significant increases in enzymes of the central nervous system, the effect of higher dosages of the hormone for shorter periods of time was investigated. Groups of rats were injected for 3 days with T_3 at 2 different doses (25 or 250 µg/100 g/day) and sacrificed on the 4th day.

The data in Table VI demonstrate that administration of 25 µg dose of T_3 for 3 days produced an increase in all cerebro-cortical enzymes investigated although the increase was statistically significant only in the case of HK. In contrast, the higher dosage of the hormone (250 µg/100 g) resulted in a significant increase in the activity of all enzymes studied with the exception of pyruvate kinase. The concentration of DNA in the cerebral cortex remained unaffected by hormone treatment.

The effects of the administration of T_3 on various enzymes and DNA in the cerebellum are shown in Table VII. Whereas the lower dosage of the hormone produced significant increases in only PK and 6-PGDH, the higher level of T_3 increased significantly the activity of all enzymes investigated except that of PFK. The table also shows

TABLE VI

INFLUENCE OF T_3 ON ENZYME ACTIVITIES AND DNA CONCENTRATION IN THE CEREBRAL CORTEX OF NEONATALLY THYROIDECTOMIZED RATS

Each value represents the mean ± S.E. based on three determinations of enzyme activity and DNA concentration in tissues pooled from 1-3 rats. Starting at 27 days of age, neonatally thyroidectomized animals were injected subcutaneously with varying doses of T_3 daily for 3 days and killed 24 hr after the last injection. DNA concentration is expressed as mg/g tissue. Enzyme activities are calculated on a per mg DNA basis. Values in parentheses indicate the percentage of control which is taken as 100%.

DOSAGE OF T_3 (μg/100 g/day)	HK	PFK	G-3PDH	PK	α-GPDH	G-6PDH	6-PGDH	DNA
CONTROL	123±3 (100)	182±7 (100)	120±7 (100)	3331±150 (100)	48.6±6.1 (100)	34.7±3.4 (100)	21.7±1.5 (100)	1.99±0.04 (100)
25	156±7 (127)*	226±12 (124)	169±9 (141)	39.6±163 (118)	63.4±4.3 (130)	40.3±1.5 (116)	29.1±2.0 (134)	1.69±0.08 (85)
250	198±10 (161)*	276±14 (152)*	191±10 (159)*	4137±182 (124)	88.0±0.9 (181)*	53.1±1.7 (153)*	31.9±0.6 (147)*	1.82±0.04 (91)

*Statistically significant difference as compared to the control values ($p = <0.05$).

TABLE VII

EFFECT OF T_3 ON ENZYME ACTIVITIES AND DNA CONTENT IN THE CEREBELLUM OF NEONATALLY THYROIDECTOMIZED RATS

Each value represents the mean ± S.E. based on three determinations of enzyme activity and DNA content in tissues pooled from 1-3 rats. Starting at 27 days of age, neonatally thyroidectomized animals were injected subcutaneously with varying dosages of T_3 daily for 3 days and killed 24 hr after the last injection. DNA concentration is expressed as mg/g tissue. Enzyme activities are calculated on a per mg DNA basis. Values in parentheses indicate the percentage of control which is taken as 100%.

DOSAGE OF T_3 (μg/100 g/day)	HK	PFK	G-3PDH	PK	α-GPDH	G-6PDH	6-PGDH	DNA
CONTROL	23.9±1.4 (100)	55±4 (100)	44±1 (100)	727±49 (100)	16.7±1.2 (100)	14.4±1.5 (100)	5.25±0.00 (100)	9.76±0.18 (100)
25	28.2±1.8 (118)	55±8 (100)	49±2 (111)	984±34 (135)*	21.1±0.7 (126)	18.7±0.9 (130)	7.36±0.47 (140)*	8.86±0.28 (91)
250	32.1±0.4 (134)*	72±2 (131)	54±1 (123)*	1095±55 (151)*	30.7±2.8 (184)*	23.1±0.9 (160)*	8.22±0.49 (157)*	8.90±0.12 (91)

*Statistically significant difference as compared to the control values (p = <0.05).

that the concentration of cerebellar DNA was not altered by this treatment. These data indicate that high dosages of T_3, when given for relatively short periods of time, are capable of producing marked increases in various brain enzymes.

(iii) Effects of delayed T_3 treatment in neonatally thyroidectomized animals

Since administration of T_3 to cretinous rats in early life exerted marked effects on the impaired body and brain growth and decreased enzyme activities in the brain, it was of interest to investigate the influence of delaying the initiation of T_3 treatment until adulthood. Groups of neonatally thyroidectomized animals were treated with T_3 (10 µg/100 g/day) for 25 days starting at either 5 or 120 days of age.

The data in Fig. 28 demonstrate that whereas T_3 treatment, initiated at 5 days of age, resulted in significant increases in body and brain weight of neonatally thyroidectomized animals, the hormone had little or no effect on the body and brain weight of adult cretinous rats. Fig. 28 also shows that administration of T_3 failed to exert any effect on DNA in the cerebral cortex and cerebellum of adult thyroidectomized animals.

The influence of delayed T_3 treatment on enzyme activities in the cerebral cortex and cerebellum of neonatally thyroidectomized animals is shown in Figs. 29-31. In all cases, administration of T_3 to young animals resulted in significant enzyme increases in both brain regions. In contrast, T_3 treatment of adult cretinous rats produced no such

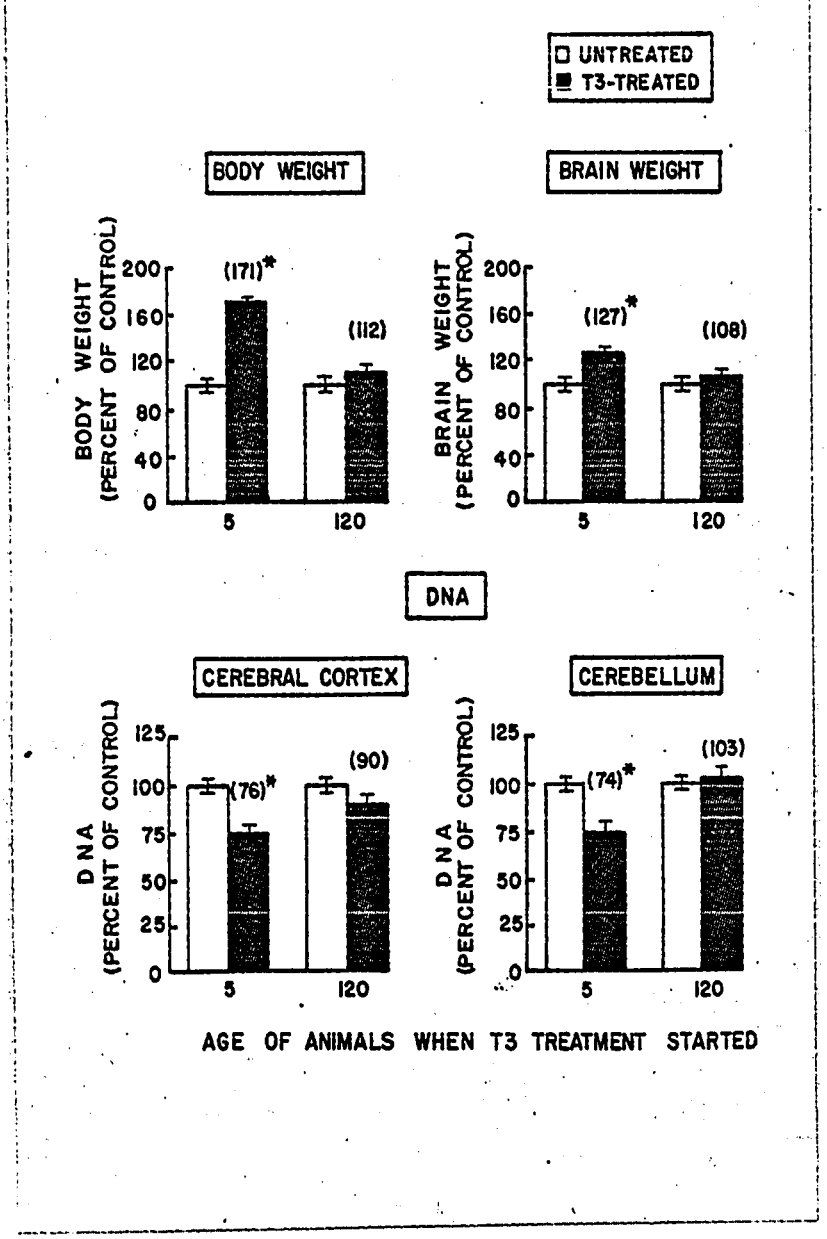

Fig. 28. Influence of delayed T_3 administration on body and brain weight and DNA concentration in the cerebral cortex and cerebellum of neonatally thyroidectomized rats. Bars represent the means ± S.E. of three values in each group. Thyroidectomized animals were injected daily with T_3 (10 µg/100 g) for 25 days starting at either 5 or 120 days of age and killed 24 hr after the last injection. Values are expressed in percentages taking the values of control animals as 100%. The asterisks denote statistically significant differences when compared with the control values ($p = <0.05$). T_3=L-triiodothyronine.

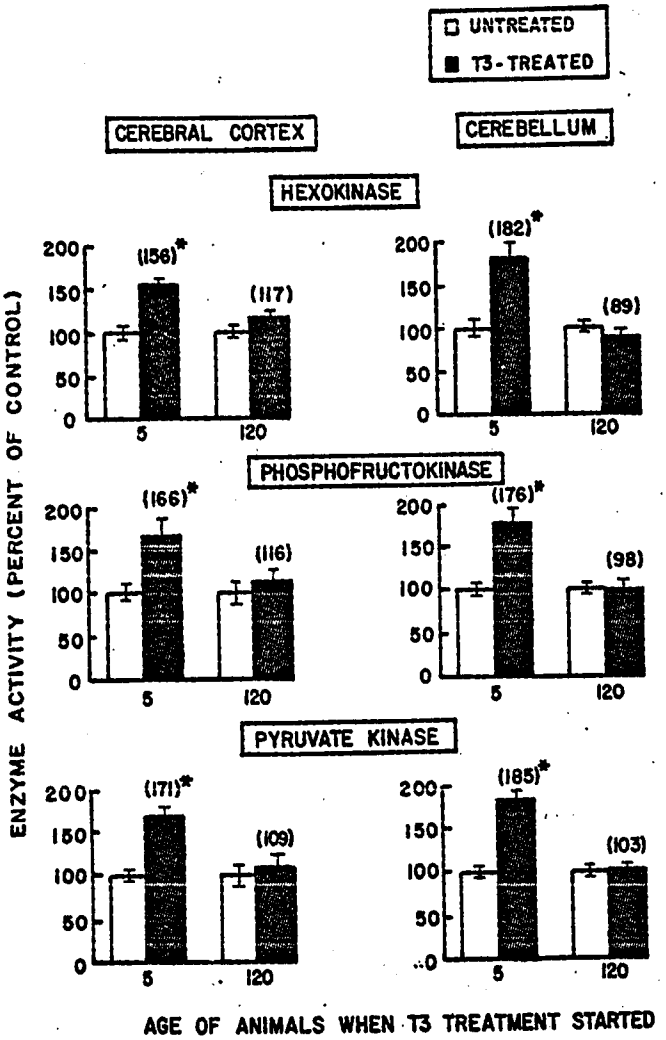

Fig. 29. Effect of delayed T_3 administration on the activities of key glycolytic enzymes in the cerebral cortex and cerebellum of neonatally thyroidectomized rats. Bars represent the means and S.E. of three determinations of enzyme activity in tissues pooled from 1-3 rats. Thyroidectomized rats were given daily injections of T_3 (10 µg/100 g) for 25 days starting at either 5 or 120 days of age and killed 24 hr after the last injection. Enzyme activities were calculated per mg DNA and are expressed in percentages taking the values of control animals as 100%. The asterisks denote statistically significant differences when compared with the values of control rats ($p = <0.05$). T_3=L-triiodothyronine.

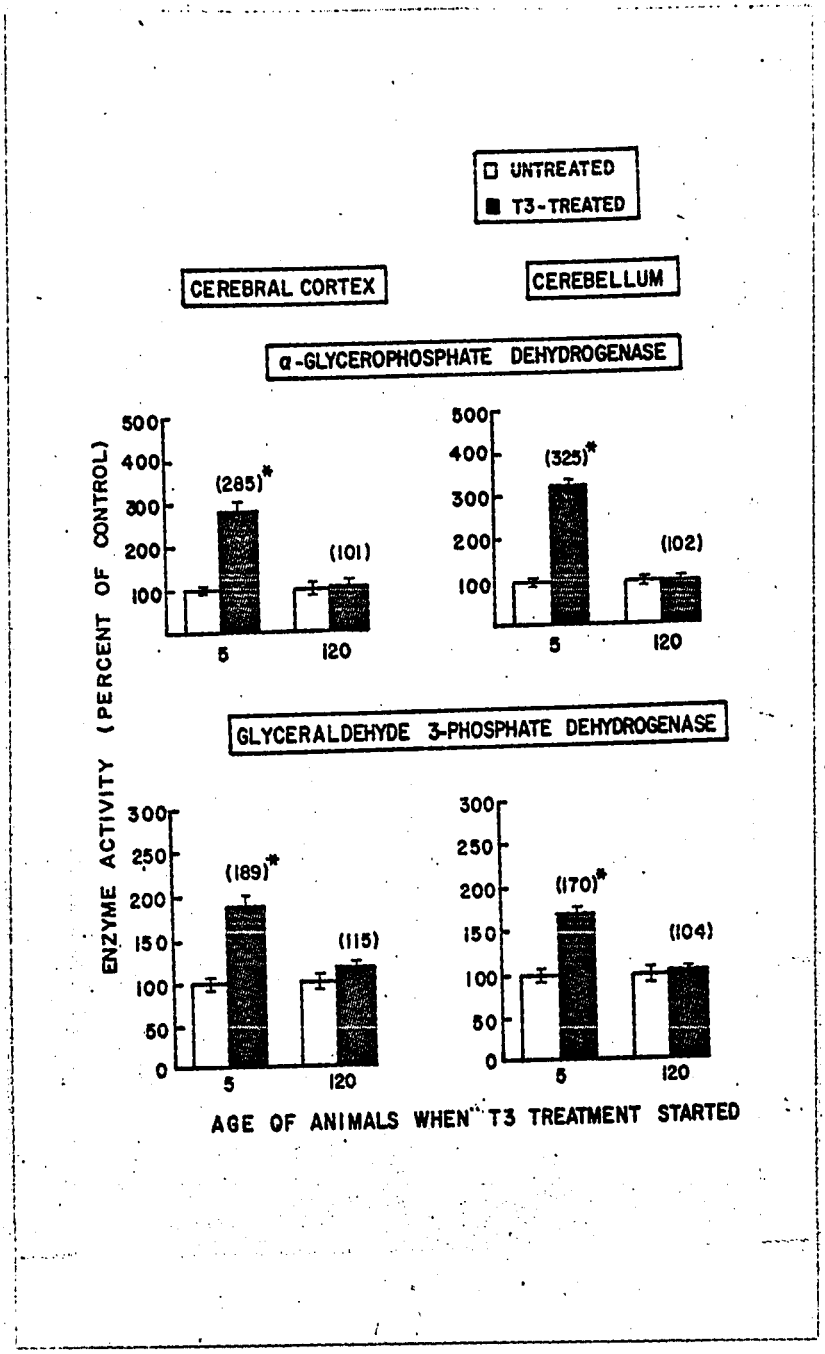

Fig. 30. Effect of delayed T_3 administration on the activities of α-glycerophosphate and glyceraldehyde 3-phosphate dehydrogenase in the cerebral cortex and cerebellum of neonatally thyroidectomized rats. Experimental conditions were the same as described in Fig. 29. Enzyme activities were calculated per mg DNA and are expressed in percentages taking the values of control animals as 100%. The asterisks denote statistically significant alterations when compared with the values of control rats ($p = <0.05$). T_3=L-triiodothyronine.

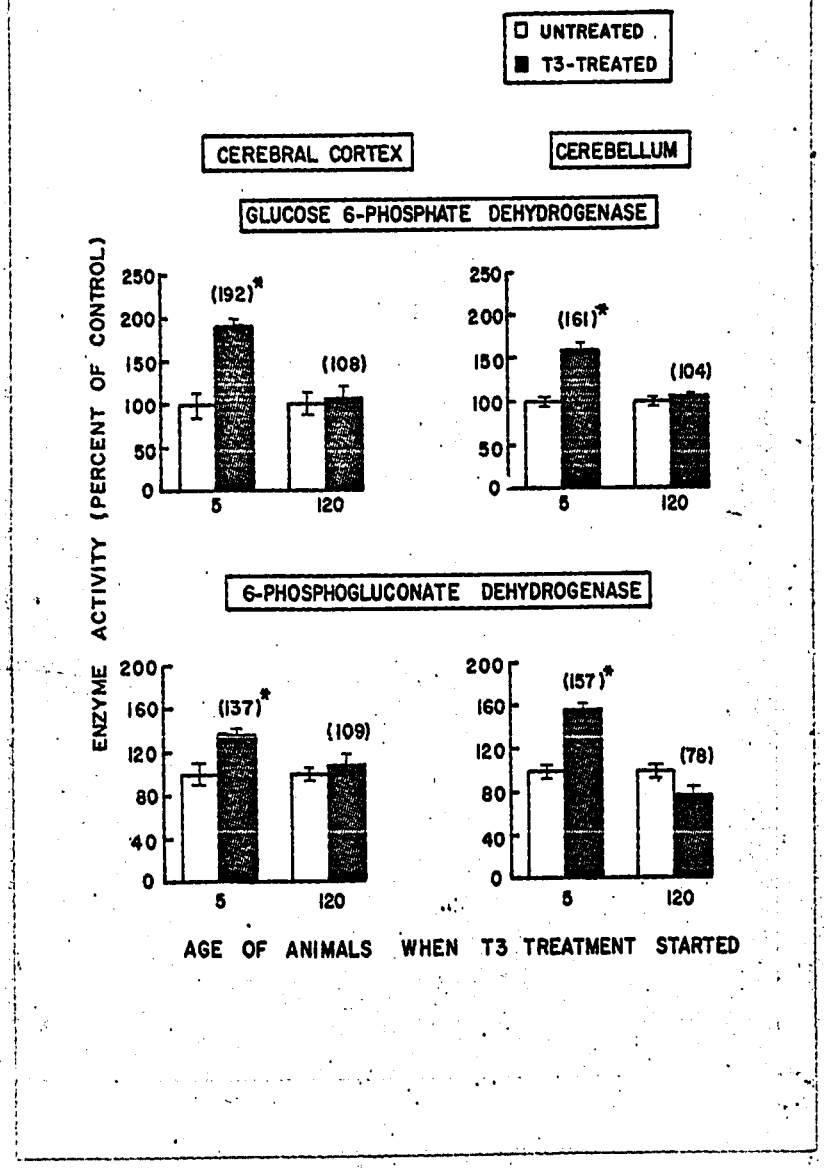

Fig. 31. Effect of delayed T_3 administration on the activities of pentose phosphate cycle enzymes in the cerebral cortex and cerebellum of neonatally thyroidectomized animals. Experimental conditions were the same as described in Fig. 29. Enzyme activities were calculated per mg DNA and are expressed in percentages taking the values of control animals as 100%. The asterisks denote statistically significant differences when compared with values of control rats ($p = <0.05$). T_3=L-triiodothyronine.

effects on any of the investigated enzymes. The data indicate that the age at which thyroid replacement therapy is instituted in cretinous animals is important in determining the effects produced by this hormone on the metabolism of the central nervous system.

(iv) Influence of cycloheximide

The nature of the T_3-stimulated increases in the activities of various enzymes in the cerebral cortex and cerebellum of neonatally thyroidectomized rats was investigated by using cycloheximide, an antibiotic which is known to inhibit protein synthesis (180).

The results presented in Tables VIII and IX demonstrate that administration of T_3 (100 µg/100 g) for 5 days produced significant increases in the activities of all enzymes investigated with the exception of HK and PK in the cerebral cortex and of G-3PDH in the cerebellum. In contrast, cycloheximide alone exerted no appreciable effect on any of the enzymes in either brain region. Concurrent administration of cycloheximide with T_3 inhibited the hormone-stimulated increases in cerebro-cortical and cerebellar enzymes although, in certain cases, the inhibition obtained was statistically insignificant. These results raise the possibility that T_3-induced increases in the activities of various enzymes in the brain of thyroidectomized animals may be the result of de novo protein synthesis.

(D) MODULATION OF BRAIN PYRUVATE KINASE AND α-GLYCEROPHOSPHATE DEHYDROGENASE ACTIVITY

The above studies suggested that several of the carbohydrate-

TABLE VIII

EFFECT OF CYCLOHEXIMIDE ON T_3-STIMULATED INCREASES IN CEREBRO-CORTICAL ENZYMES IN HYPOTHYROID RATS

Each value represents the mean ± S.E. based on three determinations of enzyme activity in tissues pooled from 1-2 rats. Neonatally thyroidectomized animals were injected with T_3 (100 µg/100 g/day) subcutaneously for 5 days starting at 25 days of age and killed 24 hr after the last hormone injection. Cycloheximide (10 µg/rat/day) was given by the intraperitoneal route concurrently with the hormone. Enzyme activities are expressed per mg DNA. Data are also given in percentages taking the values of control rats as 100%.

TREATMENT	HK	PFK	G-3PDH	PK	α-GPDH	G-6PDH	6-PGDH
CONTROL	94±3 (100)	226±6 (100)	114±1 (100)	2713±253 (100)	45±2 (100)	35.5±0.9 (100)	17.3±1.3 (100)
T_3	107±2 (114)	299±14 (132)*	143±5 (125)*	3294±125 (121)	62±2 (138)*	45.0±0.0 (127)*	24.2±0.4 (140)*
CYCLOHEXIMIDE	91±11 (97)	198±16 (88)	94±6 (82)	2515±121 (93)	43±2 (96)	32.0±2.1 (90)	17.8±0.4 (103)
T_3 + CYCLOHEXIMIDE	89±4 (95)	215±10 (95)†	87±6 (76)†	2313±213 (85)	41±2 (91)†	33.0±2.3 (93)†	17.9±0.9 (103)†

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

†Statistically significant difference when compared with the values of rats given T_3 without cycloheximide administration ($p = <0.05$).

TABLE IX

INFLUENCE OF CYCLOHEXIMIDE ON T_3-INDUCED INCREASES OF ENZYME ACTIVITY IN THE CEREBELLUM OF HYPOTHYROID RATS

Each value represents the mean ± S.E. based on three determinations of enzyme activity in tissues pooled from 1-2 rats. Neonatally thyroidectomized animals were injected with T_3 (100 µg/100 g/day) subcutaneously for 5 days starting at 25 days of age and killed 24 hr after the last hormone injection. Cycloheximide (10 µg/rat/day) was given by the intraperitoneal route concurrently with the hormone. Enzyme activities are expressed per mg DNA. Data are also given in percentages taking the values of control rats as 100%.

TREATMENT	HK	PFK	G-3PDH	PK	α-GPDH	G-6PDH	6-PGDH
CONTROL	17.8±0.6 (100)	47±2 (100)	31.4±2.6 (100)	618±29 (100)	17.6±0.9 (100)	12.8±0.9 (100)	4.67±0.21 (100)
T_3	22.0±0.6 (124)*	65±3 (138)*	39.1±1.7 (125)	844±4 (137)*	29.7±2.0 (169)*	18.9±0.7 (148)*	7.40±0.15 (158)*
CYCLOHEXIMIDE	18.0±1.0 (101)	54±2 (115)	30.4±0.4 (97)	635±26 (103)	19.5±1.2 (111)	15.6±0.4 (122)	4.44±0.34 (95)
T_3 + CYCLOHEXIMIDE	19.1±1.2 (107)	46±3 (98)†	32.4±3.2 (103)	706±31 (114)†	20.2±0.4 (115)†	15.0±0.4 (117)†	5.52±0.66 (118)

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

†Statistically significant difference as compared with the values of rats given T_3 without cycloheximide administration ($p = <0.05$).

metabolizing enzymes in the rat central nervous system are subject to *in vivo* regulation by thyroid hormone. It also was of interest to investigate the *in vitro* modulation of certain enzyme activities in rat brain. Since pyruvate kinase plays a pacemaker role in the control of central nervous system glycolysis (48), the modulation of this enzyme in the cerebral cortex was examined. In addition, some aspects of the control of brain α-glycerophosphate dehydrogenase, the enzyme believed to be important for myelin formation in nervous tissue (68), were studied.

(a) Modulation of Pyruvate Kinase Activity in the Rat Cerebral Cortex

(i) Effects of L-alanine

Since L-alanine is known to inhibit the activity of PK in hepatic tissue (181), the effect of this amino acid on cerebro-cortical PK was investigated. The results in Table X demonstrate that direct addition of increasing concentrations of L-alanine to the reaction mixture produced a progressive activation of PK. In the presence of 100-200 mM of the amino acid, approximately 30% activation of enzyme activity was observed.

(ii) Influence of L-phenylalanine and some phenylalanine analogs and metabolites

The effect of the related amino acid, L-phenylalanine on cerebro-cortical PK activity was also examined since this amino acid

TABLE X

EFFECT OF L-ALANINE ON PYRUVATE KINASE ACTIVITY IN THE CEREBRAL CORTEX

Various concentrations of L-alanine were added directly to the cuvette just prior to addition of the supernatant fluid.

L-ALANINE (mM)	PYRUVATE KINASE ACTIVITY (μmoles/g/hr)	PERCENT OF CONTROL
NONE (control)	9,804	100
1.25	10,614	108
2.5	10,990	112
5.0	10,990	112
12.5	10,903	111
25	11,250	115
50	11,347	116
100	12,638	129
200	12,985	132

is known to accumulate in the blood and tissues of phenylketonuric patients. The results in Fig. 32 (left) demonstrate that addition of L-phenylalanine to the assay system produced a dose-dependent inhibition of PK activity; 20-25 mM L-phenylalanine inhibited the enzyme almost completely when assayed in the presence of 2 μmoles of the substrate (phosphoenolpyruvate). The degree of enzyme inhibition decreased as the concentration of phosphoenolpyruvate was increased. Since the Lineweaver-Burk plots obtained for PK activity in the presence and absence of L-phenylalanine extrapolate to the same point on the ordinate (Fig. 32; right), the data suggest that L-phenylalanine is a competitive inhibitor of this enzyme in the cerebral cortex. The Ki for L-phenylalanine calculated from these data was 4.8×10^{-4} M.

The results presented in Fig. 33 compare the inhibitory effects of L-phenylalanine on PK activity with those produced by several analogs and metabolites of this amino acid. The analog, p-chloro-phenylalanine, which has been used for producing an experimental model of phenylketonuria (182,183), resulted in inhibition of PK similar to that observed with the parent compound. o-Chlorophenylalanine was somewhat less inhibitory and a 20 mM concentration of this analog inhibited PK to only 67% of the control value. Phenyllactic and phenylacetic acid, two phenylalanine metabolites which are known to accumulate in the body fluids of phenylketonuric patients (184), as well as chloroacetyl phenylalanine exerted little or no effect on the enzyme activity. Lineweaver-Burk plots suggested that the o- and p-chloro analogs of phenylalanine also produced a competitive inhibition

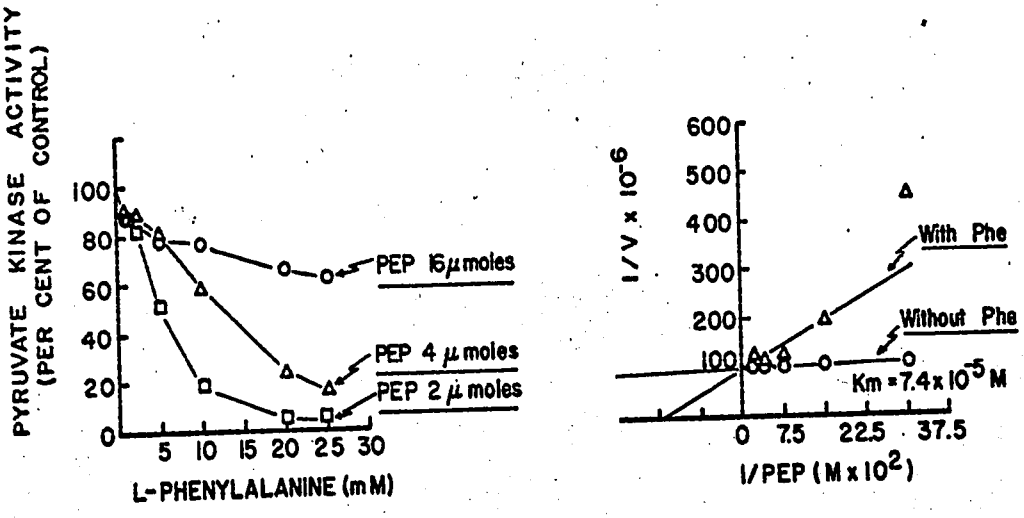

Fig. 32. (Left). Activity of pyruvate kinase in rat cerebral cortex as a function of the concentration of L-phenylalanine. Enzyme activity was measured in the presence of three different concentrations of the substrate, phosphoenolpyruvate (PEP). Pyruvate kinase activity is expressed as percentage of the control value, taken as 100%. (Right). Lineweaver-Burk plot illustrating competitive inhibition of cerebrocortical pyruvate kinase by L-phenylalanine (Phe). The final concentration of L-phenylalanine in the reaction mixture was 5 mM.

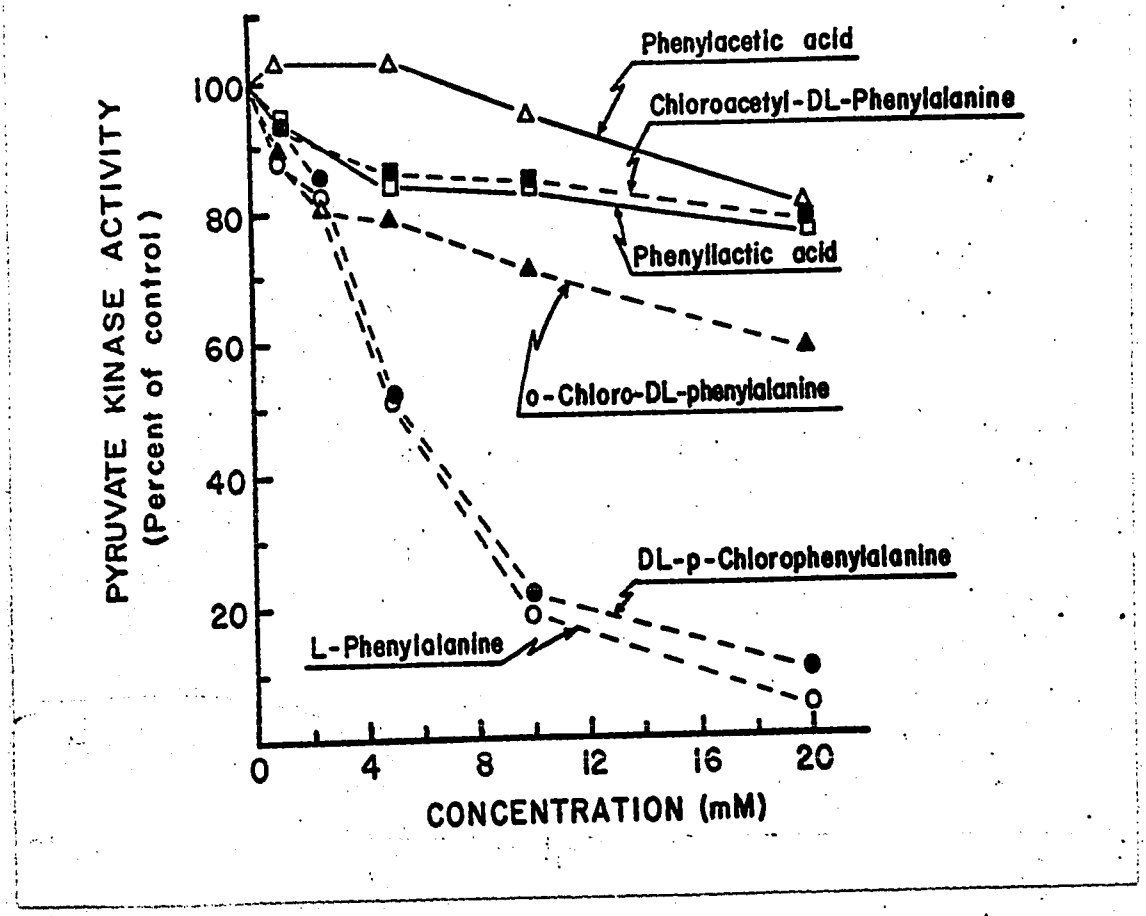

Fig. 33. Influence of varying concentrations of L-phenylalanine and several phenylalanine analogs and metabolites on pyruvate kinase activity of rat cerebral cortex. The enzyme activity is expressed as percentage of the control value taken as 100%.

of PK with an apparent Ki of 1.1×10^{-3} M for p-chlorophenylalanine and 1.3×10^{-2} M for the o-chloro analog.

Since L-alanine and L-phenylalanine produced opposite effects on cerebro-cortical PK, it was of interest to investigate the influence of L-alanine on enzyme inhibition produced by L-phenylalanine and its analogs. The protective action of L-alanine against PK inhibition is shown in Table XI. Addition of L-phenylalanine and p-chlorophenylalanine resulted in 43% and 36% inhibition of PK, respectively. However, when L-alanine was added simultaneously with these compounds, the inhibition was almost completely prevented and the activity remained within the control range. It is of interest that L-alanine, even in concentrations as high as 50 mM, failed to provide any protection against PK inhibition produced by the o-chloro analog.

The ability of L-alanine to reverse the inhibitory effects of L-phenylalanine and its analogs was also investigated. The results in Table XII demonstrate that L-alanine reversed L-phenylalanine inhibition from 58% to 92% and from 50% to 87% in the case of p-chlorophenylalanine. In contrast, the inhibition of enzyme activity by o-chlorophenylalanine could not be reversed by L-alanine. These results indicate that L-alanine can effectively prevent and reverse cerebro-cortical PK inhibition produced by L-phenylalanine and p-chlorophenylalanine.

(iii) Thermal inactivation of pyruvate kinase

The ability of L-alanine to protect the activity of PK against

TABLE XI

PROTECTIVE ACTION OF L-ALANINE AGAINST INHIBITION OF PYRUVATE KINASE ACTIVITY BY L-PHENYLALANINE AND ITS ANALOGS

L-Phenylalanine, phenylalanine analogs as well as L-alanine were added directly to the reaction mixture just prior to the addition of the supernatant fluid.

ADDITIONS	PYRUVATE KINASE ACTIVITY (μmoles/g/hr)	PERCENT OF CONTROL
NONE (control)	10,903	100
L-ALANINE (12.5 mM)	12,696	116
L-PHENYLALANINE (5.0 mM)	6,189	57
L-PHENYLALANINE (5.0 mM) + L-ALANINE (12.5 mM)	10,006	92
DL-p-CHLOROPHENYLALANINE (5.0 mM)	6,999	64
DL-p-CHLOROPHENYLALANINE (5.0 mM) + L-ALANINE (12.5 mM)	10,296	94
o-CHLORO-DL-PHENYLALANINE (20.0 mM)	6,767	62
o-CHLORO-DL-PHENYLALANINE (20.0 mM) + L-ALANINE (50.0 mM)	6,738	62

TABLE XII

REVERSAL BY L-ALANINE OF PYRUVATE KINASE INHIBITION BY L-PHENYLALANINE AND ITS ANALOGS

0.5 ml of the supernatant fluid was incubated with L-phenylalanine (5.0 mM) or DL-p-chlorophenylalanine (5.0 mM) at 0° for 10 min. The mixture was then added to either distilled water or L-alanine (12.5 mM) and an aliquot was used for the assay of pyruvate kinase. In the case of o-chloro-DL-phenylalanine, 0.5 ml of the supernatant fluid was incubated with this analog (20.0 mM) at 0° for 10 min. This mixture was added to either distilled water or L-alanine (50.0 mM) and an aliquot was used for the enzyme assay.

ADDITIONS	PYRUVATE KINASE ACTIVITY (μmoles/g/hr)	PERCENT OF CONTROL
NONE (control)	10,990	100
L-PHENYLALANINE (5.0 mM)	6,420	58
L-PHENYLALANINE (5.0 mM) + L-ALANINE (12.5 mM)	10,093	92
DL-p-CHLOROPHENYLALANINE (5.0 mM)	5,524	50
DL-p-CHLOROPHENYLALANINE (5.0 mM) + L-ALANINE (12.5 mM)	9,601	87
o-CHLORO-DL-PHENYLALANINE (20.0 mM)	6,652	61
o-CHLORO-DL-PHENYLALANINE (20.0 mM) + L-ALANINE (50.0 mM)	6,131	56

thermal inactivation was also examined. The supernatant fluid was incubated at three different temperatures for varying periods of time with and without the addition of L-alanine (Fig. 34). Slight inactivation of enzymatic activity occurred at 37°C. Increasing the incubation temperature to 45°C resulted in 30% inhibition and the enzyme was inhibited by 60% when incubated at 55°C for a 60 min period. L-Alanine failed to protect PK completely from thermal inactivation although, in all cases, enzymatic activity was higher when L-alanine was included in the preincubation mixture.

(iv) Effect of copper

Since large amounts of copper are known to accumulate in the brain of individuals suffering from hepatolenticular degeneration or Wilson's disease (185), the effect of this cation on cerebro-cortical PK was investigated (Fig. 35). In the presence of 16 μmoles of phosphoenolpyruvate, a definite inhibition of enzymatic activity was observed with concentrations of Cu^{++} as low as 0.01 mM, whereas 50% inhibition of PK was produced by 0.25 mM Cu^{++}. The extent of inhibition became more pronounced as the concentration of phosphoenolpyruvate in the assay system was decreased. Complete inhibition of PK activity was achieved with 1 mM Cu^{++}, regardless of the concentration of phosphoenolpyruvate used. Lineweaver-Burk plots of these data suggested that the inhibition of cerebro-cortical PK by Cu^{++} was of the competitive type.

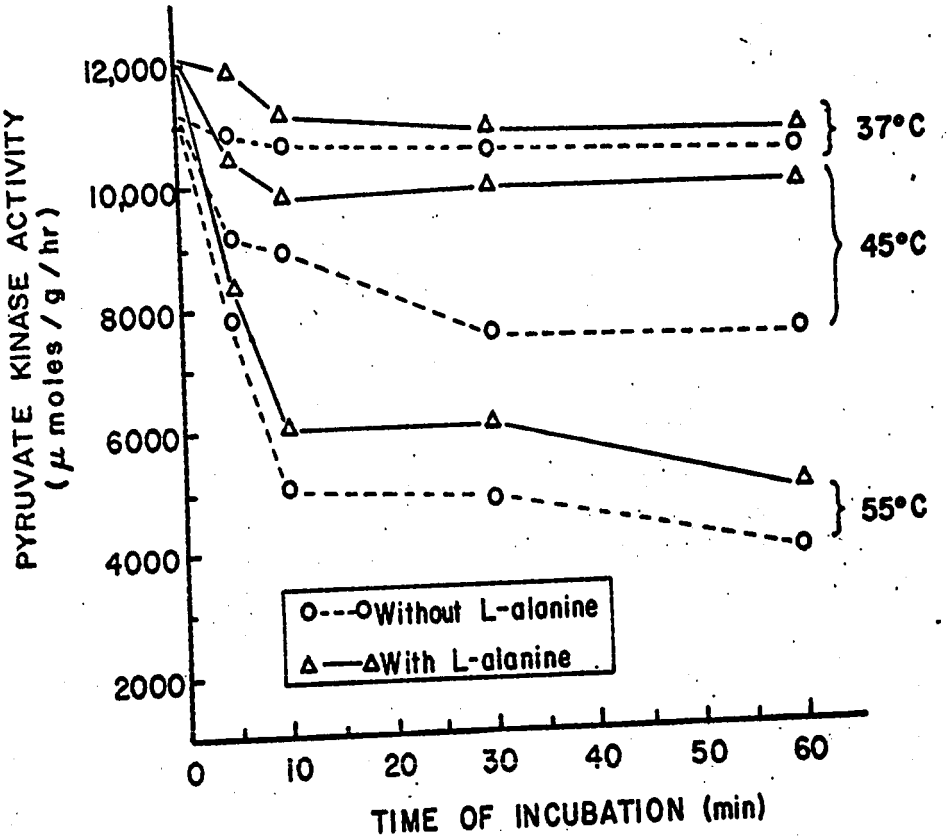

Fig. 34. Effect of L-alanine on the thermal inactivation of cerebrocortical pyruvate kinase. A 0.5 ml portion of the supernatant fluid was preincubated at 37°, 45° or 55°C with either 0.5 ml distilled water or a 0.075 M solution of L-alanine for varying periods. From this mixture, 0.1 ml samples were withdrawn and added to the cuvette so that the reaction mixture contained 12.5 mM L-alanine.

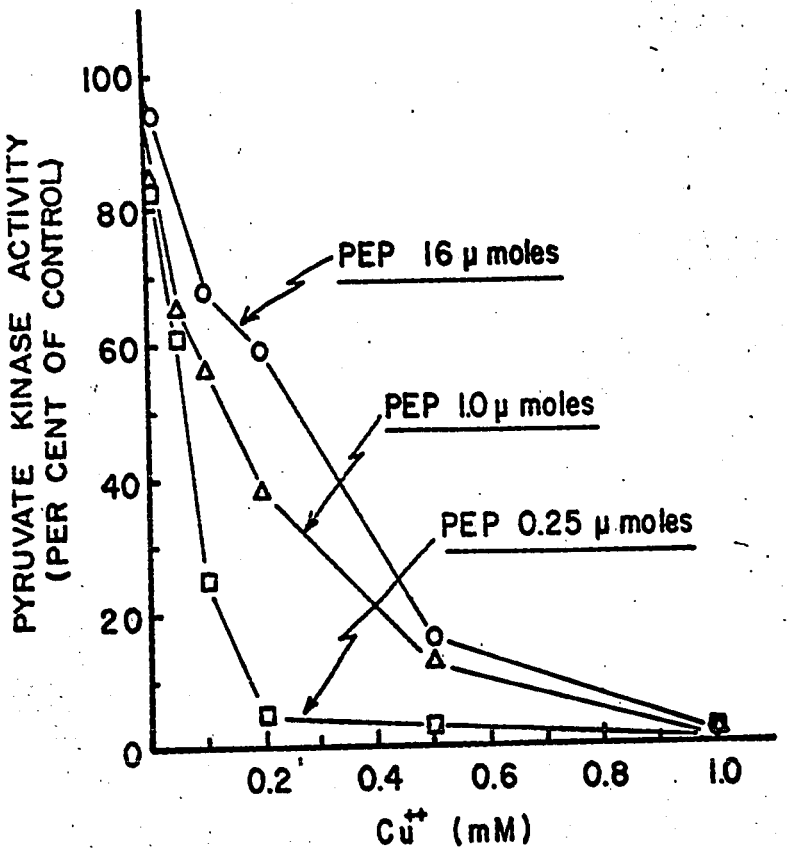

Fig. 35. Activity of pyruvate kinase in the cerebral cortex as a function of the concentration of copper (as $CuSO_4$). Enzyme activity was measured in the presence of three different concentrations of the substrate, phosphoenolpyruvate (PEP). Pyruvate kinase activity is expressed as percentage of the control value taken as 100%.

Since L-alanine provided some protection against thermal inactivation and prevented as well as reversed L-phenylalanine and p-chlorophenylalanine inhibition of PK, the effect of L-alanine on Cu^{++}-induced inhibition of this enzyme was also investigated. The results presented in Table XIII demonstrate that L-alanine completely prevented the inhibitory effects of the cation on enzyme activity. In addition, substantial reversal of Cu^{++} inhibition of PK activity was observed when L-alanine was added to the enzyme preparation subsequent to the addition of Cu^{++}.

(v) Effects of calcium and EDTA

The influence of calcium and EDTA on PK activity in the cerebral cortex was also studied (Table XIV). A definite inhibition of enzymatic activity was produced by 0.1 mM Ca^{++}; increasing the Ca^{++} concentration to 5.0 mM resulted in 79% inhibition of PK. Lineweaver-Burk plots (Fig. 36) indicated that Ca^{++} produced a non-competitive inhibition of cerebro-cortical PK with an apparent Ki of 1.15×10^{-3} M. In contrast to the inhibitory effect of Ca^{++}, low concentrations of EDTA produced slight activation of PK and maximal activation (37%) was observed with 0.75 mM EDTA (Table XIV). However, at concentrations of EDTA above this level, there was a gradual decline in PK activity.

Since Ca^{++} and EDTA exerted contrasting effects on PK, the influence of EDTA on Ca^{++} inhibition of the enzyme was investigated (Table XV). Simultaneous addition of the chelating agent with Ca^{++} to the reaction mixture resulted in a substantial protection against

TABLE XIII

PREVENTION AND REVERSAL BY L-ALANINE OF INHIBITION OF PYRUVATE KINASE INDUCED BY Cu^{++}

In prevention studies, $CuSO_4$ (0.25 mM) and L-alanine (5.0 mM or 12.5 mM) were added to the reaction mixture immediately prior to the addition of the enzyme preparation (supernatant fluid from brain homogenate). For reversal experiments, 1.5 ml of enzyme preparation were mixed with 1.5 ml of $CuSO_4$ (0.001 mM) and incubated at 0° for a period of 5 min. 1 ml of this mixture was then added to either 0.5 ml distilled water or L-alanine solution (5.0 mM or 12.5 mM) and 0.3 ml of this mixture was used in each case for assaying the activity of pyruvate kinase. Data in parentheses indicate percentage of control values (=100%).

ADDITIONS	PYRUVATE KINASE ACTIVITY (μmoles/g/hr)	PERCENT OF CONTROL
PREVENTION		
NONE (control)	9,775	100
L-ALANINE (5.0 mM)	10,006	102
L-ALANINE (12.5 mM)	11,706	113
Cu^{++} (0.25 mM)	5,292	54
Cu^{++} (0.25 mM) plus L-ALANINE (5.0 mM)	8,965	92
Cu^{++} (0.25 mM) plus L-ALANINE (12.5 mM)	9,254	95
REVERSAL		
NONE (control)	8,618	100
Cu^{++} (0.001 mM)	4,830	56
Cu^{++} (0.001 mM) plus L-ALANINE (5.0 mM)	6,536	76
Cu^{++} (0.001 mM) plus L-ALANINE (12.5 mM)	7,461	87

TABLE XIV

EFFECT OF Ca^{++} AND EDTA ON PYRUVATE KINASE ACTIVITY IN RAT CEREBRAL CORTEX

Various concentrations of $CaCl_2$ and disodium EDTA were added directly to the cuvette just prior to the addition of the supernatant fluid. Values in parentheses indicate percentage of control which is taken as 100%.

CALCIUM (mM)	ENZYME ACTIVITY (μmoles/g/hr)	EDTA (mM)	ENZYME ACTIVITY (μmoles/g/hr)
NONE (control)	9,052 (100)	NONE (control)	9,920 (100)
0.1	7,519 (83)	0.1	9,775 (99)
0.25	6,738 (74)	0.25	12,002 (121)
0.5	6,160 (68)	0.5	13,361 (135)
1.0	4,569 (50)	0.75	13,621 (137)
1.25	4,020 (44)	1.0	12,031 (121)
2.5	2,545 (28)	2.0	10,209 (103)
5.0	1,880 (21)	2.5	8,011 (81)

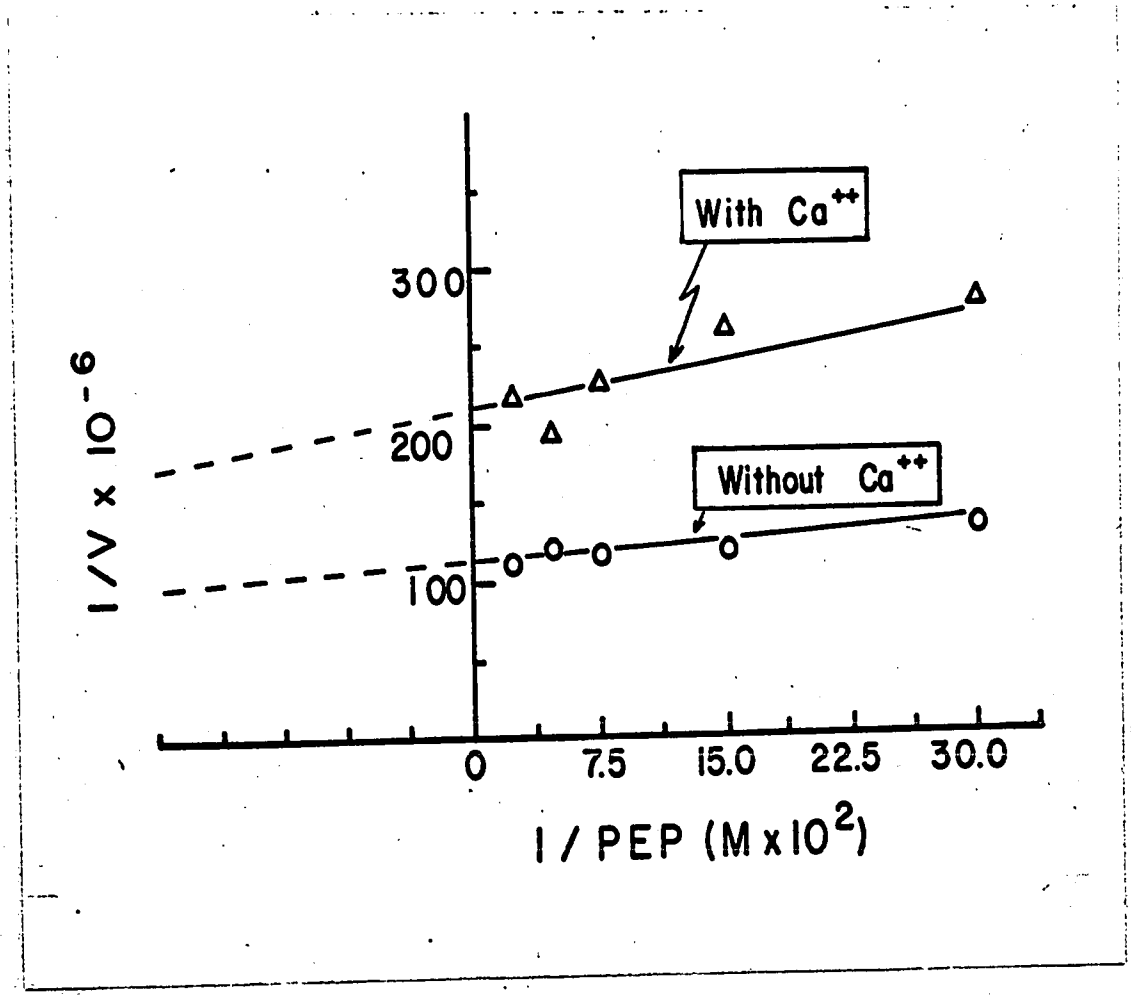

Fig. 36. Lineweaver-Burk plot illustrating non-competitive inhibition of cerebro-cortical pyruvate kinase by calcium. The final concentration of calcium in the reaction mixture was 1.0 mM.

TABLE XV

PREVENTION AND REVERSAL BY EDTA OF Ca^{++} INHIBITION OF PYRUVATE KINASE

For prevention experiments, $CaCl_2$ (1.0 mM) and disodium EDTA (0.5 or 2.0 mM) were added to the reaction mixture immediately prior to the addition of the supernatant fluid. For studying the ability of EDTA to reverse pyruvate kinase inhibition by Ca^{++}, 1.5 ml of supernatant fluid and 1.5 ml of $CaCl_2$ solution (0.5 mM) were mixed and incubated at 0^{o} for 10 min. 1 ml of the mixture was then added to either 0.5 ml distilled water or disodium EDTA solution (0.5 or 2.0 mM) and 0.3 ml of this preparation was used in each case for the assay of pyruvate kinase. Data in parentheses indicate percentage of control values which are taken as 100%.

ADDITIONS	PYRUVATE KINASE ACTIVITY (μmoles/g/hr)	
	Prevention	Reversal
NONE (control)	9,920 (100)	9,920 (100)
Ca^{++}	4,222 (43)	5,177 (52)
Ca^{++} plus EDTA (0.5 mM)	6,449 (65)	5,871 (59)
Ca^{++} plus EDTA (2.0 mM)	8,878 (89)	7,577 (76)

Ca^{++} inhibition of enzymatic activity. In addition, Table XV shows that EDTA reversed enzyme inhibition from 52 to 76% of the control value when EDTA was added to the enzyme preparation after PK was inhibited by Ca^{++}.

(vi) Influence of p-chloromercuribenzoate (p-CMB) and penicillamine

In order to investigate the importance of sulphydryl groups in the regulation of cerebro-cortical PK activity, the influence of p-CMB, a known sulphydryl inhibitor (186), was studied. Table XVI shows that addition of increasing concentrations of p-CMB to the reaction mixture led to a progressive inhibition of PK activity; 0.25 mM p-CMB inhibited enzyme activity by 42%. In contrast, penicillamine, a donor of sulphydryl groups, led to slight activation of PK. In the presence of 5 mM penicillamine, enzymatic activity was increased by 20%.

The results in Table XVII demonstrate that simultaneous addition of penicillamine and p-CMB to the reaction mixture effectively prevented the enzyme inhibition produced by p-CMB. In addition, the table shows that penicillamine reversed the p-CMB inhibition of PK from 70% to 26% when it was added to the enzyme preparation subsequent to p-CMB.

(b) Control of Brain α-Glycerophosphate Dehydrogenase Activity

Since recent studies have demonstrated that the free fatty acid, octanoate, may be of importance in the regulation of α-GPDH

TABLE XVI

INFLUENCE OF p-CMB AND PENICILLAMINE ON PYRUVATE KINASE ACTIVITY IN RAT CEREBRAL CORTEX

Various concentrations of p-CMB and D-penicillamine hydrochloride were added directly to the reaction mixture just prior to the addition of the supernatant fluid. Data in parentheses indicate percentage of control values which are taken as 100%.

p-CMB (mM)	PYRUVATE KINASE ACTIVITY (μmoles/g/hr)	PENICILLAMINE (mM)	PYRUVATE KINASE ACTIVITY (μmoles/g/hr)
NONE (control)	8,098 (100)	NONE (control)	8,502 (100)
0.01	7,404 (91)	0.5	8,647 (102)
0.025	6,709 (83)	1.0	8,821 (104)
0.05	6,102 (75)	1.5	9,052 (107)
0.1	4,887 (60)	2.0	9,977 (117)
0.25	4,685 (58)	5.0	10,180 (120)

TABLE XVII

PREVENTION AND REVERSAL BY PENICILLAMINE OF p-CMB INHIBITION OF PYRUVATE KINASE

In prevention experiments, p-CMB (0.25 mM) and penicillamine (2.0 mM) were added to the reaction mixture just prior to the addition of the supernatant fluid. In order to examine the ability of penicillamine to reverse pyruvate kinase inhibition by p-CMB, 1 ml of supernatant fluid and 1 ml of p-CMB (0.25 mM) were mixed and incubated at 0^{o} for 10 min. 0.75 ml of the mixture was then added to 0.75 ml of penicillamine (1.0 mM) and 0.4 ml of this preparation was used for determining the activity of pyruvate kinase. Data in parentheses indicate percentage of control values which are taken as 100%.

ADDITIONS	PYRUVATE KINASE ACTIVITY (μmoles/g/hr)	
	Prevention	Reversal
NONE (control)	7,866 (100)	7,866 (100)
p-CMB	3,962 (50)	2,343 (30)
p-CMB + PENICILLAMINE	7,577 (96)	5,784 (74)

in heart and adipose tissue (187), the effect of octanoate on the activity of this enzyme in the cerebral cortex and cerebellum was examined.

The results presented in Table XVIII show the effects of various concentrations of octanoate on the activity of α-GPDH in the cerebral cortex and cerebellum. A definite inhibition of the enzyme activity was observed with a 10 mM concentration of the free fatty acid in both regions of the brain. The enzyme activity declined further when the concentration of octanoate was increased and was almost completely inhibited in the presence of 80 mM octanoate.

The nature of the octanoate-induced inhibition of α-GPDH was examined by determining enzyme activity in the presence of varying amounts of the substrate, fructose 1,6-diphosphate, with or without the addition of octanoate. Lineweaver-Burk plots of the results are shown in Fig. 37. These plots, which extrapolate to the same point on the ordinate, indicate that octanoate produced a competitive inhibition of α-GPDH in the cerebral cortex and cerebellum. The Ki values were 9.7×10^{-4} M for the cerebro-cortical and 1.2×10^{-3} M for the cerebellar enzyme.

It has been demonstrated that preincubation of supernatant fluids with octanoate produces marked inhibition of α-GPDH in heart and adipose tissue (187). Fig. 38 shows the influence of preincubation of the supernatant fluids with octanoate on the activity of α-GPDH in the cerebral cortex and cerebellum. Incubation at 37°C without octanoate for 30 min decreased enzyme activity to 80% of the control value in the cortex and to 91% in the cerebellum. When 1 mM octanoate was included in the incubation

TABLE XVIII

EFFECT OF SODIUM OCTANOATE ON α-GLYCEROPHOSPHATE DEHYDROGENASE ACTIVITY IN RAT CEREBRAL CORTEX AND CEREBELLUM

Various concentrations of sodium octanoate (pH 7.4) were added directly to the reaction mixture just prior to the addition of the substrate, fructose 1,6-diphosphate. Each value is the mean ± S.E. of 3 determinations of enzyme activity.

SODIUM OCTANOATE (mM)	ENZYME ACTIVITY (μmoles/g/hr)	
	Cerebral cortex	Cerebellum
NONE (control)	284±20 (100)	365±2 (100)
2.5	245±22 (86)	340±3 (93)
10.0	178±12 (63)*	255±8 (70)*
20.0	133±11 (47)*	196±9 (54)*
40.0	84±14 (30)*	108±4 (30)*
80.0	12.0±0 (4)*	11±2 (3)*

*Statistically significant difference as compared with the control values ($p = <0.05$).

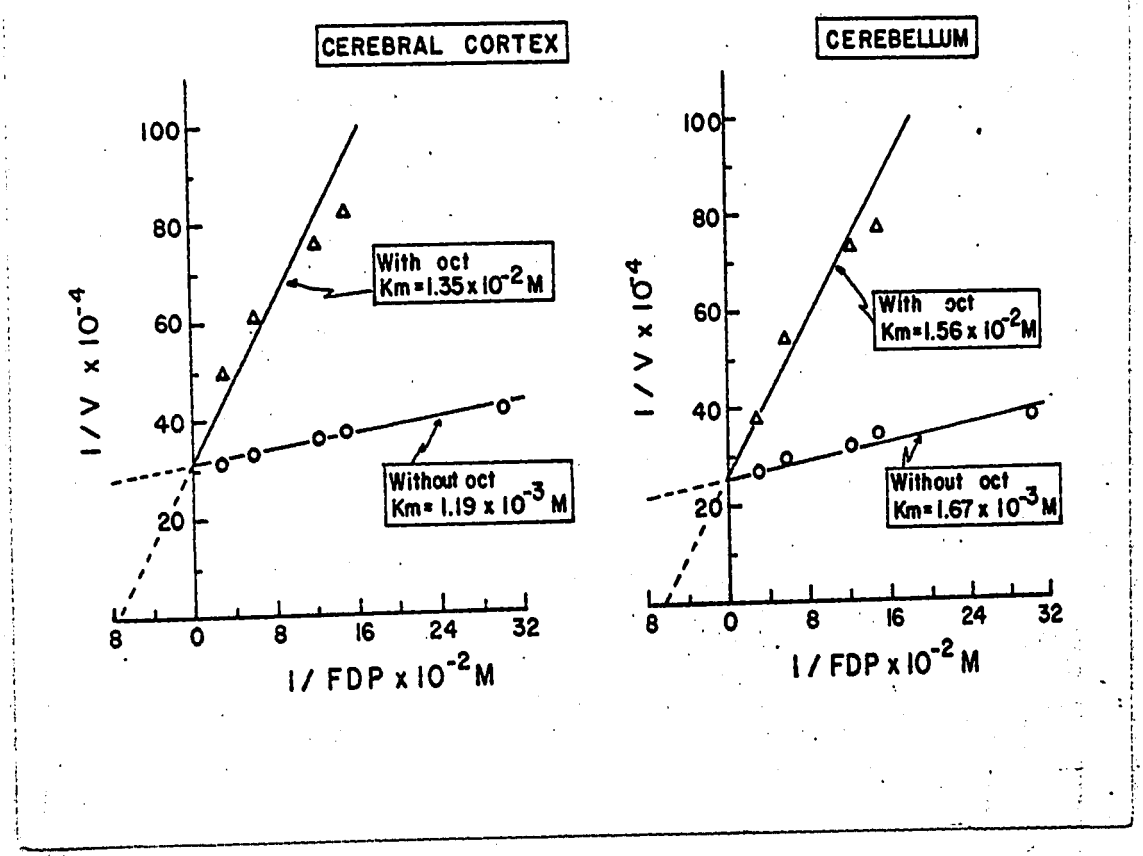

Fig. 37. Lineweaver-Burk plots showing competitive inhibition of α-glycerophosphate dehydrogenase by sodium octanoate in rat cerebral cortex and cerebellum. The final concentration of sodium octanoate in the reaction mixture was 10 mM. FDP=fructose 1,6-diphosphate; oct=octanoate.

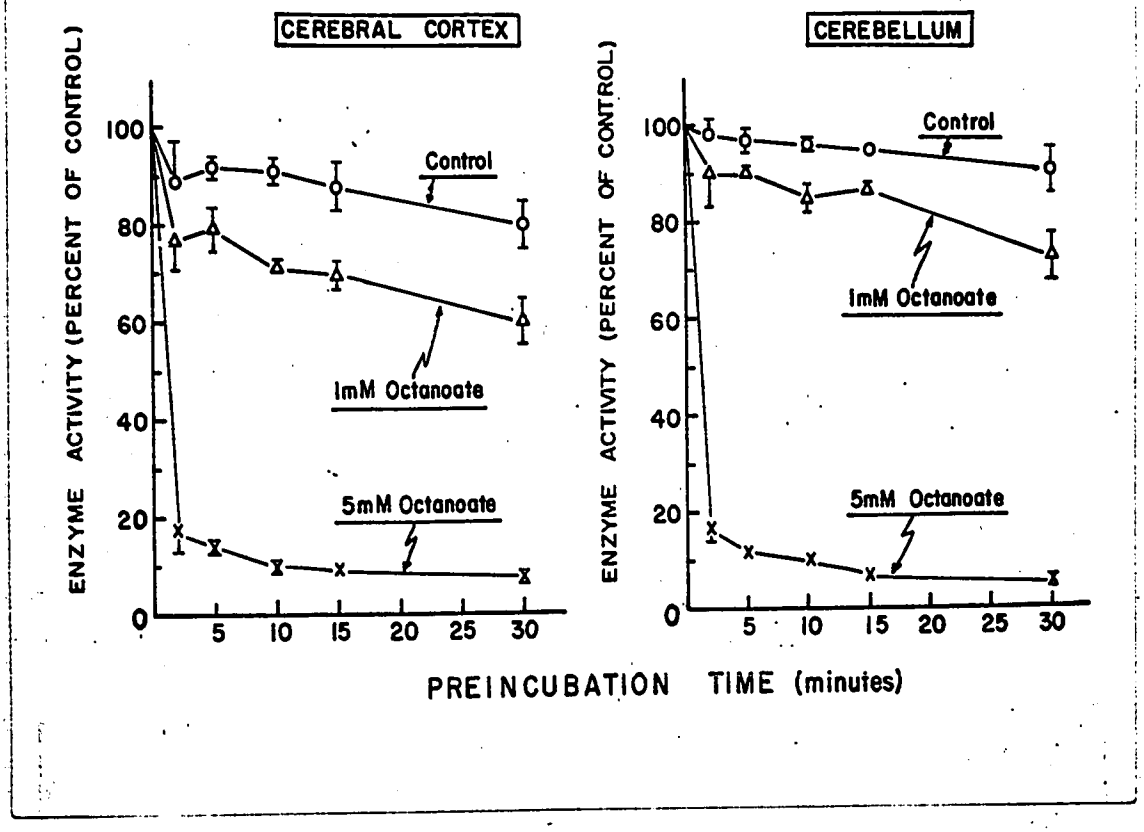

Fig. 38. Effect of preincubation of supernatant fluids with octanoate on α-glycerophosphate dehydrogenase activity in cerebral cortex and cerebellum. A 0.9 ml aliquot of the supernatant fluid was incubated at 37°C for various periods of time with either 0.3 ml distilled water or sodium octanoate (1 or 5 mM). At the end of incubation, a 0.4 ml aliquot was added to the assay system for determining enzyme activity. Each point represents the mean ± S.E. of 2 or 3 determinations. Enzyme activity is expressed in percentages taking the control values as 100%.

mixture, the enzyme activity was inhibited to 60% in the cerebral cortex and to 73% of the control value in the cerebellum following 30 min of incubation. However, in the presence of 5 mM octanoate, a more rapid and pronounced inhibition of the enzyme activity was observed. In this case, α-GPDH was inhibited by 80% in both cerebral cortex and cerebellum with 2 min preincubation and by 90% when the supernatant fluids were incubated with octanoate for a period of 5 min.

V. DISCUSSION

(A) ENZYME DISTRIBUTION AND ONTOGENESIS IN THE CENTRAL NERVOUS SYSTEM

Considerable variations were observed in the activities of carbohydrate-metabolizing enzymes in different regions of the central nervous system. The activity of the glycolytic enzymes, when expressed per g of tissue, was lower in the spinal cord than in more rostral regions which were studied and this may be associated with the lower rate of glycolysis in the spinal cord (25). The high concentration of DNA in the cerebellum reflects the high cellularity in this brain region (188) and results in low enzyme activities in the cerebellum when calculated on a per mg DNA basis.

The mammalian central nervous system is a highly complex structure, consisting of different areas with specific morphological and functional characteristics, and neurochemical investigations of discrete regions can offer important information about brain function (87). In the present study, attention was focussed on the regulation of enzyme activity in two regions of the nervous system, the cerebral cortex and cerebellum. These areas were selected since: (i) samples fromtthese regions can be readily obtained, even in neonatal animals; (ii) the cerebral cortex and cerebellum represent phylogenetically different areas of the central nervous system and (iii) these brain regions influence different functions in the animal.

The data on the changes in the amount of DNA in the developing rat cerebral cortex and cerebellum agree with those reported previously by other investigators for rats and rabbits (130,189). The postnatal decrease in DNA concentration in the cerebral cortex may be due to

proliferation of nerve cell processes with a consequent "dilution" of DNA-containing cell nuclei (130,189). The marked increase in DNA of the cerebellum during early postnatal life is indicative of the substantial DNA synthesis and cellular multiplication which occurs at this time (190,191).

In order to examine the developmental changes in cerebrocortical and cerebellar enzymes, their activities were calculated on both a per g tissue basis and a per mg DNA basis. Fresh tissue weight has commonly been employed as a reference but changes in enzyme activity expressed in this manner do not take into account the signfficant decrease in the water content of nervous tissue during maturation (84). McCaman and Aprison (189) and Pasquini et al (130) have suggested that the use of DNA as a reference material may relate changes in enzyme activity more accurately to cellular maturation in the nervous system.

The activities of the glycolytic enzymes increased during postnatal development of the cerebral cortex and cerebellum. Sydow (192) also observed a significant rise in hexokinase and phosphofructokinase activity in the rat brain during maturation and, more recently, Weber (172) reported marked increases in pyruvate kinase activity during development of the rat and human brain. The data are in accord with those of Lowry and Passonneau who found that the level of glycolytic enzymes was considerably lower in the 10-day-old mouse brain than in the adult (37). The observed increases in hexokinase, phosphofructokinase and pyruvate kinase may lead to an enhanced glycolysis and thus increase in the supply of energy required for various bio-

synthetic processes occurring in the developing central nervous system (172).

The developmental increases in α-glycerophosphate dehydrogenase activity of the cerebral cortex and cerebellum are of interest and support data reported by Laatsch who studied the ontogenesis of this enzyme in the rat forebrain and spinal cord (68). The rapid rise in the activity of this enzyme which begins 10 days after birth parallels closely the progression of myelination in the central nervous system; in the rat, myelination begins at 10-20 days of age, progresses rapidly between 20-40 days and is completed by approximately 90 days (193). Since α-glycerophosphate dehydrogenase is responsible for providing glycerol phosphate for the synthesis of myelin lipids, it is thought that the development of this enzyme in the central nervous system is important to the process of myelinogenesis (68).

In contrast to α-glycerophosphate dehydrogenase, as well as the glycolytic enzymes, the two pentose phosphate shunt dehydrogenases exhibited relatively minor changes during development of the cerebellum and cerebral cortex. The observed ontogenic changes in the activity of glucose 6-phosphate dehydrogenase of the rat cerebral cortex are similar to those reported previously by Kuhlman and Lowry and by Hunter and Hagy (95,194). Guerra et al found higher activities of the pentose phosphate shunt enzymes in the fetal rat brain and suggested that this pathway of carbohydrate metabolism may play an important role in the prenatal development of the central nervous system (58).

The growth and maturation of the mammalian central nervous system is accompanied by morphological and functional changes that are closely associated with alterations in the biochemical make-up of nervous tissue (84). The activities of many enzymes are known to increase markedly during development of the nervous system (39,84,195). Flexner described a "critical period" which is of importance to the normal maturation of the central nervous system and which is characterized by significant alterations in the activities of many important enzymes (96). The present study demonstrates that several key enzymes of brain carbohydrate metabolism increase during postnatal life although the magnitude of increase and the time at which these changes occur differ for individual enzymes. The increased activity of these enzymes may lead to an enhanced activity of the specific pathway and provide energy and precursor materials for the growth and maturation of the central nervous system.

(B) REGULATION OF BRAIN ENZYMES BY THYROID HORMONE

The observed effects produced by neonatal radio-thyroidectomy indicate the importance of thyroid hormone in mammalian growth and maturation. Thyroidectomy of the rat in early life led to an impairment of growth of both the body and brain and these observations agree well with the earlier studies of Pasquini *et al* and Balazs *et al* (130,131). Behavioural abnormalities observed in cretinous rats, such as the delayed appearance of certain reflexes, were also noted by Eayrs and Lishman and were indicative of the defective development of the central nervous system in hypothyroid animals (120).

Time course studies of the effects of neonatal thyroidectomy demonstrated that extensive neurochemical alterations occur in the cerebral cortex and cerebellum during development. The observed increase in the concentration of DNA in the brain of cretinous rats is in agreement with previous reports (129-131) and may be the result of impaired development of nerve cell processes with a resultant increase in both the volume and the surface area of DNA-containing cell bodies (130). The inhibition of carbohydrate-metabolizing enzymes following neonatal thyroidectomy is also in accordance with studies which have demonstrated alterations in several enzymes in the central nervous system (135-137). The results indicate that thyroid hormone plays an important role in the regulation of various enzymes of carbohydrate metabolism in developing nervous tissue. Additional support for this view was also obtained by carrying out dose response studies with ^{131}I. Increasing dosages of the radioisotope, which resulted in increasing degrees of thyroid gland destruction (112) and thyroid hormone deficiency, produced a progressive inhibition of most enzymes investigated.

The marked inhibition of various enzymes observed in the nervous tissue of neonatally thyroidectomized rats may be of importance in the defective development of the central nervous system in these animals. The possibility exists that impairment of ontogenic increase in the activities of hexokinase, phosphofructokinase and pyruvate kinase may interfere with the development of the glycolytic pathway of carbohydrate

metabolism and thus the energy requirements which are necessary for normal maturation of the brain are not completely met. Since the developmental increases in α-glycerophosphate dehydrogenase are believed to be important for the process of myelinogenesis (68), the observed inhibition of this enzyme may lead to an impaired myelin formation in the central nervous system of cretinous rats. Indeed, Walravens and Chase (133) and Balazs et al (134) have recently demonstrated abnormal myelinogenesis in the brain of neonatally thyroidectomized animals.

The ability of T_3 to increase enzyme activity in the cerebral cortex and cerebellum of neonatally thyroidectomized rats provides additional evidence for the involvement of thyroid hormone in the control of these important enzymes in nervous tissue and is in agreement with the studies of Hamburgh and Flexner (135) and Krawiec et al (138). These investigators have demonstrated that, in cretinous rats, replacement therapy with thyroid hormone can produce remedial effects on impaired brain growth and maturation as well as on the neurochemical abnormalities (135,138).

In contrast to the marked influence of T_3 on enzymes in the cerebral cortex and cerebellum of neonatally thyroidectomized rats, this hormone exerted little effect on the brain enzymes of normal neonatal animals. These results are in concert with the well known ability of hormones to produce pronounced effects on the metabolism of responsive tissues that have been deprived of specific hormonal influences. An exception to the relative ineffectiveness of T_3 on

the normal brain was the significant stimulation of cerebellar α-glycerophosphate dehydrogenase activity. At present, no explanation can be offered for this unique response; however, the enhanced activity of this myelin-synthesizing enzyme seems to be in line with the work of Hamburgh who demonstrated that in vitro addition of thyroid hormone to cerebellar cultures obtained from normal newborn animals leads to an accelerated formation of myelin in this tissue (196,197).

Previous studies had suggested that the early postnatal life of the animal constituted a critical period during which thyroid hormone must be present to permit normal development of the central nervous system (102,139). Support for this suggestion was gained in the present study from two types of experiments. Delaying the administration of ^{131}I for prolonged periods after birth resulted in less marked inhibition of brain enzymes, suggesting that the nervous system is more susceptible to thyroid hormone deficiency in early life. Similar conclusions were reached by Geloso et al (198) and by Eayrs (121), since thyroid hormone deprivation of older rats produced minor neurohistological and behavioural abnormalities as compared to the pronounced effects observed after neonatal thyroidectomy. These results also correlate with the known consequences of thyroid hormone deficiency at different stages of human life. Hypothyroidism in the newborn child leads to a profound mental retardation whereas relatively mild signs of brain dysfunction are observed in the adult (108,109). Experiments wherein T_3 treatment of cretinous rats was initiated either in early life or in adulthood provide further evidence for the existence of a critical period for

thyroid hormone effects on the maturation of nervous tissue. Whereas T_3 treatment produced significant increases in various enzyme activities in the central nervous system of young cretinous animals, it exerted no measurable effects on brain enzymes of adult cretinous rats. These results also are comparable with the clinical condition since thyroid hormone replacement therapy instituted early in the life of the cretinous child can usually prevent mental retardation whereas initiation of this treatment later in life produces little or no remedial effects (140).

The nature of the thyroid hormone regulation of brain enzymes was investigated with the use of cycloheximide. This compound, which inhibits protein biosynthesis by preventing the transfer of aminoacyl-transfer RNA to ribosomes (180,199), generally blocked the T_3-induced increases in the activity of various brain enzymes in cretinous rats. The data suggest that thyroid hormone may regulate enzyme activities in the central nervous system by inducing enzyme synthesis de novo. The observations that thyroid hormone stimulates protein synthesis in the immature brain (200) and that the nervous tissue from thyroid hormone deficient animals has a reduced capacity for protein biosynthesis (128) also indicate that thyroid hormone affects protein (and enzyme) formation in the central nervous system.

It is therefore clear that thyroid hormone plays a vital role in the maturation of the mammalian central nervous system. However, the molecular mechanisms by which this is achieved have not yet been clarified. Our knowledge of the processes which govern the normal

development of the central nervous system is rather limited; yet, studies during recent years have indicated that alterations in the metabolic activity of nervous tissue are extremely important to its growth and maturation. Changes in the metabolic capacity of the developing nervous system may, in turn, be related to ontogenic changes in the activity of certain enzymes which propel a variety of metabolic processes. The present investigation.demonstrates that thyroid hormone is involved in the regulation of enzymes which catalyze several important pathways of carbohydrate metabolism in the developing brain. It also suggests that thyroid hormone may affect the development of the central nervous system by virtue of its regulatory influences on a number of enzymes and, consequently, on the metabolic events which underlie the maturation of nervous tissue.

(C) MODULATION OF α-GLYCEROPHOSPHATE DEHYDROGENASE AND PYRUVATE KINASE IN THE CENTRAL NERVOUS SYSTEM

The results of the present study also demonstrate that the activities of α-glycerophosphate dehydrogenase and pyruvate kinase in the central nervous system are subject to acute modulation by specific metabolites. The observed competitive inhibition of cerebro-cortical and cerebellar α-glycerophosphate dehydrogenase by octanoate is in accordance with a recent report on the inhibitory effects of this free fatty acid on the cardiac and adipose tissue enzyme (187). Free fatty acids have also been demonstrated to inhibit several glycolytic enzymes and it was suggested that they function physiologically in acute

adaptation as a "metabolic directional switch" to restrict the flow of glycolysis (171,201). While the physiological significance of the observed inhibition of brain α-glycerophosphate dehydrogenase by octanoate remains to be elucidated, it is conceivable that the free fatty acids may affect the process of myelination by regulating the activity of this myelin-synthesizing enzyme (202).

The inhibitory effects of L-phenylalanine on cerebro-cortical pyruvate kinase agree with the observations of Weber who also indicated that concentrations of L-phenylalanine which inhibit the activity of pyruvate kinase are comparable to those found in the plasma of phenylketonuric patients (172). It is of interest that the p-chloro analog of phenylalanine, which has been used for the production of an experimental model of phenylketonuria (182,183), also inhibited pyruvate kinase activity in the cerebral cortex. The inhibitory effects of calcium on pyruvate kinase are in accord with the results of Takagaki who suggested that the concentrations of this cation required to inhibit pyruvate kinase are similar to those found physiologically in the nervous tissue (48).

L-Alanine was also found to be a modulator of pyruvate kinase in the cerebral cortex. Although this amino acid, by itself, produced only a slight activation, it was capable of preventing and reversing the inhibition of the brain enzyme by L-phenylalanine, p-chlorophenylalanine and copper. L-Alanine has been shown to completely protect pyruvate kinase activity against thermal inactivation in liver and the prostate (181,203); however, it provided only a partial protection against heat

inactivation of the brain enzyme. The precise mechanism by which L-alanine modulates pyruvate kinase activity in the cerebral cortex is unknown but slight activation by L-alanine and its ability to reverse L-phenylalanine, p-chlorophenylalanine and copper inhibition suggests the involvement of an allosteric regulatory mechanism (204,206).

Mildvan and Cohn have shown that the inhibition of pyruvate kinase by calcium may involve a competition with magnesium, one of the co-factors for this enzyme (207,208). The observed prevention and reversal of the calcium-induced inhibition of cerebro-cortical pyruvate kinase by EDTA may involve removal of calcium from the assay system by chelation. Although the mechanism by which penicillamine prevents and reverses p-CMB inhibition of cerebro-cortical pyruvate kinase is unknown, two possibilities merit consideration. Sulphydryl donors such as cysteine and glutathione reverse p-CMB inhibition of hexokinase in brain and of lactic dehydrogenase in cardiac tissue (209,210). Since penicillamine is also a sulphydryl donor, it may reverse the p-CMB inhibition of brain pyruvate kinase by making sulphydryl groups available to the enzyme. Secondly, penicillamine can chelate heavy metals and may thus reverse the p-CMB-induced inhibition of pyruvate kinase by chelating the Hg^{++} ions bound to sulphydryl groups on the enzyme molecule (206).

A variety of neurological disorders are associated with accumulations of abnormal quantities of metabolites in body fluids and tissues of the affected individual. However, relatively little is known about the cellular events that are disrupted by toxic levels

of the metabolite and how interruption of these processes leads, ultimately, to the manifestation of disorders of neurological function. The inhibitory effects of copper on brain pyruvate kinase may have some relevance to Wilson's disease where large amounts of copper accumulate in the liver and brain (185). Although the accumulation of this cation in the brain occurs primarily in the basal ganglia, it seems unlikely that the present results with cerebro-cortical pyruvate kinase would differ greatly from those with the basal ganglia enzyme. Extremely low concentrations of copper are required to markedly inhibit cerebro-cortical pyruvate kinase; hepatic pyruvate kinase is also inhibited by this cation (211). Data on the concentration of copper in normal and pathological tissues are needed before any direct correlation can be made between the observed inhibition of brain pyruvate kinase by copper and the clinical condition.

The present study also demonstrates that L-phenylalanine is a competitive inhibitor of cerebro-cortical pyruvate kinase. Weber has suggested that accumulation of L-phenylalanine in phenylketonuric patients may inhibit the activity of this key glycolytic enzyme, curtail brain glycolysis and interfere with vital cellular processes (172). Indeed, a recent study demonstrated that L-phenylalanine and phenylpyruvate (another metabolite which accumulates in phenylketonurics) markedly inhibit glycolysis in the brain and lead to an impaired synthesis of lipids, proteins and nucleic acids in this tissue (212). While the relevance of L-phenylalanine-induced inhibition of cerebro-cortical pyruvate kinase to phenylketonuric brain damage remains to

be elucidated, the extremely low levels of pyruvate kinase present in the neonatal brain may increase the vulnerability of this enzyme to the inhibitory effects of L-phenylalanine (172,206).

Weber proposed that in addition to low L-phenylalanine diet, it may be beneficial to include high levels of carbohydrates in the diets of phenylketonuric patients since a product of carbohydrate metabolism, phosphoenolpyruvate, can readily reverse L-phenylalanine-induced inhibition of pyruvate kinase (172). The present study demonstrates that L-alanine can also reverse inhibition of pyruvate kinase produced by L-phenylalanine and supports the suggestion that the use of diets high in carbohydrate (or carbohydrate precursor such as L-alanine) but low in L-phenylalanine may be beneficial in preventing the development of this mental disorder.

Although the factors which precipitate several types of mental retardation are known, the pathogenesis of the defective brain development in these conditions is poorly understood (213). The present study demonstrates enzymatic derangements which may be associated with the retarded brain development in conditions such as cretinism and phenylketonuria. The specificity of the observed changes in carbohydrate-metabolizing enzymes during experimental cretinism may be questioned since thyroid hormone is known to affect protein synthesis <u>per se</u> in nervous tissue (200). However, the demonstration that the levels of certain enzymes such as soluble aspartate aminotransferase (130), cytochrome oxidase (135) and Mg-ATPase (137) remain unaffected in the brains of cretinous animals would seem to indicate that a degree of specificity exists for the observed alter-

ations in the activities of various carbohydrate-metabolizing enzymes. The data suggest that subtle changes occurring at the molecular level in nervous tissue may be involved in the production of neurological disorders.

VI. SUMMARY

An important requirement for the maintenance of dynamic homeostasis in the mammalian organism is the ability to alter the rates of metabolic events which subserve various physiological processes. Metabolic reactions are mediated by enzyme systems and an elucidation of the factors which regulate the activity of enzymes in nervous tissue is crucial for understanding the molecular processes which underlie the functioning of the central nervous system. Since carbohydrate matabolism plays a very important role in the maintenance of normal cerebral activity, the aim of the present study was to investigate the regulatory mechanisms involved in the control of several carbohydrate-metabolizing enzymes in the central nervous system.

Considerable differences in the activity of certain glycolytic, pentose phosphate shunt and α-glycerophosphate cycle enzymes were observed in different areas of the nervous system; in general, lower enzyme levels were noted in the spinal cord than in more rostral regions. Studies on the ontogenic changes of these enzymes in the cerebral cortex and cerebellum demonstrated that extremely low levels of enzyme activity are present at birth. Maturation of these brain regions was accompanied by marked increases in the activities of hexokinase, phosphofructokinase, glyceraldehyde 3-phosphate dehydrogenase and pyruvate kinase as well as of α-glycerophosphate dehydrogenase. However, this was not a generalized phenomenon since the two pentose phosphate shunt enzymes, glucose 6-phosphate dehydrogenase and 6-phos-

phogluconate dehydrogenase, exhibited relatively minor changes from birth through 1 year of age.

Thyroid hormone deficiency, induced by radio-thyroidectomy of neonatal rats with a single dose of 100 μc of ^{131}I, produced a state of experimental cretinism which was characterized by marked impairment of physical and behavioural development of the animals. Also, neonatal thyroidectomy markedly inhibited the developmental increases in the activities of various carbohydrate-metabolizing enzymes in the cerebral cortex and cerebellum. Administration of increasing dosages (50-200 μcuries) of the radioisotope to 1-day-old rats produced a progressively greater inhibition of enzyme activity in both regions of the brain.

Additional evidence for thyroid hormone control of brain enzymes was obtained from studies in which administration of T_3 (L-triiodothyronine) was found to increase the activity of various carbohydrate-metabolizing enzymes in the cerebral cortex and cerebellum of young cretinous rats. The T_3-induced increases in glycolytic andppentose phosphate shunt enzymes as well as α-glycerophosphate dehydrogenase were both time- and dose-dependent. This treatment also produced remedial effects on the impaired growth and behavioural development of neonatally thyroidectomized animals. In addition, treatment of normal neonatal animals with T_3 resulted in an accelerated appearance of certain physical and behavioural criteria of maturation but exerted little or no effect on most of the brain enzymes investigated.

In contrast to the stimulatory effects of T_3 on brain enzymes in young cretinous animals, thyroid replacement therapy in adult rats

that had been thyroidectomized at birth produced little or no effect on the activity of various enzymes in the cerebral cortex and cerebellum. Similarly, when the thyroidectomy was delayed for 20 days after birth, little or no inhibition of carbohydrate-metabolizing enzymes was observed in these two regions of the brain. These findings support the contention that, in the rat, early post-natal life presents a critical period during which thyroid hormone exerts a marked influence on the maturation of the central nervous system. The data also indicate that the brain is particularly vulnerable to thyroid hormone deficiency during neonatal life of the animal.

Treatment of T_3-injected neonatally thyroidectomized rats with an inhibitor of protein synthesis, cycloheximide, generally blocked the ability of T_3 to stimulate the activity of various brain enzymes. The results indicate that thyroid hormone may regulate enzyme activities in the cerebral cortex and cerebellum by inducing enzyme synthesis de novo. It is suggested that thyroid hormone affects the development of the central nervous system by virtue of its regulatory influence on a number of important enzymes and, consequently, on the metabolic events which subserve the maturation of nervous tissue.

The modulation of two brain enzymes, α-glycerophosphate dehydrogenase and pyruvate kinase, by certain specific metabolites also was investigated. The free fatty acid, octanoate, produced a dose-dependent, competitive inhibition of the myelin-synthesizing enzyme, α-glycerophosphate dehydrogenase, in both the cerebral cortex and cerebellum. Preincubation of the enzyme for 5-10 minutes with octanoate

resulted in an almost complete inhibition of brain α-glycerophosphate dehydrogenase. These data suggest that free fatty acids may be involved in the control of myelin formation in the nervous system.

The activity of cerebro-cortical pyruvate kinase was modulated by L-alanine, L-phenylalanine, copper and several other metabolites. Whereas L-alanine produced slight activation of this key glycolytic enzyme, L-phenylalanine, p-chlorophenylalanine and copper induced a dose-dependent inhibition of pyruvate kinase in the cerebral cortex. The inhibition by these compounds was prevented and reversed by L-alanine. L-Alanine also provided partial protection against thermal inactivation of the enzyme. The non-competitive inhibition of pyruvate kinase activity by calcium was prevented and reversed by EDTA. The sulphydryl inhibitor, p-chloromercuribenzoate, produced a dose-dependent inhibition of cerebro-cortical pyruvate kinase; this inhibition was prevented and reversed by penicillamine. It is suggested that the observed inhibition of cerebro-cortical pyruvate kinase by copper and L-phenylalanine may be related, respectively, to the pathogenesis of neurological dysfunction seen in Wilson's disease and phenylketonuria.

In conclusion, some aspects of the regulation of carbohydrate-metabolizing enzymes have been investigated in the central nervous system of the rat. The activities of various glycolytic, pentose phosphate shunt and α-glycerophosphate cycle enzymes were shown to be influenced markedly by thyroid hormone in the developing cerebral cortex and cerebellum. In addition, brain pyruvate kinase and α-glycerophosphate dehydrogenase activities were shown to be modulated by certain specific

metabolites. The results provide additional knowledge of the molecular events which underlie the functioning of the central nervous system under normal and pathological conditions and suggest important directions along which further work may be carried out concerning metabolic regulations in the brain.

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