# CEREBRAL METABOLISM IN ANOXIA AND THE EFFECTS OF SOME NEUROTROPIC DRUGS

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ABSTRACT

The effects of tetrodotoxin (TTX) and other neurotropic drugs on anaerobic glycolysis, and on transport processes, of incubated cerebral cortex slices have been studied in an effort to understand more fully cerebral metabolic processes during anoxia, and the mode of action of certain neurotropic drugs.

The general approach has been to study the action of various drugs on the rates of anaerobic glycolysis of incubated brain slices under a variety of conditions. The cation ($Na^+$, $K^+$) contents were also studied under these conditions and the changes in these contents were related to concomitant changes in cerebral metabolism. Experiments were also carried out on the cerebral transport of amino acids and glucose under a variety of incubation conditions.

Measurement of the rates of anaerobic glycolysis in the presence of TTX showed that the drug, at low concentrations such as 2 μM, enhances the rate of anaerobic glycolysis of cerebral cortex slices two to three fold, the effects being greater in the absence of $Ca^{++}$. Such an effect of TTX is far greater than that obtained on the aerobic metabolism of the cerebral cortex slices. The anaerobic glycolysis of

kidney medulla slices, 2-day old rat brain slices or of acetone powder extracts from brain are not affected by the drug, indicating that the effects of TTX on the anaerobic glycolysis are specific for mature cerebral tissue, and requires integrity of the brain cells for its action.

TTX has little or no effect in increasing rate of anaerobic glycolysis when it is added 10 minutes, or later, after the onset of anoxia, or when high $K^+$, protoveratrine, L-glutamate or $NH_4^+$ are present in the incubation medium. Under these conditions, there is either an influx of $Na^+$ into, or ~~and~~ loss of $K^+$ from, the incubated cerebral tissue. In the presence of TTX under anoxic conditions, a much slower decline in the $K^+/Na^+$ ratio of the cerebral cortex slices is observed. These and other experiments lead to the conclusion that the effects of TTX on anaerobic glycolysis are due to its action at the brain cell membrane resulting in the prevention of the changes in brain cell permeabilities to $Na^+$ and $K^+$ brought about by the onset of anoxia. In the presence of TTX, the initial high rate of glycolysis tends to be maintained due to only a slow decline in the cellular $K^+/Na^+$ ratio. The effects of $K^+$ and $Na^+$ on the anaerobic glycolysis are thought to be mediated

largely by changes in the pyruvate kinase activity, which is enhanced by $K^+$ and diminished by $Na^+$.

TTX appears to affect the aerobic and anaerobic metabolism of brain in vitro in the same way as it affects the generation action potentials i.e. by diminishing the influx of $Na^+$ and efflux of $K^+$. These results lead to the conclusion that action potentials are generated in the incubated cerebral tissue at the onset of anoxia. These are blocked by TTX which manifests its effect by a higher rate of anaerobic glycolysis. The effect of TTX on the $Na^+$ and $K^+$ contents may be greater in the neurons than in glial cells because the former are the site of action of TTX. Consequently, the changes in the neuronal $K^+/Na^+$ ratio brought about by TTX are probably much greater than those of the $K^+/Na^+$ ratio found in the tissue as a whole.

In addition to its effects on the $Na^+$ and $K^+$ fluxes, TTX also prevents the efflux of amino acids from the incubated cerebral cortex slices that occurs at the onset of anoxia. This effect of TTX is independent of the activity of the transport processes normally operating on the amino acid uptake into the brain.

Local anesthetics, ouabain, amytal and reserpine also increase the rate of anaerobic glycolysis of cerebral cortex slices. Local anesthetics act in a manner similar to TTX, although much higher concentrations are required. The effects of ouabain in a $Ca^{++}$-free medium are much greater than in a $Ca^{++}$-containing medium. It is suggested that the increase in the rate of anaerobic glycolysis due to ouabain is possibly mediated by an increase in cell ATP concentration under anoxia, as a result of inhibition of $Na^{+}$ , $K^{+}$-ATPase. The actions of amytal and reserpine on the anaerobic glycolysis of cerebral cortex slices are possibly mediated by membrane cation changes, but further work is necessary to support this conclusion.

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# ABBREVIATIONS

| | |
|---|---|
| AMP | Adenosine monophosphate |
| ADP | Adenosine diphosphate |
| ATP | Adenosine triphosphate |
| cAMP, cyclic AMP | 3',5'-cyclic adenosine monophosphate |
| Dibutyryl cyclic AMP | $N^6$, $O^{2'}$-Dibutyryl adenosine-3'-5'-cyclic phosphate |
| DNP | 2,4-Dinitrophenol |
| EDTA | Ethylenediaminetetraacetic acid |
| EGTA | Ethanedioxybis(ethylamine) tetraacetate |
| α-KG | α-Ketoglutarate |
| $NAD^+$ | Nicotinamide adenine dinucleotide |
| NADH | Nicotinamide adenine dinucleotide, reduced form |

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# CHAPTER 1

# INTRODUCTION

## 1.1 EFFECTS OF ANOXIA ON BRAIN METABOLISM

The major aim of investigations on the brain metabolism is to gain information concerning biochemical mechanisms operating in the living brain. All the information desired can not be obtained by doing *in vivo* experiments by techniques presently available and a number of techniques have been developed to study the brain *in vitro*. Removal of the brain from the animal may result in a variety of metabolic and structural changes. Lack of oxygen* is one major factor influencing these changes. This is specially important during the first few minutes after the death of the animal during the preparation of the cerebral tissue (slices) for *in vitro* studies, even when oxygen is made available to the tissue during subsequent incubation procedure.

### (i) Effects of Hypoxia on Brain Metabolism in vivo

Many investigations have been carried out on the effects of oxygen deprivation on the brain *in vivo*. When oxygen supply to the brain is reduced or cut off, cerebral function may still be maintained for a short time through utilization of energy reserves that have, so far as is known at present, four major components- phosphocreatine, ATP, glycogen and glucose. Changes in these substances during brief periods of hypoxia have been

* Anoxia - oxygen lack; hypoxia - oxygen deficiency; ischemia - local anemia due to mechanical obstruction (mainly arterial narrowing) to the blood supply.

studied as measures of metabolic rates under these conditions.

As early as in 1944, Gurdjian, Stone and Webster[1] reported that in the dog, cerebral lactic acid level rises and phosphocreatine breakdown occurs when the oxygen saturation of the cerebral venous blood is reduced. In a later study[2], these workers showed that cerebral ATP levels remain unchanged until the phosphocreatine is almost completely decomposed.

Jilek, Fischer, Krulich and Trojan[3] studied the functional, biochemical and structural reaction of the brain to hypoxia and anoxia during development and showed that the newborn rat is more resistant than the adult to anoxia. This resistance decreases rapidly at about the 20th day of life. In younger rats (4-12 days) there is almost complete exhaustion of glycogen and a two-to three-fold rise in lactic acid in hypoxia. In older rats the decrease in glycogen and the increase in lactic acid are much less. Their results led them to the conclusion that immature nervous tissue is able to adjust to hypoxic conditions by increasing the intensity of anaerobic glycolysis, whereas the tissues of adult animals do not possess this ability. Thorn, Scholl, Pfleiderer and Mueldener[4] studied the metabolic processes of the brain under anoxia and ischemia, and observed similar changes taking place under both these conditions. For example, inorganic phosphate (Pi) and lactic acid increased, the content of creatine phosphate decreased rapidly while ATP content decreased gradually. Lolley and Samson[5] extended these studies further to include other nucleotides. They confirmed the general observation that there is a shift during anoxia of triphosphate nucleotides to monophos-

phates and a very rapid breakdown of phosphocreatine. The pattern of changes in GTP and UTP content was more complex than that of ATP. $NAD^+$ levels remained unaffected until late anoxia when they decrease to some extent.

Studies carried out by Lowry, Passonneau, Hasselberger and Schulz[6], and Stewart, Passonneau and Lowry[7], with the whole brain and with the rabbit sciatic nerve showed that during ischemia changes in the high energy compounds and energy reserves are measure of the metabolic rates. Gatefield, Lowry, Schulz and Passonneau[8], used the method of blocking blood supply to several different regions of mouse brain, and three histologically defined layers of mouse cerebellum to investigate changes in the high energy compounds. During ischemia there was depletion of glycogen, phosphocreatine and ATP from nearly all regions and there was an accumulation of lactate. The metabolic rates assessed from changes in metabolites indicated that they were about the same in cerebral cortex, cerebellar cortex and medulla but much more depressed in the Ammon's horn.

(ii) Effects of Anoxia on the Metabolism of the Cerebral Cortex Slices

Many studies have been carried out with cerebral cortex slices and some have been concerned exclusively with the effects of anoxia on cerebral morphology and chemistry. Cohen[9] developed a method whereby the morphology of the cerebral cortex slices metabolizing in vitro might be correlated with biochemical findings. Using this method Cohen[10] made detailed study of the chemical behaviour of cerebral cortex slices in anoxia and confirmed the observations of a number of workers that ATP level

declines slowly during anoxia. This was in contrast to the results of Albaum, Noell and Chinn[11] who claimed that the concentration of phosphocreatine decreases more slowly than ATP. As ATP is the immediate source of energy to meet metabolic requirements in the brain while phosphocreatine serves as a reservoir, the tendency should be for ATP to remain elevated at the expense of phosphocreatine. Cohen[10] also observed that anoxia results in widespread neuronal damage; however, scattered islands of relatively intact neurons still persist after 40 minutes. Despite morphological alterations indicating Nissl substance diminution, the amount of RNA does not decrease during a two hour period of anoxia. Other metabolic effects of anoxia, such as a decrease in the incorporation of radioactive phosphate into lipids and nucleic acids, were considered to be the result of diminished ATP synthesis. The general conclusion was that hypoxia damages cerebral mechanisms for energy production.

Swanson[12] studied the effects of oxygen deprivation on the electrically stimulated guinea pig cerebral cortex slices and he suggested that hypoxia damages the energy utilizing systems in the cerebral cortex slices. He attempted to explain the results of McIlwain[13] that decreased anaerobic glycolysis persists after cessation of electrical stimulation and the tissue loses its ability to respond aerobically, both in respiration and glycolysis, to electrical stimulation. Swanson[12] speculated that, with hypoxia, alterations in the membrane structure occurs so that the cells can no longer maintain cation gradients across their membranes or that the cell membranes

become inexcitable by interference with the transient structural changes that occur upon passage of a nerve impulse. He studied the functional deficit induced by hypoxia with the help of electrical stimulation technique and concluded that during the period of hypoxia, production of high energy phosphates, through non-oxidative pathways, is insufficient to maintain the energy utilization processes such as those involved in active cation transport. Lack of energy in the brain cell results in increased $Na^+$ influx and $K^+$ efflux. This effect is made more apparent if electrical stimulation accompanies the hypoxia. However, experiments carried out[12] with reoxygenated slices, after only short period of hypoxia (15 minutes), suggest that there is some preservation of the mechanisms of both active cation transport and energy production. Experiments with prolonged periods of hypoxia (60 minutes) suggest that an irreversible damage to the phosphorylating mechanisms takes place. Jennings, Kaltenbach and Sommers[14] have found that mitochondria, isolated from ischemic canine myocardium, show a 60 percent decrease in oxidative and phosphorylating capacity after 15 minutes of ischemia.

Other changes have also been observed in the cerebral cortex slices after periods of anaerobiosis. Thus McIlwain, Thomas and Bell[15] found that the level of cozymase in the guinea pig cerebral cortex slices decreases during anaerobiosis while it remains constant during aerobic period after an initial drop. Thomas[16] concluded that creatine phosphate is lost from the incubated cerebral cortex slices during anaerobiosis and that uptake of $K^+$, glutamate, ascorbate and creatine is diminished.

Quastel and his coworkers[137] have shown that accumulation in the brain cell, against a concentration gradient, of thiamine, ascorbic acid and amino acids is completely suppressed under anaerobic conditions.

## 1.2 GLUCOSE METABOLISM IN BRAIN

As noted earlier, lactate formation from glucose and endogenous glycogen increases several fold during anoxia. This process, the anaerobic glycolysis, and the effects thereon of various environmental conditions is the major topic of the present study. It is proposed, therefore, to discuss in the first place, mechanisms of glycolysis and its regulation.

One of the major consequences of glucose metabolism, in the mammalian tissue, is the synthesis of ATP. ATP can be obtained from glucose through well known glycolytic sequence either by formation of acetyl CoA and oxidation of this substance by operation of the citric acid cycle, or, in the absence of oxygen, through the formation of lactic acid. Whereas the former process produces 38 moles of ATP, the latter produces 2 moles of ATP per mole of glucose consumed. Most animal tissues are capable of carrying out the process of glycolysis, though to varying extents. In brain, the end product of glycolysis under anaerobic conditions is lactic acid. The process and conditions affecting carbohydrate metabolism in brain have recently been reviewed[17-20]. In this section we will be mostly concerned with the non-oxidative metabolism of glucose in brain.

Brain is characterized by the presence of high concentration of glycolytic enzymes and it has been estimated that more than 90 percent of the total glucose consumed in brain

proceeds through the glycolytic pathway[21]. The reverse formation of glucose from pyruvate is negligible in brain (see Balázs[17]).

The enzymic makeup of brain for glycolytic enzymes differs quantitatively from that of other tissues. Balázs[17] has compared the relevant data on the enzyme activities in brain with those present in liver. Thus the available data show that hexokinase activity is about twenty times higher in brain than in liver[22]. Activities of all other kinases (phosphofructokinase, phosphoglycerate kinase and pyruvate kinase) are also higher in brain than in liver. These enzymic steps are control points in glycolysis and have been discussed later. It has been mentioned that the synthesis of glucose from pyruvate is negligible in brain as compared to that in the liver or kidney. Formation of glucose from pyruvate requires reversal of certain reactions of the glycolytic pathway[23,24]. The activities of enzymes catalyzing these reactions seem to be very low in brain[17] so are those of enzymes needed for the synthesis of glycogen, and of pentose phosphate cycle[17]. A number of investigations have led to the conclusion that pentose phosphate pathway plays only a minor role in the central nervous system (CNS) of adult mammals.

It should be pointed out that for an understanding of glycolytic process in the brain, the localization of various enzymes involved in such a heterogeneous organ is most important. As pointed out by Lowry and Passonneau[25], true local concentrations of intermediates in various parts of the brain undoubtedly vary greatly and are quite different from average concentrations. An understanding, therefore, of the finer details of glycolytic changes in the brain must wait histochemical studies

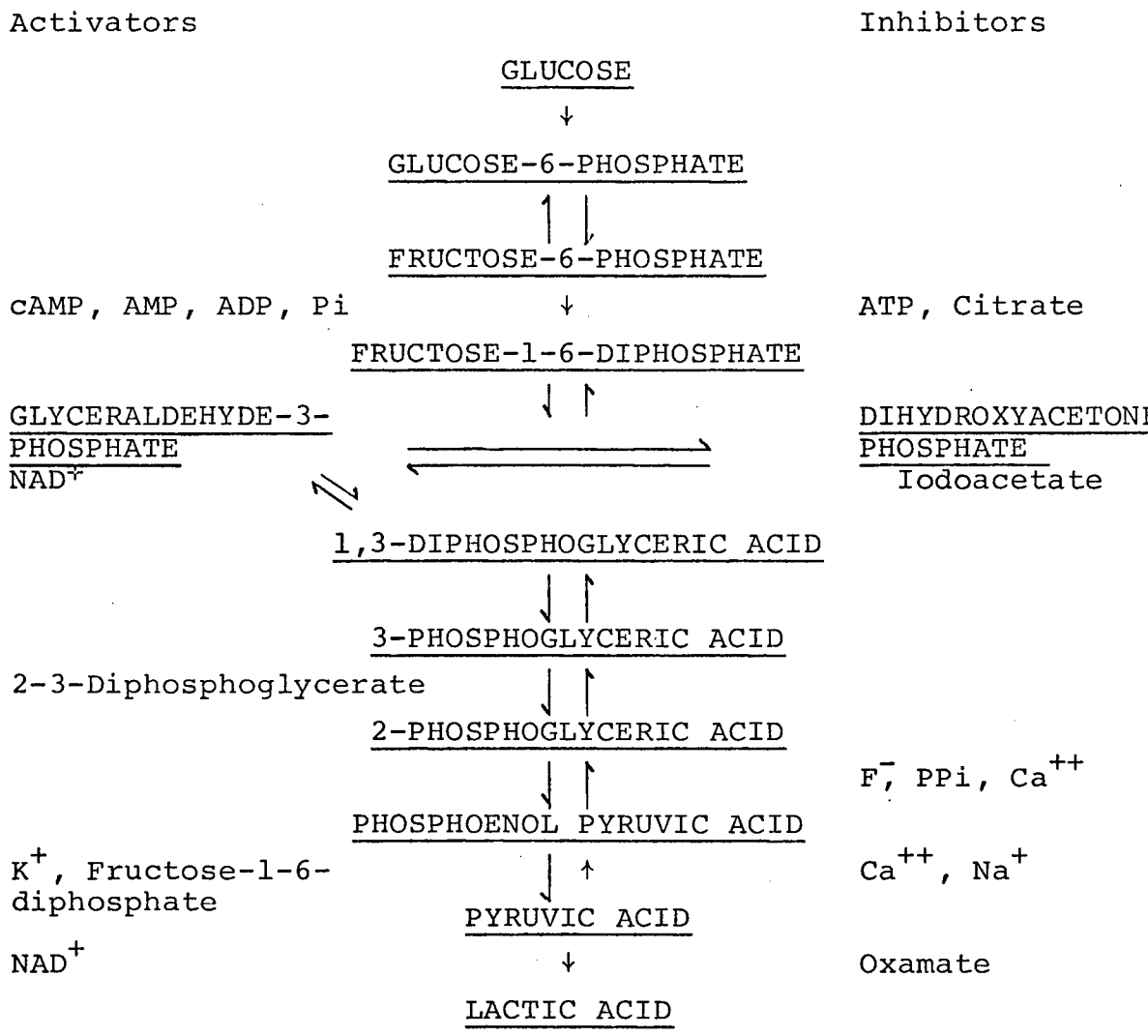
of individual cells and part of cells.

(i) Properties of Some Enzymes involved in Glycolysis of the Brain

(a) Hexokinase: Brain hexokinase (HK) requires ATP and $Mg^{++}$ for its activity and it phosphorylates several sugar substrates including glucose, fructose and mannose as well as D-glucosamine and 2-deoxyglucose[26]. It is inhibited by glucose 6-phosphate (G-6-P) and ADP[27-29]. G-6-P acts as a non-competitive inhibitor and is thought to play a regulatory role. G-6-P and ADP inhibit HK activity by competing for ATP site. G-6-P inhibition may be partially relieved by phosphate ions. Km for glucose is in the range of 0.04-0.1 mM[18]. However, enzymes with higher Km have been reported from sheep brain[32] and human brain[33]. Thompson and Bachelard[34] were able to separate both mitochondrial HK and cytoplasmic HK into two fractions but could not detect any major difference between the activities of the mitochondrial and cytoplasmic enzymes.

(b) Phosphofructokinase: Phosphofructokinase (PFK) from brain, as well as from a variety of tissues, has been extensively studied. This enzyme catalyzes the transfer of the terminal group of ATP to fructose 6-phosphate (F-6-P) in the presence of $Mg^{++}$. PFK acts specifically on F-6-P and does not catalyze any other transfer reaction. It shows kinetic properties which may account for its rate limiting effects on the glycolysis in brain. Whilst ATP and citrate are strong inhibitors of PFK, the enzyme is stimulated by F-6-P, ADP, AMP, $NH_4^+$, phosphate ions as well as cyclic AMP (see Lowry and Passonneau[35] and Balázs[17]). Lowry and Passonneau[35] have proposed a model for brain PFK that

Scheme 1 : THE GLYCOLYTIC PATHWAY



may account for a number of kinetic properties of the enzyme. They suggest that the enzyme possess at least seven, and possibly as many as twelve, separate sites for combination with inhibitors or activators. Their arrangement on the enzyme are such that binding of one inhibitor may lead to greater affinity of other inhibitors; the activators may also act synergistically: these properties of the enzyme are thought to play an important role in the regulation of glycolysis (see later in this section).

(c) Pyruvate Kinase: Pyruvate kinase (PK), which is one of the most important of the glycolytic enzymes, catalyzes the conversion of phosphoenol pyruvate (PEP) to pyruvate. Its activity in the brain, as compared to other tissues, is very high. One of the interesting properties of this enzyme is that its activity is stimulated by $K^+$, $NH_4^+$ and $Rb^{++}$ (see Axelrod[36]) and is inhibited by $Na^+$ [37-39].

(d) Lactic Dehydrogenase: Lactic dehydrogenase (LDH) is present in the brain at a very high activity[17]: there is very little pyruvate accumulation in the isolated incubated brain cortex or in vivo even during convulsions, which shows the great capacity of this enzyme[40]. LDH from brain is a tetramer and is present in several different molecular forms (isozymes)[17]. In adult mammalian brain, type I (heart type, H) is the dominant form while type V (muscle type, M) and hybrids of different subunits (H and M type) are present to a lesser extent. Excess pyruvate inhibits H type at lower concentrations than M type[17]. There is a progressive change in the LDH isozyme pattern during brain maturation which correlates with the resistance of the animal to anoxia[41].

(ii) Localization of Glycolytic Enzymes in the Brain

The glycolytic enzymes, with the exception of HK, are almost entirely present in the supernatant fractions obtained by high speed centrifugation of brain homogenates (see Quastel [19]). Several groups of workers have studied the distribution of glycolytic enzymes in the sub-cellular preparations of brain [42-45]. Cerebral HK occurs partly in the cytoplasm and partly bound to mitochondria[30,46]. However, sufficient cytoplasmic HK is available to account for normal rates of glycolysis[34]. Particulate HK activity is exclusively mitochondrial and varies from 30 to over 75 percent of the total activity[43-44,46].

Earlier workers have shown that some glycolytic activity of rat brain homogenates is associated with the mitochondrial fraction[43]. Such initial observations are related to the presence of nerve ending particles, which contains glycolytic enzymes, in this fraction[17]. Abood, Brunngraber and Taylor[49] have shown that, with hypertonic sucrose, it is possible to obtain a fraction rich in mitochondria which is almost devoid of glycolytic activity. Tanaka and Abood[45] established that the presence of glycolysis in the mitochondrial fraction, prepared conventionally from rat brain, may be due largely to contamination of this fraction with other cell components.

Studies have been done on the distribution of glycolytic enzymes in different areas of brain and in the various layers of cerebral and cerebellar cortex, and Ammons' horn. These investigations show that the contents of glycolytic enzymes vary in different parts of the brain.

(iii) Controls of Carbohydrate Metabolism in the Brain

The rate of glucose utilization in cerebral tissue *in vitro* is much less than that *in vivo*[50]. Even *in vitro*, under anoxia, the rate of glucose utilization is much less than the maximum possible by the enzymes involved. From this it is evident that the various rates must be subject to control by individual regulatory mechanisms. Most of the regulatory processes in cerebral glycolysis have been investigated with the use of brain cortex slices *in vitro*. Glycolytic rates in the cerebral tissues are controlled by two different mechanisms: (1) regulation of the concentrations of metabolic intermediates through control of enzyme activities and (2) regulation of the ionic contents.

(a) Pasteur Effect: The rate of oxygen consumption in brain slices can account for the oxidation of approximately 0.4 mmole lactate $Kg^{-1}$ $min^{-1}$. Since, under anoxia, approximately 1 mmole lactate $Kg^{-1}min^{-1}$ is formed, it would be expected that aerobic lactate production would be less than 1 mmole $Kg^{-1}min^{-1}$. However, the actual rate is only 10-20 percent of this value. The phenomena of inhibition of glycolysis by respiration is usually called the Pasteur effect.

The process of respiration alone is not, however, responsible for the Pasteur effect. It is possible to increase the aerobic rate of lactate production while still maintaining the respiratory rate at a constant value. Under these conditions there is found to be a decrease in the ATP content of the tissue while the contents of ADP, AMP and Pi increase. These changes in the concentrations are considered to bring about an increased rate of glycolysis by stimulation of some of the glycolytic

enzymes (see later). Racker[31] has, in fact, concluded that inhibition of PFK by ATP together with the rate limiting concentrations of ADP and Pi may account for the Pasteur effect.

(b) Regulation of Glycolytic Enzymes: Lowry and his coworkers[6,25] have studied the effects of ischemia on the amount of the known substrates and cofactors of the glycolytic pathway in brain in vivo. Comparison of the steady state levels of intermediates found under aerobic conditions with those found under anoxia led to the conclusion that the changes resulted largely from acceleration of the PFK step. This was attributed to the increase in ADP, AMP and Pi that are capable of overcoming the ATP inhibition of PFK. Kinetic evidence also provided supporting evidence for the controlling roles of HK and PFK.

Rolleston and Newsholme[51,52] studied the control of glycolysis in guinea pig cerebral cortex slices. They correlated the changes in concentrations of substrates for enzymes catalyzing "non-equilibrium" reactions with the changes in rates of glycolysis caused by alteration of the conditions of incubation. They concluded that HK, PFK, PK and possibly glyceraldehyde 3-phosphate dehydrogenase (G-3-PDH) are subject to metabolic control in the cerebral cortex slices. They further suggested that HK and PFK together form a regulatory system. It has been known for a long time that ATP inhibits PFK and that this inhibition is relieved by AMP[35,53-56]. Because of the presence of adenylate kinase, a small change in the ATP concentration may cause large increase in the concentration of AMP. Rolleston and Newsholme[51,52] pointed out that any condition decreasing the concentration of ATP will tend to increase the activity of PFK

while the changes in AMP concentration will amplify the effect of ATP. The resultant increased activity of PFK will tend to decrease the concentration of F-6-P which is itself an inhibitor of HK. Thus, according to these workers, the activity of HK and PFK are linked together in a regulatory system.

The controlling role of PFK has been established in a wide variety of tissues[35]. It has been already mentioned that a wide variety of compounds affect PFK activity. Inhibition of PFK by citrate may result in a modifying affect of the citric acid cycle on the rate of glycolysis[56]. The $NH_4^+$ formed during anoxia, or during electrical activity, may be expected to result in increased PFK activity[35].

The rate of glycolysis may also be affected by the concentration of phosphate in the medium[18,31b,50]. Lactate formation in cerebral extracts is dependent upon the presence of added phosphate and is proportional to the phosphate concentration up to 15-20 mM[50]. Further, phosphate is a substrate for G-3-PDH and it is an activator of PFK as well as HK[30,35].

It has been known for a long time that brain HK is sensitive to inhibition by ADP[27,29] and by G-6-P[28]. Inhibition of HK by G-6-P is non-competitive[28] and can be relieved by phosphate[30,31a,57]. G-6-P, by itself, is unlikely to suppress the enzymatic activity in the brain cell to the extent it is required because the rate of glycolysis is only 1-3% of the maximal rate possible, as calculated from the total HK activity, or about 5-6% of the maximal rate possible from the amount of HK found in the cytoplasm[18]. G-6-P, at the concentrations present in vivo, produce about 50-70% inhibition of HK under optimal

conditions[28,30,31a]. From the experimental data on the degrees of inhibition of HK by G-6-P and ADP, it is quite clear that the total inhibition of HK by these substances is not sufficient to account for the observed cerebral glycolytic rates. The localization of the enzymes and inhibitors in the brain cell may be just as important as their quantities for an explanation of the regulation of cerebral glycolysis.

It has already been pointed out that the activity of PK is greatly affected by the presence of cations, notably $K^+$ and $Na^+$: $K^+$ being stimulatory and $Na^+$ being inhibitory. $Ca^{++}$ is also known to inhibit the activity of PK, both in the brain and other mammalian tissues[58,59]. Bygrave[60] observed that the inhibition of glycolysis by added $Ca^{++}$ in extracts of Ehrlich ascites cells is largely accounted for by the inhibition of PK activity due to competition with $Mg^{++}$ and $K^+$. Considering the effects of cations on PK activity in cerebral tissues, Takagaki[59] concluded that in the presence of $K^+$ concentration normally found in the cerebral tissue, PK is fully active and a small change in the concentration of $K^+$ in this range, may not change the enzyme activity. However, the concentration of $Ca^{++}$ (1.3mM)[61], which inhibits PK *in vitro*, is similar to that found in the intact tissue and, therefore, small changes in $Ca^{++}$ concentration may bring about large changes in the enzyme activity. Takagaki[59] has further concluded that $Ca^{++}$ in the aerobically incubated cerebral tissue may, in part, be responsible for the presence of PK in a highly inhibited state, in addition to the cellular organization of the slices.

(c) Effects of Cation Contents and of Electrical Stimulation on Glycolysis in Cerebral Cortex Slices:

Quastel[19] has reviewed the effects of various ions on glycolysis in brain and has pointed out that the effects of cations must be interpreted in the light of two fold action - ion transport changes at the cell membrane causing changed ion concentration in the cell, and direct effects on the glycolytic process itself.

It has been known for a long time that carbohydrate metabolism of isolated brain tissue in vitro is dependent on the ionic environment. As early as in 1935, Ashford and Dixon [62] found that the presence of 100mM $K^+$ increase both the rate of oxygen consumption and of aerobic glycolysis of rabbit brain cortex slices, respiring in a Ringer medium at $37^o$. Dickens and Greville[63] further studied the effect of neutral salts on respiration and aerobic glycolysis of rat brain cortex slices. They[63] confirmed the observations of Ashford and Dixon and in addition found that omitting $Ca^{++}$ from the medium increases the rate of respiration. The effect of $Ca^{++}$ in lowering the respiration could be overcome by using high concentrations of $K^+$ (up to 100mM). With $Na^+$ as the only cation, the rate of aerobic glycolysis is increased but that of anaerobic glycolysis is suppressed. Dickens and Greville were unable to explain clearly the effect of neutral salts on aerobic glycolysis.

With cell-free brain preparations, Racker and Krimsky[64] observed that $Na^+$ is a strong inhibitor of respiration and glycolysis (see Utter[37]). Takagaki and Tsukada[65] studied the effects of 100mM $K^+$ on cerebral cortex slices metabolizing in a $Na^+$-free medium. They observed that in response to 100mM $K^+$, there is no increase in the rate of oxygen uptake, lactic acid formation or glucose utilization, although in the usual medium

such an increase in $K^+$ increased the metabolism considerably. When $Na^+$ is omitted from the medium, the oxygen uptake is not changed but the aerobic lactic acid formation and glucose utilization increase significantly; the omission of $K^+$ causes an increase in the glucose utilization and the lactic acid formation, while the respiration is not changed.

Anaerobic glycolysis in brain slices is increased if $Na^+$ is replaced by choline or $K^+$ but is slightly depressed in a $Li^+$ medium[66]. In contrast to aerobic glycolysis, anaerobic glycolysis is depressed by the addition of high $K^+$ to a Ringer medium[62].

The effect of $K^+$, under aerobic conditions, on the metabolism of cerebral cortex slices may be partly due to the fact that $K^+$ stimulates the interaction of PEP and ADP[67] and partly due to its stimulation of the activity of membrane ATPase, causing an increase in the concentration of ADP and phosphate[19]. The effect of high $K^+$ may also partly be the result of a diminished cell level of ATP. Brain slices metabolizing glucose or pyruvate in a normal balanced media maintain high concentration of phosphocreatine. In the presence of high $K^+$, the content of phosphocreatine is diminished[68]. Findlay, Magee and Rossiter[69] found that high $K^+$ inhibits the incorporation of $Pi^{32}$ in the various phosphate fraction of the brain cortex slices.

In 1937, Quastel and Wheatley[70] observed that the rate of anaerobic glycolysis of brain cortex slices is markedly stimulated by the addition of $Ca^{++}$, and to a lesser extent by $Mg^{++}$, to a medium in which $Na^+$ and $K^+$ are the only cations.

Adams and Quastel[71] made a detailed study of the effects of $Ca^{++}$ on the anaerobic glycolysis of guinea pig cerebral cortex and of tumour slices. They[71] confirmed the observations of Quastel and Wheatley and further observed that the inhibitory effect on the rate of brain glycolysis established in the absence of $Ca^{++}$ from the medium, in which the brain slices are suspended, is largely overcome by decreasing the pH to 7.0. The same effect is obtained by subjecting the brain slices to oxygenation after a period of anerobic treatment. This was thought to be due to pyruvate accumulation during the the period of oxygenation [71] - pyruvate being a well known accelerator of cerebral glycolysis. Quastel[19] suggested that it is quite possible that the diminished rate of cerebral anaerobic glycolysis resulting from the absence of $Ca^{++}$ is due to an inflow of $Na^{+}$ into the brain cells with corresponding suppression of glycolytic rate.

A number of organic bases such as pyrrole, pyridine and aniline accelerate anaerobic brain glycolysis in a $Ca^{++}$-free medium[71]. Their efficiency in replacing $Ca^{++}$ for brain anaerobic glycolysis can be, in general, correlated with their dissociation constants[71].

Studying aerobic glycolysis in the cerebral cortex slices, Takagaki[59] observed that $Ca^{++}$ inhibits the activities of many glycolytic enzymes including four controlling enzymes, namely HK, PFK, G-3-PDH and PK. It has already been mentioned that $Ca^{++}$ has inhibitory effect on the glycolysis of Ehrlich ascites tumour cell extracts[60] while they have no effect on the glycolysis of intact tumour cells[60,71].

Application of electrical impulses to the nerve or

cerebral tissue leads to well known movements of cations across the cell membrane. Even a few minutes excitation leads to 2-3 fold increase in the $Na^+$ content. This results in greater activity of $Na^+$, $K^+$ ATPase and hence greater utilization of ATP. There is a fall in the cell level of ATP and increase in ADP. As a result, during electrical stimulation of the tissue slices, respiration as well as aerobic glycolysis is increased. On the other hand anaerobic glycolysis is suppressed by electrical stimulation.[50]

1.3 EFFECTS OF LOCAL ANESTHETICS

Much of this thesis is concerned with the effects of tetrodotoxin (TTX) on brain metabolism. TTX has potent local anesthetic effects[128]. In this section the mode of action and the effects of local anesthetics on the nervous system will be discussed. As the primary action of the local anesthetic is the blockade of nerve impulse conduction, a short account of the transmission of the nerve impulse will follow. Effects of TTX will be discussed in a separate section.

(i) Transmission of the Nerve Impulse:

Electrochemical aspects of excitation has been extensively reviewed[72-74]. In excitable as well as in other cells, there is a steady difference of potential between the inner and outer part of the cell (the resting potential). This is in contrast to the transitory changes in this potential during propagation of a nerve impulse (the action potential). The potential difference set up across the neuronal membrane is the result of the difference in the concentration of $Na^+$ and $K^+$ across the membrane. For the cat motoneurons, the concentration of $K^+$

inside the neurons is 27 times higher than that outside while the concentrations of $Na^+$ is 10 times higher outside than that inside[75].

The resting membrane is at least 50 times more permeable to $K^+$ than to $Na^+$. During the generation of an action potential, the nerve becomes more permeable to $Na^+$. As a result $Na^+$ flushes inside the cell and almost at the same magnitude $K^+$ moves outside the cell. Hodgkin[76] has calculated that $Na^+$ and $K^+$ movements associated with the electrical activity of the unmyelinated nerve fibers are in the range of $3.5\text{-}4.5 \times 10^{-12}$M per $cm^2$. The changes in the membrane permeability during the generation of an action potential have been visualized as resulting from a change in the pore size of certain channels[77]. Using TTX as a tool, it has been calculated that there are about 13, or fewer, $Na^+$ channels per $\mu^2$ of the membrane surface[78,79]. It is still uncertain whether $Na^+$ and $K^+$ traverse the same channels[75]. $Na^+$ and $K^+$channels can be differentially blocked[75].

(ii) Calcium ions and Excitation:

It has been known for a long time that nerves fire spontaneously when the concentration of $Ca^{++}$ is reduced and this has led to the suggestion that the increase in the $Na^+$ permeability occurs because depolarization removes $Ca^{++}$ from sites or carriers in the membrane[80,81]. One can visualize that $Na^+$ cross the membrane through special channels which are blocked in the presence of $Ca^{++}$ [80].

On the other hand, if a nerve is bathed in a solution containing higher than normal $Ca^{++}$ concentration, the firing threshold is raised and the nerve may even become inexcitable[75].

It has been proposed that $Ca^{++}$ may be thought of as blocking transmembrane channels to $Na^{+}$ by loose binding to the polar heads of the external phospholipids layer; dislocation of $Ca^{++}$ from this bond would then permit rotational movement of the polar head and free passage of $Na^{+}$ [82,83].

(iii) The Sodium Pump:

As has been discussed earlier in this chapter, the generation of an impulse results in the influx of $Na^{+}$ and efflux of $K^{+}$ from the nerve cell. After the conduction of the nerve impulse, the resting potential is re-established by movement of $Na^{+}$ to outside the nerve cell, and of $K^{+}$ to inside of the cell. This is achieved by the so called "Sodium Pump" which utilizes ATP to eject $Na^{+}$ and return $K^{+}$. It is believed that the sodium pump is operated by $Na^{+}$, $K^{+}$-activated ATPase which is present in the cell membrane[84]. This will be discussed later in detail.

(iv) Site and Mode of Action of Local Anesthetics:

There is very little doubt that site of action of local anesthetics is at the nerve membrane[85]. Thus when an isolated nerve membrane is bathed with a local anesthetic solution, the action potential becomes smaller and smaller and eventually disappears. It reappears only when the drug is washed out[75]. Further, the external supporting structure of the fiber, its sheath and Schwann cell, is not required for the blocking effect. The local anesthetics may in fact block the impulse conduction quicker in unstripped nerves[86]. In contrast to non-myelinated fibers, the myelinated fibers permit access to local anesthetics only at the nodes of Ranvier; this exposure is still sufficient

to block impulse conduction[87,88].

It is generally believed that the local anesthetics block the conduction by interfering with the process fundamental to the generation of an action potential, namely the large transient rise in the permeability of the membranes to $Na^+$, which arises on depolarization of the membrane[89]. It has been well established that the effect is the result of reduction in the carrying capacity of the system to $Na^+$ during an action potential[90,96]. Local anesthetics also reduce the increase in $K^+$ conductance but the effect on $K^+$ conductance is much less than that on the $Na^+$ conductance[90,91,97,98]. Increasing anesthetic concentration has an increasing effect on the $K^+$ conductance[99]. Thus, 0.5mM lidocaine has no effect on the $K^+$ conductance but 3.5mM lidocaine lowers it to 75% of its original value; lidocaine is 100 times more effective on $Na^+$ channels than on $K^+$ channels.

The effects of local anesthetics on the $Na^+$ and $K^+$ conductance in excitable cells differ from those in inexcitable cells such as human red blood cells (RBC)[100]. High concentration of the local anesthetic causes hemolysis and it has been suggested that local anesthetic base makes the red cell membrane more pervious. In addition to nerve fibers, local anesthetics block the movements of $Na^+$ and $K^+$ in the membrane of muscle (both in the resting state and during generation of the action potential), frog skin and other tissues[89].

A number of naturally occurring substances such as acetylcholine, ATP, $Ca^{++}$, and thiamine have been thought to play key roles in the conduction of the nerve impulse and there have

been a number of reports on interaction of local anesthetics with the above agents. However, as Ritchie and Greengard[89] have pointed out, any theory on the mode of action of local anesthetics, based on an antagonism between local anesthetics and a naturally occurring substance, is weakened by the very diversity of the structures capable of antagonizing local anesthetic activity.

In many respects local anesthetics behave like $Ca^{++}$ in stabilizing the excitable membranes. They increase the time constants for the rise and fall of $Na^{+}$ conductance[102]. In addition to increasing the excitation threshold, if sufficient quantity is present, the local anesthetic will block the conductance completely with no change in the resting potential [72,73,91,103]. It has been proposed that the local anesthetic and $Ca^{++}$ both act on the same system which is responsible for carrying the $Na^{+}$ through the nerve membrane[104]. In frog desheathed myelinated nerves, depolarization in the absence of $Ca^{++}$ can be prevented by local anesthetics[105,106]. Blaustein and Goldman[92] have shown that, in lobster giant axons, $Ca^{++}$ and local anesthetics compete for the same site on the membrane. On the other hand Feistein[107] has proposed that local anesthetics may act primarily by inhibiting release of $Ca^{++}$ from the sites to which it is bound in the membrane. In this way, local anesthetics may prevent the secondary changes in the $Na^{+}$ and $K^{+}$ permeability, and thus prevent propagation of the wave of excitation along the cell membrane.

There is still a controversy as to whether the undissociated molecular form of the drug or its cationic form is

responsible for the nerve blocking action. De Jong[75] concluded that in all studies in which the nerve sheath was left intact, local anesthetics were found to be most effective in alkaline solution where the base predominates, whereas in most studies in which the nerve sheath was removed, anesthetics were more effective in neutral or slightly acid solution where the cation predominates.

(v) Biochemical Effects of Local Anesthetics:

Several studies have been carried out on the effects of local anesthetics on the metabolism of isolated nervous tissue as well as on that of homogenates. As early as in 1919, Niwa[108] showed that cocaine depressed carbon dioxide production in the sciatic nerve of the frog. Sherif[109] showed that cocaine and procaine inhibited respiration in the sciatic nerves of the rabbit. These studies led Watt[110] to study the effects of local anesthetics on the respiration of brain homogenates. He observed that a number of local anesthetics such as nupercaine, butacaine, tetracaine, metycaine, cocaine and procaine inhibited the oxidation of succinate and glucose by rat brain homogenates. Of these drugs, nupercaine was the most effective. In order to show that the inhibition of respiration was not due to the inhibition of some of the glycolytic enzymes, Watt[110] studied the effect of these drugs on the anaerobic glycolysis of rat brain homogenates and showed that they had no inhibitory effect in this system; in contrast, tetracaine and nupercaine stimulated the anaerobic glycolysis. Watt was unable to give any explanation for this and stated that the increase in the anaerobic glycolysis

might be due to the maintenance of the original high rate of glycolysis by some unexplained mechanism. He concluded that the inhibition of respiration by local anesthetics was due to inhibition possibly at the oxidation of cytochrome c-cytochrome oxidase stage.

Geddes and Quastel[111] observed that the local anesthetics procaine, lidocaine, tetracaine and dibucaine, at pharmacologically active concentrations, inhibit the $K^+$ stimulated respiration of rat brain cortex slices in the presence of glucose. They had little or no effect at these concentrations on the resting or unstimulated brain cortex respiration. Results of these workers further indicated that the potencies of these drugs as inhibitors of $K^+$ stimulation of brain cortex parallel their anesthetic activities.

Fink, Kenny and Simpson[112] found that the rate of oxygen consumption of a suspension of mouse heteroploid cells was depressed by volatile, local and barbiturate anesthetics. They compared their results with that of Geddes and Quastel[111] and concluded that the sensitivity of the respiration of the mouse cell system to local anesthetics is as great as that of the brain slices. Ryman and Walsh[113] find that certain local anesthetics (e.g., cocaine) may inhibit the condensing enzyme, blocking the entry of active acetate into the citric acid cycle. The fact that local anesthetics have little action on the metabolism of the resting nerve makes it likely that their effects are exerted by their block of ion movement in the stimulated nerve.

## 1.4 EFFECTS OF TETRODOTOXIN ON THE NERVOUS SYSTEM

TTX is one of the most toxic non-protein substances known to man and has a mechanism of action which seems very similar to local anesthetics but has potency of more than 100,000 times that of cocaine[114]. TTX is found in the Japanese puffer fish and in the Californian newt. Its chemistry and pharmacology have been reviewed[114-116].

TTX has a variety of pharmacological actions *in vivo* such as the depression of respiration leading to respiratory failure, paralysis of skeletal muscle, suppression of stimulus evoked responses such as spinal reflexes and hypotensive effects. These aspects have been discussed in detail by Kao[115]. As far as the effect of TTX *in vitro* is concerned, there is now sufficient evidence to show that it acts on electrically excitable tissues such as the nerve fibers and the muscles. In these cells, the generation of the action potential is abolished by TTX. In fact, block of the generation of the action potential is considered to be the main property of TTX.

(i) Action of TTX on the Neuromuscular Junction and on the Isolated Nerve Preparations:

Furukawa, Sasooka and Hosoya[117] studied the effects of TTX on the neuromuscular junction on the frog nerve sartorius preparation. They showed that response of the end plate region to acetylcholine is not affected by TTX at concentrations high enough to block the action potential. This study

was followed by the work of Narahasi, Deguchi, Urakawa and Ohkubo[118] in which they analysed the mode of action of TTX on frog muscle fiber membrane. With the aid of intracellular microelectrodes, they observed that TTX, at $10^{-7}$M concentration, make the applied cathodal current ineffective in producing action potential whereas the resting potential and resting membrane resistance undergoes little or no change. The authors concluded that in the presence of TTX the membrane is stabilized by inactivation of the $Na^+$ carrying mechanism. However, it remained for Narahashi, Moore and Scott[119] to do voltage clamp experiments with lobster giant axons to verify the above hypothesis. These studies confirmed the fact that TTX, at a very low concentration, blocks the action potential production through its selective inhibition of the $Na^+$ carrying mechanism and showed that it has no effect on the $K^+$ carrying mechanism. These findings have been further extended by a number of workers[122-125].

Kao and Fuhrman[120] had shown that tarichotoxin (which has now been shown to be identical with TTX) exerted a strong nerve blocking action on the frog sciatic nerves. Takata, Moore, Kao and Fuhrman[121] extended the studies of Narahashi[119] et al. with tarichotoxin and, in addition, observed that a high concentration of $Ca^{++}$, applied concomitantly with the toxin, significantly improves the reversibility of the $Na^+$ blocking and thus gives some protection against the toxin.

There seems to be little doubt that TTX acts on the outer rather than the internal surface of the axon[124,127], its effect being to block the inward movement of $Na^+$ accompanying

the generation of action potential.

Pullman, Lavender and Aho[126] studied the direct renal effects of TTX in dog. Tetrodotoxin was infused in dilute solution directly into one renal artery and caused highly significant differential increase in $K^+$ and $Mg^{++}$ excretions.

Using crayfish abdominal nerve fibers, Ogura and Mori [128] have shown that in alkaline solution TTX is more effective in the sheathed preparation, and in neutral solution it was more effective in the desheathed one, they suggested that the cationic form, due to guanidyl group, is the active form of TTX and that TTX penetrates nervous tissue more rapidly in its uncharged form.

(ii) Effects of TTX on the Excitability, Cation Content and Metabolism of Isolated Cerebral Tissues:

Until recently most of the experiments with TTX were concerned either with in vivo effects or with electrophysiological effects on the isolated nerves and muscle. In 1967, almost simultaneously, results of two studies appeared which opened the possibility of using TTX as a tool to study the metabolism and transport phenomenon in the isolated cerebral tissues.

Chan and Quastel[130] showed that 3 μM TTX inhibits the respiratory increase of the rat brain cortex slices that takes place upon the application of electrical impulses but has no effect on the $K^+$ stimulated respiration. They further observed that TTX also inhibits increase in the rate of respiration that

occurs when $Ca^{++}$ is omitted from the incubation medium. Moreover, their results demonstrated that the inhibition of acetate oxidation in brain slices by electrical impulses, due to the influx of $Na^{+}$, is completely blocked by TTX at small concentrations. Chan and Quastel concluded that TTX blocks the influx of $Na^{+}$ during electrical stimulation and that this is responsible for the potent metabolic effects of TTX on isolated brain.

McIlwain[129] similarly found that TTX inhibits the metabolic responses induced by electrical stimulation in the guinea pig cerebral cortex slices. He also showed that large increase in the respiration due to electrical stimulation are greatly diminished by 0.4 μM TTX but it has no effect on the respiration of the unstimulated tissue; $K^{+}$ stimulated respiration was found to be insensitive to TTX. Similar effects were observed with aerobic glycolysis. The $K^{+}$ content of the electrically stimulated slices is greater in the presence of TTX[129]. Because TTX is a guanidine derivative, McIlwain[129] studied a number of guanidine derivatives and showed that only some of them are as effective as TTX, but at much higher concentrations.

Swanson[131] studied the effects of TTX on the electrically stimulated guinea pig cerebral cortex slices to see if it affects the cationic shifts which normally take place with electrical stimulation. Cerebral cortex slices lose non-inulin $K^{+}$ and creatine phosphate, and gain non-inulin $Na^{+}$ during electrical stimulation. These effects were shown to be prevented

by 1 μM TTX. His experiments strongly support the conclusion that TTX directly interferes with the passive downhill movement of both $Na^+$ and $K^+$, which occur upon electrical stimulation.

It is well known that glutamate causes excitation of the nervous tissue[50]. Electrical stimulation or the addition of 5mM L-glutamate causes an influx of $Na^+$ into the incubated cerebral cortex slices[132]. McIlwain, Harvey and Rodriguez[134] confirmed that TTX almost completely prevents increase in $Na^+$, induced electrically, and that it partly inhibits, during a short initial period, the $Na^+$ influx induced by glutamate. Extrusion of $Na^+$, following electrical stimulation, is unaffected by TTX. These studies were further extended by Pull, McIlwain and Ramsay[133], who considered the possibility of glutamate acting in a way similar to chelating agents. They[133] observed that $Ca^{++}$ act synergistically with TTX in restricting the cation movements on the addition of glutamates. 5mM EDTA caused an increase in intracellular $Na^+$ and a decrease in $K^+$, and these changes were partially blocked by TTX.

Ramsay and McIlwain[134] studied the effects of TTX on $Ca^{++}$ movement in incubated guinea pig cerebral cortex slices induced by glutamate. They found that very low concentrations of TTX (66-330nM) are capable of inhibiting $^{45}Ca$ influx, both in the presence and absence of L-glutamate. TTX also caused a detectable diminution of $Ca^{++}$ efflux.

Okamoto and Quastel[135] reported that $Na^+$ influx and water uptake in rat cerebral cortex slices in the presence of 0.1 mM ouabain, or 10 μM protoveratrine, or electrical stimu-

lation or the absence of glucose is partially or wholly suppressed by 3 μM TTX. On the other hand, TTX has no effect on the $Na^+$ influx and water uptake in the presence of 30 μM dinitrophenol or of 100 mM $K^+$.

Itokawa and Cooper[136] showed that perfusion with TTX (as well as with ouabain and LSD-25) at low concentrations, promoted release of radioactive thiamine from the spinal cords and the sciatic nerves of bull-frogs and rats which had been earlier injected with $S^{35}$-thiamine.

1.5 EFFECTS OF OTHER NEUROTROPIC DRUGS ON THE NERVOUS SYSTEM

(i) Ouabain.

(a) Inhibition of $Na^+, K^+$-ATPase by Ouabain:

It has been previously stated that the process by which $Na^+$ is extruded from the cell and $K^+$ is accumulated inside the cell is described as the "Sodium Pump". It has been well established that the transfer of $Na^+$ and $K^+$, which takes place through the membrane against a concentration gradient, is energy dependent[137]. Since Skou[138,139] showed that in the peripheral nerve of crab, an ATPase is present which requires both $Na^+$ and $K^+$ for activation and this enzyme is inhibited by the cardiac glycoside ouabain, there is overwhelming evidence in support of the controlling role of this enzyme in the transport processes[137,140-145]. This ATPase and the transport mechanism for $Na^+$ and $K^+$ have a number of features in common such as their localization, in the cell, their properties of energy utilization, activation by $Na^+$ and $K^+$, and ouabain

inhibition[137].

As early as in 1953, Schatzman[146] showed that cardiac glycosides at low concentrations are specific inhibitors of cation transport. This was later confirmed by a number of workers[147-149]. Ouabain seems to act on the phosphorylated form of the $Na^+$, $K^+$-ATPase, which appear to act as an intermediate during the operation of this enzyme[150-158]. $Na^+$ activate the formation of the phosphorylated intermediate while $K^+$ stimulate its breakdown. The action of $K^+$ is prevented by ouabain. There is a stochiometric relation between the transport of $Na^+$ and $K^+$. Thus in erythrocytes 3 $Na^+$ is transported to the outside and 2 $K^+$ is transported inside the cell for each molecule of ATP hydrolyzed[159-162].

The $Na^+$, $K^+$-ATPase activity in the cerebral cortex is very high[163]. Yoshida, Nukada and Fujisawa[164] showed that 0.01mM ouabain causes an almost complete block of uptake of $K^+$ and extrusion of $Na^+$ from the guinea pig cerebral cortex slices.

In recent years many studies have been carried out concerning the binding of ouabain and other cardiac glycosides to the $Na^+$, $K^+$-ATPase. Schwartz, Matsui and Laughter[165] studied the binding of tritiated digoxin to the heart muscle enzyme and concluded that the conformational state of the enzyme is probably of primary significance in glycoside binding. Their data support the concept of an

"allosteric type" of enzyme and they state that the formation of phosphorylated enzyme may be one of a number of ways in which the conformational nature of the enzyme may be altered. Charnock and Potter[166] using enzyme from the guinea pig cortex concluded that ouabain may inhibit both phosphorylation and dephosphorylation of the enzyme depending on the nature and amount of the cations present. Yoda and Hokin [167] observed that the binding of cardiac glycosides to the beef brain enzyme is irreversible. Their results indicated that the sugar in glycoside linkage with the 3 position of the steroid plays an important role in the irreversible binding. Other studies[156,168] indicate that the enzyme can exist in several states, and that whether the binding of ouabain is reversible or irreversible depends on the temperature and other factors.

(b) Effects of Ouabain and Cations on $Na^+$, $K^+$-ATPase in Relation to Brain Metabolism and Transport:

As energy derived from glucose metabolism is utilized for $Na^+$ and $K^+$ transport, and as the activity of $Na^+$, $K^+$-ATPase is affected by the cation concentrations, this has led to the suggestion that $Na^+$, $K^+$-ATPase may be involved in the regulation of respiration in the cerebral

cortex slices. Whittam and Blond[169] found a parallelism between the activity of $Na^+$, $K^+$-ATPase and a part of the respiration of the incubated rat brain homogenates suggesting that the rate of energy production by respiration is geared to its rate of utilization. Gonda and Quastel[170] could find only little effect of ouabain on the respiration of unstimulated brain cortex slices but it partially suppressed the $K^+$ stimulated respiration, the amount of suppression depending on the concentration of ouabain. They observed a pronounced effect of ouabain on the rates of transformation of glucose into amino acids, and on amino acid and creatine transport in the cerebral cortex slices.

Using frog brain, dePiras and Zadunaisky[171] showed that the effects of ouabain on glucose metabolism differ according to the $K^+$ concentrations. At low concentrations of $K^+$, there is a small but consistent stimulation of oxygen uptake but the increase in respiration due to high $K^+$ is completely inhibited by ouabain. With guinea pig cerebral cortex slices, Swanson and McIlwain[172] observed that ouabain causes an initial increase in respiration but after 40-60 minutes respiration begin to fall. Creatine phosphate and ATP levels also fall after short periods of incubation with glycosides and these effects could be partially reversed by transferring the tissue to a fresh medium. There was also a great increase in the $Na^+$ and decrease in the $K^+$ content of the ouabain treated slices (cf. Yoshida *et al.*[164]). These effects are augmented by electrical stimulation and the recovery process is completely ineffective in the presence of ouabain[131]. Swanson[131] noted that in the presence of ouabain creatine phosphate levels

continue to drop after electrical stimulation has ceased.

Swanson[173] observed that the increased loss of $K^+$ and the rise in $Na^+$ content of the incubated guinea pig cerebral cortex slices in the presence of ouabain is most marked in a $Ca^{++}$-free medium. This was explained by the suggestion that access of ouabain to the $Na^+$, $K^+$-ATPase is more difficult when $Ca^{++}$ is present in the medium[131,173,174]. The loss of $K^+$, in the presence of ouabain, is also slow in a $Na^+$-free medium[175] (Choline Chloride was substituted for $Na^+$). These results led Swanson and Stahl[175] to conclude that in the presence of $Na^+$ there is a $Na^+$-induced structural change in the $Na^+$, $K^+$-ATPase which allows accessibility of ouabain to its site of inhibition.

Tower[176] showed that ouabain treated slices, and the mitochondrial fraction from such slices, have higher contents of $Ca^{++}$ than the controls. Stahl and Swanson[177] stated that the increased uptake of $Ca^{++}$ does not appear to be an artifact of preparation and might be due to increased membrane permeability or possibly to an inhibition of the active transport mechanism for extrusion of $Ca^{++}$.

Quastel[137], while reviewing the characteristics of cation transport and respiratory control in the brain, concluded that the fluxes at the brain cell membrane, as a result of various stimuli, may effect brain metabolism largely by their direct effect on the membrane bound ATPase. Nevertheless, it was pointed out that influx of $Na^+$ may suppress acetate oxidation by its inhibiting action on acetate conversion to acetyl CoA and that $K^+$ may affect the activity of PK.

Rolleston and Newsholme[52] observed that, in the presence of 0.1 mM ouabain, glucose utilization and aerobic lactic acid production by guinea pig cerebral cortex slices is increased. These effects are not observed with .001 mM ouabain. Measurement of the level of glycolytic intermediates in the presence of 0.1 mM ouabain led these workers to conclude that the addition of 0.1 mM ouabain to guinea pig cerebral cortex slices causes inhibition of either G-3-PDH or phosphoglycerate kinase, or both, in a manner independent of the known action of ouabain on $Na^+$, $K^+$-ATPase.

(ii) Protoveratrine

(a) Site and Mode of Action

Veratrum and related plants have been used for medicinal purposes for many years. They contain a number of hypertensive alkaloids, the first one to be obtained in crystalline preparation was protoveratrine. The major effect of protoveratrine is alteration in the permeabilities of membranes of a number of excitable cells.

Frank[178] observed that in the veratrine-treated muscle fibers, the membrane becomes permeable to $Na^+$ following an action potential and the enhanced inward movement of $Na^+$ considerably delays repolarization. Using crustacean axons, Wright and Tomita[179] concluded that veratrine action is the result of chemical or metabolic reaction by the alkaloid in the membrane. They suggested that veratrine may inhibit the $Na^+$ extrusion mechanism or may itself compete for sites in the membrane with $Ca^{++}$ or $Na^+$.

Shanes[73] suggested that an interaction takes place

between the alkaloids and membrane lipids which affect the ionic channels, affecting in turn the membrane permeability. However, Kupchan and Flacke[180] have pointed out that no specific "binding site" or "receptor" has been recognized for the veratrum alkaloids and that there are no specific antagonists known (cf. Kini and Quastel[186]).

(b) Biochemical Effects of Protoveratrine and Other Veratrum Alkaloids

Wollenberger[181] observed that veratrum alkaloids (protoveratrine and veratridine) produce significant stimulation of respiration lasting for several hours in the incubated guinea pig cerebral cortex slices. They increase aerobic glycolysis to the normal anaerobic level while inhibiting the anaerobic glycolysis. These effects are characteristic of the brain and resemble the effects of high $K^+$ reported by Ashford and Dixon[62]. Omission of $K^+$ from the medium gives rise to strong aerobic glycolysis which is not enhanced further by protoveratrine[181]. The inhibition of anaerobic glycolysis may be partially prevented by the presence of nicotinamide[182]. The net uptake of $K^+$ by washed cerebral cortex slices is inhibited by protoveratrine at concentrations which stimulate respiration and aerobic glycolysis[182].

Wollenberger[183] studied the effects of protoveratrine on electrically stimulated cerebral cortex slices and noted that simultaneous exposure of cerebral cortex slices to protoveratrine and to brief condenser pulses bring about an increase in the rate of respiration which is greater than the sum of the increases caused by the drug and by the pulses individually.

Quastel[184,185] has noted that protoveratrine stimulation of rat brain respiration is highly sensitive to narcotics and is diminished by malonate. Kini and Quastel[186] found that the addition of 5 μM protoveratrine to aerobically incubated slices of rat brain cortex in the presence of glucose-U-$C^{14}$ gives rise to a radioactive amino acid pattern which differs from that obtained in the absence of protoveratrine. These effects are antagonized by cocaine (cf. Kupchan and Flacke[180]).

(iii) Amphetamines and Nialamide

While discussing the mechanism of release of norepinephrine (NE), Brodie, Cho, Stephano and Gessa[187] stated: "It is disquieting to realize that at present we cannot describe at a physiological-biochemical level the mechansim of action of any drug that acts on the nervous system". If one knows the mechanism of action of even a single drug then it may provide a framework on which the study of action of other drugs could be based. Amphetamine is a suitable candidate for a thorough study because of its simplicity in structure and the multiplicity of its biological effects, and in recent years it has been extensively studied.

Amphetamines release catecholamines from their neuronal storage sites but they do not appear to affect the uptake or release of serotonin (5-hydroxytryptamine, 5-HT). Amphetamine strongly affects the uptake of NE by the neuronal membrane and is also an inhibitor of monoamine oxidase (MAO) which is an important enzyme in inactivating the action of monoamines in CNS. Parallelism between the stimulating effect of amphetamines and their effects on MAO was first shown by Mann and Quastel[188,189].

Other effects of amphetamines are presumably linked up with these basic effects and have recently been discussed in detail in a monograph[190].

D-Amphetamine is at least 20 times more active than l-amphetamine. A number of derivatives of these compounds have been prepared with different biological activity[191]. Usually hydroxylation of phenyl group or the side chain reduces the activity. Unlike amphetamine, p-chloroamphetamine causes a cerebral serotonin depletion in rats[192]. Amphetamine has recently been shown to increase the levels of 5-HT in mouse brain[193].

Nialamide is a MAO inhibitor and treatment of the animals with this drug causes increased accumulation of amines in the brain[194].

(iv) Barbiturates

In 1932, Quastel and Wheatley[195] demonstrated that certain barbiturates as well as other anesthetic drugs suppress the respiration of brain tissue preparations. This observation was later confirmed by other workers. The concentrations of the drug required were relatively large compared with the anesthetic doses. Barbiturates however, at anesthetic doses, suppress $K^+$ as well as electrically stimulated respiration[196, 197]. It is now well known, following the work of Michaelis and Quastel [281] and Ernster and co-workers[282-283], that the barbiturate amytal as well as certain other hypnotics suppress the oxidation of NADH and hence the generation of ATP in the cell. Thus all the processes which require ATP are affected by these drugs. These effects have been discussed by Quastel[196,197].

Barbiturates do not affect cerebral $Na^+$, $K^+$-ATPase at concentrations that suppress stimulated brain respiration, nor do they inhibit the increased $Na^+$ influx due to electrical stimulation[198]. They also do not show alleviation of the depression of oxidation of (1-$C^{14}$) acetate to $C^{14}O_2$ brought about by the application of electrical pulses[198]. They differ in this respect to local anesthetics and TTX.

Webb and Elliott[199] found that the inhibition of respiration of brain suspensions and slices by barbiturates is accompanied by a considerable increase in the rate of aerobic glycolysis. The maximum glycolytic rate, equal to or exceeding the normal rate of anaerobic glycolysis, occurs when the rate of oxygen consumption is inhibited about 50 percent by the drug. (This is consistent with the present information on the relief of Pasteur effect in the presence of respiratory chain inhibitors due to decrease in ATP.) Webb and Elliott[199] further observed that the rate of anaerobic glycolysis of brain suspensions is unaffected or slightly accelerated by low drug concentrations (1 mM in the case of amytal and pentobarbital), but is inhibited by concentrations greater than those which cause maximum aerobic glycolysis. The concentrations at which these effects occur vary from drug to drug.

(v) Chlorpromazine (CPZ)

Introduction of CPZ in the 1950's was of great therapeutic importance and since then CPZ as well as other phenothiazine derivatives have been extensively used in the clinical treatment of a number of mental disorders.

Lindan, Quastel and Sved[200] have shown that CPZ at low

concentration depresses the $K^+$ stimulation of cerebral oxygen uptake. The electrically stimulated respiration is also reduced by CPZ. CPZ differs from the narcotics in its high binding power with tissue proteins but resembles the narcotics in its uncoupling of oxidative phosphorylation at concentrations that do not affect the respiration of unstimulated brain cortex slices[194]. It has been shown that CPZ uncouples the oxidative phosphorylation in the mitochondria[202-204].

CPZ is an antagonist of flavin adenine dinucleotide (FAD)[205]. FAD is claimed to antagonize the EEG effects of CPZ[206]. Dawkins, Judah and Rees[207] have shown that CPZ inhibits electron transport between NADH and cytochrome C. This effect of CPZ, together with its suppression effect on membrane permeability[208-210], may account for its ability to uncouple oxidative phosphorylation[194,211].

CPZ inhibits the release of acetylcholine in cat brain[212] and stabilizes the acetylcholine containing synaptic vesicles[194]. Maickel[213] has shown that CPZ inhibits acetylcholinesterase. Considerable information exists on the influence of CPZ on the levels of NE, dopamine and 5-HT in brain. CPZ diminishes uptake of NE in heart and adrenals and also cause complete release of particle bound adrenalin in adrenal gland preparations[194,196].

Magee and Rossiter[214] have shown that low concentrations of CPZ bring about changes in the incorporation of $P^{32}$ into various phospholipids of guinea pig cerebral cortex slices. Mule[215] studied the effect of CPZ and others CNS acting drugs on phospholipd-facilitated $Ca^{++}$ transport which is inhibited, and concluded that an alteration in the binding of ions to

phospholipids within the neuronal membrane may be involved in the pharmacological action of CNS acting drugs. CPZ decreases the rates of penetration of $Na^{22}$ and $K^{42}$ into cerebral tissues [216], a fact that might bear on the mechanism of action of this drug.

Buzard[217] has shown that 1 mM CPZ has a marked inhibitory effect on the anaerobic glycolysis of rat brain homogenates, especially in the later period of incubation.

At high concentrations CPZ is an inhibitor of microsomal $Na^+$, $K^+$-ATPase[218]. Akera and Brody[219] observed that the exposure of CPZ solution to light enhanced the inhibition of ATPase activity. Ultraviolet (UV) light was even more effective and these authors concluded that a semiquinone free-radical of CPZ rather than CPZ itself may be responsible for the inhibition of $Na^+$, $K^+$-ATPase activity *in vitro*. In a later study[220], these workers concluded that the CPZ free-radical inhibits enzyme activity by interacting with sulphydryl (-SH) groups on the enzyme in contrast to ouabain which is bound to a different site.

(vi) Reserpine

Preparations from the plant *Rauwolfia serpentina* have been used in India for centuries as a calming drug. Reserpine has been shown to be the principal alkaloid of this plant[221] and the effects of reserpine on CNS has been extensively studied since then.

The most important biochemical effect of reserpine in the CNS found *in vivo* is the release of NE, 5-HT and dopamine from the amine storage sites in brain. Prolonged treatment by reserpine cause depletion of these amines from the brain.

Presumably these effects are due to changes in the permeabilities of certain neuronal membranes. Giachetti and Shore[222] have shown that reserpine greatly enhances the permeability of adrenergic neuronal membrane to the outward movement of amines. Reserpine interferes with the uptake of the amines by the amine storage granules[223]. Whether reserpine acts at the neuronal membrane or storage granule membrane is controversial[224].

Vascular tissue $Na^+$ and $K^+$ contents have been shown to be changed after treatment of the animal with reserpine[225].

The actions of reserpine have been reviewed in detail by a number of workers[194,223,226,227].

## 1.6 BIOCHEMISTRY OF THE DEVELOPING BRAIN

Profound biochemical changes take place in the brain of mammals during development. Here we will be concerned only with the problems which are more pertinent to the experiments reported in this thesis. Various aspects of the developing brain have been discussed by McIlwain[50] and Himwich[228].

The time at which the maximum brain growth occurs differs from species to species[229]. In rats, the growth rate is maximum at 5-15 days after birth, with a peak at 10 days. In contrast to the rat, in guinea pigs the maximum growth occurs before birth with a peak at 17 days before birth, while in man the growth rate of brain is maximum at about the time of birth[229].

Functionally, the major events in the developing nervous system are the formation of synapses and the process of myelination. The myelination period coincides with the period of

greatest net increment in the brain weight[230]. Myelination first occurs close to the nerve cell body and slowly spreads to the terminal portion of the nerve. However, even in adult life, nerve fibers are often not totally myelinated, their terminal portions usually lacking myelin (see Davison and Peters[231] for details). As with the process of myelination, the time at which synapses are formed differs from animal to animal. In the guinea pig considerable synapses are present at birth, while in the rat a sharp increase in the number of synaptic functions occurs during the 3rd and 4th week of development[232].

Concomitant with these changes, there occurs a marked change in the enzymic make up of the brain[50,244]. Glycolysis is of major importance in the infant rather than in the adult brain (except guinea pig). The citric acid cycle enzymes increase markedly during development and oxidative metabolism of brain greatly increases during maturation. The hexose monophosphate pathway is of little importance in the adult brain although considerable portions of the glucose utilized are channeled through this pathway in the infant brain.

There is a decrease in the quantities of electrolytes together with a decrease in water content during development[50]. However, several important changes occur that are related to the changes in the electrical activity during maturation. $K^+$ content increases while $Na^+$ diminishes. Chloride content and chloride space decrease with development. Activity of $Na^+$, $K^+$-ATPase is extremely low in the newborn rat and increases rapidly during neonatal development with the maximum at about 50 days after

birth[234]. The increase in the activity of this enzyme may account partly for a large portion of the increased energy metabolism noted during brain maturation. The increase in the mitochondrial enzyme system is equally important. In addition, $K^+$ is an efficient in vitro stimulator of the oxygen consumption in the mature rat brain but is inefficient in a new born rat brain[235]. This phenomenon may be directly related to the changes in ATPase activity[233].

Although $Na^+$-$K^+$ ATPase activity is very low in the infant rat brain, it may be sufficient to maintain a high cellular $K^+$ and low $Na^+$ [236].

## 1.7 TRANSPORT OF AMINO ACIDS AND SUGARS ACROSS THE BRAIN CELL MEMBRANE

Although it has been known for a long time that $Na^+$ influences the transport of solutes across the cell membrane, the influence of $Na^+$ on the transport of a variety of solutes across animal cell membranes was first identified in the five year period between 1958 and 1963[237]. Riklis and Quastel[238], in 1958, first showed that glucose absorption by isolated guinea pig small intestine depends markedly on the presence of $Na^+$. This was soon extended by a number of workers to include other compounds such as amino acids, uracil and 3-O-methyl glucose[237]. There is evidence that $Na^+$ dependent active transport systems may be driven by a parallel downhill $Na^+$ flux[239-241].

The transport reactions of amino acids and sugars at the brain cell membrane has been reviewed recently by Quastel[137, 242]. Although there is some exchange diffusion, the amino acids

are usually accumulated against a concentration gradient. Several drugs are known to affect the transport of amino acids in vivo as well as in cerebral cortex slices. L-amino acids penetrate the brain cells more easily than the corresponding D-isomers.

The uptake of amino acids by cerebral cortex slices is energy dependent and their accumulation under aerobic conditions is blocked by 2,4-dinitrophenol or iodoacetate[137, 243,244]. Uptake of glycine by cerebral cortex slices under a wide variety of experimental conditions is proportional to the level of ATP[245]. There is a reduction in cerebral uptake of one amino acid in the presence of another which may be largely due to the mutual competition for a common carrier (or site) at the brain cell membrane[137].

The active transport of amino acids in brain is also $Na^+$ dependent[245,246,247]. High concentrations of $K^+$ diminish the cerebral uptake of amino acids by lowering the ATP level [137]. Also, there is less uptake of amino acids in the absence of $Ca^{++}$ or in the presence of 10 mM $Ca^{++}$; the latter may be due to inhibition of $Na^+,K^+$-ATPase. Ouabain inhibits the uptake of amino acids, which is consistent with the role of this enzyme in amino acid transport processes[137]. In the presence of ouabain, the amino acids efflux into medium is increased[170]. Anaerobiosis, lack of $Na^+$, the presence of a respiratory chain inhibitor or chlorpromazine also increases the efflux of amino acids[137].

Although it is well established that glucose transport across the intestinal wall is $Na^+$ and energy dependent, there

is no evidence that $Na^+$ mediate or is essential for glucose transport into the brain cell[137].

Very little information is available on the specificity of carbohydrate transport processes and even less on the kinetic properties of such processes in the brain[248]. Studies on glucose transport have been difficult owing to the rapid metabolism of glucose in the brain under normal conditions and use of glucose in suboptimal concentrations renders data from such studies of doubtful significance. However, in the case of pentoses, evidence has emerged to support the view that a facilitated or carrier mediated process rather than diffusion is involved[137,248].

Bachelard[248] has studied the uptake of a number of $C^{14}$-labelled glucose analogs into the non-raffinose space of cerebral cortex slices. Kinetic properties of the uptake of 2-deoxyglucose indicate that the transport is a facilitated process rather than diffusion. Other experiments indicate that deoxyglucose may be regarded as a competitive inhibitor of glucose uptake and the apparent Km for glucose transport has been suggested to be of the order of 5 mM.

Consideration of the factors likely to operate in regulating brain carbohydrate metabolism indicate that glucose transport may be a possible control point[20,51,249]. Rolleston[51] observed that when the rate of glucose metabolism is rapid (in the presence of 50 mM $K^+$ or 3 mM cyanide), increasing the concentration of glucose in the medium from 3 to 10 mM causes increased rate of glycolysis. This was not observed under condition giving lower rate of metabolism. Rolleston[51] concluded

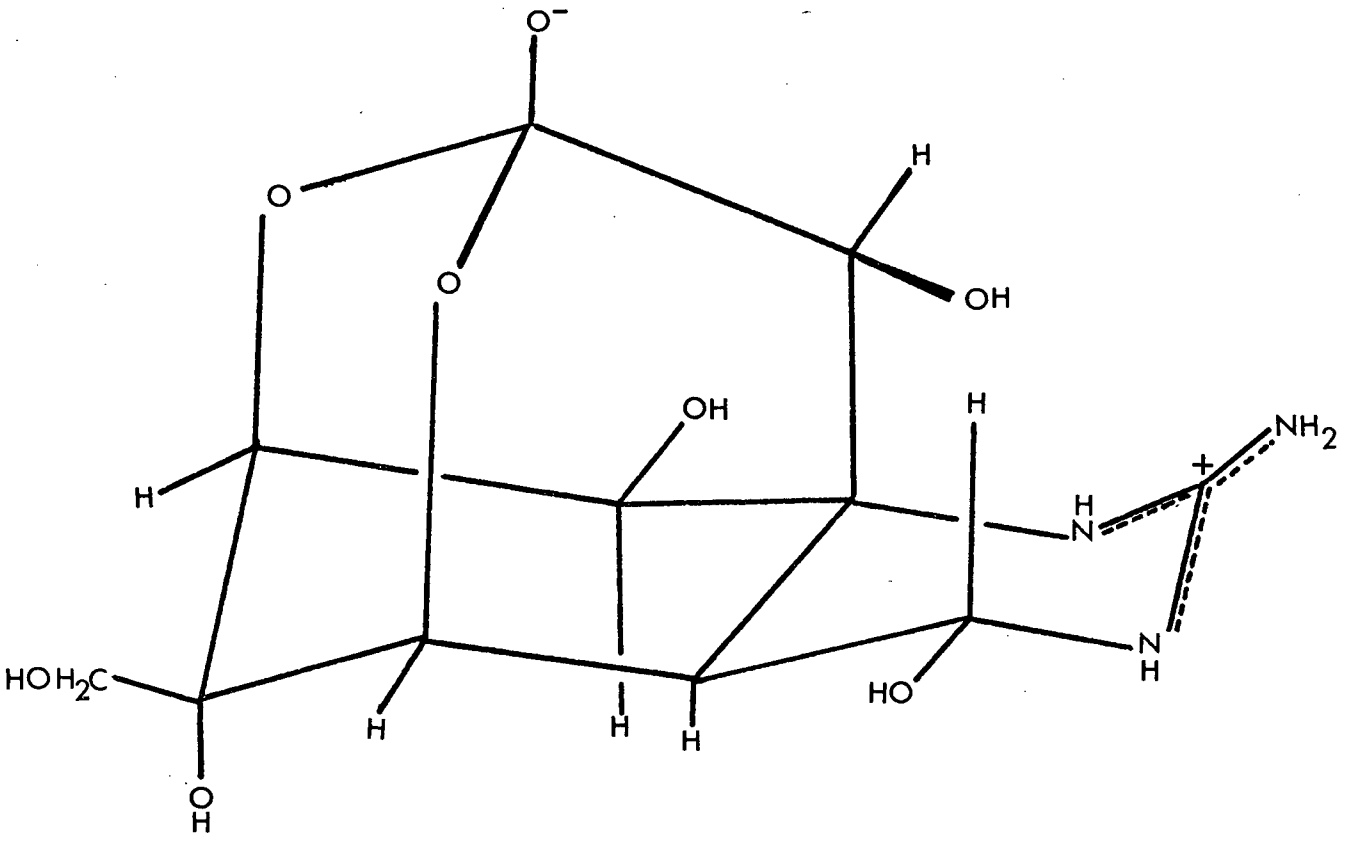
that the rate of entry of glucose into the tissue may become a limiting factor in glucose metabolism.

## OBJECTIVES

The aim of the present investigation has been to study the effect of TTX and other neurotropic drugs on cerebral anaerobic glycolysis, and on transport processes of incubated cerebral cortex slices, in an effort to understand more fully cerebral metabolic processes during anoxia and the mode of action of certain neurotropic drugs under these conditions. The general approach has been to investigate the action of various drugs on anaerobic glycolysis of brain slices and to see whether their activities may be affected by a variety of conditions. The cation contents, and rates of $Na^{22}$ transport were studied under various conditions in the presence of various drugs and these then related to the accompanying changes in the brain metabolism. Some experiments were also carried out on the transport of amino acids and glucose.

Preliminary experiments (Chapter 3) deal with the rates of anaerobic glycolysis in the cerebral cortex slices. The following two chapters (Chapter 4 and 5) deal with the effects of TTX on the transport processes and on anaerobic glycolysis of brain slices. Chapters 6 and 7 include experiments carried out with other drugs to see how they resemble or differ from TTX in affecting anoxic metabolism of the brain slices. The last chapter (Chapter 8) presents a general discussion of the experiments reported in this thesis and of the results and conclusions.

STRUCTURE OF TETRODOTOXIN




Mol. Wt. 322

For details of chemistry and pharmacology see Mosher et al[114], Kao[115] and Evans[116].

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 EXPERIMENTAL ANIMALS

The animals used in the experiments were rats and guinea pigs.

Adult Wistar rats, weighing about 200-250 g, were obtained either from the National Laboratory Co., Edmonton or the Department of Zoology Vivarium, The University of British Columbia. Infant rats were supplied by the Vivarium.

Adult male guinea pigs, weighing about 300-350 g, and newly born guinea pigs, of English short hair strain, were obtained from the Animal Unit, Faculty of Medicine, The University of British Columbia.

All the adult animals had free access to food and water until used, whereas the infant animals were separated from the mother shortly before start of the experiment.

## 2.2 CHEMICALS

All common laboratory chemicals were of "reagent grade" and were used without any further purification.

$NAD^+$, NADH, $NADP^+$, ATP, ADP, AMP, Adenosine, cAMP, phosphoenol pyruvate, lithium lactate, epinephrine, norepinephrine, histamine, tyramine, LDH (beef heart), LDH (rabbit muscle), glutamic dehydrogenase (beef liver), pyruvate kinase (rabbit muscle) and phospholipases A and C were obtained from Calbiochem, Los Angeles. Triethanolamine was the product of Sigma Chemical Co., St. Louis. Hexokinase (HK) and glucose 6-phosphate dehydrogenase (G-6-PDH) were

purchased either from Calbiochem or from Sigma Chemical Co. Dibutyryl cAMP was obtained from Schwarz BioResearch and acetyl choline was a product of Matheson Co. Inc., Norwood, Ohio.

The radiochemicals: sodium-22 (as chloride), adenine-8-$C^{14}$ sulfate and glucose-2-$C^{14}$ were the products of Radiochemical Centre, Amersham, England. U-$C^{14}$-glutamic acid and glycine-2-$C^{14}$ were obtained from Volk Radiochemical Co., Chicago.

Tetrodotoxin was purchased from Calbiochem. Ouabain and reserpine were obtained from Nutritional Biochemicals Co.; protoveratrine from K and K Laboratories, Plainview, New York; chlorpromazine from Poulenc, Montreal; d- and l-amphetamines from Smith, Kline and French Laboratories, IAC, Montreal; pentothal from Abbott Laboratories, Montreal; nialamide from Pfizer Inc., Brooklyn, N. Y. and amytal from Eli Lilly. Procaine and lidocaine were obtained from Department of Pharmacology, The University of British Columbia.

## 2.3 PREPARATION OF BRAIN SLICES

Rats were stunned by blow at the back and decapitated quickly. The brains were dissected and kept in the incubation medium (without any additions except glucose) until slicing. The cerebral cortex slices were prepared with the Stadie-Riggs tissue slicer and a moist razor. The slices were about 0.4 mm thick and thinner or thicker ones discarded. Only one top dorsal and one lateral slice from each hemisphere were used. The selected slices were kept

on an ice-cold petri dish until placed in the incubation medium in the Warburg vessel. One dorsal and one lateral slice from each hemisphere weighing about 60-75 mg, was used in each vessel.

Guinea pig cerebral cortex slices were prepared in the same way as for rats, except that for lateral slices parietal lobes were first cut out and then the slices were prepared from them. When guinea pigs were used, only one slice weighing about 40-55 mg was used in each vessel because of the high rate of glycolysis in the guinea pig cerebral cortex slices.

Infant rat brain (1-3 day old) slices were prepared by a slightly different method. After decapitation, brains were removed, and one slice was cut from temporal-parietal portion of each hemisphere. Two slices from each brain, weighing about 70-90 mg, were used for each vessel.

Cerebral cortex slices from older infant rats and newly born guinea pigs were prepared in the same way as for adult brain.

## 2.4 INCUBATION PROCEDURE

The slices were weighed quickly on a torsion balance to the nearest mg and then suspended in 3 ml precooled incubation medium in the Warburg vessel. Additions were either made at the beginning of the experiment or tipped in from the side arm during the incubation period. Incubations were carried out using a conventional Warburg manometric apparatus at 37°C in an atmosphere of 95 percent $N_2$: 5 percent $CO_2$ ($N_2$:$CO_2$) or 95 percent $O_2$: 5 percent $CO_2$

($O_2$:$CO_2$) as indicated for each experiment. In some experiments, however, the gas phase was changed during the incubation.

## 2.5 INCUBATION MEDIUM

In the experiments described in this thesis, the cerebral cortex slices were incubated in various media depending upon the nature of the experiment. The required salt solutions were made up at 5 times isotonic concentrations and stored at 4°C. The final composition of the various media are given below.

### (a) Kreb-Ringer Medium

Krebs-Ringer bicarbonate medium was used for anaerobic experiments. It contained 119 mM NaCl, 5 mM KCl, 3.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$ and 25 mM $NaHCO_3$.

Krebs-Ringer phosphate medium contained KCl, NaCl, $CaCl_2$ and $MgSO_4$ as above, but 10 mM Na-phosphate buffer (pH 7.4) was added in place of $NaHCO_3$. Phosphate buffer was added to the final incubation medium at the end to avoid the precipitation of $Ca^{++}$ and $Mg^{++}$ (as phosphates), that may occur before dilution.

### (b) $Ca^{++}$-free Medium

In most of the anaerobic experiments the $Ca^{++}$-free medium used was similar to that described by Adams and Quastel[71]. It contained 149 mM $Na^+$, 5 mM $K^+$, 125 mM $Cl^-$ and 29 mM $HCO_3^-$. $Ca^{++}$ and $Mg^{++}$ were omitted from the medium as, under these conditions, anaerobic glycolytic rates showed greatest sensitivities to various types of neuro-

tropic drugs and gave the most consistent results. Omission of phosphate had no apparent effect in our experiments.

(c) $K^+$-free Medium

$K^+$-free medium was prepared by omitting KCl from the $Ca^{++}$-free medium. It contained 154 mM $Na^+$, 125 mM $Cl^-$ and 29 mM $HCO_3^-$.

(d) $Cl^-$-free Medium

$Cl^-$-free medium was prepared by using sulfate salts of $Na^+$ and $K^+$ instead of chloride salts. Its composition for cations was the same as that of $Ca^{++}$-free medium but the $SO_4^{--}$ was used at half the concentration of $Cl^-$.

(e) $Li^+$-containing Medium

The composition of $Li^+$-containing medium was similar to that of $Ca^{++}$-free medium except that some, or all, NaCl was replaced by LiCl. The concentrations of $Li^+$ are specified in the experiments described below.

(f) High-$K^+$ Medium

High-$K^+$ medium was prepared by replacing some, or all, NaCl by KCl, or by replacing NaCl and $NaHCO_3$ by KCl and $KHCO_3$, in the $Ca^{++}$-free medium. In some cases, where $Na^+$ was also to be reduced in concentration (without further increasing $K^+$ at the same time), sucrose (twice the molarity of salt solution) was added to maintain tonicity.

(g) $Na^+$-free Medium

$Na^+$-free medium contained 260 mM sucrose, 6 mM KCl and 10 mM tris-HCl buffer (pH 7.4).

## 2.6 PREPARATION OF KIDNEY MEDULLA

Kidney medulla was used as a control tissue in some of the glycolysis experiments. The following procedure was used for the preparation of the tissue; rats were stunned by blow on the head and the viscera opened; kidneys were then removed quickly and each was cut into two equal halves. Subsequently the kidney cortex was cut off from the medulla and discarded. Two halves of the kidney medulla, each 0.5-1 mm thick and weighing about 120-140 mg, were used in each vessel.

## 2.7 PREPARATION OF CAUDATE NUCLEUS

The rats were stunned by a blow at the back and the brains quickly removed, each brain was cut into two halves along *fissura longitudinalis cerebri* and the caudate nucleus was located[250] and dissected out. Each half of the caudate nucleus was then cut into three pieces, weighed quickly and placed in the Warburg vessel.

## 2.8 PREPARATION OF ACETONE POWDER EXTRACTS

Acetone powder extracts of the rat cerebral cortex was prepared by the method of Harpur and Quastel[251,252]. Rat brain cortices were removed and ground in ice-cold acetone. After filtering and further washing with ice-cold acetone, the resulting powder was stored in a vacuum dessicator at $4^{o}C$ until used.

100 mg of the acetone powder was extracted with 10 ml of a solution, usually containing 100 mM nicotinamide, 33 mM cysteine and 156 mM NaCl. The extract was centrifuged

and 1 ml of supernatant was taken in each vessel. This, on dilution to 3 ml, gave a final concentration of 33 mM nicotinamide, 11 mM cysteine and 52 mM NaCl. Other additions were made as indicated in the results.

## 2.9 PREPARATION OF SYNAPTOSOMES

The method developed by Whittaker[253,254] as shown in the Scheme 2 was used for the preparation of synaptosomes. After separation of subcellular particles on a density gradient, the various layers were carefully removed with the help of a pasteur pipette. After centrifugation of the fraction having density between 0.8 and 1.2 M of sucrose, the residue (synaptosomes) were resuspended and used for experiments and protein determination.

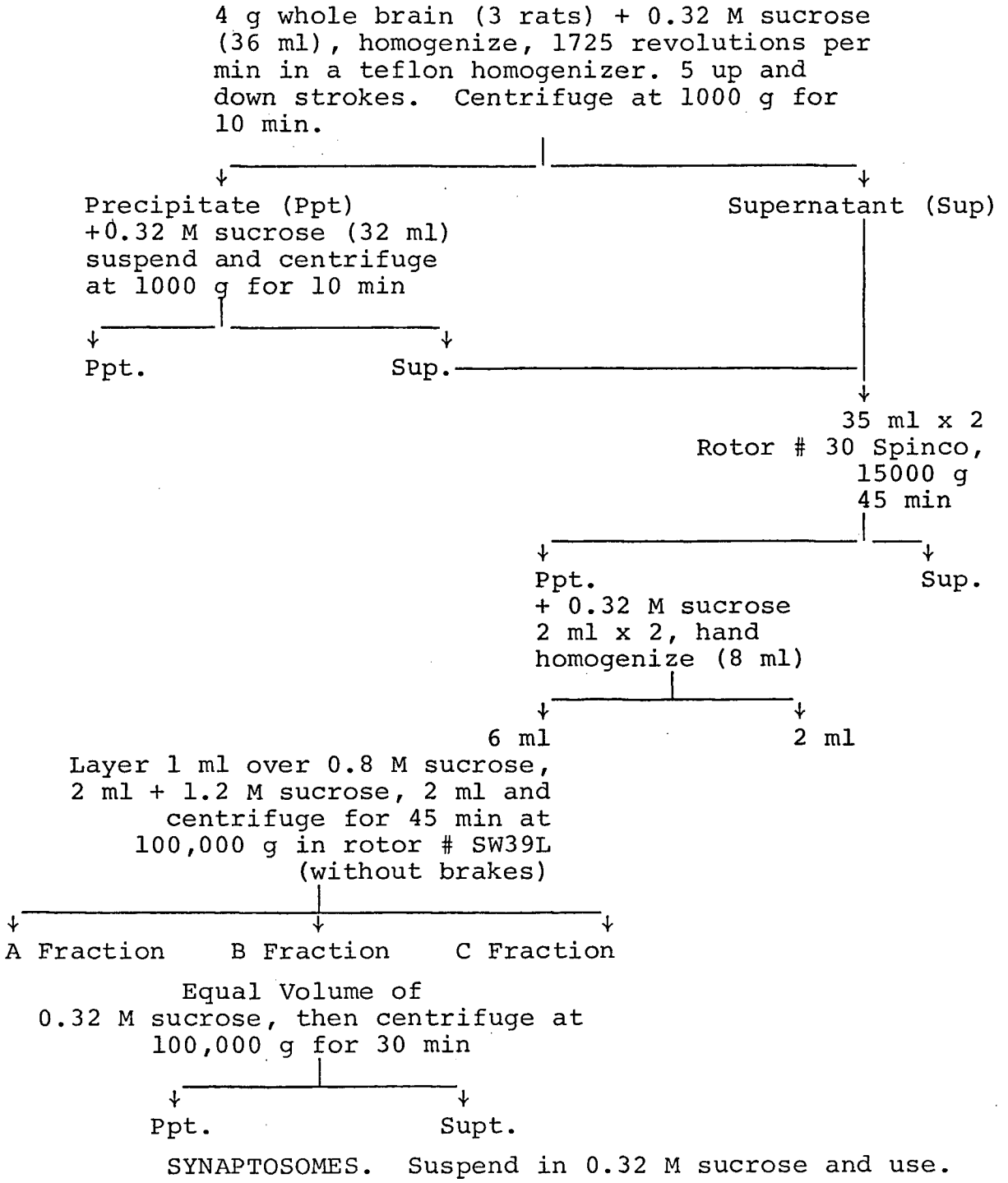
## 2.10 PREPARATION OF MICROSOMES AND ASSAY OF $Na^+$, $K^+$-ATPase

Microsomes from guinea pig brain were prepared by the method of Hokin and Yoda[255]. Brains were removed quickly after decapitation and cerebral hemispheres were homogenized in an all glass homogenizer in Sucrose-EDTA (0.25 M sucrose + 2 mM EDTA). The homogenate was first centrifuged for 5 min at 700 g and the sediment discarded. The supernatant was centrifuged at 105000 g in rotor 30 in a Spinco model L preparative ultracentrifuge for 10 min. The residue was resuspended in 0.25 M sucrose with hand homogenization.

The suspended residue was centrifuged at 8500 g for 15 min. The residue was discarded and the supernatant was again centrifuged for 20 min at 105000 g. The resulting residue (microsomal fraction) was suspended in sucrose-

Scheme 2

Fractionation of Synaptosomes by Sucrose Gradient Centrifugation



EDTA corresponding to 0.5 ml/g of original brain and was used either for assay immediately or stored frozen at -20°C.

The tubes for assay of ATPase contained 0.5 ml of the medium containing microsomal suspension (25 µg protein), 80 mM imidazole-HCl buffer (pH 7.1), 2 mM $MgCl_2$, 2 mM $Na_2ATP$, 80 mM NaCl and 60 mM KCl. Incubation period was 20 min at 37°C. All assays were carried out in duplicates and other additons were made as indicated in the results. The reaction was terminated by the addition of 0.5 ml of 10% trichloroacetic acid (TCA), the tubes were cooled, centrifuged and phosphate was measured in the supernatant by the method of Stanton[256]. From optical density (OD) vs time data, the zero time intercepts were determined and actual amounts of phosphate liberated were then calculated from these values and standards.

## 2.11 MEASUREMENT OF GLYCOLYSIS

### (a) Manometric

Lactate production during anaerobic glycolysis was estimated by measuring carbon dioxide ($CO_2$) evolution from a bicarbonate medium in the Warburg apparatus in an atmosphere of $N_2$:$CO_2$. It is well known that when there is equilibrium between $CO_2$ in the medium and in the gaseous phase, each molecule of lactic acid produced will release one molecule of $CO_2$ in the gas phase. $CO_2$ so evolved can be easily measured with the help of the manometer. The manometric method is much more convenient than the actual lactate assays, since one can measure lactate production

continuously over the entire incubation period.

Unless otherwise stated, the experiments were of 80 min duration. The vessels were gassed with $N_2$:$CO_2$ for the first 10 min, while shaking, in the incubation bath. After gassing periods the stoppers were closed and equilibration was carried out for another 10 min. Manometric readings were taken after this 20 min period for one hour. Results obtained as µl $CO_2$ of lactate produced (by calculation from the manometric readings and flask constants) were converted to µmoles of lactate by dividing by 22.4 (1 mole of a gas corresponds to 22.4 litres at NTP). It has been well established that the major product during anaerobic glycolysis of cerebral cortex slices is lactate and that values obtained by manometric and enzymatic assays closely agree.

(b) Enzymatic

For the measurement of lactate production in a $Na^+$-free medium, and aerobically, the enzymatic method was used. In this method, lactate is oxidized to pyruvate in the presence of $NAD^+$ and LDH and the pyruvate so formed is trapped by hydrazine-glycine buffer. NADH produced can be measured spectrophotometrically[257] or fluorimetrically.

For assay, the sample was deproteinized with 6 percent (v/v) perchloric acid and the supernatant neutralized with $K_2CO_3$ using phenol red as indicator. The reaction mixture, in a total volume of 2.94 ml, contained 1 ml of diluted sample, 1.3 ml of hydrazine-glycine buffer (0.4 M hydrazine, 1 M glycine pH 9.5), 0.11 ml of $NAD^+$ (40 mg/ml) and .03 ml

of LDH (1250 I.U./ml). The mixture was incubated at room temperature in cuvettes with 1 cm light path and the increase in OD was followed at 340nm using a Beckman model DU spectrophotometer. A control cuvette, without the sample, was included every time. The amount of lactate was calculated from the extinction coefficient of NADH.

In some experiments lactate was measured fluorimetrically using an Aminco-Bowman spectrophotofluorometer. In these cases, in 2.1 ml, the reaction mixture contained 2 ml of hydrazine-glycine buffer (as above), 25 µl $NAD^+$ (2 mg /ml), 20 µl LDH (20 I.U. per assay), and 50 µl of samples or standards in Bausch and Lomb colorimeter tubes. NADH produced after the addition of enzyme was measured fluorimetrically until the increase in fluorescence levelled off. The excitation wavelength was 365 nm and fluorescent wavelength was 460 nm. The amount of lactate was calculated with the help of the standards.

## 2.12 MEASUREMENT OF AMINO ACID EFFLUX AND UPTAKE

### (a) Amino Acid Efflux

The cerebral slices were incubated for 30 min in a $Ca^{++}$-free medium. At the end of the incubation, the slices were homogenized with 2.5 ml of 5% (w/v) cold TCA. The homogenate was left in cold for 30 min and then centrifuged; the supernatant was transferred to another tube, the precipitate washed with 1 ml of cold TCA and centrifuged. The two supernatants were mixed and extracted thrice with 3 ml of diethyl ether to remove TCA. Ether was removed

from the water phase by blowing air and the TCA free extract, after suitable dilution, was used for amino acid analyses.

The incubation medium was transferred quantitatively in a centrifuge tube, deproteinized with TCA and the supernatant removed. TCA was removed from the supernatant fraction as above and the extract was used after suitable dilution for amino acid analyses.

The amino acids (taurine, aspartic acid, glutamic acid, glutamine + serine, glycine and alanine) were separated on a Beckman model 120 B amino acid analyzer, using a 50 cm sulfonated polystyrene - 8% divinyl - benzene copolymer ion exchange resin (type 50 A - particle size 25-31 μ) at 50°C; the eluant used was 0.2 N sodium citrate buffer, pH 3.3. The column was regenerated with 0.2 N NaOH, and equilibrated with the buffer, before each use.

(b) Amino Acid Influx

Conditions of incubation are given with the results and the samples were prepared as for $Na^{22}$-influx studies.

## 2.13 MEASUREMENT OF $NAD^+$ AND NADH

The method of Lowry and co-workers[261-263] was used for the extraction and assay of $NAD^+$ and NADH.

(a) Extraction of NADH

At the end of incubation, the tissue was homogenized at a dilution of at least 1:100 in ice-cold 0.02N NaOH containing 0.5 mM cysteine. The homogenate was heated for 10 min at 60°C with one hour of homogenization. Cysteine was added to the NaOH solution slightly before use. Usually 10-25 μl

of the extract after 1:1000 dilution of the tissue was used for each assay.

(b) Extraction of $NAD^+$

At the end of incubation, the tissue was homogenized, in at least 1:50 dilution, in a mixture of 0.01 M $H_2SO_4$ and 0.1 M $Na_2SO_4$ with subsequent heating for 45 min at 60°C. This reduces NADase activity to a point at which it is ordinarily not disturbing although some activity may persist[263]. Usually 15 µl of the extract after 1:1000 dilution was used for each assay.

(c) Assay of $NAD^+$ and NADH

To a 200 µl of the cycling mixture, containing 0.2 M tris HCl pH 8.4, 100 mM Li-lactate, 0.3 mM ADP, 5 mM α-KG, 0.05 M $NH_4Ac$, 400 µg/ml bovine liver L-glutamic dehydrogenase and 45 µg/ml beef heart LDH in a Bausch and Lomb colorimeter tube, 0-30 µl of tissue extract was added; cycling was continued for 1 hr at 37°C. After cycling, the tubes were transferred to a boiling water bath for 2 min. The tubes were then cooled in ice and 200 µl of phosphate:LDH solution (0.65 M Na $H_2PO_4$, 0.15 M $K_2HPO_4$ and 1.5 µgm of rabbit muscle LDH) and 200 µg of NADH was added to each tube. Subsequently the tubes were incubated for 15 min at room temperature, cooled in ice and then 50 µl of 5N HCl was added. After a few min at room temperature, 2 ml of 6 N NaOH was added with immediate mixing. The tubes were then heated at 60°C for 10 min, brought at room temperature and the fluorescence was read in an Aminco-

Bowman spectrophotofluorometer. After the addition of NaOH, the tubes were kept in dark till readings were taken, since the fluorescent form of $NAD^+$ is sensitive to destruction by light.

As pyruvate formation during cycling is not strictly linear with $NAD^+$ or NADH concentrations, standards were included with each set of determinations.

## 2.14 MEASUREMENT OF cAMP PRODUCTION

The method of Shimizu, Daly and Creveling[264] was used to measure the production of cAMP in the incubated cerebral cortex slices.

Cerebral cortex slices were incubated for 40 min in a Warburg vessel, under an atmosphere of $O_2$:$CO_2$ in a Krebs-Ringer bicarbonate medium containing 2 μC of adenine-8-$C^{14}$ sulfate and 20 mM glucose. After aerobic incubation, the slices were washed in a cold medium, transferred to another Warburg vessel containing drugs, and were incubated for a desired period, as described with the results.

After the incubation, slices were homogenized in 1.5 ml of 5 percent (w/v) ice-cold TCA and centrifuged. The supernatant was removed to another tube and 150 μg (100 μl) of cold cAMP was added. 50 μl of this solution was taken for determining total radioactivity while the rest of it was extracted twice with ether to remove TCA, excess of ether was removed by blowing air and 100 μl was spotted on a PEI cellulose plate (Brinkman). The chromatograms were developed in 0.25 M LiCl[302], the spots were visualized by

UV and the radioactivity of cAMP spots determined, after scrapping, in 10 ml of scintillation medium, by a Nuclear-Chicago Mark I liquid scintillation counter. The scintillant medium contained, in 3 litres, equal volumes of toluene, dioxan and 95% (v/v) ethanol, 15 g 2,5-diphenyloxazole, 150 mg 1,4-bis-(4-methyl-5-phenoxaxol-2-yl) benzene and 240 g naphthalene.

## 2.15 MEASUREMENT OF ATP LEVELS

At the end of the incubation, the slices were quickly homogenized in 6% $HClO_4$. The homogenate was left over ice for 30 min. The supernatant obtained after centrifugation of the homogenate was neutralized with a precalculated volume of 5 M $K_2CO_3$, the $KClO_4$ precipitate was centrifuged off and ATP was assayed in this supernatant either by the spectrophotometric method of Lamprecht and Trautschold[265] or by the fluorimetric method of Greengard[266]. Both of these methods utilize the hexokinase-glucose-6-phosphate dehydrogenase system coupled to $NADP^+$ reduction in the presence of glucose and other cofactors. The amount of $NADP^+$ reduced is proportional to the amount of ATP present.

## 2.16 MEASUREMENT OF $Na^{22}$ INFLUX

0.5 μC $Na^{22}$ was tipped in from the side arm of the Warburg vessel after a short period of preincubation, as indicated in the experiments described below. After a desired period of incubation, the slices were removed and quickly washed twice, in a non-radioactive incubation medium, and homogenized in 2.5 ml of 5% (w/v) cold TCA.

After one hour in the cold, the homogenate was centrifuged and 0.5 ml of the supernatant was placed in 10 ml of scintillation medium for counting radioactivity. A Nuclear Chicago Mark I liquid scintillation counter was used.

The results of $Na^{22}$ influx experiments are expressed as $Na^+$, μequivalent/gm initial wet weight, and were calculated by multiplying the tissue $Na^{22}$ volume $\left(\frac{\text{cpm/g tissue}}{\text{cpm/μl of medium}}\right)$ by the $Na^+$ content of the incubation fluid (μequiv/μl)[267].

## 2.17 MEASUREMENTS OF $Na^+$ AND $K^+$

The tissue contents of unlabelled $Na^+$ and $K^+$ were determined with an atomic absorption spectrophotometer. Samples for these assays were prepared as described below: the tissue slices were removed from Warburg vessels with pointed forceps and the adhering fluid was removed with filter paper strips; the slices were homogenized in 3 ml of 5% cold TCA and left in cold for an hour. The precipitate was then centrifuged off and the supernatant suitably diluted (2 mg of tissue/ml for $K^+$ and 0.2 mg of tissue/ml for $Na^+$). The atomic absorption was measured at 294.3 and 383.3 nm for $Na^+$ and $K^+$, respectively, with a Perkin-Elmer model 303 atomic-absorption spectrophotometer. The contents of $Na^+$ and $K^+$ were calculated from standards run with each set of determinations.

## 2.18 MISCELLANEOUS METHODS

### (a) Determination of Protein

Protein was determined by the method of Lowry, Rosebrough, Farr and Randall[48].

(b) Determination of Water Uptake

At the end of the incubation, slices were removed from the medium and excess of fluid was completely removed with filter paper strips touching the slices. The slices were then weighed on a torsion balance; the difference between the initial weight and the final weight provided the measure of water uptake.

(c) Use of Cyanide

Whenever cyanide was used, its solution was neutralized with HCl to pH 7.0-7.5 before use, care being taken not to let pH go below neutrality.

(d) Measurement of Acid Labile Phosphate[258]

The slices were homogenized quickly in ice-cold 5 percent (w/v) TCA and the homogenate was left in ice for 30 min for complete extraction of acid soluble material. Adenine nucleotides were absorbed by treatment with approximately 50 mg Norit A (purified by successive treatments with pyridine, HCl and distilled water) for 10 min at 20°C according to the method of Crane and Lipmann[259].

The labile phosphate of the nucleotide fraction in the supernatant was assayed after treatment of the washed charcoal with 4 ml of N HCl in a boiling water bath and subsequent cooling and centrifuging, by the method of Bartlett[260].

(e) Measurement of Phosphoenol pyruvate (PEP) and Pyruvate

PEP and pyruvate were determined by fluorimetric

adaptation of the method of Czok and Eckert[303].

## 2.19 REPRODUCIBILITY OF THE RESULTS

Each experiment was carried out several times and were reproducible. There were variations in the absolute values found in experiments carried out on different days and with different batches of animals. Control experiments were, therefore, always carried out. The results given below are averages of several values obtained in typical experiments. The deviations from the mean are given.

# CHAPTER 3

## RATE LIMITING FACTORS IN ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES AND ACETONE POWDER EXTRACTS

The rate limiting factors operating in anaerobic cerebral glucose metabolism, such as $NAD^+$, ATP, AMP, cAMP, citrate, glutamate, aspartate and cations, will now be considered. Experiments were carried out both with brain slices and acetone powder extracts of brain.

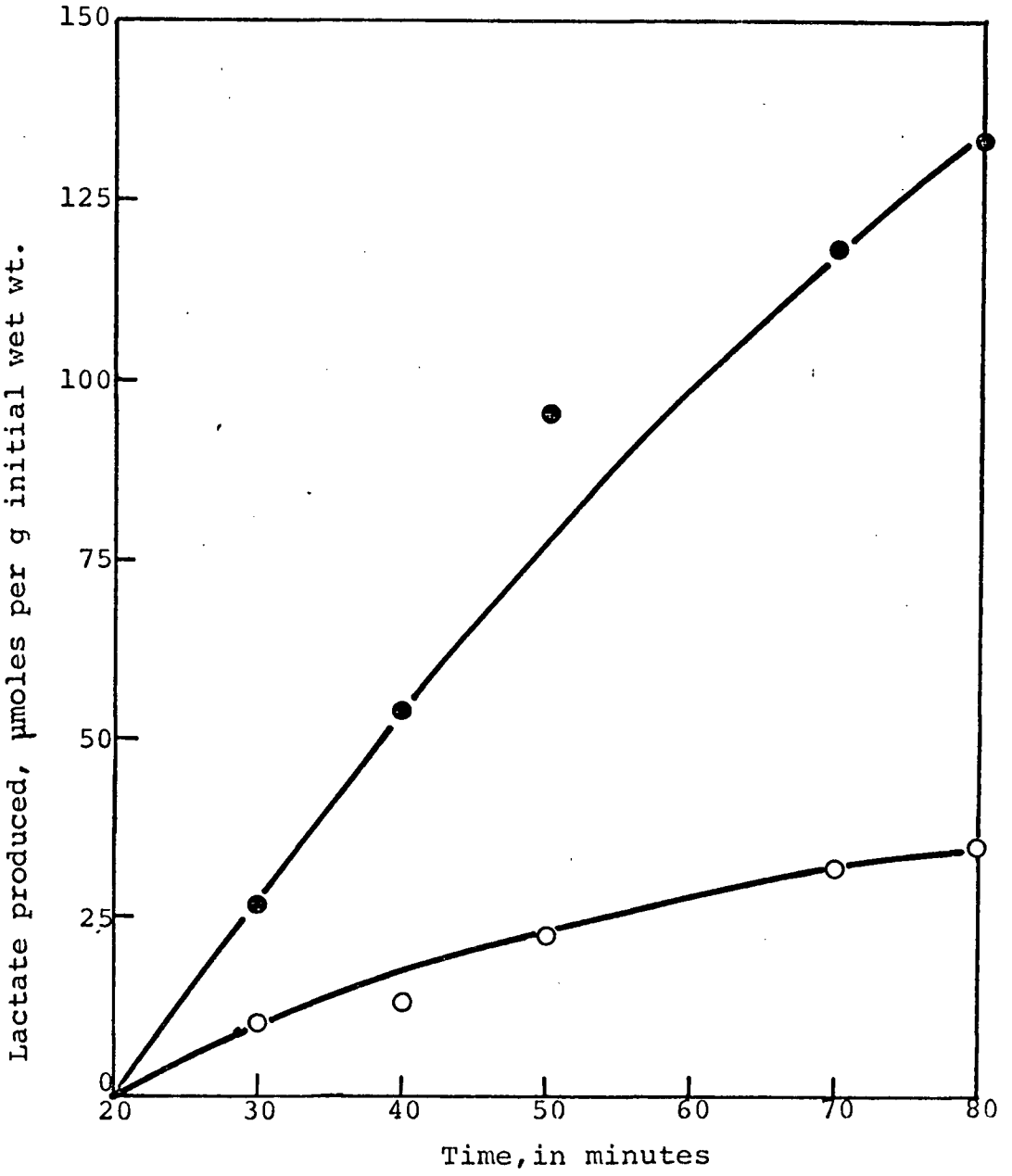
### 3.1 EFFECTS OF CALCIUM ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

The effect of $Ca^{++}$ on cerebral glycolysis has been discussed in Chapter 1. Results given in Figure 1 show that the rate of anaerobic glycolysis decreases progressively with time. Furthermore, the addition of 4 mM $Ca^{++}$ to a $Ca^{++}$-free medium has a marked stimulatory effect on the rate of anaerobic glycolysis of guinea pig cerebral cortex slices. This is in agreement with earlier findings of Quastel and co-workers[70,71].

The effects of the addition of different concentrations of $Ca^{++}$ to a $Ca^{++}$-free medium, on anaerobic glycolysis of rat or guinea pig cerebral cortex slices are given in Figure 2. It is clear from these results that $Ca^{++}$ has a greater stimulatory effect on the anaerobic glycolysis of guinea pig cerebral cortex slices than on that of rat. With guinea pig brain the presence of even 1 mM $Ca^{++}$ has a marked effect on the rate of anaerobic glycolysis whereas in rat it

FIGURE 1

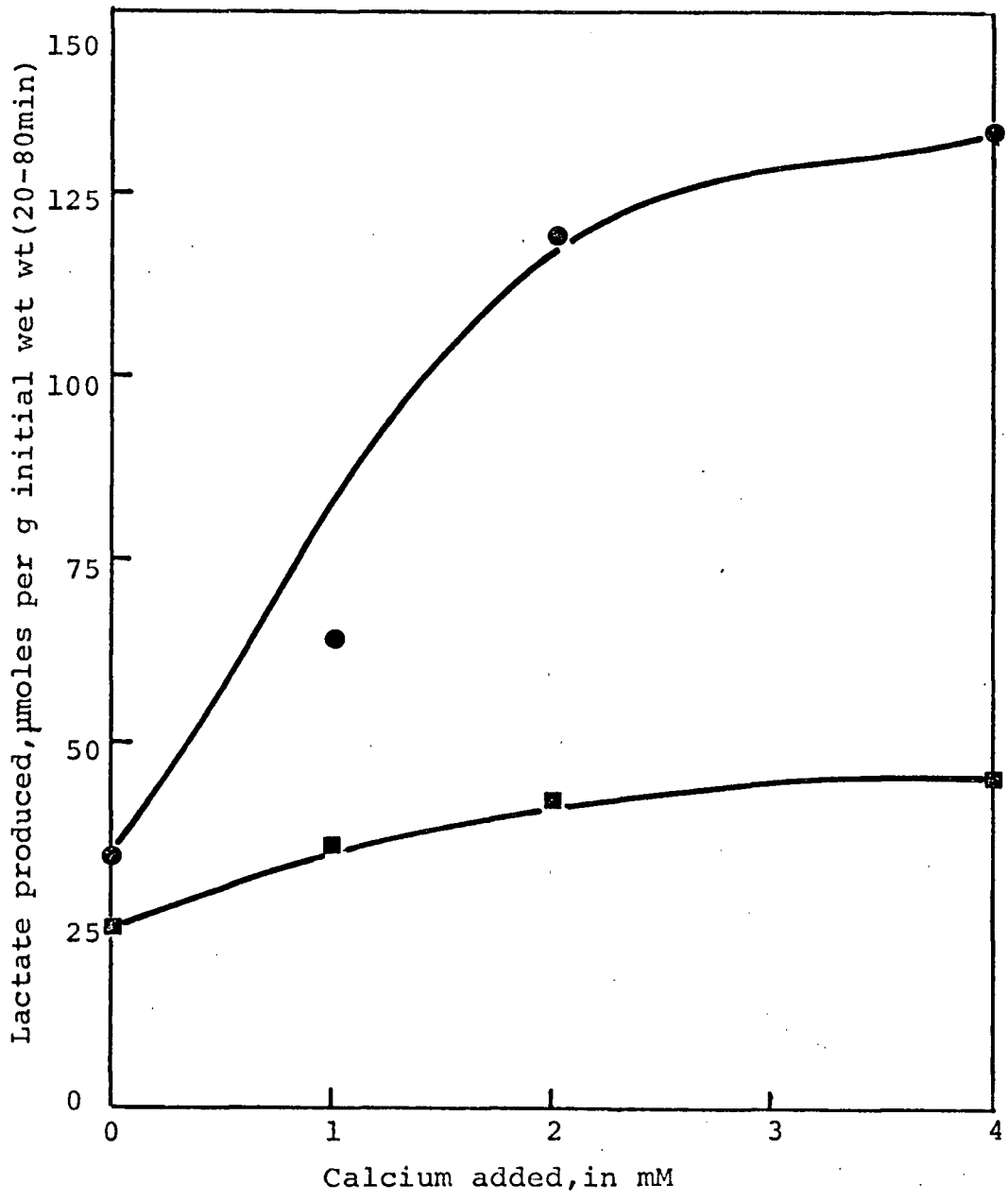
EFFECT OF CALCIUM ON THE ANAEROBIC GLYCOLYSIS OF GUINEA PIG CEREBRAL CORTEX SLICES




All vessels contained 20 mM glucose. Additions were made at zero time and lactate production was measured manometrically as given in the materials and methods. (○) $Ca^{++}$-free medium; (●) 4 mM $Ca^{++}$ added to a $Ca^{++}$-free medium.

FIGURE 2

EFFECT OF DIFFERENT CONCENTRATION OF CALCIUM ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES




Incubations were carried out in a $Ca^{++}$ free medium containing 20 mM glucose. Additions were made at zero time and lactate production was measured manometrically as given in the materials and methods. (■) Rat; (●) Guinea pig.

is less effective. The accelerating action of $Ca^{++}$ on the anaerobic glycolysis of brain slices is in contrast to that on other tissues, such as tumour slices[71]. This effect of $Ca^{++}$ is the reverse of that on the aerobic glycolysis of brain slices. Addition of $Ca^{++}$ to a $Ca^{++}$-free medium reduces the rate of aerobic glycolysis in incubated rat or guinea pig brain slices.

The results of the addition of different concentrations of $Ca^{++}$ on the anaerobic glycolysis of immature rat, and newly born guinea pig cerebral cortex slices are shown in Table 1.

Results shown in Table 1 show that the rate of anaerobic glycolysis of 1-3 day old rat brain is practically insensitive to the addition of different concentrations up to 4 mM of $Ca^{++}$. Some stimulation by $Ca^{++}$ occurs in 14-day old rat brain. With newly born guinea pig cerebral cortex slices, the presence of $Ca^{++}$, however, has a very striking accelerating effect on the rate of anaerobic glycolysis. Stimulation at 2 mM $Ca^{++}$ is more than 300%, with values approaching those obtained for the adult guinea pig cerebral cortex slices. This finding is consistent with our knowledge that the brain of a newly born guinea pig is more or less like a mature brain. It is well known that infant rat brain is very immature in its neurophysiological character compared with the adult rat brain, and it would be expected that major differences in metabolism would exist between infant and adult brain. This will be further discussed in Chapter 8.

TABLE 1

EFFECT OF CALCIUM ON THE ANAEROBIC GLYCOLYSIS OF INFANT RAT AND GUINEA PIG CEREBRAL CORTEX SLICES

| Additions | Lactate produced, µmoles per g initial wet wt (20-80 min) | | | |
|---|---|---|---|---|
| | 2-day old rat | 7-day old rat | 14-day old rat | Newly born guinea pig |
| Control | 24.6 ± 1.8 | 22.8 ± 1.4 | 24.5 ± 1.4 | 32.2 ± 4.5 |
| $Ca^{++}$, 1mM | 24.1 ± 1.4 | 24.5 ± 3.5 | 30.5 ± 5.3 | 92.5 ±12.5 |
| $Ca^{++}$, 2mM | 32.2 ± 4.5 | 25.0 ± 2.7 | 42.0 ± 5.8 | 137.5 ± 6.7 |
| $Ca^{++}$, 4mM | 28.6 ± 1.7 | 29.2 ± 5.3 | 33.5 ± 3.5 | 117.5 ± 4.1 |

Incubations were carried out in a $Ca^{++}$ free medium containing 20mM glucose. Additions were made at zero time and lactate production was measured manometrically as given in the materials and methods.

## 3.2 EFFECTS OF EXTERNALLY ADDED NAD+ AND ATP ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

Various coenzymes, i.e. NAD+, NADH, ATP and ADP, are needed for the operation of various steps of the glycolytic pathway. The established fact that the rate of anaerobic glycolysis of incubated brain slices decreases with time may be due to the loss of such coenzymes with time of incubation or to the loss or gain of cations which also exert rate limiting effects. The ATP level in the brain falls rapidly during anoxia and may become a rate limiting factor for the phosphorylation of glucose and fructose 6-phosphate. Moreover, McIlwain[13] has shown that the level of $NAD^+$ + NADH diminishes during anoxia but stays constant under aerobic conditions. In view of these facts, the effect of addition of $NAD^+$ and ATP to the incubation medium, on the rate of anaerobic glycolysis of cerebral cortex slices was studied. The experiments were carried out both in the presence as well as in the absence of $Ca^{++}$. Results shown in Table 2 demonstrate that the presence of 0.5 mM $NAD^+$ has a marked stimulating effect on the anaerobic glycolysis of both rat and guinea pig cerebral cortex slices, while the addition of 2.5 mM ATP has little or no effect. ATP + $Ca^{++}$, however, together have a marked inhibitory effect on the anaerobic glycolysis of both rat and guinea pig cerebral cortex slices.

TABLE 2

EFFECT OF EXOGENOUS NUCLEOTIDES ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

| Additions | Lactate produced µmoles per g initial wet wt (30-90 min) | |
|---|---|---|
| | Rat | Guinea pig |
| Control | 26.4 | 45.5 |
| ATP, 2.5mM | 27.0 | 56.0 |
| ATP, 2.5mM + $Ca^{++}$, 1mM | 12.1 | 36.8 |
| $NAD^{+}$, 0.5mM | 62.7 | 60.0 |
| $NAD^{+}$, 0.5mM + $Ca^{++}$, 1mM | 47.8 | 57.0 |

Incubations were carried out in a $Ca^{++}$-free medium containing 20mM glucose. Additions were made at 30 min and lactate production was measured enzymatically. Results are mean of 2-4 determinations with values within ± 7%.

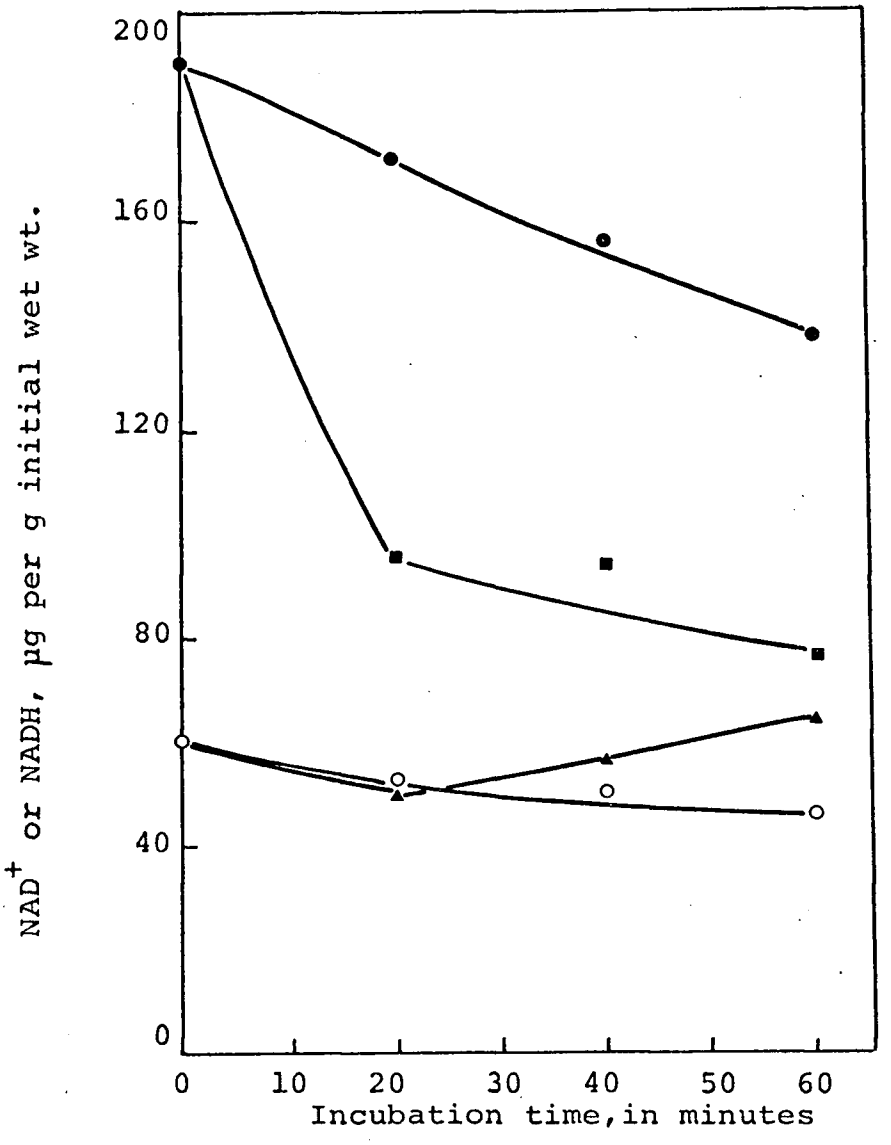
## 3.3 MOVEMENT OF $NAD^+$ ACROSS THE CELL MEMBRANE

Results reported in Chapter 3.2 show that $NAD^+$ concentration, in the cerebral cortex slices, may in fact exert a limiting effect on the rate of anaerobic glycolysis. It also appears that $NAD^+$, added to the incubation medium, may penetrate the brain cell. As it is believed that nucleotides are unable to cross the cell membrane barrier, experiments were carried out to observe whether the transport of NAD+ into the rat cerebral cortex slices can take place under anaerobic as well as aerobic conditions. Results of these experiments are shown in Figures 3 and 4.

The effects of added 0.5 mM $NAD^+$, on the total $NAD^+$ as well as NADH content of the rat cerebral cortex slices incubated under anaerobic conditions, are shown in Figure 3. It can be seen that, in brain slices, incubated in absence of added $NAD^+$, there is a marked decrease in the $NAD^+$ content during first 20 min of anaerobiosis; during the later period it remains relatively constant. On the other hand, the NADH content decreases slightly but steadily during anoxia. In the presence of 0.5 mM $NAD^+$ in the incubation medium, the $NAD^+$ concentration (measured as total $NAD^+$ present in the slice), remains at a relatively high level and shows only a slow decline. The NADH level however rises during the later period of incubation. It cannot be ascertained from these results

FIGURE 3

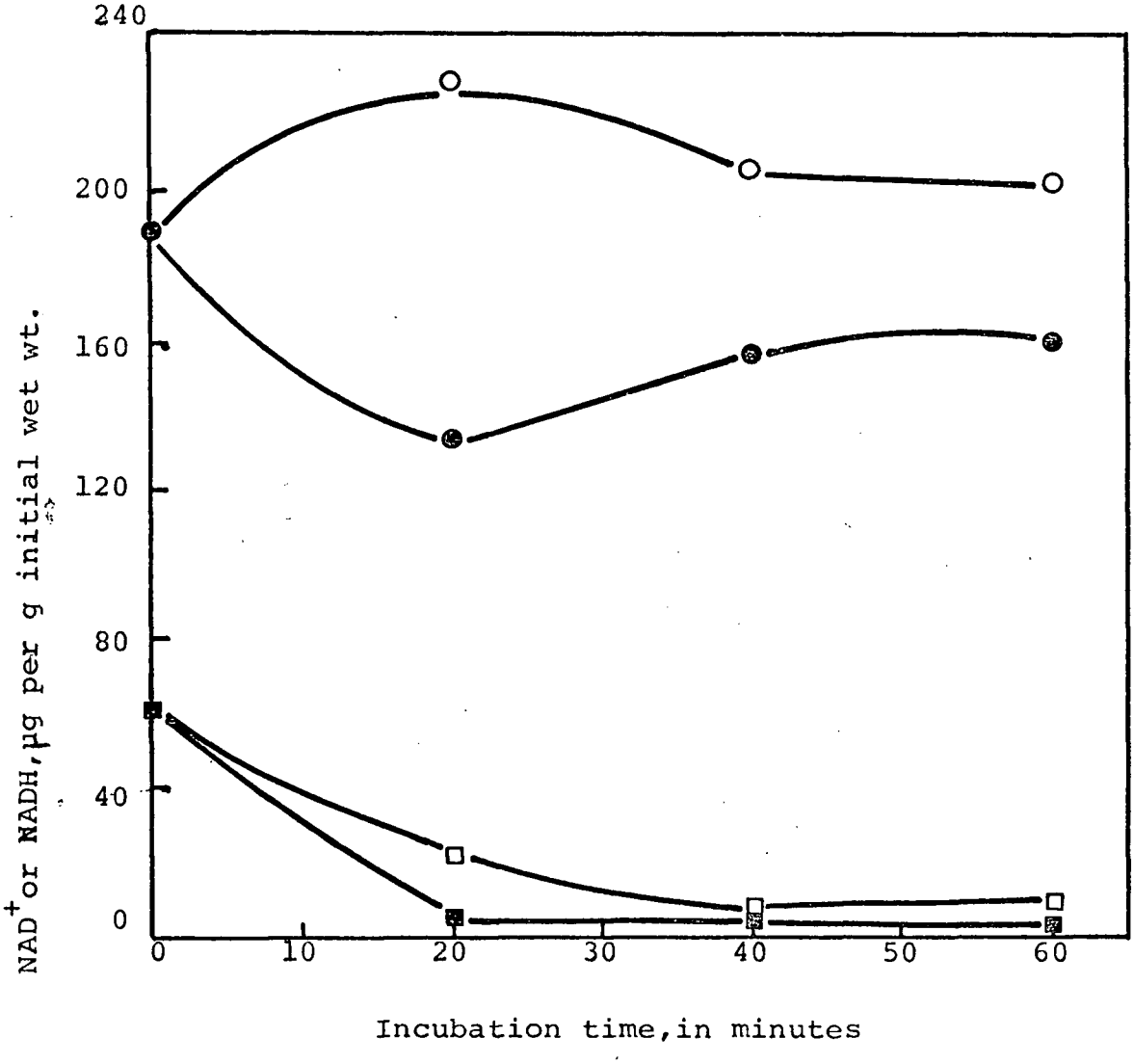
EFFECT OF EXOGENOUS $NAD^+$ ON THE $NAD^+$ AND NADH CONCENTRATIONS OF RAT CEREBRAL CORTEX SLICES UNDER ANOXIA




Incubations were carried out under $N_2$-$CO_2$ in a $Ca^{++}$-free medium containing 20mM glucose. $NAD^+$, when present in the incubation medium, was added at zero time. (●) $NAD^+$ conc., 0.5 mM $NAD^+$ was added to the medium; (■) $NAD^+$ conc., no exogenous $NAD^+$ was added to the medium; (▲) NADH conc, 0.5 mM $NAD^+$ was added to the medium; (○) NADH conc., no exogenous $NAD^+$ was added to the medium.

FIGURE 4

EFFECT OF EXOGENOUS $NAD^+$ ON THE $NAD^+$ AND NADH CONCENTRATIONS OF AEROBICALLY INCUBATED CEREBRAL CORTEX SLICES FROM RAT




Incubations were carried out under $O_2$:$CO_2$ in a $Ca^{++}$-free medium containing 20mM glucose. $NAD^+$, when present in the incubation medium, was added at zero time. (●) $NAD^+$ conc., no exogenous $NAD^+$ was added to the medium; (○) $NAD^+$ conc., 0.5 mM $NAD^+$ was added to the medium; (■) NADH conc., 0.5 mM $NAD^+$ was added to the medium; (□) NADH conc., no exogenous $NAD^+$ was added to the medium.

whether some $NAD^+$ is externally bound to the cerebral cortex slices or whether it is wholly intracellular (an ATP binding protein has been isolated from brain[268]). The facts that there is a rise in the NADH level as well as an increased rate of anaerobic glycolysis show that some $NAD^+$ must enter the cell under anaerobic conditions.

During aerobic conditions in the absence of added $NAD^+$ (Figure 4), the $NAD^+$ content of the tissue falls in the first 20 min of incubation. It then increases slowly with concomitant decrease in NADH. In the presence of 0.5 mM exogenous $NAD^+$, the cerebral level of $NAD^+$ is higher than those of the controls, while the NADH level is slightly lower during the initial period.

It has been shown by Weidemann, Hems and Krebs[269] that externally added AMP and ATP affects the metabolism of rat kidney. Presumably these nucleotides are able to penetrate the membrane barrier. Permeability of brain cell membrane to ATP has not been investigated by the present author, as substantial amounts of ATP bind to the brain membrane proteins[268]. Moreover, washing the slices to remove the bound ATP may result in a diminution of the content of endogenous ATP and give lower observed values.

## 3.4 EFFECTS OF $NAD^+$ ON AEROBIC GLYCOLYSIS

It has been shown in Chapter 3.2 that exogenous $NAD^+$ stimulates the rate of anaerobic glycolysis of

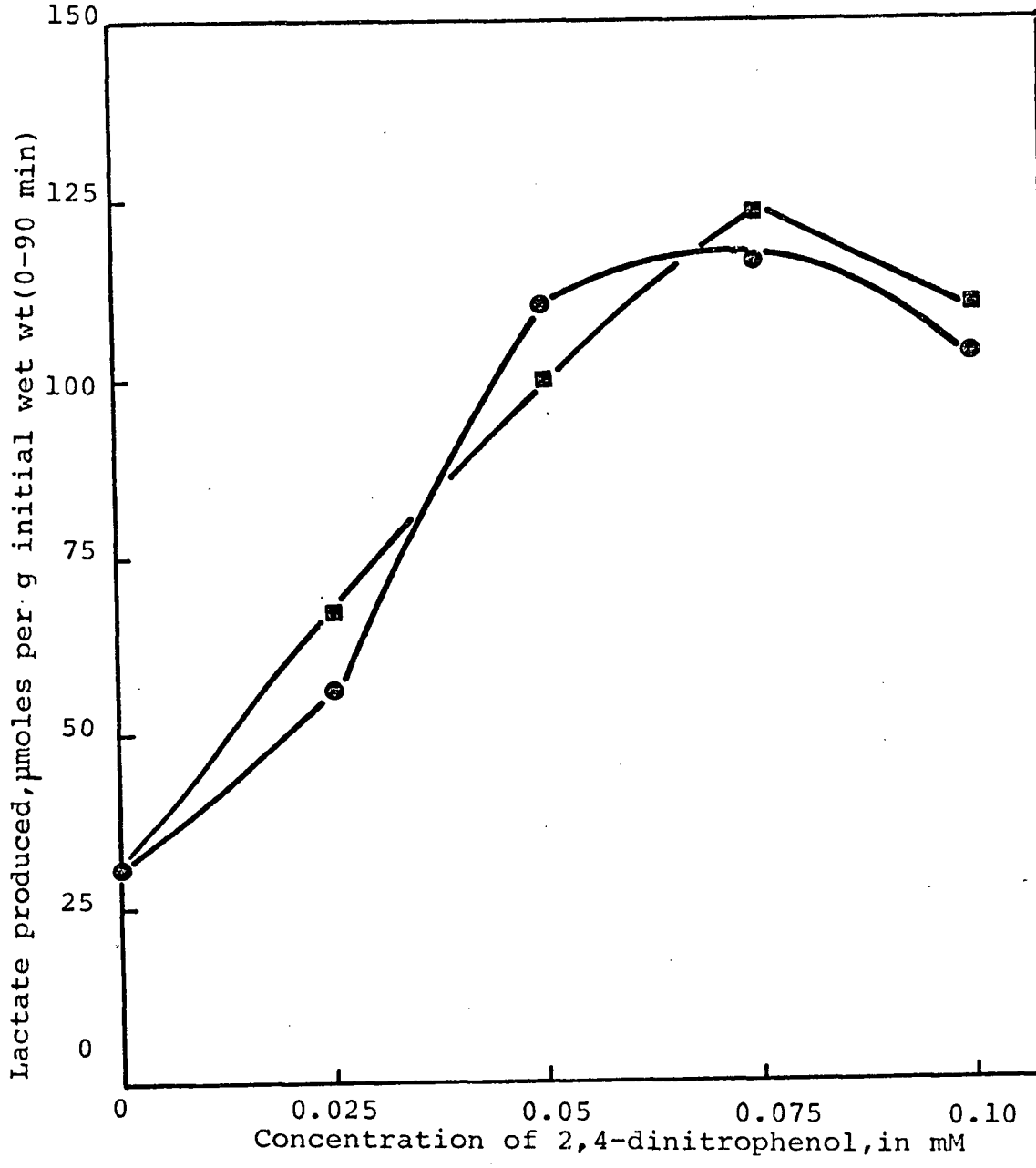
cerebral cortex slices. We also studied the effect of $NAD^+$ on the rate of aerobic glycolysis in incubated brain slices. It is known that the rate of aerobic glycolysis is reduced under aerobic conditions due to operation of the Pasteur effect (Chapter 1.2). However, in the presence of respiratory inhibitors or uncoupling agents such as 2,4-dinitrophenol (DNP), the rate of aerobic glycolysis is considerably increased. Results given in Figure 5 show the effects of external $NAD^+$ on DNP-stimulated aerobic glycolysis. It can be seen that, under these conditions, $NAD^+$ has no stimulating effect on the rate of glycolysis.

## 3.5 EFFECTS OF CITRATE AND AMP ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

Citrate is known to exert inhibitory effects on the glycolytic process and it has been reported that it markedly inhibits the activity of phosphofructokinase in isolated systems[35] (Chapter 1.2). The effect of 5 mM and 15 mM citrate on the rate of anaerobic glycolysis of rat cerebral cortex slices is shown in Figure 6. The inhibition of anaerobic glycolysis by citrate is consistent with the known suppressive activity of citrate on phosphofructokinase activity.

The effect of AMP on the anaerobic glycolysis of the rat cerebral cortex slices, as shown in Figure 6, is of importance in connection with its stimulation of the

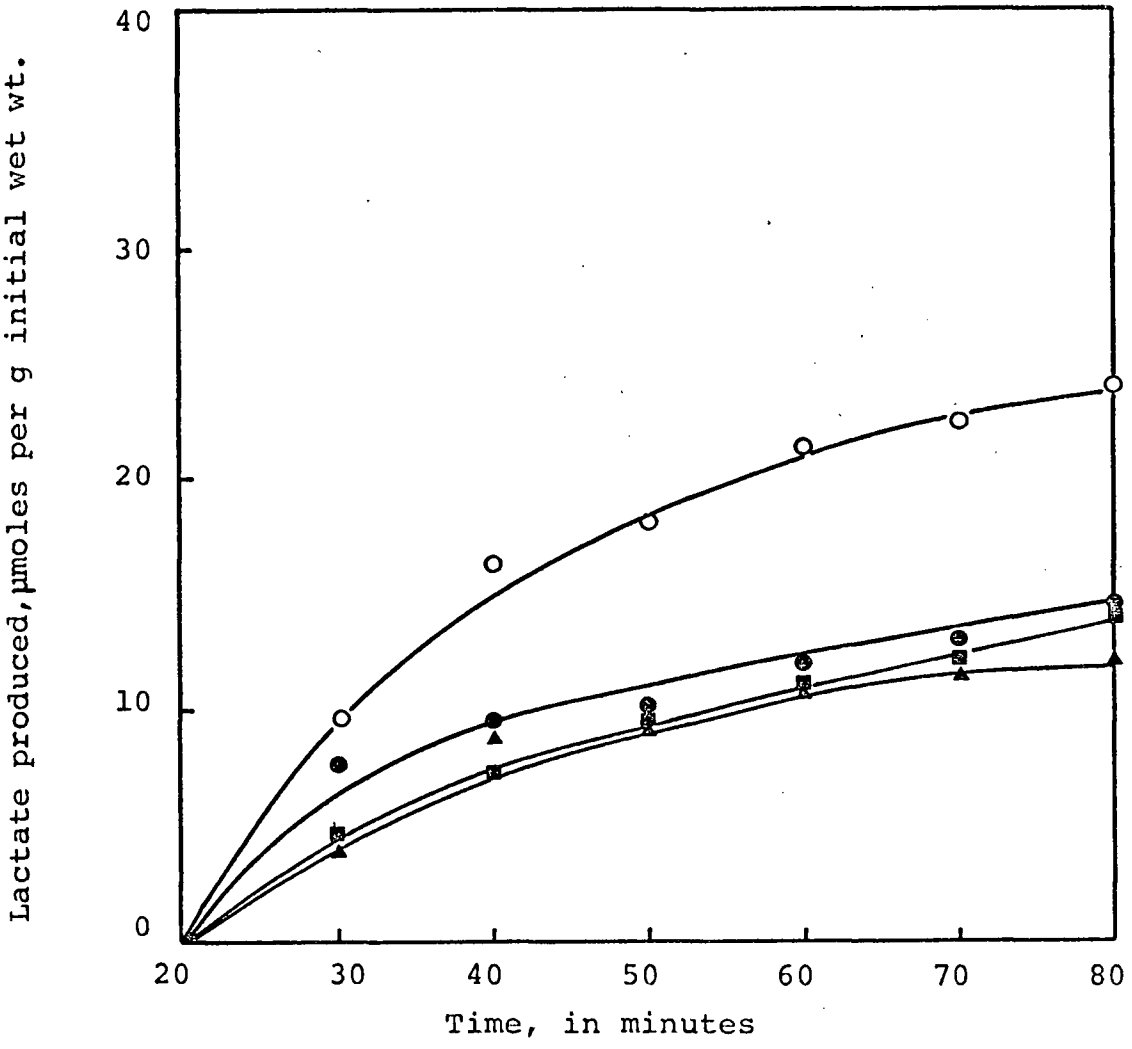
FIGURE 5
EFFECT OF $NAD^+$ IN THE PRESENCE OF VARYING CONCENTRATION OF 2,4-DINITROPHENOL ON THE AEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES




Incubations were carried out in a Krebs-Ringer bicarbonate medium containing 20 mM glucose, under $O_2$:$CO_2$. Additions were made at 30 min and lactate produced was determined enzymatically at the end of 90 min incubation period. (●) $NAD^+$ was not added to the medium; (■) 0.5 mM $NAD^+$ was added to the incubation medium.

FIGURE 6

EFFECTS OF CITRATE AND AMP ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES




Incubations were carried out in a $Ca^{++}$-free medium containing 20 mM glucose. Additions were made at zero time and lactate production was measured manometrically, as given in the materials and methods. (○) control; (●) 5mM citrate; (▲) 15 mM citrate and (■) 2 mM AMP.

activity of phosphofructokinase[36]. The data indicate an inhibition of the anaerobic glycolysis of the cerebral cortex slices. It is evident that AMP has other effects that mask its stimulation of phosphofructokinase.

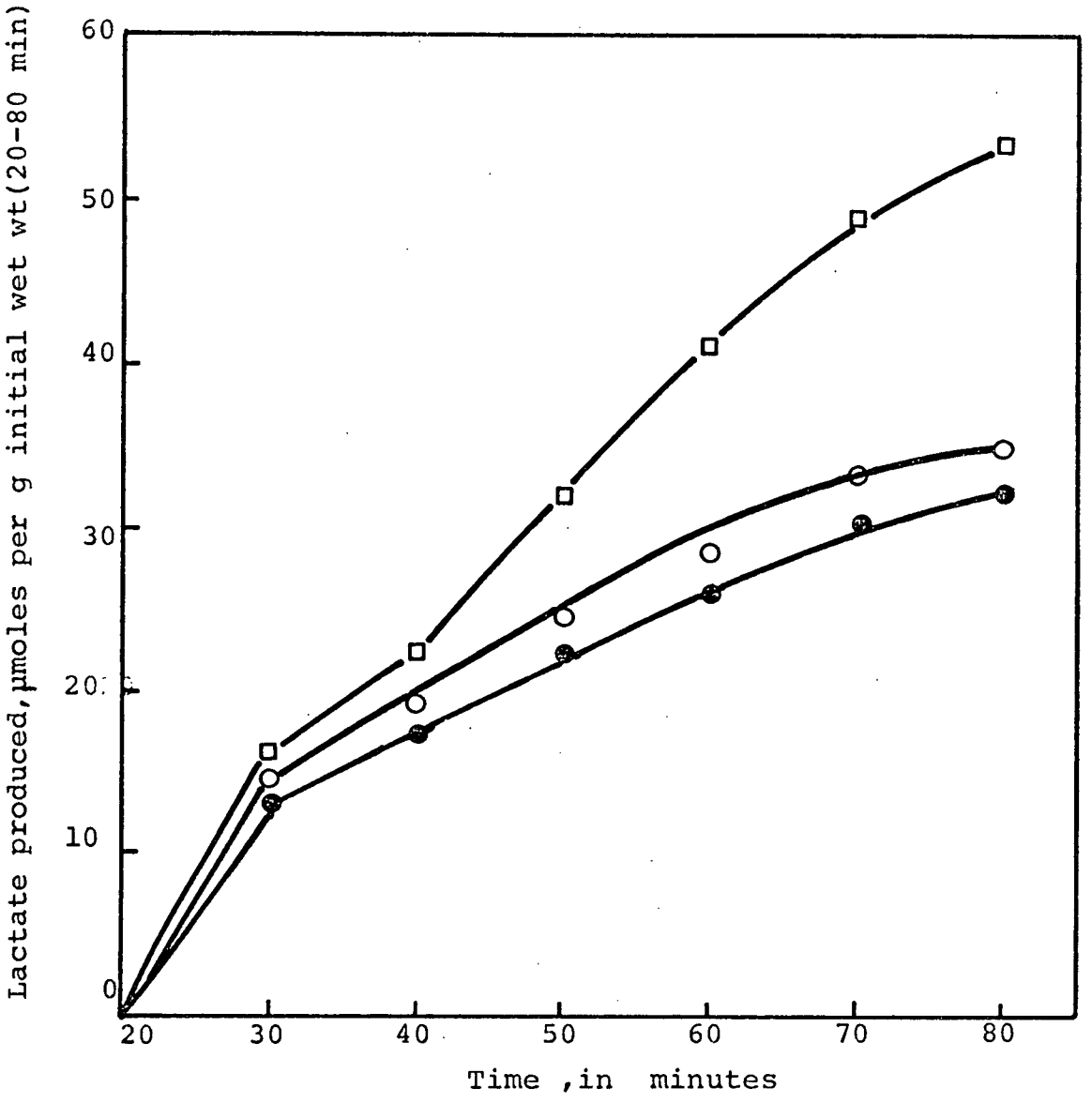
## 3.6 EFFECT OF CYCLIC AMP AND DIBUTYRYL CYCLIC AMP ON THE ANAEROBIC GLYCOLYSIS OF THE CEREBRAL CORTEX SLICES

Cyclic AMP (cAMP) is known to affect a number of enzyme systems in mammalian tissues, bacteria[270] as well as those in plants[271]. There is evidence that the action of a number of hormones might be mediated through cAMP[272], cAMP is an activator of phosphorylase[273] and it also diminishes the inhibitory effect of ATP on phosphofructokinase, causing a stimulation of phosphofructokinase activity[35]. Brain has the highest ability among mammalian tissues to synthesise cAMP[274]. Since cAMP is known to enhance the activity of phosphofructokinase, we studied the effects of cAMP on the anaerobic glycolysis of the guinea pig cerebral cortex slices and the results are shown in Figure 7. It can be seen that 1 mM cAMP enhances the rate of anaerobic glycolysis of the cerebral cortex slices. Under similar conditions, dibutyryl cAMP, whose action is similar to cAMP in a number of systems, has no effect.

Dittmann and Herrmann[275] have shown that in the presence 0.5-1.0 mM cAMP, the rates of aerobic glycolysis and respiration of rabbit cerebral cortex slices are increased.

FIGURE 7

EFFECTS OF CYCLIC AMP AND DIBUTYRYL CYCLIC AMP ON THE ANAEROBIC GLYCOLYSIS OF GUINEA PIG CEREBRAL CORTEX SLICES




Incubations were carried out in a $Ca^{++}$-free medium containing 20 mM glucose. Additions were made at zero time and lactate production was measured manometrically, as given in the materials and methods. (○) control; (□) 1mM cyclic AMP; (●) 1 mM dibutyryl cyclic AMP.

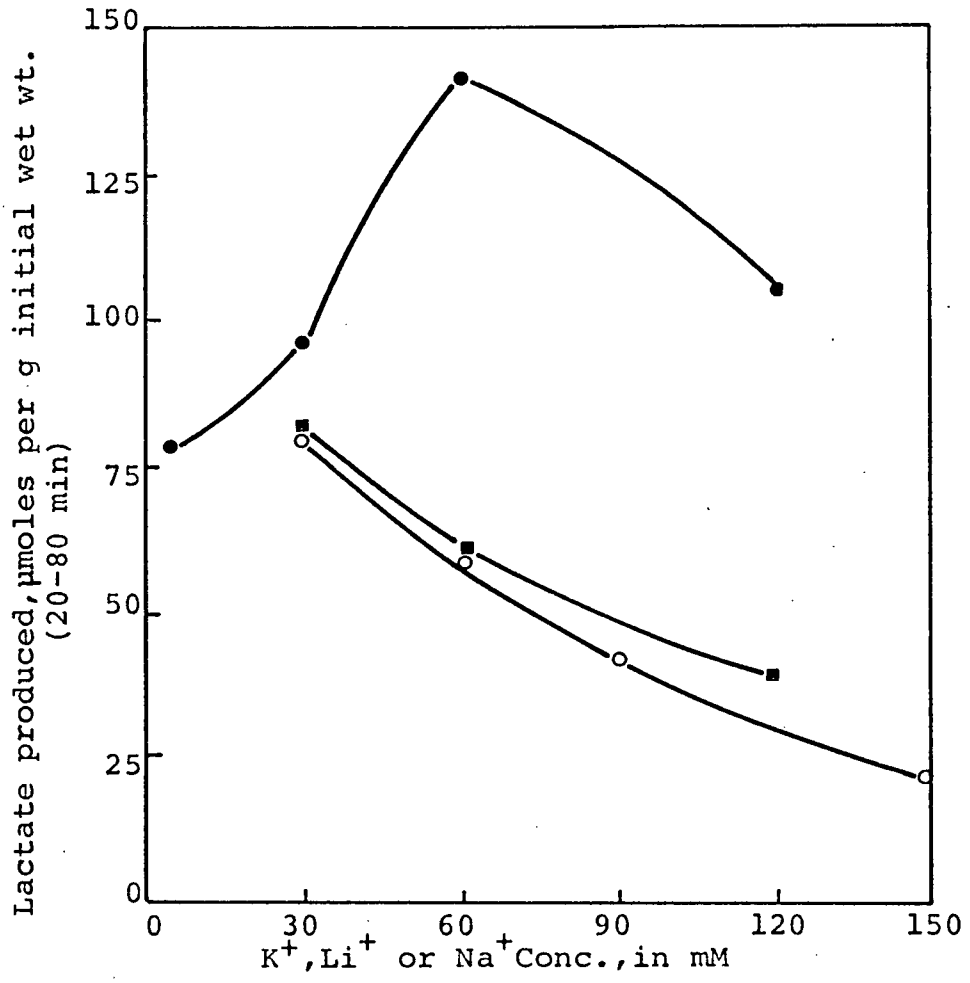
## 3.7 EFFECTS OF VARYING CATION CONCENTRATIONS ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

It has been mentioned in Chapter 1 that cerebral metabolism *in vitro* is greatly influenced by the concentration of cations in the incubation medium. $K^+$ is required for the activity of pyruvate kinase[36,301]. The activity of pyruvate kinase increases greatly with increase in $K^+$ concentration up to 50 mM[59] but a high concentration of $K^+$ has been shown to have an inhibitory effect on the anaerobic glycolysis of cerebral cortex slices[62]. However, in the latter study, the concentration of $Na^+$ in the incubation medium was not changed when the $K^+$ content was increased. Sodium ions are known to inhibit the activity of hexokinase[53a] and pyruvate kinase[37,38,39,301]. Hence the effects of varying concentrations of $Na^+$ and $K^+$ on the rates of anaerobic glycolysis of cerebral cortex slices were studied. The effect of replacing $Na^+$ with $Li^+$ was also studied. The results of these experiments are shown in Figure 8.

The evidence indicates that increasing the concentration of $K^+$, while at the same time decreasing the concentration of $Na^+$, has a marked stimulating effect on the rate of cerebral anaerobic glycolysis. At very high concentrations of $K^+$, some inhibition was observed. $Na^+$ has marked inhibitory effect on the glycolysis of brain slices. $Li^+$ acts in a manner similar to $Na^+$. These data

FIGURE 8

EFFECT OF VARYING CONCENTRATIONS OF $K^+$, $Li^+$ AND $Na^+$ ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES




Incubations were carried out in the presence of 20 mM glucose. The medium contained either $Na^+$, $K^+$ + 29 mM $Na^+$ or $Li^+$ + 29 mM $Na^+$. When required, the medium was made isotonic with sucrose. Lactate production was measured manometrically, (○) $Na^+$ conc.; (●) $K^+$ conc. + 29 mM $Na^+$; (■) $Li^+$ conc. + 29 mM $Na^+$.

show that while considering effects of a particular cation on cerebral anaerobic glycolysis, the concentration of other cations present in the incubation medium must also be taken into account.

## 3.8 EFFECTS OF L-GLUTAMATE AND OF AMMONIUM IONS ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES.

During his studies on glutamine formation, Krebs[277] observed that L-glutamate inhibits the anaerobic formation of lactic acid. This phenomenon was further investigated in greater detail by Weil-Malherbe[278] who showed that both D- and L-glutamate are active in inhibiting anaerobic glycolysis. This effect is reversed on addition of pyruvate. Because of the possible role of glutamate as an excitatory amino acid in the central nervous system, and its possible effects on cation movements, its effect on the rate of anaerobic glycolysis was reinvestigated.

Results, which are shown in Figure 9, demonstrate that glutamate inhibits the rate of anaerobic glycolysis of cerebral cortex slices in a Krebs-Ringer bicarbonate medium as well as in a $Ca^{++}$-free medium. However, the effect of glutamate in a $Ca^{++}$-free medium is less than that in a $Ca^{++}$-containing (Krebs-Ringer bicarbonate) medium. D-glutamate was also effective in diminishing the rate of anaerobic glycolysis but to a lesser extent.

Weil-Malherbe[278] noted that $NH_4^+$ inhibits the anaerobic glycolysis of guinea pig cerebral cortex slices.

FIGURE 9

EFFECTS OF GLUTAMATE AND $NH_4^+$ ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES

Incubations were carried out in a medium containing 20 mM glucose. Additions were made at zero time and lactate production was measured manometrically as given in the materials and methods. ( O ) $Ca^{++}$-free medium, control; ( ● ) Krebs-Ringer bicarbonate medium, control; ( ▲ ) Krebs-Ringer bicarbonate medium, with 5 mM L-glutamate; ( Δ ) $Ca^{++}$-free medium, with 5 mM L-glutamate; ( ■ ) Krebs-Ringer bicarbonate medium, with 5 mM D-glutamate; ( □ ) $Ca^{++}$-free medium, with 5mM $NH_4^+$.

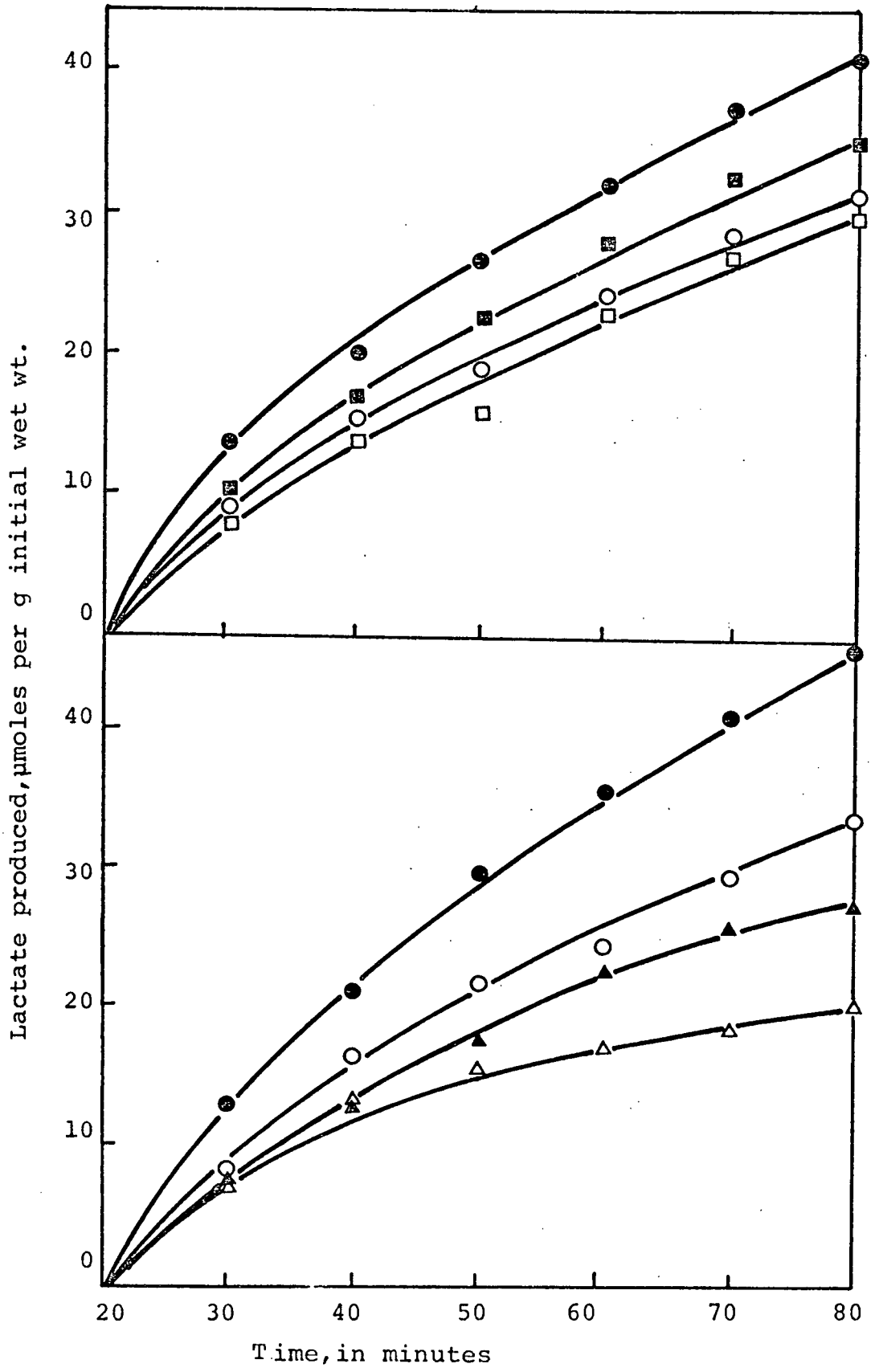



FIGURE 9 : EFFECTS OF GLUTAMATE AND $NH_4^+$ ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES

However, his results were not consistent; in some experiments 1-3 mM $NH_4^+$ had little or no effect while, in some experiments, considerable inhibition of anaerobic glycolysis was observed at 1 mM $NH_4^+$.

The effects of addition of 5mM $NH_4^+$ to a $Ca^{++}$-free medium on cerebral anaerobic glycolysis is shown in Figure 9 Under the given experimental conditions, it has little or no effect on the rate of anaerobic glycolysis. It will be shown later, in Chapter 4, that $NH_4^+$ has a marked effect on the rate of anaerobic glycolysis when the latter is stimulated by certain drugs, such as tetrodotoxin.

## 3.9 EFFECT OF $NAD^+$ AND ATP, IN A SODIUM-FREE MEDIUM, ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

The effects of $NAD^+$ and ATP on the anaerobic glycolysis of cerebral cortex slices in a $Na^+$-free medium are shown in Table 3. It can be seen that these nucleotides have no effect on the rate of anaerobic glycolysis in a $Na^+$-free medium, either in the presence or in the absence of $Ca^{++}$. Further, the rate of glycolysis, in control slices, is itself greatly reduced as compared to a medium containing normal concentration of $Na^+$ and $K^+$. Thus the anaerobic glycolysis shows a marked dependence on the presence of cations in the incubation medium. As has been shown earlier (Chapter 3.7), replacing $Na^+$ by $K^+$ in the incubation medium has a stimulatory effect on the rate of anaerobic glycolysis.

TABLE 3

EFFECTS OF CALCIUM AND $NAD^+$ ON THE ANAEROBIC GLYCOLYSIS OF GUINEA PIG CEREBRAL CORTEX SLICES IN A SODIUM FREE MEDIUM

| Addition | Incubation period | Lactate produced, µmoles per g initial wet wt |
|---|---|---|
| Control | 0-30 min | 18.1 |
| Control | 0-90 min | 20.8 |
| $Ca^{++}$, 1mM | 0-90 min | 23.0 |
| $Ca^{++}$, 4mM | 0-90 min | 20.2 |
| $NAD^+$, 0.5mM | 0-90 min | 24.5 |
| $NAD^+$, 0.5mM + $Ca^{++}$, 1mM | 0-90 min | 23.5 |

Incubations were carried out in a medium containing 260mM sucrose, 6mM KCl, 20mM glucose and 10mM Tris-HCl buffer (pH 7.4). Lactate production was measured enzymatically. Additions were made at 30 min. Results are averages of 2-4 determinations with values within ± 7%.

## 3.10 RATE LIMITING FACTORS OF GLYCOLYSIS IN THE ACETONE POWDER EXTRACTS

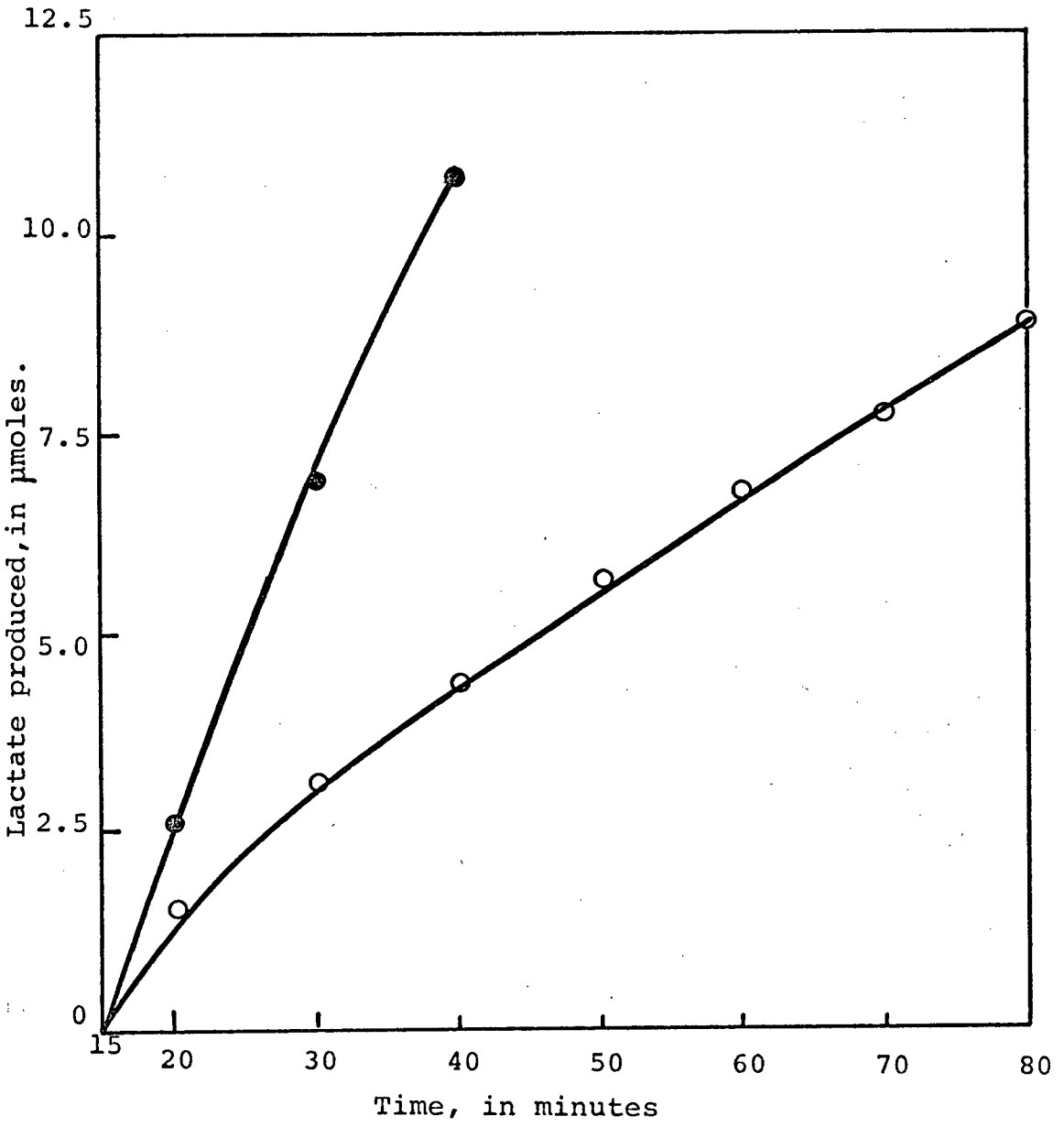
To maintain optimal rates of glycolysis by acetone powder extracts, or ground cerebral tissues, it is necessary to add various coenzymes and co-factors to the suspensions. This is necessary because $NAD^+$, ATP, $K^+$ and phosphate are lost quickly from the tissue during preparation of the brain acetone powder. Although ATPase is very low in the acetone powders[279], there is a high NADase activity[280] in the brain tissue which persists even in the acetone powders. For this reason, it is necessary to add nicotinamide, which is a strong inhibitor of NADase[280], in the extracts.

Results given in Figure 10 show the time course of glycolysis by acetone powder extracts fortified with $NAD^+$, ATP, $Mg^{++}$ and $K^+$. Under the given experimental conditions, the rate of anaerobic glycolysis by the extracts is approximately constant and it is proportional to the quantity of extract taken. Moreover, the magnitude of the rate of glycolysis by the acetone powder extracts is much higher than that of a corresponding quantity of the brain slices. This will be further discussed in Chapter 8.

The effects of changing concentration of the different cofactors and such rate limiting componenets as $K^+$, $Na^+$, $NAD^+$ and ATP are shown in Figure 11. When the concentration of ATP, or $NAD^+$ is increased, without change of the $Na^+$ concentration, the rate of glycolysis is increased.

FIGURE 10

ANAEROBIC GLYCOLYSIS BY ACETONE POWDER EXTRACTS OF RAT BRAIN




Incubations were carried out in a medium containing 52 mM $Na^{+}$, 28 mM $K^{+}$, 4 mM $Mg^{++}$, 33 mM cysteine, 33 mM nicotinamide, 0.5 mM $NAD^{+}$ and 0.8 mM ATP. Lactate production was measured manometrically. All the vessels contained 20 mM glucose. (○) 20 mg acetone powder extract per vessel; (●) 40 mg acetone powder extract per vessel.

Increase of $Na^+$ has an inhibitory effect and increasing $K^+$ concentration has a slight stimulating effect. The effect of increased $K^+$ is more marked when the concentration of $Na^+$ is low. These experiments demonstrate that different cofactors may exert rate limiting effects on the speed of anaerobic glycolysis by the acetone powder extracts.

SUMMARY OF CHAPTER 3

Results given in this chapter describe the action of various compounds that have rate limiting effects on the process of glycolysis on the rates of anaerobic glycolysis of the cerebral cortex slices and of extracts of acetone powder of brain.

(1) In confirmation of the earlier work of Quastel and his coworkers[70,71], it is found that $Ca^{++}$ has a marked stimulatory effect on the rate of anaerobic glycolysis of adult rat and guinea pig cerebral cortex slices.

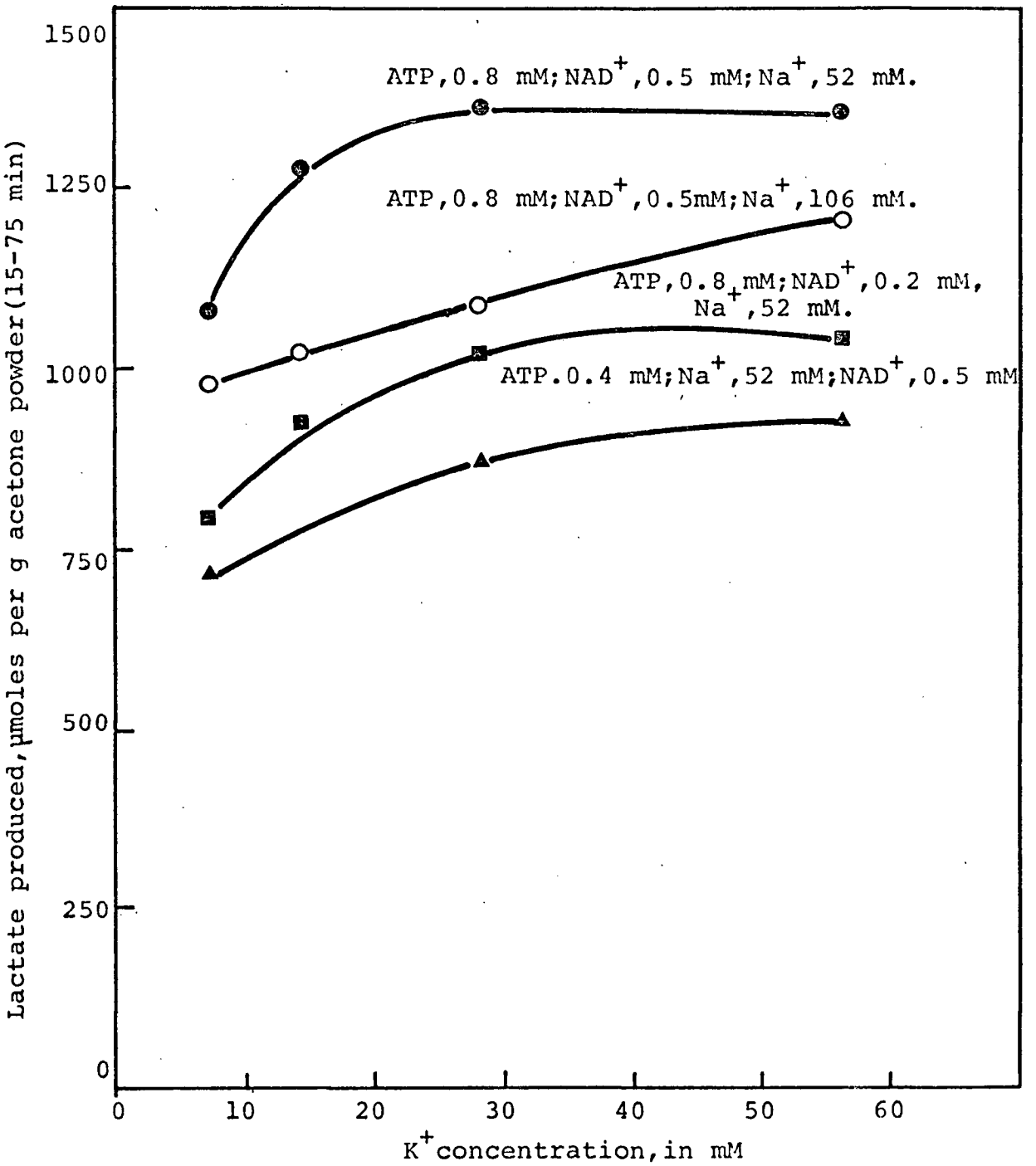
(2) $Ca^{++}$ has little effect on the rate of anaerobic glycolysis of infant rat brain, whereas the marked response of infant guinea pig brain to $Ca^{++}$ is similar to that of the adult brain.

(3) Externally added $NAD^+$ stimulates the rate of cerebral anaerobic glycolysis while additon of ATP has little or no effect in a $Ca^{++}$-free incubation medium. The addition of ATP in presence of $Ca^{++}$ inhibits the rate of anaerobic glycolysis. It is thought that this is due to ATP chelation of $Ca^{++}$.

(4) Movement of $NAD^+$ across the brain cell membrane has been studied. It appears that $NAD^+$ cross the brain cell

FIGURE 11

EFFECT OF VARYING CONCENTRATIONS OF RATE LIMITING FACTORS ON THE ANAEROBIC GLYCOLYSIS OF BRAIN ACETONE POWDER EXTRACTS




Incubations were carried out as given with Figure 10 except that the concentrations of $K^+$, $Na^+$, $NAD^+$ and ATP were varied.

membrane under both anaerobic and aerobic conditions.

(5) $NAD^+$ has no effect on the aerobic glycolysis that has been stimulated by 2,4-dinitrophenol.

(6) Citrate and AMP are inhibitors of the rate of anaerobic glycolysis of cerebral cortex slices. Cyclic AMP has a stimulatory effect.

(7) It is known that a high concentration (100 mM) of $K^+$, when added to a Ringer medium, inhibits the rate of cerebral anaerobic glycolysis. It is shown that when the concentration of $Na^+$ in the incubation medium is decreased at the time when $K^+$ is raised, $K^+$ has a marked stimulatory action on the rate of anaerobic glycolysis.

(8) The effects of $Na^+$ and $K^+$ are also apparent during the glycolysis brought about by the acetone powder extracts of brain. The effects of these ions are not as large as those shown in anaerobic glycolysis by brain tissue slices.

(9) Whilst it is confirmed that L-glutamate is an inhibitor of the rate of anaerobic glycolysis of the cerebral cortex slices, $NH_4^+$ at equivalent concentrations has no effect.

(10) It is evident that a variety of factors exert rate limiting effects on anaerobic glycolysis of cerebral cortex slices. In particular, attention is drawn to the marked effects of $Na^+$, $K^+$ and $Ca^{++}$.

# CHAPTER 4

# EFFECTS OF TETRODOTOXIN ON CEREBRAL METABOLISM AND TRANSPORT IN ANOXIA

It has been mentioned in Chapter 1 that TTX, the puffer fish neurotoxin, in low concentrations, suppresses the generation of action potentials in a variety of excitable tissues. It has also been shown that TTX blocks the increase in respiration brought about by application of electric impulses as well as by omission of $Ca^{++}$ from the incubation medium[130]. Moreover, $Ca^{++}$ increases the rate of anaerobic glycolysis of guinea pig, as well as of rat cerebral cortex slices (Chapter 3). In view of the possibility that $Ca^{++}$ and TTX might act similarly on the brain tissue, the effect of TTX on the anaerobic glycolysis of cerebral cortex slices was investigated. In addition, kidney medulla and acetone powder extracts of brain have been used to ascertain whether the effect of TTX is a specific property of excitable tissue and if the integrity of the brain cell membrane is also required for action. Results of experiments carried out, to throw light on these problems, will be described below.

## 4.1 EFFECTS OF TETRODOTOXIN ON THE ANAEROBIC GLYCOLYSIS OF GUINEA PIG AND RAT CEREBRAL CORTEX SLICES

Results of a typical experiment, showing the effect of 2 μM TTX on the rate of anaerobic glycolysis of rat cerebral

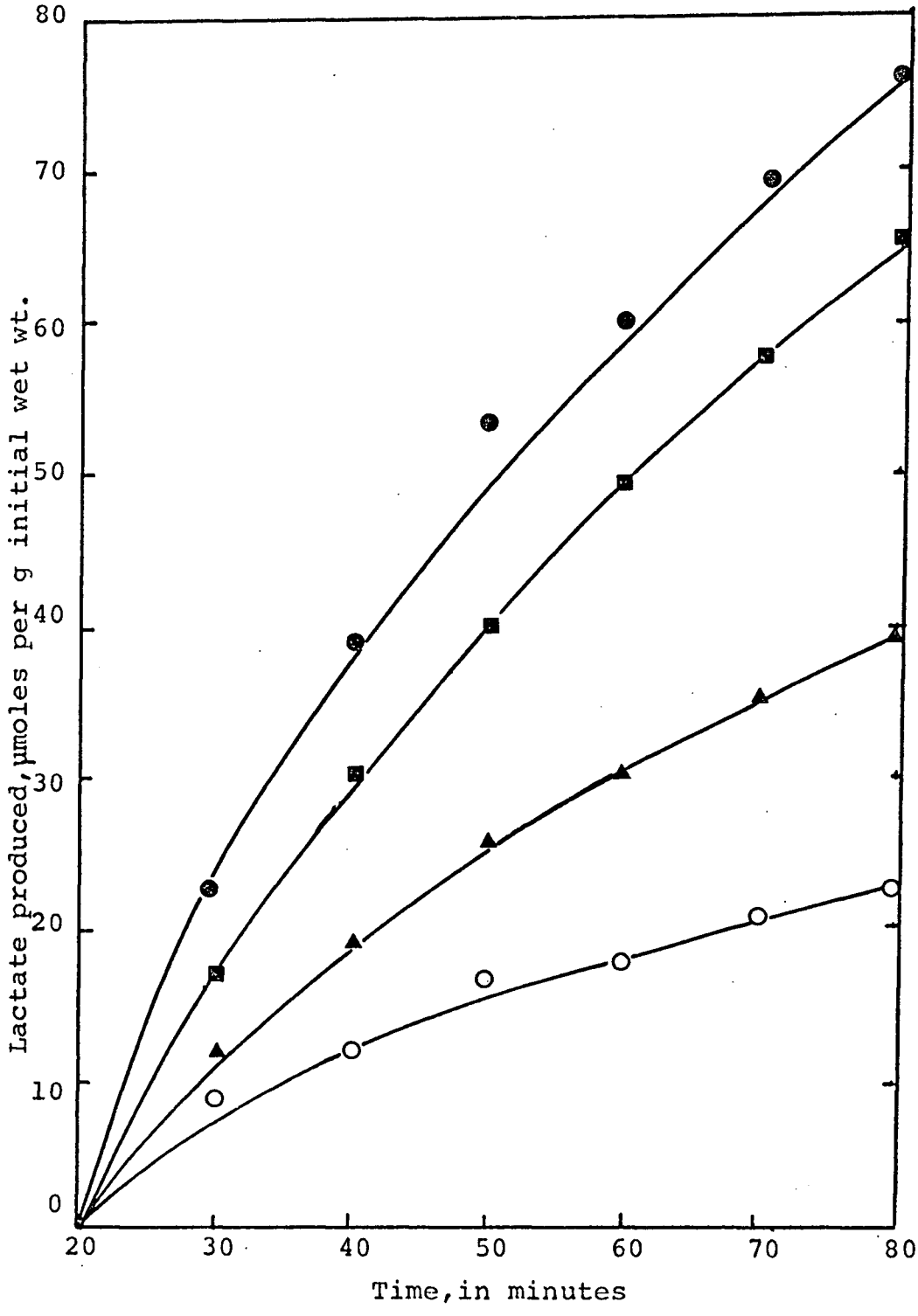
cortex slices in a $Ca^{++}$-free, as well as in a Krebs-Ringer bicarbonate medium, are given in Figure 12. It can be seen that TTX increases the rate of anaerobic glycolysis in both the media. However, the percentage increase is greater in the $Ca^{++}$-free medium than in that containing $Ca^{++}$. This is partly due to the fact that the control values of glycolysis (without TTX) are greater in a Krebs-Ringer bicarbonate medium than in the $Ca^{++}$-free medium (Figure 12). The same effects of TTX on anaerobic glycolysis are observed with guinea pig cerebral cortex slices.

The effects of three different concentrations of TTX on the rates of anaerobic glycolysis of guinea pig and rat cerebral cortex slices are shown in Figure 13. It is evident that the rate of anaerobic glycolysis increases with increased concentration of TTX in both the animals, although the rate of glycolysis in the absence of TTX is greater in guinea pig cerebral cortex slices than in rat cortex slices; the maximum rates obtained for the two animals, in the presence of TTX, are about the same.

## 4.2 EFFECTS OF CALCIUM ON TETRODOTOXIN STIMULATED ANAEROBIC GLYCOLYSIS

The effects of addition of different concentrations of calcium, to a $Ca^{++}$-free medium in the presence of 2μM TTX, on the rate of anaerobic glycolysis of cerebral

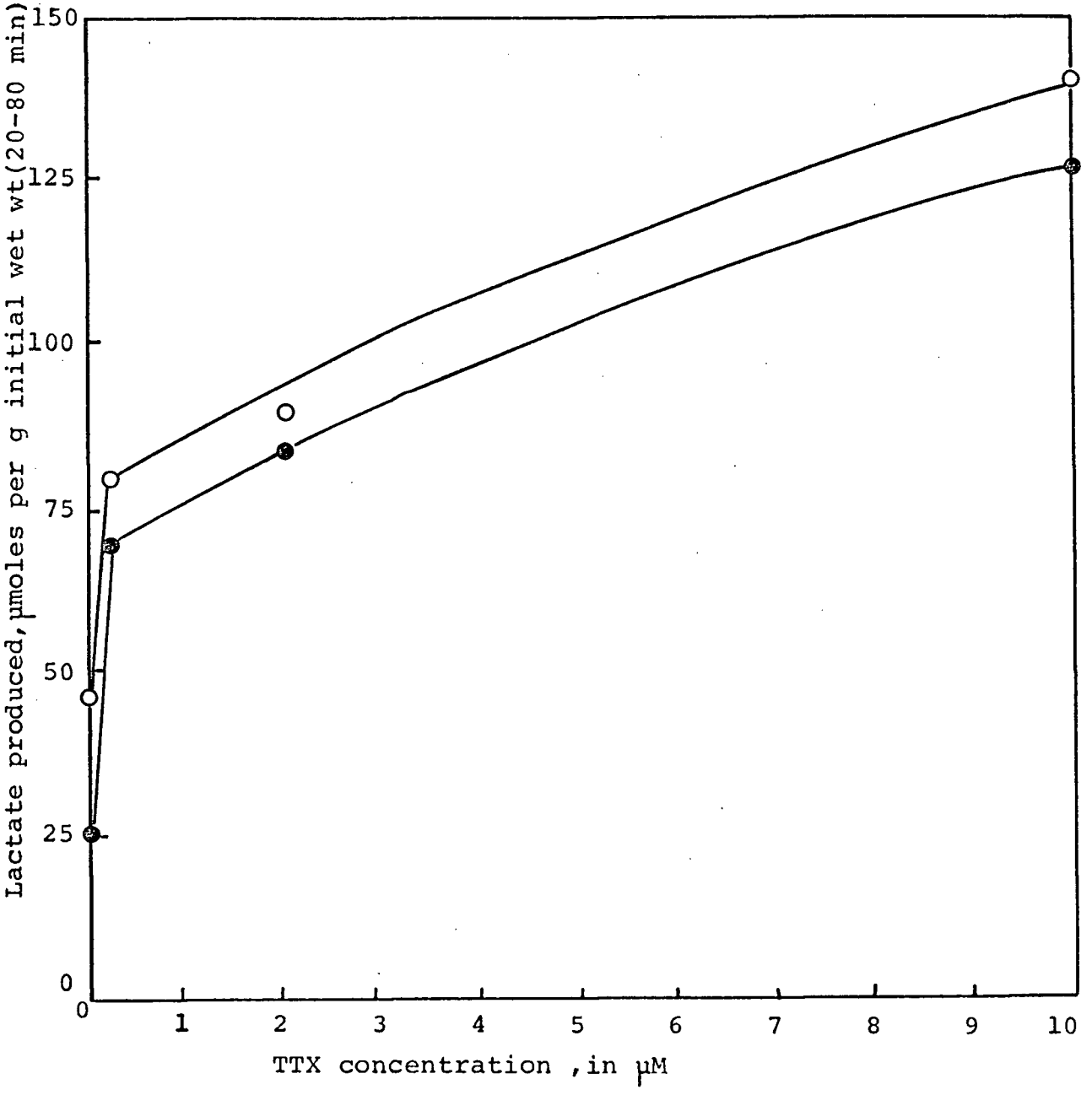
FIGURE 12
EFFECTS OF TETRODOTOXIN ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES




Incubations were carried out in the presence of 20 mM glucose. TTX(2 µM), when present, was added at zero time and lactate production was measured manometrically as given in the materials and methods. (○) control, $Ca^{++}$-free medium; (▲) control, Krebs-Ringer bicarbonate medium; (●) 2 µM TTX present, $Ca^{++}$-free medium; (■) 2 µM TTX present, Krebs-Ringer bicarbonate medium.

FIGURE 13

EFFECTS OF DIFFERENT CONCENTRATIONS OF TETRODOTOXIN ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES




Incubations were carried out in a $Ca^{++}$-free medium containing 20 mM glucose. TTX was present from the start of the experiment and lactate production was measured manometrically as given in the materials and methods. (O) Guinea pig cerebral cortex slices; (●) rat cerebral cortex slices.

cortex slices are shown in Figure 14. With guinea pigs, the presence of $Ca^{++}$, under these conditions, causes further increase in the rate of anaerobic glycolysis with values approaching that obtained with 4 mM $Ca^{++}$ alone. However, with rats, no such increase is observed; the rates of glycolysis either remain the same (as obtained with TTX alone) or are slightly decreased.

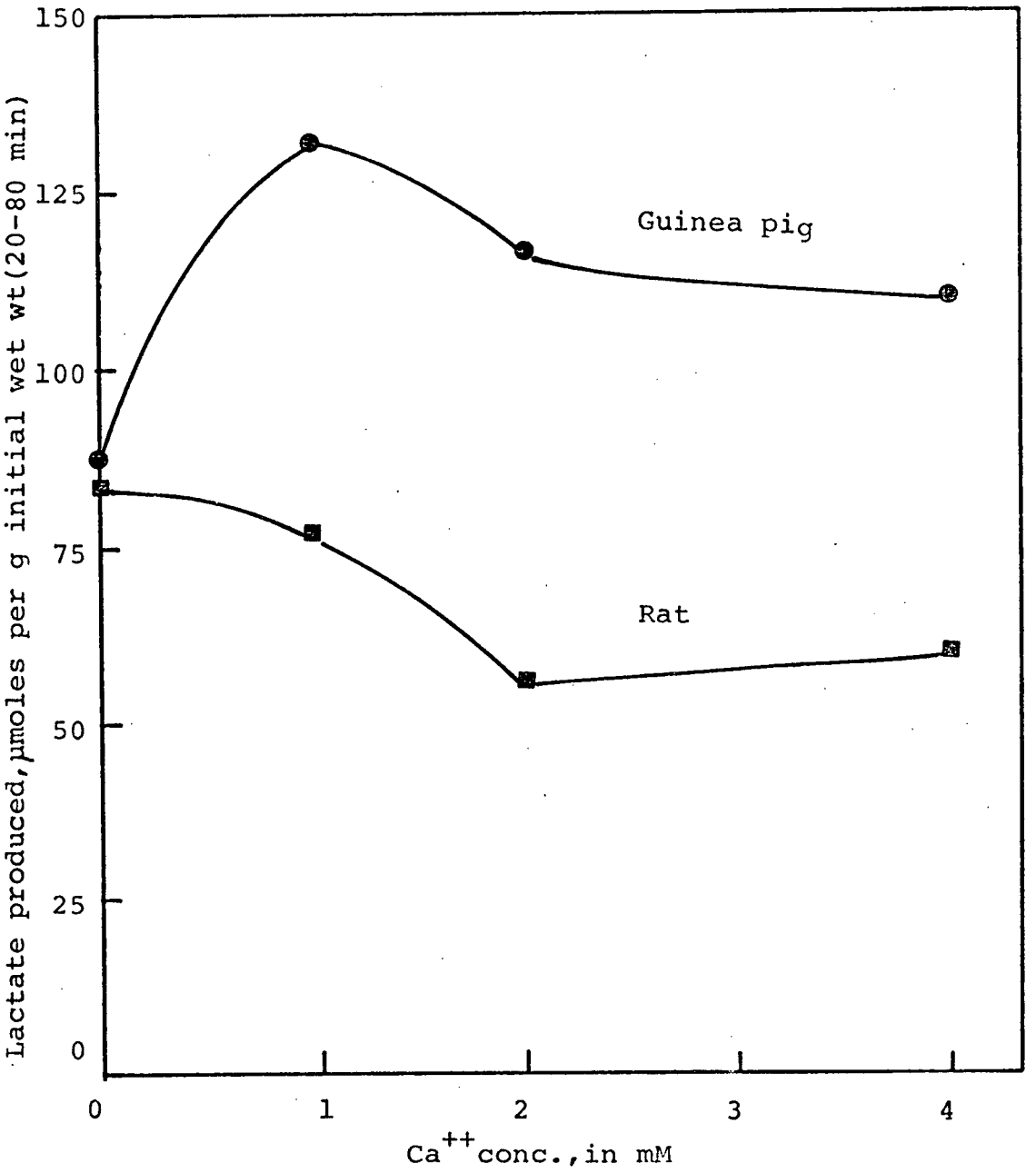
Under the same experimental conditions, the amount of acid labile phosphate was also measured (not shown), since this gives some indication of the energy status of the cell. The values obtained in the presence of TTX were higher than the corresponding controls. ATP concentrations in these tissues were also measured and the results will be discussed in Chapter 5.6.

## 4.3 EFFECT OF TETRODOTOXIN ON $NAD^+$ MOVEMENTS ACROSS THE CELL MEMBRANE

It has been shown in Chapter 3 that the rate of anaerobic glycolysis of cerebral cortex slices decreases with time of incubation. At the same time there is decrease in the $NAD^+$ concentration under anoxia. As $NAD^+$ is one of the cofactors required for glycolysis, it seemed to us that the decline of the rate of glycolysis might be due to loss of $NAD^+$ from the tissue into the medium. It seemed possible that TTX might increase the rate of glycolysis by blocking the efflux of $NAD^+$ during the incubation. Although the decline of $NAD^+$ level observed by us was only 20% during the

FIGURE 14

EFFECTS OF DIFFERENT CONCENTRATIONS OF CALCIUM ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES IN THE PRESENCE OF TETRODOTOXIN




Incubations were carried out in a $Ca^{++}$-free medium containing 20 mM glucose. 2 µM TTX and $Ca^{++}$, when present, were added at zero time and lactate production was measured manometrically as given in the materials and methods.

20-60 min incubation period, this loss may still be significant, if it occurs only from the extramitochondrial compartment (cytoplasm) where glycolysis is taking place. However, in the presence of 2 μM TTX, contrary to the above assumption, no increase in the $NAD^+$ level of the cerebral cortex slices was observed. We also assayed the $NAD^+$ level in the medium at the end of the incubation period, by the cycling method of Lowry[261-263], but no $NAD^+$ could be detected in the incubation medium (results not shown).

The presence of $NAD^+$ in the incubation medium was also tested by the method of Quastel and Wheatley[284] but attempts to show its presence were not successful. However, these experiments led to the finding that some lactic dehydrogenase appears in the medium in which the cerebral cortex slices are incubated. Stern, Eggleston, Hems and Krebs[285] have shown that glutamic-aspartic transaminase diffuses from incubated guinea pig cortex slices into the medium. It thus appears that certain enzymes may leak into the medium from incubated brain slices but it is uncertain whether this is simply due to the breakdown of damaged cells.

## 4.4 EFFECTS OF TETRODOTOXIN ON THE AMINO ACID EFFLUX FROM THE CEREBRAL CORTEX SLICES

As has been shown in Chapter 4.3, the possibility that the effect of TTX on the anaerobic glycolysis might be due to

blocking of $NAD^+$ efflux has been ruled out. However, under conditions of anoxia, owing to the fall in cell concentration of ATP, active transport mechanisms diminish in activity. Hence, the possibilities exist that some of the intermediates of glycolysis might leak out into the incubation medium and thus reduce the rate of anaerobic glycolysis. For example, compounds like pyruvate are known to increase the rate of anaerobic glycolysis of cerebral cortex slices[71]. If TTX blocks the efflux of such compounds from the slices, then it may exert, indirectly, an accelerating effect on the rate of anaerobic glycolysis.

Energy dependent transport processes diminish in activity during anoxia and as a result amino acids concentrated against a concentration gradient in the brain tend to leak into the incubation medium. The active transport of amino acids is associated with the cation movements at the brain cell membrane and because these movement are affected by TTX, it is possible that TTX may also influence the cerebral efflux of amino acids. This effect may be more pronounced in the case of aspartic acid and glutamic acid as these are present in brain slices in large amounts. The results reported in this section show that this conclusion is correct.

The amino acid concentrations in the tissue, as well as in the incubation medium, in the presence and absence of

2 μM TTX are given in Table 4. It can be seen that the concentrations of a number of amino acids are higher in the incubated slices in the presence of TTX than in its absence. The most marked effect occurs with glutamic and aspartic acids. Thus, for these acids, the tissue/medium ratios, in the presence of glucose are 1, whilst in the additional presence of TTX the ratios are 2.5 and 2.0 respectively. The same effect of TTX was observed when glucose is absent from the incubation medium although to a lesser extent. This will be discussed further in Chapter 8.

## 4.5 EFFECT OF TETRODOTOXIN ON THE UPTAKE OF AMINO ACIDS UNDER ANAEROBIC CONDITIONS

As TTX diminishes the efflux of amino acids from incubated cerebral cortex slices under anaerobic conditions (previous section), it was decided to study the rates of uptake of amino acids (glutamic acid and glycine) under the same conditions. $C^{14}$-labelled amino acids were used for these experiments, results of which are shown in Table 5. When no carrier (unlabelled) amino acid is present in the incubation medium, the radioactivity taken up by the slices is more than doubled by the presence of TTX.

A similar effect is obtained in the presence of carrier amino acids. Glutamic acid is metabolized in the brain tissue but the increased uptake of radioactive glutamic acid in these experiments can not result wholly from the metabolism,

TABLE 4

EFFECTS OF TETRODOTOXIN ON AMINO ACID CONTENT OF RAT CEREBRAL CORTEX SLICES UNDER ANOXIA

| Amino Acids | Additions | +Glucose -TTX | +Glucose +TTX | -Glucose -TTX | -Glucose +TTX |
|---|---|---|---|---|---|
| TAURINE | Tissue | 2.25 ± .01 | 2.3 ± .05 | 2.34 ± .03 | 2.39 ± .03 |
| | Medium | 3.21 ± .01 | 3.76 ± .34 | 3.26 ± .34 | 2.97 ± .20 |
| ASPARTIC ACID | Tissue | 0.96 ± .05 | 1.65 ± .02 | 1.11 ± .04 | 1.55 ± .20 |
| | Medium | 1.24 ± .2 | 0.82 ± .17 | 1.47 ± .20 | 1.14 |
| GLUTAMINE + SERINE | Tissue | 1.31 ± .07 | 1.60 ± .10 | 1.01 | 1.16 ± .1 |
| | Medium | 3.07 ± .22 | 2.52 ± .10 | 3.27 ± .07 | 2.8 ± .5 |
| GLUTAMIC ACID | Tissue | 6.67 ± .5 | 9.1 ± .20 | 5.63 ± .06 | 7.68 ± .80 |
| | Medium | 6.10 ± .34 | 3.54 ± .44 | 7.25 ± .65 | 4.60 ±1.2 |
| GLYCINE | Tissue | 0.41 ± .05 | 0.57 ± .02 | 0.41 ± .07 | 0.42 ± .02 |
| | Medium | 0.62 ± .04 | .46 ± .04 | 0.72 ± .18 | 0.56 ± .02 |
| ALANINE | Tissue | 0.36 ± .05 | 0.56 ± .02 | 0.36 ± .23 | 0.33 ± .11 |
| | Medium | 0.64 ± .02 | 0.61 ± .01 | 0.83 ± .17 | 0.54 ± .10 |

Incubations were carried out for 30 min in a $Ca^{++}$ free medium under $N_2$:$CO_2$. 2μM TTX or 20mM glucose, when present, were added at zero time. At the end of incubation, tissue as well as medium was analyzed for amino acids, as given in the materials and methods. Results are expressed as μmoles of amino acid per g initial wet wt of the slices.

TABLE 5

EFFECTS OF TETRODOTOXIN ON THE UPTAKE OF AMINO ACIDS UNDER ANAEROBIC CONDITIONS BY RAT CEREBRAL CORTEX SLICES

A. Uptake of U-$C^{14}$ L-Glutamate

| Additions | Amino acid uptake | 15 min Incubation | | 30 min Incubation | |
|---|---|---|---|---|---|
| | | No carrier | with 5mM L-Glutamate | No carrier | with 5mM L-Glutamate |
| NO TTX | cpm per g wet wt | 377,000 | 178,750 | 466,000 | 221,500 |
| | µmoles per g wet wt | 0.0019 | 4.18 | 0.0024 | 5.16 |
| WITH 2µM TTX | cpm per g wet wt | 815,000 | 241,700 | 1080,000 | 293,000 |
| | µmoles per g wet wt | .0042 | 5.56 | .0055 | 6.85 |

B. Uptake of 2-$C^{14}$ Glycine

| Additions | Amino acid uptake | 15 min Incubation | |
|---|---|---|---|
| | | No carrier | with 2mM Glycine |
| NO TTX | cpm per g wet wt | 186,500 | 133,750 |
| | µmoles per g wet wt | 0.022 | 1.85 |
| WITH 2µM TTX | cpm per g wet wt | 377,500 | 207,000 |
| | µmoles per g wet wt | 0.044 | 2.86 |

TABLE 5

(Continued)

Incubations were carried out for 15 min in a $Ca^{++}$ free medium containing 20mM glucose under $N_2$:$CO_2$. Labelled amino acids, with or without carrier were tipped in from the side arm of the Warburg vessel. TTX, when present, was added at zero time. Total cpm per ml of the medium were 214000 for $C^{14}$-glutamate and 145000 for $C^{14}$-glycine. Amino acid uptake was calculated by dividing cpm/g by specific activities and were not corrected for swelling or intracellular space. Each value represent averages of duplicate determinations within ± 7%.

because similar results are obtained with glycine which metabolizes at a much slower rate than an equivalent quantity of glutamate. Moreover, the metabolism of glutamic acid (e.g., conversion to glutamine) is greatly reduced under anaerobic conditions, and there is normally little or no uptake of amino acids against a concentration gradient under such conditions.

## 4.6 EFFECT OF TETRODOTOXIN AT DIFFERENT GLUCOSE CONCENTRATIONS ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

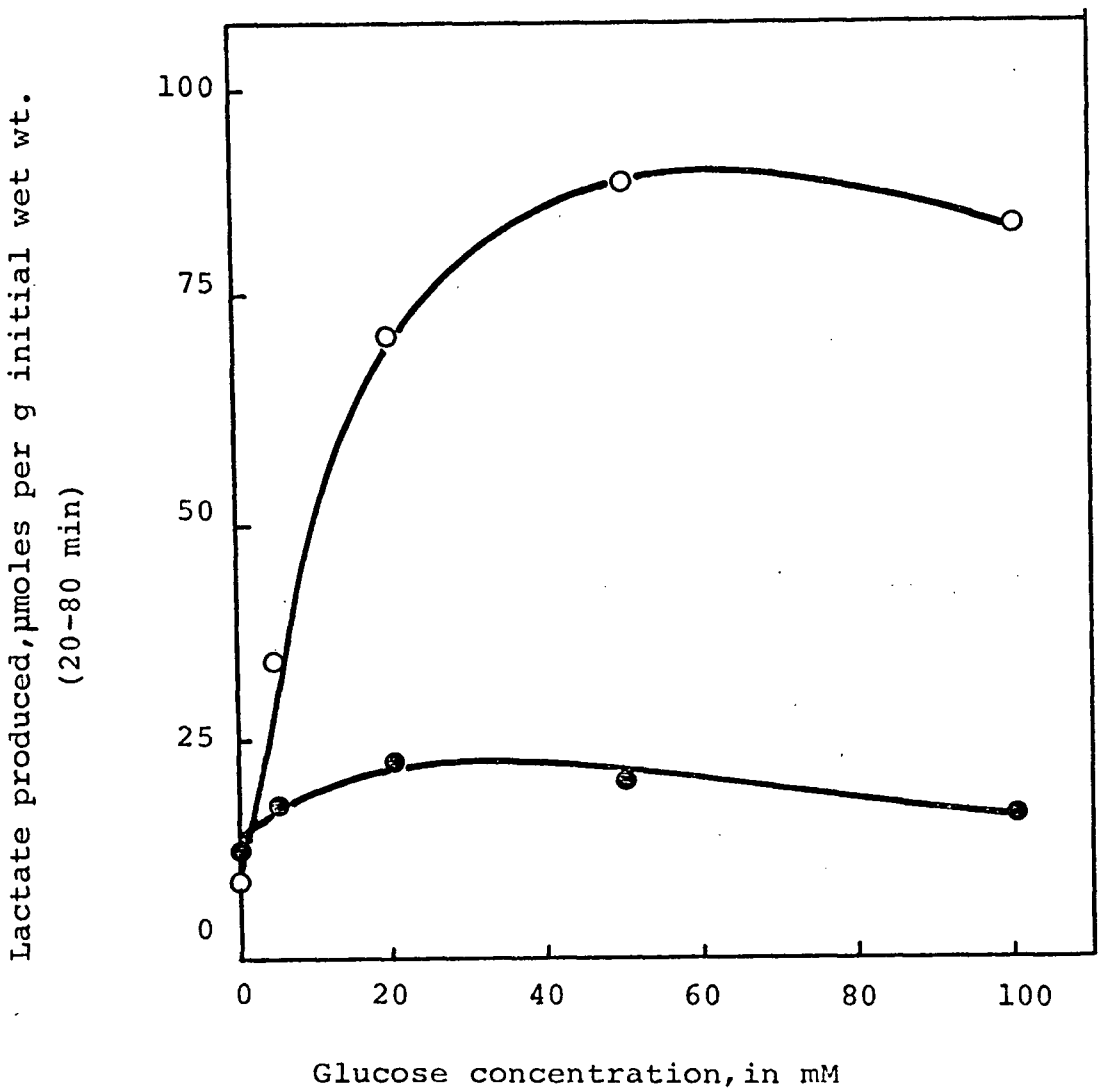
When the rate of glycolysis is high, the rate of glucose entry in the cell may become rate limiting. This can be true under anaerobic conditions, as a considerable amount of glucose is almost exclusively metabolized through the glycolytic pathway. The effect of TTX on the anaerobic glycolysis of cerebral cortex slices was, therefore, investigated at different concentrations of glucose. The results of these experiments are shown in Figure 15. It can be seen that, when the concentration of glucose is increased, the rate of anaerobic glycolysis in the presence of TTX progressively increases until a maximum is obtained at about 50 mM. The rates of anaerobic glycolysis, in the control slices (i.e. without TTX) do not show any significant increase with varying glucose concentrations above about 5 mM.

## 4.7 GLUCOSE TRANSPORT IN CEREBRAL CORTEX SLICES

The effects of 2 μM TTX on the glucose-2-$C^{14}$ transport

FIGURE 15

EFFECT OF VARYING GLUCOSE CONCENTRATION ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES IN THE PRESENCE AND ABSENCE OF TETRODOTOXIN




Incubations were carried out in a $Ca^{++}$-free medium. TTX ( 2 µM) and glucose, when present, were added at zero time and lactate production was measured manometrically, as given in the materials and methods. (●) TTX absent; (○) 2 µM TTX present.

in the rat cerebral cortex slices are given in Table 6. It is evident that the amount of radioactivity present is greater in slices which have been incubated with TTX. However, in the presence of iodoacetate which blocked glycolysis, the presence of TTX brings about no increased radioactivity in the slices. It may be concluded, therefore, that the increased radioactivity, in the absence of iodoacetate, in the TTX treated slices is due to the presence of glycolytic metabolites in the tissue. These experiments demonstrate the fact that the greater rate of glycolysis obtained in the presence of TTX at high glucose concentrations is not due to unspecific facilitation of glucose entry into the slices. It is probably due to the fact that with high rates of glycolysis, the increased glucose gradient with increased external concentrations of glucose results in a greater saturation of the glycolytic enzymes and therefore in an optimal rate of glycolysis.

## 4.8 EFFECTS OF TETRODOTOXIN, IN THE PRESENCE OF SOME AMINO ACIDS, ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

It is well known that, of all the amino acids present in the brain, glutamate and its immediate metabolites are of outstanding importance to the brain[286]. The effect of glutamate on the anaerobic glycolysis of the cerebral cortex slices has been discussed earlier in Chapter 3. L-glutamate is known to cause excitation and thus depolarization of the nerve cell[286] while it has no such effect on the glial cells.

TABLE 6

EFFECTS OF TETRODOTOXIN ON THE GLUCOSE TRANSPORT IN RAT CEREBRAL CORTEX SLICES UNDER ANOXIA

| Additions | 5mM glucose | | 20mM glucose | |
|---|---|---|---|---|
| | cpm per g wet wt | Glucose uptake µmoles/g | cpm per g wet wt | Glucose uptake µmoles/g |
| None | 183,000 | 3.2 | 174,000 | 12.1 |
| 2µM TTX | 201,000 | 3.5 | 198,000 | 13.7 |
| Iodoacetate, 0.2mM | 183,000 | 3.2 | 185,000 | 12.8 |
| Iodoacetate, 0.2mM + TTX, 2µM | 173,000 | 3.0 | 166,000 | 11.9 |

Incubations were carried out anaerobically in a $Ca^{++}$ free medium for 15 min. TTX, iodoacetate or cold glucose, when present, were added at zero time. 0.5µC of glucose-2-$C^{14}$ was added and incubation was carried out for another 5 min. Total cpm in the incubation medium was 289000/ml. Glucose uptake was calculated by dividing cpm/g by specific activities and were not corrected for swelling or intracellular space. Each value represent averages of duplicate determinations within ± 5%.

McIlwain and his coworkers[132] studied the effects of TTX on the cationic changes of the incubated cerebral cortex slices induced by glutamate. These authors showed that TTX partly inhibits the increase in $Na^+$ caused by 5 mM glutamate. It was thought desirable, therefore, to examine the effects of glutamate and other amino acids on the anaerobic glycolysis of cerebral cortex slices in the presence of TTX. The results are shown in Table 7. It is evident that in the presence of 5 mM L-glutamate, which inhibits anaerobic glycolysis, the accelerating effect of TTX on the rate of anaerobic glycolysis is diminished, both in a $Ca^{++}$-free as well as in a Krebs-Ringer bicarbonate medium. D-Glutamate also inhibits the accelerating action of TTX, while 5 mM L-aspartate has no effect.

## 4.9 EFFECTS OF CITRATE, AMP AND $NH^+_4$ ON THE TETRODOTOXIN STIMULATION OF GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES

As pointed out in Chapter 3, citrate, AMP and $NH^+_4$ have inhibitory effects on the rate of anaerobic glycolysis of cerebral cortex slices. Experiments were carried out, therefore, to ascertain the effects of TTX on the rate of anaerobic glycolysis in the presence of these compounds. The results are given in Table 8. It can be seen that 15 mM citrate and 5 mM $NH^+_4$ have marked inhibitory effects on the TTX stimulated anaerobic glycolysis, whereas AMP, under these conditions, had only a slight effect.

TABLE 7

EFFECTS OF TETRODOTOXIN, IN THE PRESENCE OF SOME AMINO ACIDS, ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES FROM RAT

| Additions | Lactate produced µmoles per g initial wet wt (20-80 min) | |
|---|---|---|
| | $Ca^{++}$ free medium | Krebs-Ringer bicarbonate medium |
| None | 31.7 ± 4.3 | 38.0 ± 4.4 |
| 5mM L-Glutamate | 20.9 ± 2.3 | 28.6 ± 3.7 |
| 5mM D-Glutamate | - | 34.4 ± 3.8 |
| 5mM L-Aspartate | 38.4 ± 2.0 | - |
| 2µM TTX | 72.8 ±17.0 | 65.0 |
| 5mM L-Glutamate + 2µM TTX | 22.4 ± 2.5 | 38.4 ± 4.4 |
| 5mM D-Glutamate + 2µM TTX | - | 43.5 ± 2.4 |
| 5mM L-Aspartate + 2µM TTX | 76.5 ±15.0 | - |

All vessels contained 20mM glucose. Additions were made at zero time and lactate production was measured manometrically, as given in the materials and methods.

TABLE 8

EFFECTS OF CITRATE, AMP AND $NH_4^+$ IN THE PRESENCE OF TETRODOTOXIN ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES

| Additions | Lactate produced µmoles per g initial wet wt (20-80 min) | |
|---|---|---|
| | No TTX | with 2µM TTX |
| None | 25.0 ± 4.9 | 63.0 ± 6.7 |
| 15mM citrate | 17.8 ± 3.5 | 16.9 ± 2.0 |
| 2mM AMP | 15.5 ± 5.0 | 50.5 ± 3.1 |
| 5mM $NH_4Cl$ | 29.9 ± 5.8 | 37.0 ± 4.3 |

Incubations were carried out in a $Ca^{++}$ free medium containing 20mM glucose. Additions were made at zero time and lactate production was measured manometrically, as given in the materials and methods.

## 4.10 EFFECTS OF PHOSPHOLIPASES ON THE TETRODOTOXIN STIMULATED GLYCOLYSIS OF THE RAT CEREBRAL CORTEX SLICES

The site of action of TTX appears to be located at the outer surface of the cell membrane[124,127]. Phospholipids are known to be major and important membrane constituents. Cuthbert[287] has shown the importance of membrane lipids in some aspects of drug action. Heilbronn[288] studied the effect of phospholipases on the uptake of atropine and acetyl choline by mouse brain cortex slices. His results demonstrate that the phospholipases decrease the uptake of atropine and particularly that of acetylcholine by the slices. The effect of the enzyme was time-dependent, and, up to a certain limit, concentration-dependent.

In view of the fact that site of action of TTX might be at the cell membrane of cerebral cortex cells, and that TTX might be acting by interaction with the membrane constituents, the effect of TTX on the anaerobic glycolysis of the phospholipase treated cerebral cortex slices was studied. Results of these experiments are shown in Table 9. It can be seen that, with progressively higher concentrations of phospholipase A, the percentage stimulation of anaerobic glycolysis by TTX is decreased. TTX is, however, still effective during the early period (20-50 minutes) of the experiment. This is true, even at phospholipase A concentration of 40 units/vessel (3 ml), present from zero time up

TABLE 9

EFFECTS OF TETRODOTOXIN IN THE PRESENCE OF PHOSPHOLIPASES ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES

| Additions | Amount of Phospholipase, I.U. per vessel (3 ml) | Lactate produced µmoles per g initial wet wt 20-50 min | 50-80 min |
|---|---|---|---|
| None | - | 22.5 ± 4.8 | 10.5 ± 1.4 |
| 2µM TTX | - | 44.9 ±13 | 30.8 ±10 |
| Phospholipase A | 1 | 12.1 ± 3.1 | 5.8 ± 2.6 |
| Phospholipase A + 2µM TTX | 1 | 51.8 ± 1.4 | 33.9 ± 2.3 |
| Phospholipase C | 1 | 20.9 ± 0.4 | 7.4 ± 1.5 |
| Phospholipase C + 2µM TTX | 1 | 33.5 ± 1.3 | 11.8 ± 2.9 |
| Phospholipase A | 5 | 13.6 ± 0.5 | 7.8 ± 1.1 |
| Phospholipase A + 2µM TTX | 5 | 37.5 ± 2.7 | 28.0 ± 2.7 |
| Phospholipase A | 20 | 25.0 ± 1.7 | 12.9 ± 0.5 |
| Phospholipase A + 2µM TTX | 20 | 45.1 ± 4.0 | 12.3 ± 1.6 |
| Phospholipase A | 40 | 22.3 ± 1.3 | 8.5 ± 1.3 |
| Phospholipase A + 2µM TTX | 40 | 50.5 ± 1.4 | 14.3 ± 1.8 |
| Phospholipase A | 60 | 20.3 ± 0.7 | 11.2 ± 0.5 |
| Phospholipase A + 2µM TTX | 60 | 22.1 ±11.8 | 17.9 ± 1.0 |

Incubations were carried out in a $Ca^{++}$ free medium containing 20mM glucose. Additions were made at zero time and lactate production was measured manometrically, as given in the materials and methods.

to the end of experiment. Phospholipase C, at the concentration tested, is more potent than phospholipase A in affecting the TTX stimulated glycolysis of cerebral cortex slices. These results lead to the conclusion that TTX may act by combining with phospholipid constituents of the cell membrane which are slowly attacked by phospholipases. However, it is possible that the products of phospholipase activity may inhibit the action of TTX. This is yet to be resolved.

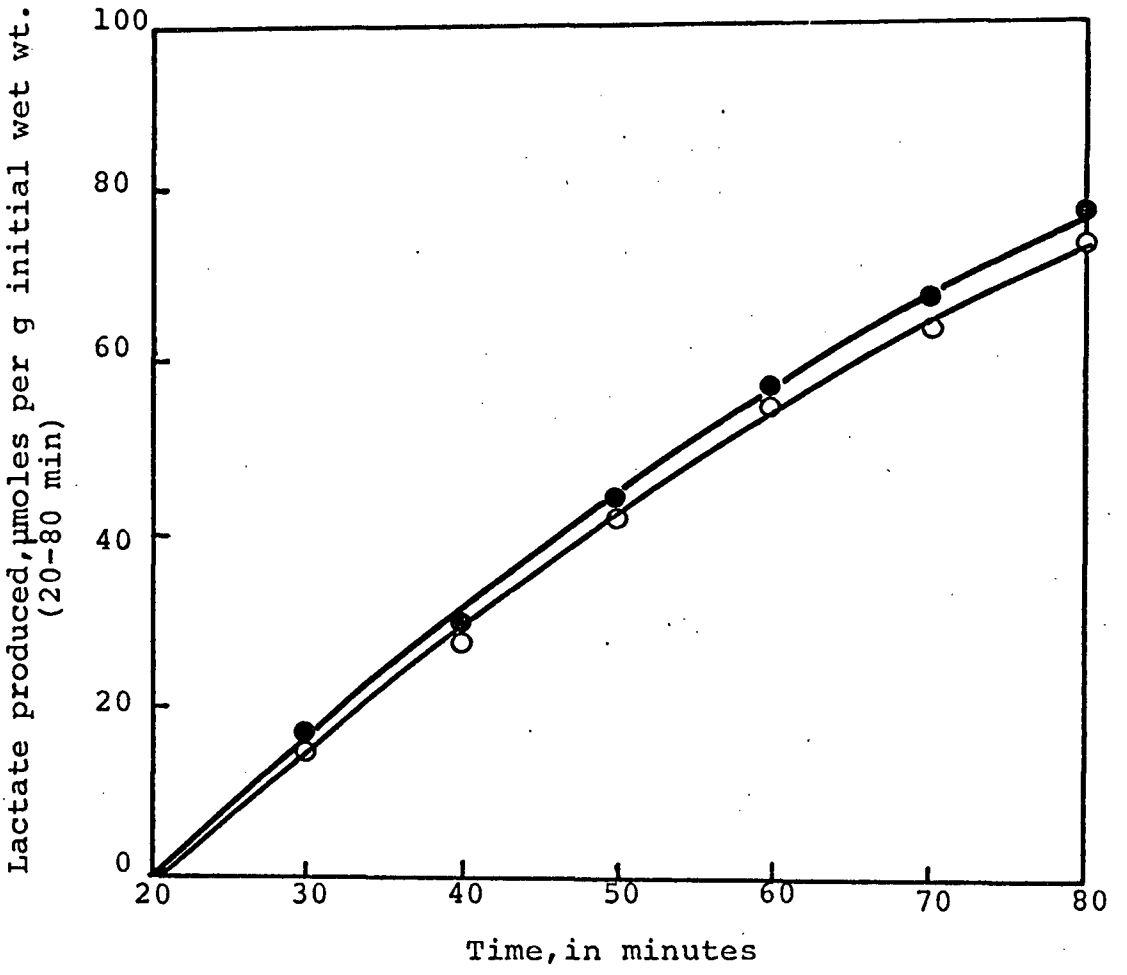
## 4.11 EFFECTS OF TETRODOTOXIN ON ANAEROBIC GLYCOLYSIS OF KIDNEY MEDULLA SLICES AND ACETONE POWDER EXTRACTS

Experiments were carried out to see if the accelerating effect of TTX on anaerobic glycolysis of cerebral cortex slices is specific for the brain tissue. It is known that kidney medulla slices have high glycolytic rates and hence it was selected for examination. The effects of TTX on the anaerobic glycolysis of kidney medulla slices are given in Figure 16. From this data it can be concluded that TTX has absolutely no effect on the anaerobic glycolysis of kidney medulla. This observation demonstrates that the effect of TTX on cerebral cortex slices is specific, and that the anaerobic glycolysis of all tissues is not sensitive to it.

The effect of TTX and ouabain on the anaerobic glycolysis of acetone powder extracts is shown in Figure 17. The results prove that TTX has no effect on the process of cell free anaerobic glycolysis and that a membrane phenomenon is

FIGURE 16

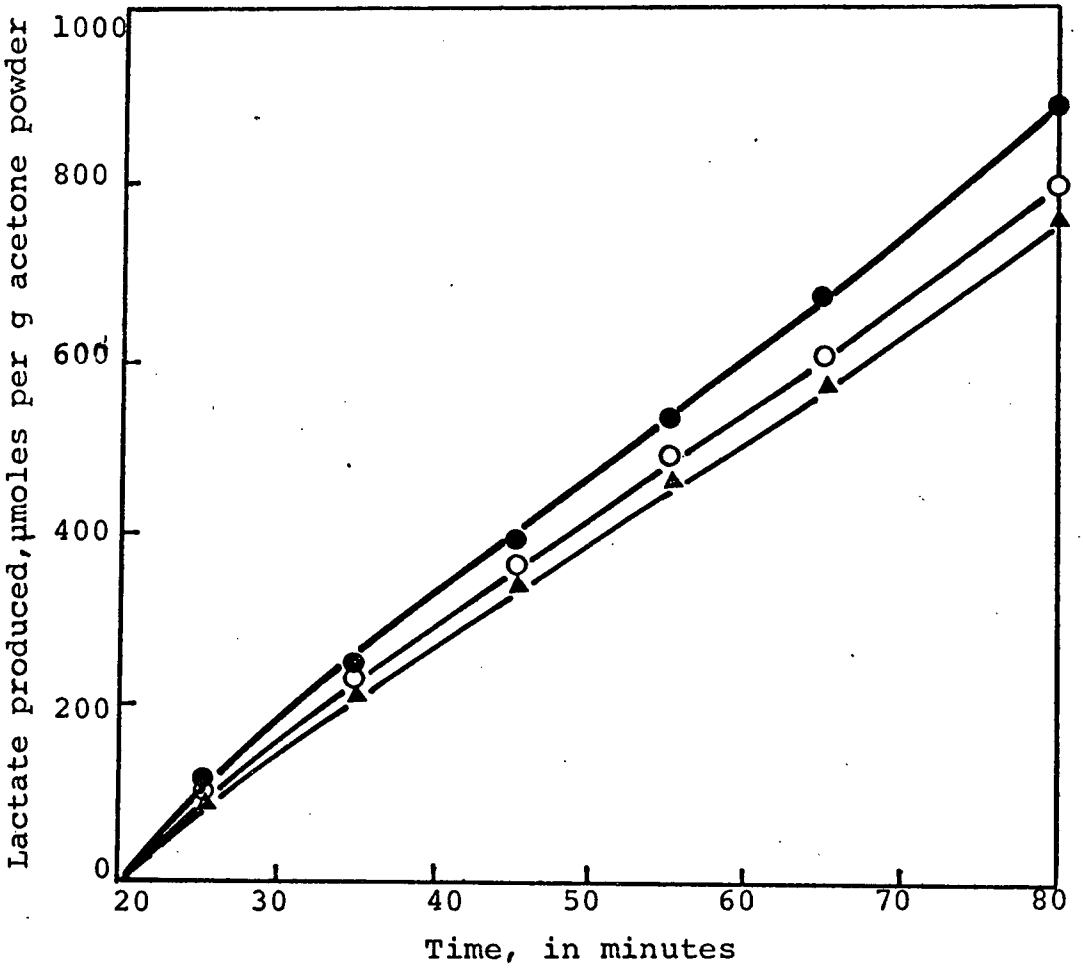
EFFECTS OF TETRODOTOXIN ON THE ANAEROBIC GLYCOLYSIS OF RAT KIDNEY MEDULLA SLICES




Incubation conditions were same as in Figure 13. (O) control; (●) with 2 µM TTX.

FIGURE 17

EFFECTS OF TETRODOTOXIN AND OUABAIN ON THE ANAEROBIC GLYCOLYSIS OF ACETONE POWDER EXTRACTS FROM RAT BRAIN




Incubation conditions were same as in Figure 10. (▲) control; (○) with 2 µM TTX; (●) with 10 µM ouabain.

involved in the mechanism of action of TTX. Intact cells are clearly necessary for the action of TTX.

4.12 EFFECTS OF TETRODOTOXIN ON THE ANAEROBIC GLYCOLYSIS OF DEVELOPING BRAIN CORTEX SLICES

Effects of TTX on the anaerobic glycolysis of cerebral cortex slices from rats of different ages are given in Table 10. It is evident that with 2 or 7-day old rat brain slices the glycolytic behaviour is unaffected by TTX. However, slices from 14-day old animals show an appreciable response.

The effects of different concentrations of TTX on the anaerobic glycolysis of newly born guinea pig cerebral cortex slices are ~~also~~ shown in Figure 18. The anaerobic glycolysis of infant guinea pig cerebral cortex slices are extremely sensitive to TTX and even 0.2 μM TTX is as effective as 10 μM TTX. Moreover, the rate of glycolysis in the presence of TTX is constant during the time period tested. Experiments with some other drugs, which will be discussed later, show that the sensitivity of the infant guinea pigs to drugs is much greater than the adult brain tissues tested. It is important in considering these results to appreciate the well known fact that newly born guinea pigs are very mature compared with newly born rats.

4.13 EFFECT OF TETRODOTOXIN IN PRESENCE OF GLUTAMATE, ASPARTATE AND $NH_4^+$ ON THE ANAEROBIC GLYCOLYSIS OF DEVELOPING CORTEX SLICES

We have shown earlier in this chapter (4.8 and 4.9)

TABLE 10

EFFECT OF TETRODOTOXIN ON THE ANAEROBIC GLYCOLYSIS OF INFANT RAT CEREBRAL CORTEX SLICES

| Additions | Lactate produced µmoles per g initial wet wt (20-80 min) | | |
|---|---|---|---|
| | 2-day old rat | 7-day old rat | 14-day old rat |
| None | 25 ± 1.5 | 21.2 ± 0.45 | 25.4 ± 3.1 |
| 0.2µM TTX | 28.6 ± 1.8 | 26.0 ± 2.2 | 42.5 ± 1.3 |
| 2µM TTX | 29.9 ± 5.9 | 27.7 ± 1.3 | 48.3 ± 9.9 |
| 10µM TTX | 29.8 ± 0.9 | 30.0 ± 0.5 | 51.4 ± 6.6 |
| 2µM TTX + 1mM $Ca^{++}$ | 35.8 ± 3.5 | 31.3 ± 1.3 | 45.3 ± 4.4 |
| 2µM TTX + 2mM $Ca^{++}$ | 31.7 ± 0.9 | 34.4 ± 0.4 | 54.5 ± 6.2 |
| 2µM TTX + 4mM $Ca^{++}$ | 29.5 ± 3.5 | 29.0 | 55.8 ± 6.7 |

Incubations were carried out in a $Ca^{++}$ free medium containing 20mM glucose. Additions were made at zero time and lactate production was measured manometrically, as given in the materials and methods.

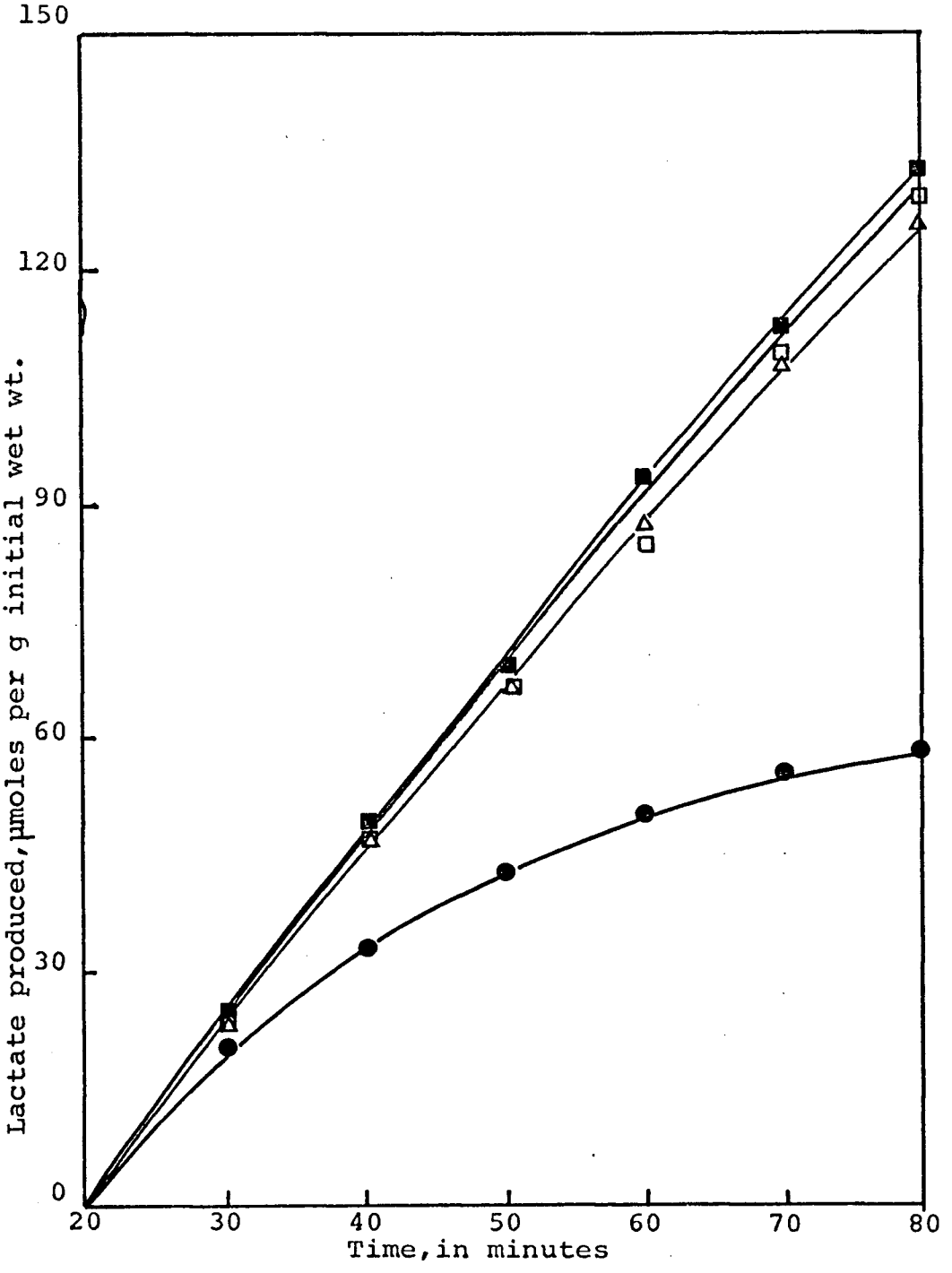



FIGURE 18

EFFECTS OF DIFFERENT CONCENTRATIONS OF TETRODOTOXIN ON THE ANAEROBIC GLYCOLYSIS OF NEWLY BORN GUINEA PIG CEREBRAL CORTEX SLICES

Incubations were carried out in a $Ca^{++}$-free medium containing 20 mM glucose. TTX, when present, was added at zero time and lactate production was measured manometrically, as given in the materials and methods. (●) control; (■) 0.2 µM TTX; (□) 2 µM TTX; (△) 10 µM TTX.

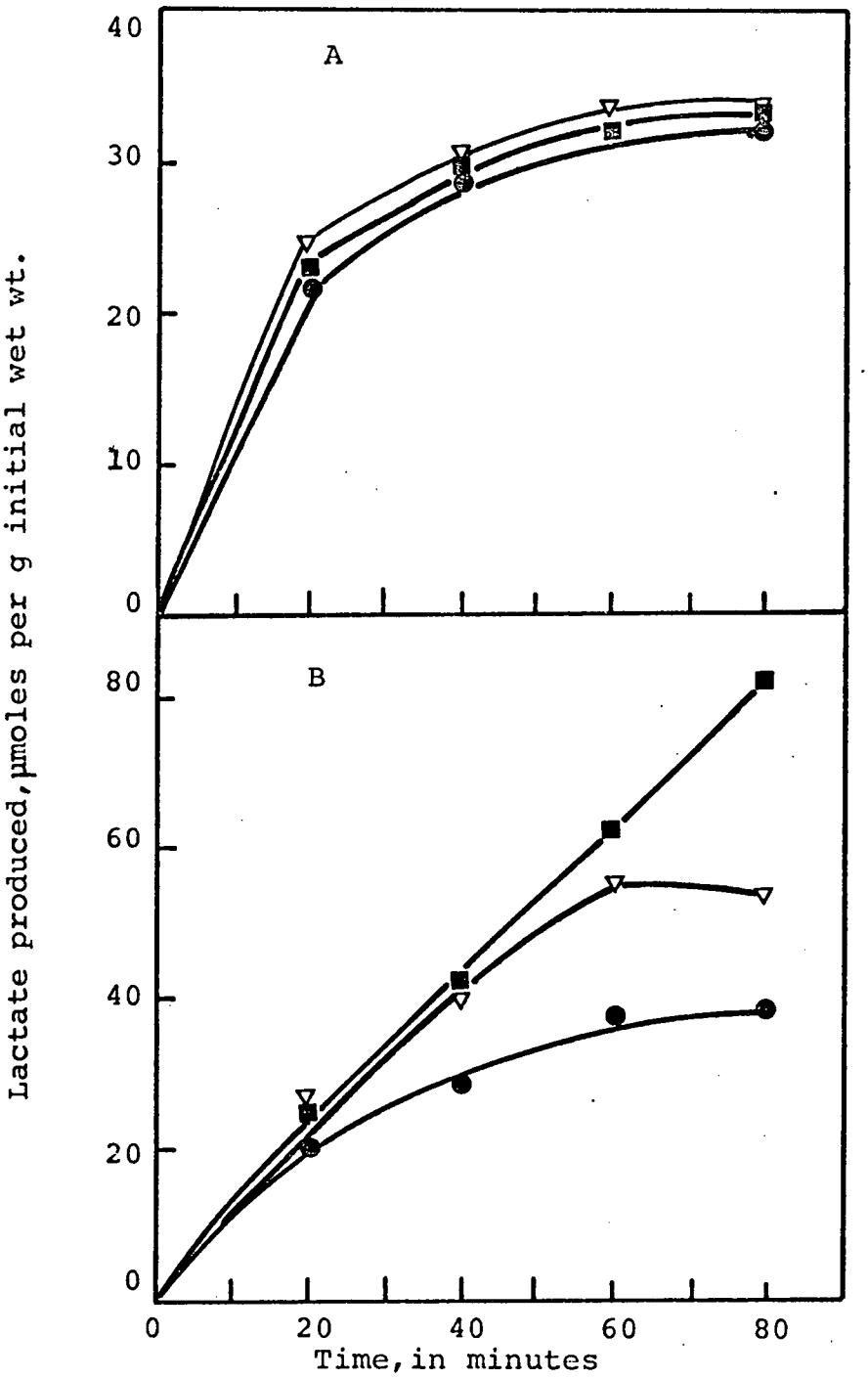
that the TTX stimulated glycolysis of cerebral cortex slices shows sensitivity to glutamate as well as to $NH_4^+$. It was desirable, therefore, to study the effects of these and allied substances on TTX stimulated glycolysis of 2-week old rat and newly-born guinea pig cerebral cortex slices to throw more light on the mechanism of action of TTX. Results of these experiments are shown in Table 11. It is clear that these compounds have inhibitory effects on the rate of TTX accelerated glycolysis. The 2-week old rat brain is more sensitive to $NH_4^+$ than that of the adult.

4.14 EFFECTS OF TETRODOTOXIN ON THE AEROBIC GLYCOLYSIS OF ADULT RAT CEREBRAL CORTEX SLICES

The effects of TTX on the aerobic glycolysis of rat cerebral cortex slices are shown in Figure 19. TTX has no effect on the rate of aerobic glycolysis of the rat cerebral cortex slices when the latter are incubated in a Krebs-Ringer bicarbonate medium. Moreover, in a $Ca^{++}$-free medium, TTX has an inhibitory effect on the aerobic glycolysis. In a $Ca^{++}$-free medium, under aerobic conditions the rate of lactate production, in the absence of the drug, is greater than that in a Krebs-Ringer medium. The inhibitory effect of TTX on the rate of aerobic glycolysis in a $Ca^{++}$-free medium is similar to that of $Ca^{++}$ and analogous to that on the respiration of rat brain cortex slices incubated under similar conditions (Chan and Quastel[130]).

Results given in Table 11A show the effects of TTX on the aerobic glycolysis in the presence of cyanide. In

FIGURE 19
EFFECTS OF TETRODOTOXIN AND OUABAIN ON THE AEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES




All vessels contained 20 mM glucose. A: Krebs-Ringer bicarbonate medium. B: $Ca^{++}$-free medium. Additions were made at zero time and lactate production was measured enzymatically. (▽) control; (●) 2 µM TTX; (■) 10 µM ouabain.

TABLE 11

EFFECTS OF TETRODOTOXIN IN THE PRESENCE OF SOME AMINO ACIDS OR $NH_4^+$ ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES FROM INFANT ANIMALS

| Additions | Lactate produced µmoles per g initial wet wt (20-80 min) | |
|---|---|---|
| | 2-week old rat | newly born guinea pig |
| None | 25.5 ± 3.1 | 34.4 ± 2.7 |
| 5mM L-glutamate | 19.0 ± 3.1 | 22.3 ± 0.9 |
| 5mM D-glutamate | 19.2 ± 1.8 | 32.2 ± 2.3 |
| 2µM TTX | 48.3 ± 9.9 | 129.5 ± 6.7 |
| 5mM L-Aspartate | 17.4 ± 1.3 | 25.9 ± 3.1 |
| 5mM $NH_4Cl$ | 13.9 ± 2.2 | - |
| 2µM TTX | | |
| + 5mM L-glutamate | 25.5 ± 0.5 | 51.5 ± 1.8 |
| + 5mM D-glutamate | 29.5 ± 4.0 | 98.1 ± 3.1 |
| + 5mM L-Aspartate | 28.6 ± 4.0 | 113.7 ± 3.5 |
| + 5mM $NH_4Cl$ | 17.0 ± 3.1 | - |

Incubation conditions were same as in Table 10.

TABLE 11A

EFFECTS OF TETRODOTOXIN ON THE AEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES IN THE PRESENCE OF CYANIDE

| Medium | Lactate produced µmoles per g initial wet wt (20-80 min) | |
|---|---|---|
| | No TTX | with 2µM TTX |
| Krebs-Ringer bicarbonate | 112.9 ± 6.7 | 117.2 ± 9.6 |
| $Ca^{++}$-free | 63.8 ± 8.9 | 103.1 ± 2.5 |

All vessels contained 20mM glucose. Additions were made at zero time and lactate production was measured manometrically, as given in the materials and methods. (gaseous phase was $O_2$:$CO_2$ in these experiments)

a $Ca^{++}$-free medium, under aerobic conditions, when the electron transport chain is blocked by cyanide, TTX is effective in further enhancing the rate of glycolysis. However, in a Krebs-Ringer bicarbonate medium, TTX is not effective in further enhancing the glycolysis, possibly due to optimal rates of glycolysis in the controls itself.

SUMMARY OF CHAPTER 4

1. Tetrodotoxin (TTX) markedly accelerates the rate of anaerobic glycolysis of rat and guinea pig cerebral cortex slices. These effects of TTX are similar to those reported by Quastel and coworkers[71] on $Ca^{++}$ with the guinea pig cerebral cortex slices.

2. The rate of anaerobic glycolysis in presence of 2 μM TTX is further enhanced by the addition of $Ca^{++}$ in the guinea pig cerebral cortex slices. In rats the addition of $Ca^{++}$ has either no effect or it has a slightly inhibitory effect.

3. The acceleration of anaerobic glycolysis of cerebral cortex slices, by the presence of TTX, is not due to suppression of the efflux of $NAD^{+}$ under anoxic conditions.

4. The efflux of glutamic acid or of aspartic acid from the incubated cerebral cortex slices, due to anaerobiosis, is markedly suppressed by 2 μM TTX. TTX also causes increased uptake of radioactive glutamic acid and glycine, from the incubation medium, by the cerebral cortex slices under anoxia.

5. In the presence of increasing concentrations of

glucose, the stimulation of anaerobic glycolysis, due to the presence of TTX, is enhanced, indicating that when the rate of anaerobic glycolysis is high, the glucose concentration may become rate limiting. The above effect is not due however, to an unspecific facilitation of glucose entry into the cerebral cortex slices by the presence of TTX as shown by experiments with labelled glucose.

6. The TTX stimulation of glycolysis is partially, or completely reversed by the addition of L-glutamate, D-glutamate, citrate or $NH_4^+$.

7. TTX is effective in accelerating the rate of anaerobic glycolysis even in the presence of phospholipases, although the percentage stimulation during the later period is considerably decreased.

8. TTX has no effect on the anaerobic glycolysis of kidney medulla slices or of acetone powder extracts of brain.

9. TTX has little or no effect on the anaerobic glycolysis of 2- or 7-day old rat brain but its effectiveness increases markedly at about 14th day. This period coincides with that of maximum brain growth and myelination. The anaerobic glycolysis by slices of newly born guinea pigs, which shows many mature characteristics,is very sensitive to TTX. The TTX stimulated glycolysis of infant brain is inhibited like that of the adult brain, by L-glutamate, D-glutamate or $NH_4^+$.

10. The rate of aerobic glycolysis of the cerebral cortex

slices in a Krebs-Ringer bicarbonate medium is unaffected by TTX, whilst in a $Ca^{++}$-free medium it is slightly depressed by TTX.

The rate of aerobic glycolysis in the presence of cyanide in a $Ca^{++}$-free medium is increased by TTX. However, in a Krebs-Ringer medium under the same conditions, TTX has no effect.

# CHAPTER 5

## FURTHER STUDIES ON THE MECHANISM OF ACTION OF TETRODOTOXIN ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

It has been shown that the presence of TTX, at low concentrations, greatly enhances the rate of anaerobic glycolysis of cerebral cortex slices and, moreover, that this phenomenon is characteristic of mature cerebral tissue. The results of experiments, carried out to throw further light on the mode of action of TTX on the brain tissue, will now be described.

### 5.1 EFFECTS OF PRE-INCUBATION IN OXYGEN ON THE TETRODOTOXIN STIMULATED GLYCOLYSIS OF CEREBRAL CORTEX SLICES

As early as 1928, Rosenthal and Lasnitzki[289] showed that a brief period of incubation in presence of oxygen results in a marked increase in the subsequent rate of anaerobic glycolysis. This phenomenon was further studied in liver, tumours and other normal tissues, including brain,[290-294] and from these studies it was concluded that the stimulation of anaerobic glycolysis by previous incubation in oxygen is a phenomenon of general importance and occurs in all normal adult tissues.[291] However, it has not been possible to define definitely the changes associated with the aerobic pre-incuba-

tion of the tissues.[292]

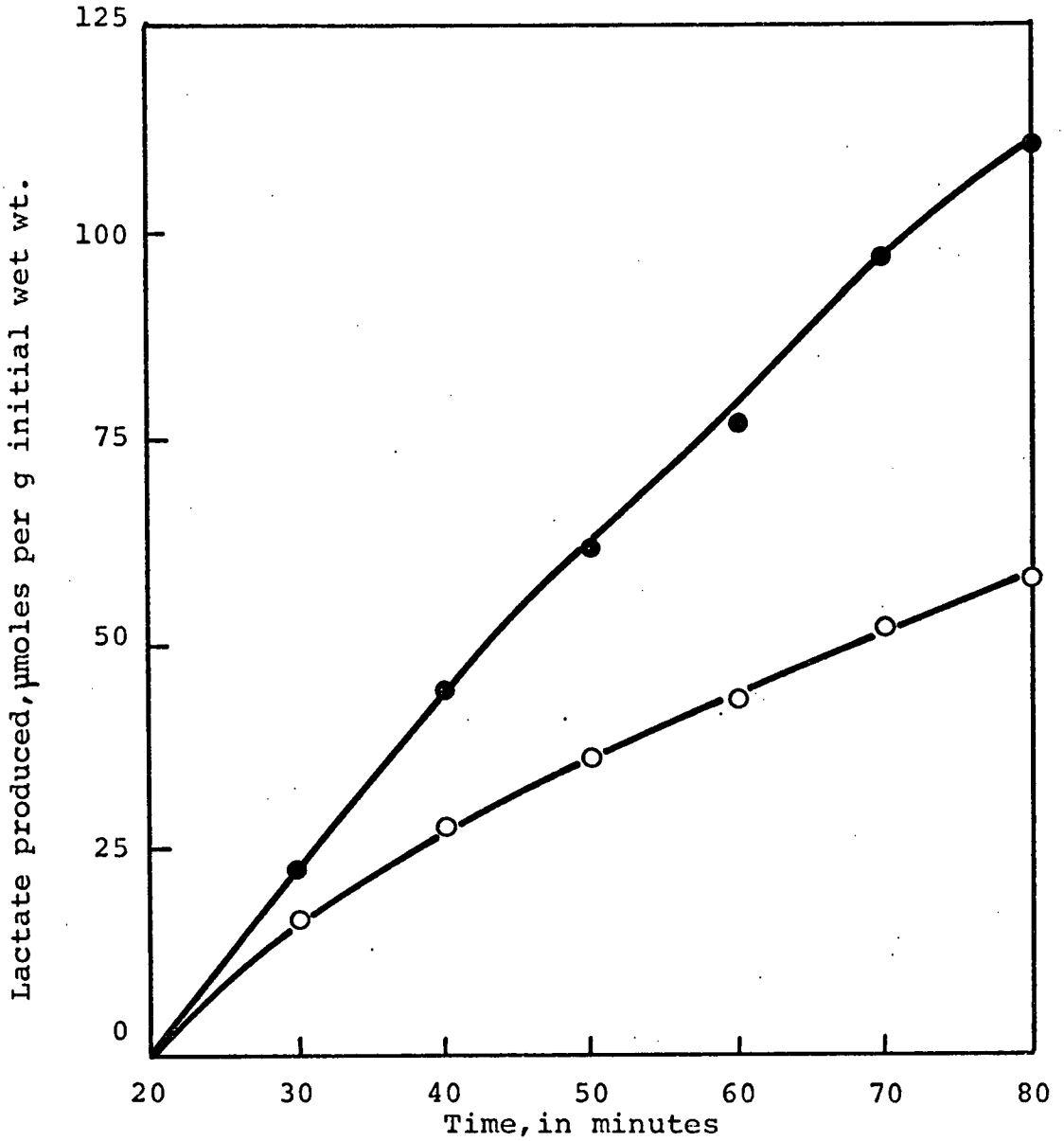
In view of the above, it was considered desirable to see if the anaerobic glycolysis, which has been stimulated by previous oxygenation, can be further enhanced by TTX. The results of these experiments are shown in Figure 20. It can be seen that when the slices are pre-incubated aerobically, in the absence of TTX, the drug is still effective in enhancing the rate of anaerobic glycolysis. Longer periods of aerobic pre-incubation (40 minutes) lower the total amount of the lactate produced during the subsequent anaerobic period, both in the control as well as in the TTX-treated slices. However, the stimulation of anaerobic glycolysis in the presence of TTX was still very apparent.

## 5.2 EFFECTS OF TETRODOTOXIN AFTER VARIOUS PERIODS OF ANAEROBIOSIS ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

The results of experiment shown in Figure 20 demonstrate that TTX is effective after the preliminary pre-incubation of the slices in oxygen. The rate of anaerobic glycolysis attained with the aerobically incubated slices is greater than that found with the corresponding slices which were not exposed to oxygen. Hence it was thought desirable to carry out experiments, in which the slices have been pre-incubated for varying periods in nitrogen, to see if TTX is still effective in enhancing the rate of anaerobic glycolysis. Results of a typical

FIGURE 20

EFFECT OF AEROBIC PREINCUBATION ON THE TETRODOTOXIN STIMULATION OF ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES




Cerebral cortex slices were preincubated in a Krebs-Ringer bicarbonate medium containing 20 mM glucose for 20 min under $O_2$:$CO_2$. They were then transferred to another set of vessels containing, in addition, 2 µM TTX. Lactate production was measured manometrically under subsequent anaerobic period, as given in the materials and methods. (○) control; (●) 2 µM TTX.

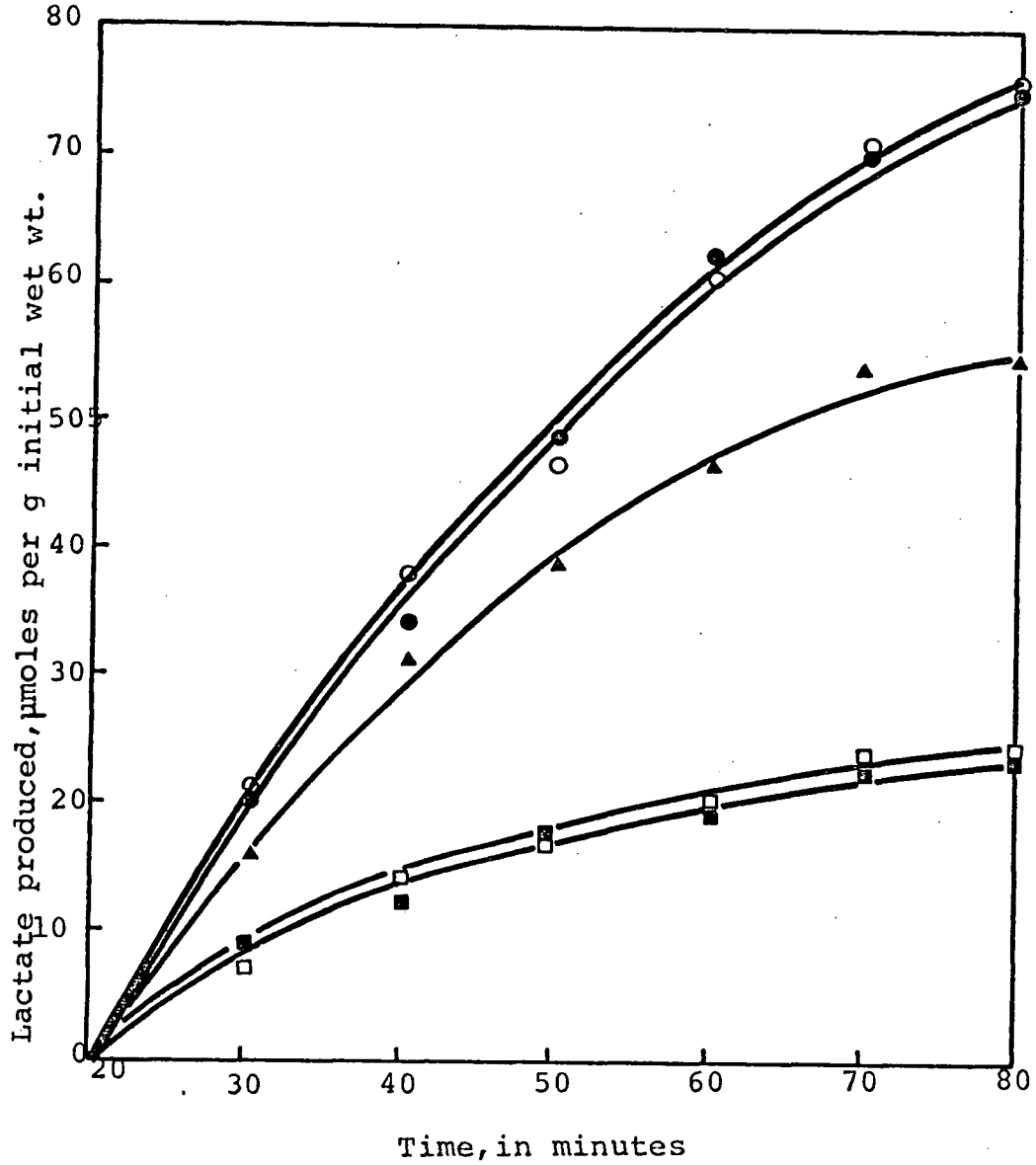
experiment, in which TTX has been "tipped in" after varying periods of anaerobiosis, are shown in Figure 21. This experiment shows that the ability of TTX in enhancing the anaerobic glycolysis, of the cerebral cortex slices, decreases progressively as the period of anaerobiosis, before the addition of TTX, is increased. Thus, although 2 min of anoxia had no effect on the stimulation of anaerobic glycolysis by TTX, the 5 min period considerably reduced the ability of cerebral cortex slices to respond to TTX and after a 10 min period, it was ineffective. This experiment demonstrates that changes in the cerebral cortex slices, during the first few minutes of anoxia, are very important for the enhancing effect of TTX on the rate of anaerobic glycolysis.

## 5.3 EFFECTS OF TETRODOTOXIN IN THE PRESENCE OF PYRUVATE ON THE ANAEROBIC GLYCOLYSIS OF THE CEREBRAL CORTEX SLICES

The fact that TTX is not effective in stimulating the rate of anaerobic glycolysis of the cerebral cortex slices after brief periods of anoxia raises the question as to the changes that occur in the cerebral cortex slices under anaerobic conditions. The major effect of anoxia on a living tissue is that the oxidative mechanisms dependent on the presence of oxygen are no longer operative. In brain, the cell ATP level drops quickly. A number of ATP dependent processes are impaired

FIGURE 21

EFFECT OF ADDITION OF TETRODOTOXIN AFTER VARYING TIME PERIODS OF ANOXIA ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES




Incubations were carried out in a $Ca^{++}$-free medium containing 20 mM glucose. (■) No addition; (○) 2 µM TTX, from zero time; (●) 2 µM TTX added at 2min; (▲) 2 µM TTX added at 5 min; (□) 2 µM TTX added at 10 min. The gas phase was anaerobic throughout the experiment, and lactate production was measured manometrically as given in the materials and methods.

and this results, for example, in changes in the cerebral cation contents, because of the inability of the sodium pump to maintain cation gradients and solute concentrations.

We have shown in Chapter 4.4 that the presence of TTX markedly suppresses the efflux of amino acids from the incubated cerebral cortex slices that occurs under anaerobic conditions. Efflux of various metabolites and cations, as a result of anoxia, may reduce the rate of glycolysis. Tetrodotoxin was, therefore, thought to have stimulating effect on anaerobic glycolysis, by preventing the efflux of such rate regulating substances, thereby raising their concentration in the cell. One such compound might be pyruvate, as the addition of pyruvate to the incubation medium greatly enhances the rate of anaerobic glycolysis of the cerebral cortex slices. Moreover, it is thought that aerobic pre-incubation results in accumulation of pyruvate in the tissue[71] and, hence, in subsequently higher rates of anaerobic glycolysis. Therefore, experiments were carried out to see if TTX could act by preventing the efflux of pyruvate from incubated cerebral cortex slices.

Results given in Table 12 show the effects of TTX on the anaerobic glycolysis of cerebral cortex slices in the presence of pyruvate; the rate of anaerobic glycolysis is further increased by TTX, the stimulatory effect in a $Ca^{++}$-free medium

TABLE 12

EFFECTS OF TETRODOTOXIN IN THE PRESENCE OF PYRUVATE ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES

| Additions | Lactate produced µmoles per g initial wet wt (20-80 min) | |
|---|---|---|
| | Krebs-Ringer bicarbonate medium | $Ca^{++}$ free medium |
| None | 38.0 ± 4.4 | 31.7 ± 4.3 |
| 2µM TTX | 65 | 76 |
| 1mM Pyruvate | 113.4 ± 6.3 | 75 ± 14.9 |
| 1mM Pyruvate + 2µM TTX | 124.1 ± 5.4 | 117.9 ± 14.3 |
| 10mM Pyruvate | - | 70.1 ± 12.7 |
| 10mM Pyruvate + 2µM TTX | - | 128.1 ± 6.5 |

All vessel contained 20mM glucose. Additions were made at zero time and lactate production was measured manometrically, as given in the materials and methods.

being almost additive. These results show that the effect of TTX on the anaerobic glycolysis can not be due to inhibition of the efflux of pyruvate from the incubated cerebral cortex slices, since in that case, an additive effect on anaerobic glycolysis would not have been observed.

## 5.4 EFFECTS OF GLUCOSE ADDITION UNDER VARIOUS CONDITIONS ON THE STIMULATION OF ANAEROBIC GLYCOLYSIS BY TETRODOTOXIN

Experiments were carried out to obtain more information concerning the conditions that might affect the TTX stimulation of anaerobic glycolysis in cerebral cortex slices. It is well known that very little endogenous energy reserves are present in the brain tissue and that exogenous glucose is the major source of energy (see Chapter 1). It is possible to deplete slices of ATP by incubating them in the absence of glucose or in the presence of uncoupling agents. Experiments were carried out to see if TTX is effective, under such conditions, in enhancing the anaerobic glycolysis of such slices.

Results in Table 13 show the effects of addition of glucose after periods of anaerobiosis and aerobiosis on the TTX stimulation of glycolysis. It can be seen from these experiments that glucose should be present before the start of anaerobiosis to obtain an effect of TTX (Table 13A). Once the slices have been exposed to anoxia, the glycolysis of the cere-

TABLE 13

EFFECTS OF ADDITION OF GLUCOSE UNDER VARIOUS CONDITIONS ON THE TETRODOTOXIN STIMULATION OF ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES

A.

| 2μM TTX from start | Time of addition of Glucose | Lactate produced μmoles per g initial wet wt (20-80 min) | |
|---|---|---|---|
| | | 1mM Pyruvate present from start | No Pyruvate |
| Absent | 0 min | 60.3 ± 4.5 | 29.0 ± 4.9 |
| Present | 0 min | 129.5 ± 5.0 | 83.5 ± 10.7 |
| Absent | 15 min | 18.8 | 17.6 ± 1.8 |
| Present | 15 min | 22.3 | 16.5 ± 4.0 |

B.

| 2μM TTX from start | Time of addition of Glucose | 1mM Pyruvate from start | Lactate produced μmoles per g initial wet wt (30-90 min) |
|---|---|---|---|
| Absent | 0 min | Present | 68.8 ± 4.5 |
| Present | 0 min | Present | 190.2 ± 0.9 |
| Absent | 10 min | Present | 54.9 ± 2.7 |
| Present | 10 min | Present | 180.8 ± 4.4 |

TABLE 13

(Continued)

| C. | 2μM TTX from start | Time of addition of Glucose | 1mM Pyruvate from start | Lactate produced μmoles per g initial wet wt (40-100 min) |
|---|---|---|---|---|
| | Absent | 0 min | Present | 79.0 ± 2.7 |
| | Present | 0 min | Present | 174.6 ± 3.6 |
| | Absent | 25 min | Present | 85.7 ± 1.3 |
| | Present | 25 min | Present | 153.1 ± 1.0 |

When present, final concentration of glucose was 20mM. Incubations were carried out in a $Ca^{++}$-free medium. (A) Incubation was anaerobic. (B) First 10 min period was aerobic followed by anaerobiosis. (C) First 15 min period was anaerobic followed by 10 min aerobic period and subsequent anaerobiosis. Lactate production was measured manometrically.

bral cortex slices is not affected by TTX, irrespective of whether TTX is present from the very beginning or is added to the medium later on. However, when the preliminary incubation, without glucose is aerobic, and is in the presence of pyruvate, and then glucose is added to the incubation medium, a stimulation of glycolysis by TTX takes place (Table 13B). The loss of response of anaerobic glycolysis to TTX, after a period of anaerobiosis in the absence of glucose, does not appear to be due to a permanent damage to the tissue. Thus, after a period of anaerobiosis, if the slices are exposed to oxygen briefly, then the ability of the slices to have increased rate of glycolysis, in the presence of TTX, during subsequent anaerobic periods is regained (Table 13C). The significance of these experiments will be discussed in Chapter 8.

## 5.5 EFFECT OF AEROBIC INCUBATION WITH DINITROPHENOL ON THE TETRODOTOXIN STIMULATION OF ANAEROBIC GLYCOLYSIS, AND ON THE ATP LEVEL OF THE RAT CEREBRAL CORTEX SLICES

From the experiments just reported it appears that the ATP concentration in the slices might be important for the activity of TTX, since their ability to respond to TTX (after a brief period of anaerobiosis) is regained by aerobic incubation. Hence experiments were carried out to define more clearly the exact role of ATP in the stimulation of anaerobic glycolysis of cerebral cortex slices by TTX. In these experi-

ments, ATP levels were measured under a variety of conditions and these were then related to the rates of anaerobic glycolysis.

Table 14A shows that the ATP content of aerobically incubated cerebral cortex slices decreases in the presence of 2,4-dinitrophenol (DNP). When the slices are incubated anaerobically for 15 min followed by 10 min aerobic incubation, the subsequent ATP level is relatively high (Table 14B). A further anaerobic period reduces the ATP level significantly (Table 14C). However, the ATP level in the slices exposed to TTX, is higher both in the presence or absence of DNP than in those not so exposed. From the data on subsequent rates of anaerobic glycolysis under these as well as under slightly different conditions (see Table 14C), it can be seen that the rate of anaerobic glycolysis of the DNP-treated slices is slightly lower than those which were not exposed to it. The rates of glycolysis are higher when pyruvate is present from the beginning of the experiment before the addition of glucose. When glucose is added after the aerobic incubation period, the rates of glycolysis are lower than that of those slices, incubated in a medium, to which glucose had been added before the aerobic period. The significance of these results will be discussed in Chapter 8.

TABLE 14

EFFECTS OF INCUBATION WITH 2,4-DINITROPHENOL (DNP) ON THE TETRODOTOXIN STIMULATION OF ANAEROBIC GLYCOLYSIS AND THE ATP CONTENTS OF RAT CEREBRAL CORTEX SLICES

A. Effect of DNP on the ATP concentration of cerebral cortex slices incubated aerobically for one hour in a Krebs-Ringer phosphate medium. DNP was added at zero time and the medium contained 20mM glucose.

| Additions | ATP µmoles per g initial wet wt |
|---|---|
| None | 1.57 |
| 0.1mM DNP | 0.66 |

B. Effects of DNP on the ATP concentration in the presence of TTX.

Incubations were carried out for 25 minutes in a $Ca^{++}$ free medium containing 20mM glucose. First 15 min period was anaerobic followed by 10 min aerobic period. Additions were made at zero time.

| Additions | ATP µmoles per g initial wet wt |
|---|---|
| None | 1.48 |
| 0.1mM DNP | 1.16 |
| 2 µM TTX | 1.42 |
| 0.1mM DNP + 2µM TTX | 1.17 |

TABLE 14

(Continued)

C. Effects of DNP and TTX on the ATP content and anaerobic glycolysis of cerebral cortex slices.

Incubations were carried out in a $Ca^{++}$ free medium. First 15 min period was anaerobic followed by 10 min aerobic period and subsequent anaerobiosis. Lactate production was measured manometrically from 40-100 min. TTX and DNP were present from zero time. Lactate produced is expressed as µmoles of lactate per g initial wet wt of the slices.

| Addition | No Pyruvate | | 1mM Pyruvate from start | |
|---|---|---|---|---|
| | ATP content at 35 min µmoles per g initial wet wt | Lactate produced Glucose at zero time | Lactate produced Glucose added at 15 min | Lactate produced Glucose added at 25 min |
| None | 0.45 | 37.9 ± 1.78 | 114.7 ± 3.1 | 79.5 ± 8.0 |
| DNP, 0.1mM | 0.43 | 26.8 ± 1.3 | 86.6 ± 9.8 | 49.6 ± 7.1 |
| TTX, 2µM | 0.64 | 146 ± 1.3 | 178.6 ± 7.0 | 159.8 ± 6.7 |
| TTX, 2µM + DNP, 0.1mM | 0.59 | 117 ± 6.3 | 136.2 ± 2.2 | 101 ±16.9 |

## 5.6 EFFECTS OF TETRODOTOXIN AND OUABAIN ON THE ATP CONTENT OF GUINEA PIG CEREBRAL CORTEX SLICES

As has been mentioned in Chapter 4, the concentrations of acid labile phosphates in the incubated cerebral cortex slices are raised in the presence of both TTX and ouabain. Under these conditions the rates of glycolysis are also higher. Table 15 shows the effect of ouabain and TTX on the ATP contents of the incubated cerebral cortex slices. It can be seen that in the presence of both these drugs the level of ATP in the slices is increased. These results will be further discussed in Chapters 6 and 8.

## 5.7 EFFECTS OF RAISING ATP LEVEL BY AEROBIC INCUBATION WITH ADENOSINE ON THE TETRODOTOXIN STIMULATED GLYCOLYSIS OF THE RAT CEREBRAL CORTEX SLICES

Abadom and Scholefield and others[245,295] have reported an increase in the ATP (7 min phosphate) content when the cerebral cortex slices are incubated aerobically in the presence of adenosine. Experiments were carried out, therefore, to see if slices treated in such a manner show more responsiveness to the effects of TTX on the anaerobic glycolysis. In these experiments (see Table 16), the slices were pre-incubated aerobically for 40 min in a Krebs-Ringer bicarbonate solution containing 1 mM adenosine and 20 mM glucose; subsequently the slices were transferred to another set of vessels containing TTX.

TABLE 15

EFFECTS OF TETRODOTOXIN AND OUABAIN ON THE ATP CONTENT OF GUINEA PIG CEREBRAL CORTEX SLICES UNDER ANOXIA

| Addition | ATP μmoles per g initial wet wt. |
|---|---|
| None | 0.46 ± 0.06 |
| ouabain, 10μM | 0.71 ± 0.05 |
| TTX, 2μM | 0.68 ± 0.02 |
| ouabain, 10μM + 1mM $Ca^{++}$ | 0.50 ± 0.09 |
| TTX, 2μM + 1mM $Ca^{++}$ | 0.60 ± 0.11 |

Incubations were carried out for 90 min under $N_2$:$CO_2$ in a $Ca^{++}$ free medium containing 20mM glucose. ATP was measured enzymatically, as described in the materials and methods.

TABLE 16

EFFECT OF AEROBIC PREINCUBATION IN ADENOSINE ON THE TETRODOTOXIN STIMULATION OF ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES

| Addition | Lactate produced µmoles per g initial wet wt (20-80 min) | |
|---|---|---|
| | slices not incubated with adenosine | slices incubated with adenosine |
| None | 80.4 ± 6.7 | 102.1 ± 4.5 |
| 2µM TTX, present from zero time | 132 ± 1.5 | 136.1 ± 4.0 |
| TTX added after 15 min anaerobiosis, 2µM final concentration | 84.8 | 92.2 |

Cerebral cortex slices were incubated for 40 min under $O_2$:$CO_2$ in a Krebs-Ringer bicarbonate medium containing 20mM glucose. They were then transferred to a $Ca^{++}$ free medium containing 20mM glucose, with or without TTX. Anaerobic glycolysis was then measured manometrically during subsequent 20-80 min anaerobic period, as described in the materials and methods.

The anaerobic glycolysis was then measured. These results with the adenosine treated slices show that there is no increase in response to TTX, although the glycolytic rates are higher than that of the non-treated slices. When TTX is added, after 15 min anaerobiosis to the adenosine treated slices, there is no accelerating effect on the rate of anaerobic glycolysis.

## 5.8 EFFECTS OF TETRODOTOXIN ON cAMP PRODUCTION IN CEREBRAL CORTEX SLICES

Cyclic AMP has been shown to increase the rate of aerobic as well as anaerobic glycolysis in brain (Chapter 3). Further, Mansour and Stone[296] have shown that some drugs such as LSD-25 increase the production of cAMP in liver flukes, and this in turn increases the rate of lactate production, presumably by activation of phosphofructokinase activity. McIlwain and his coworkers[300] established that when the cerebral cortex slices are electrically stimulated, formation of cAMP in the slices is considerably increased. As has been mentioned in Chapter 1, electrical stimulation also increases the rate of aerobic glycolysis.[50] Hence experiments were carried out to see if the stimulation of anaerobic glycolysis of cerebral cortex slices by TTX is due to increased formation of cAMP. If TTX increases the formation of cAMP then it may facilitate the phosphofructokinase step, especially at the beginning of the experiment, by decreasing the degree of inhibition of phosphofructokinase

by ATP; at a later stage, when ATP level decreases, it should not have any effect. The increased rate of glycolysis at the start of the experiment will tend to keep the rate of decline of ATP content slow, and this may result in an increased rate of glycolysis.

We used the method of Shimizu et.al.[264] to measure the production of cAMP. In this method, as described in Chapter 2, the cerebral cortex slices are first pre-incubated in oxygen in the presence of $C^{14}$-adenine to get a pool of $C^{14}$-ATP. The effect of drugs on cAMP can be easily measured by observing the rates of conversion of $C^{14}$-ATP to $C^{14}$-cAMP under various conditions.

Results in Table 17A show that the rates of incorporation of adenine-8-$C^{14}$ in the cerebral cortex slices as well as in its ATP pool increases with time. The observation of Shimizu et.al.[264] that histamine greatly increases the production of cAMP, was confirmed. However, any increased production of cAMP in the presence of TTX could not be observed (Table 17B). This shows that the effect of TTX on the anaerobic glycolysis of the cerebral cortex slices is not due to an increase in the concentration of cAMP in the brain cells.

## 5.9 EFFECT OF PROTOVERATRINE ON THE TETRODOTOXIN STIMULATED GLYCOLYSIS

It is known that protoveratrine increases the influx of $Na^+$ into excitable cells.[72,135] On the other hand, TTX is

TABLE 17

EFFECTS OF TETRODOTOXIN ON THE cAMP FORMATION IN THE CEREBRAL CORTEX SLICES

A. Incorporation of adenine-8-$C^{14}$ sulfate into rat cerebral cortex slices and its ATP pool.

Cerebral cortex slices were incubated in Krebs-Ringer bicarbonate containing 20mM glucose and 2µc of adenine-8-$C^{14}$ sulfate (51.5 mc/mM). At the end of the incubation slices were homogenized in 5% TCA. After deproteinization, TCA was removed with ether and a portion was counted for total radioactivity while another portion was spotted with carrier ATP on a PEI cellulose plate and developed in 1M LiCl; radioactivity in ATP spots were determined, after scrapping, in a Mark I liquid scintillation counter.

| Incubation time in min | Adenine-8-$C^{14}$ incorporated, x$10^4$ c.p.m. per g initial wet wt | |
|---|---|---|
| | Total | ATP |
| 5 | 171 | 5.0 |
| 10 | 288 | 16.9 |
| 20 | 471 | 42.7 |
| 40 | 1043 | - |

TABLE 17

(Continued)

B. Conversion of $C^{14}$ ATP to cAMP in guinea pig cerebral cortex slices.

Cerebral cortex slices were incubated as in A. After 40 min, the pulse labelled slices were transferred to a $Ca^{++}$ free medium containing 1mM caffeine and 20mM glucose, with and without 2μM TTX. Incubations were carried out for another 10 min in $N_2$:$CO_2$ and total radioactivity in the slices as well as that in cAMP was determined as given in the materials and methods.

| Additions | c.p.m. per g initial wet wt | |
|---|---|---|
| | Total, $x10^5$ | cAMP, $x10^3$ |
| 1mM caffeine | 103 ± 7 | 159 ± 26 |
| 1mM caffeine + 2μM TTX | 102 ± 8 | 95 ± 45 |

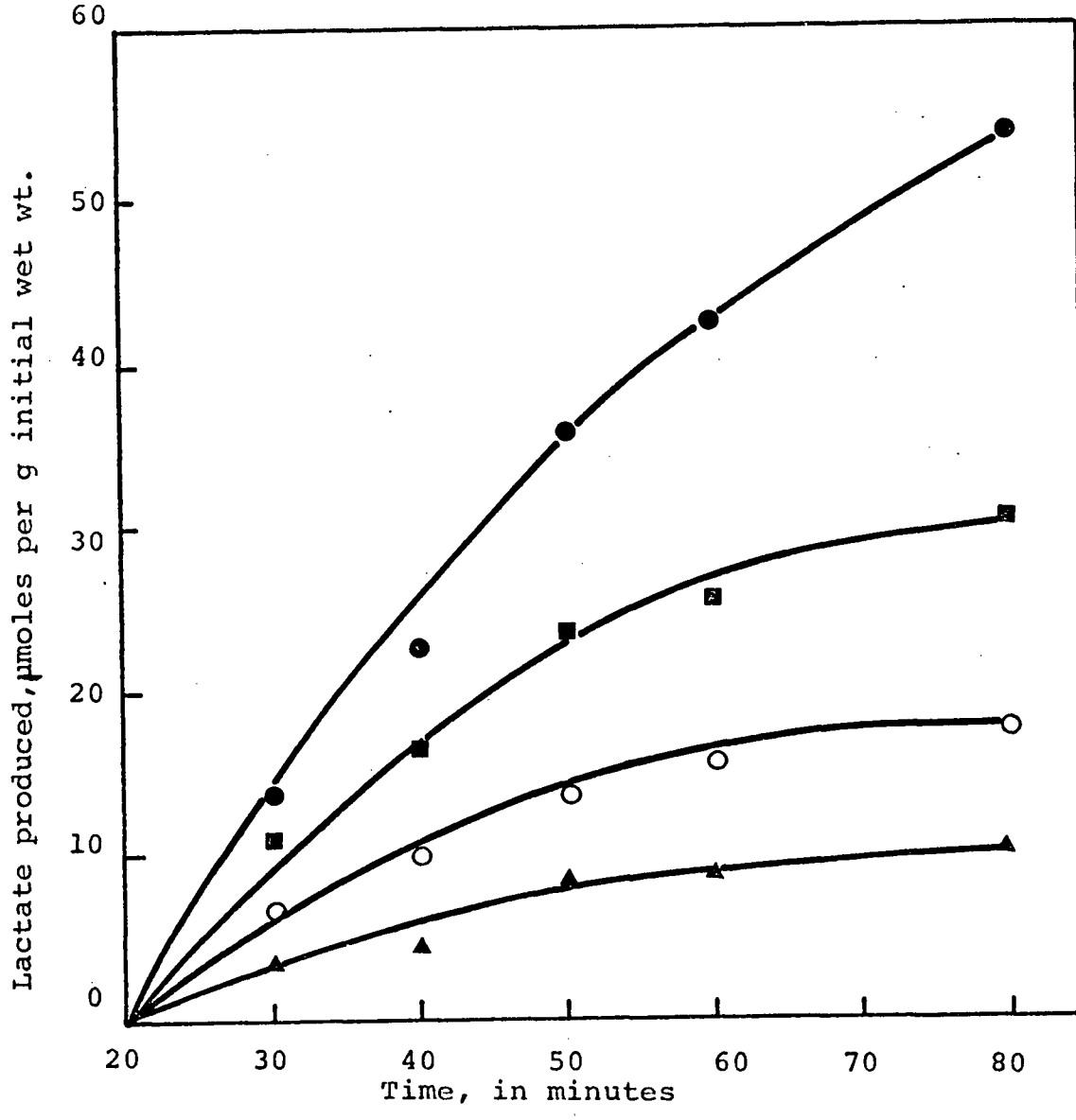
known to block the generation of the action potentials in such cells (Chapter 1). As noted by Wollenberger,[181-183] protoveratrine inhibits the rate of anaerobic glycolysis of cerebral cortex slices. In view of their opposite effects on the anaerobic glycolysis of cerebral cortex slices, it was decided to study their effects together. Results of such an experiment are shown in Figure 22. It is evident that the effects of these drugs are antogonistic. Thus 5 μM TTX protoveratrine inhibits the enhanced glycolysis caused by 2 μM TTX to a considerable extent. When the concentration of protoveratrine was further increased, up to 20 μM, the effect of TTX is completely abolished (results not shown).

## 5.10 EFFECTS OF TETRODOTOXIN ON THE Na-22 TRANSPORT IN THE RAT CEREBRAL CORTEX SLICES

It has been well established that the movement of $Na^+$ during the generation of an action potential is blocked by TTX (Chapter 1). Further, both glutamate and protoveratrine reverse the stimulating effect of TTX on the anaerobic glycolysis (Chapters 4.8 and 5.9). These two agents are known to increase the influx of $Na^+$ in the cerebral cortex slices. Moreover, $Na^+$ has inhibitory effect on the rate of anaerobic glycolysis (Chapters 1.2 and 3.7). Hence, it appeared to us that TTX might act by preventing the influx of $Na^+$, in the

FIGURE 22

EFFECTS OF TETRODOTOXIN, IN THE PRESENCE OF PROTOVERATRINE, ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES




Incubations were carried out in a $Ca^{++}$-free medium containing 20 mM glucose. Additions were made at zero time and lactate production was measured manomtrically as given in the materials and methods. (○) control; (▲) 5 µM protoveratrine; (●) 2 µM TTX; (■) 5 µM protoveratrine and 2 µM TTX.

cerebral cortex slices, at the onset of anoxia. It was decided, therefore, to study the influx of $Na^{22}$ in the presence and absence of TTX. The results of these experiments are summarized in Table 18. These results show that TTX has very little effect on the $Na^{22}$ influx under the given experimental conditions.

If the effect of TTX is due only to the suppression of $Na^+$-influx, then replacing the chloride of the incubation medium by sulfate should result in greater rate of anaerobic glycolysis (it is known that the influx of $Na^+$ due to anaerobiosis is suppressed when $Cl^-$ is replaced by $SO_4^{--}$[298]); TTX should have less effect under these conditions. The results of these experiments are given in Table 19. It is seen that TTX is still effective in increasing the rate of anaerobic glycolysis. Moreover the rate of anaerobic glycolysis in the control slices is not very large as compared to that in the $Cl^-$ medium. This experiment thus rules out the possibility that the effect of TTX on cerebral anaerobic glycolysis is solely due to suppression of the $Na^+$-influx. Furthermore, the effect of TTX on $Na^{22}$ transport in a sulfate medium is negligible. There is very little water uptake by the cerebral cortex slices, under these conditions, indicating the suppression of $Na^+$ influx (not shown).

TABLE 18

EFFECT OF SOME NEUROTROPIC DRUGS ON $Na^{22}$ INFLUX IN RAT CEREBRAL CORTEX SLICES

| Additions | μequivalents $Na^+$, corresponding to $Na^{22}$ in slices, per g initial wet wt | | |
|---|---|---|---|
| | 10 min | 15 min | 60 min |
| None | 170 ± 13 | 177 ± 4 | 208 ± 12 |
| 0.2μM TTX | 162 ± 10 | 151 ± 8 | - |
| 2μM TTX | 151 ± 7 | 149 ± 5 | - |
| 10μM TTX | - | 143 | - |
| 0.1mM Lidocaine | - | - | 189 ± 2 |
| 0.25mM Amytal | - | - | 186 ± 2 |
| 10μM ouabain | - | 179 ± 2 | - |

Cerebral cortex slices were incubated in a $Ca^{++}$ free medium containing 20mM glucose under $N_2$:$CO_2$. Additions were made at zero time. After 15 min, 0.5 μc of $Na^{22}$ was added from the side arm and $Na^{22}$ influx was determined after various intervals, (10 min, 15 min or 60 min), as given in the materials and methods. The above values have not been corrected for swelling.

TABLE 19

EFFECTS OF TETRODOTOXIN ON THE ANAEROBIC GLYCOLYSIS AND $Na^{22}$ TRANSPORT IN RAT CEREBRAL CORTEX SLICES IN A CHLORIDE FREE MEDIUM

| Additions | Lactate produced µmoles per g initial wet wt (20-80 min) | µequivalent $Na^{+}$ corresponding to $Na^{22}$, per g initial wet wt |
|---|---|---|
| None | 14.7 ± 1.0 | 61.5 ± 1.5 |
| 2µM TTX | 38.0 ± 1.8 | 60.0 ± 1.7 |

Cerebral cortex slices were incubated in a $Cl^{-}$ free medium containing 20mM glucose. Lactate production was determined manometrically as given in the materials and methods. For $Na^{22}$ influx experiments, conditions were same as Table 18 except that incubation time in $Na^{22}$ was 15 min.

## 5.11 EFFECTS OF TETRODOTOXIN, AT VARIOUS CATION CONCENTRATIONS OF THE MEDIUM, ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

Ashford and Dixon[62] showed that, in the presence of 100 mM $K^+$, both respiration and aerobic glycolysis of the cerebral cortex slices are increased. However, in contrast to aerobic glycolysis, anaerobic glycolysis is depressed (see Chapter 1.2). We have seen in Chapter 3 that when $K^+$ concentration is increased with a corresponding decrease in $Na^+$ concentration, there is a considerable increase in the rate of anaerobic glycolysis. Experiments were carried out, therefore, to observe whether under these varying cation concentrations, TTX is still effective in stimulating the rate of anaerobic glycolysis of the cerebral cortex slices.

Table 20 shows the effect of TTX on the anaerobic glycolysis of rat cerebral cortex slices in a $Ca^{++}$-free medium, to which additional $K^+$ has been added. It is evident from these results that, when the $K^+$ concentration is increased without decreasing the corresponding $Na^+$ concentration, TTX is not effective in increasing the rate of anaerobic glycolysis. However, when $Na^+$ concentration is decreased in proportion to the increase of $K^+$ concentration, then the rate of glycolysis is increased by TTX (Table 21). Under these conditions, TTX has progressively less effect as $Na^+$ is decreased until a

TABLE 20

EFFECT OF PRESENCE OF DIFFERENT CONCENTRATION OF $K^+$ ON THE TETRODOTOXIN STIMULATION OF ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES

| $K^+$, added to $Ca^{++}$ free medium, in mM | Lactate produced µmoles per g initial wet wt (20-80 min) | |
|---|---|---|
| | No TTX | 2µM TTX |
| 0 | 26.3 ± 0.4 | 77.7 ± 1.8 |
| 33.3 | 19.7 ± 1.3 | 21.0 ± 1.3 |
| 66.6 | 20.1 ± 3.6 | 24.5 ± 2.4 |
| 100 | 21.0 ± 0.9 | 23.2 ± 2.7 |

Cerebral cortex slices were incubated in a $Ca^{++}$ free medium containing 20mM glucose. Additions were made at zero time and lactate production was measured manometrically as given in the materials and methods.

TABLE 21

EFFECTS OF TETRODOTOXIN AT VARYING CATION CONCENTRATION OF THE MEDIUM ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES

| $Na^+$ | $K^+$ | 2μM TTX | Lactate produced μmoles per g initial wet wt (20-80 min) |
|---|---|---|---|
| 149 | 5 | 0 | 21 |
| 149 | 5 | + | 72 |
| 89 | 60 | 0 | 59 |
| 89 | 60 | + | 70 |
| 29 | 60 | 0 | 127 |
| 29 | 60 | + | 144 |
| 29 | 125 | 0 | 107 |
| 29 | 125 | + | 130 |
| 0 | 154 | 0 | 115 |
| 0 | 154 | + | 114 |

High potassium medium was prepared by replacing some or all $Na^+$ by $K^+$ as described in Chapter 2.5 f. Medium contained 20mM glucose, TTX was added at zero time and lactate production was measured manometrically. Values are averages of two determinations within ± 7%.

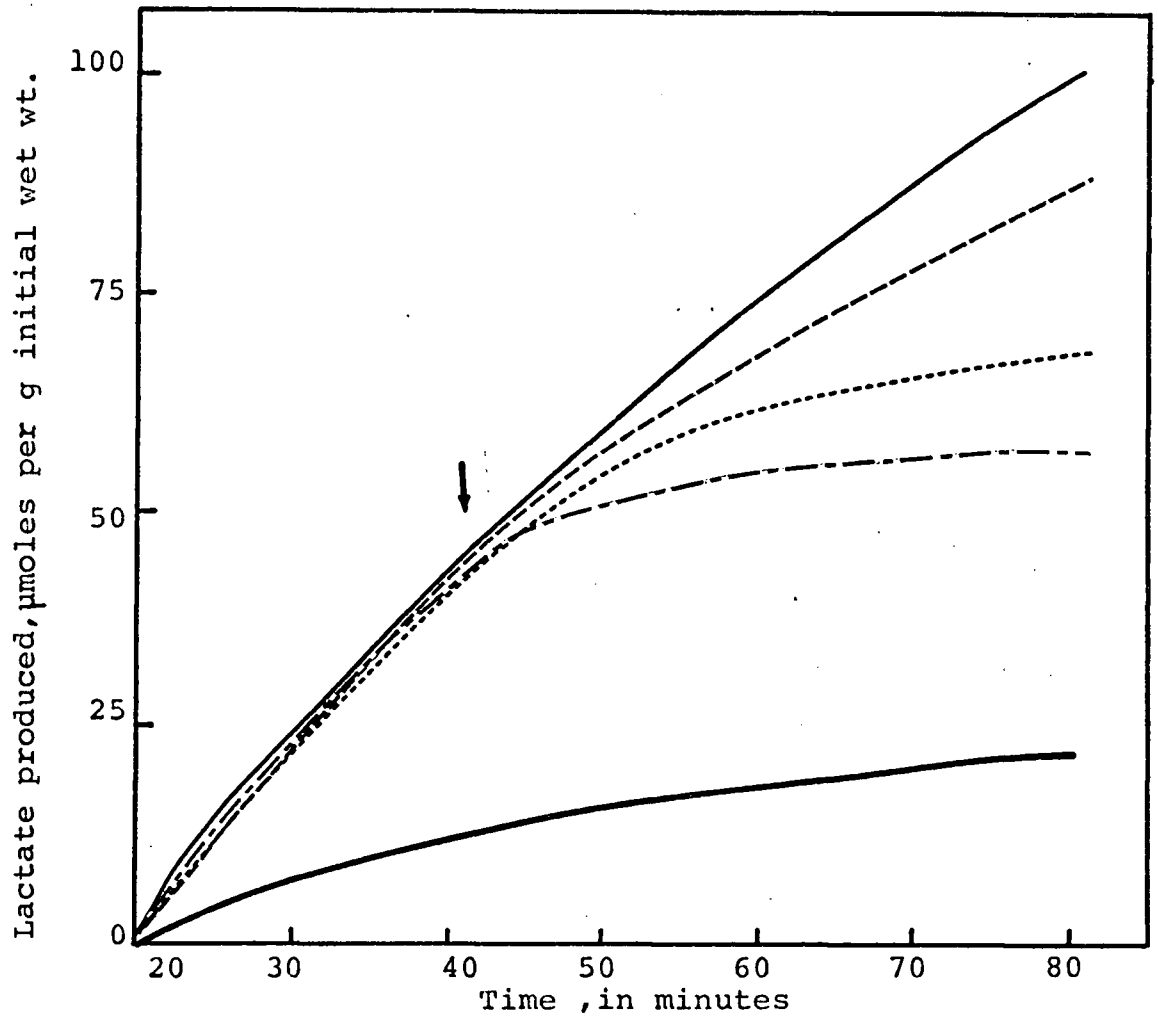
maximum rate of glycolysis is obtained when most of the $Na^+$ was replaced by $K^+$(Table 21). When the rate of glycolysis is very high (i.e., $Na^+$ is completely replaced by $K^+$), then other agents such as $Ca^{++}$ showed no stimulatory effect.

As the rate of anaerobic glycolysis in the presence of high concentrations of $K^+$ (in presence of 149 mM $Na^+$) is not influenced by TTX, it was decided to study their effects at a later stage i.e., when the effect of TTX has already been established. The results of these experiments are shown in Figure 23. It is evident that, when solutions of $K^+$, $Na^+$ or $Li^+$ are tipped in from the side arm of the Warburg vessel giving a final concentration of 100 mM (in addition to that already present), the rate of glycolysis, which is high due to the presence of TTX, is reduced significantly. Of the cations tested, $K^+$ is the most effective, followed by $Li^+$ and $Na^+$, in depressing the TTX-stimulated glycolysis. Under same conditions there is an increased influx of $Na^{22}$ (preliminary results). The effect of cations on anaerobic glycolysis does not appear to be due to changes in the tonicity since additions of sucrose, at 66, 133, or 200 mM final concentrations, have little or no effect on the rate of anaerobic glycolysis of brain slices (preliminary experiments).

The lack of effect of TTX in the presence of high $K^+$ and 149 mM $Na^+$ may be due to increased influx of $Na^+$. This will be further discussed in Chapter 8.

FIGURE 23

EFFECTS OF ADDITION OF HIGH CONCENTRATIONS OF CATIONS ON THE TETRODOTOXIN STIMULATED ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES




Incubations were carried out in a $Ca^{++}$-free medium containing 20 mM glucose. Arrow points out time at which 1 M solution of KCl, NaCl or LiCl was tipped in so as to give a final concentration of 100 mM. Lactate production was measured manometrically, as given in the materials and methods. (▬) control, (——— ) 2 µM TTX from zero time, no cation was added; ( - - - - - ) 2 µM TTX from zero time, $Na^{+}$ was tipped in; (·····) 2 µM TTX from zero time, $Li^{+}$ was tipped in; (— — — ) 2 µM TTX from zero time, $K^{+}$ was tipped in.

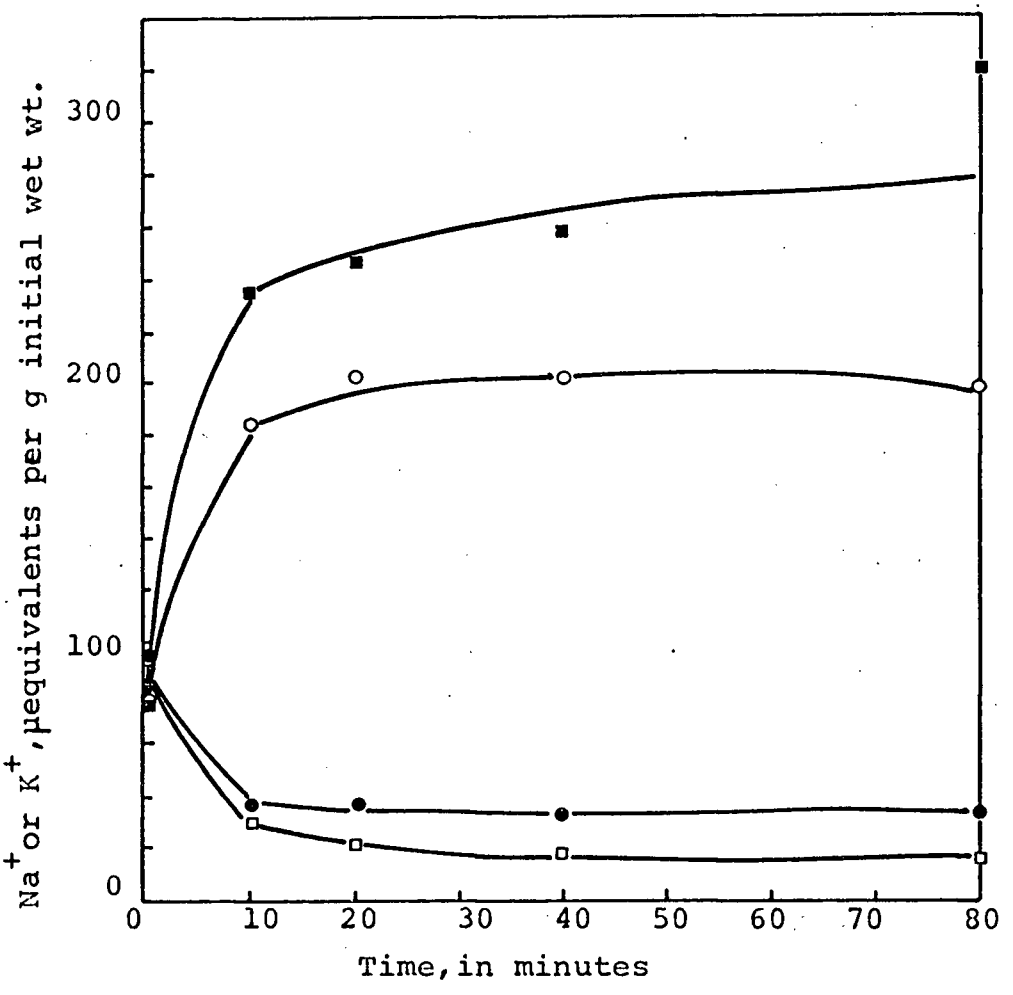
## 5.12 EFFECTS OF TETRODOTOXIN ON THE $Na^+$ and $K^+$ LEVELS OF INCUBATED CEREBRAL CORTEX SLICES UNDER ANOXIA

It is well known that the effects of electrical stimulation on cation movements in incubated brain slices are blocked by TTX, as shown by its metabolic effects[129,130] and its effects on the influx of $Na^+$[132] and on the efflux of $K^+$[129,131]. Also, as noted (see Chapter 1), $K^+$ stimulate pyruvate kinase[67,301] while $Na^+$ inhibits both hexokinase[53a] and pyruvate kinase.[37-39]. In view of these marked effects of these cations on the metabolism of cerebral tissue, it seemed quite possible that these might play an important role, under the conditions of anoxia, when action potentials might be generated. TTX could greatly influence the $K^+/Na^+$ ratio of the cerebral cortex slices by blocking the movements of these cations. An increased $K^+/Na^+$ ratio in the cell will result in a greater rate of anaerobic glycolysis. However, as already mentioned (see 5.9), we have been unable to detect large changes in the $Na^{22}$ influx under anoxia in the presence of TTX. In view of this, it was considered necessary to measure both the $K^+$ and $Na^+$ contents of incubated cerebral cortex slices under anoxia. The results of these experiments are described below.

The effects of 2 μM TTX on the $Na^+$ and $K^+$ level in the guinea pig cerebral cortex slices, incubated under anoxic conditions, are shown in Figure 24. It will be seen that, in

FIGURE 24

EFFECTS OF TETRODOTOXIN ON THE SODIUM AND POTASSIUM CONCENTRATIONS OF GUINEA PIG CEREBRAL CORTEX SLICES UNDER ANOXIA




Incubations were carried out in a $Ca^{++}$-free medium containing 20 mM glucose. TTX, when present, was added at zero time. $Na^+$ and $K^+$ contents were determined as given in the materials and methods. (■) $Na^+$ control; (○) $Na^+$, with 2 μM TTX; (□) $K^+$, control; (●) $K^+$, with 2 μM TTX.

the presence of TTX, the amount of $K^+$ retained in the slices is twice the concentration in the controls. At the same time, there is a reduction in the amount of $Na^+$. Similar results on the $K^+$ content are obtained with rat cerebral cortex slices. These results make it reasonable to conclude that the effect of TTX on the anaerobic glycolysis of the cerebral cortex slices may be due to an increase in the $K^+/Na^+$ ratio.

## 5.13 EFFECTS OF TETRODOTOXIN ON THE ANAEROBIC GLYCOLYSIS AND $Na^{22}$ TRANSPORT IN THE CAUDATE NUCLEUS OF RAT

As TTX is believed to act only on excitable cells, it is likely that the effect of TTX will be confined to the neurons. Caudate nucleus is a part of brain which has relatively few glial cells.[135] Experiments were carried out, therefore, on the effects of TTX on the rate of anaerobic glycolysis and on $Na^{22}$ transport in the caudate nucleus. Results of these experiments are shown in Table 22. The anaerobic glycolysis of caudate nucleus is increased by the presence of TTX but the magnitude of stimulation is not greater than that observed with the cerebral cortex slices. Moreover, TTX has no effect on the $Na^{22}$ influx. These results will be further discussed in Chapter 8.

## 5.14 EFFECTS OF TETRODOTOXIN ON THE ANAEROBIC GLYCOLYSIS OF SYNAPTOSOMAL PREPARATIONS OF RAT BRAIN

As mentioned earlier, 2 μM TTX accelerates the rate of anaerobic glycolysis of adult rats and guinea pigs but not

TABLE 22

EFFECTS OF TETRODOTOXIN ON THE ANAEROBIC GLYCOLYSIS AND $Na^{22}$ TRANSPORT IN CAUDATE NUCLEUS OF RAT

| Additions | Lactate produced µmoles per g initial wet wt (20-80 min) | µequivalent Na, corresponding to $Na^{22}$, per g initial wet wt |
|---|---|---|
| None | 26.8 ± 1.0 | 101 ± 2 |
| 2µM TTX | 62.5 ± 5.8 | 104 ± 2 |

Incubations were carried out in a medium containing 20mM glucose. TTX was added at zero time and lactate production was measured manometrically, as given in the materials and methods. For $Na^{22}$ experiments, conditions were same as in Table 18 except that incubation time in $Na^{22}$ was 15 min.

of 2-day old rat brain. The sensitivity of the rat brain slices to TTX increases considerably at about 14th day after birth, which coincides with the time of maximum brain growth and myelination. It has also been mentioned (Chapter 1.6) that the nerve endings are not myelinated. Whittaker[253,254] has developed techniques by which it is possible to separate the nerve endings from other subcellular particles. As the nerve endings and receptors are developed in the brain during maturation, experiments were carried out to see whether the anaerobic glycolysis of the nerve ending particles is affected by TTX and other drugs. Results of these experiments are shown in Figure 25. These experiments showed that TTX and ouabain have little or no effect on the rate of anaerobic glycolysis of synaptosomes, while 4 mM $Ca^{++}$ has an inhibitory action.

## 5.15 EFFECTS OF PRE-INCUBATION IN OXYGEN ON THE $K^+$ AND $Na^+$ CONTENTS OF CEREBRAL CORTEX SLICES UNDER ANOXIA IN THE PRESENCE OF TETRODOTOXIN

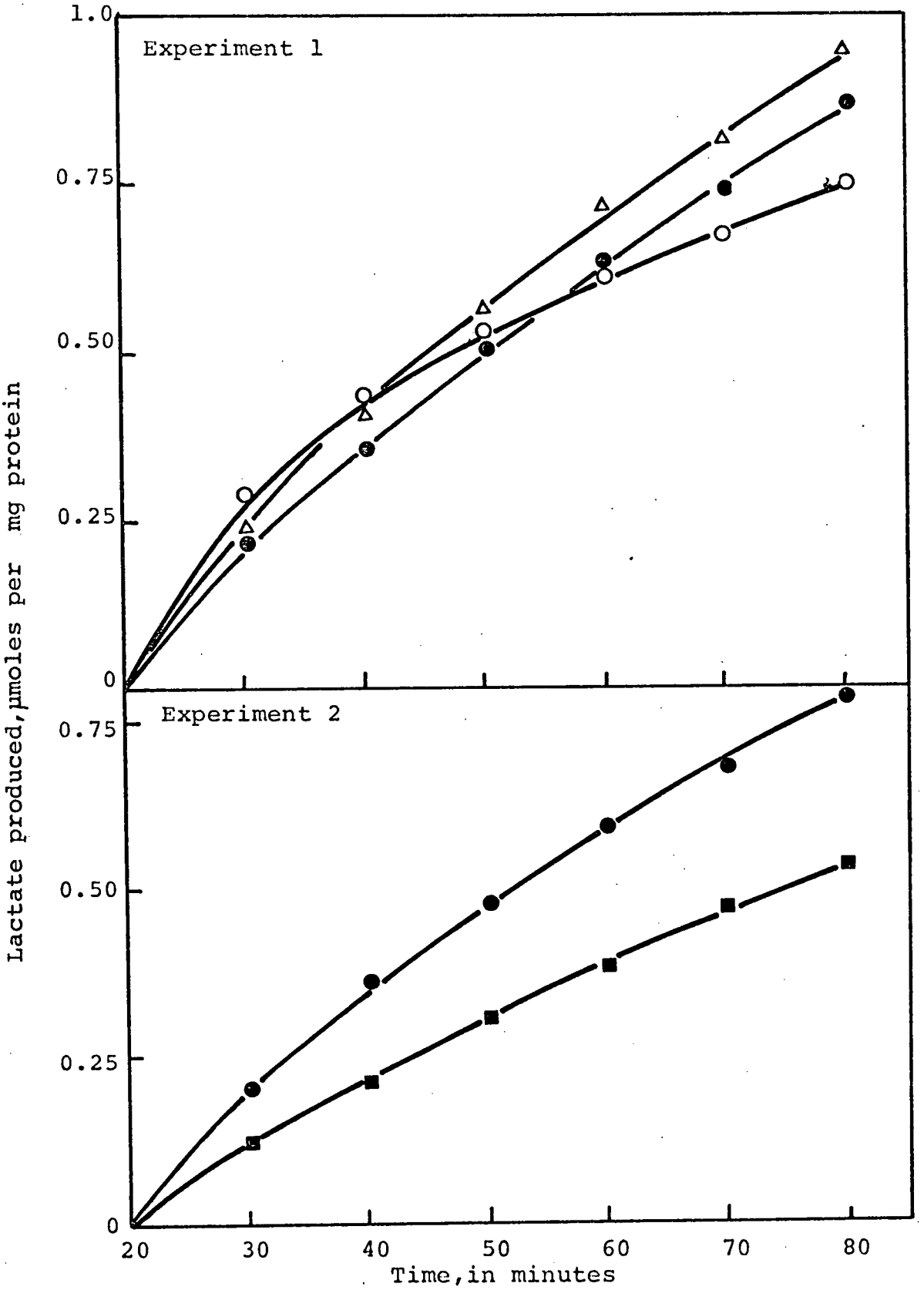
Brief aerobic pre-incubation of brain slices increases the subsequent rate of anaerobic glycolysis, both in the presence and absence of TTX, as compared to that obtained with the non-oxygenated slices (Section 5.1). Experiments were carried out, therefore, to evaluate the effects of short (10 min) aerobic pre-incubation on the $Na^+$ and $K^+$ content of the cerebral cortex slices in the presence of TTX. It is evident from the results

FIGURE 25

EFFECTS OF TETRODOTOXIN, CALCIUM AND OUABAIN ON THE ANAEROBIC GLYCOLYSIS OF RAT SYNAPTOSOMES

Incubations were carried out in a $Ca^{++}$-free medium containing 20 mM glucose. Additions were made at zero time and lactate production was measured manometrically as given in the materials and methods. (●) control; (△) 10 µM ouabain; (○) 2 µM TTX ; (■) 4 mM $Ca^{++}$.

FIGURE 25


Experiment 1
Experiment 2
Lactate produced, µmoles per mg protein
1.0
0.75
0.50
0.25
0
0.75
0.50
0.25
0
20
30
40
50
60
70
80
Time, in minutes

shown in Figure 26 that the retention of $K^+$ in the oxygenated slices during the subsequent period of anoxia is much greater. In the presence of 2 μM of TTX, there is very little loss of $K^+$ and less gain of $Na^+$ during the anaerobic incubation. There is an initial drop in the $K^+$ content during the preliminary aerobic period. The effect of $Ca^{++}$ on the $K^+$ level is also shown in the same figure. It is clear that there is some increased retention of $K^+$ by the slices in the presence of $Ca^{++}$, but it was not as effective as TTX.

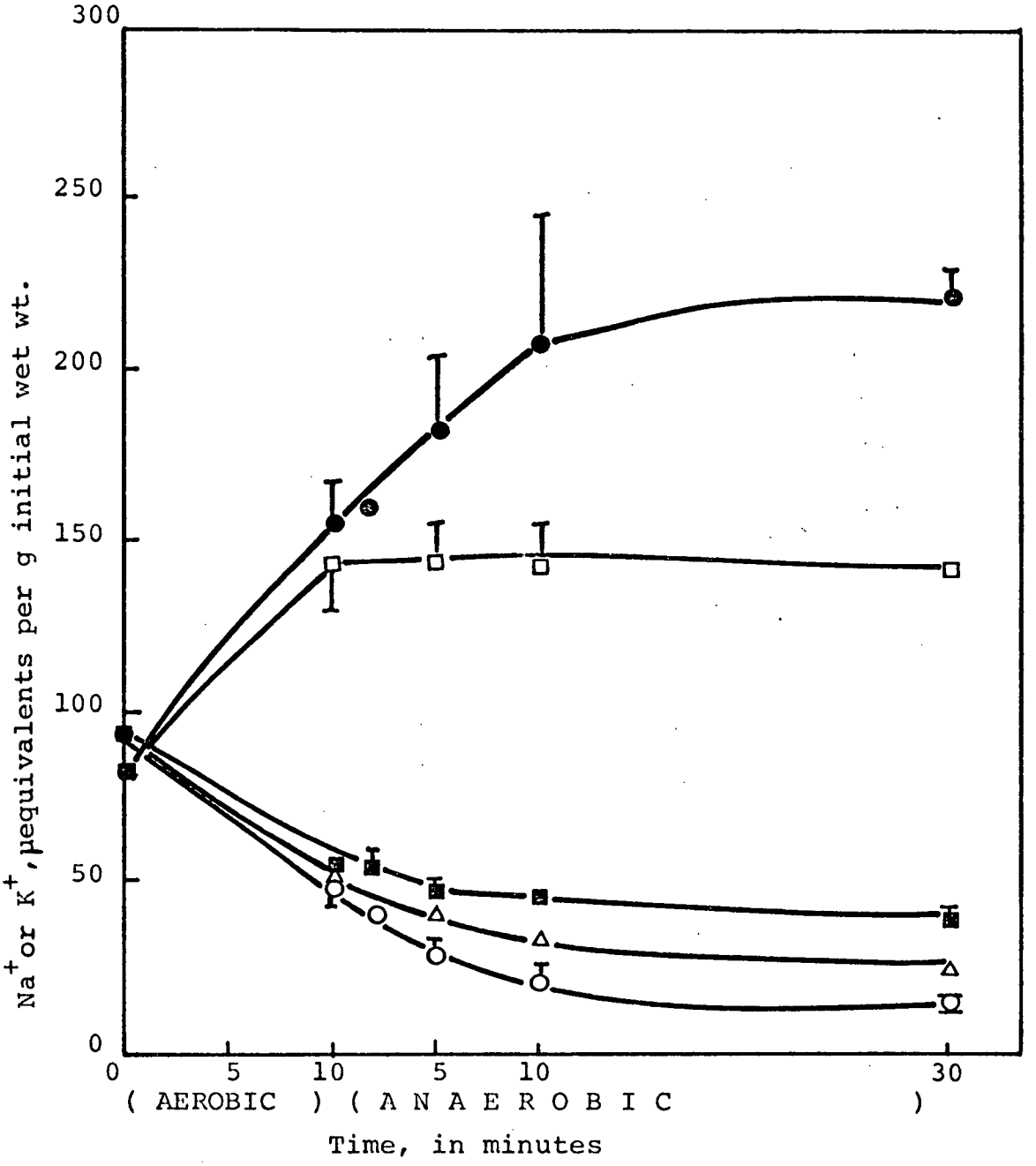
Because the results with cation contents are more clear cut in the oxygenated slices, in all subsequent experiments reported below the technique of prior oxygenation was used to study the retention of $K^+$ and uptake of $Na^+$ by incubated brain slices.

## 5.16 EFFECTS OF TETRODOTOXIN ON THE $Na^+$ AND $K^+$ LEVELS OF INFANT RAT AND GUINEA PIG CEREBRAL CORTEX SLICES

Results of experiments on the effect of TTX on the anaerobic glycolysis of developing brain cortex slices have been given in Chapter 4.12. It was of interest, therefore, to observe whether TTX affects $Na^+$ and $K^+$ contents of the infant rat, as well as infant guinea pig, cerebral cortex slices. The results of these experiments are shown in Figures 27 and 28. It is evident that TTX has little or no effect on the cation content of infant (2-day old) rat brain slices but the infant

FIGURE 26

EFFECTS OF TETRODOTOXIN AND CALCIUM ON THE SODIUM AND POTASSIUM CONCENTRATIONS OF RAT CEREBRAL CORTEX SLICES




Incubations were carried out in a $Ca^{++}$-free medium containing 20 mM glucose. Initial 10 min period was aerobic($O_2$:$CO_2$) followed by anaerobic period($N_2$:$CO_2$). Additions were made at zero time. Vertical bars represent standard deviations. (●) $Na^+$, control; (□) $Na^+$, with 2 μM TTX; (○) $K^+$, control; (■) $K^+$, with 2 μM TTX. (△) 4mM $Ca^{++}$, $K^+$.

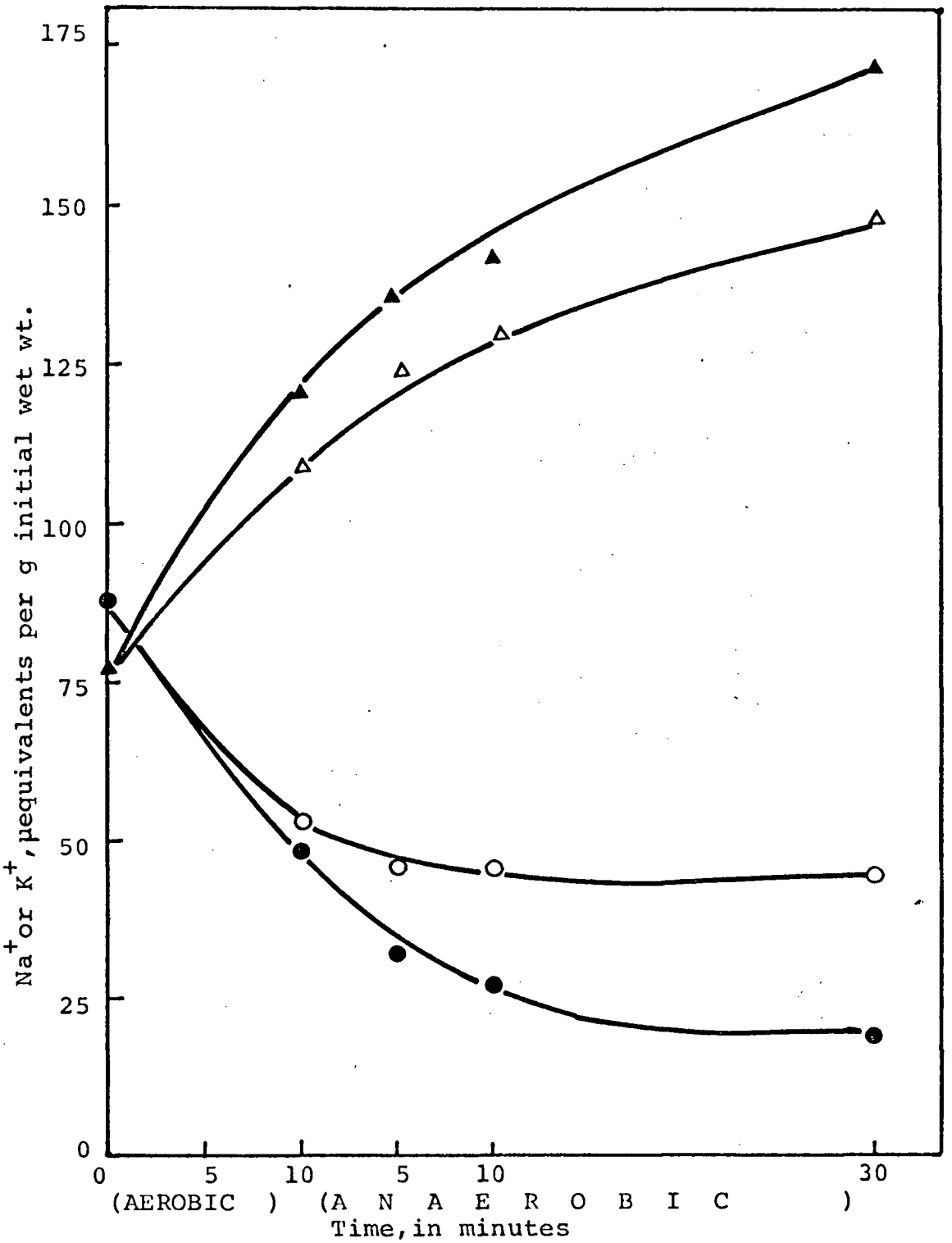
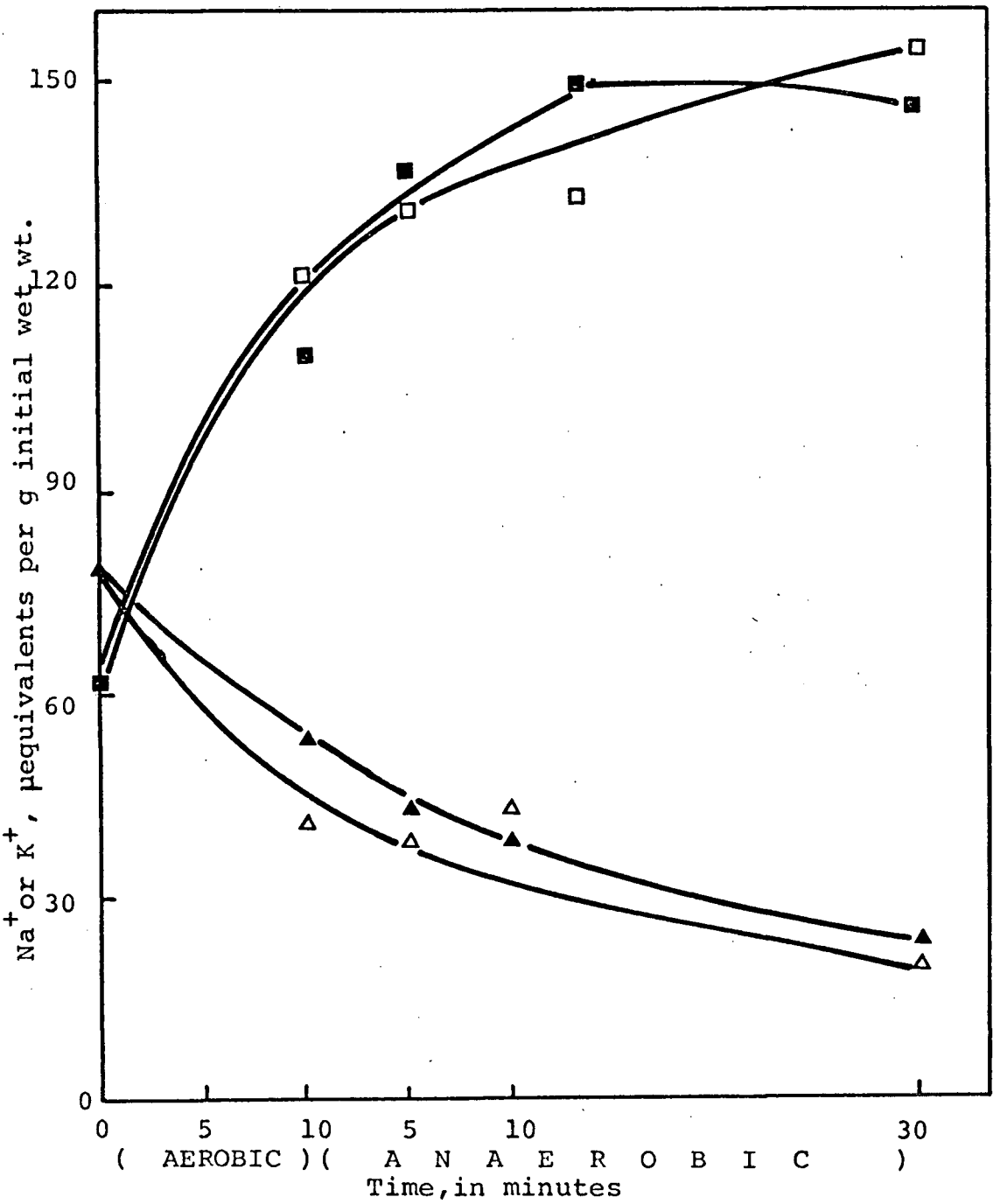



FIGURE 27 : EFFECTS OF TETRODOTOXIN ON THE SODIUM AND POTASSIUM CONCENTRATIONS OF NEWLY BORN GUINEA PIG CEREBRAL CORTEX SLICES.
Incubation conditions were same as in Figure 26. (▲) $Na^+$, control; (△) $Na^+$, with 2 µM TTX; (●) $K^+$, control; (○) $K^+$, with 2 µM TTX.

FIGURE 28

EFFECTS OF TETRODOTOXIN ON THE SODIUM AND POTASSIUM CONCENTRATIONS OF TWO DAY OLD RAT CEREBRAL CORTEX SLICES




Incubation conditions were same as in Figure 26. (■) $Na^+$, control; (□) $Na^+$, with 2 μM TTX; (△) $K^+$, control; $K^+$, with 2 μM TTX (▲).

guinea pig shows a marked increase in the $K^+$ content, and a decrease in $Na^+$ content, in the presence of TTX.

5.17 EFFECTS OF TETRODOTOXIN ON THE $Na^+$ and $K^+$ LEVELS OF KIDNEY MEDULLA SLICES

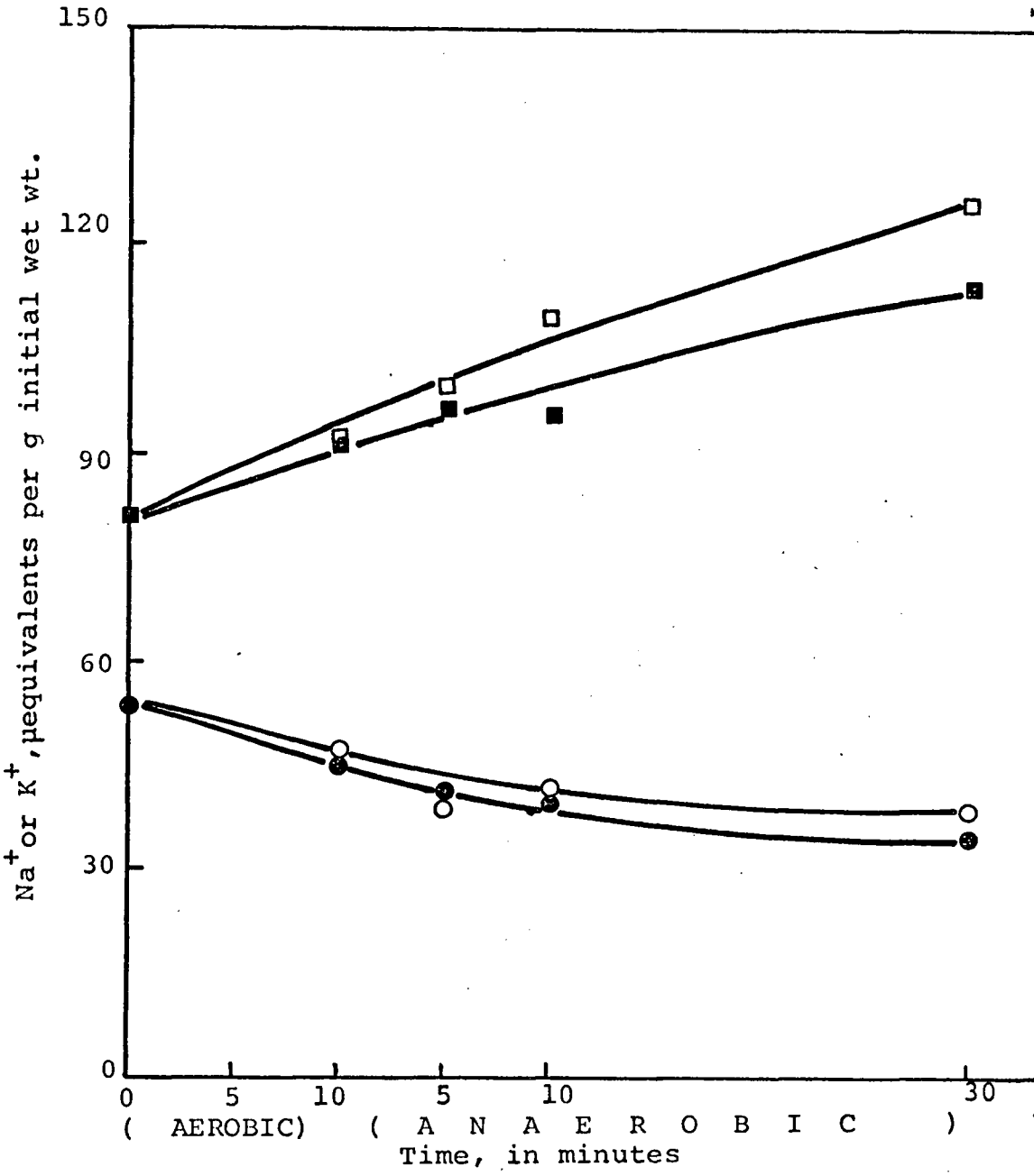
Kidney medulla was used as a control tissue (Chapter 4.11). As has been shown in Figure 29, TTX has no effect on the $K^+$ and $Na^+$ contents of the kidney medulla slices. This result is consistent with the conclusion that the effect of TTX is specific to the nervous tissue.

5.18 EFFECTS OF TETRODOTOXIN IN THE PRESENCE OF CHELATING AGENTS ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

Chan and Quastel[130] have shown that the increase in respiration brought about by the absence of $Ca^{++}$ is prevented by TTX. We have also seen, in Chapter 4, that TTX acts like $Ca^{++}$ in increasing the anaerobic glycolysis and suppressing the enhanced aerobic glycolysis caused by the absence of $Ca^{++}$. It has been known for a long time, that $Ca^{++}$ stabilizes the biological membranes. It is possible that TTX may act by inter-acting with membrane-bound $Ca^{++}$ and thus change its permeability. To test this possibility, it was considered desirable to study the effects of TTX on anaerobic glycolysis in the presence of chelating agents EDTA and EGTA (EDTA chelates both $Ca^{++}$ and $Mg^{++}$ while EGTA is more specific in binding $Ca^{++}$).

FIGURE 29

EFFECTS OF TETRODOTOXIN ON THE SODIUM AND POTASSIUM CONCENTRATIONS OF RAT KIDNEY MEDULLA SLICES




Incubation conditions were same as in Figure 26.( □ )$Na^+$,control; ( ■ )$Na^+$,with 2 μM TTX; ( ● )$K^+$,control ; ( ○ )$K^+$,with 2 μM TTX.

Results of experiments, given in Table 23, show that TTX is ineffective in the presence of these chelating agents. It is thought that this is due to the very large influx of $Na^+$ brought about by the complete chelation of $Ca^{++}$ which masks any effect of TTX or that there occurs excessive depolarization in which condition TTX is ineffective.

## 5.19 EFFECTS OF AEROBIC PRE-INCUBATION WITH ETHANOL ON THE TETRODOTOXIN STIMULATION OF ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

Israel, Kalant and LeBlanc[299] have shown that, in the presence of ethanol, cerebral slices which have lost $K^+$ do not regain it to the same extent as that obtained with the slices which have not been exposed to ethanol. This action was stated to be mediated through partial inhibition of $Na^+$, $K^+$-ATPase by ethanol. If the effects of TTX on anaerobic glycolysis are due to the retention of $K^+$, then the slices which have been incubated, under oxygen, in the presence of ethanol should show less response to TTX. Results of typical experiments are shown in Table 24; they show that this is indeed the case. However, this does not rule out the possibility that ethanol may directly interfere with the mode of action of TTX. Further work is necessary to settle this.

TABLE 23

EFFECTS OF EDTA AND EGTA ON THE TETRODOTOXIN STIMULATION OF ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

| Additions | Lactate produced µmoles per g initial wet wt (20-80 min) | |
|---|---|---|
| | Rat | Guinea Pig |
| None | 24.4 ± 2.8 | 30.7 ± 7.6 |
| 2µM TTX | 81.1 ± 7.6 | 141.8 ±14.5 |
| 1mM EDTA | 11.3 | 11.8 ± 0.8 |
| 1mM EGTA | 24.9 ± 3.5 | 17.7 ± 2.9 |
| 1mM EDTA + 2µM TTX | 17.8 ± 4.3 | 14.9 ± 4.6 |
| 1mM EGTA + 2µM TTX | 27.3 ± 1.9 | 37.5 ± 8.0 |

Cerebral cortex slices were incubated in a $Ca^{++}$ free medium containing 20mM glucose. Additions were made at zero time and lactate production was measured manometrically as given in the materials and methods.

TABLE 24

EFFECT OF ETHANOL ON THE TETRODOTOXIN STIMULATION OF ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES

| Additions | Lactate produced µmoles per g initial wet wt (30-90 min) |
|---|---|
| None | 23.7 ± 0.5 |
| 200mM Ethanol | 36.2 ± 4.5 |
| 2µM TTX | 107.2 ± 2.0 |
| 2µM TTX + 200mM Ethanol | 82.5 ± 3.1 |

Incubations were carried out in a $Ca^{++}$ free medium containing 20mM glucose. First 10 min incubation was aerobic ($O_2$:$CO_2$) followed by anaerobiosis. Additions were made at zero time and lactate production was measured manometrically as given in the materials and methods.

## 5.20 EFFECTS OF TETRODOTOXIN ON THE $Na^+$ AND $K^+$ CONTENT OF THE CEREBRAL CORTEX SLICES IN THE PRESENCE OF GLUTAMATE, ASPARTATE, HOMOCYSTEIATE AND $NH_4^+$

We have shown in Chapter 4.8 that in the presence of glutamate and $NH_4^+$, TTX is much less effective in increasing the anaerobic glycolysis of the cerebral cortex slices. Under the same conditions aspartate was found to have no effect. Hence experiments were carried out to see if the cation concentration of the slices, in the presence of TTX, is affected by glutamate, $NH_4^+$ and other amino acids. The results of these experiments are given in Table 25. It will be seen that when L- or D-glutamate is present, the retention of $K^+$ is less in the slices than in those incubated in the absence of the amino acids. In the presence of $NH_4^+$, TTX had a significant but smaller effect on the $K^+$ levels. Under the same conditions, when L-aspartate or DL-homocysteate is present, increased retention of $K^+$ in the presence of TTX is evident. These results demonstrate that the effects of glutamate and $NH_4^+$ on the TTX stimulation of anaerobic glycolysis may be mediated through changes in the cerebral concentrations of $Na^+$ and $K^+$.

## 5.21 EFFECTS OF TETRODOTOXIN ON THE PYRUVATE AND PHOSPHOENOL-PYRUVATE CONTENTS OF CEREBRAL CORTEX SLICES UNDER ANOXIA

We have seen, in sections 5.12 and 5.15, that in the

TABLE 25

EFFECTS OF TETRODOTOXIN ON THE SODIUM AND POTASSIUM CONCENTRATIONS OF RAT CEREBRAL CORTEX SLICES IN THE PRESENCE OF SOME AMINO ACIDS AND $NH_4^+$

| Addition | Cation | Aerobic | Anaerobic | | |
|---|---|---|---|---|---|
| | | 10 min | 5 min | 10 min | 30 min |
| 5mM L-Glutamate | $Na^+$ | 160 | 170 | 195 | 220 |
| | $K^+$ | 42 | 25 | 21 | 16 |
| 5mM L-Glutamate + 2μM TTX | $Na^+$ | 130 | 155 | 175 | 220 |
| | $K^+$ | 48 | 42 | 37 | 23 |
| 5mM D-Glutamate | $Na^+$ | 147 | 175 | 200 | 232 |
| | $K^+$ | 46 | 29 | 20 | 15 |
| 5mM D-Glutamate + 2μM TTX | $Na^+$ | 150 | 155 | 165 | 215 |
| | $K^+$ | 49 | 43 | 36 | 23 |
| 5mM L-Aspartate | $Na^+$ | 145 | 180 | 190 | 220 |
| | $K^+$ | 40 | 27 | 21 | 15 |
| 5mM L-Aspartate + 2μM TTX | $Na^+$ | 140 | 135 | 170 | 210 |
| | $K^+$ | 48 | 42 | 38 | 30 |
| 5mM DL-Homocysteiate | $Na^+$ | 153 | 200 | 200 | 223 |
| | $K^+$ | 48 | 27 | 20 | 14 |
| 5mM DL-Homocysteiate + 2μM TTX | $Na^+$ | 150 | 160 | 175 | 235 |
| | $K^+$ | 47 | 46 | 38 | 45 |

TABLE 25

(Continued)

| Addition | Cation | Aerobic | Anaerobic | | |
|---|---|---|---|---|---|
| | | 10 min | 5 min | 10 min | 30 min |
| 5mM $NH_4Cl$ | $Na^+$ | 125 | 145 | 170 | 210 |
| | $K^+$ | 38 | 24 | 16 | 15 |
| 5mM $NH_4Cl$ + 2μM TTX | $Na^+$ | 120 | 125 | 140 | 185 |
| | $K^+$ | 40 | 35 | 30 | 18 |

Incubation conditions were same as in Figure 26. Each value represent averages of two experiments within ± 7%. For controls, see Figure 26. Results are expressed as μequivalents per g initial wet wt.

presence of 2 μM TTX, there is an increase in the $K^+/Na^+$ ratio of the incubated cerebral cortex slices after the onset of anoxia. If the effect of TTX on the anaerobic glycolysis is due to the facilitation of pyruvate kinase step because of an increase in the $K^+/Na^+$ ratio, then in the presence of TTX there should be a decrease in the phosphoenol-pyruvate (PEP) content and an increase in the pyruvate content.

Experiments were carried out therefore to test the above hypothesis. For these experiments, conditions under which maximum effect on the $K^+/Na^+$ ratio is obtained, were selected. As shown in Table 26, in the presence of TTX the concentration of pyruvate in the cerebral cortex slices is indeed increased. Phosphoenol-pyruvate contents in the incubated slices were extremely low and accurate determination was therefore not possible.

The increase in pyruvate contents in the presence of TTX thus supports the view that the effects of TTX on the anaerobic glycolysis is due to the facilitation of the pyruvate kinase step; presumably it is not due to block of pyruvate efflux from the slices during incubation, as TTX is effective in increasing the rate of anaerobic glycolysis even in the presence of pyruvate (Section 5.3).

TABLE 26

EFFECTS OF TETRODOTOXIN ON THE PYRUVATE AND PHOSPHOENOL PYRUVATE CONTENT OF RAT CEREBRAL CORTEX SLICES

| Additions | mμmoles per g initial wet wt | |
|---|---|---|
| | Pyruvate | PEP |
| Zero time | 107 ± 6 | 62 ± 5 |
| None | 52 ± 8 | LOW |
| 2 μM TTX | 79 ± 6 | LOW |

Incubations were carried out in a $Ca^{++}$ free medium containing 20mM glucose for 10 min aerobically followed by 20 min anaerobiosis. Pyruvate and PEP were measured at the beginning and end of the incubation period, as given in the materials and methods.

SUMMARY OF CHAPTER 5

1. Prior oxygenation of the cerebral cortex slices increases the subsequent rate of anaerobic glycolysis, both in the absence and presence of TTX, to a value greater than that found in the non-oxygenated slices.

2. TTX is not effective in increasing the anaerobic glycolysis of the cerebral cortex slices if it is added 10 min, or later, after the onset of anoxia.

3. The effects of TTX on the anaerobic glycolysis of the cerebral cortex slices do not seem to be mediated through the retention of pyruvate. Its effect on anaerobic glycolysis by directly influencing ATP levels, is unlikely. (See Chapter 8). The ATP contents of the cerebral cortex slices are higher in the presence of TTX under anoxia than in the absence of TTX.

4. During aerobic incubation, uptake of adenine into the cerebral cortex slices and its incorporation into ATP increases with time. The formation of cAMP, from the pulse labelled ATP of the incubated slices, is not increased by TTX, under anaerobic conditions.

5. Protoveratrine reverses the effects of TTX on the anaerobic glycolysis of the cerebral cortex slices. Moreover, the inhibitory effect of protoveratrine on cerebral anaerobic glycolysis is blocked by TTX.

6. TTX has little effect on the $Na^{22}$ influx into the cerebral cortex slices under anoxia.

7. TTX has no accelerating effect on anaerobic glycolysis when high $K^+$ is present in the incubation medium containing normal $Na^+$ concentration. If the $Na^+$ is reduced at the same time that $K^+$ is increased, the rate of anaerobic glycolysis increases and TTX has progressively less effect with increasing $K^+$ concentration.

8. $K^+$, $Li^+$ or $Na^+$ (100 mM final concentration) when added to the incubation medium, in which the anaerobic glycolysis of cerebral cortex slices has been accelerated by TTX, has an inhibitory effect on the TTX stimulated glycolysis.

9. $K^+$ content of the anaerobically incubated cerebral cortex slices is increased in the presence of TTX. There is decrease in the $Na^+$ content at the same time.

10. TTX increases the anaerobic glycolysis of caudate nucleus but $Na^{22}$ influx is not suppressed.

11. TTX and ouabain have no effect on the anaerobic glycolysis of synaptosomes but $Ca^{++}$ has an inhibitory action.

12. The effects of TTX on the $K^+$ and $Na^+$ content is much more apparent if, after the addition of TTX, the slices are incubated for a short period in oxygen preliminary to the onset of anoxia.

13. TTX has little or no effect on the $Na^+$ and $K^+$ contents of 2-day old rat cerebral cortex slices but infant guinea pigs are similar to adult animals in retaining more $K^+$ and taking up less $Na^+$ in the presence of TTX.

14. TTX has no effect on the anaerobic glycolysis of cerebral cortex slices in the presence of chelating agents, EDTA and EGTA.

15. Aerobic pre-incubation of the slices in the presence of ethanol decreases the subsequent rate of anaerobic glycolysis in the presence of TTX.

16. Ammonium ions, L-glutamate and D-glutamate reduce the effect of TTX on the retention of $K^+$ by the cerebral cortex slices under anoxia (see Table 25), while L-aspartate and DL-homocysteiate have no effect.

17. In the presence of TTX, there is an increase in the pyruvate content of the cerebral cortex slices under anoxia. This supports the hypothesis that the effects of TTX on anaerobic glycolysis might be due indirectly to the facilitation of the pyruvate kinase step.

CHAPTER 6

## EFFECTS OF OUABAIN AND LOCAL ANESTHETICS ON THE CEREBRAL METABOLISM AND TRANSPORT UNDER ANOXIA

It was shown in Chapters 4 and 5 that TTX greatly increases the rate of anaerobic glycolysis as well as $K^+/Na^+$ ratio of the incubated cerebral tissue. It was concluded that this increase in the $K^+/Na^+$ ratio is responsible for the stimulating action of TTX on the anaerobic glycolysis. In view of the fact that some of the effects of local anesthetics on cerebral processes resemble those of TTX (Chaper 1), the effects of local anesthetics on anaerobic glycolysis were investigated.

Ouabain is a strong inhibitor of $Na^+$, $K^+$-ATPase, which is responsible for maintaining ion gradients across the brain cell membrane. As noted, ouabain has marked effects on the metabolism of aerobically incubated cerebral tissue. Most of these effects are due to inhibition of membrane bound ATPase. Rolleston and Newsholme [52] showed that 100μM ouabain increases the formation of lactic acid in the aerobically incubated cerebral cortex slices. Furthermore, they concluded that ouabain may be an inhibitor of glyceraldehyde 3-phosphate dehydrogenase. Wollenberger[181] found that some digitalis alkaloids inhibit the anaerobic glycolysis of cerebral cortex slices in a manner similar to that of protoveratrine. The effect of ouabain on the anaerobic glycolysis was investigated in the light of present knowledge as to its mode of action. The

results of experiments carried out with ouabain and local anesthetics will be considered in this Chapter.

## 6.1 EFFECTS OF OUABAIN ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

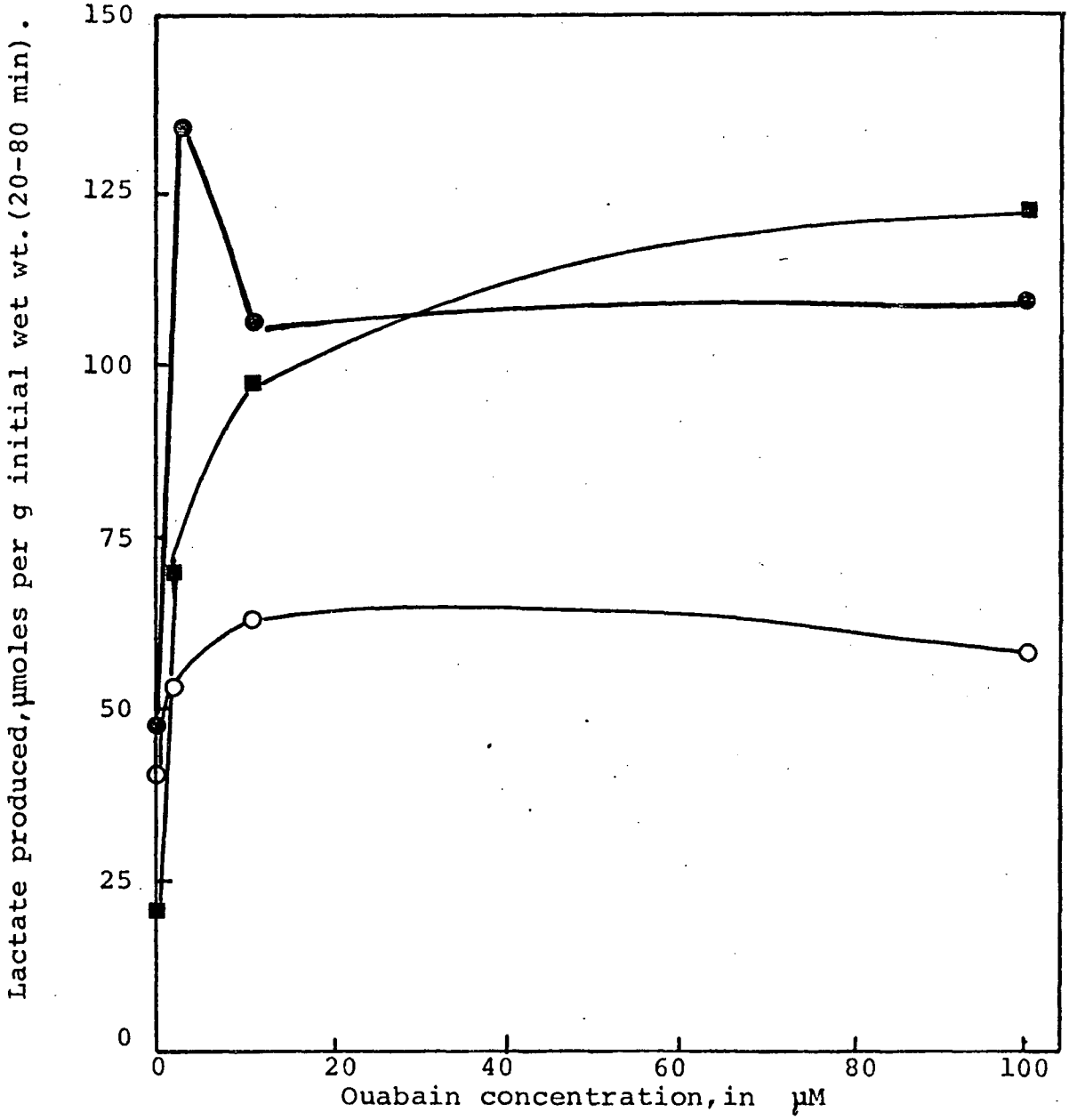
Experiments carried out with 10 μM ouabain showed that it greatly enhances the rate of anaerobic glycolysis in a $Ca^{++}$-free medium. A dose response curve for different concentrations of ouabain is shown in Figure 30. It is evident that, with rat cerebral cortex slices, the rate of anaerobic glycolysis increases progressively with increasing ouabain concentrations in a $Ca^{++}$-free medium. However, with guinea pig brain a high rate of glycolysis is obtained even with 1 μM ouabain, and further increase in its concentration had no additional stimulatory effect.

These results are contrary to those reported by Wollenberger using other digitalis alkaloids and a $Ca^{++}$-containing medium for incubation of the brain slices. It was decided, therefore, to carry out experiments using a Krebs-Ringer bicarbonate incubation medium in place of a $Ca^{++}$-free medium. These results show that in a normal balanced Krebs-Ringer bicarbonate medium, ouabain has much less stimulatory effect than that taking place in a $Ca^{++}$-free medium. This stands in contrast to the effect of TTX on the anaerobic glycolysis in a Krebs-Ringer bicarbonate medium, where considerable stimulation is observed (Chapter 4.1).

In view of these results, it was decided to study the effects of ouabain, in the presence of different concentra-

FIGURE 30

EFFECTS OF DIFFERENT CONCENTRATIONS OF OUABAIN ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES




Cerebral cortex slices were incubated in a medium containing 20 mM glucose. Additions were made at zero time and lactate production was measured manometrically as given in the materials and methods. (●) Guinea pig, $Ca^{++}$-free medium; (■) rat, $Ca^{++}$-free medium; (○) rat, Krebs-Ringer bicarbonate medium.

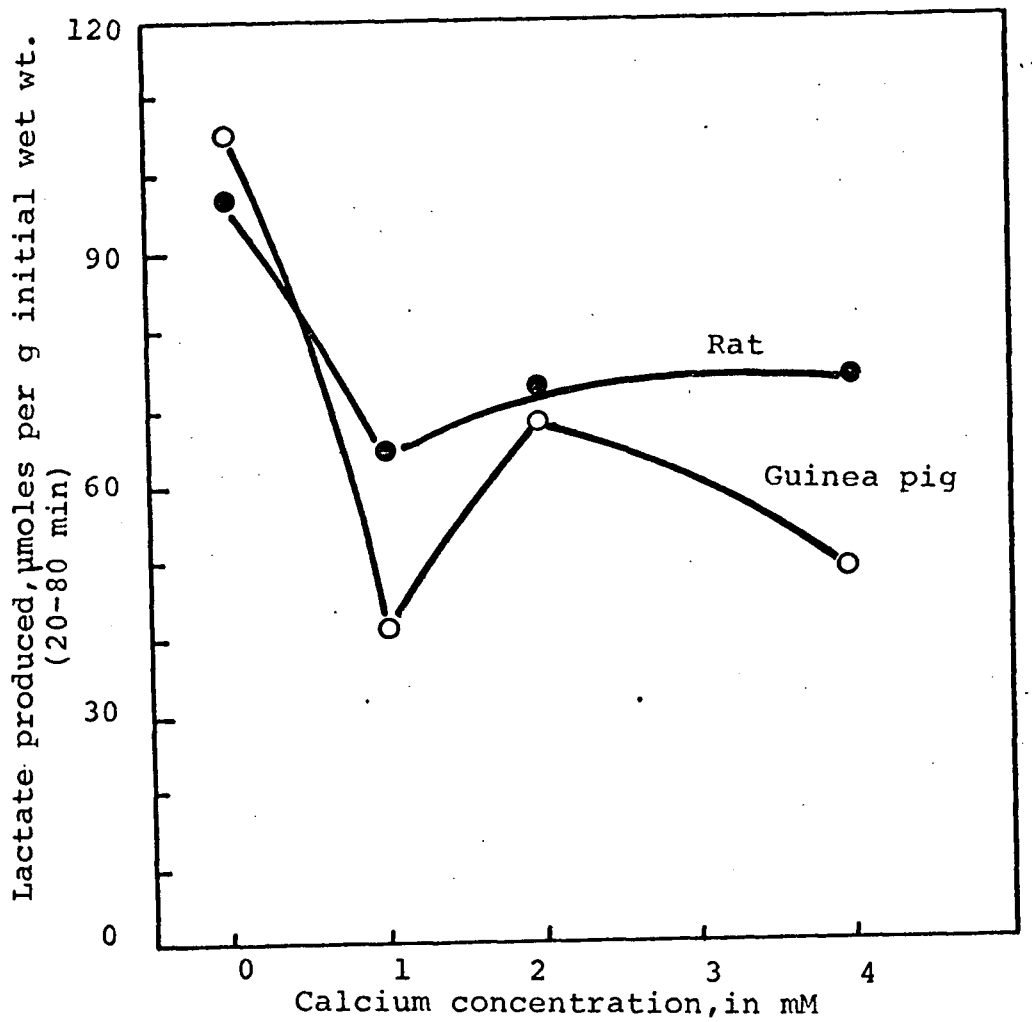
tions of $Ca^{++}$, on the anaerobic glycolysis of cerebral cortex slices. In these experiments, results of which are shown in Figure 31,different concentrations of $Ca^{++}$ (1-4 mM) were added to a $Ca^{++}$-free medium from the start of the experiment (see Figure 2 for results with $Ca^{++}$ alone). These results demonstrate that when $Ca^{++}$ is present in addition to ouabain, the glycolytic rate is not as high as with ouabain alone. Thus, when both these substances are present together they have an antagonistic action on the rate of anaerobic glycolysis of the cerebral cortex slices.

## 6.2 EFFECTS OF OUABAIN ON THE ANAEROBIC GLYCOLYSIS OF INFANT RAT BRAIN CORTEX SLICES

The primary action of ouabain is known to be on the $Na^+$, $K^+$-ATPase. In infant rats, the $Na^+$,$K^+$-ATPase activity is very low but increases rapidly during the 2-3 week period after birth. The effects of ouabain on the anaerobic glycolysis of developing brain was, therefore, investigated and the results are given in Table 27. These results indicate that ouabain has little or no effect on the anaerobic glycolysis of 2-day old rat brain. However, the anaerobic glycolysis of 2-week old rat brain is considerably stimulated in the presence of ouabain. The effect of ouabain in the presence of $Ca^{++}$ is very similar to that for adult brain, i.e. the anaerobic glycolysis of infant rat brain in the presence of ouabain is also diminished in the presence of $Ca^{++}$.

FIGURE 31

EFFECT OF VARYING CALCIUM CONCENTRATION IN THE PRESENCE OF OUABAIN ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES




Cerebral cortex slices were incubated in a $Ca^{++}$-free medium containing 10 µM ouabain, 20 mM glucose and different concentrations of $Ca^{++}$. Additions were made at zero time and lactate production was measured manometrically as given in the materials and methods.

TABLE 27

EFFECT OF OUABAIN ON THE ANAEROBIC GLYCOLYSIS OF DEVELOPING RAT CEREBRAL CORTEX SLICES

| Addition | Lactate produced µmoles per g initial wet wt (20-80 min) | | |
|---|---|---|---|
| | 2 day old | 7 day old | 14 day old |
| None | 25.0 ± 1.5 | 21.2 ± 0.45 | 25.4 ± 3.1 |
| 1µM ouabain | 38.4 ± 4.4 | 30.8 ± 1.7 | 31.7 ± 1.9 |
| 10µM ouabain | 41.1 ± 1.8 | 38.8 ± 5.8 | 64.7 ± 2.3 |
| 100µM ouabain | 32.6 ± 1.4 | 46.1 ± 8.5 | 87.0 ± 8.0 |
| 10µM ouabain + 1mM $Ca^{++}$ | 36.1 ± 1.9 | 29.4 ± 1.3 | 39.7 ± 3.6 |
| 10µM ouabain + 2mM $Ca^{++}$ | 36.0 ± 3.1 | 35.3 ± 6.2 | 46.2 ± 7.1 |
| 10µM ouabain + 4mM $Ca^{++}$ | 40.1 ± 2.3 | 35.7 ± 3.0 | 46.0 ± 2.7 |

Cerebral cortex slices were incubated in a $Ca^{++}$ free medium containing 20mM glucose. Additions were made at zero time and lactate production was measured manometrically as given in the materials and methods.

## 6.3 EFFECTS OF OUABAIN AND TETRODOTOXIN ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES AT DIFFERENT CONCENTRATIONS OF $Na^+$ IN THE INCUBATION MEDIUM

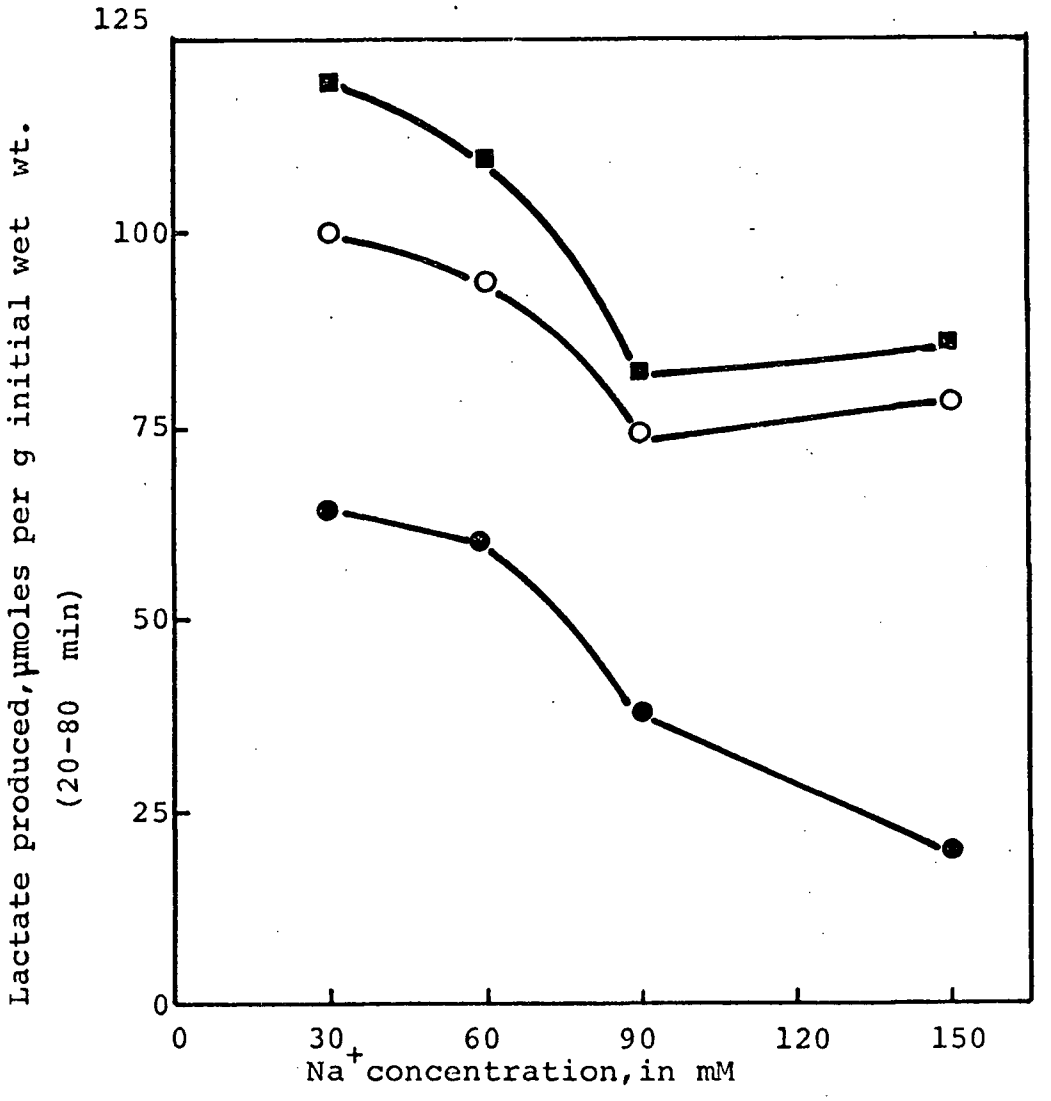
Sodium ions are known to influence the activity of pyruvate kinase. As shown in Chapter 3.7, an increase in the concentration of $Na^+$ decreases the rate of anaerobic glycolysis of cerebral cortex slices. As ouabain induces an influx of $Na^+$ into cerebral cortex slices, investigations were made of the effects of ouabain at different concentrations of $Na^+$ (30-150 mM) on rates of cerebral anaerobic glycolysis. The results of these investigations as well as of those carried out with TTX are shown in Figure 32. It is evident that in the presence of ouabain, or of TTX, at low concentrations of $Na^+$, the rates of anaerobic glycolysis attained are higher than those obtained with normal $Na^+$ concentrations (See Chapter 8).

## 6.4 EFFECTS OF OUABAIN ON ANAEROBIC GLYCOLYSIS OF ACETONE POWDER OF BRAIN

Results of experiments carried out on the effects of ouabain on the anaerobic glycolysis of brain acetone powder extract are shown in Figure 17. These results show that, as for TTX, ouabain is also not effective in increasing the rate of anaerobic glycolysis of brain acetone powder extracts. Hence the effect of ouabain seems to require the integrity of the cell.

FIGURE 32

EFFECTS OF OUABAIN AND TETRODOTOXIN ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES AT VARYING CONCENTRATIONS OF SODIUM




Incubation medium contained 5 mM $K^+$, 20 mM glucose and varying concentrations of $Na^+$. 29 mM $Na^+$ was present as $HCO_3^-$. When $Na^+$ concentration was below 149 mM, sucrose was used to replace $Na^+$. Additions were made at zero time and lactate production was measure manometrically as given in the materials and methods. (●) control; (■) 2 µM TTX and (○) 10 µM ouabain.

## 6.5 EFFECTS OF OUABAIN IN THE PRESENCE OF L-GLUTAMATE, CITRATE, AMP AND $NH_4^+$ ON THE ANAEROBIC GLYCOLYSIS OF THE CEREBRAL CORTEX SLICES

We have noted earlier, in Chapters 4 and 5, that the rate of TTX stimulated glycolysis is suppressed in the presence of $NH_4^+$, citrate or of glutamate. Experiments were, therefore, carried out to observe whether in the presence of these substances ouabain is effective in increasing the anaerobic glycolysis. Results of these experiments (Table 28) indicate that citrate has a strong inhibitory effect on the ouabain stimulated glycolysis and this is explained by the inhibition of phosphofructokinase by citrate while glutamate is less inhibitory. The inhibitory effect of glutamate may be due to the lowering of ATP concentration under our experimental conditions. AMP and $NH^+_4$ have little or no inhibitory effect. This is in contrast to that obtained with TTX, which has very little stimulating action in the presence of $NH^+_4$. In the presence of ouabain the $Na^+$ influx and $K^+$ efflux is already high, and thus under these conditions $NH^+_4$ has no effect on the glycolysis (See Chapter 4.9).

## 6.6 EFFECTS OF OUABAIN ON THE AEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

The effects of 10 μM ouabain on the aerobic glycolysis of rat cerebral cortex slices incubated in a $Ca^{++}$-free as well as in a Krebs-Ringer bicarbonate medium was investigated (Figure 19). It is evident that at 10 μM concentration,

TABLE 28

EFFECTS OF OUABAIN IN THE PRESENCE OF GLUTAMATE, CITRATE, $NH_4^+$ AND AMP ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES

| Additions | Lactate produced µmoles per g initial wet wt (20-80 min) | |
|---|---|---|
| | $Ca^{++}$ free medium | Krebs-Ringer bicarbonate medium |
| None | 31.7 ± 4.3 | 38.0 ± 4.4 |
| 10µM ouabain | 95.9 ± 3.6 | 58.5 ± 6.3 |
| 10µM ouabain + 5mM $NH_4^+$ | 99.1 ± 3.1 | - |
| 5mM L-Glutamate | 20.9 ± 2.3 | 28.6 ± 3.7 |
| 10µM ouabain + 5mM L-Glutamate | 63.8 ± 3.0 | 41.7 ± 4.5 |
| 5mM D-Glutamate | - | 34.4 ± 3.8 |
| 10µM ouabain + 5mM D-Glutamate | - | 56.7 ± 2.7 |
| 100µM ouabain | 102.6 ± 3.0 | - |
| 100µM ouabain + 5mM L-Glutamate | 82.6 ± 1.8 | - |
| 5mM citrate | 13.8 ± 2.3 | - |
| 15mM citrate | 12.5 ± 0.9 | - |
| 10µM ouabain + 5mM citrate | 52.2 ± 3.1 | - |
| 10µM ouabain +15mM citrate | 22.3 ± 4.0 | - |
| AMP 2mM | 15.5 ± 5.0 | - |
| 10µM ouabain + AMP 2mM | 83.9 ±16.5 | - |

Incubation conditions were same as in Table 27.

ouabain has little or no effect on the rate of aerobic glycolysis. In a $Ca^{++}$-free medium, however, aerobic glycolysis is slightly suppressed by TTX which is in contrast to the effects of ouabain (See Chapter 8).

## 6.7 EFFECTS OF OUABAIN ON THE ATP CONTENTS OF CEREBRAL CORTEX SLICES

Measurement of ATP contents show that under anaerobic conditions there is in fact an increase in its concentration (Table 15) in the presence of ouabain.

As the ATP content of the tissue is very much reduced under anoxia, the tissue concentration of ATP may become rate limiting for the phosphorylation of glucose and fructose 6- phosphate. It may thus exert a rate limiting effect on the speed of anaerobic glycolysis. As a considerable amount of ATP is consumed by $Na^{+}$, $K^{+}$-ATPase, the effect of ouabain on the anaerobic glycolysis may be the result of the increased concentration of ATP due to its decreased utilization. However, the possiblility cannot be ruled out that increase in the ATP content may be due to a greater rate of anaerobic glycolysis in the presence of ouabain. This will be discussed further in Chapter 8.

## 6.8 EFFECTS OF ADDITION OF OUABAIN AFTER VARIOUS PERIODS OF ANAEROBIOSIS ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES

It has been shown earlier that addition of TTX, 10 min or

more after the onset of anoxia, has no stimulating action on the rate of anaerobic glycolysis. This effect was attributed to the loss of $K^+$ from the brain cortex slices during the first few min of anoxia. Investigations were therefore made on the effects of the addition of ouabain, at various time intervals of anoxia, in order to compare its action with TTX. These results show that ouabain (Figure 33) has progressively less effect on the rate of anaerobic glycolysis if it is added to the incubation medium after the onset of anoxia. This shows that changes occurring in the cerebral cortex slices during the early period of anoxia are important for the enhancing effect of ouabain on the anaerobic glycolysis.

## 6.9 EFFECTS OF ADDITION OF OUABAIN AND TETRODOTOXIN TOGETHER ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

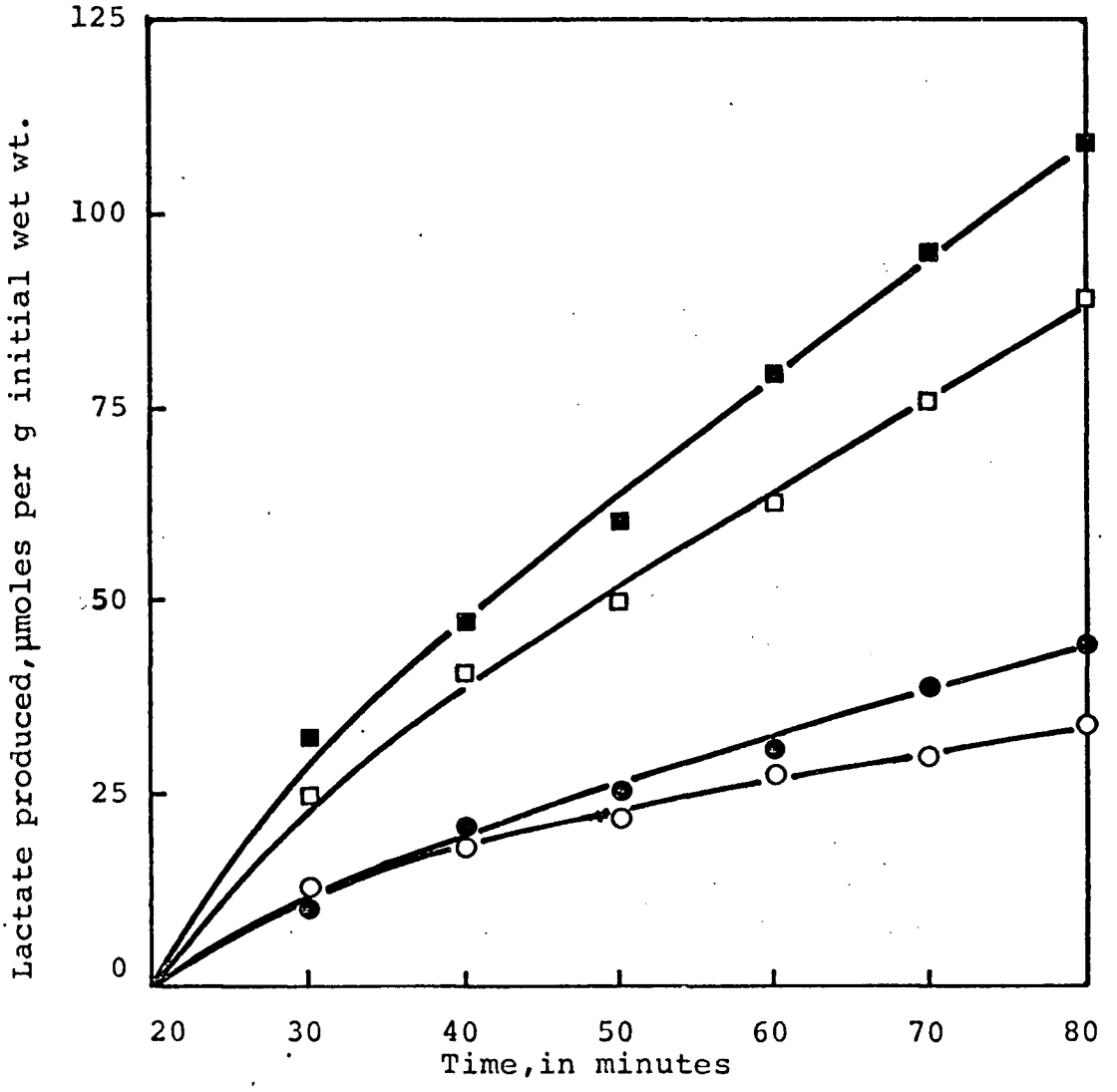
Since both ouabain and TTX were found to increase the rate of anaerobic glycolysis in a $Ca^{++}$-free medium, the combined effects of these drugs were studied. These results (Figure 34) show that, together, these drugs increase the rate of glycolysis to a value higher than that obtained when either drug is present alone.

## 6.10 EFFECTS OF OUABAIN ON THE $Na^+$ and $K^+$ CONTENTS OF RAT CEREBRAL CORTEX SLICES UNDER ANOXIA

It has been shown that in the presence of TTX, there is

FIGURE 33

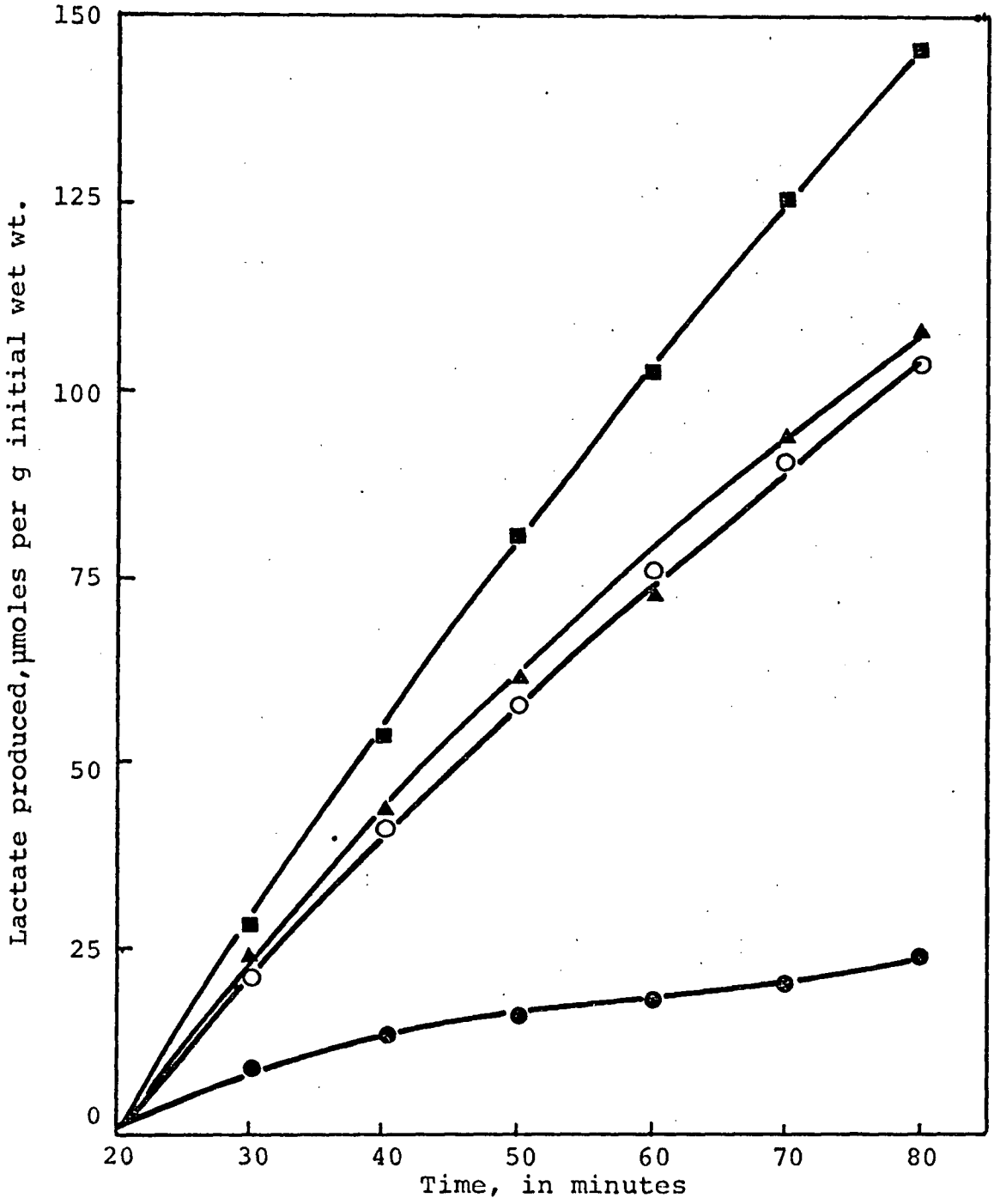
EFFECT OF ADDITION OF OUABAIN AFTER VARYING TIME PERIODS OF ANOXIA ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES




Incubations were carried out in a $Ca^{++}$-free medium containing 20 mM glucose. Ouabain was added after varying periods of anaerobiosis so as to give a final concentration of 10 µM. Lactate production was measured manometrically as given in the materials and methods. Ouabain added at (■) 0 time, (□) 5 min or (●) 10 min after onset of anoxia. (○) control.

FIGURE 34

EFFECTS OF OUABAIN AND TETRODOTOXIN TOGETHER ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES




Incubation conditions were same as in Figure 22. (●) control; (○) 2 µM TTX; (▲) 10 µM ouabain; (■) 2 µM TTX and 10 µM ouabain.

an increase in $K^+$ but a decrease in $Na^+$ content of the cerebral cortex slices under anoxia. Ouabain is not known to diminish either the efflux of $K^+$ or the influx of $Na^+$; it has in fact, just the opposite effects since $Na^+$, $K^+$-ATPase is blocked by ouabain. Studies were, therefore, made of the cation contents of cerebral cortex slices under anoxia in the presence of ouabain.

The results shown in Figure 35 indicate that, in the presence of ouabain under anoxia, there is no increased retention of $K^+$ and there is either no effect, or only a slight increase in the $Na^+$ content. On the other hand, when ouabain and TTX are present together, there is an increase in $K^+$ and a slight drop in the $Na^+$ content. Under anoxia, the influx of $Na^+$ in cerebral tissue is very large, and any effect on $Na^{22}$-influx could not be seen (Table 18).

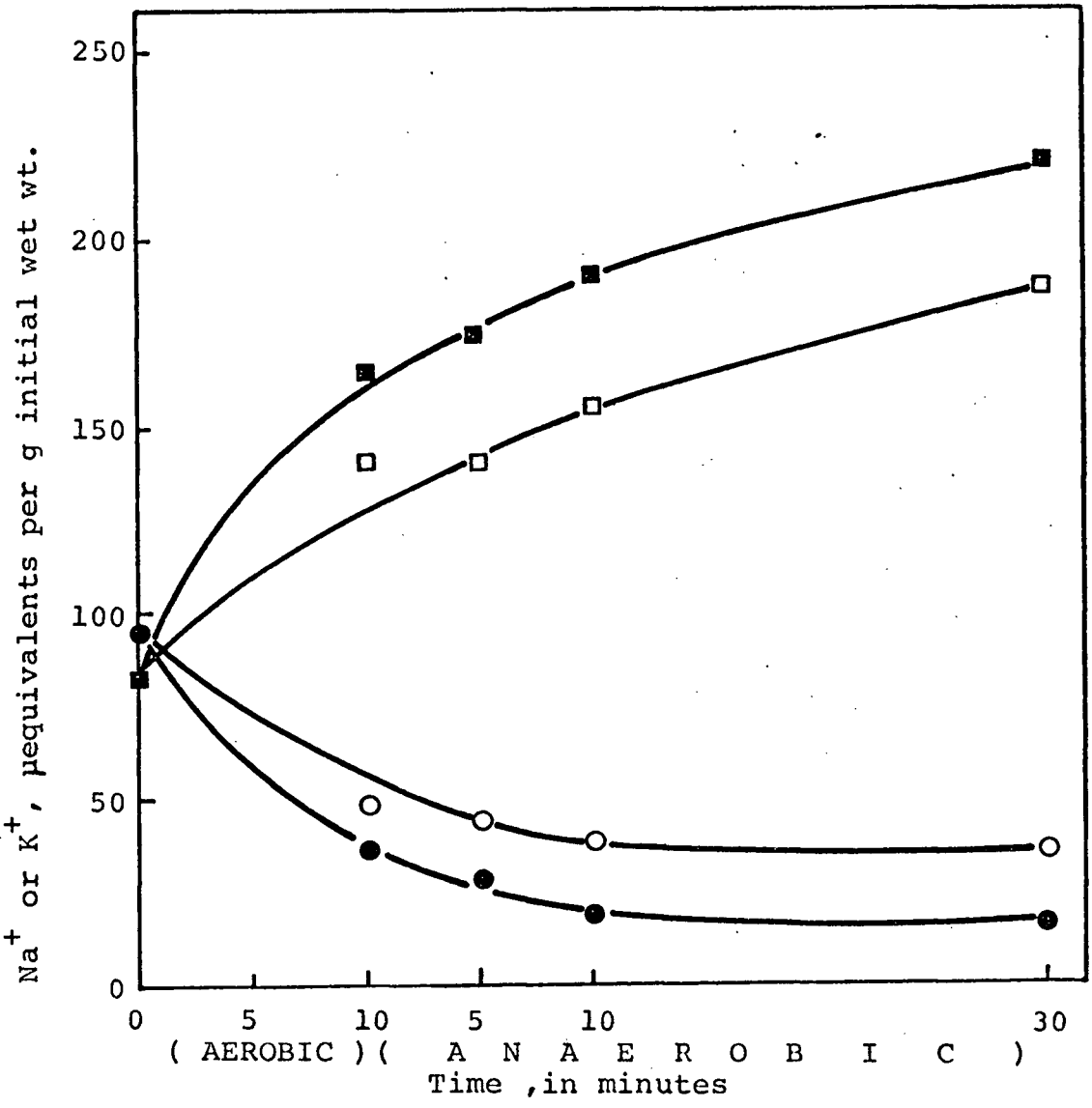
It is evident from these experiments that the effect of ouabain on anaerobic glycolysis is not mediated through its action on the $Na^+$ and $K^+$ contents of the brain cells. In this respect, its mode of action differs from that of TTX.

## 6.11 EFFECTS OF OUABAIN, $Ca^{++}$ AND $NAD^+$ ON THE MICROSOMAL $Na^+$, $K^+$-ATPase

Results reported in Section 6.1 show that, in the presence of $Ca^{++}$, ouabain is not very effective in increasing the rate of anaerobic glycolysis of cerebral cortex slices. Furthermore, ouabain and $Ca^{++}$ seem to

FIGURE 35

EFFECTS OF OUABAIN AND TETRODOTOXIN AND OUABAIN TOGETHER ON THE SODIUM AND POTASSIUM CONCENTRATIONS OF RAT CEREBRAL CORTEX SLICES




Incubation conditions were same as in Figure 26. Additions were made at zero time. (■) $Na^+$, with 10 µM ouabain; (□) $Na^+$, with 10 µM ouabain and 2 µM TTX; (●) $K^+$, with 10 µM ouabain; (○) $K^+$, with 10 µM ouabain and 2 µM TTX. Controls are same as in Figure 26.

antagonize each other. It is well established that the major effect of ouabain is the inhibition of $Na^+$, $K^+$-ATPase (see Chapter 1.5). Under anaerobic conditions, as already pointed out (Section 6.7), the ATP concentration falls and it may become rate limiting for anaerobic glycolysis. It was further suggested that ouabain might act by decreasing the rate of utilization of ATP by $Na^+$, $K^+$-ATPase.

Our results on the antagonism between ouabain and $Ca^{++}$ would be explained if, in the presence of $Ca^{++}$, the inhibitory effect of ouabain on the membrane bound $Na^+$, $K^+$-ATPase is diminished. While studying the ouabain induced changes in the $Na^+$ and $K^+$ content, as well as on the respiration of the cerebral cortex slices, Swanson[131, 173] came to a similar conclusion and stated that in the presence of $Ca^{++}$, all the $Na^+$, $K^+$-ATPase of the membrane is not accessible to ouabain.

In view of these considerations, it was decided to study the effects of $Ca^{++}$ and ouabain when present together on the activity of $Na^+$, $K^+$-ATPase. The results obtained indicate that, under our experimental conditions in brain microsomal preparations, the effects of ouabain and $Ca^{++}$ are not antagonistic (Table 29). This was true even when microsomal prep-

TABLE 29

EFFECTS OF OUABAIN, $Ca^{++}$ AND $NAD^{+}$ ON THE MICROSOMAL $Na^{+}$, $K^{+}$-ATPase

| Additions | Activity of $Na^{+}$, $K^{+}$-ATPase µmoles Pi per mg protein per hour |
|---|---|
| Experiment 1 | |
| None | 76.8 |
| 1mM $Ca^{++}$ | 31.9 |
| 2mM $Ca^{++}$ | 30.8 |
| 4mM $Ca^{++}$ | 27.5 |
| 10µM ouabain | 49.6 |
| 10µM ouabain + 1mM $Ca^{++}$ | 28.6 |
| 10µM ouabain + 2mM $Ca^{++}$ | 26.7 |
| 10µM ouabain + 4mM $Ca^{++}$ | 25.9 |
| Experiment 2 | |
| None | 57.6 |
| 100µM ouabain | 30.1 |
| 0.2mM $NAD^{+}$ | 60.4 |
| 0.4mM $NAD^{+}$ | 72.5 |
| 0.6mM $NAD^{+}$ | 72.5 |
| 0.8mM $NAD^{+}$ | 63.2 |

Microsomal preparations were prepared and ATPase activity assayed as given in the materials and methods. Additions were made before the addition of the enzyme to the incubation mixture. Each value represent averages of duplicate determination.

arations are preincubated in the presence of either ouabain or $Ca^{++}$.

Other results given in Table 29 show that $NAD^{+}$ does not inhibit the $Na^{+}$, $K^{+}$-ATPase activity. This aspect will be discussed further in Chapter 8.

6.12 EFFECTS OF OUABAIN ON AMINO ACID EFFLUXES FROM THE CEREBRAL CORTEX SLICES UNDER ANOXIA

TTX blocks the efflux of amino acids from the cerebral cortex slices under anoxia (Chapter 4.4). Experiments were, therefore, carried out to see if ouabain also affects the efflux of amino acids from the incubated slices. Results of these experiments are shown in Table 30. It is evident that in the presence of ouabain, considerable amounts of amino acids are released from the incubated brain tissue into the incubation medium. However, the efflux of amino acids in the presence of ouabain is considerably reduced by the presence of TTX.

The above findings indicate that the inhibition of amino acid efflux by TTX cannot be due to an increase in the rate of anaerobic glycolysis. TTX presumably acts directly on the brain cell membrane by abolishing the generation of the action potentials which results in increased release of tissue amino acids.

6.13 EFFECTS OF PROCAINE AND LIDOCAINE ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES

It has been pointed out earlier (Chapter 1.3) that in many respects, the mode of action of local anesthetics are similar

TABLE 30

EFFECTS OF OUABAIN AND OUABAIN + TETRODOTOXIN TOGETHER ON THE AMINO ACID EFFLUX FROM RAT CEREBRAL CORTEX SLICES UNDER ANOXIA

| Amino Acids | Additions | -Glucose +ouabain | +Glucose +ouabain | -Glucose +ouabain +TTX | +Glucose +ouabain +TTX |
|---|---|---|---|---|---|
| TAURINE | Tissue | 2.00±0.01 | 2.47±0.25 | 1.58±0.36 | 2.11±0.11 |
| | Medium | 3.55±0.11 | 3.30±0.08 | 3.68±0.34 | 3.00±0.03 |
| ASPARTIC ACID | Tissue | 0.94±0.08 | 0.98±0.19 | 0.86±0.22 | 1.53±0.13 |
| | Medium | 1.88±0.36 | 1.65±0.07 | 2.18±0.27 | 0.97±0.15 |
| GLUTAMINE + SERINE | Tissue | 0.70±0.06 | 1.03±0.11 | 0.42±0.07 | 1.49±0.28 |
| | Medium | 3.37±0.13 | 2.80±0.08 | 3.54±0.37 | 2.74±0.18 |
| GLUTAMIC ACID | Tissue | 3.82±0.40 | 5.41±1.05 | 3.10±0.46 | 8.24±0.61 |
| | Medium | 8.2 ±0.40 | 6.95±0.10 | 8.05±0.60 | 3.59±0.15 |
| GLYCINE | Tissue | 0.23±0.04 | 0.37±0.05 | - | 0.45±0.03 |
| | Medium | 0.93±0.21 | 0.85±0.05 | 1.08±0.08 | 0.47±0.04 |
| ALANINE | Tissue | 0.17±0.02 | 0.25±0.05 | - | 0.48±0.04 |
| | Medium | 1.28±0.21 | 0.88±0.03 | 1.05±0.16 | 0.56±0.02 |

Incubation conditions were same as in Table 4. For controls without any drug, see Table 4. Concentration of ouabain was 10μM and of TTX was 2μM.

to that of TTX. These drugs block the generation of action potential and in doing so they affect the movements of $Na^+$ and $K^+$. Experiments were, therefore, carried out to see if local anesthetics affect the rate of anaerobic glycolysis of cerebral cortex slices in a manner similar to that of TTX.

The results given in Table 31 show that, in the presence of local anesthetics procaine or lidocaine, the rate of anaerobic glycolysis of rat cerebral cortex slices is increased. However, a higher concentration of local anesthetic is required to obtain the same stimulation as that obtained with TTX. The presence of $Ca^{++}$ diminishes the stimulatory effects of local anesthetics, as in the case of TTX.

Additional similarities between TTX and local anesthetics may be seen when the latter are added to the incubation medium after 15 min of anoxia. Under these conditions, these drugs have no effect on the rate of anaerobic glycolysis of the cerebral cortex slices.

## 6.14 EFFECTS OF LIDOCAINE ON THE ANAEROBIC GLYCOLYSIS OF INFANT RAT AND GUINEA PIG CEREBRAL CORTEX SLICES

Further experiments were carried out to observe whether the local anesthetic lidocaine will increase the anaerobic glycolysis of brain slices obtained from infant animals (Figure 36). It will be seen that lidocaine has no effect on the anaerobic glycolysis of 2-day old rat brain slices but it markedly increases the rate of anaerobic glycolysis of newly born guinea pig cerebral cortex slices. As with the results found with TTX, this must be related to the maturity of the

TABLE 31

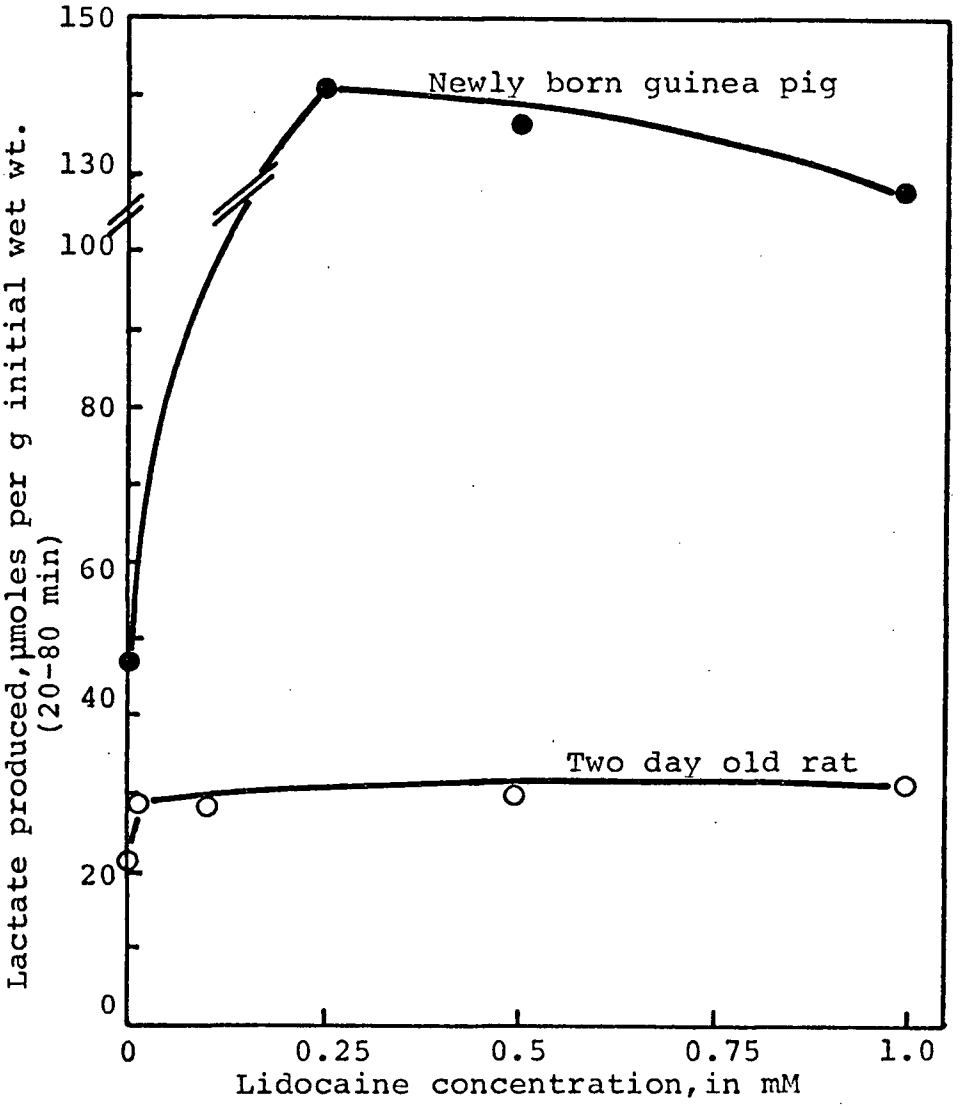
EFFECTS OF PROCAINE AND LIDOCAINE ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES

| Additions | Lactate produced µmoles per g initial wet wt (20-80 min) | |
|---|---|---|
| | $Ca^{++}$ free medium | Krebs-Ringer bicarbonate medium |
| None | 24.6 ± 1.4 | 42.4 ± 6.7 |
| 0.01mM Procaine | 40.6 ± 6.8 | - |
| 0.01mM Lidocaine | 45.6 ± 2.0 | - |
| 0.1 mM Procaine | 66.0 ± 1.0 | 58.0 ± 8.9 |
| 0.1 mM Lidocaine | 93.7 ±17.0 | 70.5 ± 3.1 |

All vessels contained 20mM glucose. Additions were made at zero time and lactate production was measured manometrically, as given in the materials and methods.

FIGURE 36

EFFECT OF LIDOCAINE ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES




Cerebral cortex slices were incubated in a $Ca^{++}$-free medium containing 20 mM glucose. Additions were made at zero time and lactate production was measured manometrically as given in the materials and methods.

brain tissue.

6.15 EFFECTS OF LIDOCAINE ON THE $Na^+$ AND $K^+$ CONTENTS OF CEREBRAL CORTEX SLICES

Since the action of local anesthetics on anaerobic glycolysis is similar to that of TTX and since in the presence of the latter there is an increase in the $K^+/Na^+$ ratio, it was thought desirable to observe the effects of local anesthetics on the $Na^+$ and $K^+$ contents of incubated cerebral cortex slices. A relatively high concentration (0.5 mM) of lidocaine was used in these experiments (See Table 32). It is evident that there is an increase in $K^+/Na^+$ ratio of brain slices in the presence of lidocaine. The results with adult rat cerebral cortex slices are to be expected as the local anesthetics block the generation of action potentials that develop on the onset of anoxia. However, the anaerobic glycolysis of 2-day old rat is not affected by lidocaine but in its presence there is still an increase in tissue $K^+/Na^+$ ratio. Possibly, this is an unspecific effect of the high concentration of local anesthetic, or in infant rat brain slices the rate limiting step in glycolysis is not $K^+$-dependent, as appears to be the case in the brains of mature animals. This will be further discussed in Chapter 8.

SUMMARY OF CHAPTER 6

1. Ouabain, at low concentrations (1-100 μM) greatly increases the rate of anaerobic glycolysis of both rat and guinea pig cerebral cortex slices in a $Ca^{++}$-free medium.

TABLE 32

EFFECTS OF LIDOCAINE ON THE SODIUM AND POTASSIUM CONTENTS OF CEREBRAL CORTEX SLICES

| Age of Rats | Cation | Aerobic | Anaerobic | | |
|---|---|---|---|---|---|
| | | 10 min | 5 min | 10 min | 30 min |
| 2-day old | $Na^+$ | 100 | 111 | 120 | 142 |
| | $K^+$ | 56 | 57 | 49 | 43 |
| Adult | $Na^+$ | 127 | 130 | 132 | 155 |
| | $K^+$ | 54 | 48 | 40 | 35 |

Incubation conditions were same as in Figure 26. Each value represent averages of two experiments within ± 7%. For controls see Figure 26 and 28. Results are expressed as μequivalents per g initial wet wt. 0.5mM lidocaine was present from the start of the experiment.

2. Ouabain has very little stimulating effect on the anaerobic glycolysis of cerebral cortex slices incubated in a Krebs-Ringer bicarbonate medium. Moreover, when $Ca^{++}$ is added to a $Ca^{++}$-free medium, the enhancing effect of ouabain on the anaerobic glycolysis of cerebral cortex slices is considerably reduced. Thus, $Ca^{++}$ and ouabain are antagonistic in their effects on the cerebral anaerobic glycolysis.

3. Ouabain has little or no effect on the anaerobic glycolysis of 2-day old rat brain slices in a $Ca^{++}$-free medium. Its effectiveness in enhancing the anaerobic glycolysis of infant rat brain increases considerably at about the second week.

4. Both ouabain and TTX significantly increase the rate of anaerobic glycolysis, even when most of the $Na^{+}$ is replaced by sucrose. The degree of stimulation decreases with increasing $Na^{+}$ concentration.

5. Ouabain has no effect on the anaerobic glycolysis of acetone powder extracts.

6. Citrate (15 mM) inhibits the ouabain stimulated glycolysis while glutamate has some inhibitory effect, possibly due to lowering of ATP. AMP and $NH_4^{+}$ have little or no effect on the stimulation of anaerobic glycolysis by ouabain.

7. Ouabain (10 μM) has little or no effect on the aerobic glycolysis of incubated brain slices.

8. In the presence of ouabain in a $Ca^{++}$-free medium, under anoxia, the cell content of ATP is increased.

9. If ouabain is added to the incubation medium after increasing periods of anoxia, it has progressively lesser effect on the rate of anaerobic glycolysis in a $Ca^{++}$-free medium.

10. When ouabain and TTX are both present in a $Ca^{++}$-free medium, the rate of anaerobic glycolysis is higher than that obtained when either drug is present alone.

11. Ouabain has no effect on the $K^+$ or $Na^+$ content of the cerebral cortex slices in a $Ca^{++}$-free medium under anoxia. TTX, however, reduces the $K^+$ loss and the $Na^+$ gain by the tissue in presence of ouabain in a $Ca^{++}$-free medium. Thus TTX acts independently of ouabain.

12. Ouabain and $Ca^{++}$ both inhibit the $Na^+$, $K^+$-ATPase in microsomal preparations. When both are present together, no antagonism on the inhibition of $Na^+$, $K^+$-ATPase is observed.

$NAD^+$ does not inhibit the $Na^+$, $K^+$-ATPase.

13. Ouabain does not affect the efflux of amino acids from the cerebral cortex slices under anoxia. However, TTX still blocks the efflux of amino acids in the presence of ouabain, indicating that the the increased amino acid content of the cerebral cortex slices in the presence of TTX is not due to greater operation of the $Na^+$-pump, as a result of an increased rate of anaerobic glycolysis.

TTX is presumably acting by blocking the generation of action potentials induced by anoxia, that result in an increased efflux of amino acids.

14. Procaine and lidocaine act like TTX in increasing the rate of anaerobic glycolysis of cerebral cortex slices from mature brain. In the presence of 0.5 mM lidocaine, the cellular ratio of $K^+/Na^+$ is increased. Thus the local anesthetics resemble TTX in their metabolic effects on brain during anoxia.

CHAPTER 7

# EFFECTS OF VARIOUS NEUROTROPIC DRUGS ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

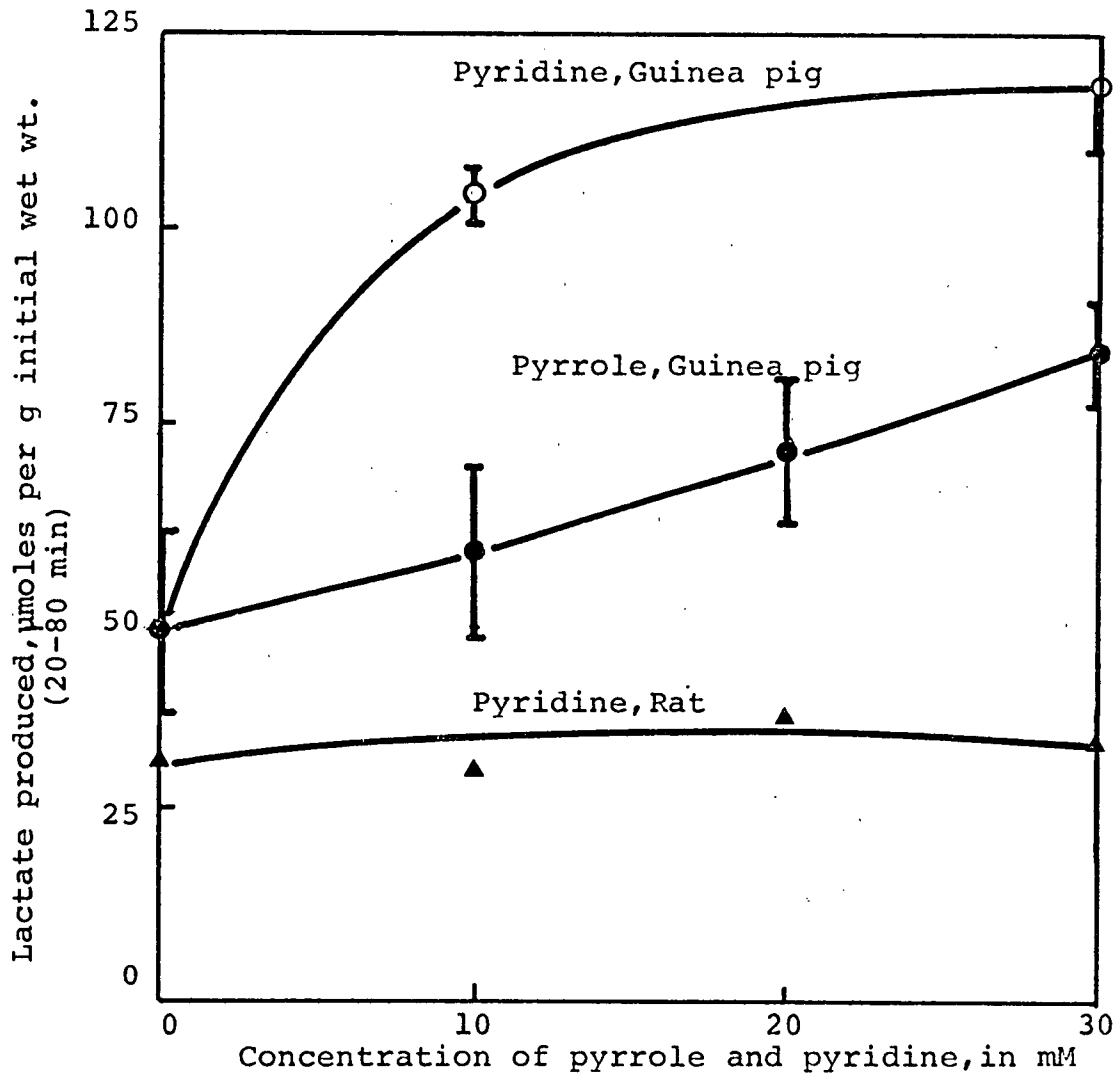
It was pointed out in Chapter 1 that $Ca^{++}$ and a number of bases, such as pyrrole and pyridine, greatly enhance the rate of anaerobic glycolysis of guinea pig cerebral cortex slices (Adams and Quastel[71]). The findings of Adams and Quastel on the effects of $Ca^{++}$ on anaerobic glycolysis, were confirmed and further extended to infant cerebral tissues (Chapter 3). In addition, effects of various drugs such as TTX, local anesthetics and ouabain were reported (Chapters 4-6). It became clear from these studies that TTX and local anesthetics may act on anaerobic glycolysis by affecting the $K^+/Na^+$ ratio in the brain cells. In view of these results, it was decided to study the effects of various organic bases on cerebral anaerobic glycolysis, to see whether their action too is mediated through changes in the cation concentrations in the brain cell. In addition, effects of other drugs such as barbiturates, reserpine and amphetamine as well as biogenic amines were also studied. The results of these experiments are described below.

## 7.1 ACTION OF PYRROLE AND PYRIDINE ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

The effects of some organic bases on the anaerobic glycolysis of guinea pig and rat cerebral slices are shown in Figure 37. It is seen that the rate of anaerobic glycolysis

FIGURE 37

EFFECTS OF PYRROLE AND PYRIDINE ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES




Incubations were carried out in a $Ca^{++}$-free medium containing 20 mM glucose. Additions were made at zero time and lactate production was measured manometrically as given in the materials and methods.

of guinea pig cerebral cortex slices is enhanced in the presence of pyrrole and pyridine, confirming the findings of Adams and Quastel[71]. It is also evident that pyridine has no effect on the anaerobic glycolysis of rat cerebral cortex slices in contrast to its effect on that of guinea pig. In general, it appears that rat cerebral cortex slices are less responsive than guinea pig cerebral cortes slices to drugs, at equivalent concentrations.

The effect of 30 mM pyrrole, in the presence of 5 mM L-glutamate, D-glutamate or L-aspartate, is shown in Table 33. It is evident from these results that stimulation of glycolysis by pyrrole is similar to that of TTX in showing response to different amino acids. Thus, in the presence of 5 mM L-glutamate, 30 mM pyrrole has little or no stimulatory effect on the anaerobic glycolysis. D-glutamate is partially effective while L-aspartate has no effect in preventing the stimulation of glycolysis by pyrrole. Moreover, if pyrrole is added to the incubation medium after 15 min anaerobiosis, it has no stimulatory effect.

## 7.2 EFFECTS OF PYRROLE ON THE $Na^+$ AND $K^+$ CONTENTS OF GUINEA PIG CEREBRAL CORTEX SLICES UNDER ANOXIA

In the previous section, it has been shown that, in several respects, the action of pyrrole on anaerobic glycolysis is similar to that of TTX. As the action of TTX on anaerobic glycolysis is presumably mediated through its effect on the $Na^+$ and $K^+$ contents of cerebral cortex slices,

TABLE 33

EFFECT OF PYRROLE ON THE ANAEROBIC GLYCOLYSIS OF GUINEA PIG CEREBRAL CORTEX SLICES IN THE PRESENCE OF SOME AMINO ACIDS

| Additions | Lactate produced µmoles per g initial wet wt (20-80 min) |
|---|---|
| None | 48.4 ± 12.5 |
| 30mM Pyrrole | 94.3 ± 16.7 |
| 5mM L-Glutamate | 25.5 ± 4.0 |
| 30mM Pyrrole + 5mM L-Glutamate | 47.4 ± 5.7 |
| 5mM L-Aspartate | 51.7 ± 5.0 |
| 5mM L-Aspartate +30mM Pyrrole | 85.9 ± 3.8 |
| 5mM D-Glutamate | 31.5 ± 6.9 |
| 5mM D-Glutamate +30mM Pyrrole | 66.5 ± 1.5 |
| 30mM Pyrrole, tipped in after 15 min anoxia | 43.3 ± 1.0 |

Cerebral cortex slices were incubated in a $Ca^{++}$ free medium containing 20mM glucose. Additions were made at zero time (except one case, as shown) and lactate production was measured manometrically as given in the materials and methods.

it was desirable to study the effects of pyrrole on the cationic concentrations. Results of these experiments are given in Figure 38. It can be seen that, in the presence of 30 mM pyrrole, there is an increase in the retention of $K^+$ but a decrease in the uptake of $Na^+$. These results indicate that the action of pyrrole on anaerobic glycolysis may also be mediated through changes in the cation concentration.

## 7.3 EFFECTS OF AMYTAL AND PENTOTHAL ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

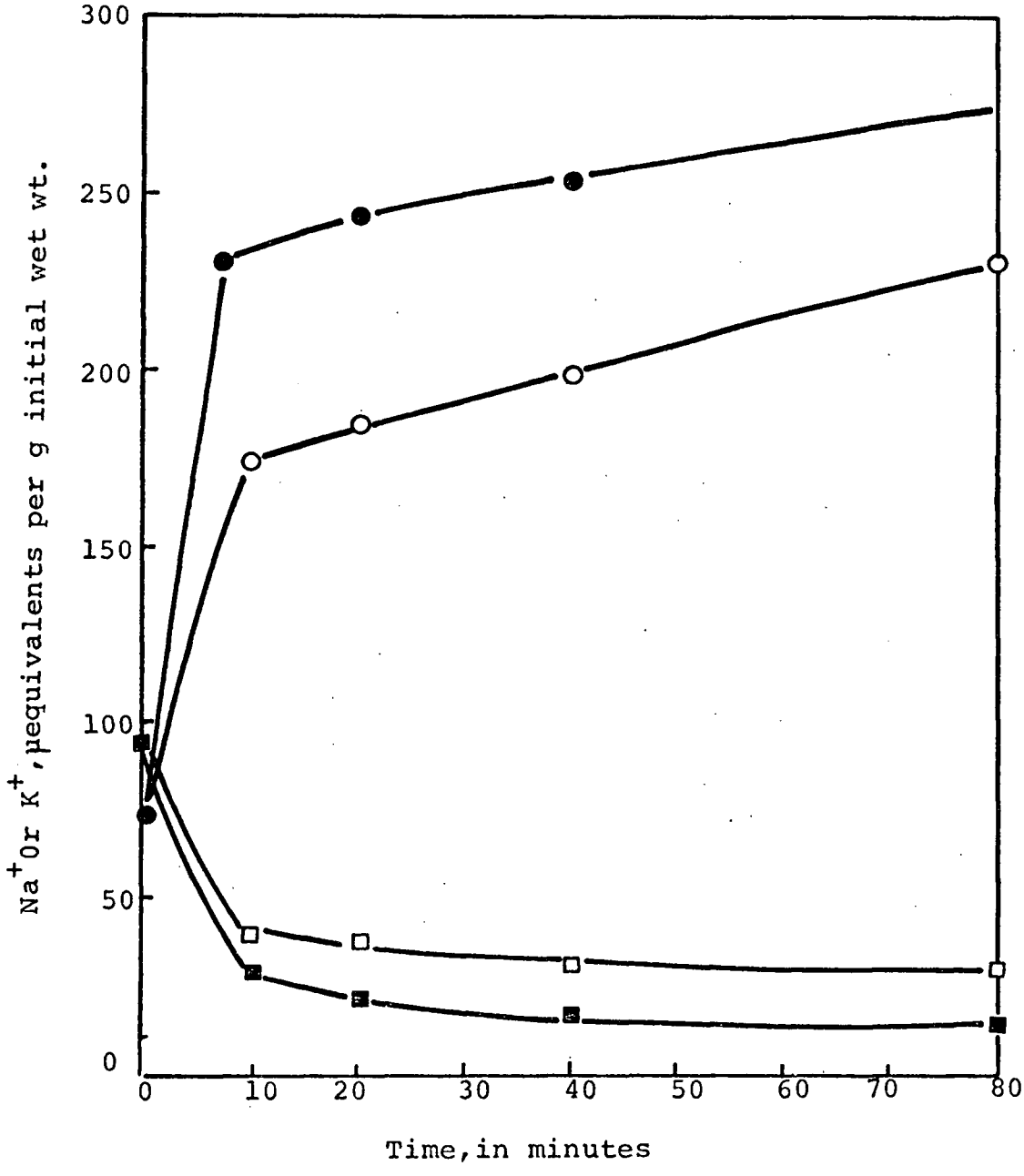
Barbiturates have long been shown to diminish the brain tissue respiration[195]. As noted, in Chapter 15 both the barbiturates (amytal and pentothal) as well as certain hypnotics, suppress the oxidation of NADH, and hence the generation of ATP in the cell[281-283]. Using suspensions of cerebral tissue, Webb and Elliott[199] showed that, amytal enhances the rate of aerobic glycolysis and suppresses the respiration. This is in agreement with present knowledge on the effects of amytal on oxidative processes. However, these workers could only observe very little effect of amytal on the anaerobic glycolysis of the cell suspensions.

The mechanism of barbiturate action on the nerve cell is still not fully understood. They are known to suppress electrical activity of excited nerve cells but so far no significant effect of the barbiturates, at anesthetic concentration, on $Na^+$ movement in the cerebral cortex slices have been observed [198].

As it was found that TTX, and local anesthetics,

FIGURE 38

EFFECT OF PYRROLE ON THE SODIUM AND POTASSIUM CONCENTRATIONS OF GUINEA PIG CEREBRAL CORTEX SLICES UNDER ANOXIA




Incubation conditions were same as in Figure 24. (●) $Na^+$, control; (○) $Na^+$, with 30 mM pyrrole; (□) $K^+$, with 30 mM pyrrole; (■) $K^+$, control.

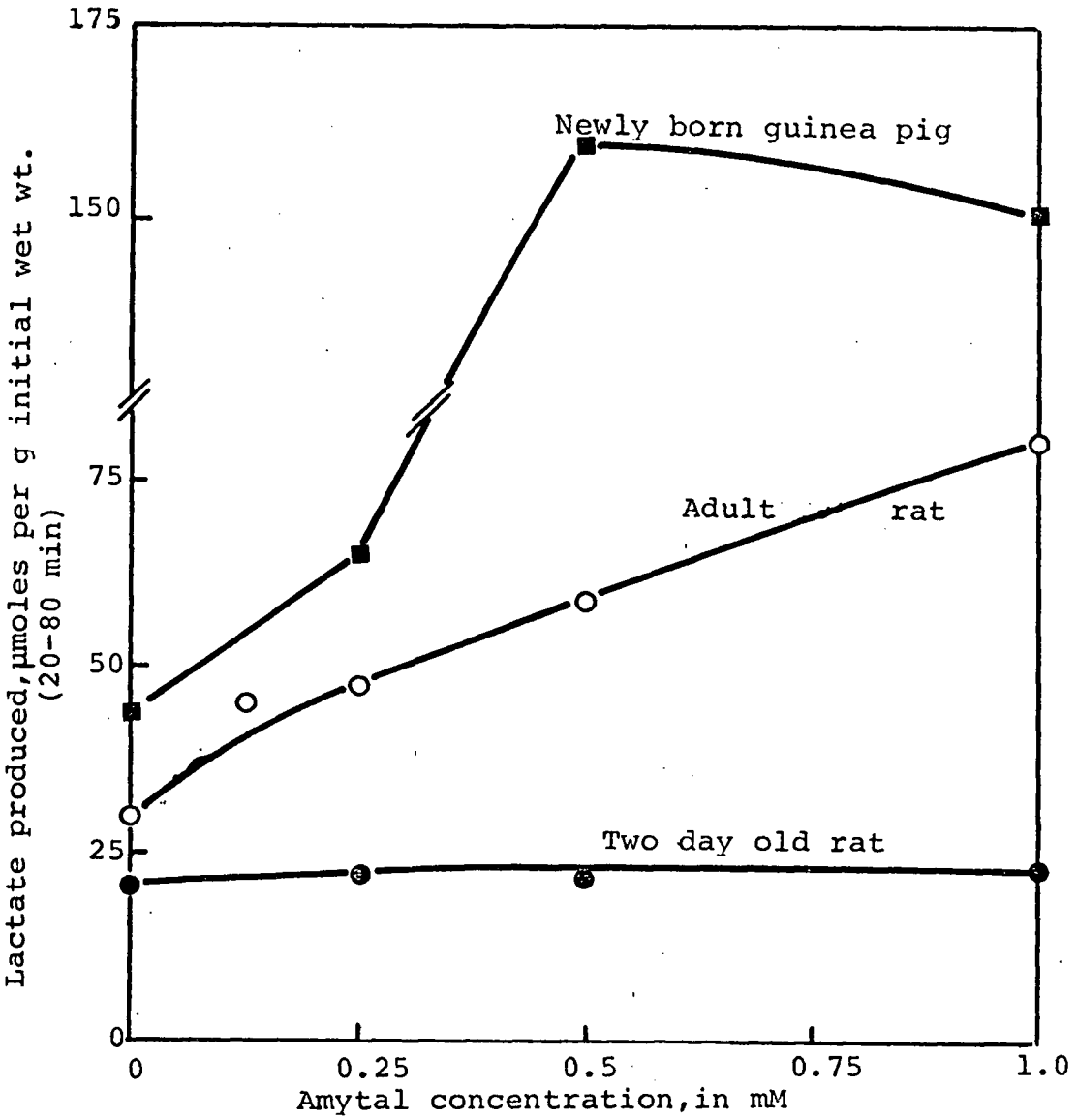
greatly increase the rate of anaerobic glycolysis of rat cerebral cortex slices, it was thought desirable to study the effects of barbiturate anesthetics on the anaerobic glycolysis of cerebral tissue. The effects of different concentrations of sodium amytal on the anaerobic glycolysis of adult rat and infant rat, as well as on infant guinea pigs are given in Figure 39. It is seen that amytal, at concentrations exceeding the anesthetic, greatly enhances the anaerobic glycolysis of cerebral cortex slices from adult rat and infant guinea pig but it has no effect on slices from infant rat brain. In adult rat brain slices, at anesthetic doses (0.25 mM) amytal has a relatively small effect but infant guinea pigs (that have mature characteristics) show very considerable sensitivity to the drug.

The presence of traces of oxygen in the $N_2$:$CO_2$ mixture used may be enough to oxidize NADH formed and thus suppress glycolysis by increasing the ATP level. Amytal under these conditions can increase the glycolytic rate by blocking the oxidation of NADH. However, this possibility is unlikely since preliminary experiments have shown that the presence of 0.5 mM azide, which blocks the respiratory chain, has no effect on the rate of anaerobic glycolysis of cerebral cortex slices. Moreover, when amytal is added 15 min after the onset of anoxia, it has no effect on the anaerobic glycolysis. This rules out the possibility that amytal might act by blocking the oxidation of NADH or any unidentified metabolite.

Other experiments showed that amytal is less effective in

FIGURE 39

EFFECT OF AMYTAL ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES




Incubation conditions were same as in Figure 37.

a Krebs-Ringer bicarbonate medium when compared with its action in a $Ca^{++}$-free medium. Effects of pentothal on anaerobic glycolysis resembles that of amytal (preliminary experiments).

From the above it appears that the effects of amytal on anaerobic glycolysis might be mediated through changes in cationic concentrations. Further experiments were carried out to examine this possibility. The results (Table 34) showed that at anesthetic doses, amytal (or pentothal) has no effect on the $K^+$ contents. However, there is some reduction in the $Na^+$ contents of the slices under our experimental conditions.

With infant guinea pigs, a slight increase in the $K^+$ contents in the presence of amytal was observed (Table 35). It appears that while anesthetic concentrations of amytal (0.25 mM) have but little effect on anaerobic glycolysis of rat brain - especially when compared with the relatively large effects due to the local anesthetics or TTX - increasing concentrations of the barbiturates do bring about ionic changes that result in increased rates of anaerobic glycolysis. These doubtless are related to the modification by barbiturates but more work is required to establish the nature of these changes.

## 7.4 EFFECTS OF CHLORPROMAZINE ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

Chlorpromazine is known to suppress the anaerobic glycolysis in the cell free extracts from brain[217]. The effects of

TABLE 34

EFFECTS OF SOME NEUROTROPIC DRUGS ON THE SODIUM AND POTASSIUM CONTENTS OF RAT CEREBRAL CORTEX SLICES

| Addition | Cation | Aerobic | Anaerobic | | |
|---|---|---|---|---|---|
| | | 10 min | 5 min | 10 min | 30 min |
| 0.25mM Amytal | $Na^+$ | 125 | 140 | 145 | 175 |
| | $K^+$ | 44 | 31 | 26 | 20 |
| 0.1mM Pentothal | $Na^+$ | 120 | 140 | 147 | 170 |
| | $K^+$ | 42 | 30 | 24 | 17 |
| 0.25mM Chlorpro-mazine | $Na^+$ | 132 | 125 | 145 | 175 |
| | $K^+$ | 45 | 31 | 25 | 16 |
| 12μM Reserpine | $Na^+$ | 115 | 140 | 135 | 147 |
| | $K^+$ | 46 | 40 | 32 | 34 |

Incubation conditions are same as in Figure 26. Each value represent averages of two experiments within ± 7%. For controls see Figure 26. Results are expressed as μequivalents per g initial wet wt.

different concentrations of chlorpromazine on the anaerobic glycolysis of adult rat cerebral cortex slices are shown in Figure 40. It is evident that chlorpromazine does not have any significant stimulating effect on the rate of anaerobic glycolysis.

Effects of chlorpromazine on the $Na^+$ and $K^+$ contents of adult rat and infant guinea pig cerebral cortex slices are given in Tables 34 and 35. Unlike the anesthetics, chlorpromazine, at the concentration tested, has no effect on the $Na^+$ and $K^+$ contents of incubated cerebral cortex slices.

## 7.5 EFFECTS OF AMPHETAMINE AND NIALAMIDE ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES

Amphetamine is one of the most potent of the central nervous system stimulating drugs. It inhibits the uptake of norepinephrine by the storage granules and releases the amines from their storage sites. As noted in Chapter 1.5, it also inhibits monoamine oxidase (MAO). Nialamide is an inhibitor of MAO. The net result of application of these drugs to the central nervous system is an increase in the amount of free biogenic amines.

The effects of both d- and l-amphetamine as well as nialamide on the anaerobic glycolysis of cerebral cortex slices was studied (Figure 41). As is evident, these drugs have relatively small or no effects on the rate of anaerobic glycolysis of cerebral cortex slices.

TABLE 35

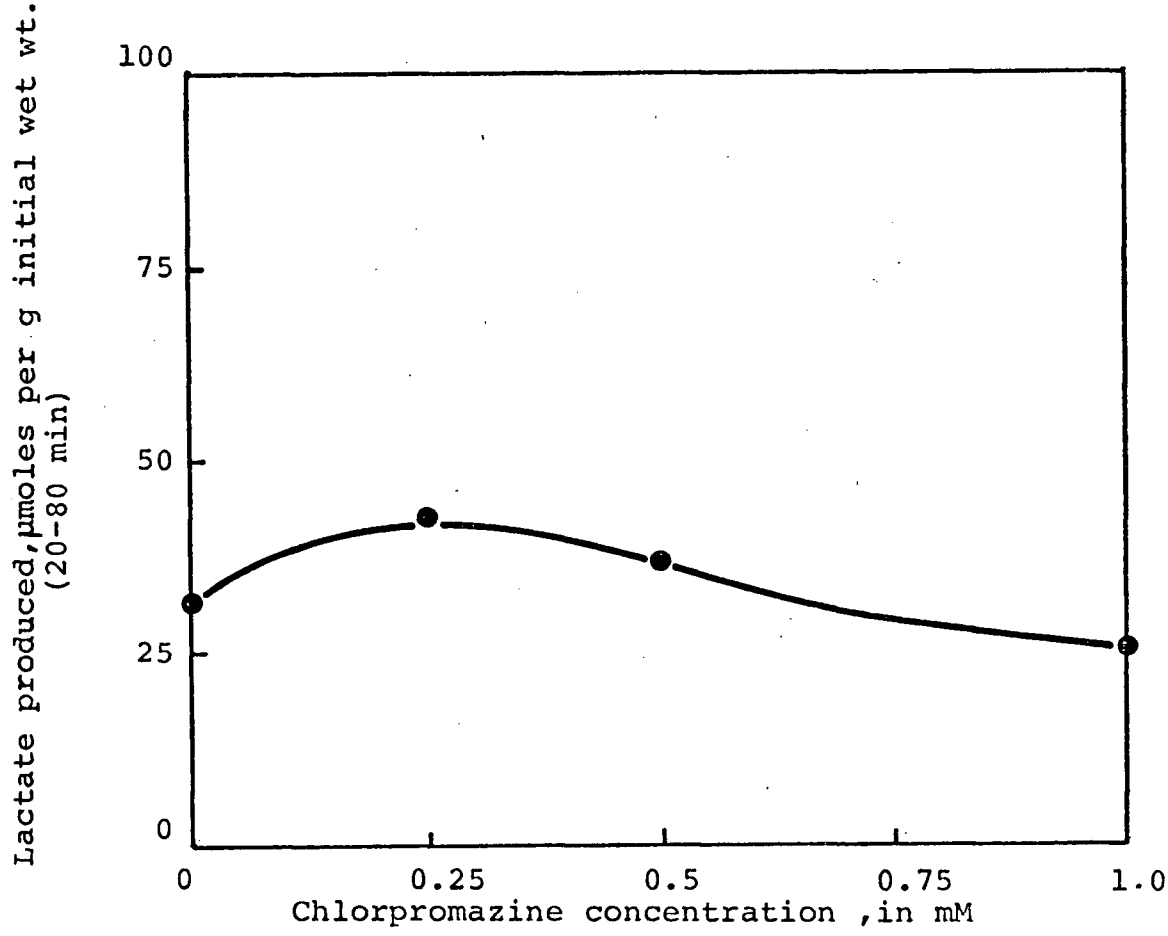
EFFECTS OF SOME NEUROTROPIC DRUGS ON THE SODIUM AND POTASSIUM CONTENTS OF NEWLY BORN GUINEA PIG CEREBRAL CORTEX SLICES

| Addition | Cation | Aerobic | Anaerobic | | |
|---|---|---|---|---|---|
| | | 10 min | 5 min | 10 min | 30 min |
| 0.25mM Amytal | $Na^+$ | 125 | 140 | 145 | 175 |
| | $K^+$ | 47 | 42 | 38 | 36 |
| 0.25mM Chlorpromazine | $Na^+$ | 127 | 145 | 167 | 175 |
| | $K^+$ | 44 | 33 | 29 | 21 |

Incubation conditions are same as in Figure 27. Each value represent averages of two experiments within ± 7%. For controls, see Figure 27. Results are expressed as μequivalents per g initial wet wt.

FIGURE 40

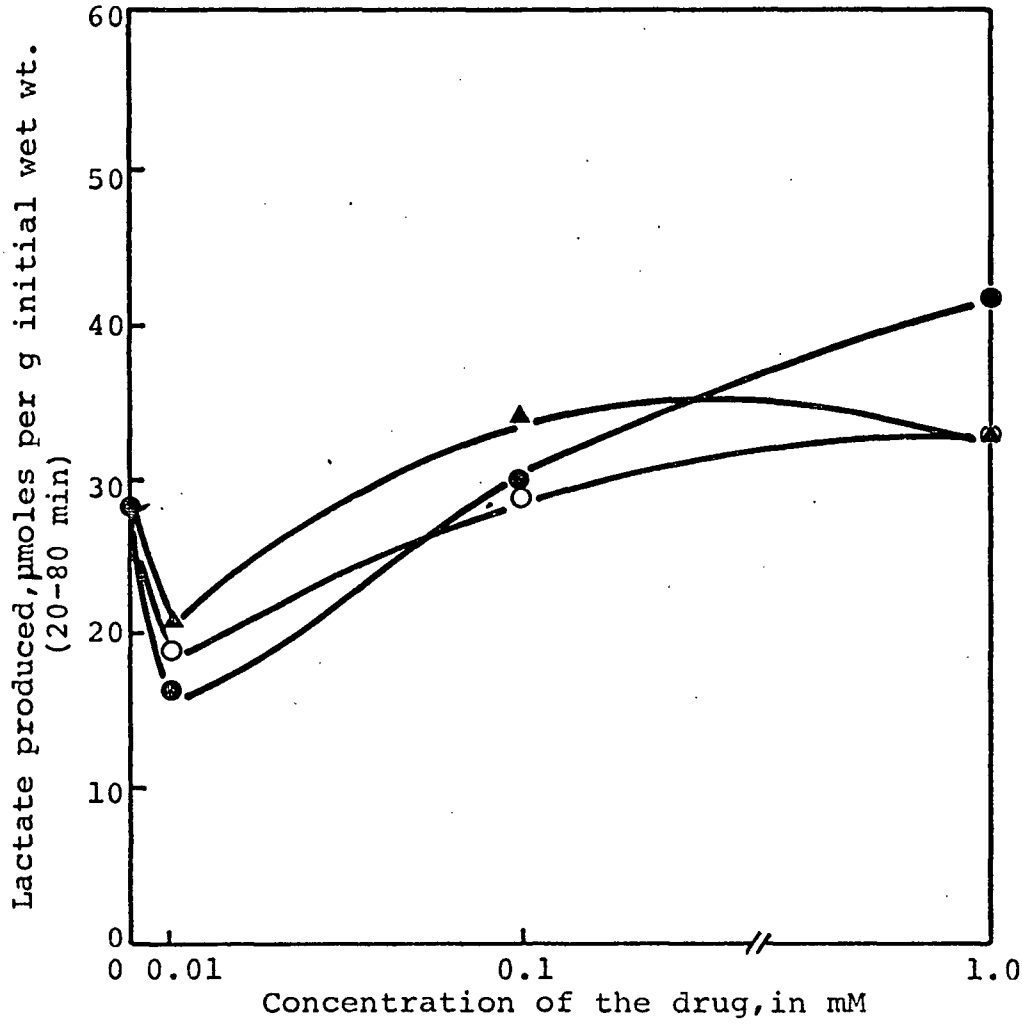
EFFECT OF CHLORPROMAZINE ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES




Incubation conditions were same as in Figure 37.

FIGURE 41

EFFECTS OF AMPHETAMINE AND NIALAMIDE ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES




Incubation conditions were same as in Figure 37. (●) l-Amphetamine; (▲) d-amphetamine; (○) nialamide.

## 7.6 EFFECT OF RESERPINE ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES

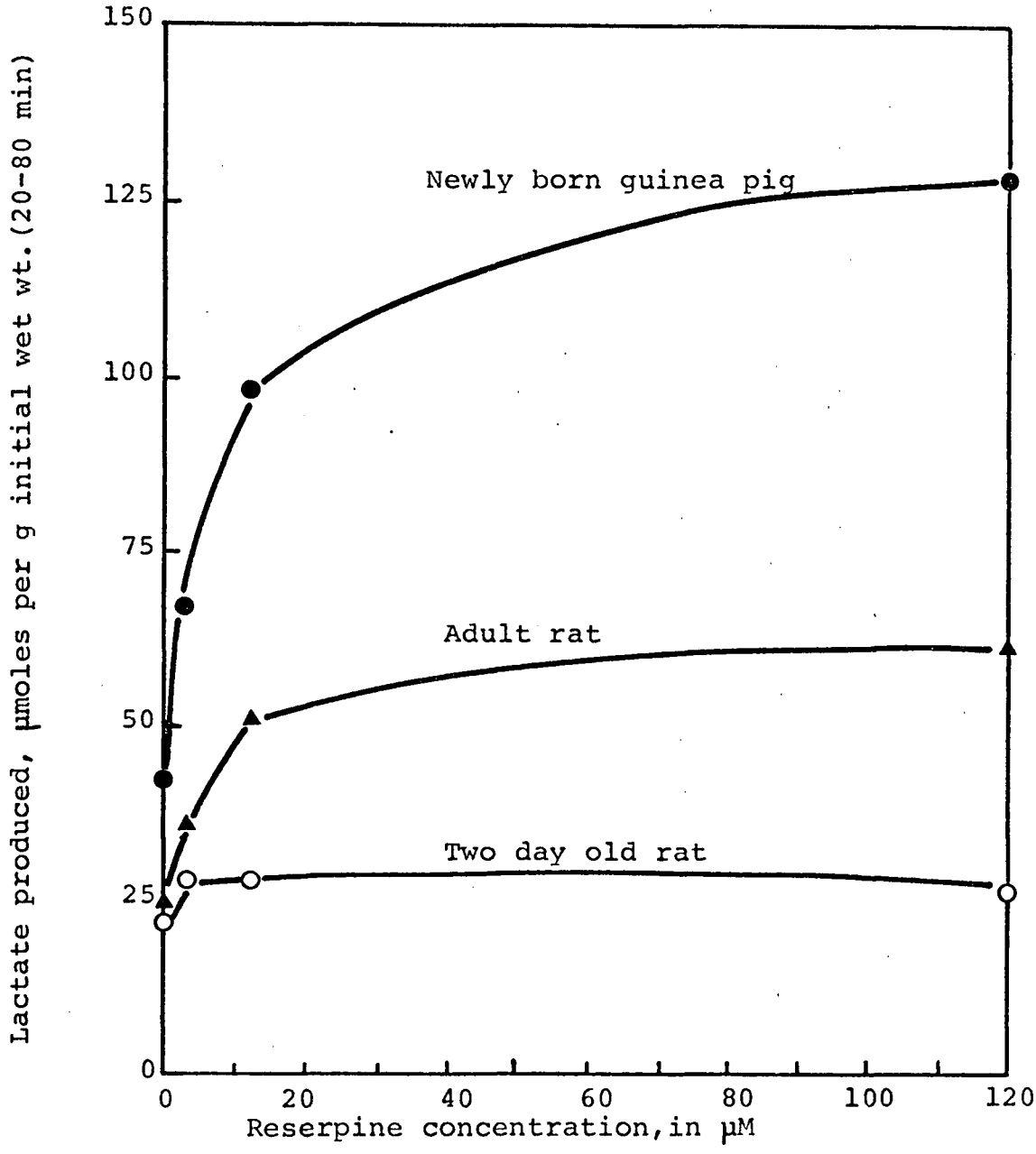
Reserpine causes release of amines from their storage sites so that they are more susceptible to degradation by monoamine oxidase (Chapter 1.5). However, it has no inhibitory effect on MAO. Prolonged treatment with reserpine may therefore cause depletion of amines from cerebral tissue. It is uncertain whether reserpine affects $Na^+$ and $K^+$ fluxes across the brain cell membrane.

The effects of reserpine on the rate of anaerobic glycolysis were investigated (Figure 42). It was found that low concentrations of reserpine markedly enhance the rate of anaerobic glycolysis of adult rat and infant guinea pig cerebral cortex slices.

Since a number of drugs affect anaerobic glycolysis by modifying the cationic fluxes, the effects of reserpine on the $Na^+$ and $K^+$ contents of the cerebral cortex slices under anoxia were investigated. These results (Table 34) showed that in the presence of 12 μM reserpine, there is an increase in the $K^+/Na^+$ ratio of the incubated rat cerebral tissue. It was thought possible that reserpine may exert this effect indirectly by amine release. Investigations were, therefore, carried out as to the effects of biogenic amines on the anaerobic glycolysis. The results indicate that unlike reserpine, these amines have no large effects on the rate of anaerobic glycolysis (Table 36). These experiments show that the action of reserpine is unlikely to be mediated through the release of amines. It is therefore concluded that reserpine

FIGURE 42

EFFECTS OF RESERPINE ON THE ANAEROBIC GLYCOLYSIS OF CEREBRAL CORTEX SLICES




Incubation conditions were same as in Figure 37.

TABLE 36

EFFECTS OF SOME AMINES ON THE ANAEROBIC GLYCOLYSIS OF RAT CEREBRAL CORTEX SLICES

| Addition | Lactate produced µmoles per g initial wet wt (20-80 min) |
|---|---|
| None | 25.6 ± 2.3 |
| 0.01mM Epinephrine | 21.0 |
| 0.1 mM Epinephrine | 29.0 ± 2.4 |
| 1 mM Tyramine | 30.6 ± 1.0 |
| 0.1 mM Norepinephrine | 28.6 ± 4.5 |
| 1 mM Norepinephrine | 30.8 ± 5.0 |
| 1 mM Histamine | 22.8 ± 2.4 |
| 50 µM Paraxon | 26.8 ± 1.3 |
| 50 µM Paraxon + 1mM Acetylcholine | 29.0 ± 3.6 |

Incubations were carried out in a $Ca^{++}$ free medium containing 20 mM glucose. Additions were made at zero time and lactate production was measured manometrically as given in the materials and methods.

may exert effects on cation transport, as well as on its well-known effects on amine storage.

SUMMARY OF CHAPTER 7

1. Observations of Adams and Quastel, that pyrrole increases the rate of anaerobic glycolysis of guinea pig cerebral cortex slices in a $Ca^{++}$-free medium, have been confirmed.

2. In the presence of 30 mM pyrrole, the incubated guinea pig cerebral cortex slices lose less $K^+$ and gain less $Na^+$ under anoxia. It is concluded that the action of pyrrole on anaerobic glycolysis is mediated through increase in the $K^+$/ $Na^+$ ratio of the brain cell.

3. Pyrrole stimulated glycolysis resembles TTX stimulated glycolysis by being inhibited by 5 mM glutamate but not by 5 mM aspartate.

4. In a $Ca^{++}$-free medium amytal increases the rate of anaerobic glycolysis of adult rat as well as infant guinea pig cerebral cortex slices while it has no effect on the anaerobic glycolysis of infant rats. Its effects, at anesthetic concentrations, are less than those of TTX or local anesthetics. Infant guinea pig cerebral cortex slices were found to be very sensitive to the presence of amytal.

5. Amytal has no effect on the $K^+$ content of the incubated rat cerebral cortex slices under anoxia, while the $Na^+$ content is slightly decreased.

6. The effect of amytal on the anaerobic glycolysis of cerebral cortex slices seems to be independent of its action as a respiratory inhibitor.

7. Chlorpromazine has little or no effect on the anaerobic glycolysis or $Na^+$ and $K^+$ content of the incubated cerebral cortex slices under anoxia.

8. D- and L-amphetamine, as well as nialamide, has little or no effect on the anaerobic glycolysis of cerebral cortex slices.

9. Reserpine, at low concentrations, increases the anaerobic glycolysis of cerebral cortex slices. The $K^+/Na^+$ ratio of the incubated cerebral cortex slices, in the presence of 12 μM reserpine, is increased under anoxia.

10. Epinephrine, norepinephrine, histamine,tyramine and acetylcholine have little or no stimulatory action on anaerobic glycolysis of cerebral cortex slices. Presumably the action of reserpine is not mediated through the release of amines from storage sites.

# CHAPTER 8

# DISCUSSION

## 8.1. EFFECT OF CALCIUM IONS ON CEREBRAL ANAEROBIC GLYCOLYSIS

Results described in Chapter 3 show that a variety of compounds, e.g. cations, nucleotides etc., exert rate limiting actions on the anaerobic glycolysis of cerebral cortex slices as well as of acetone powder extracts from brain. Thus the presence of $Ca^{++}$ in the incubation medium greatly enhances the rate of anaerobic glycolysis of cerebral cortex slices (Figures 1 and 2). The effect of $Ca^{++}$ on the cerebral cortex slices differs markedly from that on Ehrlich ascites tumour extracts, where it inhibits glycolysis[60]. On the other hand, $Ca^{++}$ has no effect on the glycolysis of intact tumour cells[60,71].

The effect of $Ca^{++}$ on the rate of anaerobic glycolysis of guinea pig cerebral cortex slices has been studied in some detail by Adams & Quastel[71] but they could not offer a satisfactory explanation for this phenomenon at that time. These workers, however, found that a number of organic bases act in a similar way to $Ca^{++}$, and correlated these effects with their dissociation constants. It was also found that a mixture of bases with $Ca^{++}$ gave no additive stimulatory effect suggesting a common site of action.

It is possible, as pointed out by Adams & Quastel, that the effects are associated with changes in membrane permeability brought about by the ions. In the presence of $Ca^{++}$, there is some increased retention of $K^{+}$ by the incubated cerebral cor-

tex slices (Figure 26) and this may be one of the reasons why $Ca^{++}$ has a stimulatory effect on the anaerobic glycolysis of cerebral cortex slices.

A view that $Ca^{++}$ acts by becoming part of the structural pattern of the cell membrane in such a way that active transport of glucose into the neuron is facilitated[71] is unlikely to be true as there is no evidence that there is active (i.e. energy assisted) transport of glucose into brain in vitro, and as the rate of cerebral anaerobic glycolysis is not affected by increasing the glucose concentration from 5 mM to 50 mM.

Under anaerobic conditions, the ATP content of the cell is much diminished and may become a rate limiting factor for the phosphorylation of glucose and fructose 6-phosphate. $Ca^{++}$ is a strong inhibitor of microsomal $Na^{+},K^{+}$-ATPase, and it may therefore stimulate the anaerobic glycolysis by decreasing the loss of ATP by $Na^{+},K^{+}$-ATPase activity. However, it is uncertain as to whether external $Ca^{++}$ inhibits the enzyme in brain slices.

It is now well known that omission of $Ca^{++}$ from the incubation medium results in an influx of $Na^{++}$ in the nerve cell with concomitant depolarization[304]. In the presence of $Ca^{++}$ this ($Na^{+}$) influx is prevented and may, along with the retention of $K^{+}$, and possible increase in the cell ATP concentration, be responsible for the increased rate of glycolysis of the cerebral cortex slices.

The effects of $Ca^{++}$ on the glycolysis of developing brain (Table 1) provide some interesting features. Infant rat brain is not known to respond to the presence of high $K^{+}$ or electrical

stimulation[235]. Results reported herein show that the anaerobic glycolysis of 2-day old rat brain does not respond to $Ca^{++}$. The responsiveness increases during the period of maximum brain growth and myelination (ie. during the 2nd week), Infant guinea pig brain was found to be extremely sensitive to the presence of $Ca^{++}$ in the incubation medium; this is not unexpected, as is well known that the brain of infant guinea pig is more mature than that of infant rat brain and behaves like an adult brain (Chapter 1).

Although there is evidence that $Ca^{++}$ inhibits some of the glycolytic enzymes in cell free extracts, this is not the case with the brain slices as in that event removing $Ca^{++}$ from the incubation medium will result in decline in the intracellular $Ca^{++}$, and this would result in greater rate of glycolysis. However, this is not true. The glycolysis of cell-free extracts and synaptosomes behave similarly in the sense that both are inhibited by $Ca^{++}$.

## 8.2. EFFECTS OF EXOGENOUS NUCLEOTIDES ON THE ANAEROBIC GLYCOLYSIS

It is known that the rate of anaerobic glycolysis of cerebral cortex slices decreases progressively with time. As has been pointed out in Chapter 3, this decrease may be due to the loss of co-enzymes or due to a change in the cationic composition of the cerebral cortex slices. McIlwain has found that tissue $NAD^+$ + NADH level decreases during anoxia[15]. In view of this fact, experiments were carried out to examine

whether the $NAD^+$ concentration of the cerebral cortex slices becomes rate limiting for anaerobic glycolysis. Results given in Table 2 indicate that the addition of $NAD^+$ to the incubation medium increases the rate of anaerobic glycolysis of the cerebral cortex slices. ATP, under the same conditions, has no effect but possibly it is hydrolyzed before reaching the site of glycolysis. Transport of $NAD^+$ has been investigated (Figures 3 and 4) and it has been found that considerable exogenous $NAD^+$ can penetrate the cell. The possibility that these results are an artifact due to mere binding of $NAD^+$ to the outer membrane, is ruled out because, under the same conditions, there is an increase in the concentration of NADH within the cell with concomitant increase in the rate of anaerobic glycolysis. This occurs in spite of the fact that considerable NADase is present in the brain cell and no inhibitor of NADase was used during these experiments. Possibly in the intact cell, $NAD^+$ is not degraded as rapidly as it is in homogenates or acetone powder extracts. This might be due to the localization of NADase in subcellular structures such as lysosomes. $NAD^+$ has no effect on the rate of aerobic glycolysis in presence of DNP. This lack of effect of $NAD^+$ in the presence of DNP may be due to the fact that an optimal rate of glycolysis is obtained in the presence of this substance.

The effect of $NAD^+$ on the anaerobic glycolysis of cerebral cortex slices is not due to inhibition of ATPase because

$NAD^+$ has no effect on the activity of this enzyme. These results point to the possibility that under anaerobic conditions the cell $NAD^+$ concentration may in fact be rate-limiting for anaerobic glycolysis.

In the presence of $Ca^{++}$, 2.5 mM ATP has an inhibitory effect on the rate of anaerobic glycolysis (Table 2). This is possibly due to chelation of $Ca^{++}$ in the incubation medium by ATP, which is well known to combine with $Ca^{++}$. Alternatively, it is possible (but less likely) that in the presence of $Ca^{++}$, ATP may penetrate the tissue unhydrolyzed and inhibit the phosphofructokinase activity.

8.3. EFFECTS OF CATIONS ON THE ANAEROBIC GLYCOLYSIS

The rate of anaerobic glycolysis of cerebral cortex slices shows strong dependence for the presence of cations in the incubation medium (Table 3).

Addition of a high concentration of $K^+$ to a Ringer medium inhibits the rate of anaerobic glycolysis of cerebral cortex slices. However, results, shown in Figure 8, indicate that when the concentration of $Na^+$ is reduced and $K^+$ is increased at the same time, the rate of anaerobic glycolysis is enhanced. This is explained by the fact that an increased concentrations of $K^+$ in the incubation medium result in considerable influx of $Na^+$ into the brain cell[135] and the inhibitory effect of the increased cell concentration of $Na^+$ may then outweigh the stimulatory action of $K^+$. When the $Na^+$ concentration in the incubation medium is also decreased at the time when $K^+$ is increased,

less $Na^+$ enters the brain cell in the presence of a high concentration of $K^+$. There results little increase in the intracellular $Na^+$ compared with the increase in cellular $K^+$ and this presumably results in a greater rate of anaerobic glycolysis. Decreasing the extracellular concentration of $Na^+$, without increasing the $K^+$ concentration, itself results in greater rate of anaerobic glycolysis (Figure 32). Thus the $K^+/Na^+$ ratio has a controlling effect on the rate of anaerobic glycolysis of cerebral cortex slices. The increased rate of anaerobic glycolysis in the presence of high $K^+/Na^+$ ratio is presumably mediated through changes in the activity of pyruvate kinase. Over 0-50 mM range, doubling the $K^+$ concentration results in a two-fold increase in the pyruvate kinase activity[59]. $Na^+$ inhibits the activation by $K^+$[301].

It is confirmed that L-glutamate inhibits the anaerobic glycolysis of cerebral cortex slices. It is now well known that L-glutamate causes a large $Na^+$-influx and this is doubtless responsible for its inhibitory action on the anaerobic glycolysis.

## 8.4. ANAEROBIC GLYCOLYSIS OF ACETONE POWDER EXTRACTS OF BRAIN

From the rates of anaerobic glycolysis in the acetone powder extracts, it can be observed that with such extracts, a much higher rate of glycolysis is obtained as compared to the corresponding amount of the brain slices (Figures 10 & 11). This may be due to the fact that in the cell-free extracts the glycolytic intermediates and coenzymes may be readily

available to the enzymes as compared to the brain slices, where the specific localization of the enzymes and compartmentalization of the metabolites must be playing an important role in determining the overall rate of glycolysis. When the rate of glycolysis is high, as in the acetone powder extracts, $K^+$ does not have very large effects on the rate of anaerobic glycolysis.

8.5. EFFECTS OF TETRODOTOXIN ON ANAEROBIC GLYCOLYSIS OF BRAIN

TTX exerts marked effects on the metabolism of brain cortex slices incubated aerobically under a variety of conditions. It was shown by Chan and Quastel[130], and by McIlwain[129], independently and at about the same time, in 1967, that the drug suppresses the increased respiration induced by application of electrical impulses. Moreover, the drug at small concentrations, suppresses the electrically induced influx of $Na^+$ as shown directly by measurements of the influx of labelled $Na^+$ and indirectly by the stimulatory action of the drug on the cerebral oxidation of acetate which is inhibited by the influx of $Na^+$ due to electrical stimulation. Recently, it has been shown by Okamoto and Quastel[135] that tetrodotoxin inhibits both the increased water uptake and influx of $Na^+$ that occurs under a variety of aerobic incubation conditions, for example in the presence of protoveratrine or of ouabain or in the absence of glucose. It was concluded that under such conditions action potentials are generated in the incubated brain slices, or that there is some

activation of a specific sodium current system, to account for the marked effects of tetrodotoxin in vitro.

Results shown in Figure 12 show that the presence of low concentrations of TTX markedly stimulates the rate of anaerobic glycolysis of the incubated cerebral cortex slices, both in a $Ca^{++}$- containing as well as in a $Ca^{++}$-free medium. This phenomenon is of considerable interest. The anoxic condition can be regarded as one leading to the generation of action potentials, as under these conditions there is stimulation of the cation carrying system resulting in depolarization of the cell.

The effect of TTX on anaerobic glycolysis differs in rat and guinea pig cerebral cortex slices. Thus, with rat the values obtained in the presence of a mixture of $Ca^{++}$ and TTX both together are either the same or slightly reduced while in the guinea pig the rate is further increased (Figure 14). This is possibly due to the fact that $Ca^{++}$ exerts qualitatively different effects on cerebral anaerobic glycolysis according to the animal species.

$Na^+$ Influx:

Because TTX is a specific inhibitor of the $Na^+$-carrying system during an action potential, it was thought that it may act on anaerobic glycolysis by abolishing the generation of action potentials and hence the concomitant movement of $Na^+$ at the onset of anoxia. However, larger effects of TTX on the influx of labelled $Na^+$ during anoxia were not observed

(Table 18). Thus, when the influx of $Na^{22}$ was studied, the percentage suppression of $Na^+$ influx is not large enough to assign it the major role in enhancing the pyruvate kinase activity by its decreased concentration.

$Cl^-$-free Media

Substitution of $Cl^-$ by $SO_4^{--}$ does not result in increased rates of anaerobic glycolysis - either in the absence as well as in the presence of TTX (Table 19). It is well known that when $Cl^-$ is replaced by the relatively impermeable $SO_4^{--}$, there is reduced influx of $Na^+$ into the incubated cerebral cortex slices under a variety of conditions. TTX is found to be effective in increasing the rate of anaerobic glycolysis of the brain slices in a $Cl^-$-free medium in spite of the reduced influx of $Na^+$. Moreover, the rate of glycolysis in a $Cl^-$-free medium is not greater than in a medium in which normal concentration of $Cl^-$ is present. It is difficult to conclude from these results that changes in the cell $Na^+$ are solely responsible for the increase in the rate of anaerobic glycolysis of the cerebral cortex slices in the presence of TTX.

A number of experiments were carried out to test other possibilities as to the mode of action of TTX. As it has been mentioned earlier in this chapter, a number of factors may become rate limiting for the rate of anaerobic glycolysis of the cerebral cortex slices. Changes in the concentration of each of these factors may result in an inhibition or activation of the rate of glycolysis, and hence these were investi-

gated in an effort to throw more light on the mechanism of enhancement of anaerobic glycolysis by the presence of TTX. These experiments will now be discussed.

Role of Nucleotides. As has been mentioned, there is a marked decline in the concentration of $NAD^+$ + NADH of the cerebral cortex slices under anoxia and it may have a rate-limiting effect on the anaerobic glycolysis. The exact reason for the decline in the $NAD^+$ + NADH level is not known but possibly it may be due to a variety of causes such as (i) rapid breakdown of $NAD^+$ under anoxia, (ii) loss of $NAD^+$ from the tissue during anaerobic incubation.

The second possibility has been ruled out by us as there is no evidence if there is a leakage of $NAD^+$ (or NADH) into the incubation medium. If it does leak out in the medium under the given experimental conditions then it is degraded so quickly that it can not be detected in our experiments. Furthermore, there is no increase in the $NAD^+$ level of the cerebral cortex slices in the presence of TTX (Chapter 4.3). Hence, the possibility that the effect of TTX on anaerobic glycolysis is due to an increase in the cell $NAD^+$ level due to reduced efflux is considered most unlikely.

As early as 1928 it was demonstrated that a short pre-incubation in oxygen markedly increases the subsequent rate of anaerobic glycolysis[289]. This has been shown for a number of adult tissues, including brain, but the reason for this is not clear[290-294]. It has been stated that the increased rate of anaerobic glycolysis of the cerebral cortex slices after an

aerobic preincubation might be the result of accumulation of pyruvate during the aerobic period[71]. However, the role of ATP concentration, which can exert a rate limiting effect on anaerobic glycolysis, has not been discussed.

One of the major consequences of anoxia is the loss of the capacity of the tissue to carry out mitochondrial phosphorylation resulting in decreased ATP concentration, and many of the other effects of anoxia are indirectly due to a decrease in the ATP content. Under anaerobic conditions, the ATP content falls to a very low level and, as pointed out earlier, it may exert a rate limiting effect on the glycolysis during subsequent anaerobiosis.

It was observed by us that there is an increase in the acid labile phosphate and the ATP content (Table 15) of the cerebral cortex slices in the presence of TTX under anaerobic conditions, and hence the possibility existed that TTX may act by increasing the ATP content of the incubated slices. Experiments were carried out, therefore, to ascertain the extent to which the cell level of ATP is responsible for the stimulating action of TTX on the anaerobic glycolysis of cerebral cortex slices. In these experiments, conditions were made such that there was induced either a decrease or an increase in the ATP content of the cerebral cortex slices in the presence of TTX in the incubation medium (Tables 13 and 14).

When TTX is added to the incubation medium after 10 or

15 minutes of anoxia, then it has no stimulating effect on the anaerobic glycolysis (Figure 21). This shows that some changes, taking place during the first few min of anoxia, such as decrease in ATP content and/or loss of $K^+$, are responsible for the effects of tetrodotoxin on the anaerobic glycolysis. If anaerobiosis is carried out for 15 min in the absence of glucose but in the presence of TTX, and then glucose is added to the incubation medium, TTX is not effective in increasing the rate of anaerobic glycolysis. However, if the first 10 min incubation period is made aerobic, in the absence of glucose but in the presence of pyruvate, then TTX is still effective in increasing the subsequent rate of anaerobic glycolysis (Table 13). A similar result is obtained if the preliminary anoxic period (15 min) is followed by a 10 min aerobic period and then by the addition of glucose. When pyruvate is present from zero time but glucose is added immediately after a period of anoxia, then TTX is not effective in increasing the rate of anaerobic glycolysis.

These results show that either the presence of glucose from the beginning of the experiment or an aerobic preincubation is necessary for the slices to show any response to TTX. One that would be greatly influenced under these conditions should be the ATP concentration of the cerebral cortex slices. Experiments carried out, in the presence of DNP, which uncouples the oxidative phosphorylation and hence decreases the ATP contents (Table 14), show that when the aero-

bic preincubation is carried out in the presence of DNP, then the percent stimulation of anaerobic glycolysis in the presence of TTX is not affected. These experiments lead to the conclusion that the cell ATP is not responsible for the effect of TTX on the speed of anaerobic glycolysis.

Experiments carried out with adenosine-preincubated slices show that although with such slices, there is an increase in the rate of lactate production in controls, possibly due to a rise in ATP, the effect of TTX is unaffected. These slices also respond in a manner similar to the non-adenosine preincubated slices in showing the effects of TTX when it is added after 15 min of anoxia.

The higher content of ATP in the incubated cerebral cortex slices, in the presence of TTX, may be the result of a higher rate of glycolysis itself rather than that of an interference of TTX with the energy utilization processes of the brain cells.

Role of Pyruvate

Pyruvate is known to increase considerably the rate of anaerobic glycolysis and it has been stated that it might act by affecting the $NAD^+$/NADH ratio. As $NAD^+$/NADH ratio is important for the regulation of the rate of anaerobic glycolysis, TTX may act by preventing the efflux of pyruvate and hence affecting the $NAD^+$/NADH ratio. However, when pyruvate and TTX are present together, their effect on anaerobic glycolysis is additive. This shows that both these agents must

be affecting the anaerobic glycolysis of cerebral cortex slices by different mechanisms. These experiments rule out the possibility that the effect of TTX is due to the suppression of pyruvate efflux from the incubated slices.

Role of Glucose Concentration

The percentage stimulation of anaerobic glycolysis by TTX falls when the external glucose concentration is decreased. The rate of anaerobic glycolysis, in the presence of TTX, progressively increases until about 50 mM glucose concentration, when a maximum rate of glycolysis is obtained. This effect is not due to facilitation of glucose entry into the slices by TTX (Table 6). These results demonstrate that, under conditions when the rate of anaerobic glycolysis is high, the concentration of glucose becomes a rate limiting factor. This is doubtless due to the fact that with high rates of glycolysis, high external concentration of glucose are required to saturate the glucose utilization system in the brain cell or cell compartments.

Role of cAMP

As shown in Chapter 5.8, the cAMP formation in the cerebral cortex slices in the presence of TTX is not increased; instead a decrease is observed. These results demonstrate that the effect of TTX on the rate of anaerobic glycolysis is not due to increased formation of cAMP.

Recently, it has been shown that under a variety of conditions, when action potentials are generated and depolariz-

ation occurs, such as in the presence of high $K^+$, protoveratrine, batrachotoxin or electrical stimulation, the production of cAMP in the cerebral cortex slices is increased (297,300); the increase caused by batrachotoxin is blocked by TTX. Direct effects of TTX on the cAMP contents of cerebral cortex slices have not been reported.

Effects of TTX in the Presence of L-glutamate, $NH_4^+$ and Protoveratrine

A number of experiments show, indirectly, that the effects of TTX on the anaerobic glycolysis might be due to effects on the movements of cations rather than to direct effects on the transport and/or cellular level of metabolites. Thus, protoveratrine, known to generate action potentials and to stimulate influx of $Na^+$ and efflux of $K^+$, also inhibits the rate of anaerobic glycolysis of cerebral cortex slices. Experiments carried out on the effects of TTX on the anaerobic glycolysis, in the presence of protoveratrine, show that these drugs are antagonistic to each other (Figure 22). Thus, in the presence of 5 μM protoveratrine, 2 μM TTX has less stimulatory effect on the rate of anaerobic glycolysis and the effect of TTX progressively decreases with increasing concentrations of protoveratrine.

Similarly, L-glutamate is known to stimulate the entry of $Na^+$ into brain cells[286]. The effect of TTX on the anaerobic glycolysis of the cerebral cortex slices is abolished if glutamate is also present in the incubation medium from the

start of the experiment. In the presence of 5mM $NH_4^+$, TTX has no effect on anaerobic glycolysis. $NH_4^+$ is known to increase the efflux of $K^+$ from the cerebral cortex slices[305]. These experiments point out that the effect of TTX on the anaerobic glycolysis is presumably due to its effect on cerebral cationic movements at the onset of anoxia.

Effects of TTX on Contents of $Na^+$ and $K^+$

The effects of TTX on the $Na^+$ and $K^+$ content of the cerebral cortex slices were directly investigated. These results (Figures 24,26,27) show that in the presence of TTX, the cellular level of cations are definitely affected; thus in the presence of TTX the $K^+/Na^+$ ratio of the cerebral cortex slices is increased. Because $Na^+$ is an inhibitor of anaerobic glycolysis while $K^+$ is an activator[37,64], it is therefore likely that TTX stimulates the anaerobic glycolysis of cerebral cortex slices by increasing the $K^+$ content and decreasing the $Na^+$ content.

As has been mentioned earlier, the metabolic effects of TTX are best seen if the slices are preincubated in oxygen before the onset of anoxia. When the $K^+$ level of the cerebral cortex slices is measured under such conditions, the results show that there is very little loss of $K^+$ in the presence of TTX during the subsequent anoxic period (Figures 26,27). This is probably due to the facts (a) that there is decreased loss of $K^+$ during preliminary aerobic incubating period, and (b) that TTX takes some time to bind to the sites at the brain cell membrane before it may affect the cation movements. Thus, if

the initial period is wholly anoxic, there is considerable loss of $K^+$ and gain of $Na^+$ by the slices before TTX could be very effective in affecting the cation movements. The concentration of cations, measured in the presence of glutamate or $NH_4^+$, showed that there is less increase in the retention of $K^+$ and less decrease in the gain of $Na^+$ by the slices under these conditions (Table 25). These results are further evidence that cation movements are involved when the rate of anaerobic glycolysis in the presence of TTX is affected by a variety of agents.

Qualitatively, the increase in the $K^+/Na^+$ ratio of the cerebral cortex slices in the presence of TTX, under anoxia, is sufficient to explain the increased rate of anaerobic glycolysis. Can the changes in the $Na^+$ and $K^+$ concentrations observed explain the results quantitatively? If it is assumed that the major site of glycolysis is in the neurons and since TTX acts only on the electrically excitable neurons, it follows that the changes in the $Na^+$ and $K^+$ found in the whole tissue are proportionately much greater if they occur only in the neurons. As in our experiments, the $Na^+$ and $K^+$ concentrations were measured in the whole tissue, they give the average composition of these cations and the actual concentration of $Na^+$ and $K^+$ may vary considerably in the neurons and glial cells. The large effects of TTX on the anaerobic glycolysis of cerebral cortex slices may then be readily explained on this basis.

Results of experiments on the caudate nucleus (Table 22)

indicate that its anaerobic glycolysis is also increased in the presence of TTX, but the magnitude of stimulation is about the same as that observed with the cerebral cortex slices.

The conclusion that the effect of TTX on the anaerobic glycolysis is mediated through increase in the $K^+/Na^+$ ratio is further supported by the fact that in the presence of TTX there is an increase in the pyruvate content which would be expected if there is facilitation of the pyruvate kinase step due to increased $K^+$ in the brain cell (Table 26).

Results of experiments carried out, to explain the effects of TTX on the anaerobic glycolysis at different $Na^+$ and $K^+$ concentrations indicate that the concentration of both cations are important for the regulation of anaerobic glycolysis of cerebral cortex slices. Thus, when $K^+$ concentration is raised with a corresponding decrease in the $Na^+$ content then a higher rate of glycolysis is obtained and the degree of stimulation in the presence of TTX progressively decreases until an optimal rate of glycolysis is obtained. Under these conditions, inhibition by $Na^+$ is not observed since its concentration in the incubation medium is low. When the concentration of $Na^+$ is decreased without increase in the $K^+$ concentration ($Na^+$ is replaced by sucrose), then the rate of glycolysis is higher than the controls having normal (149 mM) concentrations of $Na^+$ (Figure 32). The rate of glycolysis under these conditions is markedly stimulated by the presence of TTX in the incubation medium. Under these conditions, presumably,

prevention of the efflux of cellular $K^+$ plays a very important part. We have seen (Table 20) that when the concentration of $K^+$ in the incubation medium is high, in addition to 149 mM $Na^+$, then TTX is not effective in increasing the rate of anaerobic glycolysis of the cerebral cortex slices. It is known that high $K^+$ causes increased influx of $Na^+$ into the cerebral tissue[135] and when 149 mM $Na^+$ is present in the incubation medium, there is no stimulation of glycolysis because of increased $Na^+$ influx. This increase in the influx of $Na^+$ is presumably not inhibited by TTX and thus, in this respect, it differs from the $Na^+$ influx caused by protoveratrine, electrical stimulation or anaerobiosis.

Results obtained with kidney medulla slices on the effects of TTX on anaerobic glycolysis and $Na^+$ and $K^+$ contents show that TTX had no effect on either of them (Figures 16 and 29). This shows that the effect of TTX on anaerobic glycolysis is not an unspecific phenomenon and perhaps is confined only to cerebral tissue. The results with acetone powder extracts of brain demonstrate that the integrity of brain cell membrane is required for TTX to show any effect on the rate of anaerobic glycolysis and TTX does not act by affecting any of the enzymes of the glycolytic pathway.

Effects on Amino Acid Transport Under Anaerobic Conditions

TTX markedly suppresses the efflux of amino acids from the incubated cerebral cortex slices. This effect is very pronounced in the case of glutamic and aspartic acids which are present in relatively large amounts (Table 4). Abadom

and Scholefield[245] have shown that when the ATP content of the cerebral cortex slices is higher, there is greater uptake of glycine from the incubation medium. One can argue that in the presence of TTX, greater amounts of ATP are present in the slices due to a higher rate of anaerobic glycolysis and as a result the transport system works more efficiently, resulting in greater uptake of amino acids. However, such a possibility is ruled out by the fact that there is an increased retention of amino acids even in the presence of a mixture of ouabain and TTX (Table 30). Ouabain is known to block the active uptake of amino acids and its action is not antagonized by TTX. Thus TTX must be acting independently of the ouabain sensitive amino acid transport system. The increased tissue contents of amino acids in the presence of TTX is even apparent in the absence of glucose, although to a lesser extent.

Experiments with labelled amino acids (Table 5) showed that there is a significant increase in the uptake by the anaerobically incubated slices in the presence of TTX. Presumably this is due to two reasons: (i) increased uptake of amino acids by the slices because of the greater rate of anaerobic glycolysis and hence the higher ATP level and (ii) because of inhibitory effect of TTX on the efflux of amino acids from the slices.

Effects of TTX in the Presence of Chelating Agents and Phospholipases. TTX has little or no effect on the rate of anaerobic glycolysis in the presence of EDTA and EGTA (Table 23). In

the presence of these substances there is a rapid influx of $Na^+$ into and a rapid efflux of $K^+$ from the cerebral tissue. This influx of $Na^+$ is only partly inhibited by TTX[133]. Thus it appears that the action of TTX, in the presence of chelating agents, must be mediated through their actions on the $Na^+$ and $K^+$ movements. This conclusion further supports our view that the action of TTX on the cerebral anaerobic glycolysis is mediated through its effects on the $K^+$ and $Na^+$ contents.

Results on the effects of TTX in the presence of phospholipases (Table 9) show that during the early period of incubation, phospholipase A has no marked effect on the anaerobic glycolysis of cerebral cortex slices. However, during the later part of the incubation (50-80 min), the anaerobic glycolysis is considerably reduced in the presence of 20 or more International Units of phospholipase per vessel. These results indicate that possibly TTX acts at the membrane and binds to the receptors which has phospholipid constituents, and which is slowly attacked by the phospholipases. This finding further points out the importance of membrane constituents in the action of TTX on the cerebral metabolism.

Effects of TTX on Developing Brain

Results of experiments carried out on the developing rat brain shows that TTX has little or no effect on the anaerobic glycolysis of 2-day old rat brain. However, the brain from 2-week old rat is sensitive to TTX. Investigations on the $Na^+$ and $K^+$ contents of 2-day old rat cerebral cortex slices show

that there is no significant increase in the $K^+/Na^+$ ratio in the presence of TTX. These results show that maturity of the brain is required for TTX to have any effect on its metabolism. The receptors for TTX are not developed in the 2-day old rat brain and they are formed during the later period of life.

The infant guinea pig brain slices are extremely sensitive to TTX and 0.2 μM TTX is as effective in increasing anaerobic glycolysis as 10 μM. Furthermore, in the presence of TTX, there is an increased retention of $K^+$ and less uptake of $Na^+$ by the slices. This shows that with the infant guinea pig brain, too, the increase in anaerobic glycolysis in the presence of TTX is mediated through increase in the $K^+/Na^+$ ratio.

Effects of TTX on Aerobic Glycolysis

The rates of aerobic glycolysis of brain slices are increased when $Ca^{++}$ is omitted from the incubation medium. This is presumably due to greater $Na^+$ influx and the corresponding increase in the activity of $Na^+$, $K^+$-ATPase. This results in a decrease in ATP concentration of the cell and the inhibitory effect of ATP on glycolysis is diminished. In a Krebs-Ringer medium (containing $Ca^{++}$) 2 μM TTX has no effect on the aerobic glycolysis, but in a $Ca^{++}$-free medium it is suppressed by TTX, the resulting values approaching those obtained in a Krebs-Ringer medium. Thus, in this regard, TTX acts like $Ca^{++}$ and this is analogous to the results obtained by Chan and Quastel[130] who showed that the increase in respiration, brought about by omission of $Ca^{++}$ from the incubation medium, is suppressed by TTX.

## 8.6 EFFECTS OF OUABAIN ON CEREBRAL METABOLISM AND TRANSPORT

As has been discussed earlier, the effects of TTX on the anaerobic glycolysis of cerebral cortex slices is mediated through changes in the $Na^+$ and $K^+$ concentrations. However, the effect of ouabain on the anaerobic glycolysis cannot be due to its effects on the cation fluxes since ouabain is an inhibitor of $Na^+$, $K^+$-ATPase and, under aerobic conditions, the presence of this drug leads to a great influx of $Na^+$ into the cerebral cortex slices and an efflux of $K^+$. Under anaerobic conditions, the $Na^+$, $K^+$ -ATPase is not operative to the same extent as under aerobic conditions due to a fall in ATP concentration and a considerable amount of $Na^+$ enters in and $K^+$ comes out of the brain slices. Further, under anaerobic conditions ouabain has little or no effect on the $Na^+$ and $K^+$ contents (Figure 35), a result which favours the view that under these conditions, the operation of $Na^+$, $K^+$-ATPase is limited. These results indicate that the stimulation of anaerobic glycolysis of cerebral cortex slices in a $Ca^{++}$ free medium by ouabain cannot be due to its effects on the cationic contents, and therefore, under these conditions, the $Na^+$ and $K^+$ contents do not play a rate limiting role on the speed of anaerobic glycolysis.

Under anaerobic conditions, the ATP concentration falls to a very low level (Table 15) and it may become rate limiting for the phosphorylation of glucose and fructose 6-phosphate. Since a considerable amount of ATP is consumed by $Na^+$, $K^+$-ATPase in the brain cell, the inhibition of this en-

zyme in the presence of ouabain will result in a greater cell concentration of ATP due to a decrease in its utilization. This may in turn make a greater amount of ATP available for sugar phosphorylations resulting in greater rate of anaerobic glycolysis. An increase in the ATP content in the presence of ouabain is in fact observed (Table 15) but it cannot be ascertained whether this increase is due to the inhibition of $Na^+$, $K^+$-ATPase or due to a higher rate of glycolysis itself, as noted with TTX. Possibly with ouabain, an increased rate of glycolysis, due to inhibition of $Na^+$, $K^+$-ATPase, leads to diminished fall in the ATP concentration, and this results in higher observed values for ATP.

The effects of ouabain in the presence of $Ca^{++}$ show that when $Ca^{++}$ is present in the incubation medium, it is not as effective in increasing the anaerobic glycolysis as it is in the absence of $Ca^{++}$. If the action of ouabain on the anaerobic glycolysis of cerebral cortex slices is due to the inhibition of $Na^+$, $K^+$-ATPase, then these observations lead to the conclusion that in the presence of $Ca^{++}$, ouabain does not inhibit $Na^+$, $K^+$-ATPase completely. This conclusion is in agreement with the data of Swanson and his co-workers who observed that when ouabain is added to guinea pig cerebral cortex slices incubating in a medium in the presence of $Ca^{++}$, there is an initial rise in respiration followed after 45 or 60 min by a slight depression. When $Ca^{++}$ is omitted from the incubation medium, a decrease in respiration in the presence of ouabain was

observed[172]. These results were explained as resulting from the incomplete inhibition of $Na^+$, $K^+$-ATPase in the presence of $Ca^{++}$ by ouabain, which allowed the uninhibited portion of this enzyme to respond to cation shifts by consuming high energy phosphates and thereby stimulating respiration[131,173,174].

The effects of ouabain on the inhibition of $Na^+$,$K^+$-ATPase in the presence of $Ca^{++}$ were studied (Table 29), but the results show that, in microsomal preparations, $Ca^{++}$ and ouabain do not show any antagonism. Perhaps in brain tissue slices, the accessibilities of $Ca^{++}$ and ouabain to $Na^+$, $K^+$-ATPase is different as compared from those in the microsomal preparations.

When ouabain is added to the incubation medium after increasing periods of anaerobiosis, then it has progressively less effects on the rate of anaerobic glycolysis. This may be due to a fall in the ATP concentrations so that when ouabain is added later it has no more stimulating effect.

A dose response curve for the effects of different concentrations of ouabain on the anaerobic glycolysis show that the guinea pig cerebral cortex slices are more sensitive than the rat cerebral cortex slices to ouabain. This is probably because the sensitivities of $Na^+$, $K^+$-ATPase to ouabain differs with the species of the animal used. Bonting, Cravaggio and Hawkins[163] have in fact shown that ouabain inhibition curves of $Na^+$, $K^+$-ATPase differ with the species of the animal, and tissue used.

Results of experiments carried out with developing rat brain cortex slices show that the response of infant rat anaer-

obic glycolysis to ouabain increases with age and possibly this is related to the rise in $Na^+$, $K^+$-ATPase and general energy metabolism during development. Other experiments show that the effect of ouabain is a membrane phenomenon since a stimulation of anaerobic glycolysis of acetone powder extracts of brain in the presence of ouabain, is not observed.

The ouabain stimulated glycolysis differs from the TTX stimulated glycolysis by the fact that the former is not at all affected by the presence of $NH_4^+$ in the incubation medium. This is in accord with the conclusion that $K^+$ and $Na^+$ movements play a minor role with ouabain stimulated glycolysis. However, a partial inhibition was observed in the presence of L-glutamate and this is yet to be resolved. Like TTX stimulated glycolysis, ouabain stimulated glycolysis is inhibited by citrate, due probably to inhibition of phosphofructokinase activity (Table 28).

When the $Na^+$ concentration of the incubation medium is reduced, the rate of anaerobic glycolysis is markedly stimulated by ouabain. When ouabain and TTX both are present together in the incubation medium, then the rate of anaerobic glycolysis is further increased when compared with the rate found when either of these drugs is present alone (Figure 34). Under these conditions, an increase in $K^+/Na^+$ ratio may still be observed (Figure 35). These results demonstrate that TTX affects the passive downhill movements of $Na^+$ and $K^+$ independently of the operation of the sodium pump ($Na^+$, $K^+$-ATPase). In the presence of both ouabain and TTX, a combination of decreased ATP utilization and increased $K^+/Na^+$ ratio must be

responsible for the very high rates of anaerobic glycolysis obtained.

Furthermore, the efflux of amino acids, which takes place on the onset of anaerobiosis, either in the presence or absence of ouabain, is partially blocked by TTX (Table 30). These results further point out that the action of TTX on the movement of substances across the brain cell membrane is independent from the action of ouabain on $Na^+$, $K^+$-ATPase.

No significant effect of 10 μM ouabain was observed on aerobic glycolysis and this is in agreement with the findings of Rolleston and Newsholme[52].

## 8.7 EFFECTS OF LOCAL ANESTHETICS ON THE ANAEROBIC GLYCOLYSIS

The modes of action of local anesthetics and TTX on the nervous tissue seem to be identical. Results obtained with local anesthetics on the anaerobic glycolysis of cerebral cortex slices shows further similarities between these drugs. Thus, like TTX, local anesthetics increase the rate of anaerobic glycolysis of cerebral cortex slices (Table 31). When local anesthetics are added to the incubation medium after the onset of anoxia, they are ineffective in increasing the rate of anaerobic glycolysis. As observed with TTX, the $K^+/Na^+$ ratio of the cerebral cortex slices is also increased in the presence of lidocaine and presumably this is responsible for the observed increased rates of anaerobic glycolysis. In the presence of 0.5 mM lidocaine, an increase in the $K^+/Na^+$ ratio was also observed with infant rat cerebral cortex slices (Table

32); however, no stimulation of anaerobic glycolysis was observed with infant rat cerebral cortex slices (Figure 36). Perhaps the infant (2-day old) rat cerebral cortex glycolysis is not as sensitive as that of the adult to changes in the $K^+$ and $Na^+$ concentrations. Further work is necessary to clarify this point.

8.8. EFFECTS OF OTHER NEUROTROPIC DRUGS ON THE ANAEROBIC GLYLYSIS

Results of experiments carried out with pyrrole show that possibly its action on the anaerobic glycolysis of guinea pig cerebral cortex slices is mediated through an increase in $K^+$/$Na^+$ ratio resulting in stimulation of pyruvate kinase. The cerebral cortex slices from rats were found, however, to be more resistant to the organic bases tested than those of guinea pig slices and the reason for this difference between these two animals is not clear.

Preliminary results obtained with amytal and reserpine in their effects on the anaerobic glycolysis of cerebral cortex slices, are of some interest. Thus it was discovered that the anaerobic glycolysis of adult rat brain slices, and especially those from the infant guinea pig brain slices is enhanced by amytal. At anesthetic doses (0.25 mM), however, the anaerobic glycolysis of rat brain slices is not much affected by amytal (Figure 39).

The accelerative effects of amytal on anaerobic glycolysis of brain does not seem to be an artifact due to its well known action in suppressing NADH oxidation. Thus, as has been

pointed out earlier, the presence of oxygen as an impurity in the $N_2$:$CO_2$ mixture could give lower rates of glycolysis due to Pasteur effect, and amytal by preventing the oxidation of NADH under these conditions, would increase the rate of anaerobic glycolysis; however, this is unlikely since azide, under the same conditions, has no effect on the anaerobic glycolysis. (See Chapter 7).

Barbiturates have been stated to inhibit the transmission of impulses in the sympathetic ganglia. They have been found to reduce the $Na^+$ and $K^+$ conductances in lobster axons during excitation. It has been suggested that barbiturates in their anionic forms dissolve in the membrane lipids and thereby affect the binding of $Ca^{++}$ and thus affect ionic permeabilities.[276]

An increase in the $K^+$ concentration was observed with infant guinea pig cerebral cortex slices in the presence of 0.25 mM amytal. At this concentration, the anaerobic glycolysis of rat cerebral cortex slices is not much affected by the drug. Perhaps the infant guinea pig brain is more sensitive to amytal when compared with adult rat brain and a higher concentration of amytal could have a greater effect on the cationic contents of adult rat brain slices. Further study is necessary to settle the mode of action of barbiturates on the anaerobic glycolysis of cerebral cortex slices.

Reserpine was also found to increase the rate of anaerobic glycolysis of brain slices, and in its presence, too, an increase in the $K^+/Na^+$ ratio was observed. The effect of reserpine does not appear to be due to its known effect on the

release of amines from their storage sites as amines alone have very little effect on the rates of anaerobic glycolysis. The effects of reserpine on the $Na^+$ and $K^+$ contents are unexpected since it is not known to block the generation of action potential and thereby the movements of cations across the neuronal membrane. Further work is in progress to clarify the mode of action of reserpine on the anaerobic glycolysis of cerebral cortex slices. Chlorpromazine, amphetamine and nialamide have no significant stimulatory effects on the rates of anaerobic glycolysis of cerebral cortex slices.

From the results discussed in this thesis, it can be concluded that a number of neurotropic drugs can affect the rates of anaerobic glycolysis of the cerebral cortex slices. With some drugs such as TTX and local anaesthetics, the experimental results can be explained on the basis that in the presence of these drugs there is a specific increase in the cell $K^+/Na^+$ ratio. Preliminary results with pyrrole, amytal and reserpine have shown that these drugs too may affect the ionic fluxes across the brain cell membrane, but further work is necessary to settle this matter.

## 8.9 GENERAL CONCLUSIONS

1. TTX, at low concentrations, abolishes the generation of action potentials in a variety of excitable tissues. At similar concentrations, such as 2 μM, it enhances the rate of anaerobic glycolysis of cerebral cortex slices two to three-fold. Such an effect of TTX is much greater than that obtained on the aerobic metabolism of cerebral cortex slices.

2. The anaerobic glycolysis of kidney medulla or 2-day old rat brain slices are not affected by TTX. This shows that the effect of TTX is specific for mature cerebral tissue. Furthermore, since the anaerobic glycolysis of acetone powder extracts from brain is not affected by TTX, it follows that integrity of the cell is required for its action on the cerebral metabolism.

3. TTX has no accelerating effect on the rate of anaerobic glycolysis when high $K^+$ is present in the incubation medium containing normal $Na^+$ concentration. If the $Na^+$ is reduced at the same time when $K^+$ is increased, the rate of anaerobic glycolysis increases and TTX has a progressively smaller percentage acceleration. The lack of effect of TTX in a medium containing normal $Na^+$ and high $K^+$ may be due to the increased Na-influx that occur in cerebral cortex slices in presence of high external $K^+$ concentration.

4. When TTX is added after 15 min of anoxia, it no longer affects the anoxic cerebral metabolism. These experiments lead to the conclusion that there is an influx of $Na^+$ into, and efflux of $K^+$ from the incubated cerebral tissue at the onset of anoxia and if TTX is added after establishment of the steady state, it is not effective in increasing the rate of anaerobic glycolysis. The effects of TTX are due to changes in the $Na^+$ and $K^+$ contents by diminishing the cationic fluxes that occur at the onset of anoxia. These effects of TTX are due to its action at the brain cell membrane, possibly

involving phospholipids, resulting in changes in the permeability to cations. Thus, in the presence of TTX, the initial high rate of glycolysis tends to be maintained due to only a slow decline in the cellular $K^+/Na^+$ ratio. The effects of $Na^+$ and $K^+$ on the anaerobic glycolysis are considered to be mediated by changes in pyruvate kinase activity which is enhanced by $K^+$ and diminished by $Na^+$.

5. In the presence of TTX, under anoxia, the cell ATP concentration is also increased but this may be due to maintenance of original high rate of glycolysis rather than due to direct effects of TTX on the energy utilizing processes of the cell.

6. TTX affects the aerobic and anaerobic metabolism of brain in vitro in the same way as it effects the action potentials. This shows that action potentials are generated in the incubated cerebral tissue at the onset of anoxia. This is blocked by TTX and manifests itself in the higher rate of anaerobic glycolysis.

7. The above conclusion is further supported by the fact that in the presence of agents such as protoveratrine or L-glutamate, which lead to larger influx of $Na^+$ in isolated incubated brain, TTX does not affect the anoxic metabolism. Furthermore, in the presence of chelating agents such as EDTA and EGTA, which also results in greatly increased influx of $Na^+$, TTX is ineffective in enhancing the rate of anaerobic glycolysis of cerebral cortex slices.

8. The effect of TTX on the $Na^+$ and $K^+$ contents may be

greater in the neurons than in glial cells, because the former are regarded as the site of action of TTX. Thus, changes in the neuronal $K^+/Na^+$ ratio brought about by TTX may be much greater than the changes in the $K^+/Na^+$ ratio found in the tissue as whole.

9. In addition to its effects on the $Na^+$ and $K^+$ fluxes, TTX also prevents the efflux of amino acids from the incubated cerebral cortex slices at the onset of anoxia. This effect of TTX is independent of the operation of the amino acid transport system.

10. The accelerating effect of $Ca^{++}$ on the anaerobic glycolysis is presumably mediated by changes in the $K^+$ and $Na^+$ cell contents, by a mechanism resembling that of TTX.

11. Local anesthetics act like TTX on cerebral glucose breakdown in anoxia but at much higher concentrations. The effects of some organic bases, such as pyrrole, which also accelerate anaerobic glycolysis of guinea pig cerebral cortex slices may be explained on similar lines.

12. The rate of anaerobic glycolysis of cerebral cortex slices in a $Ca^{++}$-free medium is increased in the presence of 10 µM ouabain. In a $Ca^{++}$-containing medium, the stimulation of anaerobic glycolysis is not observed to the same extent as in a $Ca^{++}$-free medium.

13. The effect of ouabain is presumably due to inhibition of the $Na^+$, $K^+$-ATPase, which consumes much of the ATP in the brain cell. Under anoxic conditions there is a fall in

ATP concentration and it may become rate limiting for the phosphorylation of glucose and F-6-P. The inhibition of $Na^+$, $K^+$-ATPase results in an enhanced ATP level. This in turn results in greater availability of ATP for sugar phosphorylation reactions. Under these conditions, cation changes may play a less important part in the regulation of anaerobic glycolysis.

14. Amytal and reserpine also may increase the rate of anaerobic glycolysis of cerebral cortex slices. Preliminary results indicate that their action may be mediated through changes in the cation contents. Larger than anesthetic concentration of amytal are needed to produce a significant effect. It is less effective than TTX or local anesthetics. Further work is necessary to establish the mode of action of amytal or of reserpine.

15. Chlorpromazine, amphetamines and malamide have little or no stimulatory effects on the rate of anaerobic glycolysis of brain slices.

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