ON THE CONTROL OF BRAIN RESPIRATION BY NA^{+}, K^{+}-ADENOSINE TRIPHOSPHATASE

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ABSTRACT

ON THE CONTROL OF BRAIN RESPIRATION BY
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By

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Cellular metabolism in nervous tissue provides energy for the transport of sodium and potassium across cell membrane. Conversely, active transport of cations serves as a regulator of cellular energy utilization and respiration. By monitoring the respiration of tissue slices, effects of various drugs on Na^+,K^+-ATPase activity may be studied in intact cells. It has been posulated that the pharmacologic actions of cardiac glycosides, phenothiazines and monovalent cations such as lithium and rubidium involve the inhibition or stimulation of Na^+,K^+-ATPase activity. Most of the evidence supporting this hypothesis has been obtained with isolated ATPase preparations. The purpose of the present investigation was to formulate a method which accurately estimates the portion of tissue respiration associated with Na^+,K^+-ATPase activity, and test the hypothesis that cardiac glycosides, phenothiazine derivatives, lithium and rubidium alter Na^+,K^+-ATPase activity in intact brain cells.

The involvement of membrane Na^+,K^+-ATPase in the oxygen consumption of rat brain cortical slices was studied *in vitro*. Ouabain, a

specific inhibitor of Na^+,K^+-ATPase, markedly decreased both K^+-stimulated and non-stimulated brain slice respiration in Ca^{2+}-free Krebs Henseleit medium containing either glucose or pyruvate. The magnitude of inhibition by 100 μM ouabain was greater than the K^+-stimulated portion of respiration. In a Na^+-free medium, addition of 100 mM K^+ caused a depression of brain slice respiration while addition of 100 mM choline, or 100 μM ouabain had no effect. The replacement of Na^+ by choline in Ca^{2+}-free medium did not influence stimulation of slice respiration by 2,4-dinitrophenol. In a Na^+-free high K^+ medium, addition of Na^+ caused a stimulation of brain slice respiration. The magnitude of Na^+ stimulation was decreased in the presence of ouabain. The magnitude of Na^+-induced stimulation in a high K^+ medium was equal to that of the K^+-stimulation of slice respiration in Ca^{2+}-free Krebs medium, plus the K^+-depression of respiration in a Na^+-free medium. In Ca^{2+}-free Krebs medium, ouabain at 100 μM partially inhibited the 2,4-dinitrophenol stimulation of respiration. In this high Na^+ medium, ouabain significantly altered intracellular Na^+ and K^+ concentrations in a dose and time-dependent manner. The resulting increase in intracellular Na^+ produced by ouabain was similar to that causing a significant inhibition of optimal respiration of brain cortical homogenates. This suggests that ouabain might depress brain slice respiration by increasing intracellular Na^+ concentrations as well as by inhibiting Na^+,K^+-ATPase activity. A 40% portion of brain slice respiration observed in the presence of Na^+ and K^+ has been suggested to be associated with sodium-pump activity since it requires the simultaneous presence of Na^+ and K^+ analogous to the requirement of Na^+

and K^+ for ATP hydrolysis in the Na^+,K^+-ATPase system. The use of ouabain, however, results in an overestimation of the portion of respiration dependent on the sodium pump. The differences between slice respiration in high sodium high potassium medium, and that in sodium-free, high potassium medium has been shown to be a better estimate of sodium pump related respiration.

Among monovalent cations, only the combinations of those which stimulate Na^+,K^+-ATPase activity *in vitro*, stimulated brain slice respiration. Although lithium inhibited and rubidium stimulated brain slice respiration, these effects were observed only with concentrations markedly higher than those which would be achieved during lithium or rubidium treatment of patients.

Chlorpromazine markedly inhibited the portion of brain cortical slice respiration associated with Na^+,K^+-ATPase activity, but failed to affect that not related to the enzyme activity. Two chlorpromazine metabolites, 7-hydroxychlorpromazine and 7,8-dihydroxychlorpromazine, had no significant effects on either Na^+,K^+-stimulated or non-stimulated brain slice respiration *in vitro*. Chlorpromazine had no significant effect on either Na^+,K^+-stimulated slice respiration or Na^+,K^+-ATPase activity following a large dose of 30 mg/kg i.p. or chronic treatment of rats for 12 to 22 days with a daily dose of 30 mg/kg.

Intraperitoneal injection of digitoxin (0-30 mg/kg) in rats caused inhibition of both Na^+,K^+-stimulated brain slice respiration and brain homogenate Na^+,K^+-ATPase activity. No significant effects on Mg^{2+}-ATPase activity or non-Na^+,K^+-stimulated brain slice respiration was observed. Intraperitoneal administration of digitoxin at

high doses inhibits brain Na^+,K^+-ATPase activity in the rat, but pharmacological doses of chlorpromazine, lithium, or rubidium do not appear to affect Na^+,K^+-ATPase activity in intact brain cells.

ON THE CONTROL OF BRAIN RESPIRATION BY NA^+,K^+-ADENOSINE TRIPHOSPHATASE

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

A. Introduction

Cellular metabolism in nervous tissue provides energy for the transport of sodium and potassium against the electrochemical gradient existing across the cell membrane. Conversely, active transport of cations serves as a regulator of cellular energy utilization and respiration. Cardiac glycosides, phenothiazines and monovalent cations can alter rates of metabolism and respiration presumably via effects on the transport adenosine triphosphatase (Na^+,K^+-ATPase) enzyme. The purpose of the present studies was to determine if the pharmacologic actions of these agents involve the alteration of Na^+,K^+-ATPase. A method was developed to accurately estimate the portion of cellular respiration associated with membrane transport, and then the effects of cardiac glycosides, phenothiazines and monovalent cations on that portion of cellular respiration associated with Na^+,K^+-activated adenosine triphosphatase activity in brain were studied.

B. Review of Oxidative Phosphorylation and Factors Controlling Respiration

It is known from the work of many investigators (Engelhardt, 1930, 1932; Runnstrom *et al.*, 1934; Kalckar, 1937, 1939, 1944; Belitzer and Tsibakowa, 1939; Colowick, Welch and Cori, 1940; Colowick, Kalckar and Cori, 1941; Ochoa, 1941, 1943, 1947) that the

free energy of oxidative processes can be used for the synthesis of high energy organic phosphates. The most important high energy phosphate compound formed by these oxidative processes is adenosine triphosphate (ATP) (Krebs *et al.*, 1953). It is also widely known that the rate of mitochondrial respiration is primarily controlled by the concentration of phosphate acceptors, particularly adenosine diphosphate (ADP) produced by the hydrolysis of adenosine triphosphate (ATP) (Lardy and Wellman, 1952; Chance and Williams, 1955; Brody, 1955). Lardy and Wellman (1952, 1953) emphasized the role of microsomal ATPase as a phosphate acceptor-generating mechanism, and based on previous data by Kielley and Kielley (1951) suggested that if all ATPase activity was depressed and the system were tightly coupled, no respiration could occur unless phosphate acceptors were added (Lardy and Wellman, 1952). Thus, ATPase activity plays a major role in regulating energy generation, and hence cell respiration.

C. Brain Slice Respiration and the Role of Na^+,K^+-ATPase

The mechanism by which Na^+,K^+-ATPase regulates brain respiration involves the generation of ADP (McIlwain and Gore, 1951; Gore and McIlwain, 1952; Minakami *et al.*, 1963). Upon electrical stimulation of respiration brain slice inorganic phosphate increases, while creatine phosphate decreases (McIlwain and Gore, 1951; Gore and McIlwain, 1952; Heald, 1956). The increase in slice respiration is secondary to the breakdown of high energy organic phosphates (McIlwain and Gore, 1951). The resulting increase in phosphate acceptor is thought to be the factor stimulating slice respiration. Addition of potassium to brain slices stimulates respiration, increases ADP and

decreases cellular ATP (Minakami *et al.*, 1963). Whittam (1961), in experiments with homogenates, and Whittam and Blond (1964), in experiments with mitochondria and homogenates, demonstrated that a ouabain-sensitive extramitochondrial Na^+,K^+-ATPase served as a pacemaker for brain tissue respiration. Whittam and his colleagues (Whittam, 1962a; Whittam and Blond, 1964) estimated that the respiration associated with active transport and Na^+,K^+-ATPase activity in brain slices is about 40% of the respiration seen in calcium-free Krebs Henseleit medium. The method used by Whittam to estimate this portion of brain slice respiration, however, involved inhibition of so-called "non-stimulated" slice respiration by the Na^+,K^+-ATPase inhibitor ouabain. The method used by Whittam has obvious problems, since ouabain inhibition has been shown previously in Krebs Henseleit medium to increase with time (Wollenberger, 1947). In this dissertation, therefore, a new method using the difference between slice respiration in high sodium, high potassium medium, and that in a sodium-free, high potassium medium as a measure of Na^+,K^+-ATPase associated respiration, is proposed.

The rationale for this new method is based on the known sodium and potassium requirements for microsomal Na^+,K^+-ATPase activity (Skou, 1957; Hess and Pope, 1957; Skou, 1960; Deul and McIlwain, 1961). Both sodium (Gore and McIlwain, 1952) and potassium (Cummins and McIlwain, 1961) are necessary for electrical stimulation of brain slice respiration. This phenomenon is characteristic only in systems with intact cell membranes, and does not occur in homogenates (Whittam, 1962a; Quastel, 1965). In electrically depolarized guinea pig cortical slices, increases in sodium concentration in the medium are associated

with increases in intracellular sodium (Bachelard *et al.*, 1962). These are also associated with increases in slice respiration. In high sodium medium, increasing extracellular potassium concentration along with depolarizing brain slice membranes (McIlwain, 1951a; Li and McIlwain, 1957; Hillman and McIlwain, 1961) has been demonstrated to increase inorganic phosphate and decrease phosphocreatine (McIlwain, 1952). It has been hypothesized that the stimulation of respiration associated with potassium addition is the result of increasing intracellular sodium concentration, stimulation of sodium for potassium exchange, and stimulation of the generation of phosphate acceptor (Whittam, 1961, 1962a; Whittam and Blond, 1964). Thus there is reason to believe that a method using the difference between slice respiration in high sodium, high potassium medium, and that in a sodium-free, high potassium medium provides an accurate measure of the Na^+,K^+-ATPase regulated respiration in brain.

D. Monovalent Cations and Na^+,K^+-ATPase

Monovalent cations, particularly sodium and potassium, are important activators of enzymes in many living systems. The different ways in which cellular enzymes interact with these cations has been an important topic of scientific investigation for over a century (Grandeau, 1864). Nowhere have the subtle differences between sodium and potassium enzyme interactions been more extensively studied than with the membrane transport system Na^+,K^+-ATPase (Skou, 1957, 1960, 1965). Most of the significant work with this enzyme system to date has been limited to 1) studies of phosphorylation (Post *et al.*, 1965) and the Na^+,K^+-ATPase reaction cycle (Lindenmayer *et al.*,

1970), 2) studies on sodium and potassium transport (Glynn, 1957; Whittam and Wheeler, 1970), or 3) studies of the effects of drugs on Na^+,K^+-ATPase activity (Schwartz *et al.*, 1975). The Na^+,K^+-ATPase system has many other important functions in living systems. The preferential concentrating of potassium rather than sodium within the cell, the control of a large portion of the ATP utilized in the cell, the generation of electrochemical gradients across cell membrane, and indirectly the excitability of certain cell membranes are all consequences of the function of this enzyme (Post *et al.*, 1969). All of these effects, however, depend upon the selective interaction of this enzyme system with monovalent cations.

Since lithium or rubidium can alter the interaction of sodium and potassium with this important enzyme system, it is of pharmacological interest to study the effect of lithium, rubidium and other monovalent cations on Na^+,K^+-ATPase activity. In the presence of magnesium, ATPase activity is stimulated by sodium but not by potassium. In the presence of sodium and magnesium, ATPase activity is further stimulated by potassium. Potassium can be substituted for by rubidium, cesium and, to a lesser extent, lithium, but sodium cannot be substituted for by other monovalent cations (Skou, 1960).

In a later paper, Skou (1962) demonstrated that the abilities of monovalent cations to stimulate microsomal ATPase enzyme are similar in brain and kidney preparations from either rat, guinea pig or rabbit. He further showed that g-strophantin inhibits the activity of the ATPase enzyme in the presence of sodium, potassium and magnesium, but it has no effect on the activity with magnesium alone.

Baker and Connelly (1966) have shown that the sodium pump in crab nerve which is activated by potassium ions can be activated by a variety of other cations. Rang and Ritchie (1968) have extended this observation to rabbit desheathed vagus nerve. They have shown that thallium and rubidium are about as effective in stimulating respiration as potassium, while lithium and cesium are about half as effective. Akera and Brody (1971) have shown that the ability of the monovalent cations lithium, potassium, rubidium, cesium, and ammonium to inhibit the dissociation of ouabain from its ATPase complex can be roughly correlated with the reported ability of these cations to stimulate the ATPase activity in the presence of sodium and magnesium. Rubidium, cesium, and potassium were about equipotent inhibitors of dissociation of the ouabain-enzyme complex; thallous ion was less effective, while sodium and lithium were relatively ineffective.

In experiments studying partial reactions of Na^+,K^+-ATPase, Post *et al.* (1972) have shown that rubidium or lithium can replace potassium. They have shown that in a steady state, with concentrations of lithium and rubidium that produce equal accelerations of dephosphorylation, the level of dephosphoenzyme was higher in the presence of rubidium ion than in the presence of lithium ion. They interpret these higher levels of dephosphoenzyme to the fact that rephosphorylation is relatively inhibited in the presence of rubidium. According to the theory of Post *et al.* (1972), rubidium ions bind to ATPase similarly to potassium; it splits off a phosphate group from the enzyme, and then remains bound to the dephosphoenzyme as a relatively stable complex. This Rb^+-ATPase complex resists

rephosphorylation. This complex has been isolated kinetically by addition of a high concentration of sodium ion with oligomycin.

Tobin *et al.* (1974) have demonstrated that lithium, although a relatively poor substitute for potassium in the Na^+,K^+-ATPase reaction, can consistently activate the Na^+,K^+-ATPase in the presence of sodium and potassium. Rubidium, a more effective substitute for potassium than lithium, has been shown to be inhibitory in the presence of sodium and potassium. Lithium stimulates ATPase turnover by triggering dephosphorylation, and then rapidly dissociating from the dephosphoenzyme. In the opposite way, rubidium stabilizes the dephosphoenzyme, delays rephosphorylation of the ATPase enzyme, and inhibits its turnover.

The Na^+,K^+-ATPase enzyme system sharply discriminates between sodium, potassium, lithium, and rubidium (Hegyvary and Post, 1971). This transport enzyme system constitutes, then, one of the more probable points of biochemical interaction for monovalent cations (Ritchie and Strauss, 1957; Tobin *et al.*, 1974). Since lithium and rubidium ions respectively stimulate and inhibit Na^+,K^+-ATPase relative to its activity in the presence of sodium and potassium, it has been suggested that they may hyperpolarize or depolarize nerve cell membranes, respectively (Tobin *et al.*, 1974). Clinically lithium is useful as an anti-manic agent (Gershon, 1970), while rubidium is currently being investigated for its anti-depressant actions in man (Fieve *et al.*, 1973). It has been suggested that the actions of lithium and rubidium on the turnover of Na^+,K^+-ATPase may be their primary mechanism of pharmacological action (Tobin *et al.*, 1974). Na^+,K^+-ATPase, however, is exposed to asymmetric ionic environments

in vivo and data obtained with isolated enzyme systems, where such an environment is not reproducible, may not be extrapolated into *in vivo* situations. Thus, if lithium or rubidium's action involves Na^+,K^+-ATPase is not established.

E. Chlorpromazine

Chlorpromazine (10(3-dimethylaminopropyl)-2-chlorphenothiazine hydrochloride) was originally developed in the early 1950s in France as a drug useful for the management of anxiety, agitation and manic states in psychoneurotic and in psychotic patients (Courvoisier *et al.*, 1953; Delay *et al.*, 1952; Lehmann and Hanrahan, 1954). Winkelman (1954) concluded that chlorpromazine:

> can reduce severe anxiety, diminish phobias and obsessions, reverse or modify a paranoid psychosis, quiet manic and extremely agitated patients and change the hostile, agitated, senile patient into a quiet, easily managed patient.

More recently, chlorpromazine at extremely high doses has been widely used for the treatment of schizophrenia (see Goodman and Gilman, 1975).

Several investigators studying the mechanism of action of chlorpromazine initially observed that chlorpromazine inhibited carbohydrate metabolism in the central nervous system (Decourt, 1953; Laboritt, 1954). Norman and Hiestand (1955) reported that chlorpromazine increased blood sugar levels in mice and hamsters, while Lindaur (1956) reported a mild hyperglycemic effect in rabbits. Chlorpromazine was observed to intensify epinephrine-induced hyperglycemia and reduce insulin-induced hypoglycemia (Pravotorova and Smirnova, 1958). These authors also found that chlorpromazine significantly reduced the increase in *in vivo* oxygen consumption caused by injection of thyroxin in rats.

In vitro, chlorpromazine has been shown to inhibit the activity of various enzymes, including cholinesterase and acid phosphatase (Cruz, 1955), hyaluronidase (Mashkovskii *et al.*, 1955), hexokinase (Bernsohn *et al.*, 1956), and at high concentrations, mitochondrial ATPase (Abood, 1955; Century and Horwitt, 1956). Courvoisier *et al.* (1953) have reported that chlorpromazine diminishes the oxygen uptake of brain tissue *in vitro*. Several investigators have shown that chlorpromazine at certain concentrations will inhibit phosphorylation without affecting oxygen consumption, while at higher concentrations it inhibits respiration as well (Abood, 1955; Bernsohn *et al.*, 1956; Century and Horwitt, 1956). Abood (1955) was the first to report inhibition of phosphorylation and decrease in P:O ratios in rat brain mitochondria by chlorpromazine in concentrations of 50 μM. Brain mitochondrial respiration was inhibited by chlorpromazine only in higher concentrations of 0.2 mM (Abood, 1955). Chlorpromazine at high concentrations of 0.5 to 1 mM was subsequently shown to inhibit brain homogenate respiration (Bernsohn *et al.*, 1956). Chlorpromazine at a high concentration of 1 mM produced significant inhibition of cytochrome oxidase, hexokinase and Mg^{2+}-ATPase. Chlorpromazine in a concentration of 0.1 mM or less had no significant effect on the activity of any of these enzymes.

In vivo administration of 10 mg of chlorpromazine per kg had no significant effect on oxidative phosphorylation when measured subsequently in rat brain homogenates (Century and Horwitt, 1956). It also had no effect on non-stimulated brain slice respiration thirty minutes following treatment (Grenell *et al.*, 1955). A partial explanation for this may be that although chlorpromazine is readily adsorbed

to brain tissue, it is also readily desorbed (Kwant and Seeman, 1971). Alternatively, chlorpromazine may act by some other mechanism such as effects on phospholipids (Magee *et al.*, 1956) or membrane stabilization (Seeman and Bialy, 1963). Kaul *et al.* (1965) have demonstrated that chlorpromazine significantly lowers brain ATP levels *in vivo* at 3 hours following injection, but significantly raises brain ATP levels at 6 hours following injection. Therefore, the mechanism of action of chlorpromazine may indirectly involve energy-dependent processes or Na^+,K^+-ATPase. Since *in vitro* inhibition of Na^+,K^+-ATPase by chlorpromazine and chlorpromazine-free radical has been reported (Akera and Brody, 1968, 1969), it was decided to investigate if brain Na^+,K^+-ATPase is inhibited by chlorpromazine in intact cells, particularly after repeated *in vivo* administration.

Clinically the anti-psychotic actions of chlorpromazine develop slowly and dissipate slowly. Little or no direct relationship has been demonstrated to exist between anti-psychotic effects and plasma levels of the drug. This suggests that perhaps metabolites of the drug are active *in vivo*. In humans (Posner *et al.*, 1963) as well as in various animal species (Forrest *et al.*, 1968; Fishman and Goldenberg, 1963), chlorpromazine is metabolized to 7-hydroxy-, 8-hydroxy- and 7,8-dihydroxychlorpromazine. Goldenberg and Fishman (1964a, 1965) have shown that the 7-hydroxychlorpromazine is one of the principal metabolites in schizophrenic patients receiving chlorpromazine, while Manian *et al.* (1971) have demonstrated similar findings in the rat. Since some of these metabolites have been shown

to be potent inhibitors of Na^+,K^+-ATPase, their action was also studied (Akera *et al.*, 1974; Brody *et al.*, 1974).

F. Digitoxin

The importance of the central nervous system (CNS) as a site of digitalis action has been recognized since the work of William Withering (1785). Many of the toxicities associated with digitalis including blurred or abnormal vision (Smith, 1938; Hueper, 1945), vertigo (Weiss, 1932), headache (Luten, 1936), disorientation, delerium and coma (Willus, 1937) are known to be of central origin (Levitt *et al.*, 1970). Emesis as a toxic side effect of digitalis has been shown to be of central origin (Hatcher and Eggleston, 1912; Borison and Wang, 1951). Hatcher and Eggleston (1912) first demonstrated that digitalis could induce emesis in eviscerated animals, while Borison and Wang (1951) demonstrated that ablation of the chemoreceptor trigger zone abolishes digitalis-induced vomiting. Batterman and Gutner (1948) reported that digitalis produces additional neurological side effects including diplopia, amblyopia, scotomata, aphasia and epileptiform convulsions in man. Digitalis has been shown to depress the respiratory center in the CNS (Traube, 1851; Gross, 1914) as well as alter the response to stimulation of sites within the peripheral nervous system (Konzett and Rothlin, 1952). Temperature control and skeletal muscle tension have been shown to be affected by digitalis in the rat (Lendle and Oldenberg, 1950). Perhaps the most important potential neurological toxicities associated with digitalis, however, involve effects on cardiac rhythm.

The degree of neural involvement in digitalis-induced arrhythmias is still controversial. Erlij and Mendez (1964) have shown that sympathectomy increases the dose of digitoxin required to produce fatal arrhythmias. Similarly Boyajy and Nash (1966) have demonstrated that cats with sectioned spinal cords are resistant to the arrhythmogenic actions of ouabain. Levitt and Roberts (1966) have shown that drugs which normally counter digitalis-induced arrythmias fail to do so when the heart is deprived of sympathetic influences, while Gillis (1969) reports that ouabain produces changes in spontaneous activity in sympathetic nerves and suggests that this can be correlated with the development of cardiac arrhythmias. Subsequently, ouabain was reported by Gillis *et al.* (1972) to enhance traffic in vagus, sympathetic and phrenic nerves; and this enhancement was shown to be associated with the development of ventricular arrhythmias and respiratory hyperactivity. A number of investigators, however, have questioned these results and have reported that surgical or pharmacological interruption of the neural influences on the heart does not alter the induction of cardiac arrhythmias by digitalis (Morrow *et al.*, 1963; Koch-Weser, 1971). These workers subscribe to the hypothesis that all digitalis-induced rhythm changes in the heart are the result of direct effects of digitalis on myocardial tissue. At the present time it has not been definitively proven to what degree or by what mechanism cardiac glycosides affect cardiac rhythm.

The specific inhibition of Na^+,K^+-ATPase by cardiac glycosides administered *in vivo* has been studied in various tissues by a number of investigators (Hook, 1969; Akera *et al.*, 1969, 1970; Besch *et al.*, 1970; Allen *et al.*, 1970, 1971, 1975; Goldman *et al.*, 1973). Akera

et al. (1969, 1970) were the first to demonstrate inhibition of Na^+,K^+-ATPase *in vivo* in heart. They, however, reported that at the concentrations of ouabain studied, dog brain Na^+,K^+-ATPase was not inhibited. Not all species have been demonstrated to show inhibition of Na^+,K^+- ATPase *in vivo*. In experiments by Schwartz *et al.* (1974), Na^+,K^+-ATPase from cat heart was shown to be inhibited relative to controls at the time of peak inotropic response, but rabbit heart Na^+,K^+-ATPase was not shown to be inhibited. Cat brain Na^+,K^+-ATPase was shown to be unaffected by cardiac glycosides at doses of approximately 180 µg/kg (Weaver, 1975). The data of Schwartz *et al.* (1974) might be explained, based on *in vitro* data, in terms of a more rapid dissociation of cardiac glycosides from the rabbit heart enzymes. The negative results of Weaver (1975) may be due to a similar phenomenon, or may be due to an insufficiently high concentration of cardiac glycoside reaching the brain. This problem of access to the brain did not occur in experiments by Venturini and Palladini (1973), who reported significant inhibition of brain Na^+,K^+-ATPase activity in guinea pigs following direct intracranial injections of ouabain. In these experiments, ouabain produced a 52% inhibition of Na^+,K^+-ATPase activity one hour after injection, and a 40% inhibition of Na^+,K^+-ATPase activity 3 hours following intracranial injection (Venturini and Palladini, 1973). In these experiments brain Mg^{2+}-ATPase activity was unchanged. Therefore, under certain conditions, brain Na^+,K^+-ATPase activity has been reported to be inhibited by cardiac glycosides, and in other experiments no significant inhibition has been reported. It is not known, however, if administration of digitalis would cause an inhibition of rat brain Na^+,K^+-ATPase. Since it has been reported

that administration of digitalis in rats evokes signs of central excitation (Hatcher and Eggleston, 1919; Gold *et al.*, 1947), and since it is possible to administer high doses of digitoxin in this species due to low sensitivity of rat heart to digitalis, whereas brain Na^+,K^+-ATPase is relatively sensitive, the effects of high doses of digitoxin *in vivo* on rat brain Na^+,K^+-ATPase activity and sodium plus potassium-stimulated brain slice respiration were studied.

METHODS

A. Materials

Ouabain octahydrate (Strophanthin-G), digitoxin, yeast hexokinase, β-diphosphopyridine nucleotide (β-NAD), glucose, pyruvic acid, Tris-adenosine diphosphate (Tris-ADP), and Tris-adenosine triphosphate (Tris-ATP) were purchased from Sigma Chemical Company (St. Louis, Mo.). Chlorpromazine HCl was kindly supplied by Smith, Klein and French Laboratories (Philadelphia, Pa.). Chlorpromazine metabolites were the generous gift of Dr. A. A. Manian (NIMH, Rockville, Md.). 2,4-Dinitrophenol was purchased from the Olin-Matheson Chemical Company (Rutherford, N.J.). Inulin (Carboxylic acid-^{14}C-labeled) with specific radioactivity of 1.8 μCi/mg (approximately 9.5 mCi/mMole) was purchased from Amersham-Searle Corporation (Arlington Heights, Ill.). Nicotinamide, U.S.P., was obtained from Merck and Company (Rahway, N.J.). Choline chloride was obtained from Eastman Organic Chemicals (Rochester, N.Y.). Rubidium chloride, cesium chloride, and thallous nitrate (ultrapure grade) were purchased from Ventron Alfa Products (Beverly, Mass.). Sodium chloride, potassium chloride, magnesium sulfate, lithium chloride and all other reagents were of analytical reagent grade and were obtained from Mallinckrodt Chemical Works (St. Louis, Mo.).

B. Differential Respirometry Technique

Oxygen consumption was measured in microliters using the Barcroft technique (Stauffer, 1972) with a Gilson Differential Respirometer, maintaining a constant temperature by using a water bath, and shaking rapidly (100 oscillations/minute) to insure rapid exchange of oxygen between the fluid and gas phase. In order to assay the oxygen uptake a modification of the "direct method" of Warburg (1926) was employed, continuously absorbing the CO_2 with alkali during the determination. This was performed by placing 0.2 ml of 10% (w/v) KOH absorbed on filter paper in the center well of a 12.6 ml Warburg vessel. A 100% oxygen atmosphere was used in all experiments. By application of the universal gas law, $PV = n \cdot R \cdot T$, where P is pressure, V is volume, T is absolute temperature, n is the number of moles of gas, and R is a constant, respiration data were converted from microliters to micromoles of oxygen.

C. Brain Cortical Slice Technique

Male Sprague-Dawley rats usually weighing 200-300 g were obtained from Spartan Research Animals, Inc. (Haslett, Michigan). Rats were decapitated and their brains rapidly excised. The meninges were removed using a tissue moistened with medium, and brain cortical slices were cut freehand using a moistened slice and razor blade. All work was done on an ice-cooled aluminum block, and all slices when made were kept in an ice-cooled humidified chamber. Slices were immediately weighed and incubated for 20 minutes in a 100% oxygen atmosphere at 37°C for temperature and gas phase equilibration.

D. Studies on the "Potassium-Effect" and Ouabain's Effect on Brain Slice Respiration

Rat brain cortical slices were incubated in a 1.8 ml incubation medium containing 128 mM NaCl, 3 mM KCl, 1.23 mM $MgSO_4$, 15 mM Na-phosphate buffer (pH 7.40) and 24 mM glucose substrate. Oxygen uptake was measured manometrically at 37°C by the method described above. Control (basal) rates of brain slice respiration were measured for a 30-minute period. In the first series of experiments, 0.2 ml of either 1M KCl or 1M choline chloride in appropriate media was added to vessels which contained 1.8 ml of the above media with 0, 1, 10 or 100 µM ouabain (final potassium or choline concentration was 100 mM). Respiration was then assayed and expressed as µMoles of oxygen consumed/g tissue (wet weight)/half hour. In certain studies, 10 mM pyruvate was used instead of glucose as the substrate.

In other studies, 128 mM choline chloride and 15 mM Tris-phosphate buffer were substituted for NaCl and Na-phosphate buffer, respectively, to yield a sodium-free incubation medium. After a 30-minute incubation, 0.2 ml of choline chloride (1M), ouabain (1 mM), KCl (1M) or 2,4-dinitrophenol (0.5 mM) were added to slices in 1.8 ml of Na-free medium, to yield final choline or potassium concentrations of 100 mM, a ouabain concentration of 100 µM or a 2,4-dinitrophenol concentration of 50 µM. Respiration was assayed for an additional 30-minute period, and was expressed as a percent of respiration, setting the respiration observed during the pre-addition half hour period at 100%.

In further sodium-free medium studies, respiration of rat brain cortical slices was measured in a 1.8 ml medium containing 103 mM KCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate buffer (pH 7.4), 24 mM

glucose and either 0, 10 or 100 μM ouabain. Following a 30-minute incubation, 0.2 ml of either 1.28M NaCl or choline chloride (final concentration 128 mM) were added to high KCl media, and respiration was measured every 10 minutes for a half hour. In another experiment, slice respiration was measured in a sodium-free medium containing 100 mM KCl. Following a 30-minute initial incubation, sidearm additions were made yielding sodium concentrations of 0, 10, 20, 50 and 100 mM with choline chloride used as an osmotic substitute so that in all vessels cation concentration following addition was increased by 100 mM. In the final experiment in this series, 0.2 ml of 0.5 mM 2,4-dinitrophenol was added to vessels incubated in 1.8 ml of modified calcium-free Krebs Henseleit medium with or without 100 μM ouabain (final 2,4-dinitrophenol concentration was 50 μM). Post-addition half hour rates of slice respiration were again expressed as percent of pre-addition respiration.

E. Studies on Ouabain and Potassium Effects on Intracellular Cations

In the experiments studying changes in intracellular sodium and potassium concentrations, brain cortical slices were prepared and incubated in calcium-free Krebs solution with 0, 10 or 100 μM ouabain. Tracer amounts (1 μCi) of inulin (carboxylic acid-^{14}C-labeled) in 0.2 ml of incubation media were added to each vessel 15 minutes before the removal of tissue slices. Tissues were removed after 15, 25 and 35 minutes of 37°C incubation. They were blotted well, weighed, and then homogenized in 2 ml of double distilled water. Protein for each homogenate was assayed by the method of Lowry *et al.* (1951). Samples were diluted to 1 mg protein/ml concentration, and digested overnight

with equal volumes of nitric acid. Sodium and potassium content was then estimated using an Instrument Laboratory-Model 143 flame photometer according to the method of Pappius and Elliot (1956b). To estimate the amount of inulin trapped in the slices, 1 ml of each tissue homogenate was added to 0.2 ml of 50% (w/v) trichloroacetic acid (TCA), mixed well and centrifuged. One milliliter of the acid supernatant from each sample was then counted for ^{14}C radioactivity using a Beckman Model LS-100 liquid scintillation counter, with a 15 ml PCS cocktail (Amersham/Searle Corp.) as the liquid scintillation solution. One hundred microliters of each incubation medium was counted for ^{14}C. The densities of tissue slices were estimated by their sedimentation in various concentrations of sucrose; and from densities and tissue weights, total tissue slice volumes were calculated. From the ^{14}C-inulin data, extracellular volume in slices was calculated and this was subtracted from total slice volume to give intracellular volume. Knowing extracellular sodium and potassium concentrations, total extracellular and intracellular volumes, and total sodium and potassium concentrations, it was possible to calculate the intracellular brain slice sodium and potassium concentrations in milliequivalents/liter using the following formula:

$$(V_{total})(C_{total}) = (V_i)(C_i) + (V_o)(C_o)$$

V_{total} = total volume

V_i = intracellular volume

V_o = extracellular volume

C_{total} = total Na^+ or K^+ concentration

C_i = intracellular Na^+ or K^+ concentration

C_o = extracellular Na^+ or K^+ concentration

In a similar experiment studying changes in intracellular sodium in high potassium media, rat brain cortical slices were incubated for 20 minutes at 37°C. The media consisted of either calcium-free Krebs Henseleit medium with 24 mM glucose plus 100 mM potassium chloride, or a similar but calcium- and sodium-free medium, in which choline chloride and Tris-phosphate replaced sodium chloride and sodium phosphate, respectively. Tracer amounts (1 μCi) of inulin-(carboxylic acid-^{14}C labeled) were added 15 minutes before the removal of each slice, and intracellular sodium and potassium concentrations were estimated as described above.

F. Studies on the Effects of Sodium and Potassium on State 3 Brain Homogenate Respiration

In experiments designed to study the effects of Na^+ and K^+ on ADP-stimulated respiration of brain cortical homogenate, a variation of the technique outlined by Potter (1972) was used. Using a Potter-Elvehjem homogenizer and Teflon pestle driven at 800 rpm, 20% homogenates of rat brain cortical slices in 0.32M sucrose were prepared. All solutions and homogenates were kept on ice until incubation was begun. A sucrose homogenate (0.25 ml) was added to the incubation medium (1.75 ml) containing final concentrations of 2 mM Tris-ATP, 3 mM $MgCl_2$, 10 mM KH_2PO_4 buffer (adjusted to pH 7.2 with KOH), 10 mM glucose, 0.05 mM K_2-EDTA, 0.2 mM β-NAD, 40 mM nicotinamide and 40 μg of hexokinase enzyme (activity 18.5 units/mg protein; obtained from Sigma Chemical Company, St. Louis, Mo.).

In one series of experiments, sodium and potassium were added in concentrations ranging from 0 to 100 mM with background potassium

concentrations of 25 mM and background sodium concentrations near 0 mM. In these experiments, as the sodium concentration was increased, the potassium concentration was decreased. In another series of experiments, sodium and potassium concentrations were varied independently from 0 to 120 mM using choline chloride as an osmotic substitute to maintain the added cation concentration at 120 mM. The time from decapitation of the rat until the start of the incubation was approximately 17 minutes, while the total time before taking the first data point was approximately 30 minutes. The incubation was performed at 30°C with shaking speed of 100/minute in a 100% oxygen atmosphere. Homogenate respiration was measured manometrically for four 15-minute periods. Protein concentration was assayed in each homogenate using the Biuret method as described by Gornall *et al.* (1949).

G. Studies on the Effects of Monovalent Cations on Brain Slice Respiration

In the experiments studying the effects of various concentrations of monovalent cations on respiration, rat brain cortical slices were prepared and respiration was measured as previously descri-ed. Slices were incubated for 30 minutes at 37°C in 1.8 ml of a medium containing 128 mM NaCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate buffer (pH 7.4) and 24 mM glucose. Either potassium, rubidium or cesium was then added to yield 2 ml final volume, and final concentrations of 0 to 100 mM. Slice respiration was then assayed for four half-hour periods, and expressed as a percent of respiration observed during the pre-addition half-hour period.

In two series of experiments studying the effects of ouabain on rubidium and cesium stimulation of slice respiration, rat brain cortical slices were prepared and the respiration was assayed as described above. Slices were incubated for 30 minutes at 37°C in 1.8 ml of a medium containing 128 mM NaCl, 3 mM KCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate (pH 7.4), and 24 mM glucose, plus either 0, 1, 10 or 100 μM ouabain. To these media, 0.2 ml of either 1M rubidium chloride or choline chloride, or in the second series of experiments 1M cesium chloride or choline chloride was added to yield final rubidium, cesium or choline concentrations of 100 mM. Slice respiration was measured for four post-addition half-hour periods.

The initial experiment studying the effects of lithium on brain slice respiration involved a medium containing 150 mM NaCl, 1.5 mM KCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate (pH 7.4) and 24 mM glucose. Brain cortical slice respiration was assayed during an initial half-hour incubation at 37°C, followed by addition of lithium chloride in final concentrations of 0 to 30 mM. Slice respiration was measured for four post-addition half-hour periods, and expressed as μMoles of oxygen/g tissue (wet weight)/half hour.

In the experiment studying the effects of lithium on sodium stimulation of brain slice respiration, the medium contained 100 mM KCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate (pH 7.4), and 24 mM glucose with or without 20 mM lithium chloride. Respiration was measured during a half-hour period at 37°C, then sodium chloride was added yielding final concentrations of 0 to 100 mM. Respiration was measured for four succeeding half-hour periods and expressed as a percent of initial pre-addition half-hour respiration.

The techniques used in experiments studying the effects of lithium, rubidium and cesium on rat brain homogenate respiration were similar to those used in studies on the effect of sodium and potassium on optimal brain homogenate respiration. In each experiment lithium, rubidium or cesium were varied from 0 to 100 mM using choline chloride as an osmotic substitute to maintain added cation concentration at 100 mM. Background potassium concentration was approximately 25 mM; background sodium concentration was zero. Homogenate respiration was assayed at 30°C for four 15-minute periods and expressed as microliters or micromoles of oxygen consumed/mg protein/15 minutes.

In the experiments studying the effects of thallous ion on brain slice respiration, incubation media containing 128 mM NaCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate (pH 7.4) and 24 mM glucose with or without 100 mM KCl were used. Following a 20-minute preincubation, brain slice respiration was assayed for four half-hour periods at 37°C. Thallous ion concentrations ranged from 0 to 3 mM in high sodium, high potassium medium and from 0 to 10 mM in the potassium-free medium. Respiration rates were expressed in micromoles of oxygen consumed/g tissue (wet weight)/half hour.

In the final experiment studying the effects of monovalent cations on respiration in the presence of high concentrations of potassium, rat brain cortical slices were incubated at 37°C in 1.8 ml of a medium containing 100 mM KCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate (pH 7.4) and 24 mM glucose. Either sodium, lithium, rubidium, cesium or choline chloride was then added to yield final concentrations of 100 mM. Slice respiration was measured for four half-hour periods,

and expressed in micromoles of oxygen consumed/g tissue (wet weight)/ half hour.

H. Studies on the Effect of Chlorpromazine *in vivo* and *in vitro* on Brain Slice Respiration and ATPase Activity

In the experiments studying the effects of chlorpromazine or chlorpromazine metabolites on rat brain slice respiration *in vitro*, brain slices were prepared and respiration was assayed as described above. Slices were incubated for an initial half-hour period at 37°C in a medium containing 128 mM NaCl, 103 mM KCl, 1.23 mM $MgSO_4$, 15 mM Na-phosphate buffer (pH 7.4) and 24 mM glucose (high sodium, high potassium medium), or in a similar but sodium-free medium in which 128 mM choline chloride and 15 mM Tris-phosphate buffer were substituted for sodium chloride and sodium-phosphate, respectively. Chlorpromazine or its metabolites, 7-hydroxychlorpromazine and 7,8-dihydroxychlorpromazine, were then added in final concentrations of 0, 1, 10 or 100 μM, and slice respiration was assayed for an additional 2, 3, or 4 half-hour periods. Respiration was expressed as micromoles of oxygen/g tissue (wet weight)/half hour. The differences between slice respiration in high sodium, high potassium medium, and in low sodium, high potassium (sodium-free, choline containing) medium, were calculated as a measure of the sodium, potassium-stimulated respiration.

In the *in vivo* experiments studying the acute effects of chlorpromazine on slice respiration and brain ATPase activity, male Sprague-Dawley rats (250-350 g) were injected intraperitoneally with chlorpromazine hydrochloride in saline in a dose of 30 mg/kg.

Control rats received equal volumes of 0.9% saline per kg of body weight. Rats were sacrificed 30 minutes after the injection, and cortical slices and whole brain homogenates were prepared. Slice respiration was measured during a two-hour incubation period at 37°C in 2 ml of either high sodium, high potassium medium or sodium-free, high potassium medium. Slice respiration was expressed in micromoles of oxygen/g tissue (wet weight)/half hour. Differences between rates of respiration in high sodium, high potassium medium and sodium-free, high potassium medium were calculated as the sodium, potassium-stimulated portion of brain slice respiration. In the ATPase activity assays, 5% whole brain homogenates were prepared at 0°C from control and chlorpromazine-treated rats as described previously using a solution containing 250 mM sucrose, 5 mM histidine buffer (pH 7.0), and 1 mM buffered EDTA. Na^+,K^+-ATPase and Mg^{2+}-ATPase activities of the homogenates were assayed immediately from the amount of inorganic phosphate liberated from ATP during a 10-minute incubation at 37°C. Na^+,K^+-ATPase activity is the difference in ATPase activities assayed in the presence of 100 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, 5 mM Tris-ATP and 50 mM Tris-HCl buffer (pH 7.4) (total ATPase activity) and that assayed in the presence of 5 mM $MgCl_2$, 5 mM Tris-ATP and 50 mM Tris-HCl buffer (pH 7.4) (Mg^{2+}-ATPase activity). Protein was estimated using bovine serum albumin as standard by the method of Lowry *et al.* (1951).

In the series of experiments in which chronic effects of chlorpromazine were studied, male Sprague-Dawley rats (body weight between 150 and 175 g) were injected intraperitoneally with chlorpromazine hydrochloride in a daily dose of 30 mg/kg. Control rats

received equivalent volumes of saline per kg of body weight. Control and chlorpromazine-treated rats were sacrificed 30 minutes after injection on days 12, 13, 21 and 22 of the chronic treatment. Brain slice respiration was assayed and the differences between rates of respiration in high sodium, high potassium medium and those in sodium-free, high potassium medium were calculated to estimate the sodium, potassium-stimulated portion of slice respiration. Na^+,K^+-ATPase and Mg^{2+}-ATPase activities of rat brain homogenates from control and chronically chlorpromazine-treated rats were assayed as described in the acute chlorpromazine study.

I. Studies on the Effects of Digitoxin *in vivo* on Brain Slice Respiration and ATPase Activity

In the experiments in which *in vivo* effects of digitoxin were studied, male Sprague-Dawley rats weighing 200-250 g were injected intraperitoneally with various doses of digitoxin dissolved in 100% ethyl alcohol. Control rats received equal volumes of ethyl alcohol. Rats were sacrificed 30 minutes after injection, and cortical slices and whole brain homogenates were prepared. In the brain slice respiration study, cortical slices were incubated at 37°C in 2 ml of either medium containing 128 mM NaCl, 103 mM KCl, 1.23 mM $MgSO_4$, 15 mM Na-phosphate buffer (pH 7.4) and 24 mM glucose, or similar medium in which 128 mM choline chloride and 15 mM Tris-phosphate buffer replaced NaCl and Na-phosphate buffer, respectively, to yield a sodium-free incubation medium. Respiration rates in both high sodium, high potassium and sodium-free, high potassium media were assayed and expressed in terms of micromoles of oxygen/g tissue (wet weight)/

half hour. Differences between respiration rates in these two media were calculated. These sodium, potassium-stimulated respiration rates of brain slices obtained from digitoxin-treated rats were expressed as percent of control slice respiration observed with matched control animals. In the ATPase activity experiments, 5% whole brain homogenates were prepared as described in the chlorpromazine experiments. Homogenization was performed at 0°C in a medium containing 250 mM sucrose, 5 mM histidine buffer (pH 7.0), and 1 mM buffered EDTA. Na^+,K^+-ATPase and Mg^{2+}-ATPase activities of the homogenates were estimated immediately from the amount of inorganic phosphate liberated from ATP during a 10-minute incubation at 37°C. Na^+,K^+-ATPase activity is the difference in ATPase activities assayed in the presence of 100 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, 5 mM Tris-ATP and 50 mM Tris-HCl buffer (pH 7.4) (total ATPase activity), and that assayed in the presence of 5 mM $MgCl_2$, 5 mM Tris-ATP and 50 mM Tris-HCl buffer (pH 7.4) (Mg^{2+}-ATPase activity). Protein was estimated using bovine serum albumin as standard by the method of Lowry *et al.* (1951). Percent inhibition of Na^+,K^+-ATPase activity in digitoxin-treated rats was calculated relative to the Na^+,K^+-ATPase activity in matched control rats assayed concurrently. Similar calculations were made for percent inhibition of Mg^{2+}-ATPase activity.

J. Statistics

Statistical analyses, unless otherwise stated, were by random design, or randomized complete block analysis of variance (Sokal and Rohlf, 1969). The Student-Newman-Keuls (SNK) test was used to

determine significant differences between means. The accepted level of significance in all experiments was $p<.05$ (Rohlf and Sokal, 1969).

RESULTS

A. The Effects of Potassium and Ouabain on Brain Slice Respiration

In vitro brain slice studies have shown that potassium stimulates (Ashford and Dixon, 1935), and ouabain inhibits (Whittam, 1962a), tissue respiration. It has been proposed that inhibition by ouabain can be used to estimate that portion of slice respiration related to the Na^+,K^+-ATPase activity (Whittam and Blond, 1964). Therefore, the relationship between the "potassium effect", ouabain inhibition and Na^+,K^+-ATPase related respiration was investigated.

The first experiment of this series examined the effects of various concentrations of ouabain on potassium stimulated and "non-stimulated" brain slice respiration in a calcium-free medium using glucose substrate (Figure 1). Control brain slice respiration during the pre-addition half hour was 53.7 µMoles of oxygen/g tissue (wet weight). The addition of 100 mM potassium chloride stimulated brain cortical slice respiration in the absence of ouabain approximately 30%. Addition of up to 100 µM ouabain to the incubation mixtures caused a decrease in "non-stimulated" oxygen consumption. Although the decrease to 49.9 µMoles of oxygen/g tissue (wet weight)/half hour with 100 µM ouabain during the first half-hour period was not statistically significant, the magnitude of ouabain inhibition increased with time. Thus the inhibition of non-stimulated brain slice oxygen

Figure 1. Effects of potassium and ouabain on brain cortical slice respiration in modified calcium-free Krebs Henseleit medium with glucose substrate.

Respiration of rat brain cortical slices was assayed in a medium containing 128 mM sodium chloride, 15 mM sodium phosphate buffer (pH 7.4), 3 mM potassium chloride, 1.23 mM magnesium sulfate and 24 mM glucose at 37°C. Following a half-hour control incubation, 0.2 ml of 1M potassium chloride or choline chloride in respective media was added to vessels containing 1.8 ml of medium with 0, 1, 10 or 100 µM ouabain (final potassium or choline concentration was approximately 100 mM). Respiration was measured for one half hour, and expressed as µMoles of oxygen consumed/g tissue (wet weight)/half hour. Data shown by the total bars represent mean slice respiration in 4 experiments with potassium. Data shown by the shaded bars represent slice respiration in paired experiments with choline, while the open portions represent the differences between respiration with 100 mM potassium and 100 mM choline. Vertical lines indicate S.E.M. Statistical analysis was by randomized complete block analysis of variance using the Student-Newman-Keuls (SNK) test to determine significant differences between means. Respiration with 100 mM choline was significantly inhibited by 100 µM ouabain. *Denotes significant potassium stimulation ($p<0.05$).

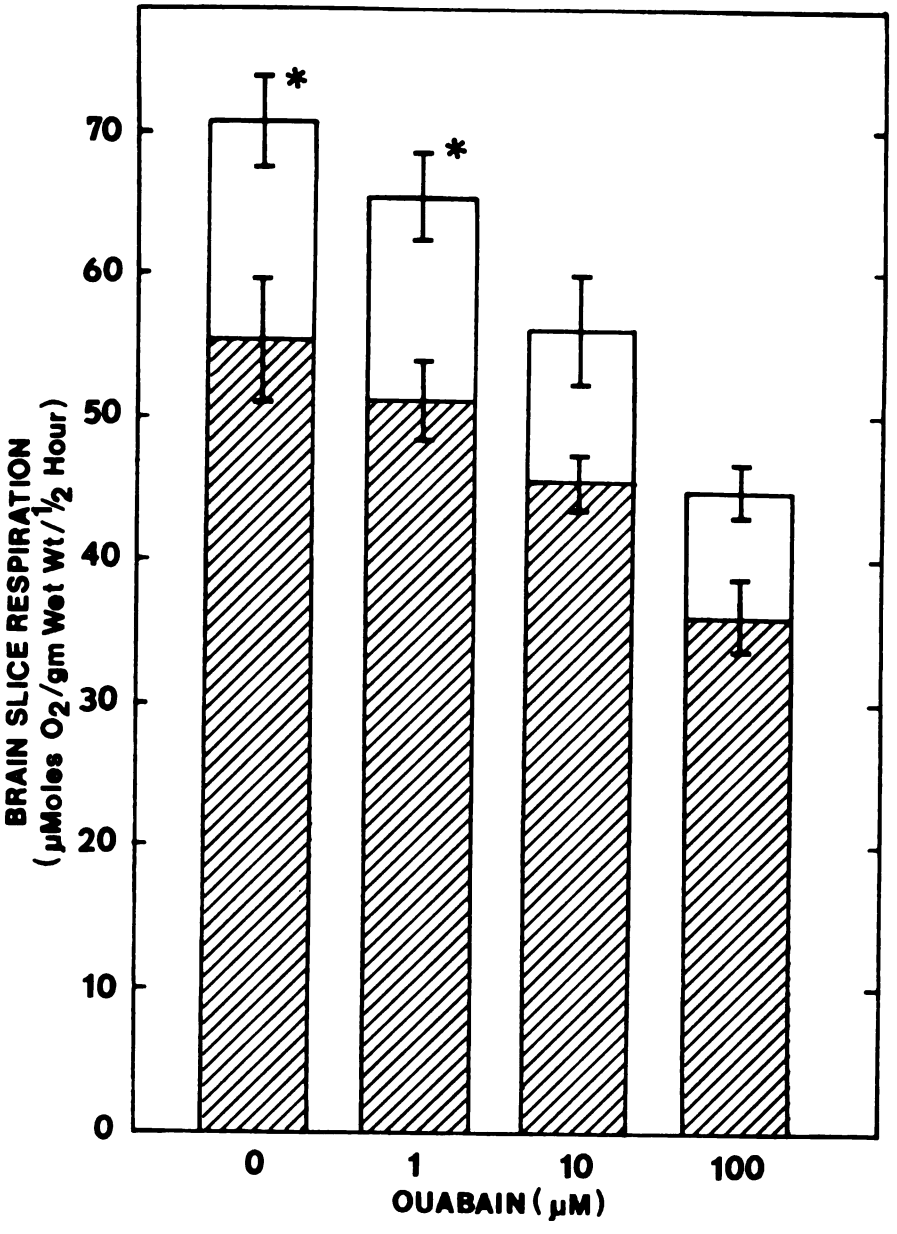
BRAIN SLICE RESPIRATION
(μMoles O_2/gm Wet Wt./½ Hour)
70
60
50
40
30
20
10
0
*
*
0
1
10
100
OUABAIN (μM)

Figure 1

consumption by 100 μM ouabain was significant by the first half hour after the addition of choline chloride, i.e., at about one hour following the addition of brain slices to incubation mixtures containing ouabain. The inhibition by 1 and 10 μM ouabain of basal respiration was not statistically significant. In the presence of 10 and 100 μM ouabain, there was a significant inhibition of brain slice respiration in the presence of 100 mM potassium chloride. In the presence of 0 and 1 μM ouabain, stimulation of brain slice respiration by 100 mM potassium was significant. Although ouabain inhibited both potassium-stimulated and non-stimulated slice respiration, the inhibition by ouabain was greater for the potassium stimulated portion of the respiration. It should be noted, however, that ouabain in high concentrations can inhibit both potassium stimulated brain slice respiration, and also basal brain slice respiration significantly (Figure 1).

Since ouabain's effects on brain slice respiration may be the result of the inhibition of sugar transport by this agent which may decrease the respiration by decreasing the amount of available substrate (glucose) for respiration, similar experiments were repeated with a similar medium containing pyruvate as substrate instead of glucose. The control pre-addition respiration was 60.5 μMoles of oxygen/g tissue (wet weight)/half hour (Figure 2). In a medium containing pyruvate as substrate, the addition of 100 mM potassium chloride again significantly stimulated brain slice respiration. The rate of respiration during the first half-hour period following potassium addition was increased by 26% relative to choline controls in the presence of 10 mM pyruvate. Ouabain at a concentration of

Figure 2. Effects of potassium and ouabain on brain cortical slice respiration in modified calcium-free Krebs Henseleit medium with pyruvate substrate.

Incubation conditions were identical to those for slices described in Figure 1, except that 10 mM pyruvate replaced glucose as substrate. Data shown by the total bars represent the means of 4 experiments with 100 mM potassium. Data shown by the shaded bars represent mean slice respiration in paired experiments with 100 mM choline, while the open portions represent the difference between slice respiration with potassium and choline. Vertical lines indicate S.E.M. Statistical analysis was as described in Figure 1. Respiration with 100 mM choline was significantly inhibited by 100 µM ouabain. *Denotes significant potassium stimulation ($p<.05$).

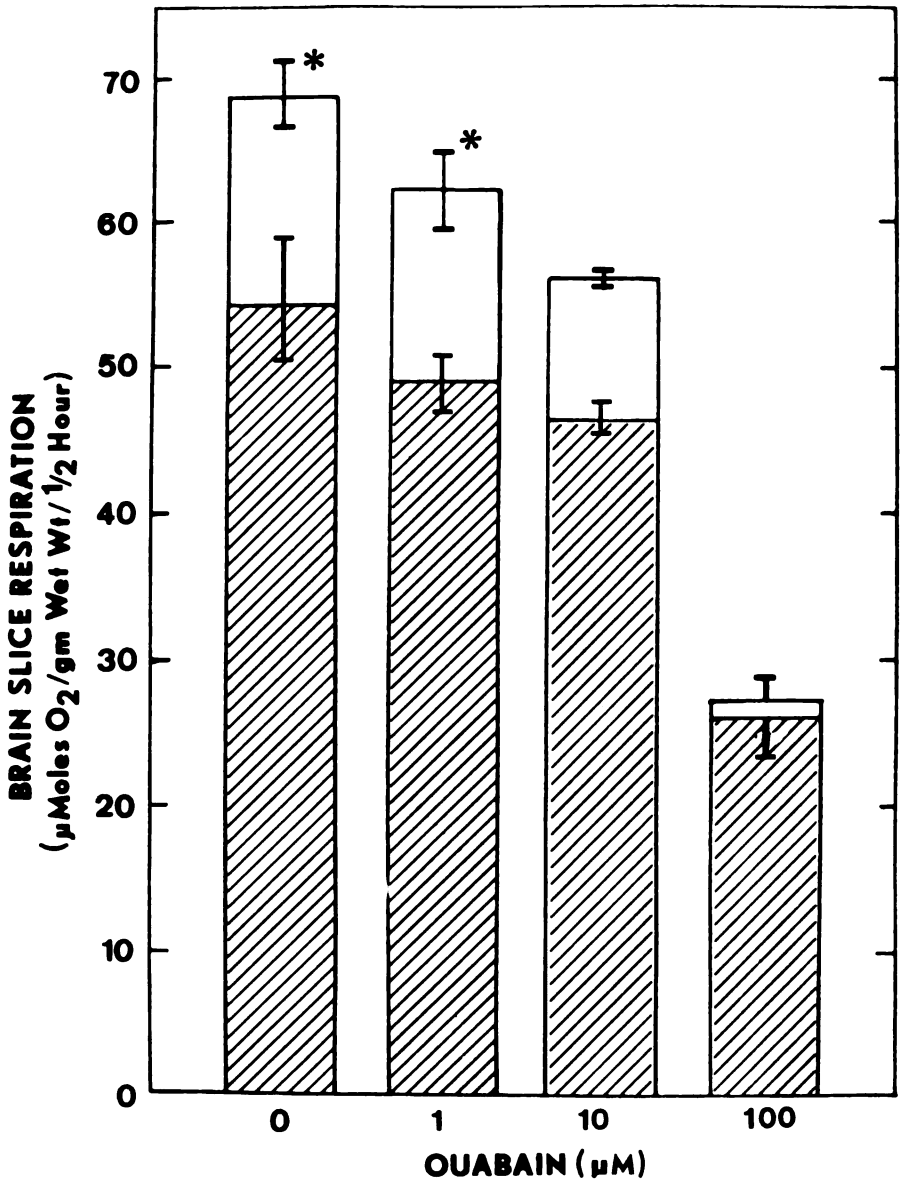
BRAIN SLICE RESPIRATION
(μMoles O2/gm Wet Wt/ 1/2 Hour)
70
60
50
40
30
20
10
0
*
*
0
1
10
100
OUABAIN (μM)

Figure 2

100 μM significantly inhibited slice respiration following choline addition in medium with pyruvate substrate, while 1 and 10 μM ouabain did not. When pyruvate was the substrate, there was no significant stimulation of brain slice respiration by 100 mM potassium in the presence of 10 and 100 μM ouabain. Therefore, it may be concluded that ouabain in high concentrations can inhibit both basal and potassium stimulated brain slice respiration in a medium with pyruvate substrate.

In order to substantiate the contention that the ouabain-induced decrease in brain slice respiration results from ouabain inhibition of Na^+,K^+-ATPase activity, studies on oxygen consumption in sodium-free medium were undertaken (Figure 3). In this medium, choline chloride replaced sodium chloride and Tris-phosphate buffer replaced sodium-phosphate buffer. The mean control level of slice respiration during the first half-hour period in sodium-free (choline) medium with glucose as substrate was 44.7 μMoles of oxygen/g tissue (wet weight)/half hour (Figure 3). This was significantly lower by Student's t-test ($p<.05$) than the 53.7 μMoles of oxygen/g tissue (wet weight)/half hour observed in high sodium medium with glucose as substrate (Figure 1). Following the addition of 100 mM choline chloride, brain slice respiration was 94.8% of pre-addition control respiration (Figure 3). In the ouabain containing medium, respiration following the addition of choline chloride was 93.4% of pre-addition respiration. Whereas addition of 100 mM potassium to high sodium medium caused a significant stimulation of brain slice respiration (Figures 1 and 2), the addition of potassium to a sodium-free medium caused a depression of slice respiration (Figure 3). The fact

Figure 3. Effects of ouabain, potassium, and 2,4-dinitrophenol on brain slice respiration in sodium-free (choline) medium.

Respiration of rat brain cortical slices was measured in medium containing 128 mM choline chloride, 15 mM Tris-phosphate buffer (pH 7.4), 3 mM potassium chloride, 1.23 mM magnesium sulfate and 24 mM glucose at 37°C. Preincubation conditions were the same as described in Figure 1. Following half-hour control incubation, either 0.2 ml of choline medium (C), 1M potassium chloride (KCl) (final concentration was approximately 100 mM), 0.5 mM 2,4-dinitrophenol (DNP) (final concentrations 50 μM) or 1 mM ouabain (Q) (final concentration, 100 μM) was added. Slice respiration was measured for one half hour, and expressed as a percent of pre-addition respiration. Data represent the means of 10 experiments. Vertical lines indicate S.E.M. Statistical analysis was by completely random design analysis of variance using the Student-Newman-Keuls (SNK) test to determine significant differences between means. *Denotes significantly different from control ($p<.05$).

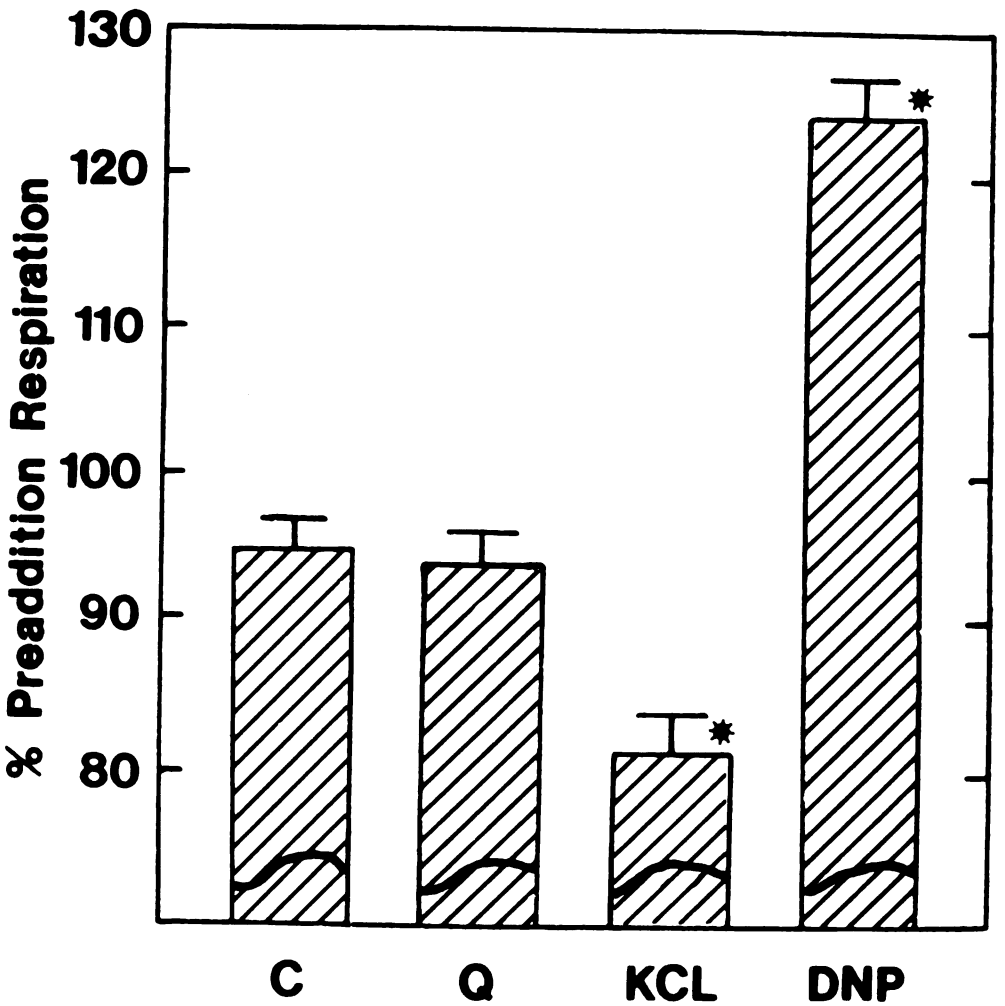

Figure 3

that there was no stimulation of brain slice respiration upon addition of 100 mM potassium to sodium-free medium suggests that there is a specific sodium requirement for the potassium effect. Addition of 2,4-dinitrophenol to brain slices in sodium-free medium resulted in a significant stimulation of respiration, similar to the stimulation by 2,4-dinitrophenol in calcium-free Krebs medium (see Figure 7), indicating that brain slices are capable of respiring at the same rate in sodium containing and sodium-free media, once respiratory control is removed (Figure 3).

Since potassium failed to stimulate brain slice respiration in sodium-free media, an attempt was made to demonstrate the requirement for the simultaneous presence of sodium and potassium for the stimulation of slice respiration. To do this, a medium in which sodium had been replaced with 103 mM potassium was used. The average pre-addition brain slice respiration in this medium with glucose as substrate was 41.6 μMoles of oxygen/g tissue (wet weight)/half hour. This was significantly lower by Student's t-test ($p<.05$) than pre-addition respiration in high sodium medium experiments shown in Figure 1. Addition of 128 mM sodium chloride in high potassium medium produced a significant stimulation of respiration when compared to brain slice respiration following the addition of choline chloride in the absence of ouabain (Figure 4). In high potassium medium, the stimulation by sodium was markedly inhibited in the presence of 10 and 100 μM ouabain. Thus, ouabain inhibited respiration associated with potassium addition to high sodium medium (Figure 1) and also slice respiration associated with sodium addition to a high potassium medium (Figure 4). Ouabain also inhibited brain slice respiration in

Figure 4. Effects of ouabain on sodium-stimulated brain cortical slice respiration in high potassium medium.

Respiration of rat brain cortical slices was measured in medium containing 103 mM potassium chloride, 15 mM Tris-phosphate buffer (pH 7.4), 1.23 mM magnesium sulfate, 24 mM glucose, and either 0, 10 or 100 μM ouabain. Preincubation conditions were as described in Figure 1. Either 0.2 ml of 1.28M sodium (Na) or choline (C) chloride (final concentrations were 128 mM) were added after the half-hour control incubation. Respiration was measured every 10 minutes for a period of one half hour. Percentages of pre-addition respiration were calculated with data representing the means of 10 experiments. Vertical lines indicate S.E.M. Statistical analysis was performed as described in Figure 3. *Denotes significant sodium stimulation ($p<.05$).

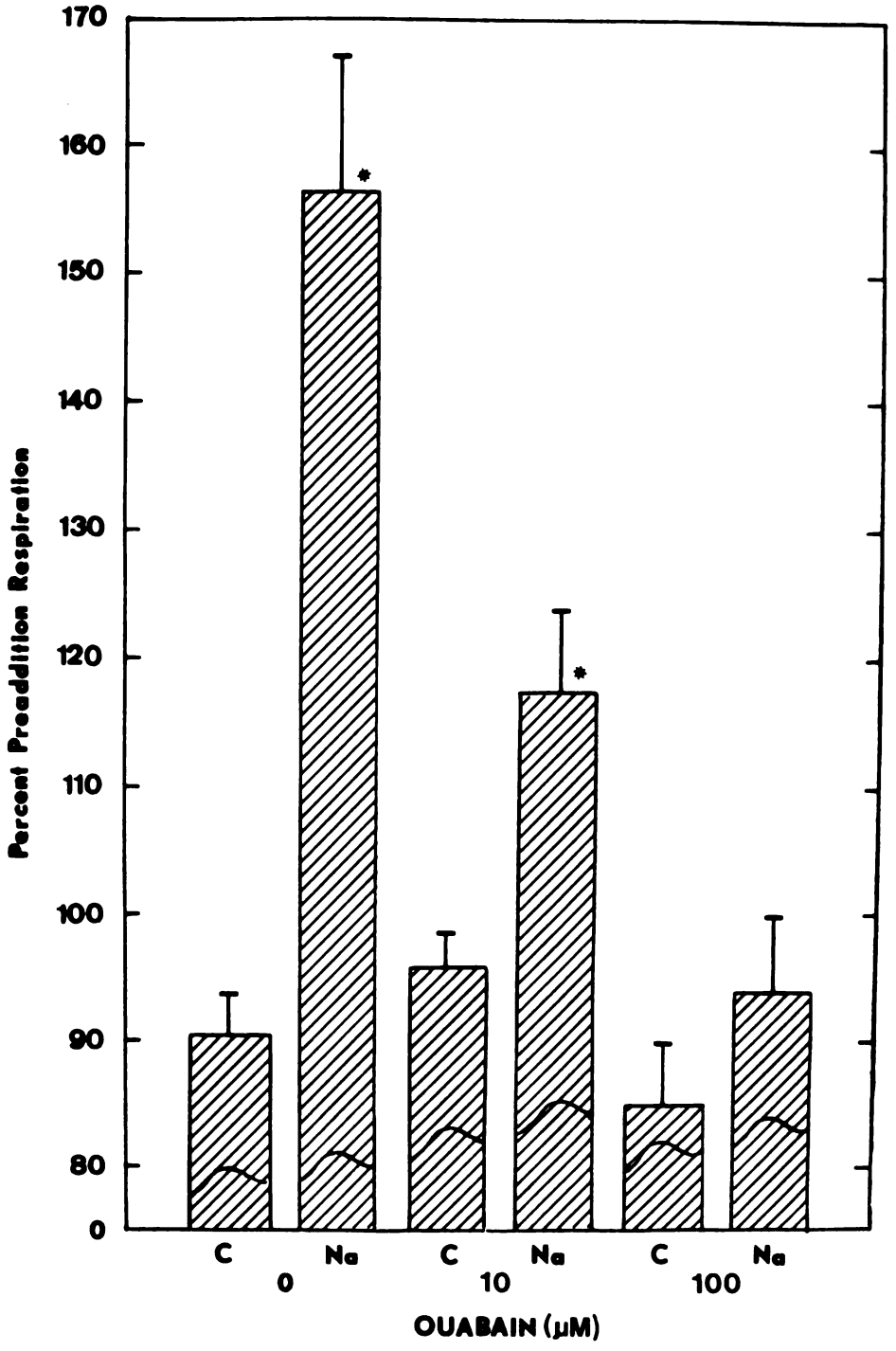
170
160
150
140
130
120
110
100
90
80
0
Percent Preaddition Respiration
*
*
C
Na
C
Na
C
Na
0
10
100
OUABAIN (μM)

Figure 4

a high sodium, low potassium medium, but failed to affect the respiration in a sodium-free, high potassium medium.

The timecourse of sodium effect in high potassium medium in the presence and absence of ouabain is shown in Figure 5, in which respiration data from these same slices during each of three consecutive ten-minute periods following sidearm addition of sodium or choline are plotted. In the absence of ouabain, slice respiration during each ten-minute period following sodium addition was significantly higher than slice respiration after choline addition. In medium containing 10 and 100 μM ouabain, slice respiration was somewhat higher following the addition of sodium than following the addition of choline during the first and second ten-minute periods. With both ouabain concentrations, slice respiration decreased with time. The decrease in the presence of 100 μM ouabain was the most rapid. By the third ten-minute period following the addition of sodium, slice respiration in the presence of 10 and 100 μM ouabain approached slice respiration rates observed in the choline control. Thus, the sodium-induced stimulation was transient and dissipated rapidly in the presence of high concentrations of ouabain. This would indicate that the action of ouabain to inhibit sodium-stimulated respiration develops relatively slowly following the addition of sodium to sodium-free, high potassium incubation media.

In the next experiment, the concentration dependency of sodium stimulation of brain slice respiration in a medium containing 100 mM potassium, 1.23 mM magnesium, 15 mM Tris-phosphate buffer (pH 7.4) and 24 mM glucose was studied. The average rate of respiration during the first half-hour period in sodium-free, high potassium medium

Figure 5. Time course of ouabain's effect on sodium-stimulated brain cortical slice respiration in high potassium medium.

Data are from the same slices as described in Figure 4, and show respiration plotted on the abscissa with time plotted on the ordinate. Preincubation conditions were as described in Figure 1. Brain slice respiration was measured in a medium containing 103 mM potassium chloride, 15 mM Tris-phosphate buffer (pH 7.4), 1.23 mM magnesium sulfate and 24 mM glucose for three 10 minute periods following the addition of 128 mM sodium chloride ●——●, or 128 mM choline chloride □——□. In certain vessels 10 μM ouabain ●— — —●, or 100 μM ouabain ●- - - -● were present prior to 128 mM sodium chloride addition. Data represent the means of 10 experiments. Vertical lines indicate S.E.M. Statistical analysis was performed as described in Figure 3. *Denotes significant sodium stimulation relative to choline control.

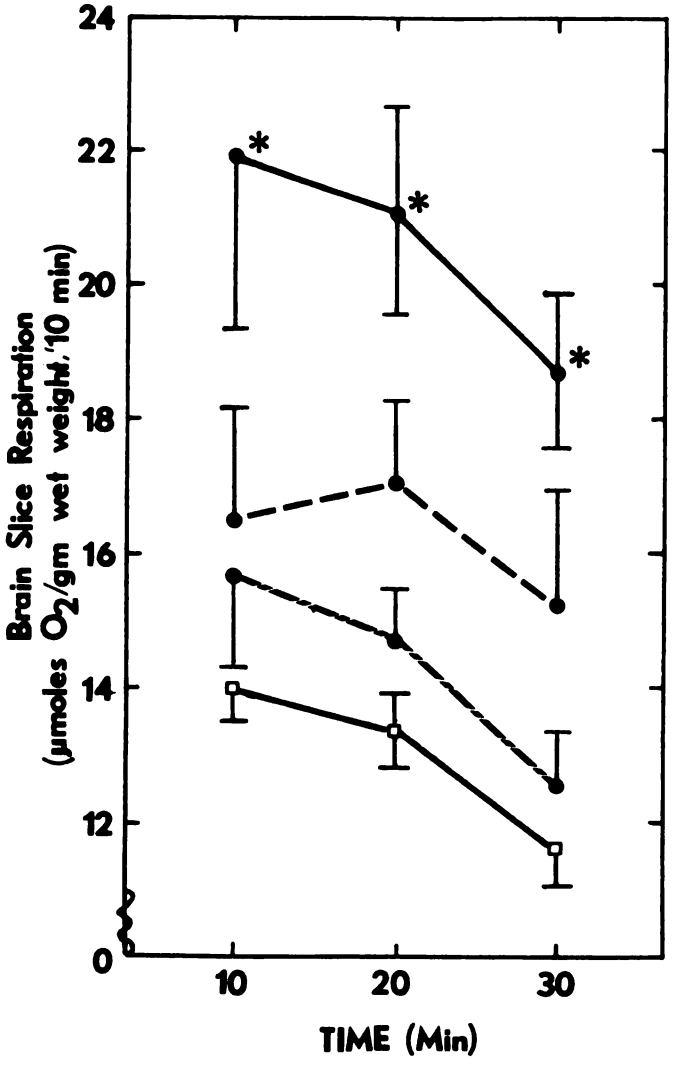
24
22
20
18
16
14
12
0
10
20
30
TIME (Min)
Brain Slice Respiration
(μmoles O_2/gm wet weight/10 min)
*
*
*

Figure 5

with glucose as a substrate was 40.5 Moles of oxygen/g tissue (wet weight)/half hour (Figure 6). Addition of 10 to 100 mM sodium stimulated slice respiration 21% to 71%, respectively. Slice respiration with 50 mM or 100 mM sodium was significantly higher than that observed with 10 mM or 20 mM sodium. This concentration dependent stimulation of slice respiration by sodium in 100 mM potassium medium decreased with time, but it continued to be significant relative to choline controls for at least two hours following the addition of sodium. The magnitude of stimulation by 100 mM sodium from approximately 40 μMoles of oxygen/g tissue (wet weight)/half hour to approximately 68 μMoles of oxygen/g tissue (wet weight)/half hour was greater than that of the potassium-induced stimulation of respiration in high sodium media (Figure 1). The magnitude of the sodium-induced stimulation was equal to that of the potassium stimulation in high sodium medium (Figure 1), plus the potassium-induced depression of respiration in sodium-free medium (Figure 3). The respiratory rate in medium containing 100 mM potassium of 52 μMoles of oxygen/g tissue (wet weight)/half hour following the addition of 20 mM sodium plus 80 mM choline (Figure 6) was approximately equivalent to the rate of slice respiration observed initially in high sodium medium with glucose as a substrate. Thus, in medium containing 100 mM potassium, the rate of brain slice respiration with sodium is dependent on the sodium concentration.

Since ouabain inhibited brain slice respiration significantly below the level of non-stimulated respiration, the action of this agent may not be limited to the reduced ADP generation resulting from Na^+,K^+-ATPase inhibition. Thus, the simultaneous effects of

Figure 6. Sodium stimulation of brain slice respiration in sodium-free, high potassium medium.

Respiration of rat brain cortical slices was measured in medium containing 100 mM potassium chloride, 15 mM Tris-phosphate buffer (pH 7.4), 1.23 mM magnesium sulfate, and 24 mM glucose. Preincubation was as described in Figure 1. Following half-hour control incubation, sidearm additions were made yielding final concentrations of sodium of 0, 10, 20, 50 and 100 mM with choline used so that in all vessels osmolarity following addition was increased equally. Data represent the means of 4 experiments. Vertical lines indicate S.E.M. Statistical analysis was as described in Figure 1. *Denotes significantly different from time-matched 0 mM Na^+ control.

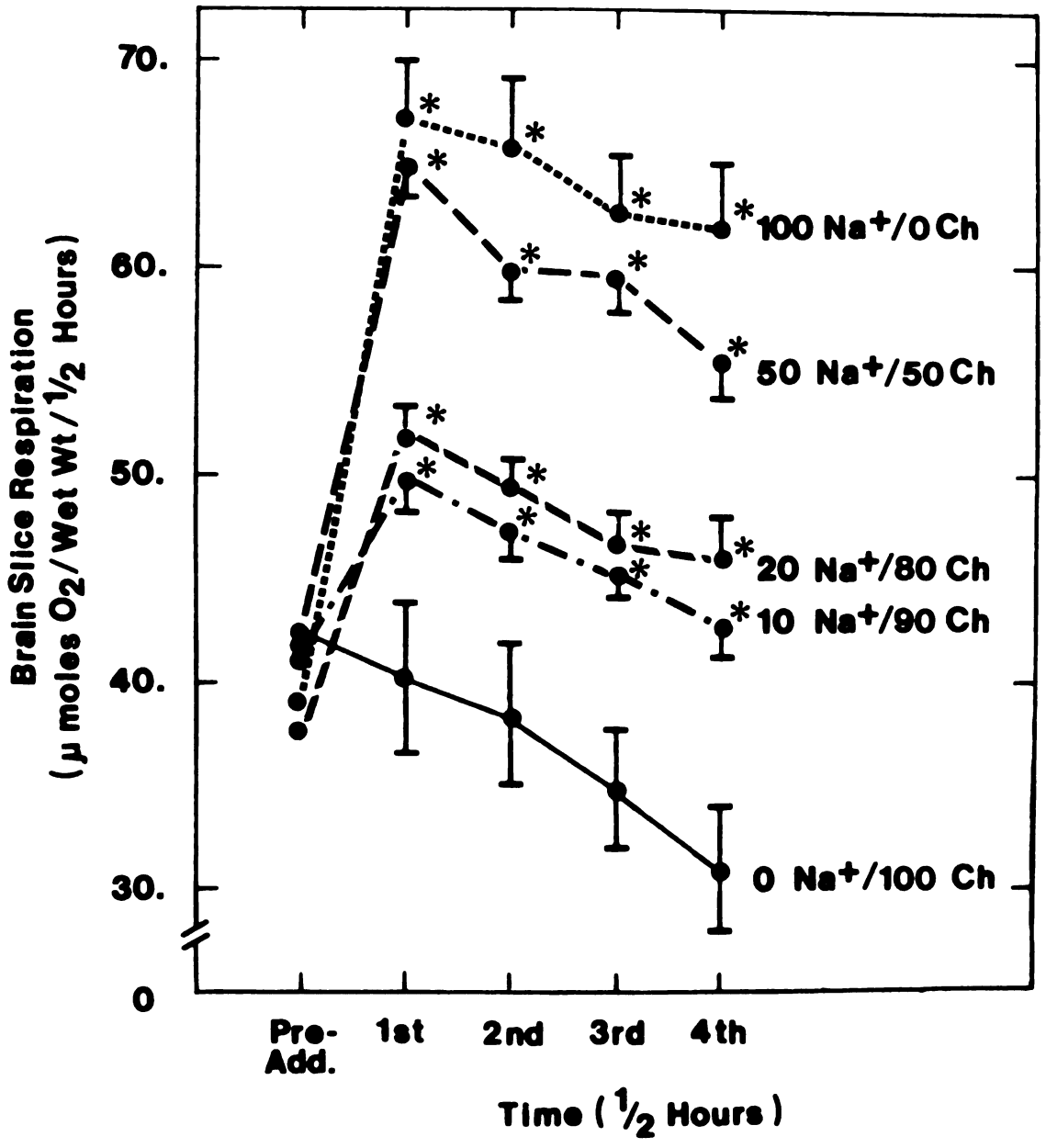
70.
60.
50.
40.
30.
0
Brain Slice Respiration
(μ moles O2/Wet Wt/½ Hours)
100 Na+/0 Ch
50 Na+/50 Ch
20 Na+/80 Ch
10 Na+/90 Ch
0 Na+/100 Ch
Pre-
Add.
1st
2nd
3rd
4th
Time (½ Hours)

Figure 6

2,4-dinitrophenol and ouabain on brain slice respiration were studied to determine the action of ouabain under the condition in which availability of ADP is not the rate limiting factor for respiration. In a sodium-free medium with glucose as the substrate, 50 μM 2,4-dinitrophenol stimulated brain slice respiration (Figure 3). The level of 2,4-dinitrophenol-stimulated respiration in sodium-free medium was similar to that produced by 50 μM 2,4-dinitrophenol in calcium-free Krebs medium which contained sodium (Figure 7). Pre-addition control respiration in this experiment was 58.5 μMoles of oxygen/g tissue (wet weight)/half hour, while pre-addition respiration with 100 μM ouabain was 50.7 μMoles of oxygen/g tissue (wet weight)/ half hour. Addition of 50 μM 2,4-dinitrophenol produced a significant stimulation of respiration to 71.7 μMoles of oxygen/g tissue (wet weight)/half hour in the absence of ouabain. In the presence of ouabain, brain slice respiration was significantly smaller than that observed in the absence of ouabain. The rate of respiration observed with 2,4-dinitrophenol in the presence of ouabain was significantly lower than that observed with 2,4-dinitrophenol in the absence of ouabain. Thus, ouabain can inhibit both basal brain slice respiration and brain slice respiration in the presence of 2,4-dinitrophenol (Figure 7).

Preliminary experiments have indicated that the concentration of 50 μM 2,4-dinitrophenol was near optimal for the stimulation of brain slice respiration under the present conditions. If ouabain's actions involve exclusively an inhibition of ATP hydrolysis inhibiting the Na^+,K^+-ATPase activity, it would be expected that the respiration in the presence of 2,4-dinitrophenol would be equal with or

Figure 7. Effects of 0.1 mM ouabain on 2,4-dinitrophenol stimulation of brain respiration in medium with glucose.

Respiration in rat brain cortex slices were measured in calcium-free Krebs Henseleit medium with glucose as described in Figure 1. Following a half-hour control incubation, 0.2 ml of control medium or 0.5 mM 2,4-dinitrophenol (DNP) (final concentration 50 μM) was added to 1.8 ml of medium. Similarly with slices in 100 μM ouabain (Q), either ouabain containing medium or dinitrophenol (final concentration 50 μM) was added. Respiration was measured for a period of a half hour and expressed as a percent of pre-addition control. Data represent the mean of 10 to 15 experiments. Vertical lines indicate S.E.M. *Denotes significant difference from the value in column C ($p<.05$ by Student's t-test). **Denotes significant difference from value in column DNP ($p<.05$ by Student's t-test).

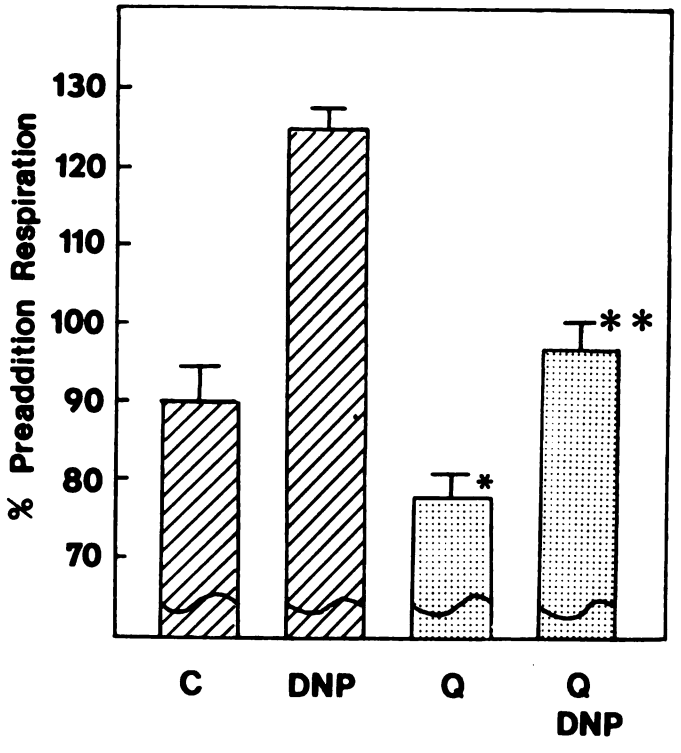

Figure 7

without ouabain. The lower respiration rate observed in the presence of 50 μM 2,4-dinitrophenol in a medium containing 100 μM ouabain appears to indicate that some other factor(s) is(are) responsible for ouabain inhibition of respiration besides reduced ADP availability.

B. Effects of Potassium and Ouabain on Intracellular Sodium and Potassium Concentrations and the Effects of Sodium and Potassium on Brain Homogenate Respiration

Since ouabain is a relatively specific inhibitor of Na^+,K^+-ATPase, and does not affect mitochondrial respiration directly, a part of ouabain's effect may be brought about secondarily by an increased brain slice sodium and/or decreased brain slice potassium concentration. Thus, experiments were conducted to determine whether the rate and magnitude of these ouabain-induced changes were sufficient in modified calcium-free Krebs Henseleit medium to explain a part of the ouabain-induced inhibition of brain slice respiration. In non-incubated slices, the concentration of sodium was 50.3 μEq/100 mg protein, and that of potassium was 93.3 μEq/100 mg protein. Incubation of slices in calcium-free Krebs medium with glucose as substrate at 37°C for 15 to 35 minutes increased intracellular sodium concentrations to approximately 100 μEq/ml, and decreased intracellular potassium concentrations to approximately 55 μEq/ml, respectively (Figure 8). These and other data were corrected for slice extracellular volume which averaged 13.6%. The presence of ouabain in the incubation media caused further increases in intracellular sodium concentration. There were also significant decreases in intracellular potassium concentrations in the presence of 10 and 100 μM ouabain. The ouabain-induced increases in intracellular sodium concentration

Figure 8. Effects of ouabain on intracellular sodium and potassium concentration in rat brain cortical slices.

Rat brain cortical slides were prepared and incubated in calcium-free Krebs Henseleit medium with 0 □—□, 10 ○—○, or 100 ●—● mM ouabain at 37°C. Tissue slices were removed after 15, 25, and 35 minute incubation periods, blotted well and weighed. Using flame photometry, sodium and potassium concentrations were measured in acid supernatants prepared from brain cortex slice homogenates. From ^{14}C-inulin data, extracellular volume was calculated, and from this, brain slice sodium and potassium concentrations were calculated in microequivalents per milliliter intracellular space. Data represent the mean of 4 experiments. Vertical lines indicate S.E.M. Statistical analysis was as described in Figure 1. *Denotes significantly different from control.

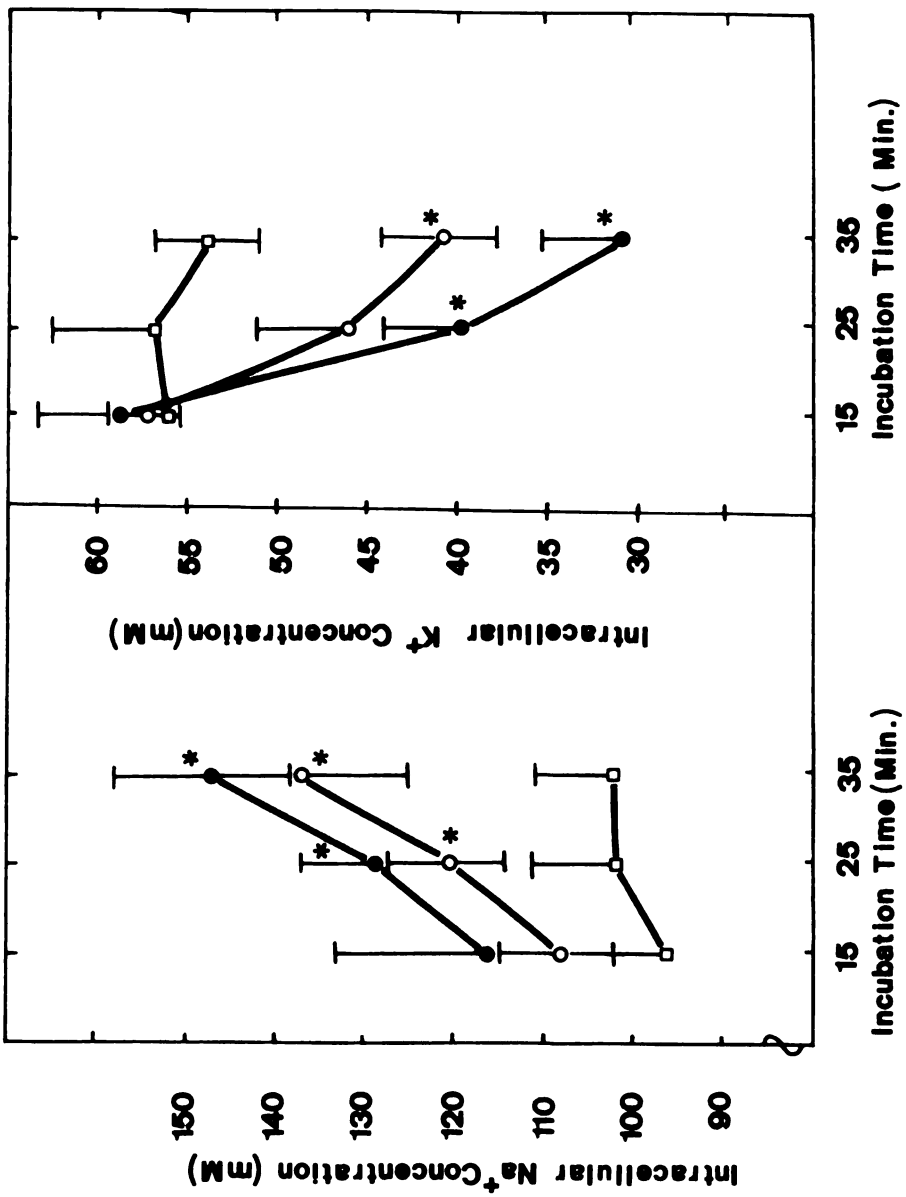
Intracellular Na+Concentration (mM)
150
140
130
120
110
100
90
Incubation Time(Min.)
15
25
35
Intracellular K+ Concentration(mM)
60
55
50
45
40
35
30
Incubation Time (Min.)
15
25
35

Figure 8

and decreases in intracellular potassium concentration were both dose and time dependent. These changes in intracellular sodium and potassium concentrations are consistent with the inhibition of Na^+,K^+-ATPase enzyme by ouabain.

Based on the data from brain slice respiration and cation concentration experiments, it was postulated that either increases in intracellular sodium or decreases in intracellular potassium might be playing a role in ouabain inhibition of brain slice respiration. The effects of either increased intracellular sodium or decreased intracellular potassium on tissue respiration were therefore studied using optimally respiring brain cortical homogenates (Figures 9 and 10). Glucose, ADP, NAD, nicotinic acid and hexokinase enzyme were added to the incubation medium to yield maximal respiration, free from respiratory control, and sodium and potassium concentrations were varied. Decreases in potassium concentration from 125 mM to 45 or 25 mM with simultaneous increases in sodium concentration from 0 to 80 or 100 mM caused a significant depression of brain homogenate respiration (Figure 9).

When sodium and potassium concentrations were varied independently with choline chloride used as an osmotic substitute, decreases in potassium concentration from 145 to 25 mM had no significant effect on brain homogenate respiration, whereas increases in sodium concentration from 0 to 60, 80, 100 and 120 mM produced dose-dependent inhibition of the homogenate respiration (Figure 10). The magnitude of increase in intracellular sodium concentration observed previously in ouabain-inhibited brain slices was similar to that producing the 30% inhibition of optimal brain homogenate respiration during the 15-minute incubation period shown in Figures 9 and 10. Thus,

Figure 9. Effects of sodium and potassium on ADP stimulated brain.

Rat brain cortical slices were homogenized in .32M sucrose at 0°C. Homogenates were then incubated at 30°C in medium containing final concentrations of 2 mM Tris-ADP, 3 mM magnesium chloride, 10 mM potassium phosphate buffer (adjusted to pH 7.2 with potassium hydroxide), 10 mM glucose, 0.05 mM EDTA, 0.2 mM NAD, 40 mM nicotinamide, and 40 µg hexokinase enzyme. Background potassium was approximately 25 mM. Within this system sodium and potassium concentrations of from 0 to 100 mM were added. Oxygen consumption was measured manometrically. Data represent the means of from 8 to 10 experiments. Vertical lines indicate S.E.M. Statistical analysis was performed as described in Figure 3. *Denotes significantly different from respiration with 0 mM sodium.

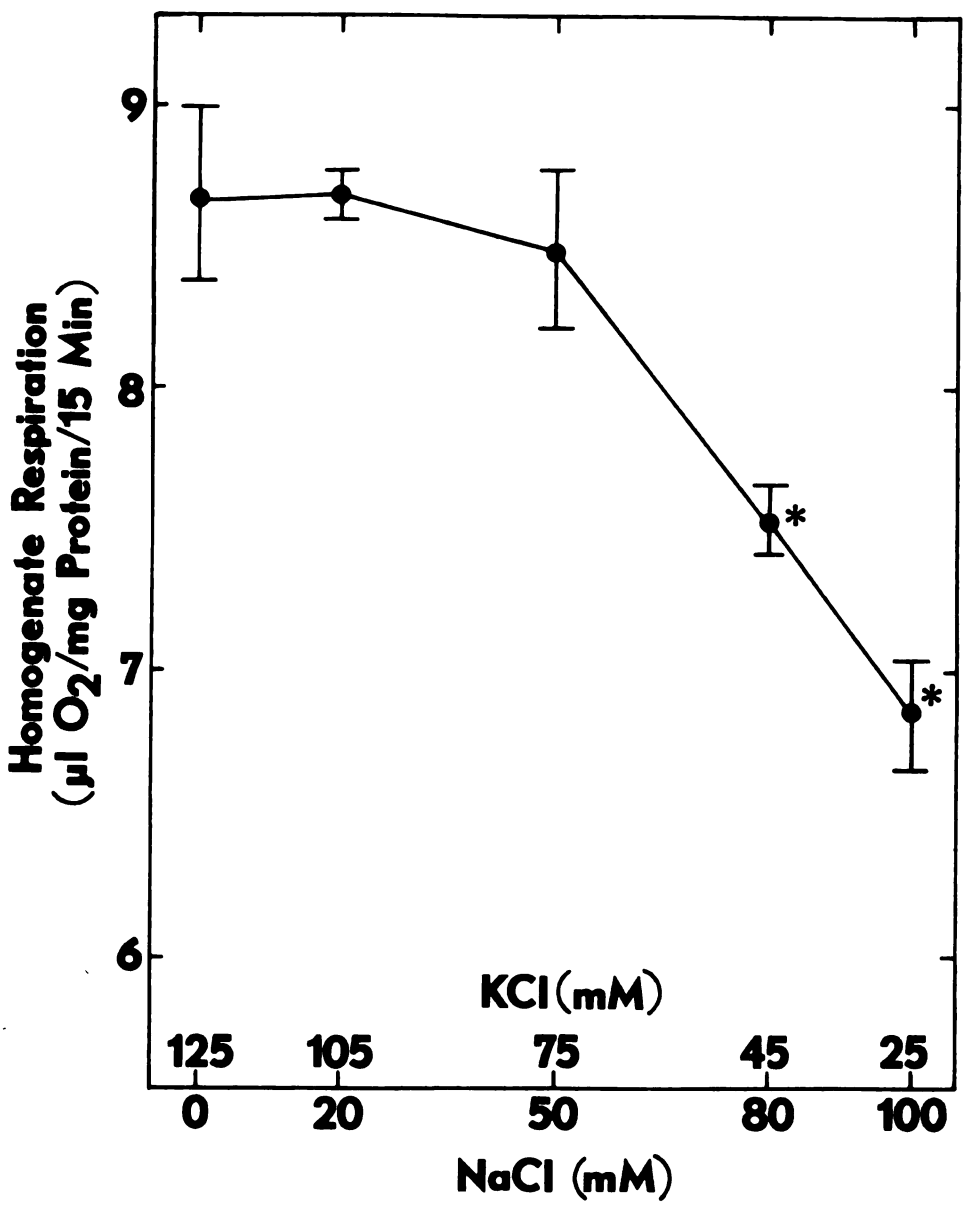
Homogenate Respiration
(µl O_2/mg Protein/15 Min)
9
8
7
6
*
*
KCl (mM)
125
105
75
45
25
0
20
50
80
100
NaCl (mM)

Figure 9

Figure 10. Effects of sodium and potassium on ADP-stimulated brain cortex homogenate respiration.

Rat brain cortical slices were homogenized in .32 mM sucrose at 0°C. Homogenate was then incubated at 30°C in medium containing final concentrations of 2 mM Tris-ADP, 3 mM magnesium chloride, 10 mM potassium phosphate buffer (adjusted to pH 7.2 with potassium hydroxide), 10 mM glucose, 0.05 mM K-EDTA, 0.2 mM NAD, 40 mM nicotinamide, and 40 µg hexokinase enzyme (activity 18.5 units per milligram). Background potassium was about 25 mM. Within this system, sodium (●—●) and potassium (○—○) cation concentrations were varied by addition of from 0 to 120 mM, using choline chloride and an osmotic substitute to maintain added cation concentration at 120 mM. Oxygen consumption was measured manometrically. Data represent the means of 5 experiments. Vertical lines indicate S.E.M. Statistical analysis was as described in Figure 3. *Denotes significantly different from respiration with 0 mM sodium.

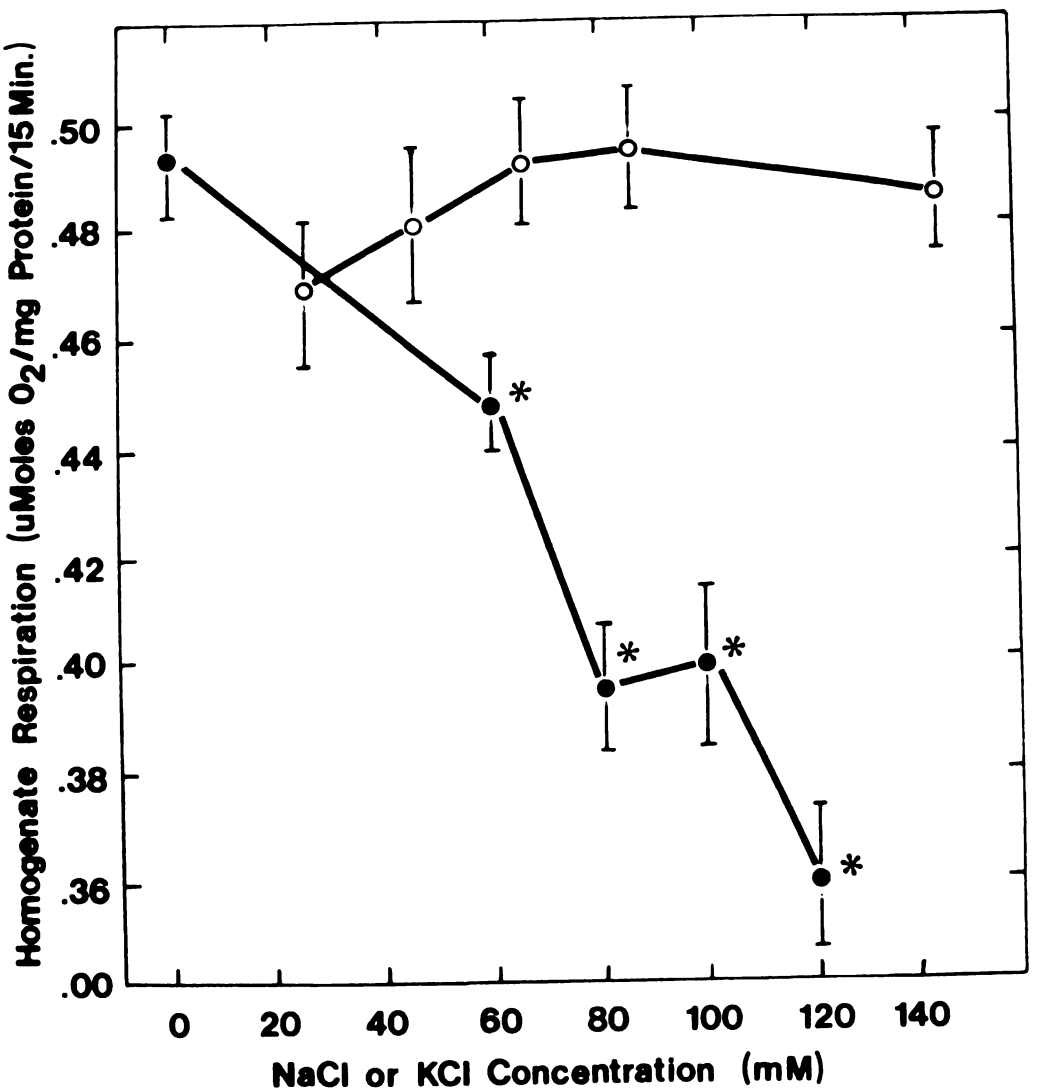
Homogenate Respiration (uMoles O_2/mg Protein/15 Min.)
.50
.48
.46
.44
.42
.40
.38
.36
.00
0
20
40
60
80
100
120
140
NaCl or KCl Concentration (mM)
*
*
*
*

Figure 10

ouabain-induced inhibition of brain slice respiration may be due to both an indirect effect of ouabain to increase intracellular sodium, as well as the direct inhibition of ADP production associated with the inhibition of Na^+,K^+-ATPase activity. Thus, the use of cardiac glycosides, such as ouabain, would overestimate the portion of respiration associated with Na^+,K^+-ATPase activity, although such a method was employed by other investigators (Whittam, 1962a; Whittam and Blond, 1964).

In the following experiment the effect of 100 mM potassium on intracellular sodium and potassium concentrations was examined in slices in high sodium and sodium-free media (Figure 11) to elucidate ionic events which occur at the time of the potassium effect. Following the incubation at 37°C for 20 minutes in the presence of 100 mM potassium, intracellular sodium and potassium concentrations of brain slices in calcium-free Krebs Henseleit medium were 109 and 142 µEq/ml of intracellular volume, respectively. Thus, the intracellular sodium concentration (109 µEq/ml) in calcium-free Krebs medium with 100 mM potassium was not different from the intracellular sodium concentration of 102 µEq/ml observed earlier in control slices incubated in similar medium with low potassium (Figure 8). In a medium in which choline replaced sodium, addition of 100 mM potassium produced a significant decrease in intracellular sodium concentration of brain slices to approximately 4.7 µEq/ml intracellular volume (Figure 11). Intracellular potassium in these slices was 133.2 µEq/ml intracellular volume. Thus, there was no significant difference between intracellular potassium concentrations of brain slices in the high sodium or in sodium-free media. In both cases,

Figure 11. Effects of incubation in sodium-free (choline) medium with 100 mM potassium on brain slice intracellular cation concentrations.

Rat brain cortical slices were incubated for 20 minutes at 37°C in either Ca^{2+}-free Krebs Henseleit medium with 100 mM KCl or sodium-free medium in which choline chloride replaced NaCl and Tris-phosphate replaced Na-phosphate buffer. Using flame photometry, cation concentrations were measured in homogenate digests of brain slices. From ^{14}C-inulin data extracellular volume was calculated, and from this brain slice cation concentrations were calculated in microequivalents per milliliter intracellular space. Data from slices in high sodium medium represent the means of 6 experiments, while data from slices in sodium-free medium represent the means of 4 experiments. Vertical lines indicate S.E.M. Statistical analysis was as described in Figure 3. *Denotes significantly different from sodium medium.

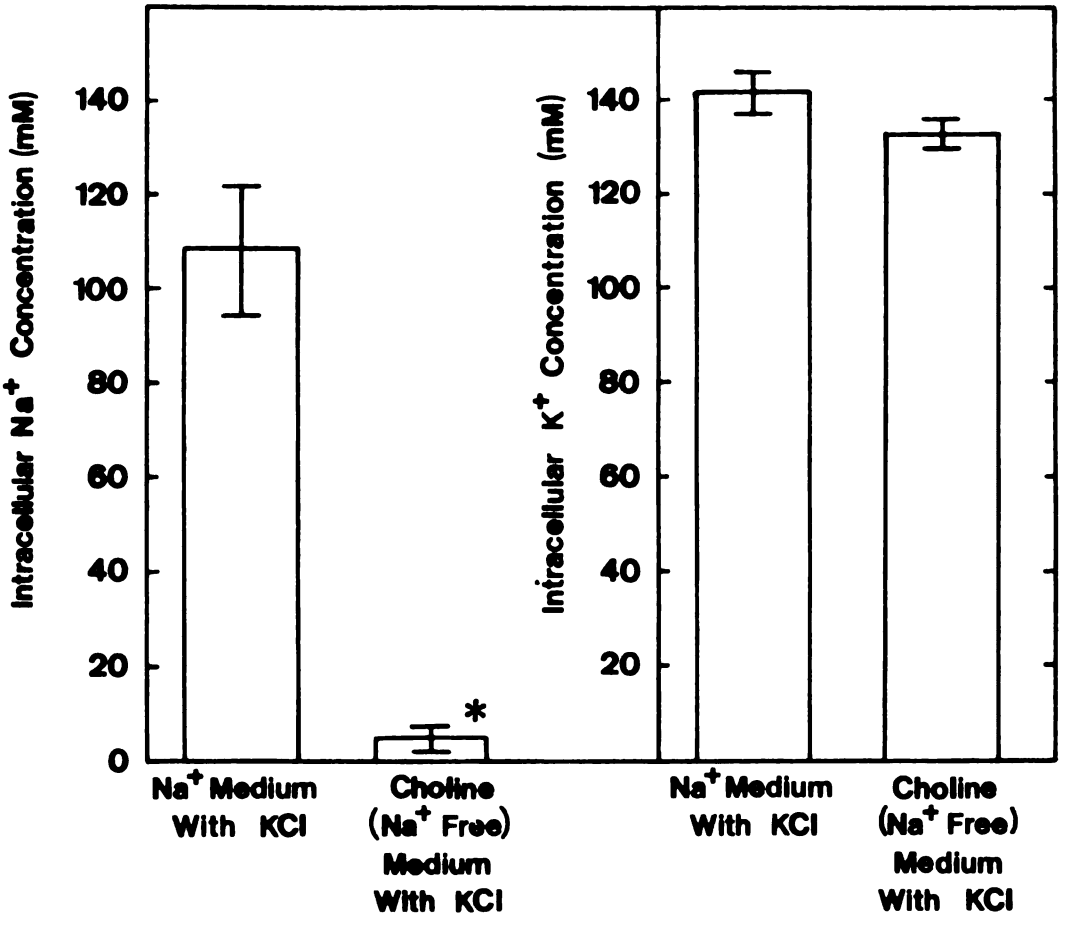
Intracellular Na+ Concentration (mM)
140
120
100
80
60
40
20
0
*
Na+ Medium With KCl
Choline (Na+ Free) Medium With KCl
Intracellular K+ Concentration (mM)
140
120
100
80
60
40
20
Na+ Medium With KCl
Choline (Na+ Free) Medium With KCl

Figure 11

intracellular potassium concentrations were markedly higher than the potassium concentrations in the extracellular medium, which contained 109 mM potassium.

Since the rates of slice respiration in sodium-free medium were significantly lower than those in modified calcium-free Krebs Henseleit medium, it would seem that the respiration observed in calcium-free Krebs medium is already "stimulated" to some extent. Thus, the potassium stimulated portion of respiration in calcium-free Krebs solution is not a good estimate of Na^+,K^+-ATPase-associated respiration. In order to estimate the portion of slice respiration associated with Na^+,K^+-ATPase activity, it is necessary to estimate the level of completely "non-stimulated" slice respiration. Since respiration inhibiting and pump stimulating intracellular sodium concentrations were minimal in slices in sodium-free medium in the presence of 100 mM potassium, respiration rates observed under this condition would accurately represent the non-stimulated slice respiration. Thus, the difference between slice respiration in a high sodium, high potassium medium, and that in a sodium-free (choline containing), high potassium medium appears to represent the Na^+,K^+-stimulated portion of respiration.

C. Effects of Monovalent Cations on Rat Brain Slice and Homogenate Respiration

As described in section "D" of the introduction, it has been postulated by several investigators (Willis and Fang, 1970; Tobin *et al.*, 1974) that the pharmacological actions of a number of monovalent cations might involve the inhibition or stimulation of Na^+,K^+-ATPase enzyme. This is based on observations that monovalent cations such

as rubidium, cesium and lithium are capable of inhibiting or stimulating under certain circumstances isolated Na^+,K^+-ATPase *in vitro*. It is not known, however, if these cations affect Na^+,K^+-ATPase activity in intact brain cells. Results from experiments in sections "A" and "B" suggest that a sodium plus potassium-stimulated portion of brain slice respiration may be used to estimate Na^+,K^+-ATPase activity in intact cells. Thus, the effects of rubidium, cesium, lithium and the monovalent thallous ion were studied on rat brain slice and homogenate respiration.

Addition of potassium, rubidium or cesium produced a concentration dependent stimulation of rat brain cortical slice respiration in a modified calcium-free Krebs medium (Figure 12). Control respiration before the addition of cations was 57.8 µMoles of oxygen/g tissue (wet weight)/half hour. In control vessels, the respiration rate following the addition of choline was about the same as before the addition of choline. Pharmacological and toxicological concentrations of rubidium and cesium (i.e., below 20 mM) had no significant effect on brain slice respiration. Addition of higher concentrations of 50, 75 and 100 mM potassium, rubidium or cesium caused significant stimulation of brain slice respiration. Addition of 20, 50 and 75 mM cesium produced somewhat larger increases in respiration than addition of comparable concentrations of potassium or rubidium. At 100 mM concentrations, stimulation with potassium was somewhat higher than with rubidium or cesium. Thus, although potassium, rubidium, and cesium were capable of producing stimulations of brain slice respiration, such effects were observed only with extremely high concentrations.

Figure 12. Effects of potassium, rubidium, and cesium on brain slice respiration in high-sodium medium.

Rat brain cortical slice respiration was measured for a control half hour in medium containing 128 mM NaCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate buffer (pH 7.4), and 24 mM glucose at 37°C. Either potassium, rubidium, or cesium was then added to yield final concentrations of from 0 to 100 mM. Choline chloride of appropriate concentrations was also added so that in each vessel osmolarity following addition was the same. Slice respiration was then measured, and expressed as a percent of respiration observed during the pre-addition control period. Each curve represents the means of 6 experiments. Statistical analysis was as described in Figure 1. *Denotes significantly different from choline chloride.

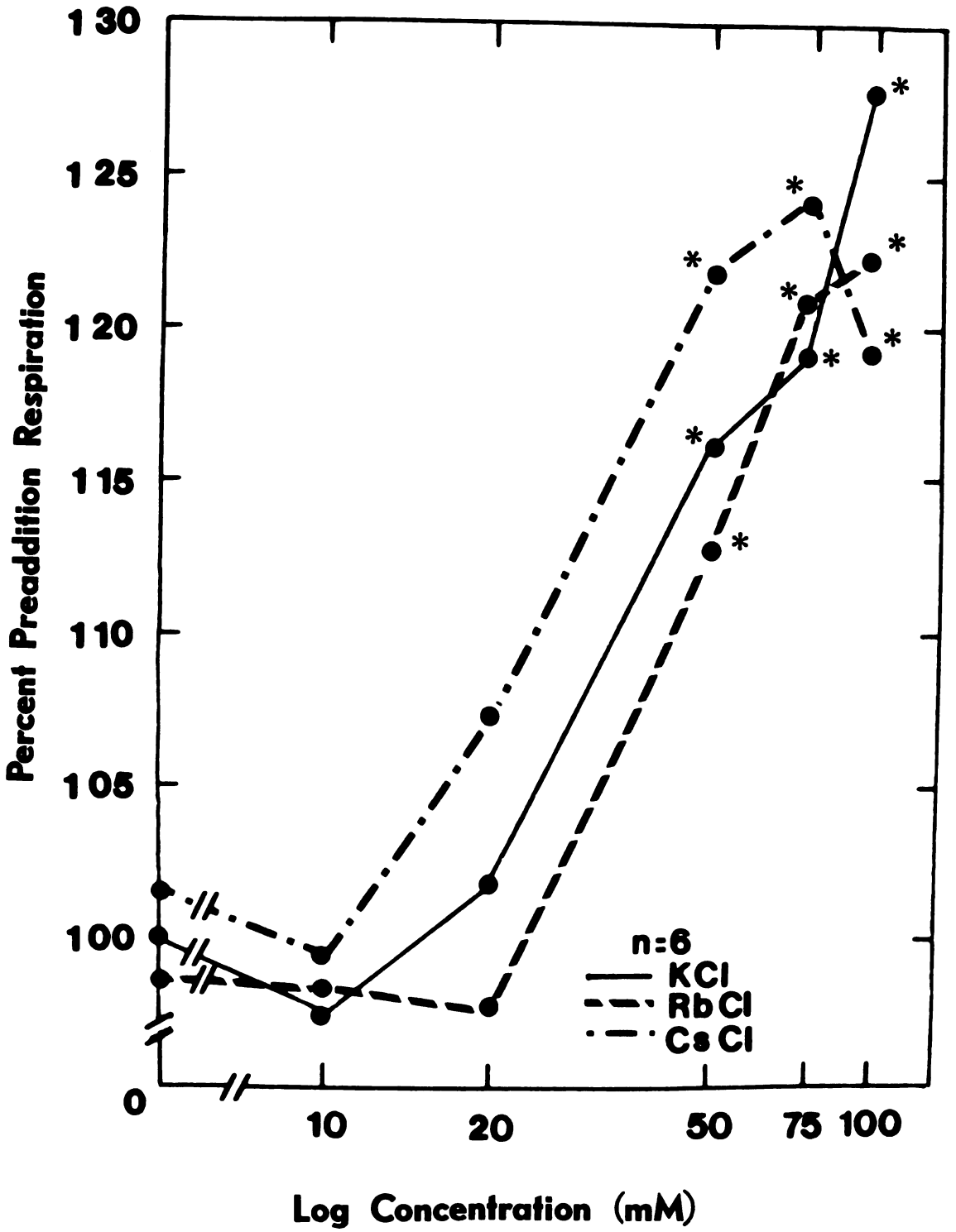
130
125
120
115
110
105
100
0
Percent Preaddition Respiration
10
20
50
75
100
Log Concentration (mM)
n:6
KCl
RbCl
CsCl

Figure 12

Studies on the inhibition of rubidium and cesium stimulation by ouabain were next conducted to determine if the rubidium and cesium stimulation of brain slice respiration was associated with a stimulation of Na^+,K^+-ATPase. Ouabain at concentrations of 10 and 100 μM significantly inhibited brain slice respiration in the presence of 100 mM rubidium in modified calcium-free Krebs Henseleit solution (Figure 13). The effects of 1 μM ouabain on brain slice respiration were not statistically significant. Sodium plus rubidium stimulation of respiration in the presence of 100 μM ouabain was 46.1% of sodium plus rubidium stimulation in the absence of ouabain during the first half hour following rubidium or choline addition. This was similar to the effect of ouabain observed earlier on sodium plus potassium stimulation, where the sodium plus potassium-stimulated portion of respiration in the presence of 100 μM ouabain during the first half hour following potassium or choline addition was 51.3% of control (Figure 1).

Similarly, ouabain at concentrations of 10 and 100 μM significantly inhibited brain slice respiration in the presence of 100 mM cesium in modified calcium-free Krebs Henseleit solution (Figure 14).

With time, 1 μM ouabain produced progressively greater inhibition of cesium-stimulated slice respiration (Figure 15). During the first half hour, sodium plus cesium stimulation was decreased to 94.5% of matched controls. During the second half hour following cesium addition, sodium plus cesium stimulation was decreased by 1 μM ouabain to 64% of matched controls, and during the third half hour to 33.5% of controls. During the fourth half hour it was further

Figure 13. Effects of rubidium and ouabain on brain slice respiration.

Rat brain cortical slices were incubated for a half hour in a medium containing 128 mM NaCl, 3 mM KCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate buffer, and 24 mM glucose, plus either 0, 1, 10, or 100 μM ouabain at 37°C. Rubidium chloride or choline chloride was then added to yield final concentrations of 100 mM. Slice respiration during the first half hour following additions is shown. Data shown in the total bars represent means of 4 experiments with 100 mM rubidium. Data shown by the shaded bars represent the means of the same 4 experiments with 100 mM choline, and the differenc erepresents the sodium, rubidium-stimulated portion of respiration. Vertical lines indicate S.E.M. Statistical analysis was as described in Figure 1. *Denotes significant rubidium stimulation.

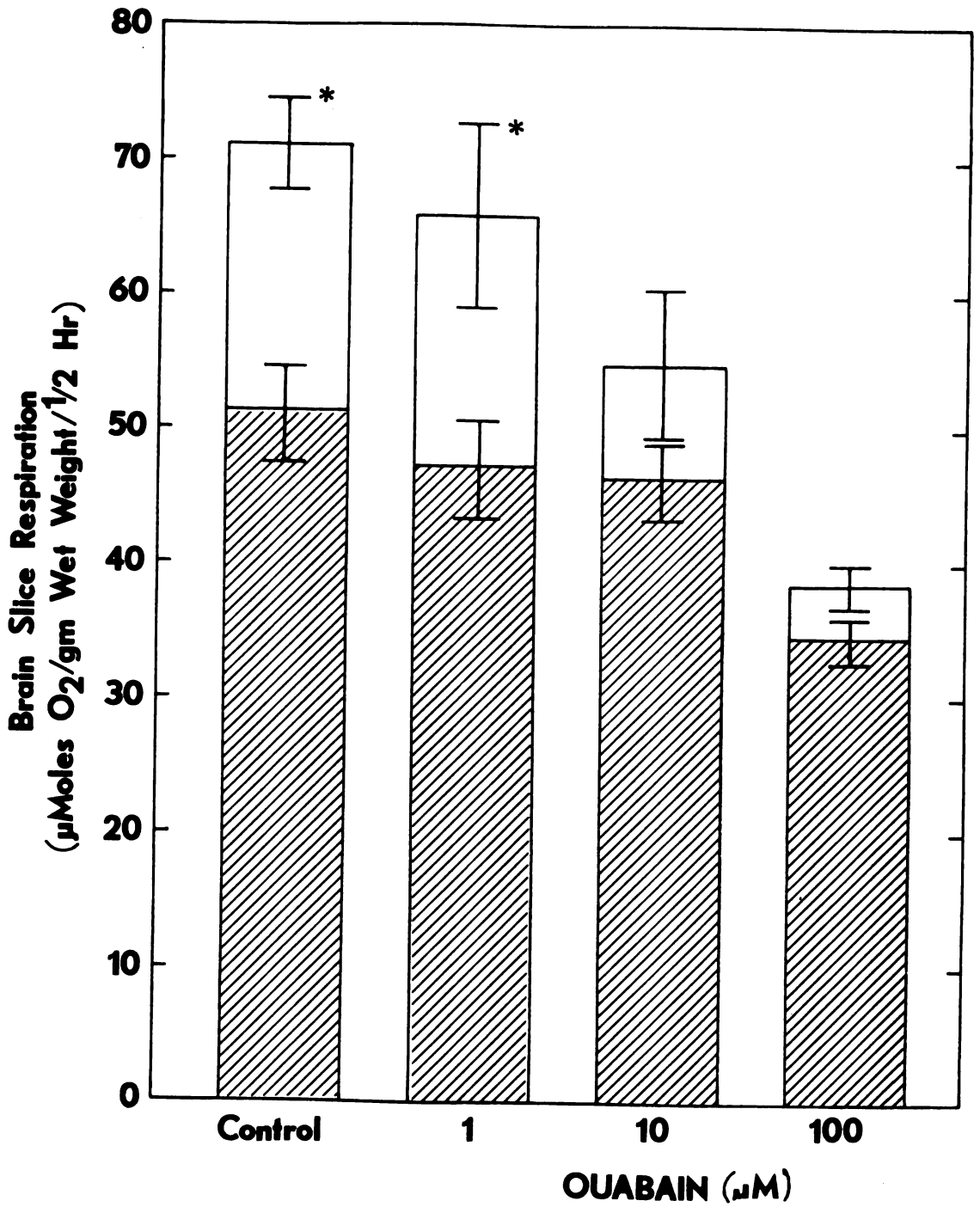
80
70
60
50
40
30
20
10
0
Brain Slice Respiration
(μMoles O2/gm Wet Weight/1/2 Hr)
*
*
Control
1
10
100
OUABAIN (μM)

Figure 13

Figure 14. Effects of cesium and ouabain on brain slice respiration.

Rat brain cortical slices were incubated as described in Figure 13. Cesium chloride or choline chloride were added after a half-hour incubation to yield final concentrations of 100 mM. Slice respiration during the first half hour following addition is shown. Data shown in the total bars represent the means respiration of 4 experiments with 100 mM cesium. Data shown by the shaded bars represent mean respiration in the same 4 experiments with 100 mM choline, and the difference represents the sodium, plus cesium-stimulated portion of respiration. Vertical lines indicate S.E.M. Statistical analysis was as described in Figure 1. *Denotes significant cesium stimulation.

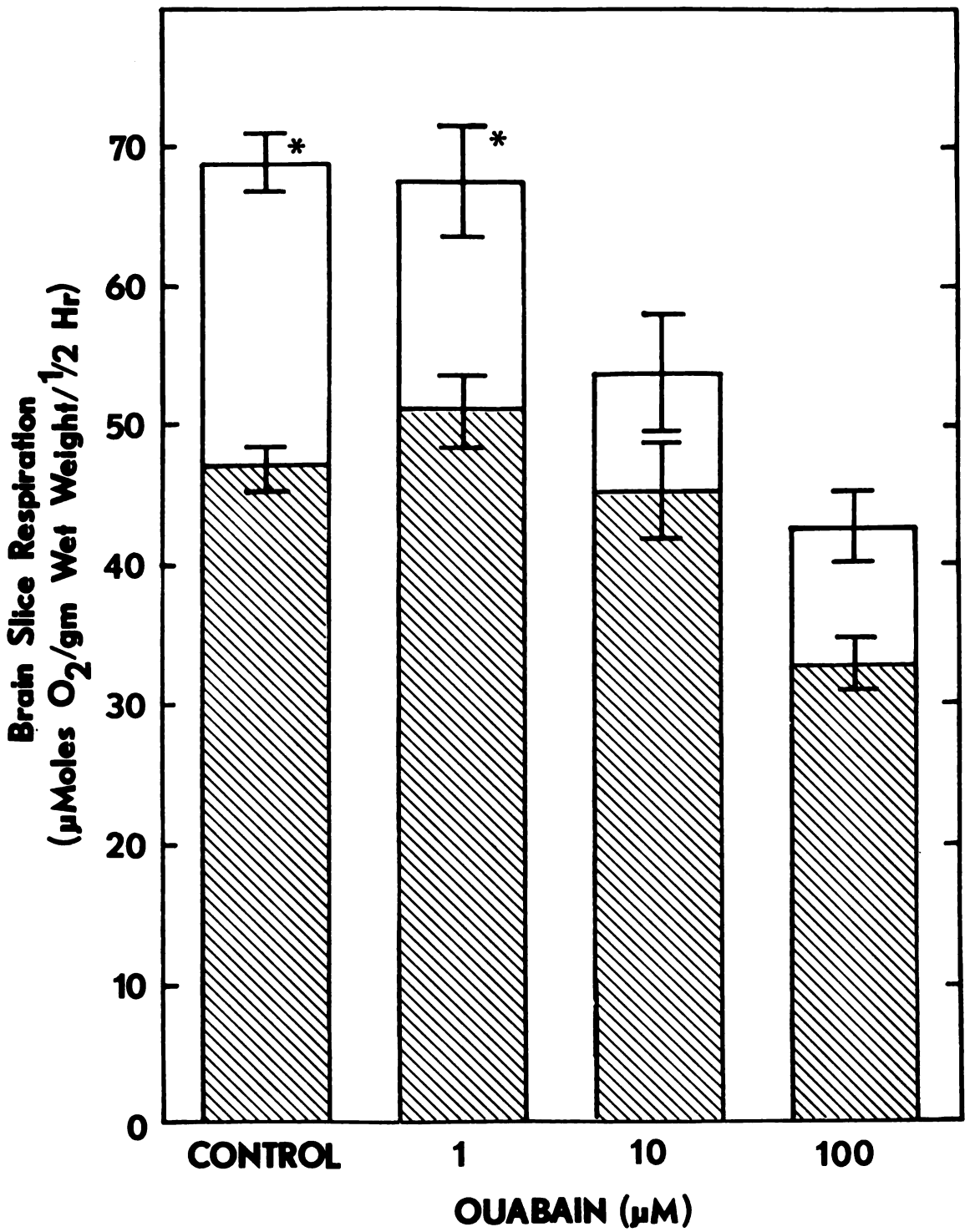
Brain Slice Respiration
(μMoles O_2/gm Wet Weight/1/2 Hr)
70
60
50
40
30
20
10
0
*
*
CONTROL
1
10
100
OUABAIN (μM)

Figure 14

Figure 15. Time course of ouabain effects on cesium-stimulated brain slice respiration.

Data are from the same slices as described in Figure 14 and show respiration plotted on the abscissa against the time plotted on the ordinate. Preincubation conditions were as described in Figure 13. Brain slice respiration was measured for 4 half-hour periods following addition of 100 mM cesium chloride ●—●—● or 100 mM choline chloride ○—○—○. In certain vessels, slice respiration was measured in the presence of 1 μM ouabain following the addition of 100 mM cesium chloride ●— — —● or 100 mM choline chloride ○— — —○. Data represent the means of 4 experiments. Vertical lines indicate S.E.M. Statistical analysis was performed as described in Figure 1. *Denotes significantly different from corresponding choline control.

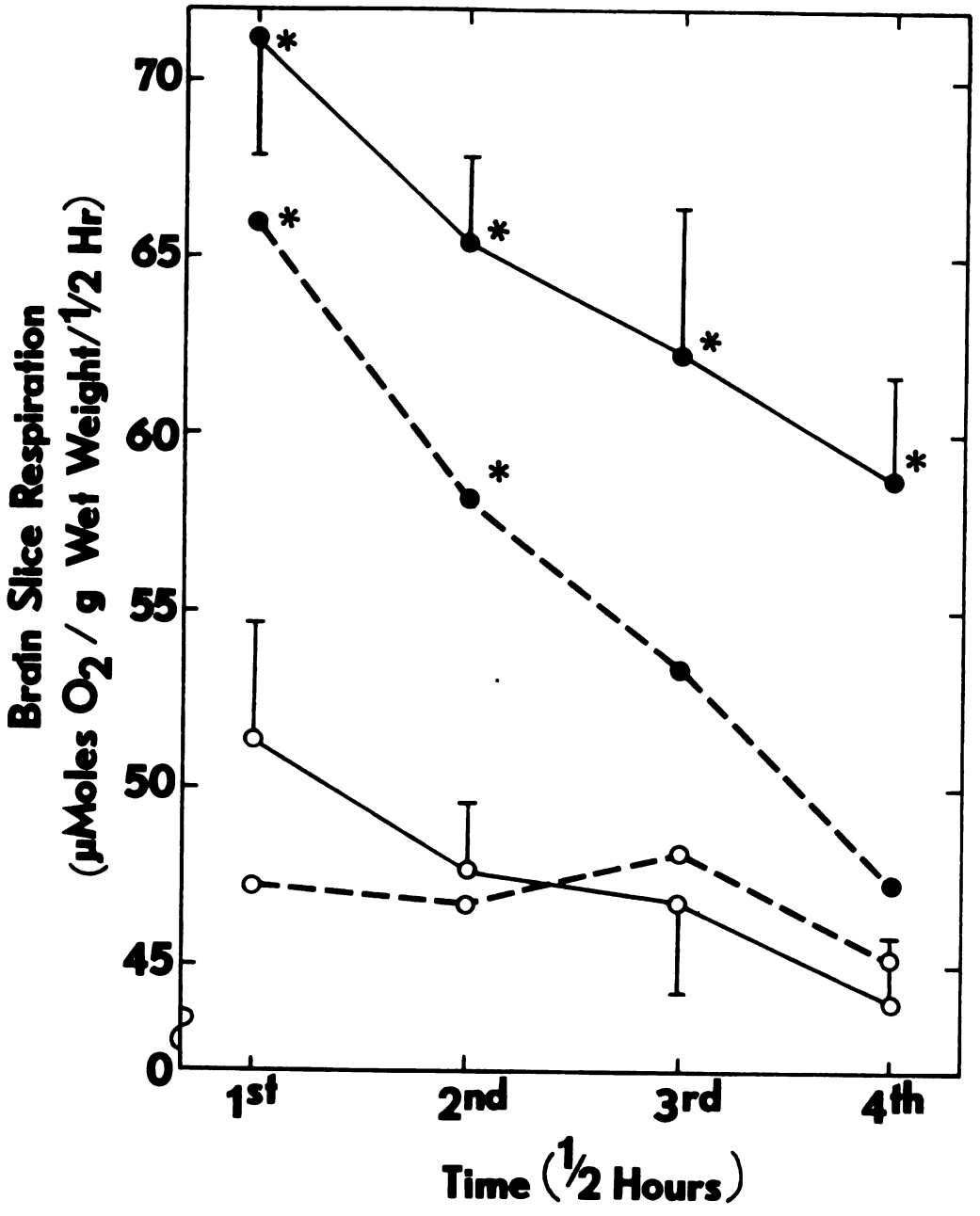
Brain Slice Respiration
(μMoles O_2 / g Wet Weight/1/2 Hr)
70
65
60
55
50
45
0
1st
2nd
3rd
4th
Time (1/2 Hours)

Figure 15

decreased to 15% of controls. It thus appears that the effect of ouabain develops slowly at low ouabain concentrations.

While the preceding monovalent cation experiments examined the effects of chiefly high concentrations of rubidium and cesium on slice respiration, the following lithium experiments attempted to study the effects of lithium in somewhat lower, more nearly pharmacological concentrations. Lithium in concentrations from 0 to 30 mM was found to have no significant effect on brain cortical slice respiration in a medium containing 150 mM sodium and 1.5 mM potassium, during four half-hour periods following lithium chloride addition (Figure 16). During the first half hour 3 mM lithium produced a small 10.7% stimulation of slice respiration relative to first half-hour controls. During the fourth half hour 30 mM lithium produced a larger 20.8% inhibition of slice respiration relative to fourth half-hour controls. These effects of lithium, however, were not significant ($p<.05$) using a Student's t-test. In a high sodium, low potassium medium, therefore, lithium in therapeutic (1 mM) and even in toxic (3-10 mM) concentrations had no significant effect on brain slice respiration. It should be noted that lithium in a concentration of 30 mM did not substitute for potassium to produce a stimulation of slice respiration in high sodium, low potassium medium.

The effects of 20 mM lithium on sodium stimulation of brain cortical slice respiration in sodium-free, high potassium medium were studied next. The slice respiration prior to the addition of sodium in the absence of lithium was 40.4 µMoles of oxygen/g tissue (wet weight)/half hour, while the respiration in the presence of 20 mM lithium was 42.5 µMoles of oxygen/g tissue (wet weight)/half hour

Figure 16. Effects of lithium on brain slice respiration in 150 mM sodium, 1.5 mM potassium medium.

Rat brain cortical slice respiration was measured for a control half hour in medium containing 150 mM NaCl, 1.5 mM KCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate buffer (pH 7.4), and 24 mM glucose at 37°C. Lithium chloride in final concentrations from 0 to 30 mM was then added. Data represent the means of 3 experiments. Vertical lines indicate S.E.M. (A) First half-hour respiration; (B) second half-hour respiration; (C) third half-hour respiration; (D) fourth half-hour respiration.

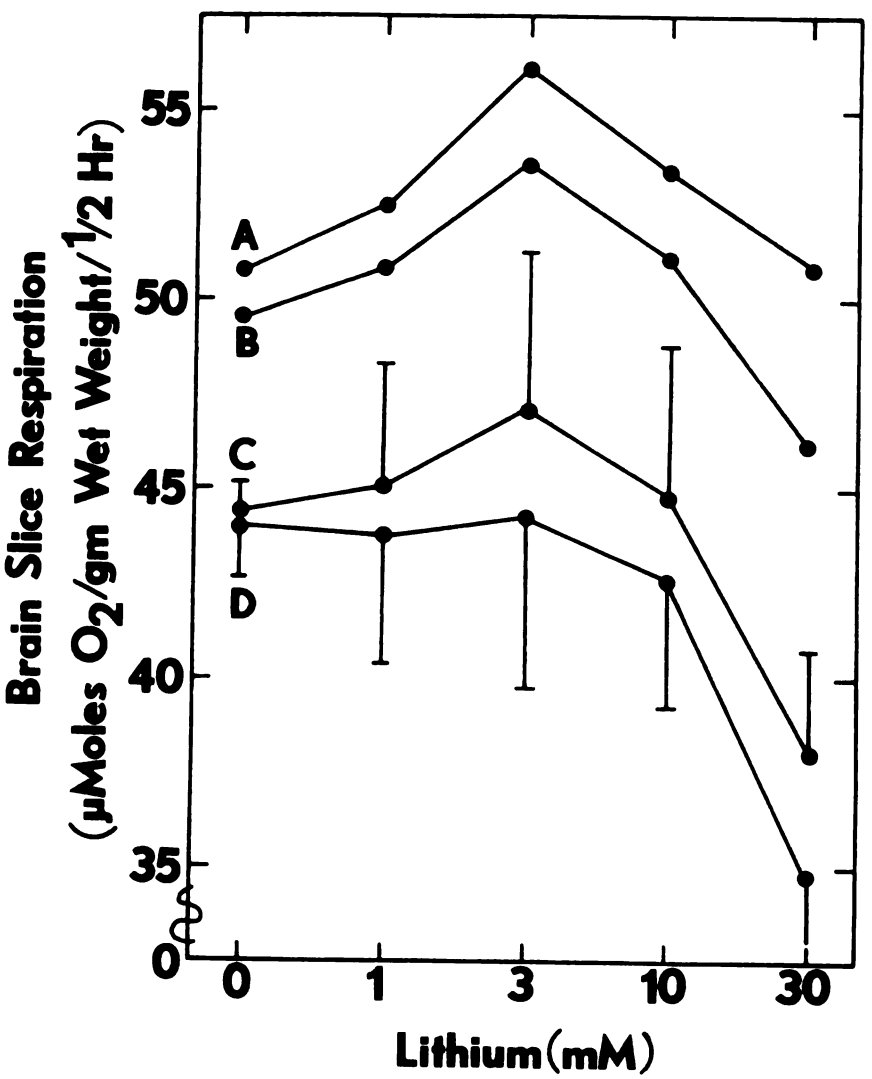
Brain Slice Respiration
(μMoles O_2/gm Wet Weight/1/2 Hr)
55
50
45
40
35
0
A
B
C
D
0
1
3
10
30
Lithium (mM)

Figure 16

(Figure 17). During the first half-hour period following sodium or choline addition, respiration in choline added controls in the absence of lithium was 40.3 μMoles of oxygen/g tissue (wet weight)/ half hour, while respiration in choline controls in the presence of 20 mM lithium was 38.6 μMoles of oxygen/g tissue (wet weight)/half hour. Since these non-sodium-stimulated rates of respiration in the presence or absence of lithium were not significantly different by Student's t-test ($p<.05$), it appears that lithium does not substitute for sodium to stimulate brain slice respiration in high potassium medium. The presence of 20 mM lithium appeared to decrease sodium stimulation. However, at the concentrations of lithium and sodium studied, it was not possible to analyze if lithium produced a change in the slope, decreased the maximum rate of respiration, or if it shifted the sodium stimulation curve in parallel to the right. It was thus impossible to determine if the lithium effect was competitive or non-competitive with respect to sodium.

In a brain homogenate, lithium in concentrations of 20 to 100 mM significantly inhibited optimal respiration (Figure 18). Lithium in concentrations of 10 mM or less, however, produced no significant effects on optimal brain homogenate respiration. In similar studies on the effects of rubidium (Figure 19) and cesium (Figure 20) no significant effects were observed with concentrations of either rubidium or cesium up to 100 mM.

Additional studies were performed with thallous ion, which has been shown to be a potent substitute for potassium in the Na^+,K^+-ATPase reaction, and a potent inhibitor of Na^+,K^+-ATPase in the presence of sodium and potassium (Skulskii *et al.*, 1973, 1975). Because of its

Figure 17. Effects of lithium on sodium-stimulation of brain slice respiration.

Rat brain cortical slice respiration was measured for a control half hour in medium containing 100 mM KCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate buffer (pH 7.4) and 24 mM glucose with or without 20 mM lithium chloride. Sodium chloride was then added yielding final concentrations of from 0 to 100 mM. Respiration was measured of slices in media with or without lithium, and expressed as a percent of pre-addition respiration. Data represent the means of 4 experiments. Vertical lines indicate S.E.M.

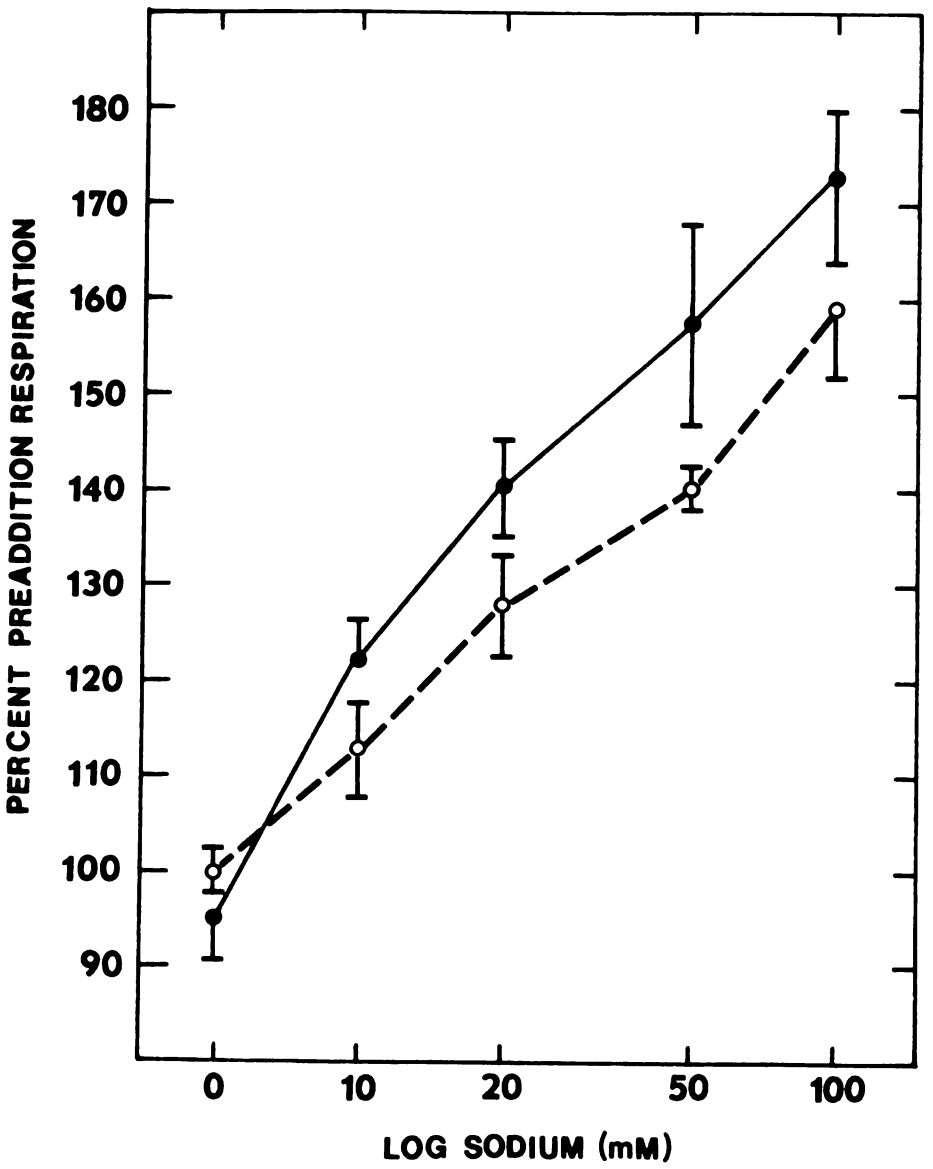
PERCENT PREADDITION RESPIRATION
180
170
160
150
140
130
120
110
100
90
0
10
20
50
100
LOG SODIUM (mM)

Figure 17

Figure 18. Effects of lithium on rat brain homogenate respiration.

Rat brain cortical slices were homogenized in .32M sucrose at 0°C. Homogenates were then incubated at 30°C in medium containing final concentrations of 2 mM Tris-ADP, 3 mM magnesium chloride, 10 mM potassium phosphate buffer (adjusted to pH 7.2 with potassium hydroxide), 10 mM glucose, 0.05 mM EDTA, 0.2 mM NAD, 40 mM nicotinamide, and 40 µg hexokinase enzyme. Lithium chloride was varied from 0 to 100 mM using choline chloride as an osmotic substitute to maintain added cation concentration at 100 mM. Background potassium was approximately 25 mM; sodium concentration was zero. Data represent the means of 5 experiments. Vertical lines indicate S.E.M. *Significantly different from control.

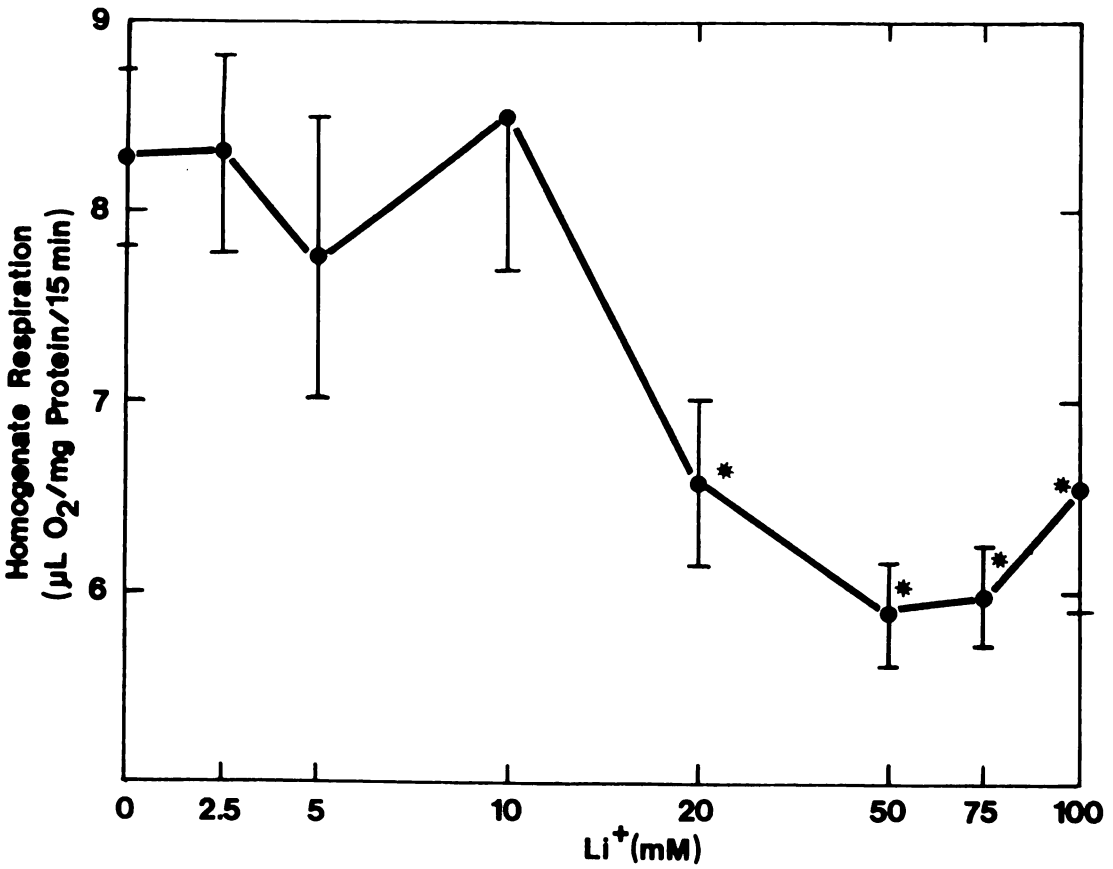
9
8
7
6
Homogenate Respiration
(µL O_2/mg Protein/15 min)
0
2.5
5
10
20
50
75
100
Li^+(mM)
*
*
*
*

Figure 18

Figure 19. Effects of rubidium on rat brain homogenate respiration.

Rat brain cortical slices were homogenized in .32M sucrose at 0°C. Homogenates were then incubated at 30°C in medium containing final concentrations of 2 mM Tris-ADP, 3 mM magnesium chloride, 10 mM potassium phosphate buffer (adjusted to pH 7.2 with potassium hydroxide), 10 mM glucose, 0.05 mM EDTA, 0.2 mM NAD, 40 mM nicotinamide, and 40 µg hexokinase enzyme. Rubidium chloride was varied from 0 to 100 mM using choline chloride as an osmotic substitute. Background potassium was approximately 25 mM; sodium was zero. Data represent the means of 5 experiments. Vertical lines indicate S.E.M.

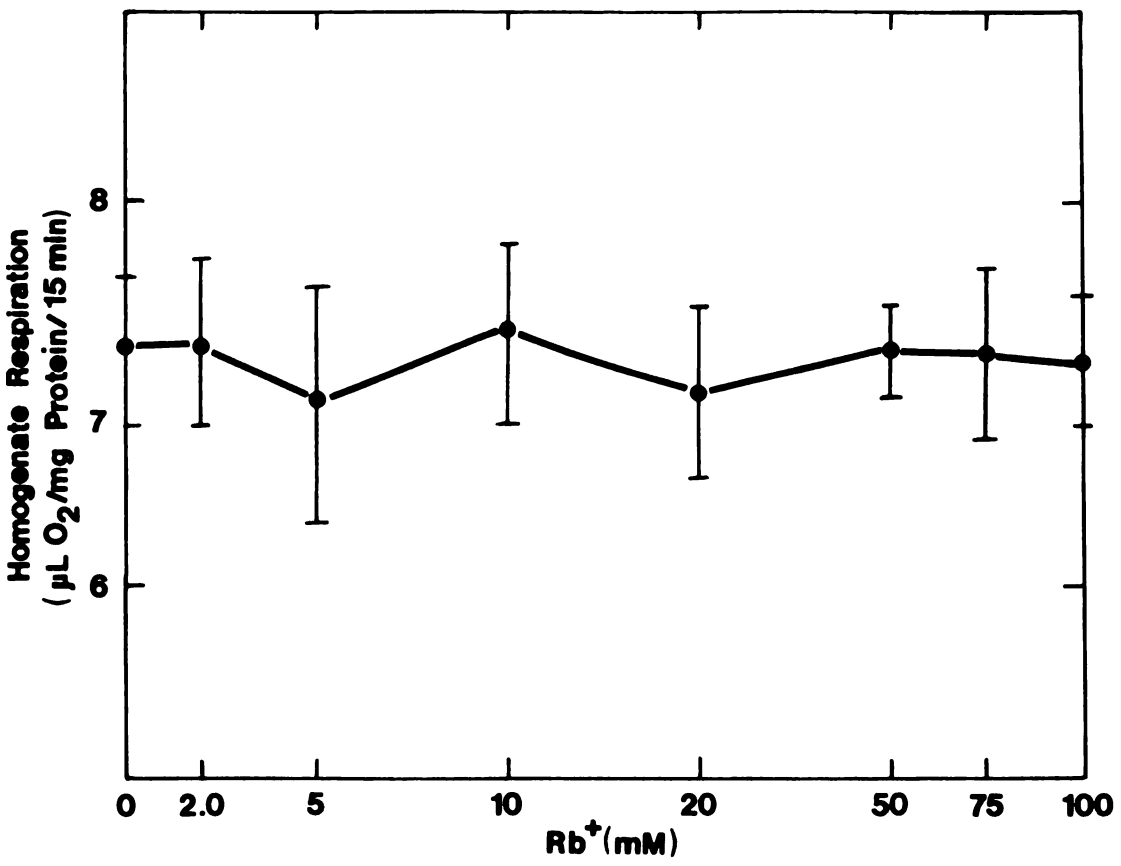
Homogenate Respiration
(µL O_2/mg Protein/15 min)
8
7
6
0
2.0
5
10
20
50
75
100
Rb^+(mM)

Figure 19

Figure 20. Effects of cesium on rat brain homogenate respiration.

Rat brain cortical slices were homogenized in .32M sucrose at 0°C. Homogenates were then incubated at 30°C in medium containing final concentrations of 2 mM Tris-ADP, 3 mM magnesium chloride, 10 mM potassium phosphate buffer (adjusted to pH 7.2 with potassium hydroxide), 10 mM glucose, 0.05 mM EDTA, 0.2 mM NAD, 40 mM nicotinamide, and 40 µg hexokinase enzyme. Cesium chloride was varied from 0 to 100 mM using choline chloride as an osmotic substitute. Background potassium was approximately 25 mM; sodium was zero. Data represent the means of 5 experiments. Vertical lines indicate S.E.M.

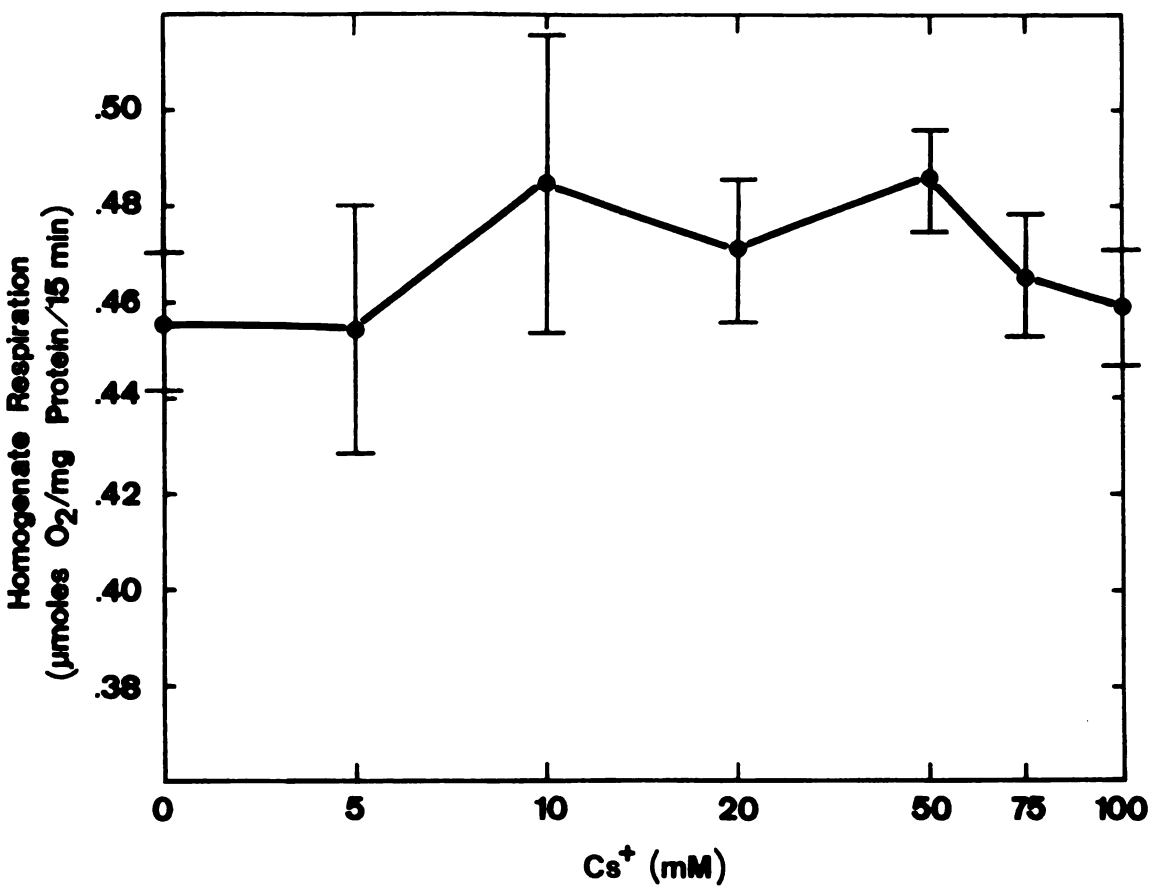
Homogenate Respiration
(μmoles O_2/mg Protein/15 min)
.50
.48
.46
.44
.42
.40
.38
0
5
10
20
50
75
100
Cs^+ (mM)

Figure 20

relatively high solubility relative to other thallous compounds, thallous nitrate was used. Thallous nitrate in a concentration of 3 mM produced significant inhibition of brain slice respiration in the presence of high concentrations of sodium and potassium (Figure 21). This inhibition of respiration by 3 mM thallous ion was significant during each of the four half-hour incubation periods tested. At lower concentrations of 0.1 to 1 mM, thallous nitrate had no significant effect on brain slice respiration with high sodium plus potassium during any of the four half-hour periods.

Since thallous ion is capable of substituting for potassium in the Na^+,K^+-ATPase reaction, the effects of thallous ions were studied in a potassium-free medium, where presumably Na^+,K^+-ATPase-associated brain slice respiration is minimal. In a potassium-free medium, however, thallous ion in concentrations of 0.3 to 10 mM produced significant inhibition of brain cortical slice respiration (Figure 22). Only during the first half hour did 0.3 and 1 mM thallous ion produce a slight stimulation of brain slice respiration. This stimulation, however, was statistically not significant. At higher concentrations of thallous ion no stimulation was observed. Therefore, thallous nitrate inhibits brain slice respiration in high sodium, potassium-free medium in both a dose and time-dependent manner. Although stimulation of slice respiration was observed during the first half hour with 0.3 and 1 mM thallous ion, such a stimulation was probably masked at higher concentrations by the inhibitory effects of thallous ions on brain respiration.

In previous experiments, it has been demonstrated that rubidium and cesium could substitute for potassium in stimulating respiration

Figure 21. Effects of thallium on sodium, potassium-stimulated brain slice respiration.

Rat brain cortical slice respiration was measured in medium containing 128 mM NaCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate buffer (pH 7.4), 100 mM KCl, and 24 mM glucose plus thallous ion concentrations ranging from 0 to 3 mM at 37°C. Data represent the means of 8 experiments. Vertical lines indicate S.E.M. *Denotes significantly different from control.

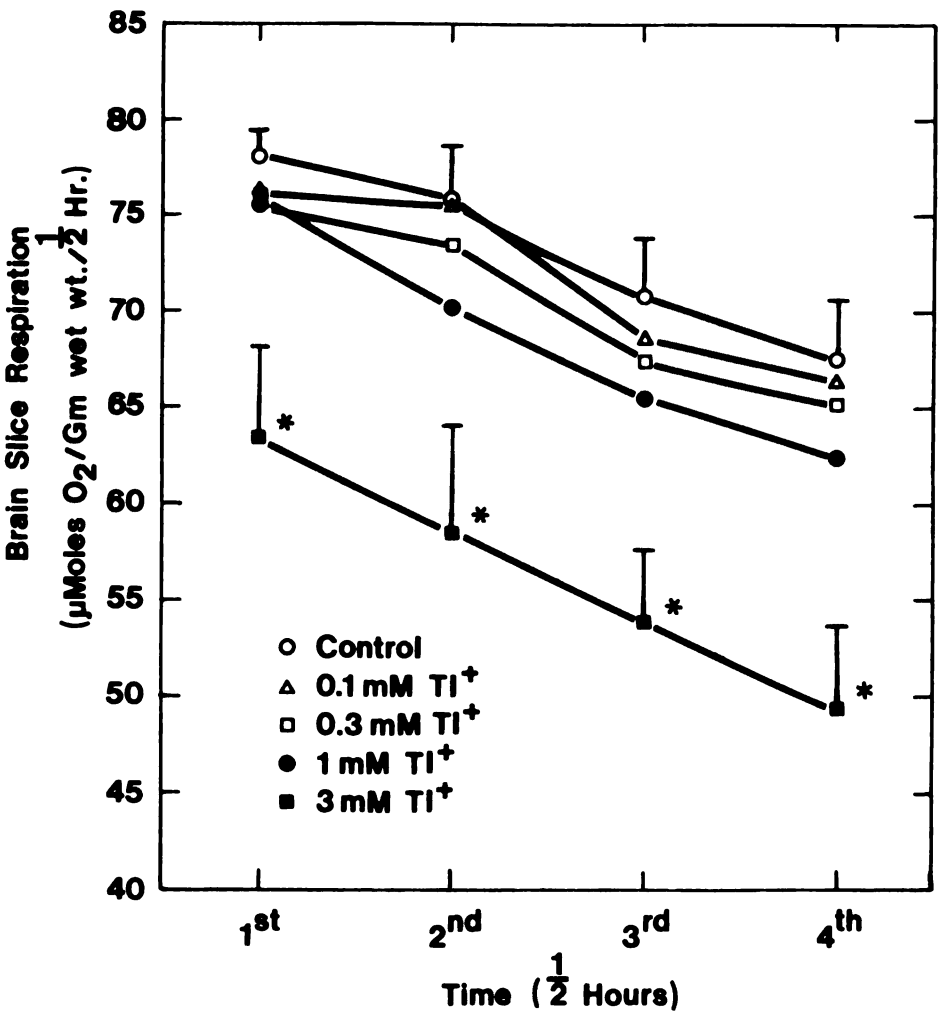
Brain Slice Respiration
(µMoles O_2/Gm wet wt./$\frac{1}{2}$ Hr.)
85
80
75
70
65
60
55
50
45
40
Control
0.1 mM Tl^+
0.3 mM Tl^+
1 mM Tl^+
3 mM Tl^+
*
1st
2nd
3rd
4th
Time ($\frac{1}{2}$ Hours)

Figure 21

Figure 22. Effects of thallium on brain slice respiration in a potassium-free medium.

Rat brain slice respiration was measured in medium containing 128 mM NaCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate buffer, and 24 mM glucose plus thallous ion concentrations ranging from 0 to 10 mM at 37°C. The background concentration of potassium was zero millimolar. Data represent the means of 4 experiments. *Denotes significantly different from control.

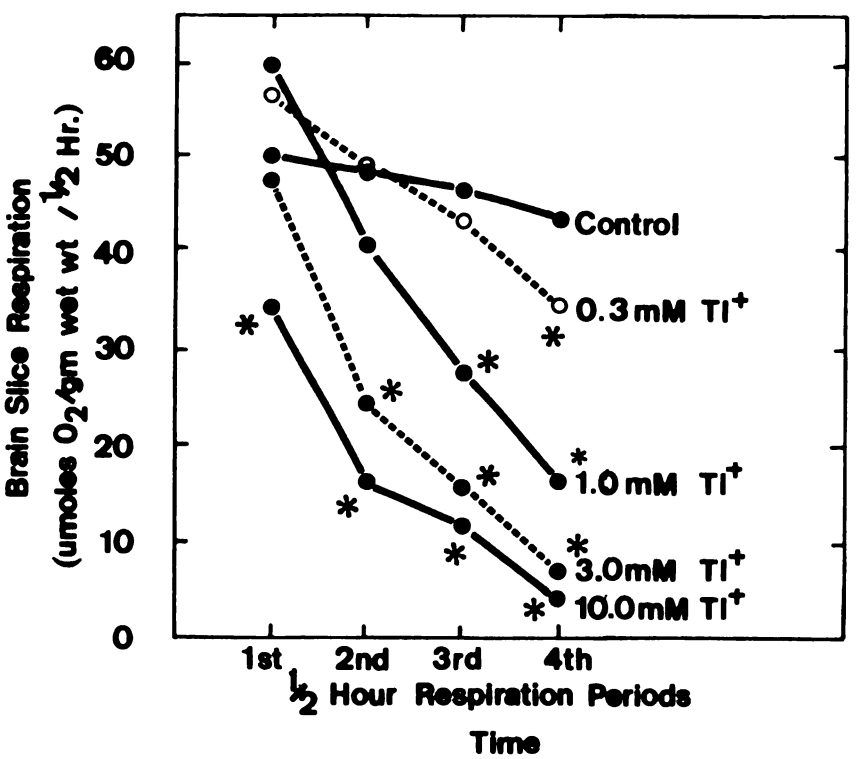
Brain Slice Respiration
(umoles O_2/gm wet wt / ½ Hr.)
60
50
40
30
20
10
0
Control
0.3mM Tl^+
1.0mM Tl^+
3.0mM Tl^+
10.0mM Tl^+
1st
2nd
3rd
4th
½ Hour Respiration Periods
Time

Figure 22

(Figures 12-15), while lithium could not (Figures 16-17). In the final series of monovalent cation experiments, whether these ions could substitute for sodium was studied. Figure 23 shows the effects of addition of 100 mM choline, sodium, rubidium, cesium or lithium on brain slice respiration in a sodium-free medium containing 100 mM potassium, 1.23 mM magnesium, 15 mM Tris-phosphate buffer (pH 7.4) and 24 mM glucose. Sodium in a concentration of 100 mM again produced a significant stimulation of brain cortical slice respiration relative to choline controls. Slice respiration was 152% and 173% of control respiration during the first and the fourth half hour following sodium addition, respectively. Lithium, rubidium, and cesium in concentrations of 100 mM failed to significantly stimulate brain slice respiration in a sodium-free medium containing 100 mM potassium. Respiration decreased somewhat in all systems with time. During the fourth post-addition half hour 100 mM lithium produced a significant depression of brain slice respiration relative to time matched choline controls. Since this significant depression was in a sodium-free medium, this effect of lithium would seem to be a direct effect of lithium on respiration, rather than a competition with sodium for activation of the Na^+,K^+-ATPase. From these data it may be concluded that only addition of sodium to high potassium media, and not addition of choline, lithium, rubidium, or cesium, yields a stimulation of brain cortical slice respiration.

Figure 23. Comparative effects of sodium, lithium, rubidium, cesium, and choline on rat brain slice respiration in a 100 mM potassium medium.

Rat brain cortical slice respiration was measured in a medium containing 100 mM KCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate buffer (pH 7.4), and 24 mM glucose, plus each of the monovalent cations as shown at 37°C. Data represent the means of 8 experiments. Vertical lines indicate S.E.M. *Denotes significantly different from choline control.

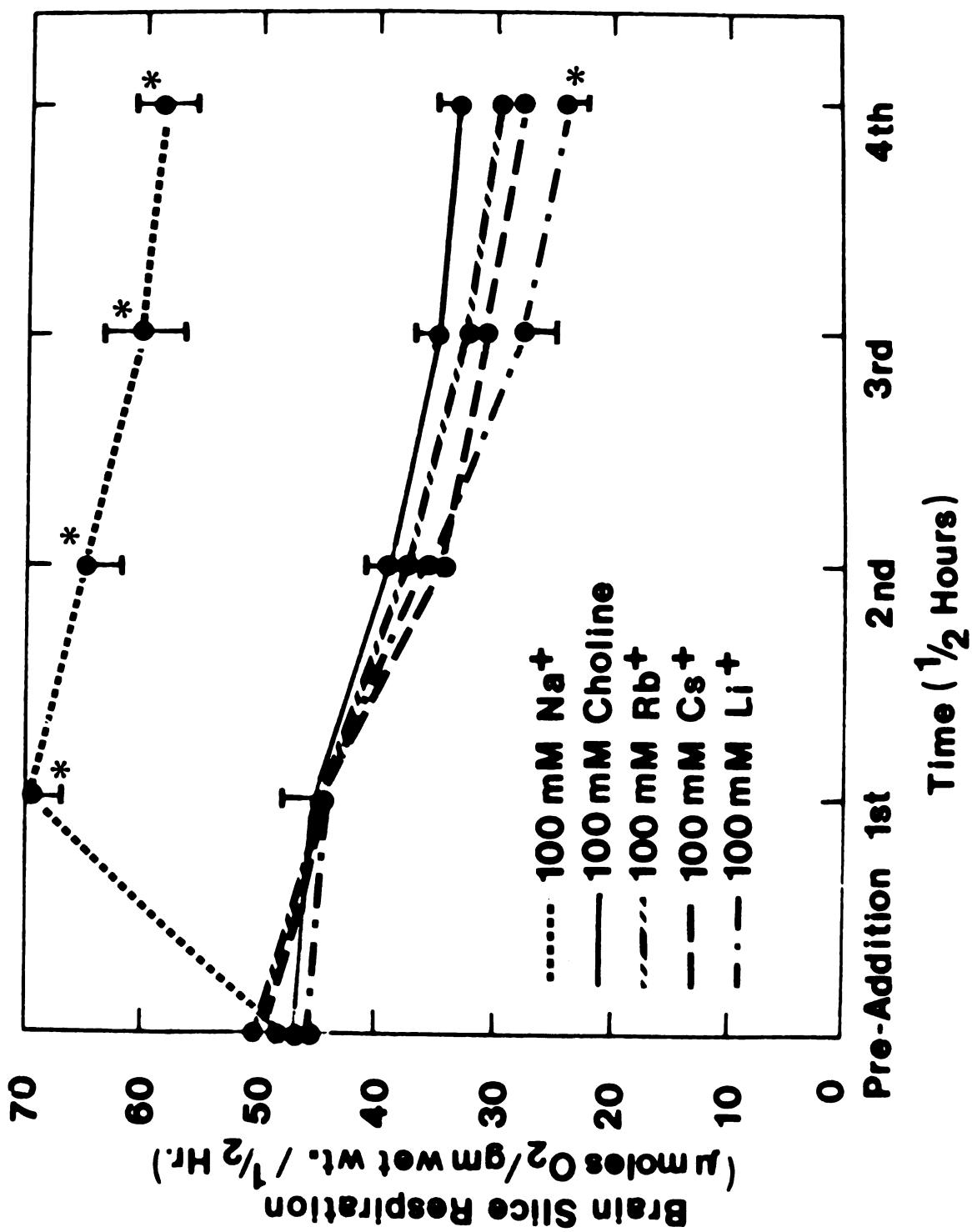
Brain Slice Respiration
(μ moles O2/gm wet wt. / 1/2 Hr.)
70
60
50
40
30
20
10
0
Pre-Addition
1st
2nd
3rd
4th
Time (1/2 Hours)
100 mM Na+
100 mM Choline
100 mM Rb+
100 mM Cs+
100 mM Li+

Figure 23

D. Effects of Chlorpromazine and Chlorpromazine Metabolites on Rat Brain Slice Respiration *in vitro* and the Effects of Chlorpromazine Administration *in vivo* on Respiration and ATPase Activity Assayed *in vitro*

Free radicals of chlorpromazine and various other substituted phenothiazines have been shown to be potent inhibitors of brain microsomal Na^+,K^+-ATPase *in vitro* (Akera and Brody, 1968, 1969; Gubitz *et al.*, 1973). Chlorpromazine and certain of its metabolites have also been shown to affect oxygen consumption and oxidative phosphorylation *in vivo* (Dawkins *et al.*, 1959a, 1959b; Løvtrup, 1963; Skinner and Spector, 1968; Tjoie *et al.*, 1972). Chlorpromazine has been demonstrated to inhibit oxygen consumption in intact cells such as polymorphonuclear leukocytes and lymphocytes (McCurrach *et al.*, 1970). The actions of chlorpromazine and several of its metabolites therefore were first studied on Na^+,K^+-stimulated and non-stimulated brain slice respiration *in vitro*.

Chlorpromazine in concentrations of 10 and 100 μM significantly inhibited brain slice respiration in a high sodium, high potassium medium, but had no significant effect on slice respiration in a sodium-free, high potassium medium in which sodium was substituted for by choline (Figure 24). The difference between respiration in high sodium, high potassium and sodium-free, high potassium media, that is, the sodium, potassium-stimulated portion of slice respiration, was significantly decreased by 10 and 100 μM chlorpromazine. The inhibition by these concentrations of chlorpromazine of this portion of respiration increased with time (Figure 24). Thus, chlorpromazine inhibition of brain slice respiration *in vitro* was

Figure 24. Chlorpromazine effects on sodium, potassium-stimulated brain slice respiration *in vitro*.

Rat brain cortical slices were incubated at 37°C in media with either 128 mM NaCl, 103 mM KCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate buffer (pH 7.4), and 24 mM glucose (total bars), or in a similar but sodium-free medium, in which choline chloride replaced NaCl (shaded bars). The difference between respiration in high sodium, high potassium, and sodium-free, high potassium media represents the sodium, potassium-stimulated portion of respiration (open bars). Data represent means of 4 experiments. Vertical lines indicate S.E.M. *Significant inhibition by chlorpromazine of sodium, potassium-stimulated respiration.

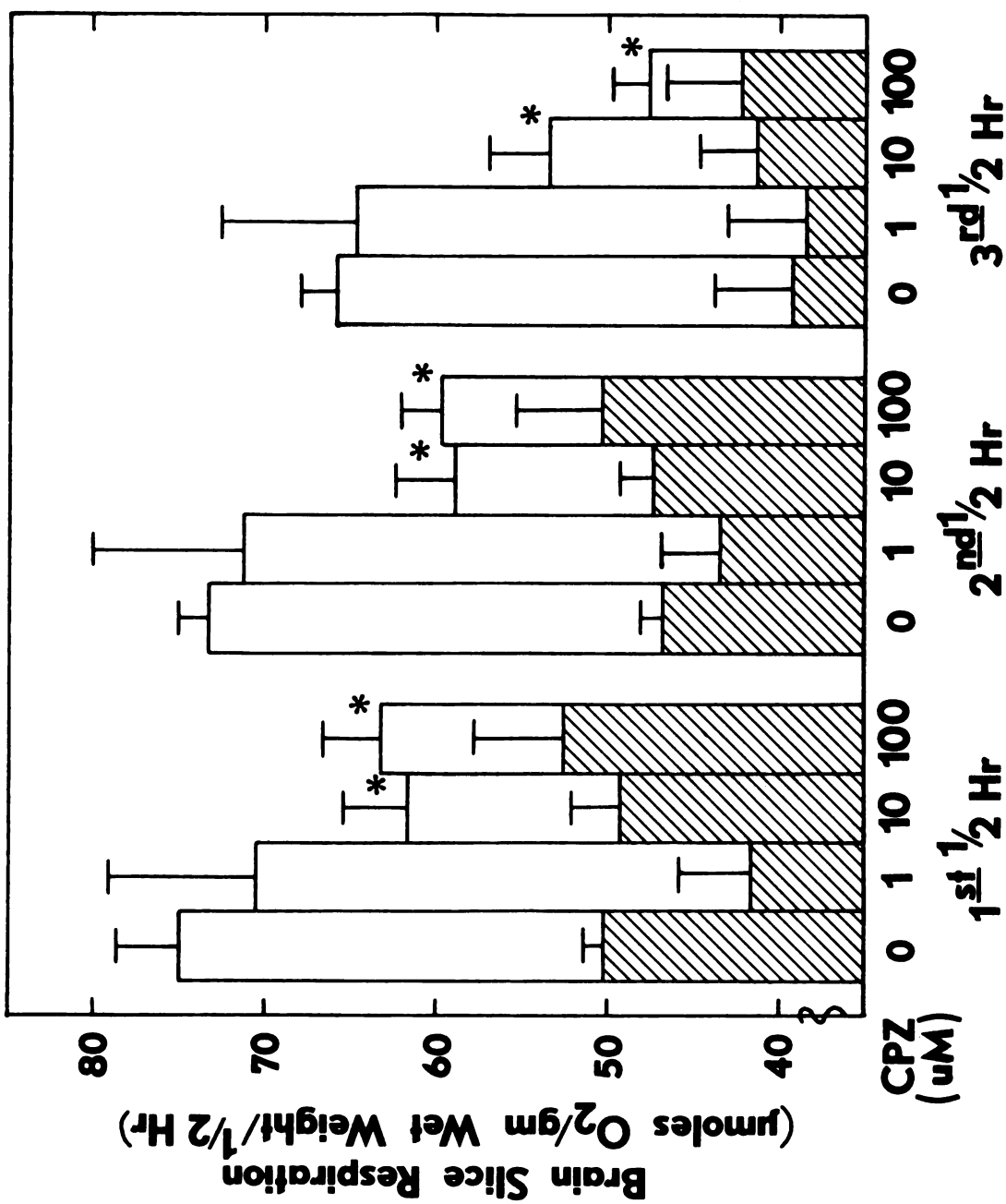
Brain Slice Respiration
(μmoles O2/gm Wet Weight/ 1/2 Hr)
80
70
60
50
40
CPZ (uM)
0 1 10 100
1st 1/2 Hr
0 1 10 100
2nd 1/2 Hr
0 1 10 100
3rd 1/2 Hr
*

Figure 24

both dose and time dependent, and was specific for the sodium, potassium-stimulated portion of respiration.

Two hydroxylated metabolites of chlorpromazine, 7-hydroxychlorpromazine and 7,8-dihydroxychlorpromazine, however, had no significant effects on brain slice respiration in either high sodium, high potassium or sodium-free, high potassium media at concentration of these agents up to 100 μM (Figures 25 and 26).

In order to study the effect of chlorpromazine administered *in vivo*, rats were injected with 30 mg of chlorpromazine hydrochloride per kg body weight for 1 to 22 days. Rats were sacrificed 30 minutes after the last injection, and brain cortical slice respiration and homogenate Na^+,K^+-ATPase activity were assayed *in vitro*. A single administration of chlorpromazine at a dose of 30 mg/kg had no significant effect on rat brain slice respiration (Figure 27). There were no significant differences in respiration of brain slices between control and acute chlorpromazine-treated rats either when slices were incubated in a high sodium, high potassium medium, or in a sodium-free, high potassium medium. Thus, acute chlorpromazine treatment *in vivo* failed to affect the sodium, potassium-stimulated portion of brain slice respiration assayed *in vitro*.

Chronic administration of chlorpromazine hydrochloride at a dose of 30 mg/kg/day for 12 to 22 days had no effect on brain slice respiration (Table 1). There was no significant difference in respiration of brain slices between control and chronic chlorpromazine-treated rats, when slices were incubated in either a high sodium, high potassium medium, or in a sodium-free, high potassium medium.

Figure 25. 7-Hydroxychlorpromazine effects on brain slice respiration *in vitro*.

Rat brain cortical slices were incubated at 37°C as described in Figure 24. Total bars represent respiration in high sodium, high potassium medium; shaded bars represent respiration in sodium-free, high-potassium medium, while the open bars represent the sodium, potassium-stimulated portion of respiration. Data represent the mean respiration of 4 experiments. Vertical lines indicate S.E.M.

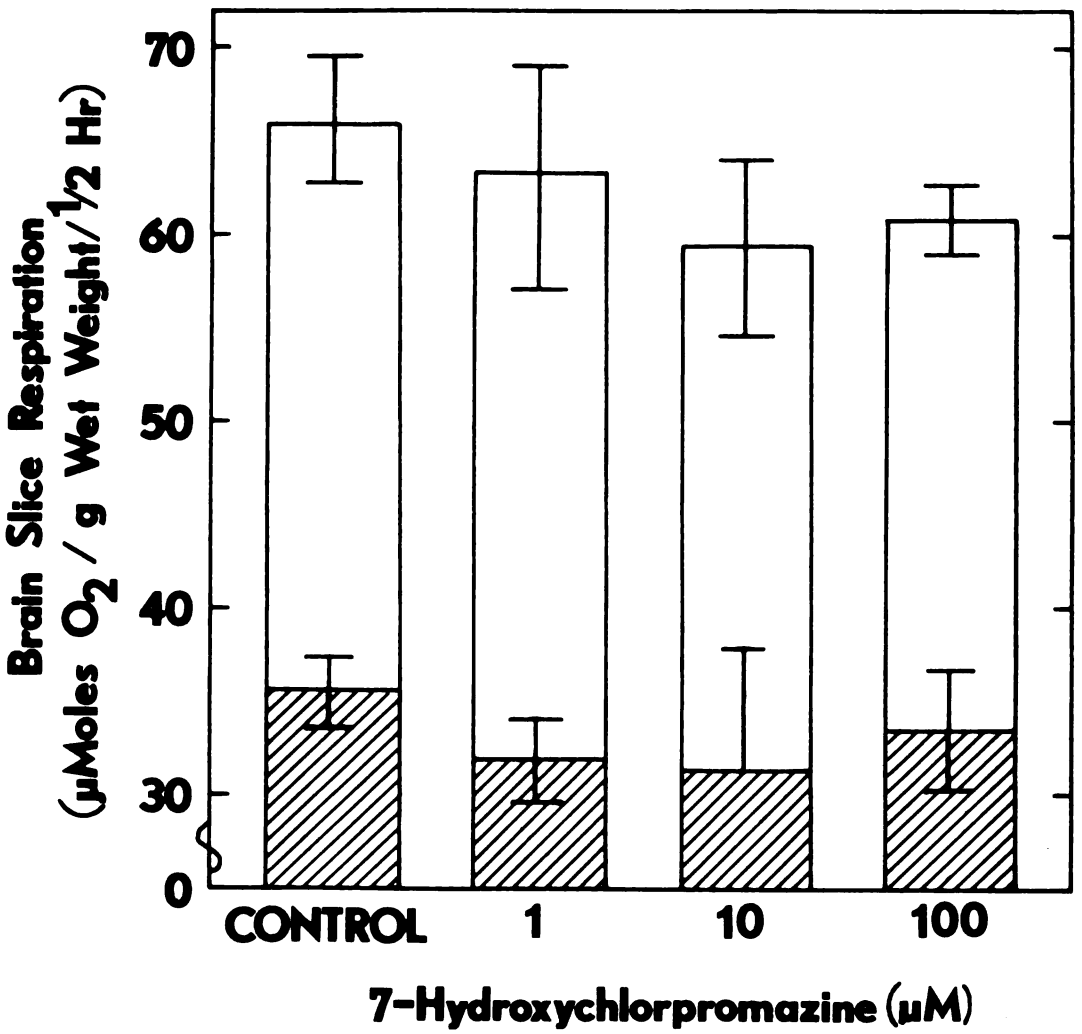
Brain Slice Respiration
(μMoles O_2 / g Wet Weight/ 1/2 Hr)
70
60
50
40
30
0
CONTROL
1
10
100
7-Hydroxychlorpromazine (μM)

Figure 25

Figure 26. 7,8-Dihydroxychlorpromazine effects on brain slice respiration *in vitro*.

Rat brain cortical slices were incubated at 37°C as described in Figure 24. Total bars represent respiration in high sodium, high potassium medium; shaded bars represent respiration in sodium-free, high-potassium medium, while the open bars represent the sodium, potassium-stimulated portion of respiration. Data represent the mean respiration of 4 experiments. Vertical lines indicate S.E.M.

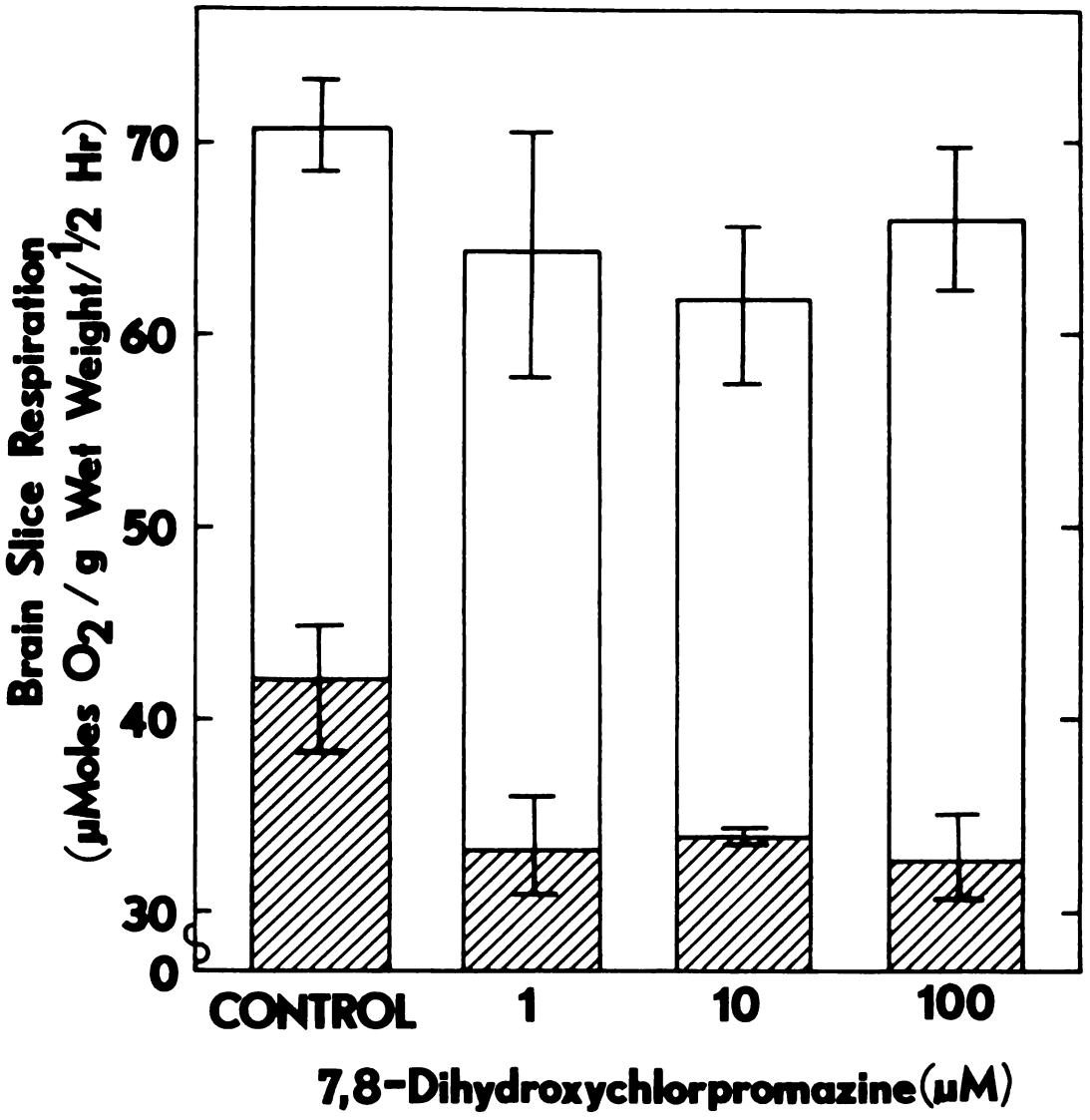
Brain Slice Respiration
(μMoles O2 / g Wet Weight/ 1/2 Hr)
70
60
50
40
30
0
CONTROL
1
10
100
7,8-Dihydroxychlorpromazine(μM)

Figure 26

Figure 27. Effects of chlorpromazine administered *in vivo* on brain cortical slice respiration.

Male 250-350 g Sprague-Dawley rats were injected intraperitoneally with chlorpromazine in a dose of 30 mg/kg. Control rats received equal volumes of 0.9% saline per kilogram of body weight. Rats were sacrificed 30 minutes after injection and brain cortical slices from these rats were incubated in 2 ml of either high sodium, high potassium medium, or sodium-free, high potassium medium at 37°C. Slice respiration was measured, and the differences between rates of respiration in high sodium, high potassium medium and that in sodium-free, high potassium medium were calculated as the sodium, potassium-stimulated portion of respiration. Data represent the means of 5 experiments. Vertical lines indicate S.E.M. The total bars represent respiration in sodium, potassium medium. The shaded bars represent respiration in sodium-free, high potassium medium, and the open portions of the bars represent the sodium, potassium-stimulated portion of slice respiration. Statistical analysis was as described in Figure 1.

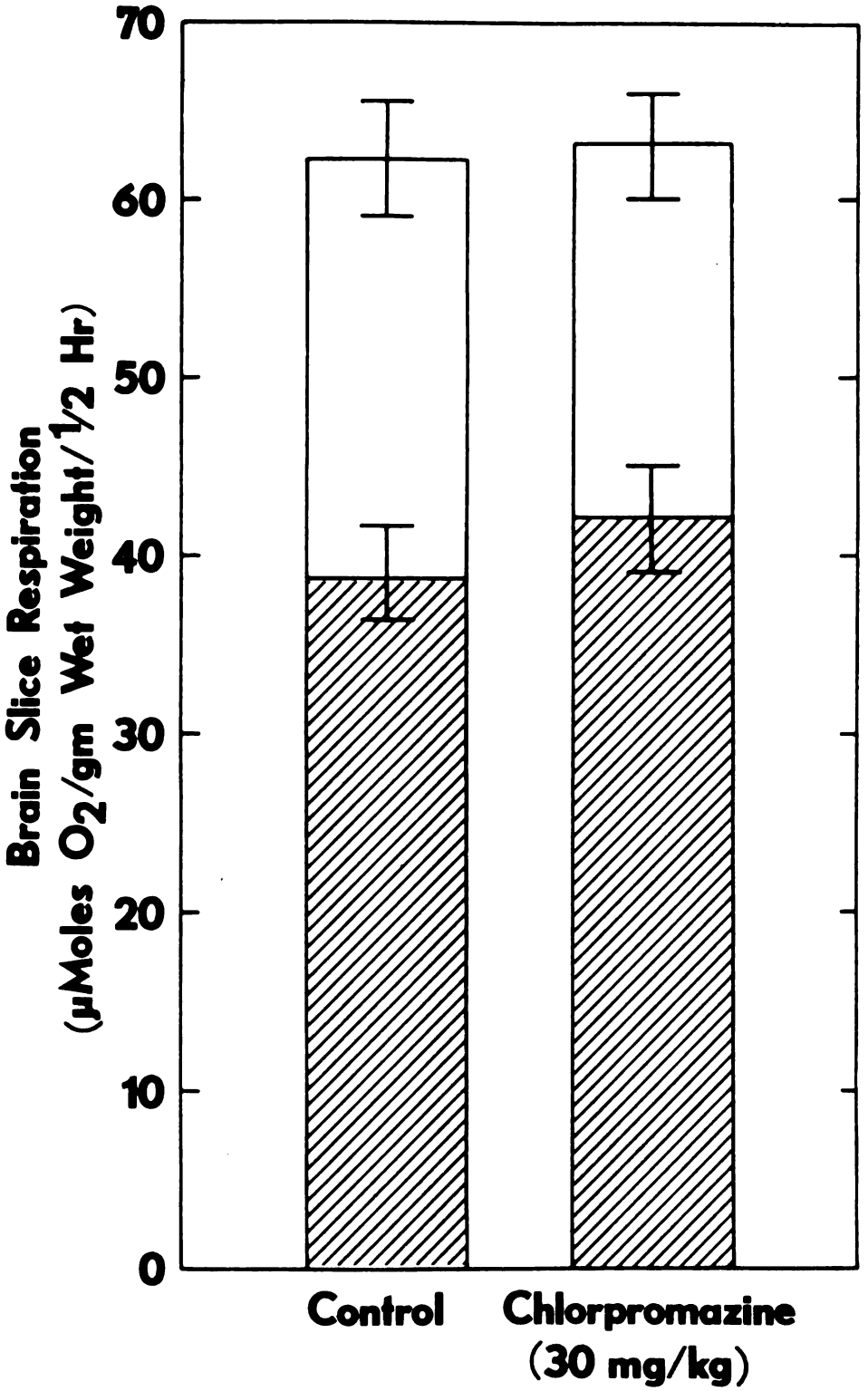
70
60
50
40
30
20
10
0
Brain Slice Respiration
(μMoles O2/gm Wet Weight/1/2 Hr)
Control
Chlorpromazine
(30 mg/kg)

Figure 27

Table 1. Chronic effects of chlorpromazine *in vivo* on brain cortical slice respiration

Male Sprague-Dawley rats with an initial weight of between 150 and 175 g were intraperitoneally injected with a daily dose of 30 mg of chlorpromazine per kg of body weight. Control rats received equivalent volumes of 0.9% saline per kg of body weight, daily. Control and chlorpromazine treated rats were sacrificed 30 minutes after injection on days 12, 13, 21, and 22 of the chronic treatment. Slice respiration was measured, and the difference between rates of respiration in high sodium, potassium medium and sodium-free, high potassium medium was calculated as the sodium, potassium-stimulated portion of respiration.

	Control			CPZ-treated (30 mg/kg/day)		
Day	Na^+,K^+	Ch,K^+	Diff.	Na^+,K^+	Ch,K^+	Diff.
12	75.0*	41.5	33.5	85.4	40.0	45.4
13	84.8	48.2	36.6	78.6	40.8	37.8
21	76.8	39.5	37.3	86.2	51.6	34.6
22	74.1	45.3	28.8	84.6	49.8	34.8
Mean ±S.E.M.	77.68 ±2.44	43.63 ±1.94	34.05 ±1.93	83.70 ±2.01	45.55 ±3.00	38.15 ±2.53

*μmol O_2/g wet weight/half hour.

Thus, there was also no significant effect of chlorpromazine treatment on the sodium, potassium-stimulated portion of respiration.

Na^+,K^+-ATPase activity of rat brain homogenates obtained from animals treated with a single dose of chlorpromazine hydrochloride (30 mg/kg i.p.) was 108% of control, while Na^+,K^+-ATPase activity in homogenates from animals chronically treated with 30 mg of chlorpromazine per kg body weight per day was 102% of control (Figure 28).

Figure 28. Acute and chronic effects of chlorpromazine administered *in vivo* on rat brain Na^+,K^+-ATPase activity assayed *in vitro*.

Na^+,K^+-ATPase activity of brain homogenate from rats treated acutely and chronically with chlorpromazine (CPZ) is shown on the ordinate. Acute and chronic animals were sacrificed 30 minutes after chlorpromazine (30 mg/kg) treatment. Chronically treated animals received 30 mg/kg for 12 to 22 days. Data for acutely treated rats represent the mean of five experiments. Data for chronically treated rats represent the means of four experiments. Vertical lines indicate S.E.M.

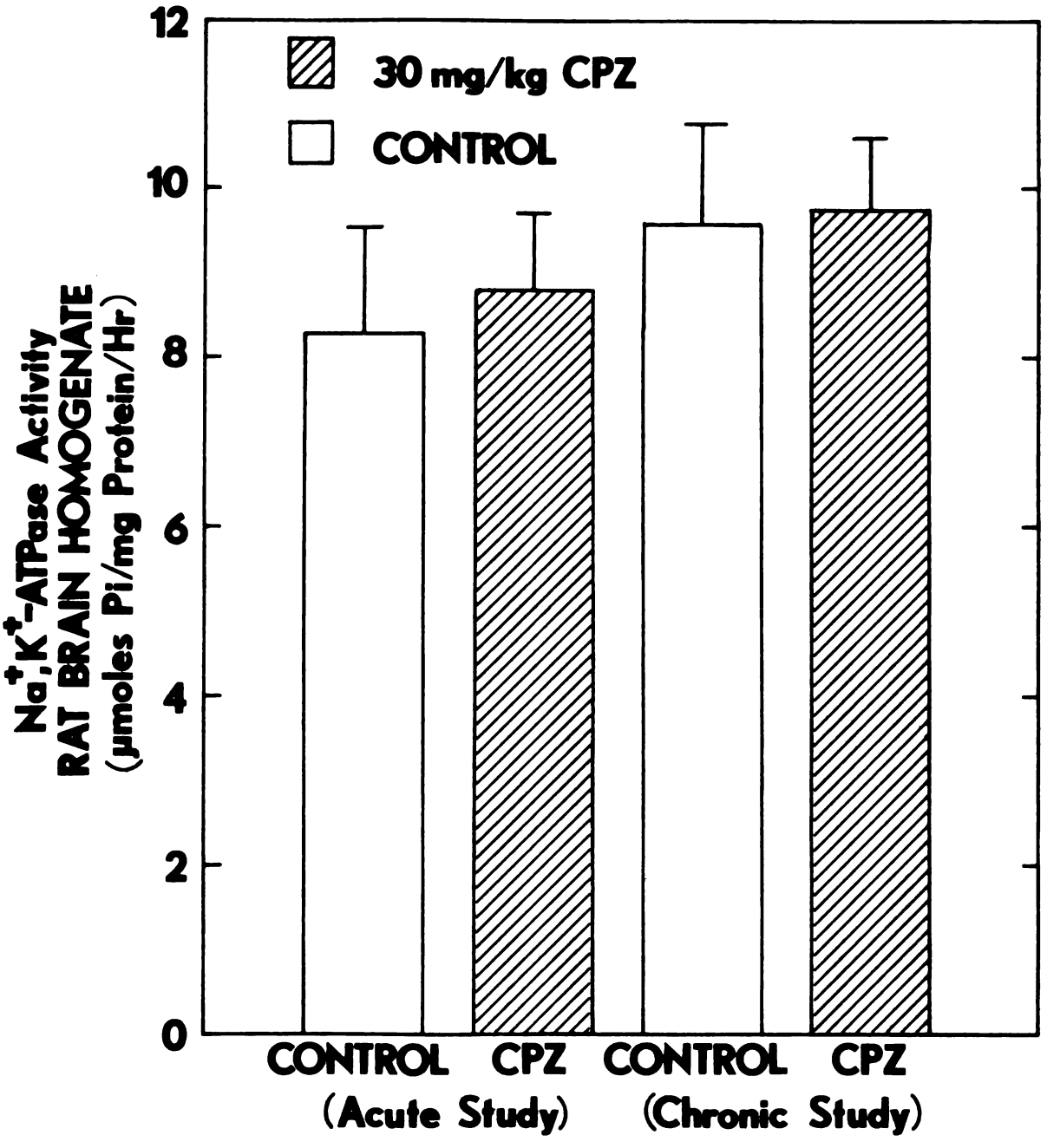
30 mg/kg CPZ
CONTROL
12
10
8
6
4
2
0
Na^+,K^+-ATPase Activity
RAT BRAIN HOMOGENATE
(μmoles Pi/mg Protein/Hr)
CONTROL
CPZ
CONTROL
CPZ
(Acute Study)
(Chronic Study)

Figure 28

Thus, neither acute nor chronic *in vivo* administration of chlorpromazine in a dose of 30 mg/kg had a significant effect on the Na^+,K^+-ATPase activity of rat brain homogenates.

E. Effects of Digitoxin Administration on Rat Brain Respiration and ATPase Activity

Cardiac glycosides, such as ouabain, digoxin and digitoxin, have been shown to inhibit both brain Na^+,K^+-ATPase and brain slice respiration *in vitro* (Whittam and Blond, 1964; Swanson and McIlwain, 1965; Ruscak and Whittam, 1967). Ouabain and digoxin administration *in vivo* have been demonstrated to inhibit Na^+,K^+-ATPase activity in dog heart and kidney (Akera *et al.*, 1969, 1970; Hook, 1969; Besch *et al.*, 1970; Allen *et al.*, 1970, 1971; Goldman *et al.*, 1973). In the following experiments the effect of digitoxin administration *in vivo* was studied on sodium, plus potassium-stimulated respiration and brain Na^+,K^+-ATPase activity. The rat was chosen because of the relatively high sensitivity of rat brain Na^+,K^+-ATPase and extremely low sensitivity of rat cardiac Na^+,K^+-ATPase to cardiac glycoside inhibition (Repke *et al.*, 1965), which makes it possible to administer relatively large doses of cardiac glycosides to affect brain Na^+,K^+-ATPase without causing the death of the animal due to cardiac toxicity. The rat was also chosen because of the relatively slow dissociation of cardiac glycosides from its brain Na^+,K^+-ATPase (Tobin and Brody, 1972). In these studies, digitoxin was used instead of ouabain, because digitoxin being one of the more lipid-soluble of the cardiac glycosides penetrates more readily across lipid cell membrane and into the brain (Repke, 1958; Kuschinsky *et al.*, 1968; Greenberger and Caldwell, 1972). Ouabain being highly water soluble and

relatively poorly lipid soluble would be more poorly absorbed and have greater difficulty entering the brain (Greenberger and Caldwell, 1972).

Following intraperitoneal administration of digitoxin in rats, sodium, potassium-stimulated respiration of brain slices obtained from these animals was significantly inhibited (Figure 29). Slices from saline-treated control rats had a mean respiration rate of 75.1 µMole of oxygen/g tissue (wet weight)/half hour in a high sodium, high potassium medium, and a mean respiration rate of 45.6 µMoles of oxygen/g tissue (wet weight)/half hour in a sodium-free, high potassium medium. Respiration of slices in high sodium, high potassium medium from rats treated with 7.5 to 30 mg of digitoxin per kg body weight was significantly lower than that of brain slices from control rats. There was no significant difference in respiration in sodium-free, high potassium medium of brain slices from control and digitoxin-treated rats. Maximum inhibition of sodium, potassium-stimulated brain slice respiration to approximately 45% of control respiration during the first half hour incubation *in vitro* occurred following treatment with 15 and 30 mg of digitoxin per kg, although the respiration of slices in a high sodium, high potassium medium from rats treated with 1 to 4 mg of digitoxin per kg body weight was not significantly different from that in slices from alcohol-treated control rats. Thus, the effect of digitoxin was dose-dependent and was specific for the sodium, potassium-stimulated portion of respiration.

Intraperitoneal injection of digitoxin significantly decreased Na^+,K^+-ATPase activity of rat brain homogenates. The inhibition was

Figure 29. Inhibition of sodium plus potassium-stimulated respiration by digitoxin treatment *in vivo*.

Male Sprague-Dawley rats were injected intraperitoneally with digitoxin or alcohol vehicle. Thirty minutes later they were decapitated, and brain cortex slices prepared. Slices were incubated in 2 ml of either calcium-free Krebs Henseleit medium with 100 mM potassium, or in sodium-free, choline media with 100 mM potassium, at 37°C. Respiration rates were measured and expressed in micromoles of oxygen per gram wet weight per half hour. Differences between rates in sodium plus potassium medium versus choline plus potassium medium were calculated. Sodium plus potassium stimulated respiration rates in slices from digitoxin treated rats were divided by sodium plus potassium stimulated rates in matched controls, and the results were expressed as percent control respiration. Data represent the means of 4 experiments. *Denotes significantly different from control.

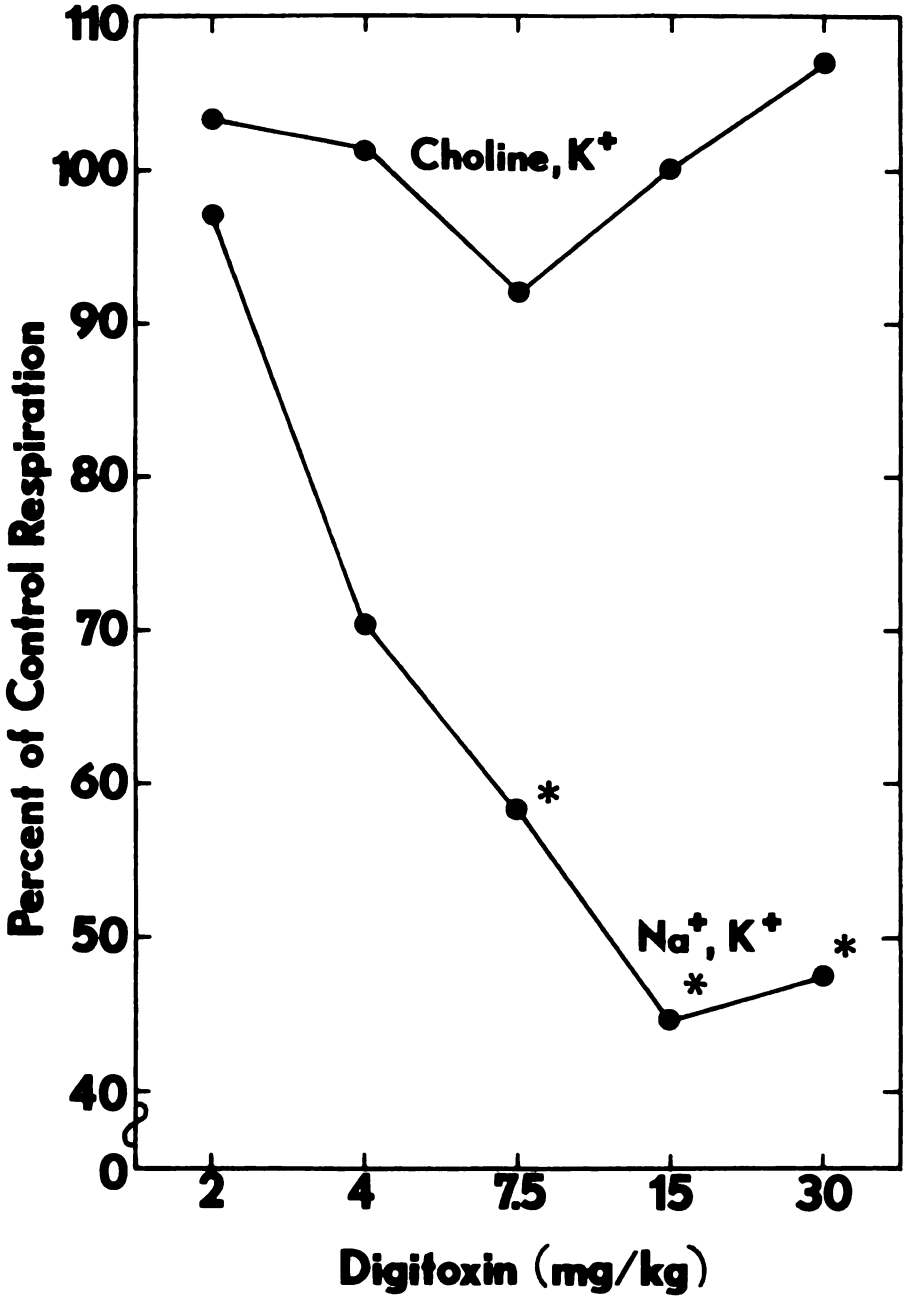
110
100
90
80
70
60
50
40
0
Percent of Control Respiration
Choline, K+
Na+, K+
*
*
*
2
4
7.5
15
30
Digitoxin (mg/kg)

Figure 29

maximal at doses of 15 and 30 mg per kg (Figure 30). Control rat brain homogenate Na^+,K^+-ATPase activity was approximately 7.72 µMoles of inorganic phosphate/mg protein/hour. Maximal inhibition of brain homogenate Na^+,K^+-ATPase was to about 65% of control activity. There were no significant effects observed on Mg^{2+}-ATPase activity in homogenates prepared from digitoxin-treated rats. Thus, inhibition of rat brain Na^+,K^+-ATPase activity by digitoxin administered *in vivo* was dose-dependent, and specific for Na^+,K^+-ATPase. This correlated well with specific inhibition of sodium, potassium-stimulated respiration of brain slices following administration of digitoxin.

Figure 30. Inhibition of sodium plus potassium-stimulated ATPase activity assayed *in vitro* by digitoxin treatment *in vivo*.

The ATPase activity in whole brain homogenates was estimated from digitoxin-treated and alcohol-treated rats from the amount of inorganic phosphate liberated from ATP during incubations at 37°C. Magnesium-dependent ATPase activity assayed in the presence of 5 mM magnesium was subtracted from total ATPase activity assayed in the presence of 100 mM sodium, 15 mM potassium, and 5 mM magnesium, to calculate the sodium, potassium-activated ATPase activity. For each experiment, percent inhibition of sodium, potassium ATPase activity in digitoxin-treated rat homogenates was calculated relative to the sodium, potassium-ATPase activity in paired control rat brain homogenates. Data represent the means of 4 experiments. *Denotes significantly different from control.

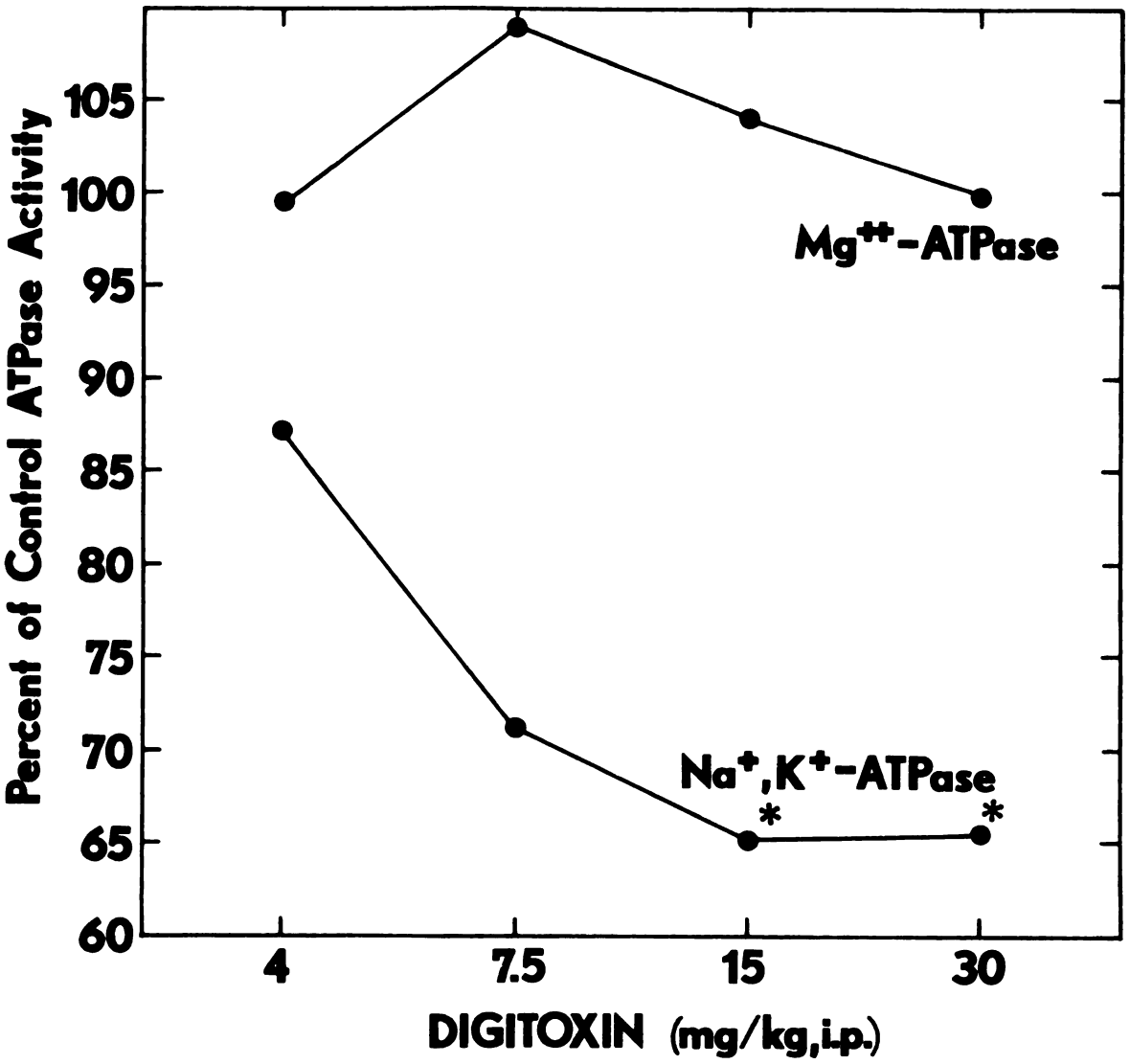
Percent of Control ATPase Activity
105
100
95
90
85
80
75
70
65
60
Mg++-ATPase
Na+,K+-ATPase
*
*
4
7.5
15
30
DIGITOXIN (mg/kg,i.p.)

Figure 30

DISCUSSION

A. Potassium and Ouabain Effects on Brain Slice Respiration

Ashford and Dixon (1935) have demonstrated that the addition of 100 mM potassium chloride stimulates respiration in rabbit brain cortical slices. This phenomenon is now known as the "potassium effect." Whittam (1961) proposed that Na^+,K^+-ATPase serves as a pacemaker of respiration in brain, and Minakami *et al.* (1963) suggested that the mechanism of the so-called "potassium effect" is to enhance respiration coupled to ADP phosphorylation, subsequent to increases in active cation transport and ADP production by the membrane ATPase enzyme. Since this work, a number of alternative proposals have been made to explain the "potassium effect." These alternative mechanisms for the "potassium effect" include: 1) potassium affecting glucose transport into the cell (Rolleston and Newsholme, 1967), 2) potassium stimulating pyruvate kinase (Takagaki, 1968), 3) potassium stimulating pyruvate dehydrogenase (Kini and Quastel, 1959; Clark and Nicklas, 1970), 4) potassium stimulating enzymes in the Krebs tricarboxylic acid cycle or oxidative phosphorylation (Hoskin, 1960; Cremer, 1967; Klicpera and Hoffmann, 1975) and 5) potassium inducing depolarization of cell membrane (Hillman and McIlwain, 1961).

When pyruvate is substituted for glucose, slice respiration is enhanced, and if 100 mM potassium is added when pyruvate is the

substrate, slice respiration is further stimulated (Figure 2). This suggests that potassium must have other actions than simply to stimulate glucose uptake or glycolysis. It suggests that the "potassium effect" is not primarily involved with stimulation of pyruvate kinase. Stimulation of slice respiration by sodium in high potassium medium where cells are already depolarized rules out the possibility that potassium-induced depolarization is the mechanism of potassium stimulation. The requirement of sodium for potassium stimulation (Figure 3), and the observation that ouabain inhibits potassium-stimulated respiration in a similar manner as it inhibits isolated Na^+,K^+-ATPase, support the hypothesis that potassium stimulation of slice respiration is associated with potassium stimulation of Na^+,K^+-ATPase. The observation that the rate of potassium-stimulated respiration never exceeds the respiration rate observed in the presence of 2,4-dinitrophenol, i.e., where the control of respiration by ADP is relieved, is consistent with this hypothesis. This hypothesis is further supported by the observation that among combinations of monovalent cations, only those which would stimulate microsomal Na^+,K^+-ATPase *in vitro* are capable of stimulating brain slice respiration. These combinations included sodium, plus either potassium, rubidium or cesium, but not lithium.

The use of ouabain to estimate the portion of respiration associated with Na^+,K^+-ATPase activity (Whittam and Blond, 1964) appears to result in an overestimation. Although ouabain has been shown in semi-purified enzyme preparations to be a specific inhibitor of Na^+,K^+-ATPase (see review by Skou, 1965), present data indicate that the inhibition of slice respiration by ouabain is not solely dependent on

its action to reduce the availability of ADP by inhibiting ATP hydrolysis associated with cation transport. Evidence for this is that 1) ouabain inhibits so-called "non-stimulated" as well as potassium-stimulated slice respiration, and 2) ouabain is able to inhibit respiration in the presence of 2,4-dinitrophenol (Figure 7).

In both the present study (Figure 7) and in previous studies by Cremer (1967), rat brain slice respiration in the presence of ouabain plus 2,4-dinitrophenol was significantly lower than with 2,4-dinitrophenol alone. If ouabain's mechanism of action is exclusively the inhibition of Na^+,K^+-ATPase resulting in reduced generation of ADP, 2,4-dinitrophenol at concentrations of 50 μM, that completely uncouple oxidative phosphorylation (Brody, 1955), would be expected to cause similar stimulation in the presence or absence of ouabain.

Further evidence for a secondary effect of ouabain is that inhibition by 100 μM ouabain of both potassium-stimulated and so-called "non-stimulated" slice respiration is larger in a medium with pyruvate substrate (Figure 2) than in comparable medium with glucose (Figure 1). This suggests that ouabain might be acting more specifically on a system where pyruvate is being oxidized (Figure 2). Neither this, nor the previous data, however, suggest a mechanism by which ouabain might be exerting its secondary effects on respiration.

One of the potential indirect factors in ouabain's inhibition of respiration with pyruvate substrate, and ouabain's inhibition in the presence of 2,4-dinitrophenol, could be a change in intracellular environment. High concentrations of ouabain increase intracellular sodium concentration and decrease intracellular potassium concentration. The magnitude of increase in intracellular sodium is similar

to that producing an approximate 30% inhibition of optimal brain homogenate respiration. This is consistent with previous reports that sodium ion significantly inhibits glycolysis and respiration of brain and other tissue homogenates (Racker and Krimsky, 1945; LePage, 1948; Utter, 1950). This is also consistent with the observation that respiration in the presence of ouabain plus 2,4-dinitrophenol was about 30% lower than that in the presence of 2,4-dinitrophenol alone (Figure 7).

Since total osmolarity in the present sodium versus potassium study is different from that in the sodium versus choline study, it is difficult from the present data to determine whether the sodium inhibition is competitive or non-competitive with respect to potassium. Although potassium has been shown to stimulate oxidation and phosphorylation in mitochondrial preparations (Pressman and Lardy, 1955; Whittam, 1964; Krall *et al.*, 1964; Nicklas *et al.*, 1971), the magnitude of decrease in intracellular potassium concentrations produced by ouabain is not such as to affect the rate of mitochondrial oxygen consumption. Thus, the increase in intracellular sodium concentration occurring with incubation of brain slices in the presence of ouabain appears to play the primary role in ouabain's inhibition of brain slice respiration.

Swanson (1968) has suggested that ouabain inhibition of slice respiration in calcium-free media is not a good indicator of the portion of the cell's metabolism used for active transport. This is based on his observation that ouabain significantly inhibits incorporation of ^{32}P-labeled phosphate into creatine phosphate. The use of ouabain overestimates the portion of slice respiration associated with Na^+,K^+-ATPase activity. Thus, the present results confirm and

extend the observation that ouabain inhibition of potassium-stimulated respiration in high sodium medium is unsuitable as a measure of the proportion of oxygen consumption of brain cells directly associated with Na^+,K^+-ATPase activity.

B. Estimation of Brain Slice Respiration Associated with Na^+,K^+-ATPase Activity

The purpose of the present experiments was to study the precise portion of brain slice respiration associated with Na^+,K^+-ATPase activity. The assumption was made that maximal brain slice respiration associated with full activation of the Na^+,K^+-ATPase occurs with potassium stimulation. This was based on two observations: 1) that potassium stimulation was similar in magnitude to that occurring with 2,4-dinitrophenol, and 2) that potassium did not significantly stimulate respiration above that occurring with 2,4-dinitrophenol. Klicpera and Hoffmann (1975) have shown in Krebs Ringer medium that 2,4-dinitrophenol (25 μM) stimulated brain slice respiration by approximately 38%. This was similar to the stimulation achieved by addition of 100 mM potassium in calcium-free medium (Figure 1). Klicpera and Hoffmann (1975) demonstrated that no further effect on respiration occurred upon addition of high potassium to brain slices already respiring at a stimulated rate with 25 μM 2,4-dinitrophenol. With this concentration of 2,4-dinitrophenol, respiration should be nearly completely uncoupled and should be proceeding at the maximum velocity permitted by the enzymes of the electron transport system (Klicpera and Hoffmann, 1975). Since potassium-stimulated respiration was similar to that with 2,4-dinitrophenol, it may be that 100 mM potassium stimulates membrane

Na^+,K^+-ATPase activity and ADP generation (Minakami *et al.*, 1963) to a point where other factors such as substrate phosphorylation or electron transport processes, rather than the ADP concentration, become rate limiting. If ADP generation far exceeds the rate of "other" rate limiting systems, then it is not possible to estimate ATPase activity from respiration. Such is probably not the case. With digitoxin treatment, Na^+,K^+-ATPase inhibition is associated with reduced respiration, indicating that under the present conditions, ADP generation can become rate limiting when Na^+,K^+-ATPase is inhibited.

Potassium stimulation alone, however, provides an underestimation of Na^+,K^+-ATPase-associated respiration, since respiration in calcium-free medium is already partially "stimulated" (Figure 1 versus Figure 6). In order to determine the full component of Na^+,K^+-ATPase-associated respiration, it is necessary to first accurately estimate the level of completely "non-stimulated" slice respiration. Even in sodium-free (choline) medium, cells still contain small amounts of intracellular sodium (Whittam, 1962a) which, together with extracellular potassium, presumably can stimulate Na^+,K^+-ATPase activity and hence respiration. Twenty-minute incubation at 37°C with 100 mM potassium added to slices in sodium-free medium decreases intracellular sodium concentration to a minimal level (Figure 11). Thus, in sodium-free medium with high potassium concentrations, both coupled sodium, potassium pump activity, and ADP-dependent pump related respiration would be expected to be minimal. Hence, slice respiration in sodium-free, high choline and high potassium medium would provide a more accurate baseline from

which to measure a Na^+,K^+-ATPase-associated portion of brain slice respiration.

In the presence of high sodium and high potassium, brain slice respiration is approximately 30% above that in high sodium, low potassium medium (Figure 31). With sodium-free, high potassium medium, there is a depression of slice respiration to levels approximately 15 to 20% below those in high sodium, low potassium medium (Figure 31). The sum of these two components, the sodium, potassium-stimulated (in high sodium medium) and the potassium-inhibited component of respiration (in sodium-free medium) yield approximately 40-50% of total brain slice respiration observed in a high sodium, high potassium medium (Figure 31). From the slice respiration data here and that of Minakami *et al.* (1963) it is postulated that this 40-50% of brain slice respiration (Figure 31) observed in high sodium, high potassium medium is associated with ATP hydrolysis resulting from sodium and potassium activation of the membrane Na^+,K^+-ATPase. Thus, the difference in respiration rates observed in high sodium, high potassium media, and those in sodium-free, high potassium media, appear to yield a more accurate measure of brain slice respiration associated with stimulated active transport. The advantage of this system is that there can be no effect of high intracellular sodium inhibition of slice respiration as produced using inhibition by ouabain.

C. Effects of Monovalent Cations on Brain Slice Respiration

The purpose of the present studies was to investigate whether monovalent cations could alter respiration or Na^+,K^+-ATPase activity

Figure 31. Components of brain slice respiration associated with Na^+,K^+-activated ATPase and non-Na^+,K^+-activated ATPase activity (hypothetical).

Brain slice respiration independent of sodium-potassium ATPase activity is shown by the shaded portion of the bar. The sodium-potassium-dependent slice respiration is shown by the open portion of the bar.

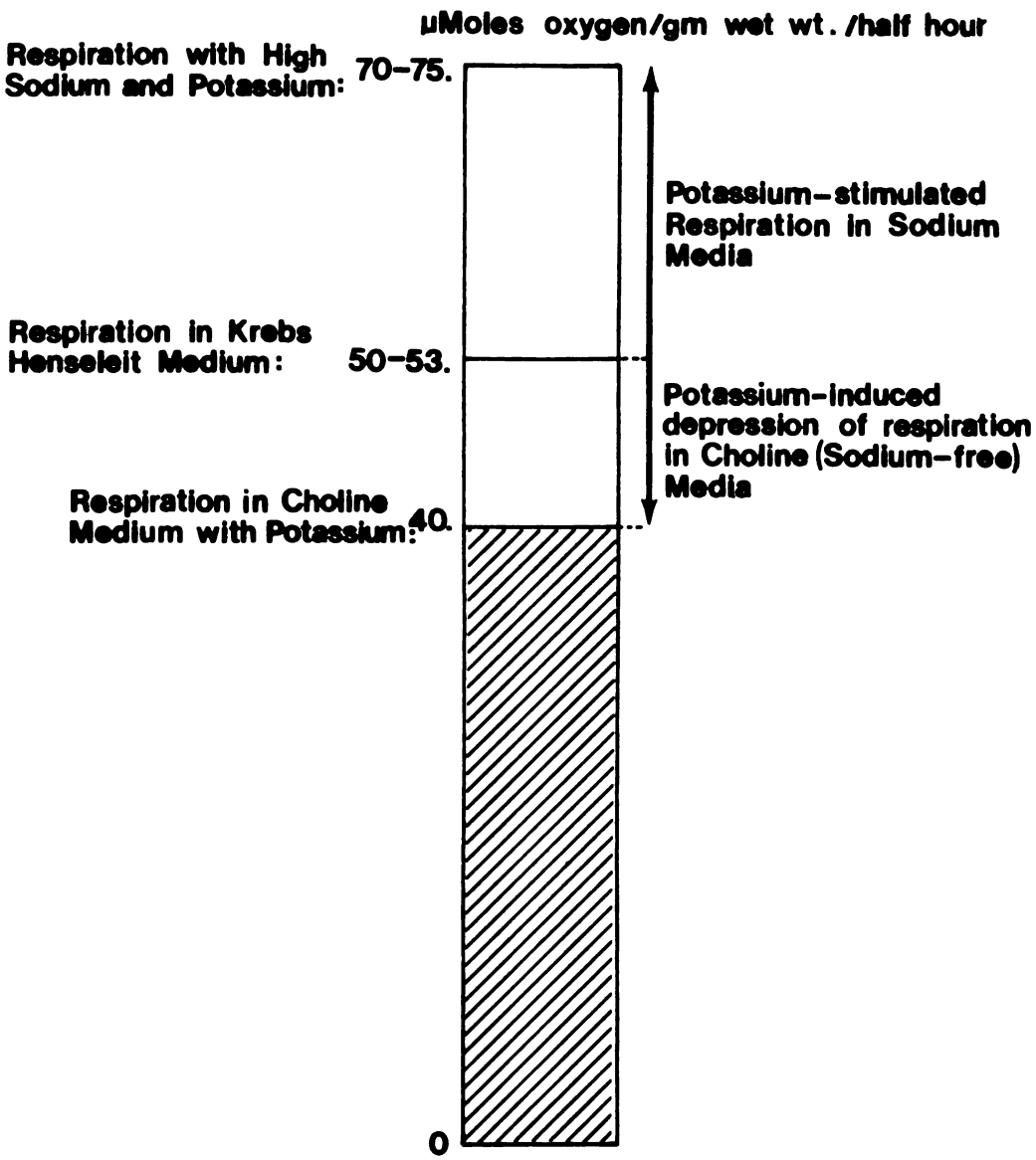
µMoles oxygen/gm wet wt./half hour
Respiration with High Sodium and Potassium:
70-75.
Potassium-stimulated Respiration in Sodium Media
Respiration in Krebs Henseleit Medium:
50-53.
Potassium-induced depression of respiration in Choline (Sodium-free) Media
Respiration in Choline Medium with Potassium:
40.
0

Figure 31

in intact brain tissue in such a manner as to produce their pharmacologic effects by this mechanism.

The dose-dependent stimulation of brain cortical slice respiration by potassium, rubidium and cesium in a modified calcium-free Krebs Henseleit medium is consistent with the known stimulation of brain slice respiration by 100 mM concentrations of these cations in physiological medium containing approximately 2 mM calcium (Dickens and Greville, 1935). The observation that cesium is somewhat more potent than rubidium or potassium in stimulating slice respiration in calcium-free medium (Figure 12) is in agreement with previous data on cesium, rubidium and potassium stimulation of slice respiration in 125 mM sodium chloride medium with 1.5 mM calcium (Hertz and Schou, 1962). The similarities between the rubidium and potassium stimulation curves, the maximum level of slice respiration being about 140-150 μMoles of oxygen/g tissue (wet weight)/hour, and the potassium stimulation being somewhat greater than the cesium or rubidium stimulations at 100 mM concentrations are also in agreement with the previous data of Hertz and Schou (1962). Based on previous data that potassium depolarizes nerve cell membrane (McIlwain, 1951a; Li and McIlwain, 1957), one might speculate that cesium and rubidium have similar actions. Based on the resemblance between the activation by potassium, rubidium and cesium of Na^+,K^+-ATPase-associated slice respiration (Figure 12) and the activation by these cations of microsomal Na^+,K^+- ATPase in the experiments of Skou (1960), one might speculate that rubidium and cesium are acting here to substitute for potassium in stimulating brain slice respiration via a stimulating of membrane Na^+,K^+-ATPase activity. The observation that ouabain, a specific inhibitor of

microsomal Na^+,K^+-ATPase (Skou, 1957), inhibits rubidium and cesium stimulation of respiration in a high sodium medium, supports the hypothesis that the rubidium plus sodium, and cesium plus sodium, stimulations of respiration are associated with stimulation of the Na^+,K^+-ATPase enzyme in intact cells. Since the dose-dependency of ouabain's inhibition is similar following stimulation by potassium, rubidium or cesium, this further supports the hypothesis that the same or similar mechanisms are involved in potassium, rubidium and cesium's stimulation of slice respiration. The fact that with prolonged incubation 1 μM ouabain inhibits sodium plus cesium stimulated brain slice respiration also supports the hypothesis that sodium and cesium are acting to stimulate membrane ATPase activity in these intact cells (Figure 15). The longer time needed to produce inhibition at this lower concentration of ouabain may be due to 1) slower ouabain binding to membrane ATPase, 2) delay in altering ATP/ADP levels controlling respiration, and/or 3) more gradual increases in inhibitory intracellular sodium concentration.

An explanation for ouabain's lack of effect in a sodium-free medium (Figure 4) could be that pump-associated respiration is minimal in sodium-free medium as suggested by Whittam (1962a). An alternative explanation could be that in the absence of extracellular sodium, ouabain can only minimally bind to the membrane Na^+,K^+-ATPase and therefore can exert only a minimal effect on any remaining active transport associated respiration. Schwartz *et al.* (1968) have demonstrated that cardiac glycoside binding to microsomal Na^+,K^+-ATPase is greatly enhanced by sodium in the presence of magnesium and ATP. The role of sodium in ouabain inhibition of active transport in red

cell ghosts has been demonstrated by Schatzmann (1965). The role of sodium in ouabain binding to and inhibition of Na^+,K^+-ATPase in other tissues, e.g., in brain slices, is less well understood. The time course of ouabain's inhibition of slice respiration after the addition of sodium to high potassium medium (Figure 5) suggests that sodium is necessary for specific binding of ouabain to brain slices. After sodium addition, ouabain at 100 μM concentrations rapidly blocks sodium-stimulated respiration, while ouabain at 10 μM concentrations blocks this respiration to a lesser degree and after greater delay (Figure 5). This gradual inhibition of sodium, plus potassium stimulation with time indicates that ouabain is probably not bound before sodium addition. The delayed binding in this system is probably similar to that observed previously in purified enzyme systems by Allen *et al.* (1970) and Akera (1971). Potentially another reason contributing to ouabain's lack of inhibitory effect on brain slice respiration in a sodium-free medium is that the increase in intracellular sodium concentration that is secondary to ouabain's inhibition of Na^+,K^+-ATPase in high sodium medium (Figure 8) cannot occur in a sodium-free medium.

The result that only sodium addition stimulates slice respiration in an initially sodium-free medium containing 100 mM potassium (Figure 23) demonstrates the absolute requirement of sodium for stimulation of brain slice respiration. It demonstrates that combinations of potassium plus rubidium, potassium plus cesium, and potassium plus lithium do not yield higher rates of respiration, and therefore probably do not have higher rates of Na^+,K^+-ATPase activity than those in potassium plus choline control. The combinations of monovalent

cations that stimulate respiration include sodium, plus in decreasing order either potassium, rubidium or cesium, but not lithium. This is the same order as seen for monovalent cation stimulation of microsomal ATPase activity in the presence of 100 mM sodium and 3 mM magnesium (Skou, 1960). The present results with monovalent cations are consistent with the hypothesis that intracellular sodium concentration is the predominant factor controlling the magnitude of Na^+,K^+-ATPase-associated respiration in slices from brain.

Previously, investigators have shown that brain or kidney slices leached and incubated thereafter in a sodium-free medium containing either 125 mM lithium (Hertz and Schou, 1962) or 140 mM lithium (Willis and Fang, 1970) show very high rates of respiration. This lithium stimulation has been shown to be inhibited by ouabain (Willis and Fang, 1970), as well as by the addition of sodium in concentrations of 50 to 125 mM (Hertz and Schou, 1962). Following an initial stimulation, there has been shown to occur a rapid and significant decline in slice respiration with very high concentrations of lithium. In human erythrocytes, lithium can substitute for sodium or potassium in stimulating ouabain-sensitive Na^+,K^+-ATPase activity, but in this system net transport of lithium is negligible (Willis and Fang, 1970). The hypothesis has therefore been advanced that lithium stimulates Na^+,K^+-ATPase activity by uncoupling the active transport of cations from the hydrolysis of ATP. Early studies of lithium's effect on brain Na^+,K^+-ATPase have shown that lithium is the poorest activator of this enzyme, having about one eighth the apparent affinity and one quarter the efficacy of potassium ions (Skou, 1960). Lithium will substitute for sodium, however, in stimulating the phosphorylation

(Skou and Hilberg, 1969), as well as substitute for potassium in stimulating the dephosphorylation of Na^+,K^+-ATPase (Tobin *et al.*, 1974). In the present studies, lithium either has no effect or tends to decrease slice respiration in high sodium, low potassium medium (Figure 16), or in high potassium medium with varying concentrations of sodium added (Figure 17). This may represent either a lack of primary effect in an intact brain cell system, or reflect simultaneous stimulation and inhibition, with inhibition predominating with time. The mechanism of inhibitory effects remains to be elucidated. If lithium has inhibitory effects on the Na^+,K^+-ATPase and the Na^+,K^+-ATPase-associated respiration, lithium would be expected to inhibit respiration in the presence of sodium, but not in sodium-free medium. In the present experiments (Figure 17) increasing inhibition by 20 mM lithium with increasing concentrations of sodium has been observed. At the concentration of lithium studied, however, it is not possible to analyze whether lithium produced a change in the slope or if it shifted the sodium stimulation curve in parallel to the right. It is thus impossible to determine if the lithium inhibition is competitive or non-competitive with respect to sodium. The present results in general do not support the lithium uncoupling hypothesis as proposed by Willis and Fang (1970), or the stimulatory role of lithium as postulated by Tobin *et al.* (1974). The therapeutic plasma lithium concentration in man is in the range of 0.6 to 1.5 mEq/liter, with toxic effects seen at blood levels of lithium above 1.5 mEq/liter (Gershon, 1970). Since concentrations of lithium from 0 to 30 mM had no significant effects on brain cortical slice respiration in a high sodium, low potassium medium, lithium at reasonable

therapeutic concentrations in an intact brain cell system, and presumably in the intact animal, probably has no significant effect on brain Na^+,K^+-ATPase.

Univalent thallous ions resemble potassium in both their chemical and biological effects. Mullins and Moore (1960) found that similarly to potassium, thallous ions accumulate in muscle fibers and depolarize membranes. Gehring and Hammond (1967) demonstrated that thallous and potassium ions are handled similarly in dog kidney, and that thallous ions in the presence of sodium can activate rat erythrocyte Na^+,K^+-ATPase activity. Subsequently, Britten and Blank (1968) have shown that thallous ion could replace potassium in the activation of Na^+,K^+-ATPase from rabbit kidney. In these experiments, thallous ion was shown to have an affinity approximately 10 times greater than potassium for the potassium activating site on the ATPase (Britten and Blank, 1968; Inturrisi, 1969a, 1969b). Maslova *et al.* (1971) have shown that thallous ion inhibits sodium transport across frog skin. Recently it has been demonstrated that concentrations of 0.5 to 1 mM thallous ion can stimulate ^{22}Na-efflux from red cells into a potassium-free medium; however, concentrations above 1.0 mM thallous ion inhibit this ^{22}Na-efflux (Skulskii *et al.*, 1973).

In the present experiments, thallous ion inhibits brain slice respiration in both a high potassium and potassium-free medium. An exception to this occurs with 0.3 to 1.0 mM thallous ion, where brain slice respiration is somewhat higher than control in potassium-free medium during the first half hour of incubation. Thus, the effect of thallous ion on slice respiration may be biphasic. At low

concentrations, it stimulates respiration in potassium-free medium by stimulating Na^+,K^+-ATPase. At high concentrations inhibition is predominant. The inhibition by thallous ion in potassium-free and high potassium media is probably the result of direct damage to mitochondria. This may be similar to that described in cultured nervous tissue by Spencer *et al.* (1972, 1973). Significant inhibition occurs with concentrations of from 0.3 to 10 mM thallous ion in potassium-free medium, while significant inhibition of brain slice respiration occurs only with 3 mM, and not with 0.1 to 1.0 mM, thallous ion in high potassium medium. The decrease in slice respiration due to thallous ion in the presence of 100 mM potassium is also slower and less marked than that observed in potassium-free medium. From these data it might be concluded that thallous ion is more potent in its inhibition of slice respiration in the absence of potassium. There may be several explanations for this: 1) high concentrations of potassium ion compete with thallous ion for binding sites within the cell, i.e., probably in mitochondria, thereby partially protecting these intracellular structures from thallous ion, and 2) with high potassium concentrations, the coupled transport of sodium out of the cell continues, whereas in a potassium-free medium it does not; therefore, in potassium-free medium, intracellular sodium concentration rises. This rise in intracellular sodium in conjunction with increased thallous ion within the cell may cause a decrease in respiration. Another possibility is that thallous ions and potassium ions compete at the cell membrane. Whereas potassium stimulates the rapid dephosphorylation of Na^+,K^+-ATPase, and then itself is released from the enzyme, thallous ion, with a much higher affinity, is slower to be

released (Inturrisi, 1969a, 1969b). Potassium stimulates a more rapid turnover of the Na^+,K^+-ATPase, yielding higher rates of respiration.

In conclusion, monovalent cations can alter brain slice respiration by presumably affecting Na^+,K^+-ATPase activity in intact cells. Such effects, however, are observed only with extremely high concentrations of these cations. Thallous ion is the only monovalent cation which affects brain slice respiration at low concentrations. Thus, it appears that pharmacologic effects of lithium and rubidium are not due to their action on Na^+,K^+-ATPase activity. The only exception is low concentrations of lithium which stimulate brain slice respiration slightly under certain conditions. The statistical and biological significance of such a stimulation should be investigated further.

D. Effects of Chlorpromazine and Its Metabolites on Brain Respiration and Na^+,K^+-ATPase

Chlorpromazine at concentrations above 100 μM has been demonstrated to inhibit oxygen consumption of brain slices and homogenates from a variety of species in Krebs Ringers medium (Courvoisier *et al.*, 1953; Ganshirt and Brilmayer, 1954; Magee *et al.*, 1956). These experiments were performed under conditions in which chlorpromazine would be inhibiting either Mg^{2+}-ATPase activity, or only the small "resting" Na^+,K^+-ATPase activity observed in high sodium, low potassium medium (Bernsohn *et al.*, 1956; Magee *et al.*, 1956). In the present experiments, 10 to 100 μM chlorpromazine markedly inhibited slice respiration associated with Na^+,K^+-ATPase activity, but not respiration in sodium-free medium. It thus appears that the sodium, potassium-stimulated portion of respiration is more sensitive to the inhibitory

action of chlorpromazine than the non-stimulated respiration. This is consistent with the differential inhibitory effect of chlorpromazine free radicals on isolated Na^+,K^+-ATPase and Mg^{2+}-ATPase (Akera and Brody, 1968, 1969).

Chlorpromazine is known to undergo 7-hydroxylation in animals (Fishman and Goldenberg, 1963). The 7,8-dihydroxychlorpromazine metabolite has been found in biological samples taken from human patients on chlorpromazine therapy (Turano *et al.*, 1973). The 7-hydroxychlorpromazine metabolite has been shown to possess pharmacological activity similar to that of chlorpromazine (Manian *et al.*, 1965; Tjioe *et al.*, 1972), and both 7-hydroxy- and 7,8-dihydroxychlorpromazine have been shown to stimulate state 4 respiration in concentrations of approximately 100 μM (Tjoie *et al.*, 1972). Tjioe *et al.* (1972) have shown that with 7-hydroxychlorpromazine state 3 mitochondrial respiration is slowed and the state 4-3 transition becomes less sharply defined. The 7,8-dihydroxychlorpromazine metabolite initially stimulates state 4 respiration with glutamate and succinate; however, with time 7,8-dihydroxychlorpromazine gradually causes a decrease in state 4 mitochondrial respiration (Tjioe *et al.*, 1972). It has been suggested that this inhibition may be due to the native 7,8-dihydroxychlorpromazine, its semiquinone free radical or its corresponding orthoquinone (Tjioe *et al.*, 1972). It has also been proposed that sulfhydryl groups in mitochondria may be the sites of interaction of these hydroxylated metabolites (Tjioe *et al.*, 1972). Additionally, hydroxylated metabolites of chlorpromazine have been shown to be potent inhibitors of Na^+,K^+-ATPase (Akera *et al.*, 1974; Brody *et al.*, 1974).

The lack of effect of 7-hydroxy- and 7,8-dihydroxychlorpromazine on brain slice respiration *in vitro* may be due to 1) their inability to penetrate cell membrane, being more polar and less lipid soluble than the native drug (Forrest *et al.*, 1968), or 2) their rapid chemical or photooxidation to inactive sulfoxide metabolites during the course of the experiment. Although the Na^+,K^+-ATPase is located on the cell membrane, the site which interacts with hydroxylated chlorpromazine or SH inhibitors may be located on the inner aspect of the cell membrane.

Chlorpromazine injected *in vivo* at a dose of 30 mg/kg produces no significant effects on rat brain slice respiration. Although Grenell *et al.* (1955) have found that chlorpromazine at doses of 10 mg/kg and 50 mg/kg significantly increases ATP levels in the brain, such an effect may be a result of sedation produced by chlorpromazine rather than the result of an inhibition by chlorpromazine of the ability to break down ATP as initially proposed by these investigators. Other central depressants such as barbiturates also may increase brain ATP levels, although they have no direct effect on Na^+,K^+-ATPase (LePage, 1946; Gerlach *et al.*, 1958). The present experiments studying Mg^{2+}-ATPase and Na^+,K^+-ATPase activity in whole brain homogenates from rats acutely or chronically treated with chlorpromazine show that chlorpromazine at a dose of 30 mg/kg *in vivo* has no significant effect on rat brain ATPase activity subsequently assayed *in vitro* (Figure 28). These data, however, might be related to the reversibility or irreversibility of chlorpromazine binding to the ATPase enzyme. If chlorpromazine free radical binding to sulfhydryl groups on the Na^+,K^+-ATPase were involved in the

chlorpromazine inhibition of Na^+,K^+-ATPase, as suggested by Akera and Brody (1968, 1969), one would expect this inhibition to be irreversible. If chlorpromazine interaction with Na^+,K^+-ATPase were reversible, this might explain why an inhibition of Na^+,K^+-ATPase could not be observed subsequently *in vitro*. Alternatively, free radical formation from chlorpromazine occurs only at certain anatomical sites within the brain, and therefore specific analysis of various areas of the brain would be necessary in order to find such an effect (Brody *et al.*, 1974).

E. Effects of Digitoxin on Brain Respiration and Na^+,K^+-ATPase Activity

The specific inhibition by digitoxin administered *in vivo* of rat brain Na^+,K^+-ATPase and sodium plus potassium-stimulated brain slice respiration is consistent with the hypothesis that cardiac glycosides specifically affect Na^+,K^+-ATPase and not Mg^{2+}-ATPase activity *in vitro* and *in vivo* (Schwartz *et al.*, 1975). It is similar to Na^+,K^+-ATPase inhibition observed following *in vivo* administration of cardiac glycosides in several other tissues, including the kidney (Palmer and Nechay, 1964; Hook, 1969) and heart (Akera *et al.*, 1969, 1970; Besch *et al.*, 1970; Allen *et al.*, 1970, 1975; Goldman *et al.*, 1973).

The present data that digitoxin injected *in vivo* in rats inhibits at high doses both sodium plus potassium-stimulated respiration of brain slices and the Na^+,K^+-ATPase activity of whole brain homogenates when assayed *in vitro* suggest that the dissociation of the glycoside from Na^+,K^+-ATPase enzyme is relatively slow in brain from this species. Tobin and Brody (1972) have shown that the dissociation half-time of

the ouabain-enzyme complex from both dog heart and rat brain is long (i.e., about 45 minutes). Akera *et al.* (1970) have observed inhibition of Na^+,K^+-ATPase activity in dog heart tissue following infusion of ouabain, but have failed to observe significant inhibition of brain Na^+,K^+-ATPase in the dog. Both canine brain and heart Na^+,K^+-ATPase are sensitive to cardiac glycoside inhibition *in vitro*, and both have been reported to have a relatively long half-life for ouabain dissociation similar to that of rat brain enzyme (Tobin and Brody, 1972). Several other factors, however, might explain the inhibition of enzyme from dog heart and rat brain, and lack of inhibition of enzyme from dog brain. These include differences in the drug or dosages used. The drug used in the dog studies of Akera *et al.* (1970) was ouabain, which penetrates less readily across lipid membranes (Greenberger and Caldwell, 1972) and presumably less well across the blood brain barrier than digitoxin (Friedman *et al.*, 1952). Further, in the studies by Akera *et al.* (1970), the average dose of ouabain used in the dogs was 27-66 µg/kg. This dose of ouabain resulted in a non-significant decrease in dog brain Na^+,K^+-ATPase activity of 16% (Akera *et al.*, 1970). In the present rat brain studies, much higher doses of digitoxin in the range of 4 to 30 mg/kg were used. Administration of these high doses was possible because the rat heart is relatively insensitive to digitalis. In the present studies in rats, high doses of digitoxin produced a significant 35% inhibition of brain Na^+,K^+-ATPase activity (Figure 30). Thus, in relatively digitalis-insensitive species such as the rat (Repke *et al.*, 1965), administration of the inotropic dose of digitalis may cause an inhibition of brain Na^+,K^+-ATPase. Events which may be associated with central

excitation were reported following the administration of digitalis in rats (Gold *et al.*, 1947). In the experiments of Gold *et al.* (1947) digitoxin and other more lipid soluble cardiac glycosides were more effective in producing convulsions in the rat than was ouabain. In highly digitalis-sensitive species, including man, it is not likely that administration of the relatively low doses of digitalis used clinically would cause significant inhibition of brain Na^+,K^+-ATPase.

In vivo administration of digitoxin in rats resulted in inhibition of brain slice respiration at lower doses than were required for significant effects on Na^+,K^+-ATPase activity. Maximal digitoxin inhibition of sodium, potassium-stimulated respiration was to about 45% of control, while maximal digitoxin inhibition of Na^+,K^+-ATPase was to about 65% of control. This greater inhibition of slice respiration in the presence of high concentrations of sodium and potassium may involve both a decrease in ADP generation and an inhibitory effect of intracellular sodium secondary to inhibition of the Na^+,K^+-ATPase. The lack of effect of digitoxin on slice respiration in the absence of sodium would be consistent with *in vitro* cardiac glycosides having no effect on respiration in sodium-free media. The lack of effect of digitoxin on Mg^{2+}-ATPase activity *in vivo* would be consistent with the lack of effect of cardiac glycosides on Mg^{2+}-ATPase *in vitro*.

In summary, the specific inhibition following digitoxin administration *in vivo* of rat brain Na^+,K^+-ATPase and sodium plus potassium-stimulated brain slice respiration assayed *in vitro* occurs only at extremely high doses of digitoxin, and may involve both changes in intracellular cations and inhibition of ADP generation.

REFERENCES

REFERENCES

Abood, L. G. "Effect of chlorpromazine on phosphorylation of brain mitochondria." Proc. Soc. Exp. Biol. Med. 88: 688-690 (1955).

Akera, T. "Quantitative aspects of the interaction between ouabain and (Na^++K^+)-activated ATPase *in vitro*. Biochim. Biophys. Acta 249: 53-62 (1971).

Akera, T.; Baskin, S. I.; Tobin, T.; Brody, T. M.; and Manian, A. A. "7,8 Dihydroxychlorpromazine: (Na^+ + K^+)-ATPase inhibition and positive inotropic effect" in *The Phenothiazines and Structurally Related Drugs*. I. S. Forrest, C. J. Carr, and E. Usdin, Eds. Raven Press, New York, N.Y. (1974).

Akera, T., and Brody, T. M. "Inhibition of brain sodium- and potassium-stimulated adenosine triphosphatase activity by chlorpromazine free radical." Mol. Pharmacol. 4: 600-612 (1968).

Akera, T., and Brody, T. M. "Interaction between chlorpromazine free radical and microsomal sodium- and potassium-activated adenosine triphosphatase from rat brain." Mol. Pharmacol. 5: 604-614 (1969).

Akera, T., and Brody, T. M. "Membrane adenosine triphosphatase. The effect of potassium on the formation and dissociation of the ouabain-enzyme complex." J. Pharmacol. Exp. Ther. 176: 545-557 (1971).

Akera, T., Larsen, F. S., and Brody, T. M. "The effect of ouabain on sodium- and potassium-activated adenosine triphosphatase from the hearts of several mammalian species." J. Pharmacol. Exp. Ther. 170: 17-26 (1969).

Akera, T., Larsen, F. S., and Brody, T. M. "Correlation of cardiac sodium- and potassium adenosine triphosphatase activity with ouabain-induced inotropic stimulation." J. Pharmacol. Exp. Ther. 173: 145-151 (1970).

Allen, J. C.; Besch, H. R. Jr.; Glick, G.; and Schwartz, A. "The binding of tritiated ouabain to sodium- and potassium-activated adenosine triphosphatase and cardiac relaxing system of perfused dog heart." Molec. Pharmacol. 6: 441-443 (1970).

Allen, J. C., Entman, M. L., and Schwartz, A. "The nature of the transport ATPase digitalis complex. VIII. The relationship between *in vivo* formed H^3-ouabain-Na+,K+-ATPase complex and ouabain-induced positive inotropism." J. Pharmacol. Exp. Ther. 192: 105-112 (1975).

Allen, J. C., Lindenmayer, G. E., and Schwartz, A. "An allosteric explanation for ouabain-induced time-dependent inhibition of sodium, potassium-adenosine triphosphatase." Arch. Biochem. Biophys. 141: 322-328 (1970).

Allen, J. C.; Martinez-Maldonado, M.; Eknoyan, G.; Suki, W. N.; and Schwartz, A. "Relation between digitalis binding *in vivo* and inhibition of sodium, potassium-adenosine triphosphatase in canine kidney." Biochem. Pharmacol. 20: 73-80 (1971).

Ashford, C. A., and Dixon, K. C. "The effect of potassium on the glucolysis of brain tissue with reference to the Pasteur effect." Biochem. J. 29: 157-168 (1935).

Bachelard, H. S., Campbell, W. J., and McIlwain, H. "The sodium and other ions of mammalian cerebral tissues, maintained and electrically stimulated *in vitro*." Biochem. J. 84: 225-232 (1962).

Baker, P. F., and Connelly, C. M. "Some properties of the external activation site of the sodium pump in crab nerve." J. Physiol. (Lond.) 185: 270-297 (1966).

Batterman, R. C., and Gutner, L. B. "Hitherto undescribed neurological manifestations of digitalis toxicity." Amer. Heart J. 36: 582-586 (1948).

Belitzer, V. A., and Tsibakowa, E. T. "The mechanism of phosphorylation associated with respiration." Biokhimya 4: 516-534 (1939).

Bernsohn, J., Namajuska, I., and Cochrane, S. G. "The effect of chlorpromazine on respiration and glycolysis in rat brain." Archives of Biochem. and Biophys. 62: 274-283 (1956).

Besch, H. R. Jr.; Allen, J. C.; Glick, G.; and Schwartz, A. "Correlation between the inotropic action of ouabain and its effects on subcellular enzyme systems from canine myocardium." J. Pharmacol. Exp. Ther. 171: 1-12 (1970).

Borison, H. L., and Wang, S. C. "Locus of the central emetic action of cardiac glycosides." Proc. Soc. Exp. Biol. Med. 76: 335-338 (1951).

Boyajy, L. D., and Nash, C. B. "Alteration of ouabain toxicity by cardiac denervation." Toxic. Appl. Pharmacol. 9: 199-208 (1966).

Britten, J. S., and Blank, M. "Thallium activation of the (Na+-K+)-activated ATPase of rabbit kidney." Biochim. Biophys. Acta. 159: 160-166 (1968).

Brody, T. M. "The uncoupling of oxidative phosphorylation as a mechanism of drug action." Pharmacol. Rev. 7: 335-363 (1955).

Brody, T. M.; Akera, T.; Baskin, S. I.; Gubitz, R.; and Lee, C. Y. "Interaction of Na,K-ATPase with chlorpromazine free radical and related compounds." Ann. N.Y. Acad. Sci. 242: 527-542 (1974).

Century, B., and Horwitt, M. K. "Actions of reserpine and chlorpromazine hydrochloride on rat brain oxidative phosphorylation and adenosine triphosphatase." Proc. Soc. Exp. Biol. Med. 91: 493-497 (1956).

Chance, B., and Williams, G. R. "Respiratory enzymes in oxidative phosphorylation." J. Biol. Chem. 217: 383-393 (1955).

Clark, J. B., and Nicklas, W. J. "The metabolism of rat brain mitochondria: Preparation and characterization." J. Biol. Chem. 245: 4724-4731 (1970).

Colowick, S. P., Kalckar, H., and Corri, C. F. "Coupling of oxidation and phosphorylation." J. Biol. Chem. 137: 343-356 (1941).

Colowick, S. P., Welch, M. S., and Cori, C. F. "Phosphorylation of glucose in kidney extracts." J. Biol. Chem. 133: 359-373 (1940).

Courvoisier, S.; Fournel, J.; Ducrot, R.; Kolsky, M.; and Koetschet, P. "Proprietes pharmacodynamiques du chlorhydrate de chloro-3-(dimethylamino-3-propyl)-10-phenothiazine (4560 R.P.): Etude experimentale d'un nouveau corps utilise dans l'anesthesie potentialisee et dans l'hibernation artificielle." Arch. Intern. Pharmacodynamie 93: 305-361 (1953).

Cremer, J. "Studies on brain-cortex slices: Differences in the oxidation of 14C-labeled glucose and pyruvate revealed by the action of triethyltin and other toxic agents." Biochem. J. 104: 212-222 (1967).

Cruz, A. "Action de la chlorpromazine sur l'activite de la phosphatase et de la cholinesterase du sang." Comp. Rend. Soc. Biol. 149: 1829-1831 (1955).

Cummins, J. T., and McIlwain, H. "Electrical pulses and the potassium and other ions of isolated cerebral tissues." Biochem. J. 79: 330-341 (1961).

Dawkins, M. J. R., Judah, J. D., and Rees, K. R. "The effect of chlorpromazine on the respiratory chain: Cytochrome oxidase." Biochem. 72: 204-209 (1959a).

Dawkins, M. J. R., Judah, J. D., and Rees, K. R. "The mechanism of action of chlorpromazine. 2. Reduced diphosphopyridine nucleotide-cytochrome C reductase and coupled phosphorylation." Biochem. J. 73: 16-23 (1959b).

Decourt, P. "Activie narcobiotique de derives de la Phenothiazine et son importance therapeutique." Compt. Rend. Ac. Sci. 236: 1195-1197 (1953).

Delay, J., Deniker, P., and Harl, J. M. "Traitement des estats d'excitation et d'agitation par une methode medicamenteuse derivee de l'hibern therapie." Ann. med. psychol. 110: 267-273 (1952).

Deul, D. H., and McIlwain, H. "Activation and inhibition of adenosine triphosphatases of subcellular particles from the brain." J. Neurochem. 8: 246-256 (1961).

Dickens, F., and Greville, G. D. "The metabolism of normal and tumor tissue. XIII. Neutral salt effects." Biochem. J. 29: 1468-1482 (1935).

Engelhardt, W. A. "Ortho-und pyrophosphat im aeroben und anaeroben Stoffwechsel der Blutzellen." Biochem. 227: 16-38 (1930).

Engelhardt, W. A. "Die Beziehungen zwischen Atmung und Pyrophosphatumsatz im vogelerythrocyten." Biochem. Z. 251: 343-368 (1932).

Erlij, D., and Mendez, R. "The modification of digitalis intoxication by excluding adrenergic influences on the heart." J. Pharmacol. Exp. Ther. 144: 97-103 (1964).

Fieve, R. H.; Meltzer, H.; Dunner, D. L.; Levitt, M.; Mendlewicz, J.; and Thomas, A. "Rubidium: Biochemical, behavioral and metabolic studies in humans." Am. J. Psychiatry 130: 55-61 (1973).

Fishman, V., and Goldenberg, H. "Metabolism of chlorpromazine. IV. Identification of 7-hydroxychlorpromazine and its sulfoxide and desmethyl derivatives." Proc. Soc. Exp. Biol. Med. 112: 501-506 (1963).

Forrest, I. S., Bolt, A. G., and Aber, R. C. "Metabolic pathways for the detoxication of chlorpromazine in various mammalian species." Agressologie 9: 259-265 (1968).

Friedman, M.; St. George, S.; Bine, R.; Byers, S. O.; and Bland, C. "Deposition and disappearance of digitoxin from the tissues of the rat, rabbit and dog after parenteral injection." Circulation 6: 367-370 (1952).

Ganshirt, H., and Brilmayer, H. "Über den Einfluss des Praparates Megaphen (Largactil) auf den Sauerstoffverbrauch von Hirnschnitten und Hirnhomogenaten." Arch. Int. Pharmacodynamie 98: 467-474 (1954).

Gehring, P. J., and Hammond, P. B. "The interrelationship between thallium and potassium in animals." J. Pharmacol. Exp. Ther. 155: 187-201 (1967).

Gerlach, E., Doring, H. J., and Fleckenstein, A. "Papierchromatographische Studien über die Adenin- und Guanin-Nucleotide sowie andere säuerlösliche Phosphorverbindungen des Gehirns bei Narkose, Ischämie und Abhängigkeit von der Technik der Gewebsentnahme." Pflüg. Arch. ges. Physiol. 266: 266-291 (1958).

Gershon, S. "Lithium in mania." Clin. Pharmacol. Ther. 11: 168-187 (1970).

Gillis, R. A. "Cardiac sympathetic nerve activity - changes induced by ouabain and propranolol." Science 166: 508-510 (1969).

Gillis, R. A.; Raines, A.; Sohn, Y. J.; Levitt, B.; and Standaert, F. G. "Neuroexcitatory effects of digitalis and their role in the development of cardiac arrhythmias." J. Pharmacol. Exp. Ther. 183: 154-168 (1972).

Glynn, I. M. "The action of cardiac glycosides on sodium and potassium movements in human red cells." J. Physiol. 136: 148-173 (1957).

Gold, H.; Modell, W.; Cattell, M.; Benton, J. G.; and Cotlover, E. W. "Action of digitalis glycosides on the central nervous system with special reference to the convulsant action of red squill." J. Pharmacol. Exp. Ther. 91: 15-30 (1947).

Goldenberg, H., and Fishman, V. "Metabolism of chlorpromazine. V. Confirmation of position 7 as the major site of hydroxylation." Biochem. Biophys. Res. Commun. 14: 404-407 (1964a).

Goldenberg, H., and Fishman, V. "Comments on 7-hydroxychlorpromazine in the urines of schizophrenics receiving chlorpromazine." Biochem. Pharmacol. 14: 365-368 (1965).

Goldman, R. H.; Coltart, J.; Friedman, J. P.; Nola, G.; Berke, D. K.; Schweizer, E.; and Harrison, D. C. "The inotropic effects of digoxin in hyperkalemia: Relation to (Na^+,K^+)-ATPase inhibition in the intact animal." Circulation 48: 830-838 (1973).

Goodman, L.S., and Gilman, A. *The Pharmacological Basis of Therapeutics*. Macmillan Publishing Company, Inc., New York (1975).

Gore, M. B. R., and McIlwain, H. "Effects of some inorganic salts on the metabolic response of sections of mammalian cerebral cortex to electrical stimulation." J. Physiol. 117: 471-483 (1952).

Gornall, A. G., Bardawill, C. J., and David, M. M. "Determination of serum protein by means of the Biuret reaction." J. Biol. Chem. 177: 751-766 (1949).

Grandeau, L. "Experiments on the physiological action of the salts of potassium, sodium, and rubidium injected into veins." J. de la Anatomie et de la Physiol. 1: 378-385 (1864).

Greenberger, N. J., and Caldwell, J. H. "Studies on the intestinal absorption of ^{3}H-digitalis glycosides in experimental animals and man" in *Basic and Clinical Pharmacology of Digitalis, Proceedings of a Symposium*. Marks, B. H., and Weissler, A. M. Eds. Charles C. Thomas Publishers, Springfield, Illinois (1972).

Grenell, R. G., Mendelson, J., and McElroy, W. D. "Effects of chlorpromazine on metabolism in central nervous system." A.M.A. Arch. Neurol. Psychiat. 73: 347-351 (1955).

Gross, E. "Über die Wirkung von Strophanthidin und Digitoxin auf die Atmung des Kaninchens." Z. Ges. Exp. Med. 4: 210-236 (1914).

Gubitz, R., Akera, T., and Brody, T. M. "Comparative effects of substituted phenothiazines and their free radicals on (Na+, K+)-activated adenosine triphosphatase." Biochem. Pharmacol. 22: 1229-1235 (1973).

Hatcher, R. A., and Eggleston, G. "The emetic action of the digitalis bodies." J. Pharmacol. Exp. Ther. 4: 113-134 (1912).

Hatcher, R. A., and Eggleston, G. "Studies in the elimination of certain of the digitalis bodies from the animal organism." J. Pharmacol. Exp. Ther. 12: 405-496 (1919).

Heald, P. J. "Effects of electrical pulses on the distribution of radioactive phosphate in cerebral tissues." Biochem. J. 63: 242-249 (1956).

Hegyvary, C., and Post, R. L. "Binding of adenosine triphosphate to sodium and potassium ion stimulated adenosine triphosphatase." J. Biol. Chem. 246: 5234-5240 (1971).

Hertz, L., and Shou, M. "Univalent cations and the respiration of brain-cortex slices." Biochem. J. 85: 93-103 (1962).

Hess, H. M., and Pope, A. "Effect of metal cations in adenosine triphosphatase activity of rat brain." Fed. Proc. 16: 196 (1957).

Hillman, H. H., and McIlwain, H. "Membrane potentials in mammalian cerebral tissues *in vitro*: Dependence on ionic environment." J. Physiol. 157: 263-278 (1961).

Hook, J. B. "A possible correlation between natriuresis and inhibition of renal Na-K-adenosine triphosphatase by ouabain." Proc. Soc. Exp. Biol. Med. 131: 731-734 (1969).

Hoskin, F. C. G. "Chemical stimulation and modification of glucose metabolism by brain." Arch. Biochem. Biophys. 91: 43-46 (1960).

Hueper, W. C. "Some toxic aspects of digitalis therapy." New York State J. Med. 45: 1442 (1945).

Inturrisi, C. E. "Thallium activation of K+-activated phosphatases from beef brain." Biochim. Biophys. Acta 173: 567-569 (1969a).

Inturrisi, C. E. "Thallium-induced dephosphorylation of a phosphorylated intermediate of the (sodium + thallium-activated) ATPase." Biochim. Biophys. Acta 178: 630-633 (1969b).

Kalckar, H. "Phosphorylation in kidney tissue." Enzymologia 2: 47-52 (1937).

Kalckar, H. "The nature of phosphoric esters formed in kidney extracts." Biochem. J. 33: 631-641 (1939).

Kalckar, H. M. "The function of phosphate in enzymatic synthesis." Ann. N.Y. Acad. Sci. 45: 395-408 (1944).

Kaul, C. L., Lewis, J. J., and Livingstone, S. D. "Influence of chlorpromazine on the levels of adenine nucleotides in the rat brain and hypothalamus *in vivo*. Biochem. Pharmacol. 14: 165-175 (1965).

Kielley, W. W., and Kielley, R. K. "Myokinase and adenosine triphosphatase in oxidative phosphorylation." J. Biol. Chem. 191: 485-500 (1951).

Kini, M. M., and Quastel, J. H. "Carbohydrate-amino acid interrelations in brain cortex *in vitro*." Nature (London) 184: 252-256 (1959).

Klicpera, J. A., and Hoffman, P. C. "Shifts in ubiquinone redox status of rat brain *in vitro* with cationic stimulation." J. Neurochem. 24: 1029-1035 (1975).

Koch-Weser, J. "Beta receptor blockade and myocardial effects of cardiac glycosides." Circ. Res. 29: 109-118 (1971).

Konzett, H., and Rothlin, E. "Effect of cardioactive glycosides on a sympathetic ganglion." Arch. Int. Pharmacodyn. Ther. 89: 343-352 (1952).

Krall, A. R., Wagner, M. C., and Gozansky, D. M. "Potassium ion stimulation of oxidative phosphorylation by brain mitochondria." Biochem. Biophys. Res. Commun. 16: 77-81 (1964).

Krebs, H.A.; Ruffo, A.; Johnson, M.: Eggleston, L. V.; and Hems, R. "Oxidative phosphorylation." Biochem. J. 54: 107-116 (1953).

Kushinsky, K., Lüllmann, H., and Van Zwiefen, P. A. "A comparison of the accumulation and release of 3H-ouabain and 3H-digitoxin by guinea pig heart muscle." Br. J. Pharmacol. Chemother. 32: 598-608 (1968).

Kwant, W. O., and Seeman, P. "Chlorpromazine adsorption to brain regions." Biochem. Pharmacol. 20: 2089-2091 (1971).

Laborit, H. "Potenzierte Narkose und künstlicher Winterschlaf." Arch. exper. Path. u. Pharmakol. 222: 41-58 (1954).

Lardy, H. A., and Wellman, H. "Oxidative phosphorylations: Role of inorganic phosphate and acceptor systems in control of metabolic rates." J. Biol. Chem. 195: 215-224 (1952).

Lardy, H.A., and Wellman, H. "The catalytic effect of 2,4-dinitrophenol on adenosinetriphosphate hydrolysis by cell particles and soluble enzymes." J. Biol. Chem. 201: 357-370 (1953).

Lehmann, H. E., and Hanrahan, G. E. "Chlorpromazine." A.M.A. Arch. Neurol. and Psychiat. 71: 227-237 (1954).

Lendle, L., and Oldenburg, D. "Prüfung extrakardialer Wirkungen an der digitalisunterempfindlichen Ratte." Arch. exper. Path. u. Pharmakol. 211: 243-263 (1950).

LePage, G. A. "Biological energy transformations during shock as shown by tissue analysis." Am. J. Physiol. 146: 267-281 (1946).

LePage, G. A. "Glycolysis in tumor homogenates." J. Biol. Chem. 176: 1009-1020 (1948).

Levitt, B.; Raines, A.; Sohn, Y. J.; Standaert, F. G.; and Hirshfeld, J. W. "The nervous system as a site of action for digitalis and antiarrhythmic drugs." J. Mt. Sinai Hospital 37: 227-240 (1970).

Levitt, B., and Roberts, J. "Effect of quinidine and pronethalol on acetylstrophanthidin-induced ventricular arrhythmia in cats treated with reserpine." Circ. Res. 19: 622-631 (1966).

Li, C. L., and McIlwain, H. "Maintenance of resting membrane potentials in slices of mammalian cerebral cortex and other tissues *in vitro*." J. Physiol. (Lond.) 139: 178-190 (1957).

Lindaur, V. "Zur Frage des Einflusses von chlorpromazine auf den Blutzucker." Arch. exper. Path. u. Pharmakol. 229: 253-257 (1956).

Lindenmayer, G. E., and Schwartz, A. "Conformational changes induced in Na^+,K^+-ATPase by ouabain through a K^+-sensitive reaction: kinetic and spectroscopic studies." Arch. Biochem. Biophys. 140: 371-378 (1970).

Løvtrup, Soren. "A comparative study of the influence of chlorpromazine and imipramine on mitochondrial activity: Oxidation and phosphorylation." J. Neurochem. 10: 471-477 (1963).

Lowry, O. H.; Rosenbrough, N. J.; Farr, A. L.; and Randall, R. J. "Protein measurement with the Folin phenol reagent." J. Biol. Chem. 193: 265-275 (1951).

Luten, D. *The Clinical Use of Digitalis*, First Ed. Charles C. Thomas Company, Springfield, Illinois (1936).

Magee, W. L., Berry, J. F., and Rossiter, R. J. "Effect of chlorpromazine and azacyclonol on the labelling of phosphatides in brain slices." Biochem. Biophys. Acta 21: 408-409 (1956).

Manian, A. A., Efron, D. H., and Goldger, M. E. "A comparative pharmacological study of a series of monohydroxylated and methoxylated chlorpromazine derivatives." Life Sciences 4: 2425-2438 (1965).

Manian, A. A., Efron, D. H., and Harris, S. R. "Appearance of monohydroxylated chlorpromazine metabolites in the central nervous system." Life Sciences 10: 679-684 (1971).

Mashkovskii, M. D., Liberman, S. S., and Polezhaeva, A. I. "Farmakologiya i toksikologiya." Pharmacology and Toxicology 18: 1-14 (1955).

Maslova, M. N., Natochin, Y. V., and Skulsky, I. A. "Inhibition of active sodium transport and activation of Na+,K+-ATPase by Tl+ ions in frog skin." Biokhimiya 36: 867- (1971).

McIlwain, H. "Metabolic responses *in vitro* to electrical stimulation of sections of mammalian brain." Biochem. J. 49: 382-393 (1951a).

McIlwain, H. "Phosphates of the brain during *in vitro* metabolism: Effects of oxygen, glucose, glutamate, glutamine, and calcium and potassium salts." Biochem. J. 52: 289-295 (1952).

McIlwain, H., and Gore, M. B. R. "Actions of electrical stimulation and of 2:4-dinitrophenol on the phosphates in sections of mammalian brain *in vitro*." Biochem. J. 50: 24-29 (1951).

Minakami, S., Kakinuma, K., and Yoshikawa, H. "The control of respiration in brain slices." Biochim. Biophys. Acta 78: 808-811 (1963).

Morrow, D. H., Gaffney, T. E., and Braunwald, E. "Studies on digitalis. VIII. Effects of autonomic innervation and of myocardial catecholamine stores on the cardiac action of ouabain." J. Pharmacol. Exp. Ther. 140: 236-242 (1963).

Mullins, L. J., and Moore, R. D. "The movement of thallium ions in muscle." J. Gen. Physiol. 43: 759-773 (1960).

Nicklas, W. J., Clark, J. B., and Williamson, J. R. "Metabolism of rat brain mitochondria: Studies on the potassium ion-stimulated oxidation of pyruvate." Biochem. J. 123: 83-95 (1971).

Norman, D., and Hiestand, W. A. "Glycemic effects of chlorpromazine in the mouse, hamster and rat." Proc. Soc. Exp. Biol. Med. 90: 89-91 (1955).

Ochoa, S. "Coupling of phosphorylation with oxidation of pyruvic acid in brain." J. Biol. Chem. 138: 751-773 (1941).

Ochoa, S. "Efficiency of aerobic phosphorylation in cell-free heart extracts." J. Biol. Chem. 151: 493-505 (1943).

Ochoa, S. "Chemical processes of oxidative recovery." Ann. N.Y. Acad. Sci. 47: 835-845 (1947).

Palmer, R. F., and Nechay, B. R. "Biphasic renal effects of ouabain in the chicken: Correlations with a microsomal Na^+,K^+-stimulated ATPase." J. Pharmacol. Exp. Ther. 146: 92-98 (1964).

Pappius, H. M., and Elliot, K. A. C. "Factors affecting the potassium content of incubated brain slices." Canad. J. Biochem. Physiol. 34: 1053-1067 (1956b).

Posner, H. S., Culpan, R., and Levine, J. "Quantification and probable structure in human urine, of the nonphenolic and phenolic metabolites of chlorpromazine." J. Pharmacol. Exp. Ther. 141: 377-391 (1963).

Post, R. L., Hegyvary, C., and Kume, S. "Activation by adenosine triphosphate in the phosphorylation kinetics of sodium and potassium, ion transport adenosine triphosphatase." J. Biol. Chem. 247: 6530-6540 (1972).

Post, R. L.; Kume, S.; Tobin, T.; Orcutt, B.; and Sen, A. K. "Flexibility of an active center in sodium-plus-potassium adenosine triphosphatase." J. Gen. Physiol. 54: 306-326 (1969).

Post, R. L., Sen, A. K., and Rosenthal, A. S. "A phosphorylated intermediate in adenosine triphosphate-dependent sodium and potassium transport across kidney membrane." J. Biol. Chem. 240: 1437-1445 (1965).

Potter, V. R. "The homogenate technique" in *Manometric and Biochemical Techniques*, 5th Edition. Umbriet, W. W., Burris, R. H., and Stauffer, J. F., Eds. Burgess Publishing Co., Minneapolis, Minn. (1972) pp. 177-195.

Pravotorova, E. L., and Smirnova, A. V. "The effect of aminazine and mepazine on carbohydrate metabolism and the activity of certain hormonal preparations" in *The Pharmacology and Clinical Aspects of Phenothiazine Derivatives*. Zakusov, V. V., Ed. Academy of Medical Sciences of the U.S.S.R., Moscow (1958).

Pressman, B. C., and Lardy, H. A. "Further studies on the potassium requirements of mitochondria." Biochim. Biophys. Acta 18: 482-487 (1955).

Quastel, J. H. "Effect of drugs on metabolism of brain." Br. Med. Bull. 21: 49-56 (1965).

Racker, E., and Krimsky, I. "Effect of nicotinic acid amide and sodium on glycolysis and oxygen uptake in brain homogenates." J. Biol. Chem. 161: 453-461 (1945).

Rang, H. P., and Ritchie, J. H. "The dependence on external cations of the oxygen consumption of mammalian non-myelinated fibers at rest and during activity." J. Physiol. (Lond.) 196: 163-181 (1968).

Repke, K., Est, M., and Portius, H. J. "Über die Ursache der species Unterschiede in der Digitalisempfindlichkeit." Biochem. Pharmacol. 14: 1785-1802 (1965).

Repke, K. "Verteilung Ausscheidung und Stoffwechwechsel von Digitoxin in der Ratte." Arch. Exp. Path. Pharmak. 233: 271-283 (1958).

Ritchie, J. M., and Strauss, W. W. "The hyperpolarization which follows activity in mammalian non-medullated fibers." J. Physiol. (Lond.) 136: 80-97 (1957).

Rohlf, F. J., and Sokal, R. R. *Statistical Tables*. W. H. Freeman and Company, San Francisco (1969).

Rolleston, F. S., and Newsholme, E. A. "Control of glycolysis in cerebral cortex slices." Biochem. J. 104: 524-533 (1967).

Runnström, J., Lennerstrand, A., and Borei, H. "Oxydation und Phosphatbildung im Hämolysat der Pferdeblutkörperchen." Biochem. Z. 271: 15-21 (1934).

Ruscak, M., and Whittam, R. "The metabolic response of brain slices to agents affecting the sodium pump." J. Physiol. (Lond.) 190: 595-610 (1967).

Schatzmann, H. J. "The role of Na+ and K+ in the ouabain-inhibition of the Na+ K+-activated membrane adenosine triphosphatase." Biochim. Biophys. Acta 94: 89-96 (1965).

Schwartz, A.; Allen, J. C.; VanWinkle, W. B.; and Munson, R. "Further studies on the correlation between the inotropic action of ouabain and its interaction with the Na^+,K^+-adenosine triphosphatase isolated perfused rabbit and cat heart." J. Pharmacol. Exp. Ther. 191: 119-127 (1974).

Schwartz, A., Lindenmayer, G. E., and Allen, J. C. "The sodium-potassium adenosine triphosphatase pharmacological, physiological and biochemical aspects." Pharmacol. Rev. 27: 3-134 (1975).

Schwartz, A., Matsui, H., and Laughter, A. "Tritiated digoxin binding to (Na+ + K+)-activated adenosine triphosphatase. Possible allosteric site. Science 160: 323-325 (1968).

Seeman, P. M., and Bialy, H. S. "The surface activity of tranquilizers." Biochem. Pharmacol. 12: 1181-1191 (1963).

Skinner, A., and Spector, R. G. "The effect of chlorpromazine on 14C-glucose metabolism in mouse liver and brain." Br. J. Pharmacol. Chemother. 33: 129-135 (1968).

Skou, J. C. "The influence of some cations on an adenosine triphosphatase from peripheral nerves." Biochem. Biophys. Acta 23: 394 (1957).

Skou, J. C. "Further investigations of a Mg++ and Na+ activated adenosine triphosphatase, possibly related to the active linked transport of Na+ and K+ across nerve membrane." Biochem. Biophys. Acta 42: 6-23 (1960).

Skou, J. C. "Preparation from mammalian brain and kidney of the enzyme system involved in active transport of Na+ and K+." Biochim. Biophys. Acta 58: 314-325 (1962).

Skou, J. C. "Enzymatic basis for active transport of Na+ and K+ across cell membrane." Physiol. Rev. 45: 596-617 (1965).

Skou, J. C., and Hilberg, C. "The effect of cations, g-strophanthin and oligomycin on the labeling from ^{32}P-ATP of the Na+,K+-activated enzyme system and the effect of cations and g-strophanthin on the labeling from ^{32}P-ITP and ^{32}P." Biochim. Biophys. Acta 185: 198-219 (1969).

Skulskii, I. A., Manninen, V., and Jarnefelt, J. "Interaction of thallous ions with the cation transport mechanism in erythrocytes." Biochim. Biophys. Acta 298: 702-709 (1973).

Skulskii, I. A., Manninen, V., and Järnefeld, J. "Thallium inhibition of ouabain-sensitive sodium transport and of the (Na^+,K^+)-ATPase in human erythrocytes." Biochim. Biophys. Acta 394: 569-576 (1975).

Smith, H. L. "Cerebral manifestations of digitalis intoxication." Proc. Staff Meeting Mayo Clinic 13: 574 (1938).

Sokal, R. R., and Rohlf, F. J. *Biometry: The Principles and Practice of Statistics in Biological Research*. W. H. Freeman and Company, San Francisco (1969).

Spencer, P. S., Raine, C. S., and Peterson, E. R. "Effects of thallium on axonal mitochondria in cord-ganglion-muscle cultures." J. Cell Biol. 55: 247a (1972).

Spencer, P. S.; Peterson, E. R.; Madrid, R. A.; and Raine, C. S. "Effects of thallium salts on neuronal mitochondria in organotypic cord-ganglia-muscle combination cultures." J. Cell Biol. 58: 79-95 (1973).

Stauffer, J. F. "Differential manometry -- The 'Barcroft'." In *Manometric and Biochemical Techniques*, 5th Edition. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Eds. Burgess Publishing Company, Minneapolis, Minnesota (1972) pp. 111-125.

Swanson, P. D. "Effects of ouabain on acid-soluble phosphates and electrolytes of isolated cerebral tissues in presence or absence of calcium." J. Neurochem. 15: 57-67 (1968).

Takagakai, G. "Control of aerobic glycolysis and pyruvate kinase activity in cerebral cortex slices." J. Neurochem. 15: 903-916 (1968).

Tjoie, S. A., Manian, A. A., and O'Neill, J. J. "Calcium efflux and respiratory inhibition in brain mitochondria: Effects of chlorpromazine metabolites." Biochem. Biophys. Res. Commun. 48: 212-218 (1972).

Tobin, T.,; Akera, T.; Han, C. S.; and Brody, T. M. "Lithium and rubidium interactions with sodium- and potassium-dependent adenosine triphosphatase: A molecular basis for the pharmacological actions of these ions." Mol. Pharmacol. 10: 501-508 (1974).

Tobin, T., and Brody, T. M. "Rates of dissociation of enzyme-ouabain complexes and K0.5 values in (Na+ + K+)-adenosine triphosphatase from different species." Biochem. Pharmacol. 21: 1553-1560 (1972).

Traube, L. "Versuche über die Wirkung der digitalis" in *Charite-Annales*, pp. 19-120 (1851).

Turano, P., Turner, W. J., and Manian, A. A. "Thin-layer chromatography metabolites: Attempt to identify each of the metabolites appearing in blood, urine and feces of chronically medicated schizophrenics." J. Chromatography 75: 277-293 (1973).

Utter, M. F. "Mechanism of inhibition of anaerobic glycolysis of brain by sodium ions." J. Biol. Chem. 18: 499-517 (1950).

Venturini, G., and Palladin, G. "ATPase activity, sodium and potassium content in guinea pig cortex after ouabain treatment *in vivo*." J. Neurochem. 20: 237-239 (1973).

Warburg, O. *Über den Stoffwechsel der Tumoren*. Springer-Verlag, Berlin (1926).

Weaver, L. C. "Digitalis toxicity: Primary sites of drug action in the sympathetic nervous system." Doctoral dissertation, Michigan State University (1975).

Weiss, S. "Effect of the digitalis bodies on the nervous system." Med. Clinics North America 15: 963 (1932).

Whittam, R. "Active cation transport as a pacemaker of respiration." Nature (Lond.) 191: 603-604 (1961).

Whittam, R. "The dependence of the respiration of brain cortex on active cation transport." Biochem. J. 82: 205-212 (1962a).

Whittam, R., and Blond, D. M. "Respiratory control by an adenosine triphosphatase involved in active transport in brain cortex." Biochem. J. 92: 147-158 (1964).

Whittam, R., and Wheeler, K. P. "Transport across cell membrane." Ann. Rev. Physiol. 32: 21-60 (1970).

Willis, J. S., and Fang, L. S. T. "Li+ stimulation of ouabain-sensitive respiration and (Na+-K+)-ATPase of kidney cortex of ground squirrels." Biochim. Biophys. Acta 219: 486-489 (1970).

Willus, F. A. "Digitalis: Its rational use." Med. Clin. North America 21: 761 (1937).

Winkelman, N. W. Jr. "Chlorpromazine in the treatment of neuropsychiatric disorders." J.A.M.A. 155: 19-21 (1954).

Withering, W. "An account of the Foxglove and some of its medical uses: With practical remarks on dropsy and other diseases" (1785) in *Medical Classics*. Robinson, C. G. J., and Robinson, J. Eds. 2: 305-443 (1937).

Wollenberger, A. "Metabolic action of the cardiac glycosides. I. Influence on respiration of heart muscle and cortex." J. Pharmacol. Exp. Ther. 91: 39-51 (1947).