ACKNOWLEDGEMENTS

The experimental work reported in this thesis was carried out in the Donner Laboratory of Experimental Neurochemistry of the Montreal Neurological Institute.

To Professor K.A.C. Elliott, I wish to express my appreciation and gratitude for the quality of his teaching. To work under his direction during these few years has been, both from the scientific and from the human point of view, a very enriching experience.

I am grateful to Dr. Hanna M. Pappius, Dr. Leonhard S. Wolfe and Mr. Richard Lovell for many helpful and interesting discussions concerning the subject of this thesis.

My thanks are due to Mrs. Anne-Marie Turner for the care she has taken in typing this text, to Mr. Charles Hodge and his department for the photography of the illustrations and to Mademoiselle Micheline Godbout for her assistance in the preparation of the bibliography.

This work was carried out during the tenure of a Studentship of the National Research Council.

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Chapter I

Historical Introduction and Literature Review

Part 1. Intracellular Occurrence of Potassium

In 1811, Nicolas Vauquelin reported the presence of potassium in brain (408). Analyzing the ash residues of muscle, Liebig found more potassium than sodium (cited by Kaunitz,211). He suggested that potassium occupies an intracellular position and sodium, an extracellular position, in muscle tissues (see Tower,392). Since that time a long series of analytical studies have shown that an analogous distribution of the two elements exist almost universally in bacterial, plant and animal cells (see Reinberg,327). Fenn has defined this phenomenon, writing: "As is well known, potassium is of the soil and not the sea; it is of the cell but not the sap" (122).

The remarkable constancy of this phenomenon along the biological evolutionary scale led the early biologists to recognize as a fundamental property of life the selective ionic permeability of cellular membranes. This is at the origin of many important biological theories, among which may be quoted the concept of "milieu intérieur" of Claude Bernard (34) and the theory of ionic antagonism of Ringer (329).

Distribution of potassium in the nervous system

Mammal brain cerebral cortex contains about 100 microequivalents of potassium and 60 microequivalents of sodium

per gram of fresh weight. Variations from one species to another are relatively small (309,242). The high potassium content of brain is built up before or early after birth. Leulier and Bernard (242) found a correlation between the relative maturity at birth of the sensorimotor functions and the potassium content of the nervous system, in various species.

Significant differences in potassium concentration have been found between regions of the nervous system. In a detailed analysis of the different regions of the dog brain, Tupikova and Gerard (398) found the highest concentration in the cerebellum (128 micromoles per gram). Midbrain and spinal cord contained less potassium than the cerebrum, and the sciatic nerves had the lowest content. Analogous observations have been made concerning the potassium distribution in the nervous systems of the cat, rabbit and rat (242). A similar gradation exists also in the potassium content of skeletal, cardiac and smooth muscles of the same species (242). Other workers have published data which confirm these results (9,105,419).

No significant difference in potassium concentrations has been found between cerebral grey and white matter of different mammal species (5,264,379). Colfer and Essex (75) obtained some results which apparently contradict these data. Autoradiographic analysis of the distribution in the brain of radioactive potassium administered by intraperitoneal injection indicates consistantly greater concentrations in grey

matter areas (cerebral and cerebellar cortex, basal ganglia, roof of the 4th ventricle). However, as certain of these areas have been shown to equilibrate more easily and rapidly with radioactive tracers of the blood and cerebrospinal fluid, these results may not give a true representation of potassium distribution. (102,45).

The potassium content of corresponding nerves has been found to vary greatly from one species to another (253,123). However within the same species, cholinergic and adrenergic nerves contain approximately equal amounts of potassium (253).

Only one region of the mammalian central nervous system was shown to contain more sodium than potassium, the posterior hypophysis - also the anterior (213).

Intracellular distribution of potassium.

Using a cytochemical technique, which apparently permitted the detection of very small quantities of potassium, Macallum, as early as 1905, was able to examine under the microscope the intracellular distribution of potassium of different animal and plant cells. He found potassium uniformly distributed in the cytoplasm, except for certain non identified regions of high concentrations. The nuclei appeared to be completely devoid of potassium (259).

Few studies have been done on the same subject since that time. Holland and Auditore, using differential centrifugation techniques, analyzed the potassium content of the mitochondrial and supernatant fractions of kidney, liver and brain homogenates (194). The supernatant fractions of kidney and liver were

found to contain between 70 and 75% of the total potassium, which amounts to about 90 micromoles per gram of liver and about 80 micromoles per gram of kidney. The mitochondrial fraction contained between 10 and 15% of the tissue potassium. These results reveal a fairly constant distribution of potassium in the whole kidney and liver cells, without obvious concentration at one particular region.

Brain tissue, however, was found different, its mitochondria containing about 30% of the total potassium of the tissue. This would suggest the existence, in vivo, of a potassium concentration gradient between these particles and the cytoplasmic sap. The possibility that potassium ions changed their location during homogenization was not considered. However this conclusion is in accord with Macallum's results, and many recent works which have suggested that the mitochondria of different tissues can concentrate potassium in vitro.

In 1956, Griswold and Pace published the results of a similar analysis bearing on liver cells (165). They found a greater proportion of the total potassium (19%) than of the sodium (7%) in the mitochondrial fraction, and the inverse relation in the microsomal fraction (15% of the Na and 12% of the K), and about 13% of each ion in the nuclei. Another analysis of the liver tissue, by Berger (1957), gave about 7% of both ions in the nuclei, 6% of the potassium and 4% of the sodium in the mitochondrial fraction, 4 and 7% respectively in the microsomal fraction (31). In spite of the low proportion of potassium found in the mitochondria, the author feels justified in concluding that these particles maintain a potassium

concentration gradient against the cellular cytoplasmic sap, in vivo. Her results obtained on the nuclei, as those of Griswold, contradict the findings of Macallum but are in accord with data obtained by Itoh and Schwartz (201,202).

These authors found the concentrations of potassium and sodium in the nuclei of thymus, liver and kidney, to be approximately equal to the content of the whole tissues. They found sodium slightly more concentrated and potassium slightly less concentrated in the nuclei than in the cytoplasm.

From these few works, it appears that both potassium and sodium are distributed in all parts of the cell. Mitochondria may contain slightly greater concentrations of potassium than the cytoplasm. Microsomes have more affinity for sodium than potassium and the inverse relation may hold in the case of the mitochondria. Other results related to this subject will be discussed in more detail in the section of this review dealing with cellular mechanisms of active ionic transport.

Stability of the brain potassium content in vivo.

A comparison of the potassium and sodium contents of brain with the normal levels of these ions in blood plasma and cerebrospinal fluid gives apparent intracellular over extracellular ratios of about 20 to 1 and 40 to 1 respectively for potassium, and about 0.4 in both cases for sodium. These ratios give an indication of the magnitude of the concentration gradients maintained by brain in vivo. Under normal physiological conditions, the potassium content of brain is remarkably constant

from the time when the organism has finished its growth until death. Aging has been shown to have no effect on the potassium level (256).

However a number of experimental and pathological conditions interfering with energy producing metabolic reactions, with the selective permeability properties of the brain cells or having unspecific general effects on the whole organism, have been found to induce significant changes in the potassium levels of brain in vivo.

Thus potassium losses from the brain have been observed when the normal metabolic activity of this organ was limited by lack of oxygen (29) or lack of oxidizable substrates following either insulin hypoglycaemia (361) or starvation (150). Chemical agents which are thought to act directly on the cellular membrane can also induce potassium losses when added to the perfusion fluids of the brain, like acetylcholine (104) or when injected into the general circulation, like deoxycorticosterone (12). Various forms of systemic physical stresses have similar effects: low atmospheric pressure (87), high oxygen pressure (209), high acceleration stress (49,143), exposure to heat or cold (29).

Lowering the sodium content of perfusion fluids below the normal level of plasma causes the release from the brain of proportional amounts of potassium (432).

Intravenous injection of various stimulant or depressant drugs may provoke changes in the potassium content of nervous tissues (95,236). Agents provoking experimental convulsions

were found to decrease the potassium content of brain: examples were carbon dioxide (428), metrazol (68), electrical stimulation (68). Colfer and Essex have detected potassium loss and sodium gain in the cortical neurons of rat, rabbit and cat, accompanying or following metrazol, electrical and audiogenic induced seizures, the normal levels being recovered after 3 to 4 hours (76). However epileptogenic foci of the human brain have been found to contain normal concentrations of potassium and sodium (309). It is probable that the number of cells discharging at any one time was too low to affect the average content of the tissue samples.

A lowering of the potassium content of brain has been reported to accompany experimental psychoses (51) and various degenerative diseases involving demyelination. Sodium replaces part of the potassium in degenerating nerves (234). Denervated muscles lose more than 50% of their intracellular potassium. This effect is reversed after reinnervation and restitution of the motor functions (103).

In many instances, drugs, stress and blood electrolyte imbalances which induce marked changes in the electrolyte content of various other organs and tissues leave the brain levels unchanged. Magnesium deficiency reduces the potassium content of erythrocytes and muscles, increases the level in liver, but does not change the potassium level of brain (316). Auditore and Holland have shown that adrenalectomy, injections of corticosteroids, of catecholamines and of various drugs affect the intracellular distribution of potassium in kidney and liver

cells without parallel effects on brain cells (15).

As in the case of the other organs and tissues of the organism which maintain large electrolyte concentration gradients (muscles, heart, liver, kidney, erythrocytes etc.), the stability of the potassium and sodium content of brain is protected to some extent through the hormonal, renal and nervous mechanisms which keep the electrolyte balance of the organism as a whole. The unique permeability properties of the hemoencephalic barrier constitute another more specific form of protection of the electrolyte content of the central nervous system (45). This has been demonstrated by many studies which have shown that the electrolytes of brain equilibrate very slowly with radioactive potassium (304,219) and sodium (263) injected into the general circulation.

These systemic mechanisms of control (kidneys, adrenal glands, blood brain barrier) may well participate in the maintenance of the electrolyte balance of brain, but as a high concentration of potassium is maintained by practically all living cells, they obviously do not constitute the fundamental element of this control. "They only support but do not create a condition antedating their existence" wrote Kaunitz (211). This observation locates the problem mentioned at the cellular level.

Part 2. Intracellular Maintenance of Potassium

Many theories have been proposed to explain the intracellular maintenance of high potassium and low sodium concentrations, the oldest one being the Ostwald-Bernstein theory

of membrane semipermeability.

Theory of membrane semipermeability.

Ostwald first suggested in 1890 that intracellular and extracellular phases are separated by thin semipermeable membranes analogous to non-living artificial membranes (306). In 1902 Bernstein developped this idea, suggesting that these membranes would be impermeable to the intracellular anions but permeable to potassium (35). He also assumed that the membrane would be impermeable to sodium except, as was suggested by Overton (307), after depolarization by the local application of stimulus. According to this hypothesis it was possible to use a simplified form of the equation originally developped by Nernst (303) to calculate the theoretical resting potential of nerve and muscle fibers. Experimental determinations proved subsequently that the resting potential of these cells is remarkably close to the predicted values, and the theory remained unchanged for over 30 years.

In 1941, Boyle and Conway proposed a modified version of this old theory (43). These authors explained the unequal distribution of potassium across the membranes of living cells as the result of a double Donnan equilibrium resulting from the presence, inside the membrane, of undiffusible anions (phosphate esters and proteins) and, outside the membrane, of large concentrations of sodium supposedly undiffusible. This theory explained the difference in the permeability of potassium and sodium by the existence in the membrane of pores whose diameter would be greater than that of hydrated potassium ions

but smaller than that of hydrated sodium ions. Various experimental findings, which have been reviewed by Ling (250) and by Ungar (401), have shown that many of the assumptions of the membrane semipermeability theory of Bernstein and Conway were false. Intracellular anions like phosphate, lactate and chloride ions which were assumed to be "impermeant" were shown to diffuse through the cellular membrane. The use of radioactive isotopes of sodium revealed that this ion diffuses through cellular membranes as does potassium. Finally it became clear that the concentration gradients of potassium and sodium maintained by the cells could not be the result of purely passive diffusion and equilibrium since they were found to be dependant on the cellular metabolic activity and energy output.

Metabolic requirements for the maintenance of intracellular potassium.

Isolated nerves incubated in vitro need oxygen to maintain at an optimal level their resting potential and their ability to conduct impulses, as was discovered by Furasawa (137) and Gerard (144). These early findings were confirmed and complemented by many more recent studies, in particular by the works of Lorente de No (255) and of Shanes (347). Early works of Fenn (122,125) and of Dean (93) had suggested that anoxia does not influence the rate of potassium losses from isolated nerves. However Shanes (349,351) and others have shown that the external perineurial connective tissues may mask electrolyte shifts occurring at the nerve cell membranes and it was definetely

demonstrated by Fenn (124), Shanes (348), Van Harrefeld (406) and others that aerobic conditions are required for the maintenance of the optimal potassium levels in isolated nerve fibers.

Interference with the oxidative metabolism of isolated nerves, by incubation at 0°C (284) or by various metabolic inhibitors (53,189,190,339) results in potassium leakage and sodium gain or limits the reabsorption of potassium and extrusion of sodium either during the post-anoxic period of restoration of oxidative metabolism or during the recovery phase after impulse conduction. The works of Caldwell (52, 53,54) have provided evidence suggesting that the levels of energy-rich phosphate compounds inside the nerve cells are closely related to the control of ionic transports across the membrane.

Experimental data related to the metabolic aspects of potassium retention and accumulation in isolated nerves have been reviewed by Keynes (216), Hodgkin (187), Brink (46) and Shanes (346).

Elliott (106) has shown that brain slices incubated in the absence of oxygen lose as much as 90% of their potassium content in 30 minutes, while aerobically and in presence of glucose the slices retain or reaccumulate about 50% of their original potassium content. Using radioactive potassium, Krebs (233) was able to show that 3.5 to 4% of the potassium content of brain cortex slices is exchanged per minute with the potassium of the external medium. He concluded that the high potassium concentrations kept within the cells cannot be due to permeability barriers but must be the result of an active or

energy-requiring transport of potassium into the tissue counteracting the effects of leakage down the gradient into the extracellular phase.

The maintenance of a potassium concentration gradient in brain slices is dependant to a large extent on the type of oxidizable substrate supplied to the tissue; it can also be affected by the presence of glutamate in the medium (106,388) and also by the ionic composition of the medium. These effects will be discussed in some detail in Chapter VII.

Besides isolated nerves and brain tissue many other types of tissues and cells maintain potassium concentration gradients in vitro. In all cases this potassium maintenance has been found to depend closely on the metabolic activity of the tissues.

Erythrocytes were the first cells in which the metabolic activity was observed to control the potassium exchanges in vitro (Danowski,86 and Harris,171). A long series of remarkable studies, particularly related to the problems of blood storage in the cold, have shown that maintenance or reaccumulation of potassium is completely dependant on the metabolic activity of erythrocytes. This is influenced by temperature (127), various drugs (particularly the glycolytic inhibitors,391), changes of pH (36) and the presence of various substrates (208).

Christensen has found that the coupled uptake of potassium and amino acids by Erlich mouse ascites carcinoma cells is largely prevented by respiratory inhibitors and reduced by uncouplers of phosphorylation like 2,4-dinitrophenol, aureomycin or chloromycetin (65).

Disturbance of the phosphorylation processes provokes also potassium losses from erythrocytes (145), skin cells (245) and bacteria (342). Correlation has been demonstrated between potassium uptake and respiratory rate of yeast (7,369) and between glucose accumulation or utilization and potassium uptake of yeast (37), various bacteria (291) or muscles treated with insulin (157,424).

Working on kidney cortex slices, Mudge (293,294) has shown that cooling or anoxia induce large losses of potassium, the reaccumulation depending on aerobic oxidation as modified by temperature, oxygen tension and various other factors. He found a correlation between depression of potassium uptake and effects of dinitrophenol on the generation of energy-rich phosphate bonds.

Davies (422,25) confirmed most of these results, but contrarily to Mudge who had not detected any substrate specificity, he showed that alpha-ketoglutarate is required for optimal retention of potassium (as Krebs had shown for glutamate in the case of brain,388).

It is interesting to observe that the energy supplied for ionic transport is derived in some tissues from glycolysis, in others from oxidation through the tricarboxylic acid cycle and in others from both sources.

Davies (212) recently showed that renal medullary slices depend on anaerobic glycolysis for their potassium accumulation while renal cortex slices depend on aerobic energy metabolism. Active accumulation by leukocytes is unaltered by respiratory

inhibitors but completely abolished by inhibitors of anaerobic glycolysis (119). Mammalian erythrocytes also depend largely on glycolysis for their ionic transport (159). However, according to Maizels (262), non-mammalian red cells derive their energy for potassium accumulation from respiration. Hastings (128) has demonstrated that liver slices can maintain in vitro 80% of their potassium content. Specific glycolytic inhibitors affect markedly the potassium retention and there is a good deal of evidence suggesting that anaerobic glycolysis is sufficient in liver from fed animals to furnish energy for potassium uptake (148).

Muscles and nerves seem to derive their energy for the maintainance of ionic gradients partly through oxidative metabolism, partly through glycolysis, since, during a period of anoxia, their potassium content can still be reduced by iodoacetate (55,351). Shanes has shown that glucose retards both anoxic depolarization and potassium loss (351).

There are some indications that brain slices may also partly rely on glycolytic metabolism for potassium transport, under certain specific conditions. Dixon (101) reported first that glucose prevents loss of potassium from brain slices incubated anaerobically. Terner et al. (388) and Pappius and Elliott (311), measuring directly the potassium content of brain tissue, could not reproduce this early finding. However these last authors found that stimulation of glycolysis by addition of pyruvate or by a previous period of aerobiosis, or both stimuli together resulted in a marked increase of the potassium content of brain slices.

The sodium pump hypothesis.

When it was demonstrated that cellular membranes are permeable to sodium, it became necessary to find another explanation of the low intracellular sodium concentrations. In 1941, Dean suggested that cellular membranes may actively extrude the sodium ions diffusing into the cells down the concentration gradient (94). This suggestion became the basis of a new modification of the old membrane permeability theory, the distribution of potassium being still considered to be the result of a passive equilibrium secondary to the active extrusion of sodium. Hodgkin expressed this idea, writing: "It makes little difference to the potassium distribution whether sodium is kept out by an active process or by a semi-permeable membrane" (188).

This working hypothesis was the origin of a long series of remarkable works on the ionic permeability and bioelectric properties of squid giant axons, by Hodgkin (186,188,189,190, 191) and by Keynes (215,218) in particular. The experimental data obtained along these lines have been reviewed extensively by Hodgkin (186,187), Ussing (402) and Keynes (216,218). The same hypothesis was also invoked to explain the ionic transports occurring in muscle by Steinbach (375), in kidney by Cort and Kleinzeller (80), in red cells by Harris (173) and by Glynn (151), in frog skin by Ussing (403) and in various other biological systems by Krogh (235) and others.

However the nature and properties of the postulated pump mechanism have remained obscure and many new problems have originated from this theory.

Enzymatic activity and ionic transport.

One of the obscure points concerns the succession of events which have been shown to occur in the membrane of excitable cells following depolarization: changes of permeability which first favor the inflow of sodium and subsequently the outflow of potassium. As these ionic shifts occur in the same direction as the concentration gradients they were assumed to be of a purely physical nature (191). However recent findings of Hill have revealed a marked increase in the heat production coinciding with these events (1). This would indicate that this process is of chemical nature.

These rapid ionic transports appear to be related to the facilitated diffusion processes described by Danielli (85). This author has suggested that the lipoid layers of cellular membranes may contain active patches of a different chemical composition through which the rapid diffusion of specific molecular species would be facilitated. These carriers are not dependant on energy supply, as they transport molecules down concentration gradients but they may be affected by chemical and physical agents and as they show a high degree of substrate specificity, they are similar to enzymes in certain of their properties.

Nachmansohn has postulated a similar mechanism which would be responsible for the changes in sodium conductance associated with nerve activity (299). According to this theory, any stimulus applied to the membrane releases free acetylcholine which reacts with a receptor protein and changes

its configuration. This provokes the observed changes in the permeability properties of the membrane. Hydrolysis of acetylcholine by the enzyme cholinesterase reverses this process and the membrane recovers its normal permeability properties. Some rather strong evidence supporting this theory has been reported recently and is discussed in recent reviews of this problem (300,301).

Acetylcholine, or inhibition of cholinesterase, provokes potassium losses from isolated nerves (125,431), heart (326), and muscles (227). Greig (163) has published a long series of studies indicating that acetylcholine is implicated in the maintenance of the permeability properties of red blood cells and other tissues. However the concentrations of drugs used in these experiments were very high and the significance of these data has been called in question in more recent works (138,153).

Strickland (382,383) has studied the effects of cholinesterase inhibitors on the potassium uptake by brain slices and came to the conclusion that these drugs cause potassium losses only at concentrations which inhibit O_2 uptake or stimulate aerobic glycolysis, both effects being indicative of some disruption of the normal metabolism. Christensen (64) working on ascites cells and Mudge (294), on kidney cortex slices, came to similar conclusions.

There is some evidence that the carbonic anhydrase enzyme of kidney and nerve cells may participate in the membrane permeability processes. Specific inhibitors of this enzyme (sulfanilamide and other sulfonamide derivatives) have been

shown to change the distribution of potassium and sodium in isolated nerves (261,338). These drugs have been found to reduce the potassium uptake of brain and kidney slices (90). However these effects were accompanied by inhibition of the O_2 uptake rates of the tissues (89). Mudge, working on kidney slices (294) and McIlwain, on brain slices (272), were unable to show any effects of these drugs on ionic transport.

Shanes has noted the importance of acid-base equilibrium in nerves as a factor in potassium retention or accumulation (261). He claims that the nerve metabolism contributes to the maintenance of the potassium concentration gradient by providing hydrogen ions which exchange with extracellular potassium. The carbon dioxide produced in aerobic metabolism and the lactic acid in anaerobic metabolism would serve as sources of hydrogen ions. In relation to these considerations, this author suggests that sodium extrusion may be secondary to potassium uptake in some cases, and the two processes may be independent in other cases. Experimental data supporting this view have been reviewed by Shanes (345,346,353). This theory is not admitted by the majority of the workers in this field (see Ussing,402). However similar mechanisms of hydrogen ion-potassium exchanges are well established in the case of yeast (78,321,333) and various unicellar organisms (402).

In yeast, the hydrogen ions produced by the respiratory reduction-oxidation system have been suggested to exchange with extracellular potassium (78). A few workers have also presented evidence of active transport of potassium independent of a sodium pump in erythrocytes (314,317), in leukocytes (119) in skin (245) and in bacteria (96).

Coupling of energy production and sodium pump.

The exact nature of the mechanism coupling the energy produced by the cellular metabolism with the active transport of sodium across the membrane constitutes another obscure point of the sodium pump theory. Goldacre (152) has suggested that active transport processes are based upon contractile protein systems analogous to the actomyosin-adenosine triphosphatase system of muscle. Danielli (84) has made a critical analysis of this theory and has suggested that the membrane contractile proteins responsible for sodium and potassium transport would not be associated with phosphatases but with other more specific enzymes such as cholinesterases.

However the possible role in membrane ionic transports of an adenosine triphosphatase, present in cell microsomal fractions, has received a great deal of attention recently. Libet (247) and Abood and Gerard (2) have found in the sheath of giant axons and in submicroscopic particles of peripheral nerves a magnesium-activated adenosine triphosphatase. From crab nerves, Skou isolated a similar enzyme and found its activity highly dependent on the relative concentrations of potassium and sodium, and inhibited by g-strophantin,a drug which is known to inhibit ionic transports (362,363).

This enzyme is located in subcellular particles similar to those identified as fragments of disintegrated membrane in the electron microscopic studies of Hanzon and Toschi (166). After a careful analysis of the properties of this enzyme, Skou has concluded that its function may be directly related

to the control of the intracellular sodium-potassium ratio. Post et al. (319) who have isolated a similar enzyme from erythrocyte fragments and Jarnefelt (204,205) in brain microsomes, have come to similar conclusions.

Ionic carriers of the membrane.

The identification of the postulated carrier molecules responsible for the transport of ions across cellular membranes constitutes the most difficult problem originating from the pump hypothesis. Theoretically, this carrier molecule must have more affinity for sodium when it is in presence of a ratio of K/Na greater than one, and more affinity for potassium in the opposite situation; it must probably be lipid-soluble and negatively charged. Theoretical analyses of this question have been made by various workers (151,173,375).

Various cellular constituents which show more or less the required properties of the hypothetical carrier have been suggested: histamine (121), pyridoxal (62), the carboxyl groups of protein, ribonucleic acid (380), polyphosphates (374). The works of Folch (130,132) have directed attention towards the acidic phospholipids as anionic membrane constituents which could fulfil the function of ionic carriers.

Solomon et al. (367) have isolated from blood various extracts which show a greater affinity for potassium than for sodium, are lipid-soluble and release their attached cations in water. Kirschner (224) has isolated from erythrocytes four phospholipidic compounds, two of which have been identified as phosphatidyl-serine and phosphatidyl-ethanolamine, which

bind sodium preferentially to potassium.

Hokin and Hokin (192,193), working on brain microsomes and on the salt glands of marine birds, have found that the formation of phosphatidic acid from ATP and diglyceride is stimulated by acetylcholine and blocked by very low concentrations of atropine. They have presented evidence suggesting that this substance may act as a membrane carrier of hydrophilic secretory materials, of sodium in particular.

In his redox pump theory, Conway has suggested that the cation carriers are the respiratory enzymes themselves in their reduced form, and that the osmotic work is performed in parallel with the electron transport. He obtained some evidence in favor of this theory from experiments on yeast (79) and on muscle (77).

Mitochondria and cellular ionic transport.

Claude found that mitochondria exhibit osmotic properties (71) and possess a structure ressembling a membrane (72). In studies on intracellular distribution of electrolytes, mitochondria were found to contain greater concentrations of potassium than the cellular sap (31,165,194). Other experiments performed in vitro, suggested that these subcellular particles can apparently maintain concentration gradients of potassium, this activity depending directly or indirectly (to conserve the membrane integrity) on oxidative phosphorylations (24,260,370). These observations have been repeatedly confirmed on mitochondria obtained from liver (357,358), kidney (373), brain (194,195), heart and skeletal muscles (400) and plants (197).

Krebs and coworkers (25) suggested that mitochondria "are the basic units responsible for the secretory and absorptive activity of cells". According to them: "the cell membrane may at least in some cases serve as a means of keeping the mitochondria together rather than a site where the secretory or absorptive work is done." Holland (195) suggested that the cell membranes are implicated in the transient permeability phenomena associated with conduction but that mitochondria are responsible for the changes occurring in resting cells or in recovery processes.

Many authors do not accept this view. Some were unable to find any evidence of active transport performed by mitochondria (170,203,341,420). Others have emphasized the fact that large amounts of potassium appear to be bound to the membranes of these particles (139).

As the ionic concentrations are not commonly expressed as a function of the water content, Amoore (11) remarked that swelling and damage may be responsible for the apparent increases of ionic content of mitochondria observed. Expressing the concentration of potassium per unit of water volume, Amoore (10) found equal concentrations of potassium in mitochondria and in the cytoplasm.

As very few authors were able to find sodium accumulation by mitochondria and as nobody has ever shown sodium extrusion, there is presently very little evidence supporting the view that these subcellular particles are directly responsible for the cellular ionic exchanges.

Intracellular potassium binding.

There is a good deal of evidence suggesting that important amounts of intracellular potassium may be bound. Thudichum (390), Koch and Pike (228) and Christensen and Hastings (63) found large amounts of potassium in various brain lipid extracts. Folch found that three different acidic lipids may combine together as much as one fourth of all the cations present in adult bovine brain (129). Stone and Shapiro (381) and Bergen et al. (30) have found by ultrafiltration experiments about 30% of the total potassium of brain in a non-filterable form. Others have also found various amounts of potassium bound to lipids (243), microsomal fractions (177,337) and mitochondrial fragments (162) of brain.

Studies on exchanges of tissue potassium with the radioactive isotope have shown slow and fast components of equilibration in different muscles (122,175,403), kidney slices (295,423) and unicellular organisms (225). Leiderman and Katzman (241) have shown that 20 microequivalents of potassium per gram of fresh brain are non-exchangeable with radio-potassium, in the adult rat. Muscles (172,282) and kidney slices (293,422) which were cooled to $0^{\circ}C$, and nerves and muscles which were allowed to die naturally or were poisoned (359) were found to retain important fractions of their potassium content. Pappius and Elliott obtained evidence of potassium binding in incubated brain slices (311). Among the intracellular compounds which were found or supposed to bind potassium may be mentioned creatine phosphate of skeletal muscles and heart (298),

hexosephosphates of liver (122), proteins (58), nucleic acids (344) and also various lipidic compounds already mentioned.

These data and other reasons have led certain authors (249,250,359) to support the theory of selective ionic adsorption as a better explanation to the ionic distribution in living cells than the pump hypothesis. Van Wazer (407) and Melchiors (286) have suggested that the molecular configuration of different polyphosphate compounds (ATP in particular) favors potassium in the formation of chemical complexes. This would be a molecular mechanism by which living cells discriminate between sodium and potassium. Snell (365) has presented evidence to the contrary. In recent reviews of this subject, Ussing (404), Hinke (185) and Solomon (342) came to the conclusion that a selective protoplasmic binding of potassium cannot account for the high intracellular concentrations of this ion found in biological systems.

Ungar (401) has reviewed other theories which tend to give more importance to the cellular cytoplasm as a whole rather than to the membrane in the phenomena of ionic transport and maintenance. Szent-Gyorgyi has recently presented a physico-chemical theory which postulates that the intracellular water is disposed in ordered lattices which tend to eject sodium and retain potassium for reasons of atomic structure and of energetics (19).

These views are not generally accepted and studies with the electron microscope which have rendered visible the cellular membranes have given more weight to the "membrane" theories.

Part 3. Metabolic Properties of Potassium

In his famous studies on the beat of isolated frog heart, Ringer found that it was necessary to add small amounts of potassium to the perfusion medium in order to maintain normal function (329). Since that time, potassium has been found to participate in a great number of physiological processes and to influence many others (122). This extensive physiological activity may be related to the depolarizing action of this ion on excitable structures, as shown by Cowan (81). Potassium has long been thought to participate in biological phenomena primarily through its influence on the hydration of protoplasmic material (360). However it became gradually apparent that this cation exerts very specific effects on the cellular general metabolism and on well-defined enzymatic reactions.

The Ashford and Dixon effect.

Studying the glycolytic activity of rabbit brain slices in presence of different substrates, Ashford and Dixon (13) found that potassium glycerophosphate decreased the anaerobic glycolysis and increased the aerobic glycolysis while the correspondent sodium salt was inactive. This finding led them to study the effects of potassium chloride on the metabolic activity of brain slices. They found that the addition to the standard medium of an amount of potassium chloride sufficient to increase the concentration of potassium by 100 millimolar could increase the oxygen uptake rate by about 100%, the

aerobic glycolysis by 200% and diminish the anaerobic glycolytic rate by 75%. They studied the effects of different concentrations of potassium and concluded: "It would appear probable that at the concentrations of normal Ringer, potassium does not exert this effect." Omission of calcium from the standard medium could also cause analogous but much less marked effects. Sodium chloride salt had no similar effects.

In the same year Dickens and Greville confirmed these results on rat brain cortex slices (98). They found that cesium and rubidium salts were also effective. The effect on oxygen uptake could be obtained with glucose, lactate, pyruvate, fructose, used as oxidizable substrates. Large amounts of sodium were required to be present in the medium for potassium to exert its effects.

In 1942, Canzanelli et al.(56) studied these effects further and found that hypertonic conditions were not necessary. When replacing sodium by potassium the effect on oxygen uptake was maximal at 40 millimolar potassium. The presence in the medium of at least 20 millimolar sodium was required. These authors concluded that potassium and calcium are the only cations influencing respiration: in a balanced medium they annul each other's effect and the result is quantitatively the same as if sodium was the only cation of the medium.

Lipsett and Crescitelli reported that the effect of potassium on respiration could not be obtained when glutamate or various intermediates of the tricarboxylic acid cycle were used as oxidizable substrates (252).

In 1949, Dixon reported that the effects on glycolysis could be obtained by replacing sodium by potassium, keeping the media isotonic. Potassium could cause a significant inhibition of anaerobic glycolysis at a minimum concentration of 20 millimolar and a significant stimulation of aerobic glycolysis at a concentration of 30 millimolar (100).

Tissue specificity.

Himwhich reported that high concentrations of potassium can stimulate the respiration of other parts of the central nervous system, besides brain cortex: the upper brain stem and medulla (184). Chain et al. also reported the stimulation by potassium of the respiration of cerebellar cortex, optic chiasm and various parts of the hypothalamus of the rabbit (60). Chang et al. had studied in 1935 the effects of potassium on the respiration of nerve preparations (61). Shanes (356) has found that the oxygen uptake of nerve fibers is maximally stimulated at a potassium concentration of 30 millimolar. From these data it appears likely that all types of nervous tissues are susceptible to the effects of potassium described by Ashford and Dixon.

Dickens and Greville had shown that kidney cortex, liver, testis, retina and tumour tissues of various types are not sensitive to the metabolic effects of potassium. The respiration of kidney slices was found to be unchanged (246) or decreased (293) by potassium. The respiratory activity of heart (288), liver (38) and erythrocytes (200) is inhibited by high concentrations of potassium. The oxidative and glycolytic metabolism

of diaphragm has been shown to react differently to potassium according to whether the muscle is denervated or not (435) and also depending on the pH of the medium of incubation (135). In normal conditions the oxygen uptake rate of this tissue was found either unchanged (435) or inhibited (135) by potassium. Apparently there are some species differences in the sensitivity to potassium of muscle tissues. The aerobic metabolism of frog and toad muscles is stimulated by potassium (296) and also the respiration of the heart of small invertebrate animals(57). Rothstein (332) and others have shown that the rates of fermentation and respiration of yeast is stimulated by potassium.

Potassium effects on homogenates.

It was recognized by Ashford and Dixon that the effects of potassium obtained on brain slices were not apparent when tissue suspensions were used. Racker and Krimsky (325) discovered that sodium inhibits the glycolytic activity of brain homogenates. Utter (405), and Muntz and Hurwitz (297) confirmed this finding and found that potassium stimulates markedly the anaerobic glycolysis of homogenates or acetone powder extracts of brain. This effect is not specific to brain and many tissue suspensions require high concentrations of potassium (plus DPN and ATP) to show a maximal glycolytic activity. On the other hand, it was found that high concentrations of potassium may inhibit the respiration (115) and aerobic glycolysis (220) of brain suspensions. These data show clearly that the effects of high concentrations of potassium on slices, mentioned earlier, are the result of changes occurring

at the cellular level (perhaps involving membrane permeability) rather than at the level of subcellular particles or of isolated enzymic reactions.

Various metabolic effects of potassium.

Under aerobic condition in presence of high potassium concentrations, brain has been reported to utilize glucose at a rate 2 to 3 times greater than under standard conditions (395,396). A stimulatory effect of potassium on glucose uptake was also shown in bacteria (332), kidney (246), liver (176) and blood polynucleocytes (27). This effect seems to result mainly from an activation of the hexokinase system of the cellular membrane (332, see also 106) and also from greater rate of glucose oxidation in brain or of glycogen formation in liver and other tissues.

The effects of potassium on glycogen metabolism vary strikingly depending on the tissue studied. Kleinzeller and Rybova (226) showed that below a concentration of 40 millimolar, potassium does not exert any effect on the glycogen metabolism of brain slices, while at higher concentrations it inhibits the glycogen formation. Hastings et al.(14,176) have clearly demonstrated that potassium, in opposition to sodium, favours the synthesis of glycogen in liver slices. A similar effect was found in the case of blood polynucleocytes (27). At all concentrations potassium inhibits the synthesis of glycogen in heart (371) and various muscles (397).

McIlwain (271) and others (395) have shown that high concentrations of potassium decrease the level of creatine

phosphate and increase the liberation of inorganic phosphate in brain slices. Heald (179) confirmed this finding and showed that the creatine phosphate levels fall by 40 to 50% one minute after the addition of the potassium to the medium. Rossiter and coworkers demonstrated that similar conditions decrease radioactive phosphorus incorporation into phospholipids, ribonucleic acid and phosphoproteins of brain slices (106,126). Tsukada has presented some rather unconvincing evidence to the contrary (394,396).

Quastel and coworkers have found that potassium, at a concentration of about 27 millimolar, stimulates markedly the synthesis of acetylcholine in brain slices (265). This effect was confirmed by Elliott and coworkers (283,112) and by Welsh and Hyde (418). Direct effects of potassium on the enzymatic system responsible for the metabolism of acetylcholine will be discussed in the last section of this review.

The stimulated respiratory activity of brain slices is highly sensitive to malonate (221,395), azide (395) and other metabolic inhibitors. Kimura (221) has shown that malonate (10 mM) reduces the stimulated oxygen uptake of brain incubated with glucose to the level obtained when the tissue is incubated without oxidizable substrate (endogenous respiration). Tsukada (395) demonstrated that malonate increases markedly the rate of lactic acid production. Ethanol (28,149,412), chlorpromazine (248) and a variety of narcotics and anaesthetics (142,273,322) have been found to inhibit the stimulated respiratory rates of brain slices, at concentrations which are without much effect on the normal respiration. This subject will be discussed more extensively in Chapter VI.

Other agents stimulating the metabolism of brain slices.

A great number of agents can mimic the effects of high concentrations of potassium on brain metabolism. Brain slices exposed to electrical stimulation (268,269,274), low concentrations of calcium (56,98), glutamate and glutamine (231,416), high concentrations of ammonia (156,417), protoveratrine (427) and 2,4,-dinitrophenol (278,279) show increased rates of aerobic metabolism and decreased rates of anaerobic glycolysis. As for potassium, all these agents (except protoveratrine) have been shown to lower the levels of creatine phosphate, to increase the levels of inorganic phosphate and to reduce the rates of phosphorylation reactions (106,126,179,270,271,278).

The parallelism of the action of high concentrations of potassium and electrical stimulation in vitro is also evident in other aspects of brain metabolism: substrate specificity (230,270,271), acetylcholine and ammonia formation (334), glycogen synthesis (226,240). As with potassium stimulation, electrical stimulation requires the presence in the medium of a minimal amount of sodium, in order to be effective (156). Stimulation by the two methods gives very close rates of glucose utilization, of oxygen uptake and lactic acid production.

The relation between the metabolic effects caused by high potassium concentrations and by low calcium concentrations is not so well defined. In some instances these effects are diametrically opposed. Like potassium, calcium favours the synthesis of acetylcholine (283). Ammonia formation in brain slices is decreased by high concentrations of potassium but not

by lowering the calcium content of the saline medium (411). Omission of calcium and increase of potassium concentration bring different and sometimes opposite effects on the turnover rates of some amino acids (222,223). This problem will be discussed further in Chapters IV and IX.

Influence of potassium on enzymic systems.

Potassium activation of a specific enzyme reaction was first described by Boyer and Lardy for the conversion of phosphoenolpyruvic acid to pyruvic acid (41,42,238). It was demonstrated later that potassium does not merely exert a stimulatory effect but constitutes as well as ADP an essential part of the enzyme system (174,207). Boyer showed that the pyruvic phospherase enzymes extracted from 19 different tissues including heart, liver, kidney and brain are all strongly activated by potassium (40). Erythrocytes of six different mammalian species were also shown to contain a similar potassium-activated enzyme (368).

Liver (182) and yeast (332) hexokinases are also activated by potassium. The brain enzyme was found to be inhibited by extremely high concentrations (700 mMolar) of potassium (376).

Various enzymes involved in the metabolism of "active acetate" are also influenced by potassium. Von Korff (409) demonstrated that potassium is required in the complex system forming citrate from acetate, ATP, coenzyme A and oxaloacetate. The author believes that the K-activated reaction is the formation of acetyl-coenzyme A by the "acetate activating enzyme" and he found no clear indication that the "condenzing enzyme" itself

would require potassium. Stadtman (372) found that a related bacterial enzyme: "phosphotransacetylase" is also K-dependent.

Nachmansohn (302) and others have found that the enzyme choline acetylase requires potassium for optimal activity. The affinity of the acetylcholinesterase enzyme for its specific substrate and for various competitive inhibitors is also influenced by potassium (287,158). Various aspects of fatty acid metabolism where acetylating reactions are involved are also influenced by potassium (147).

Other reactions of carbohydrate metabolism are affected by potassium. The "malic enzyme" of bacteria requires potassium to decarboxylate malic acid and form pyruvate (257). In presence of high concentrations of potassium heart muscle mitochondria oxidize acetate or malate more rapidly but not alpha-ketoglutarate or succinate (410). On the other hand potassium concentrations greater than 10 mMolar inhibit strongly the formation of oxaloacetate from phosphoenol pyruvate and CO_2 by the "oxaloacetate synthesizing" enzyme of the liver (384).

It is very remarkable that many of the enzyme systems which were found to be influenced by potassium are involved in transphosphorylation reactions. Various phosphatases have also been shown to be affected by potassium. The Mg-dependent adenosine triphosphatases of brain (154,204,205), nerves (362,363), muscles (285,292) and erythrocytes (319) are generally activated by low concentrations and inhibited by high concentrations of potassium. E. Coli apyrase (69) is activated either by sodium or potassium, while the brain apyrase (136) has been reported to be activated by sodium only.

The opposite effects of low and high concentrations of potassium on the phosphatases and phosphorylating enzymes are thought to be due to a greater affinity of the apoenzymes for the complex Mg-ATP-K than for the K-ATP-K complex. The antagonistic effects of sodium and potassium on many of these reactions are also believed to result from similar displacement mechanisms (237,286,363).

As a result of its effects on the various enzymic reactions described, potassium influences markedly the oxidative phosphorylation processes of many tissues, increasing the P/O ratios of liver (164), heart (410) and skeletal muscles (32,320) in particular. In many instances these effects are associated with a stimulation or a stabilization of the respiratory activity of the mitochondria (320,410).

Influence of potassium on the metabolism of amino acids.

Many years ago it was observed that protein synthesis or catabolism in liver is associated with potassium uptake or loss respectively (122). Potassium deficiency in rats results in a complete failure to utilize amino acids for protein anabolism (134). Steward et al. have suggested that potassium exerts a direct effect on the formation of peptide bonds (378). Incorporation of amino acids into the protein microsomal preparations is markedly activated by potassium (336,415). In contrast Allfrey et al. (6) found that the same process in thymus nuclei requires sodium while potassium is inactive and Breitman (44) found that potassium exerts an inhibitory action. However Brunish and Luck (47) showed that in vitro incorporation

of amino acids by the desoxypentosenucleoproteins and histones of the liver is increased by potassium. Misky et al. (305) found that various adverse conditions release both nucleotides and potassium in proportional amounts from nuclei. This depletion causes the impairment of the protein synthetic mechanisms.

Various simple reactions involving the formation of peptide bonds are stimulated by potassium or show an absolute requirement for this cation: synthesis of glutathione in yeast (366), erythrocytes (210) and liver (206), of pantothenate in bacteria (258). The conversion of ornithine to citrulline in rat liver homogenates is stimulated by potassium (74).

Happold has demonstrated that potassium is required to keep pyridoxal phosphate bound to the tryptophanase apoenzyme and he showed later that potassium activates three other enzymes whose coenzyme is pyridoxal phosphate: glutamic decarboxylase, serine dehydrase and transaminase (167,168).

Potassium influences the uptake and metabolism of individual amino acids in brain slices (222,223), ascites cells (62,64,65, 66,308,328) and other tissues. This specific effect will be discussed in some detail in Chapter VIII.

Significance of the potassium effects on brain metabolism.

Since its discovery, the Ashford and Dixon effect has been studied to a very great extent mainly because it was considered as a way of altering the metabolism of isolated brain tissues in such a way as to mimic the activity of the tissue in full activity in vivo.

Himwich has shown that the capacity of brain slices to respond to the potassium stimulatory effects is very low in

new born animals and increases gradually in the first weeks after birth (184). It is well known that the rates of glucose utilization, of oxygen consumption, lactic acid formation, the changes in the levels of high energy phosphate compounds, the substrate specificity and the increased drug sensitivity obtained in potassium-stimulated or electrically-stimulated brain slices are very similar to the metabolic characteristics of brain in situ.

However other aspects of the stimulated metabolism are not so representative of the conditions found in vivo. A good deal of evidence suggests that high K concentration impairs the anabolic capacities of the tissues, decreases glycogen synthesis (226), decreases ammonia formation (411), decreases the rates of phosphorus incorporation (126) and increases swelling (310). As a general rule the high potassium concentrations do not stimulate the formation of compounds which are known to be formed through endergonic reactions. It may be significant that maximal synthesis of acetylcholine (265) and of glutamine (60, 335) were obtained at concentrations of about 25 mMolar potassium, higher concentrations decreasing the rates of these synthetic reactions (335,418).

It has been discovered that perfusion of local areas of the cerebral cortex in situ with high concentrations of potassium transiently raises their rate of oxygen consumption (88). It is difficult to assess the significance of this finding. It may indicate that the parallel effects obtained in vitro are more representative of abnormal events occurring in the brain

than of the normal physiological conditions. Dixon, reviewing this problem, came to this conclusion (99).

The specific metabolic reactions underlying the changes in the general metabolic rates provoked by potassium or electrical stimulation of isolated brain tissues are very obscure and not many attempts have been made to clarify them.

However many purely theoretical explanations have been proposed. It has been suggested that potassium provokes a shift of the brain metabolism towards oxidative pathways which are not normally operant, or only to a small extent, in vitro (149,221). Hoskin (199) has demonstrated that potassium does not change the normal metabolic route of glucose metabolism. It does not favour a greater participation of the direct oxidative pathway but mainly increases the rates of glucose breakdown through the Embden-Meyerhof route and of oxidation through the tricarboxylic acid cycle (198). As it stimulates the symmetrical metabolism of glucose, Hoskin concluded that KCl may affect the permeability of subcellular membranes. Himwhich (184) reported that potassium does not change the respiratory quotient of the brain slices, suggesting that the stimulated respiration occurs at the expense of the usual cerebral substrates (mainly glucose).

Quastel has suggested that potassium increases the speed of the specific enzymatic reaction responsible for the formation of acetyl-coenzyme A (222,323).

Because the typical metabolic effects are obtained only with slices and not with ruptured cells, other theories propose that the primary effect of potassium is to change the permeability

properties of the membranes (98). The processes of glucose phosphorylation, which are supposed to occur at the membrane through the action of the hexokinase enzyme, have been thought to be implicated in the stimulatory phenomena (332). Changes in the ratio of potassium to calcium have been postulated to be the important factor, rather than the absolute magnitude of the potassium concentration (323).

McIlwain (271) holds that the added potassium would greatly increase the expenditure of energy necessary for the maintenance of a 20 to 1 ratio of intracellular to extracellular K concentration and that the metabolic changes observed result from this greater demand of energy.

A few authors have noted that the metabolic changes observed, which are accompanied by a depletion of the stores of energy-rich phosphate compounds (179,271,395), may be consequent to an impairment of the metabolism of brain slices (see 106,126, 226, and 335).

Evidence supporting or contradicting some of these theories will be presented in the following chapters, and the relations existing between potassium uptake and potassium influence on metabolism of brain slices will be studied. The question will be discussed in some detail in Chapter IX.

Chapter II

General Methods

Tissue Preparation.

Adult rats were decapitated with a guillotine, their brains removed and slices, about 0.5 mm. thick, of cerebral cortex were cut without moistening the tissue in a humid chamber by means of a Stadie-Riggs microtome (see Elliott,107). Eight slices of cerebral cortex, a surface and a second slice from the dorsal and lateral aspects of each hemisphere, were obtained from each brain. The slices from two or more brains were used for each set of experiment and two slices, weighing together 60-100 mg., were put into each experimental vessel. The slices were chosen so that each vessel received slices from two different brains and one of the slices was from the dorsal, one from the lateral aspect, and one of them was a surface and one a second slice (see Pappius and Elliott,310). The tissue was weighed on a torsion balance and introduced usually into erlenmeyers containing 3 ml. of medium.

Cold Pretreatment.

Usually the brain slices were first incubated in the cold for a period of 60 minutes. The purpose of this pretreatment will be explained in the next chapter. The erlenmeyers each containing 3 ml. of medium and brain slices, were filled with 100% oxygen, stoppered and shaken for one hour in an Aminco-Dubnoff incubator, the temperature of the bath being kept at

0° to 2°C with ice cubes. At the end of the period of incubation the contents of each erlenmeyer were poured onto a perforated disk in a funnel, very light suction being applied; the slices (kept on the disk) were removed from the funnel and drained on a filter paper. The slices were then reweighed and usually replaced in a Warburg flask containing 3 ml. of fresh medium for metabolic studies. Instead of potassium hydroxide, sodium hydroxide (0.2 ml. of a 20% solution) was used as CO_2 absorbant, in order to avoid possible contamination of the flask content by potassium at the end of the incubation when the slices were removed from the flask for potassium determination.

Incubation at 38°C.

After 10 minutes of equilibration at 38°C the respiratory rate (in presence of 100% oxygen) or the rate of carbon dioxide evolution (in an atmosphere of nitrogen-5% carbon dioxide) was measured manometrically by the standard Warburg procedure. At the end of the incubation period the slices were isolated on perforated disks, reweighed and analyzed for potassium and sodium, or for lactic acid or for amino acids.

Media of Incubation.

Unless otherwise noted, the medium used for cold incubation contained the following in milliatoms or millimoles per liter: Na 160, K 0, Mg 1.2, Cl 128, SO_4 1.2, PO_4 17.4, glucose 10, and its initial pH was about 7.8. The media used for aerobic

incubations at 38°C (used for determinations of oxygen uptake, lactic acid formation, potassium and sodium tissue content, amino acids levels) had the same composition except that various amounts of sodium (from 0 to 160 millimoles) were replaced by potassium. When calcium-containing phosphate-buffered media were used, the phosphate content was reduced to 11 mMolar and calcium chloride, dissolved in a sodium chloride saline, was added (0.1 ml. to 2.9 ml) immediately before the incubation at 38°C, in order to limit calcium phosphate precipitation. The final concentration was 1.7 mMolar in most cases. A few experiments were done with media containing 3.4 mMolar calcium.

The medium used for anaerobic experiments contained the following in milliatoms or millimoles: Na 0 to 151, K 0 to 151, Mg 1.2, Ca 1.3, Cl 128, SO_4 1.2, HCO_3 24.5, glucose 10. In some cases calcium was omitted. Carbon dioxide was bubbled through the medium for five minutes after its preparation.

Hypertonic conditions were obtained by adding either an extra 100 mMolar NaCl or 200 mMolar sucrose to the standard media.

Analyses.

Determination of lactic acid.

The rates of aerobic glycolysis were determined by measuring the amounts of lactic acid present in tissue plus medium after an incubation of one hour at 38°C. The analytical method of Barker and Summerson (23) was used. At the end of

the period of incubation at 38°C, 3 ml. of 10% (w/v) trichloroacetic acid were added to the content of the Warburg flasks; the tissue was then homogenized in the fluid from the vessel and the protein precipitate was separated by centrifugation. The values reported have been corrected for the average lactic acid content of a number of samples of cold incubated tissue before incubation at 38°C, and therefore, represent only the amount of lactic acid formed during the incubation at 38°C. The correction for preformed lactic acid was relatively small. The cold treated slices contained 6±2 micromoles of lactic acid per gram of brain, 8±1 micromoles per gram of liver and 5±1 micromoles per gram of kidney cortex.

In the case of experiments reported in Table 8, the interference with lactic acid analysis due to the amounts of pyruvate or malate added to saline media was corrected for, according to data given by Barker and Summerson (the color yelded by 1 microgram of pyruvic and malic acids is equivalent to 0.025 and 0.01 microgram of lactic acid, respectively). The values were also corrected for the small amount of lactic acid produced from added pyruvate. This was taken as the amount produced when pyruvate was added without glucose, during the incubation at 38°C (14±3 micromoles per gram of tissue per hour, 6 determinations). No lactic acid was found after incubation with malate alone and no correction was therefore applied.

Determinations of potassium and sodium.

The potassium and sodium contents of brain slices were determined according to the method of Pappius and Elliott (311). The slices were digested with 0.4 ml. of concentrated nitric acid in a test tube on a sand bath until the solution became clear. The digest was made up to 5 ml. with water, and 2 ml. were transferred into another test tube for sodium analysis. To the first tube was added 1.2 ml. of Molar sodium chloride, and the volume was made up to 5.4 ml. with water. The volume of the second tube (for sodium analysis) was made up to 10 ml. with water. The contents of both tubes were filtered through Whatman No. 40 paper, and the potassium and sodium content of the filtered solutions was determined by flame photometry (Beckman DU Spectrophotometer with the No. 9200 flame attachment and oxyacetylene flame was used).

Sodium is known to interfere with the determination of potassium by flame photometry. In order to avoid the effects of variations in the sodium content of solutions in which potassium was to be determined, sodium chloride was added to all unknown and standard solutions to give a final concentration of about 0.2 Molar. In the presence of this high added sodium concentration the effect of the sodium in the tissue samples was found by Pappius and Elliott (311) to be negligible.

The sodium determinations were corrected for the sodium found in a blank which was prepared with nitric acid, distilled water, and filter paper from the same batches as were used for

making up the unknowns. The standard solutions contained concentrations of sodium or potassium which closely bracketed those of the unknowns.

The results were expressed as microequivalents per gram of fresh tissue. A correction was applied (particularly important for sodium) which is based on the increase in weight of the tissue due to swelling by absorption of medium.

Pappius and Elliott (310) have shown that this fluid of swelling contains electrolytes in the same concentrations as the medium and does not constitute part of the tissue proper.

Determination of amino acids.

The methods used for determination of amino acids and activity of glutamic decarboxylase are described in the section on methods of Chapter VIII.

Presentation of Results.

In all tables the reported figures represent averages ± standard deviations when 5 or more determinations were done, ± widest deviations in other cases. The numbers of determinations made are given in parentheses.

Chapter III

Control Experiments on Cold Treatment of Brain Slices.

As our purpose in undertaking this research was to study the influence of low external potassium concentrations on metabolism and also the relation of potassium accumulation in brain slices to metabolic activity, it appeared necessary to use, as an adequate control, an experimental system (tissue and incubation medium) which would be as completely free of potassium as possible.

A preliminary experiment showed that the respiratory activity of fresh brain cortex slices incubated in a potassium-free medium is not significantly different from that of slices incubated in the standard medium, containing the usual small amount of potassium. This fact may be explained by the following observations.

Brain cortex tissue contains a relatively large amount of potassium (105 microequivalents per gram of fresh tissue). During the first minutes of an incubation at 38°C in a Ringer phosphate saline medium and under the optimal metabolic conditions (100% oxygen atmosphere and 10 mMolar glucose used as substrate of oxidation), brain slices lose more than 60% of their potassium content to the medium (106). Similar losses of potassium have been shown in the case of other tissues incubated under good metabolic conditions (resting nerves (348),

kidney cortex slices (422), liver slices (128), etc.). For obvious reasons, this loss is even greater when the tissue is incubated in a medium containing no potassium. The amount of potassium lost into the medium is sufficient to produce a concentration in the medium high enough to influence the metabolism of the brain slices, as will be shown in the next chapter. Hence, for the purposes of our study, the necessity of using potassium-depleted brain tissues became apparent.

Pappius and Elliott have shown that an anaerobic incubation of one hour at 38° causes an almost complete depletion of the potassium content of brain slices (311). However this causes appreciable damage to subsequent respiration (116).

A preliminary incubation at a low temperature in a potassium-free medium appeared to be a convenient pretreament to deplete the potassium content of brain slices without seriously impairing their metabolic capacities. A similar technique has been previously used in studies on red blood cells (208) and on kidney cortex slices (133) but not, as far as we know, in the case of brain. McIlwain (266) has used long periods of storage in the cold (from 5 to 17 hours) as an adverse condition blocking the response of brain slices to electrical stimulation. Leaf (239) has studied the changes in potassium, sodium and chloride occurring in tissues incubated at 0°C, but not the effects on metabolism or electrolytes of subsequent incubations at 38°C.

Control experiments were designed to characterize the effects of this cold pretreatment on brain slices. The time course of potassium depletion and sodium accumulation was

determined. The preservation of the normal respiratory capacity of brain slices after the period of incubation at low temperature was also examined. The results of these control experiments are reported in this chapter.

METHODS

The methods used for tissue preparation, incubations in the cold and at 38°C, oxygen uptake determination, potassium and sodium analysis, were described in the previous chapter. The medium used was a phosphate-buffered calcium-free saline containing 10 mMolar glucose and 0 or 3.6 mMolar K. When the time course of potassium reaccumulation at 38°C was studied, 0.3 ml. of a medium containing an amount of potassium sufficient to bring the final concentration to 3.6 mMolar was tipped into the main compartment at zero time and the slices were isolated for analysis after various periods of time. In other cases the slices were directly incubated in the potassium-containing saline media.

RESULTS

The results presented in Table 1 show no difference between the oxygen uptake rate of fresh brain cortex slices incubated in a K-free medium, and the respiration rate of slices in media containing the usual concentration of potassium (3.6 mMolar). Brain slices incubated at 0°C, for a period of one hour, in a K-free medium containing 10 mMolar glucose and in presence of 100% oxygen, then reincubated at 38°C in the same medium,

Table 1

Influence of an incubation in the cold on the subsequent respiration and potassium uptake of brain cortex slices.

Pre-incubation at 0°C	Incubation at 38°C Potassium added to medium (mM)*	Oxygen uptake µmoles per gram fr. tissue per hour	Potassium level in tissue after one hour µequivalents per gram fr. tissue
No cold treatment	3.6	122 ± 18 (3)	50 ± 4 (3)
	0	120 ± 11 (6)	30 ± 2 (12)
Cold treatment and slices kept in the same medium for incubation at 38°C	0	110 ± 2 (2)	29 ± 2 (2)
	3.6	112 ± 6 (3)	36 ± 2 (2)
Cold treatment and slices transferred into new medium for incubation at 38°C	3.6	91 ± 11 (20)	29 ± 1 (30)

* Does not include the amount of potassium that leaked from the tissue into the medium.

consume oxygen at a slightly lower rate than the controls (without cold treatment), but the difference does not appear to be statistically significant. Under the same conditions, if potassium is added to the medium increasing the concentration by 3.6 mMolar for the incubation at 38°C, again no difference in the oxygen uptake rate is apparent.

After the incubation for one hour at 38°C, the tissue potassium content is identical whether (a) the slices have been directly incubated at 38°C in a K-free medium or (b) pretreated in the cold in a K-free medium and left in the same medium for the following incubation at 38°C or (c) pretreated in the cold (in a K-free medium) and replaced in a new medium containing 3.6 mMolar K for the incubation at 38°C.

Figure 1 shows the time course of potassium depletion of brain slices incubated at 0°C for a period of one hour. The potassium content of the tissue falls very rapidly from 105 microequivalents per gram of fresh tissue to 25 microequivalents in the first 20 minutes of the cold incubation. The fall is much slower (from 25 to 10 microequivalents per gram) during the next 40 minutes in the cold.

Figure 2 shows the time course of sodium accumulation in the slices during the cold treatment. The sodium content passes from about 50 microequivalents per gram of fresh tissue to about 160 microequivalents during the period of incubation in the cold.

The series of curves shown in Figure 3 permits comparison of the respiratory activity of brain slices incubated under four different conditions: (a) fresh slices have been directly

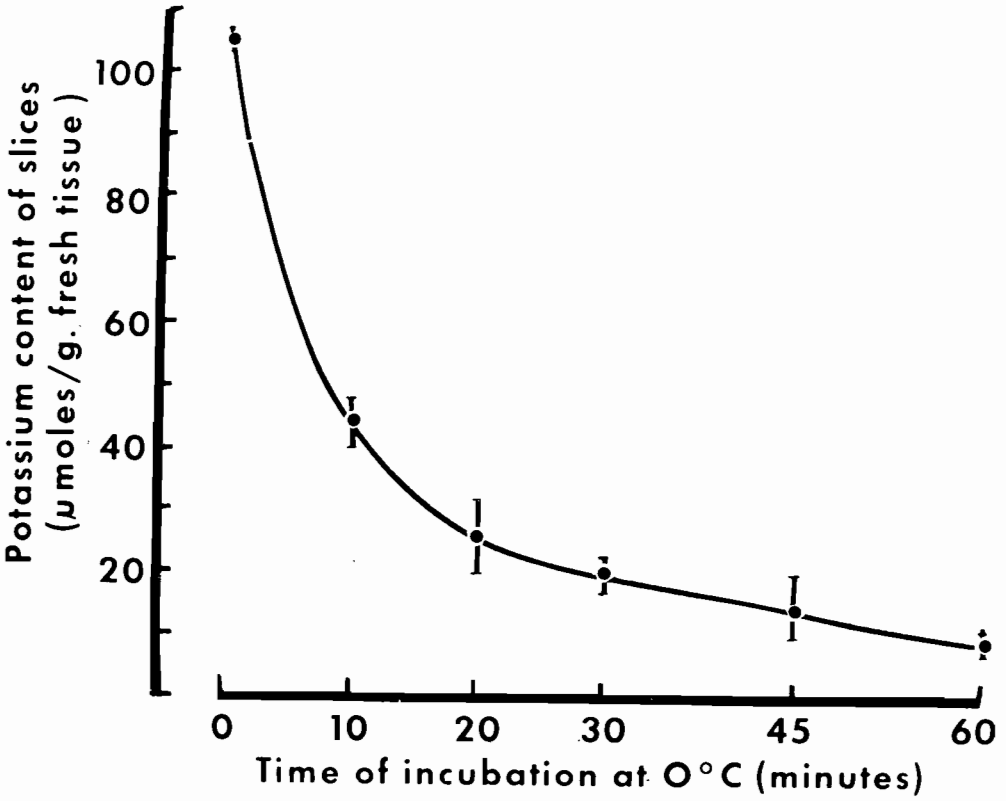

Figure 1. Time course of potassium depletion of brain slices incubated in a K-free saline medium at 0°C.

Each point is the average of 6 or more determinations and the vertical brackets indicate the standard deviations.

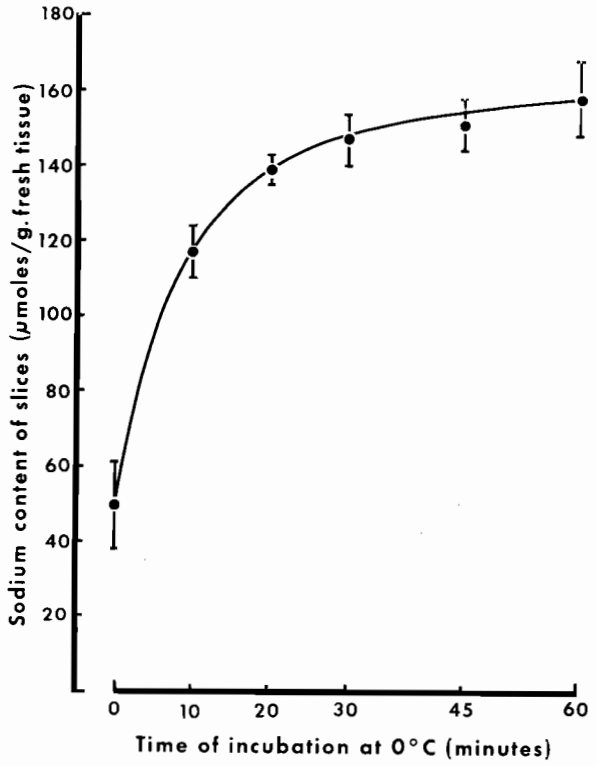

Figure 2. Time course of sodium accumulation in brain slices incubated at 0°C in a K-free saline medium containing 161 mMolar Na.

Each point is the average of 6 or more determinations and the vertical brackets indicate the standard deviations.

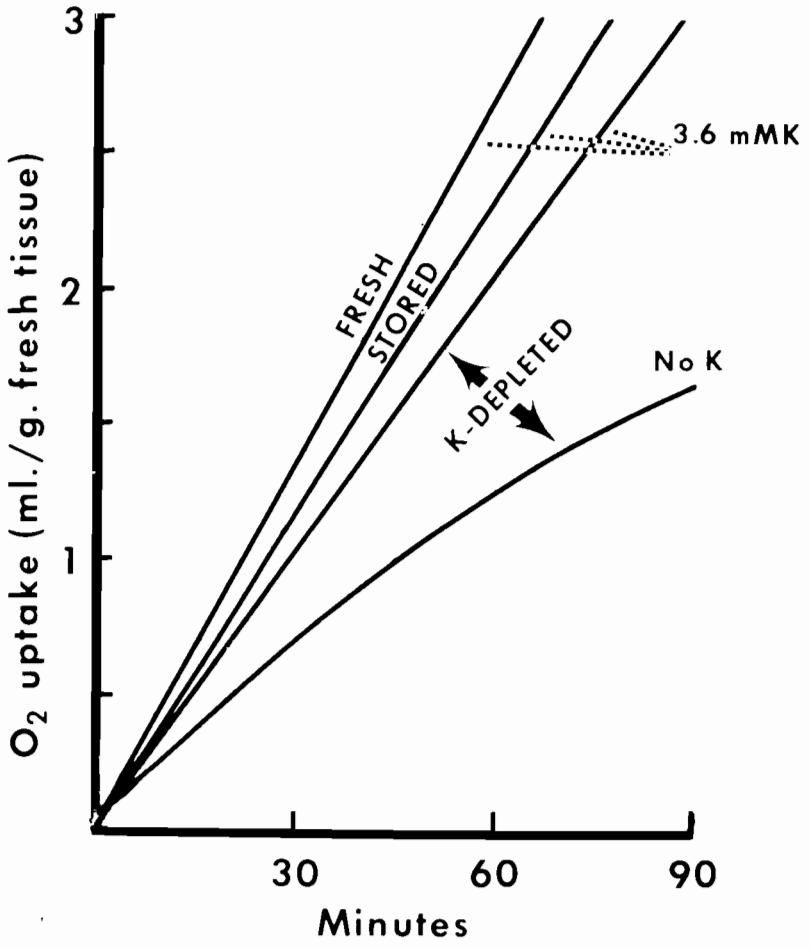

Figure 3. Influence of storage or incubation at 0°C on subsequent O2 uptake at 38°C.

brain slices incubated in the absence of K

incubated at 38°C in a medium containing the usual concentration of K (3.6 mMolar); (b) slices have been kept during 60 minutes at 0°C in humid air without medium, then placed in a medium containing 3.6 mMolar K at 38°C; (c) and (d) slices were kept at 0°C in oxygenated potassium-free medium for 60 minutes then incubated at 38°C in a medium containing 3.6 mMolar K (c), or in a potassium-free medium (d). Curves (a) and (b), representing the oxygen uptake rates of brain slices incubated in presence of K (after no treatment or a pretreatment in the cold in such a way that the tissue kept its normal K content), indicate a constant and normal respiratory activity under these different conditions. The last curve shows the low and falling respiratory rate of K-depleted brain slices kept in a medium where potassium is lacking. On the other hand, K-depleted slices which are replaced in presence of 3.6 mMolar K at 38°C (c) respire at a constant rate which is close to normal.

The experiment illustrated in Figure 4 was done to determine to what extent the falling respiratory rate of K-depleted brain slices incubated in the absence of K can be reactivated and rendered constant. Respiration of pretreated brain slices incubated in a K-free saline in which K was tipped to bring the concentration to 3.6 mMolar after 20 or 60 minutes of incubation at 38°C was compared to respiration of pretreated brain slices immediately replaced in a 3.6 mMolar K saline medium after the cold incubation. It can be seen that, even after 60 minutes in the absence of potassium at 38°C, addition of

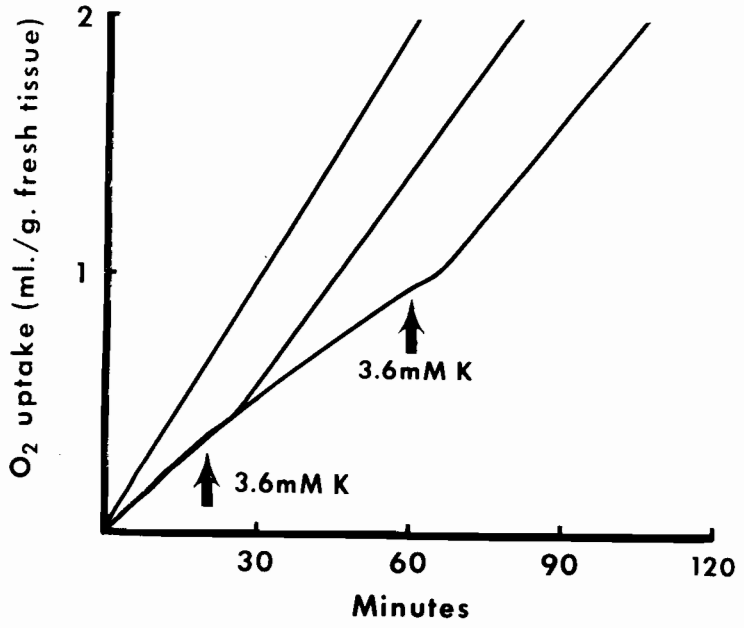

Figure 4. Effect of 3.6 mMolar K on the O2 uptake of K-depleted slices incubated for 20 or 60 minutes in a K-free saline medium.

the metabolic capacities of the tissue.

potassium to the incubation medium renders the oxygen uptake rate constant and stimulates it. However a slow irreversible process seems to take place with time which blocks the full reactivation of the respiratory activity by potassium.

DISCUSSION

Direct incubation of fresh brain cortex slices in a potassium-free medium does not provide an adequate control for the study of the influence of this ion on brain metabolism. The amount of cellular potassium leaking into the medium under the optimal metabolic conditions at 38°C is sufficient to influence the metabolic activity of brain slices. (The amount of potassium contained in a brain cortex slice weighing 100 mg. is equal to 10.5 microequivalents and is sufficient to cause an increase of concentration of about 2 mMolar in 3 ml. of incubation medium even if only 50% of the tissue content leaks into the saline medium).

The levels of potassium content found in slices either directly incubated in K-free medium, or K-depleted in the cold and replaced in a new medium containing 3.6 mMolar K for the incubation at 38°C, are approximately the same. The oxygen uptake rates of slices submitted to these different treatments are also equal or not statistically different.

It was concluded that K-depleted brain slices should be used for the purposes of our study, since an incubation of 60 minutes in the cold in an oxygenated potassium-free medium containing 10 mMolar glucose depletes the brain slices of their potassium content almost completely without seriously impairing the metabolic capacities of the tissue.

After such pretreatment the oxygen uptake rate is constant and close to normal, provided that the brain slices are replaced in presence of the usual concentration of potassium. (Krebs (232) and McIlwain (266) have shown that a storage of 4 hours in the cold does not cause significant impairment of the metabolic activity of brain slices. However the medium used for cold storage contained the usual small amount of potassium, at least in the case of Krebs' conditions).

On the other hand, if the tissue is replaced, after the cold treatment, in a potassium-free saline medium, its respiratory activity is reduced by more than 50% at the end of one hour at 38°C and is falling rapidly. After 60 minutes in this condition, the respiratory rate can still be activated and kept constant, by addition of a small amount of potassium. However, in this extreme condition, a part of the respiratory capacity of the tissue has apparently been irreversibly lost.

Two time constants seem to apply to the curve representing the time course of potassium depletion of brain slices incubated in the cold. A fraction of the potassium content is very easily released into the medium (even at 38°C, in presence of oxygen, glucose and 3.6 mMolar potassium; see Table 1). This fraction probably exists in a free or loosely bound form in the brain tissue. Another fraction requires a prolonged incubation either in the cold or under anaerobic conditions to be released. This indicates that this second fraction is bound or situated in a better protected or more remote part of the tissue. This second fraction may be calculated from the curve to be about 30 to 35 microequivalents per gram of tissue (about 30% of the total tissue content).

Folch reported that about 27% of the total potassium content of brain is combined to lipids (129). Stone (381) and Bergen (30) have found by ultrafiltration technics that between 25 and 28% of the brain potassium is non-diffusible. Holland and Auditore (194) have reported that 30% of the total potassium is present in the mitochondrial fraction of brain homogenates. These figures may or may not refer to the same fraction of the brain potassium but they are surprisingly concordant.

The curve representing the time course of sodium accumulation in the cold shows that the potassium lost by the tissue into the medium is replaced by an equivalent amount of sodium.

Chapter IV

Effects of Potassium, Sodium, Calcium and Hypertonic Conditions on the Metabolism of Brain Slices.

Several studies related to the effects of high concentrations of potassium on the respiration and glycolysis of brain slices have been reviewed in Chapter I. Practically no information is available regarding the effects of the low concentration of potassium that is normally present in extracellular fluids and in standard saline media nor of moderate changes in this concentration.

After description of the classical potassium effect, Ashford and Dixon (13) wrote: "It would appear probable that at the concentrations of normal Ringer, potassium does not exert this effect". Gore and McIlwain (156) reported that: "absence of potassium salts from the medium had no marked effects on the rate of respiration or glycolysis". Dickens and Greville (98) found that when all cations except sodium are left out of the usual medium the anaerobic glycolysis is decreased and the aerobic glycolysis is markedly increased. They did not study the effects on glycolysis of each ion separetely. Their study on respiration was more complete but in this case again they neverused media from which potassium alone was omitted. One of their conclusions was: "The action of the Ca in keeping the respiration small is not overcome by low concentration of K in the Ringer solution but it can be overcome by higher concentrations."

Contrary to Dickens and Greville who obtained the effect only when large amounts of potassium chloride were added to isotonic medium, Canzanelli et al. (56) demonstrated that stimulation of respiration occur also in isotonic solution, and Dixon (100) obtained the classical effects on glycolysis in similar conditions and at concentrations of about 40 mMolar potassium.

Many authors (56,98,385,395) have shown that the effects of high potassium concentrations on respiration and glycolysis are not obtained in the absence of sodium. Pappius et al. (313) reported that complete replacement of sodium by potassium slightly decreases the oxygen uptake and increases the rate of anaerobic glycolysis. Other workers (385,386) reported that sodium-free media do not change the respiratory rate but increase the aerobic lactic acid formation.

Various works reporting antagonistic effects of calcium and potassium on metabolic rates of brain slices were reviewed in Chapter I. Omission of calcium from saline media results in a large increase of the oxygen uptake (13,48,56,98,156,232). A similar effect on the anaerobic glycolysis has also been reported (98,156). Commenting on these results, Quastel (323) suggested that: "the stimulating effect is due to increase in the K/Ca ratio rather than to the absolute magnitude of the potassium ion concentration."

Ashford and Dixon (13) had found that omission of calcium from the normal medium reduced very slightly if at all the rates of anaerobic glycolysis. In opposition to this finding, Quastel

and coworkers (3,323) have reported that small amounts of calcium (1 mMolar) markedly increase the anaerobic formation of lactic acid while 4 mMolar potassium reduces this rate (323).

The experiments reported in this chapter were undertaken to determine as precisely as possible the metabolic effects of low potassium concentration and also to assess the influence of calcium, sodium and hypertonicity in relation to the metabolic effects of potassium. A source of confusion, the diffusion into the medium of part of the cellular potassium and the consequent increase of the external concentration, was eliminated by conducting our experiments with potassium-depleted tissues. In order to make sure that the metabolic events reported were specific to brain, other tissues (kidney cortex and liver slices) were also subjected to cold pretreatment and their metabolic activity studied in similar conditions.

METHODS

The methods used for tissue preparation, incubations in the cold and at 38°C, determinations of the oxygen uptake rates or carbon dioxide evolution or lactic acid formation were described in Chapter II. The media used for experiments on oxygen uptake or lactic acid formation (phosphate-buffered saline) or on anaerobic glycolysis (bicarbonate-buffered saline) were also previously described. The sum of sodium plus potassium concentrations was maintained at 161 mMolar in the phosphate-buffered media and at 151 mMolar in the bicarbonate-buffered media. When the potassium concentration was augmented

the sodium content of the media was proportionally decreased. Hypertonic conditions were obtained by adding either an extra 100 mMolar NaCl or 200 mMolar sucrose to the standard media. Glucose, 10 mMolar, was present in the media for all experiments reported in this chapter.

Sodium-free media or media of low sodium content were used in experiments reported in Table 3 and Figure 7. They were prepared by replacing sodium chloride by sucrose and the phosphate buffer by Tris-HCl. The slices were preincubated at 0°C in media containing zero or 36 mMolar sodium and then, at 38°C, in similar saline media. The potassium concentration was increased at the expense of sucrose, so that the isotonicity of the media was preserved.

RESULTS and DISCUSSION

1. Ionic and Osmotic Factors Affecting Respiration.

Figure 5 illustrates the effect of potassium concentration on the rate of oxygen uptake in the absence of calcium. The respiratory rate is continuously increased by augmenting the potassium concentration from 0 to 20 mMolar. The maximal respiratory activity occurs at approximately 60 mMolar K and further replacement of sodium by potassium gradually decreases the stimulatory effect of potassium. Addition of 100 mMolar NaCl markedly depresses the respiration in the absence of potassium but protects the stimulation due to potassium at concentrations greater than 60 mMolar. This protection is evidently due to the provision of sodium ions since, as Table 2 shows, the addition of 200 mMolar sucrose exerts no significant effect.

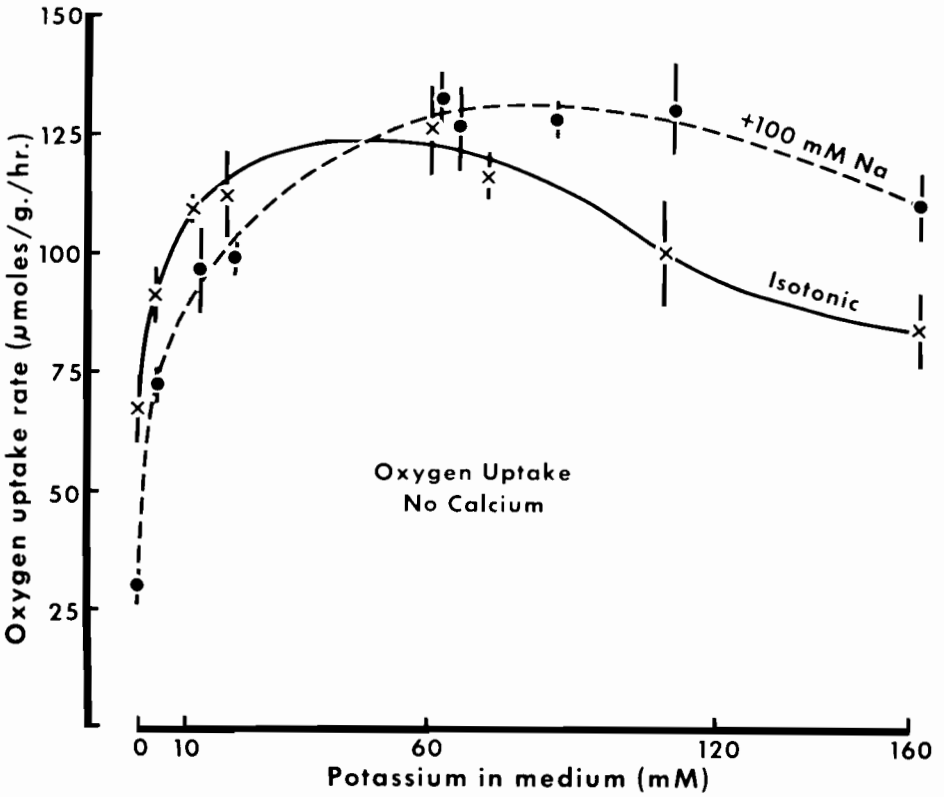

Figure 5. Effect of potassium concentration, in the absence of calcium, on the O_2 uptake rate of K-depleted brain slices.

Each point is the average of 6 or more determinations and the vertical brackets indicate the standard deviations.

Comparison of Figures 5 and 6 illustrates the effect of calcium (1.7 mMolar). Calcium exerts an inhibitory effect on respiration under all conditions but does not obviously influence the effects due to potassium. Addition of 100 mMolar NaCl has the same effect as in the absence of calcium. Table 2 shows that addition of sucrose (200 mMolar) unlike sodium does not counteract the depression that occurs when 160 mMolar potassium replaces most of the sodium in the medium. Results presented in Table 2 show that increasing the calcium concentration from 1.7 to 3.4 mMolar provokes no change in the rate of oxygen uptake at any concentration of potassium.

Figure 7 illustrates the effects of potassium concentration on the respiratory activity of brain slices which have been incubated at 0°C and then at 38°C in the absence of sodium or in presence of relatively small amounts of this ion. The results indicate that in the absence of sodium, the stimulating effect of low potassium concentrations is completely abolished and the effect of 60 mMolar potassium is markedly diminished. In presence of 36 mMolar Na the stimulating effects of potassium are close to those obtained under normal conditions. The mean values, standard deviations and number of determinations are reported in Table 3.

The experimental data reviewed suggest the following conclusions. Potassium at all concentrations and under all conditions examined (calcium and hypertonic conditions present or not) markedly stimulates the respiration of brain slices provided that a minimum amount of sodium is present in the saline medium.

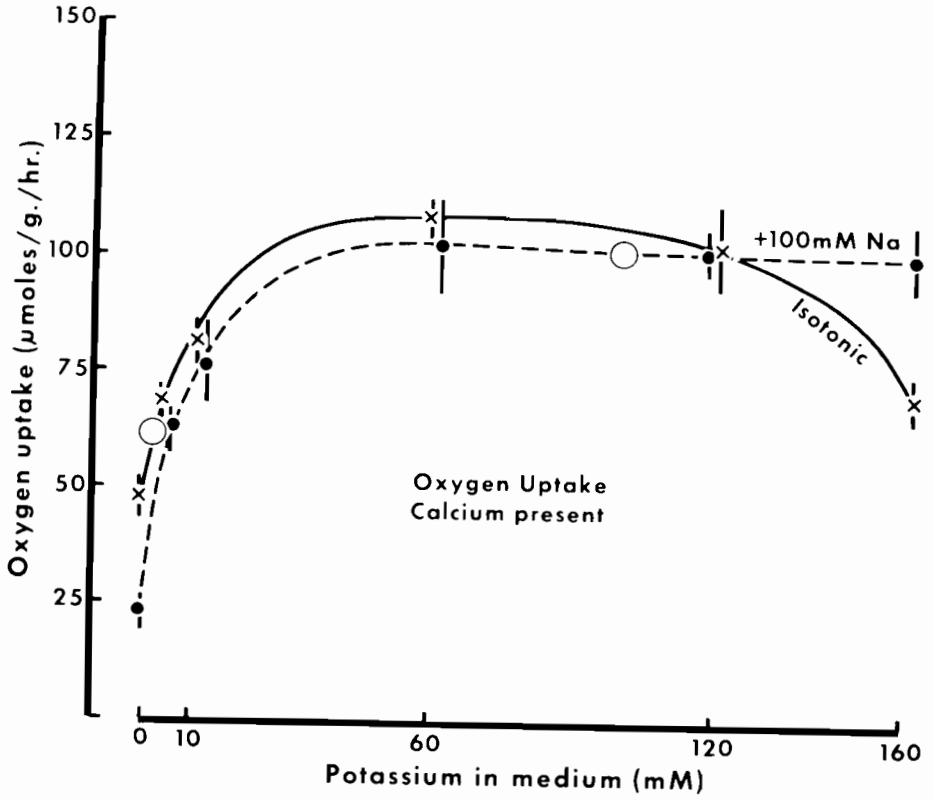

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Figure 6. Effect of potassium concentration, in the presence of 1.7 mMolar calcium, on the O_2 uptake rate of K-depleted brain slices.

The conditions of the classical stimulating effect of potassium are shown by the two large circles.

Each point is the average of 6 or more determinations and the vertical brackets indicate the standard deviations.

Table 2

Influence of hypertonicity in relation to the effects of potassium and of calcium on the rate of oxygen uptake of brain slices.

Rates expressed as μmoles of O_2 consumed / g. fresh tissue / hour.

Potassium concentration (mMolar)	0			3.6			60			160		
Calcium concentration (mMolar)	0	1.7	3.4	0	1.7	3.4	0	1.7	3.4	0	1.7	3.4
Isotonic medium	67 ±7 (24)	47 ±4 (5)	41 ±3 (6)	91 ±6 (20)	66 ±4 (5)	65 ±6 (12)	126 ±9 (17)	108 ±4 (6)	94 ±9 (7)	84 ±8 (10)	70 ±5 (6)	74 ±9 (6)
Medium made hypertonic with NaCl (100 mMolar)	30 ±4 (6)	23 ±4 (5)	21 ±2 (6)	72	62 ±5 (3)		132 ±6 (6)	102 ±10 (4)	96 ±9 (6)	110 ±7 (7)	100 ±7 (6)	97 ±10 (6)
Medium made hypertonic with sucrose (200 mMolar)							114 ±7	104 ±4		90 ±8	76 ±6	

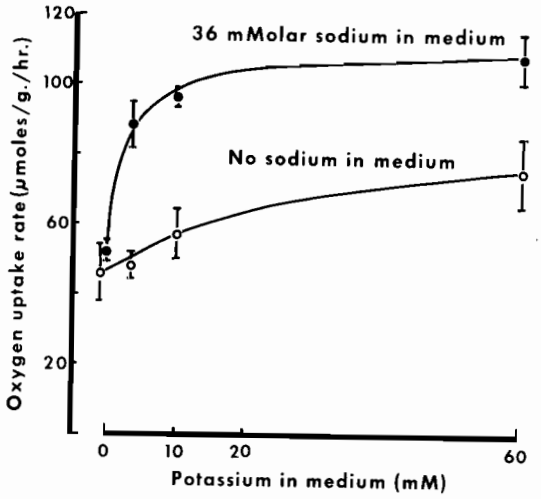

Figure 7. Influence of sodium in relation to the effect of potassium concentration on the O_2 uptake rate of brain slices.

Conditions and number of determinations are given in Table 3.

Table 3

Influence of sodium in relation to the effect of potassium on the rate of oxygen uptake of brain slices.

Rates expressed as μmoles of O_2 consumed / g. fresh tissue / hour.

Sodium content of media (mMolar)		Potassium content of media of incubation at 38°C (mMolar)			
Pre-incubation at 0°C	Incubation at 38°C	0	3.6	10	60
0	0	46 ±8 (5)	48 ±4 (9)	57 ±7 (4)	75 ±10 (5)
36	36	52 ±3 (4)	88 ±7 (3)	96 ±1 (2)	108 ±7 (4)

Calcium inhibits the respiration under all conditions but has no influence on the stimulating effect of potassium. In Figures 8 and 9 the absolute and the relative increases of oxygen uptake rate in the presence and absence of calcium are plotted against the potassium concentration of the saline media. It can be seen that the absolute increases over the control values depend on the potassium concentration and not on the presence or absence of calcium. Actually the relative increases due to potassium are greater in the presence of calcium than in its absence. The view that it is the K/Ca ratio that is effective thus cannot be supported. (The fact that a calcium concentration of 3.4 mMolar (Table 2) did not give a different effect would also be against the ratio hypothesis. However the effect of calcium in the presence of phosphate was probably limited by solubility rather than by the amount of calcium added. Lower concentrations were not tested because the solubility behaviour of calcium phosphates is too complicated to allow simple conclusions from variations in the amount of calcium added).

2. Ionic Factors Affecting the Rates of Aerobic Glycolysis.

Figure 10 illustrates the changes occurring in the rates of aerobic glycolysis when the potassium concentration of the saline medium is increased at the expense of the sodium content, in the absence of calcium. Addition to the medium of small amounts of potassium reduces markedly the very high rates of glycolysis obtained in the absence of this ion. This inhibition is progressively reversed when the potassium concentration is

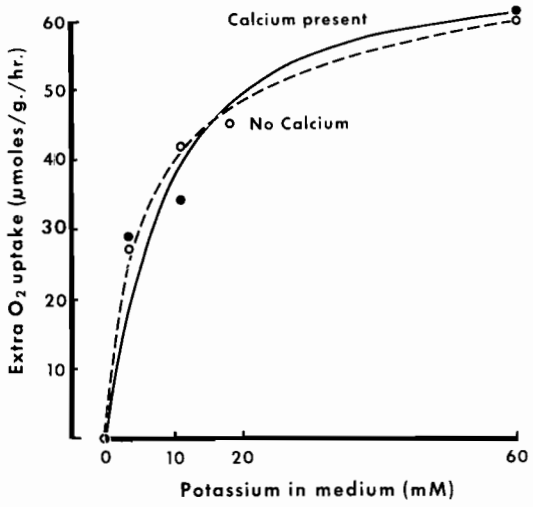

Figure 8. Influence of calcium (1.7 mMolar) on the absolute increase of the O2 uptake rate caused by potassium.

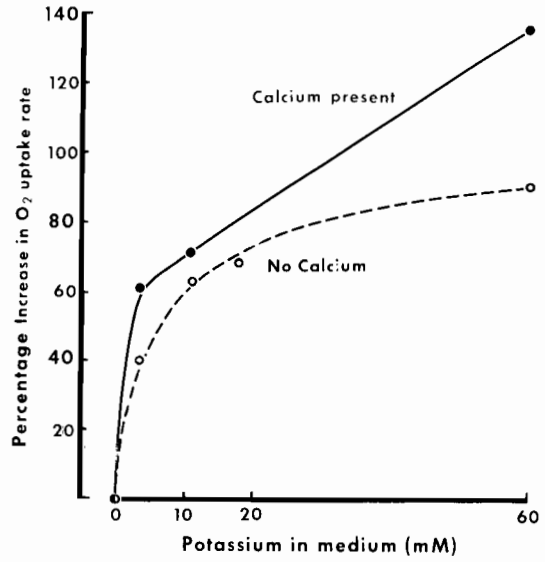

Figure 9. Influence of calcium (1.7 mMolar) on the relative increase of the O2 uptake rate caused by potassium.

Percentage increase calculated on the basis of the O2 uptake rate obtained in K-free conditions.

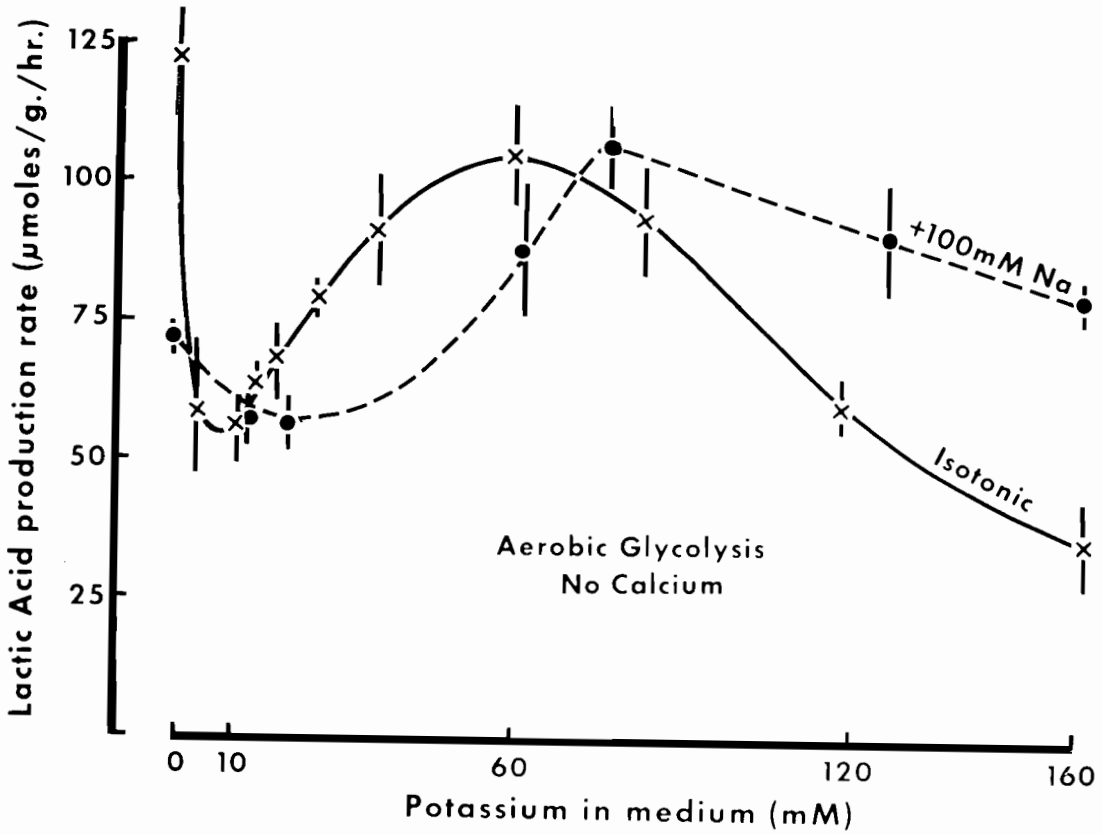

Figure 10. Effect of potassium concentration, in the absence of calcium, on the rate of aerobic glycolysis of K-depleted brain slices.

Each point is the average of 6 or more determinations and the vertical brackets indicate the standard deviations.

increased from 20 to about 60 mMolar. Further replacement of sodium by potassium gradually decreases the high rates of glucose breakdown. Addition of 100 mMolar sodium chloride markedly depresses the glycolytic activity in the absence of potassium but does not block the stimulating effect of potassium and the added sodium maintains the stimulated rates at their high level when the K concentration is augmented up to 160 mMolar.

Comparison of Figures 11 and 10 illustrates the effects of 1.7 mMolar calcium. It can be seen that calcium decreases the glycolytic rates at all potassium concentrations but more markedly in the absence than at high concentrations of potassium. On the whole, the effects of potassium are not changed by calcium. Addition of 100 mMolar NaCl greatly decreases the high aerobic activity usually obtained in the absence of potassium and reduces to some extent the magnitude of the stimulatory effect obtained at 60 mMolar K. The conditions of the classical stimulating effect of potassium are shown by the two large circles.

The aerobic glycolysis is either inhibited or activated depending on the level of concentration of potassium in the saline media. This and the effects on the rates of anaerobic glycolysis (see later) show clearly that the metabolic influence of high concentrations of potassium is completely different from that of the low concentrations of potassium. The activating effect of potassium on the aerobic glycolysis, unlike the inhibitory effect on anaerobic glycolysis (see later), is obtained whether

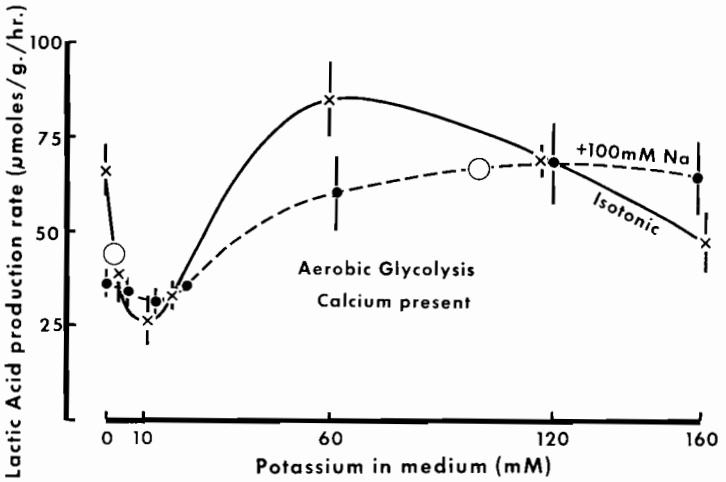

Figure 11. Effect of potassium concentration, in the presence of 1.7 mMolar calcium, on the rate of aerobic glycolysis of K-depleted brain slices.

The conditions of the classical stimulating effect of potassium are shown by the two large circles.

Each point is the average of 6 or more determinations and the vertical brackets indicate the standard deviations.

or not calcium or hypertonic conditions are present. The only requirement is the presence of high concentrations of sodium (maximal at 100 mMolar). Accordingly, it may be concluded that this stimulation is a primary effect of potassium as such.

Calcium does not interfere with the potassium effects but it exerts an inhibitory effect on glycolysis under practically all conditions. The only striking effect of hypertonic conditions is to reduce or abolish (in presence of calcium) the high rates of glycolysis obtained in the absence of potassium.

It should be noted that the effects of low concentrations of potassium on aerobic glycolysis (inhibition) are the inverse of the effects on respiration. This suggests that the two phenomena are intimately related. As it was shown by Von Korff (409) that potassium stimulates the formation of acetyl-coenzyme A, it is tempting to suggest that the fall in the rates of formation of lactic acid observed at low K concentrations is the result of greater rate of pyruvate oxidation.

The effects of high K concentration are, however, more difficult to explain since both the oxygen uptake and the lactic acid formation rates are increased under this condition. This problem will be discussed further in the following chapters.

3. Ionic and Osmotic Factors Affecting the Rates of Anaerobic Glycolysis.

Figure 12 illustrates the changes occurring in the rates of anaerobic glycolysis when, in the absence of calcium, the potassium concentration of the saline medium is increased at the expense

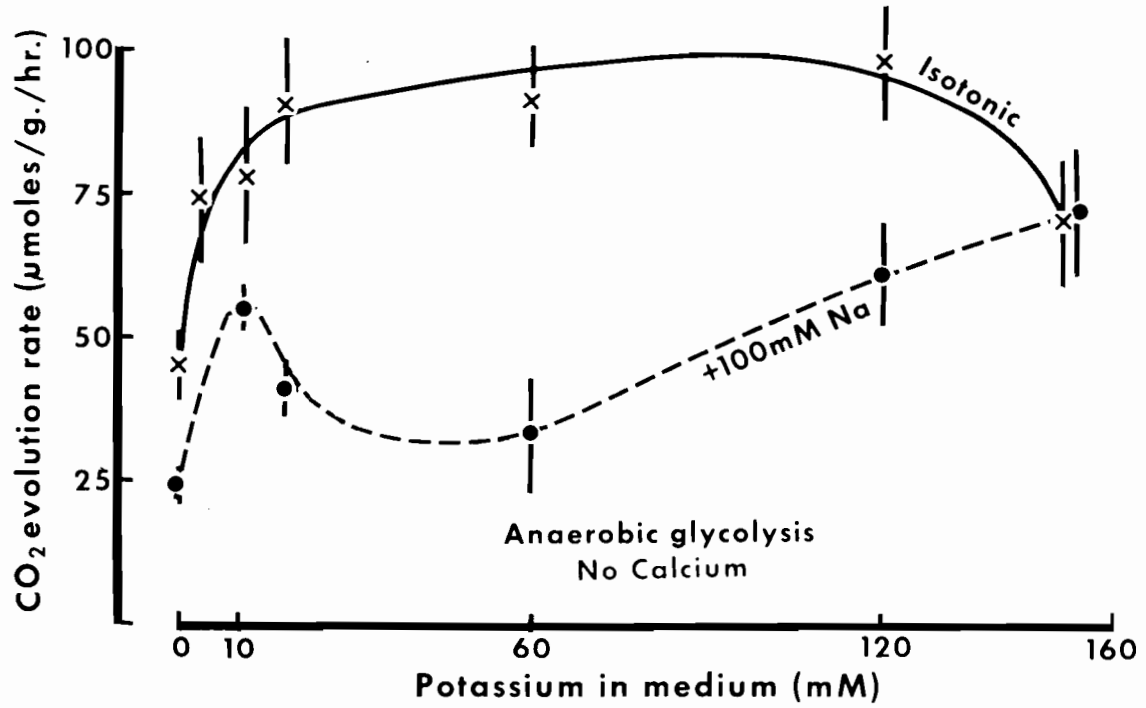

Figure 12. Effect of potassium concentration, in the absence of calcium, on the rate of anaerobic glycolysis of K-depleted brain slices.

Each point is the average of 6 or more determinations and the vertical brackets indicate the standard deviations.

of the sodium content. At all concentrations, from 3 to 150 mMolar, potassium markedly stimulates the glycolytic rates. The stimulating effect is reduced to some extent when sodium has been replaced by so much potassium that only 40 mMolar or less sodium remains.

The broken line of Figure 12 illustrates changes occurring in similar conditions except that the medium was made hypertonic by the addition of 100 mMolar NaCl. This addition causes a depression of the glycolytic rates between 0 and 10 mMolar potassium, and blocks the stimulating effect of potassium between 18 and 60 mMolar K. At higher concentrations of potassium this blocking effect is gradually removed and it is completely reversed at 150 mMolar K.

Table 4 shows that rendering the medium hypertonic by 200 mMolar sucrose instead of 100 mMolar NaCl, causes a similar inhibition of glycolysis at medium potassium concentration. Contrary to sodium, sucrose does not depress the activity below 10 mMolar K and the increased osmolarity causes a markedly increased glycolytic rate at 160 mMolar K when the medium contains almost no sodium.

When compared to Figure 12, Figure 13 illustrates the effects of calcium, 1.7 mMolar. This ion brings no obvious changes between 0 and 18 mMolar K, but above that concentration it completely blocks the stimulating effect of potassium.

This blocking effect is progressively reversed when the concentrations of potassium are increased over 100 mMolar, the sodium content of the medium being simultaneously reduced.

Table 4

Influence of hypertonicity in relation to the effects of potassium and of calcium on the rate of anaerobic glycolysis of brain slices.

Rates expressed as μmoles of CO_2 evolution / g. fresh tissue / hour.

Potassium concentration (mMolar)	0		10		18		60		120		160	
Calcium concentration (mMolar)	0	1.7	0	1.7	0	1.7	0	1.7	0	1.7	0	1.7
Isotonic medium	45 ±6 (11)	38 ±8 (14)	78 ±13 (9)	89 ±5 (3)	91 ±13 (11)	91 ±12 (6)	92 ±9 (14)	42 ±12 (4)	98 ±10 (11)	49 ±6 (2)	70 ±11 (13)	77 ±10 (4)
Medium made hypertonic with NaCl (100 mMolar)	24 ±2 (6)	23 ±6 (5)	55 ±4 (6)	67 ±9 (6)	41 ±3 (6)	52 ±10 (6)	33 ±10 (9)	25 ±8 (5)	61 ±10 (7)	15 ±2 (6)	72 ±15 (10)	16 ±4 (6)
Medium made hypertonic with sucrose (200 mMolar)	53 ±10 (3)	32 ±4 (2)	83 ±10 (5)	85 ±6 (2)			56 ±9 (6)	28 ±3 (5)			158 ±13 (7)	87 ±12 (5)

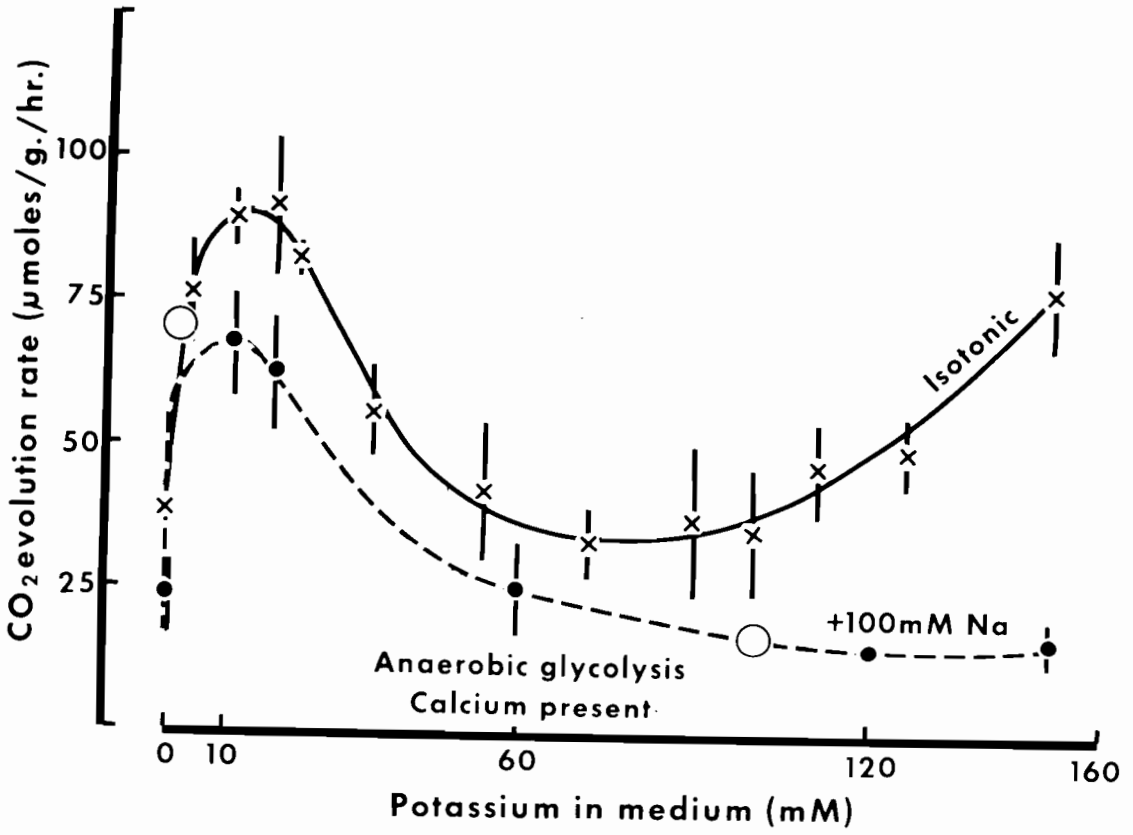

Figure 13. Effect of potassium concentration, in the presence of 1.7 mMolar calcium, on the rate of anaerobic glycolysis of K-depleted brain slices.

The conditions of the classical inhibitory effect of potassium are shown by the two large circles.

Each point is the average of 6 or more determinations and the vertical brackets indicate the standard deviations.

However in presence of an extra amount of sodium (100 mMolar NaCl added), the suppression of glycolysis is maintained as the concentration of K is increased to 150 mMolar. Table 4 shows that addition of 200 mMolar sucrose does not appreciably influence the glycolytic rates with zero to 60 mMolar potassium when calcium is present in the saline medium, but at 150 mMolar potassium, when there is very little sodium, a high rate of glycolysis occurs.

The experimental data described support the following conclusions. Potassium markedly stimulates the rates of anaerobic glycolysis. This stimulating effect increases linearly from 0 to 20 mMolar K, where it reaches a plateau. The fall of activity occurring at a concentration of potassium greater than 120 mMolar is very probably due to the lack of a minimum amount of sodium (about 50 mMolar) required for the stimulating effect of K to occur. These facts are in accord with the findings of Muntz and others on brain homogenates (297). They will be discussed further in Chapter V.

The low glycolytic activity obtained under certain conditions at high K concentrations and which is commonly referred to in the literature as an inhibitory effect of potassium is illustrated by the two large circles in Figure 13. This classical effect appears only if the following conditions are fulfilled: (1) presence of a potassium concentration greater than 20 mMolar (optimal at 60 mMolar), (2) presence of sodium (optimal at 100 mMolar), (3) presence of either small amounts of calcium or of hypertonic conditions (obtained here by addition of 100 mMolar NaCl or 200 mMolar sucrose). If only one of these three elements is lacking the effect does not appear.

This fact clearly shows that potassium alone is no more than sodium or calcium a determinant factor in this phenomenon, the conjunction of the three elements being required.

The fact that hypertonic conditions, as well as calcium, may provoke the inhibitory effect appears particularly significant. It suggests that reducing the intracellular water content increases the sensitivity of the glycolytic system (enzymes or control mechanisms) to the combined effect of sodium and potassium. This is not too surprising since the enzymes responsible for the breakdown of glucose are situated in the cytoplasm. Calcium also is known to influence the colloidal properties of the cytoplasm and its effect provokes changes ressembling a water loss (401).

The experimental data discussed here give no indication concerning the point of inhibition of the glycolytic activity and it is not possible to decide whether this phenomenon is caused by the direct inhibition of the activity of a specific enzyme or by an influence on other cellular mechanisms controlling the rate of glucose breakdown (ATP/ADP or DPN/DPNH ratios or glucose phosphorylation at a membrane site). This question will be discussed further in Chapters V and IX.

No results were obtained suggesting that calcium could have a stimulatory effect on anaerobic glycolysis. It appears that below 18 mMolar K or in absence of sodium calcium does not exert any inhibitory effect either. Hence, in the conditions described, calcium has no other effect than the reversal of the potassium stimulatory effect described above.

High concentrations of sodium inhibit the anaerobic glycolytic activity under all conditions (whether potassium or calcium are present or not). On the other hand, sodium appears to be necessary (at a concentration of about 50 mMolar) for the stimulatory effect of potassium to occur.

4. Influence of Potassium Concentration on the Metabolic Activity of Kidney and Liver Slices.

The results presented in Table 5 show the effects of different concentrations of potassium on the respiratory and glycolytic rates of kidney cortex and liver slices which had been submitted to a preincubation at $0^{o}C$ under conditions identical to the cold pretreatment of brain cortex slices. The potassium concentrations used were chosen by reference to the inflexion points of the curves relating the metabolic rates of brain cortex slices to the potassium content of the saline media.

Potassium exerts no effect on the oxygen uptake rates of kidney cortex slices while it slightly stimulates the respiration of liver slices. When potassium replaces all the sodium in the medium the respiration of kidney cortex slices is markedly inhibited while that of liver is reduced. Potassium inhibits the slight aerobic glycolysis of kidney and has no effect on that of liver slices. It has no significant effect on the anaerobic glycolysis of kidney slices. Sodium-free media inhibit both glycolytic rates of kidney.

Table 5

Effects of potassium concentration on the metabolic rates of cold-pretreated kidney cortex and liver slices.

Glucose, 10 mMolar, present in all cases.
Rates expressed as μmoles / gram of fresh tissue / hour.

Tissue studied	Metabolic rate measured	Potassium content of incubation media (mMolar)				
		0	3.6	10.8	60	160
Kidney cortex	O_2 uptake	129 ±6 (4)	139 ±5 (4)		126 ±9 (4)	79 ±1 (2)
	Aerobic lactic acid formation	14 ±3 (4)	11 ±1 (4)	4 ±1 (2)	4 ±2 (4)	- (3)
	Anaerobic lactic acid formation	16 ±2 (3)		20 ±2 (3)	25 ±6 (3)	8 ±0 (2)
Liver	O_2 uptake	43 ±8 (9)	56 ±6 (8)		69 ±11 (9)	47 ±12 (11)
	Aerobic lactic acid formation	9 ±3 (4)	14 ±2 (2)	9 ±1 (3)	9 ±1 (4)	6 ±2 (6)

These data suggest that the complex effects of potassium on the metabolic rates of brain slices described above are specific to this tissue. The need for sodium so apparent in brain appears also to some extent with kidney and liver.

Chapter V

Influence of Various Substrates on Respiration and Glycolysis in Relation to the Effects of Potassium.

Very few substrates are readily oxidized by brain (109). Glucose, pyruvate and lactate and, to a smaller extent, glutamate, are the major substrates of oxidation of brain slices. Gamma-aminobutyric acid was recently reported (393) to be also rapidly oxidized. The effects of these substrates and of a few intermediates of the tricarboxylic acid cycle on the oxygen uptake rate of cold-treated brain slices were examined in relation to the influence of potassium concentration.

As potassium is known to influence the activity of the enzyme responsible for the decarboxylation of malic acid to pyruvic acid (257,410), the changes brought by the presence of malate and pyruvate on the rate of aerobic glycolysis of pretreated brain slices were also studied in relation to the influence of potassium concentration. Malonate is known to increase further the potassium-stimulated aerobic formation of lactic acid (395). The effects of this substance on the aerobic glycolysis of pretreated slices were determined in the presence or absence of low concentrations of potassium.

The stimulating effect of catalytic amounts of pyruvate on anaerobic glycolysis has been known for a long time and it is particularly evident in the case of brain tissues (108,113). It was suggested (116) that pyruvate exerts this effect by favouring the oxidation of diphosphopyridine nucleotide. Increased levels of DPN would stimulate the activity of the glyceraldehyde dehydrogenase enzyme.

Krebs (231) demonstrated that glutamate markedly inhibits the anaerobic formation of lactic acid in brain slices and Weil-Malherbe (417), studying further this effect, found that this inhibition can be reversed by small concentrations of pyruvate. It was suggested (116) that this effect is secondary to a reduction by glutamate (271) of the concentration of high energy phosphate compounds necessary to initiate the glycolytic activity. The effects of these two compounds have now been studied in relation to the influence of potassium on the rates of anaerobic glycolysis.

These experiments were done in an attempt to clarify the points of interference of potassium on the oxidative and glycolytic metabolism of brain slices.

METHODS

The techniques and incubation media used for the determination of the rates of oxygen uptake, aerobic and anaerobic glycolysis have been described in Chapter II. The effects of pyruvate and malate on the lactic acid analysis were corrected for according to data given in Chapter II. When substances were added to the incubation media in the form of their sodium salt a corresponding amount of sodium was omitted from the saline media in order to keep the total sodium concentration constant.

The determinations of oxygen uptake reported in Table 7 were done on slices which had been incubated in the cold in a glucose-free medium. This was done with the purpose of comparing the effects of the various substrates on glucose-depleted tissues

with the effects reported in Table 6 which were obtained with tissues containing their endogenous amounts of glucose plus the amounts brought by diffusion into the tissue from the incubation medium during the cold pretreatment.

RESULTS

1. Substrates of Oxidation and the Effects of Potassium on the Oxygen Uptake Rates.

The effects of various substrates on the respiratory activity of brain slices incubated in presence of various potassium concentrations are shown in Table 6.

The endogenous respiration of pretreated brain slices is very low and is not activated by potassium. Malate and alpha-ketoglutarate are oxidized to a small extent by brain but potassium has no effect on the rates of respiration in their presence. In the absence of potassium gamma-aminobutyrate has no effect on the respiration but 3.6 mMolar potassium causes some increase of the oxygen uptake rate in presence of this substance and 10 mMolar K further increases the rate of this oxidation. In the absence of potassium glutamate has little effect but with small amounts of potassium it significantly increases the respiration rate.

In the absence of potassium, pyruvate is oxidized to a greater extent than glucose but not in presence of small amounts of K. However, when both glucose and pyruvate are added together the stimulatory effects of small concentrations of potassium are significantly increased. Addition of gamma-aminobutyrate with glucose slightly stimulates the oxygen

Table 6

Influence of various substrates of oxidation in relation to the effect of potassium on the oxygen uptake rate of brain slices.

Rates expressed as μmoles of oxygen consumed / g. fresh tissue / hour.

Substrates (*) (10 mMolar)	Potassium concentration of incubation medium (mMolar)			
	0	3.6	10.8	60
None	29 ± 6 (7)	32 ± 8 (10)	33 ± 5 (4)	31 ± 5 (13)
Glucose	67 ± 7 (24)	91 ± 6 (20)	109 ± 6 (5)	126 ± 9 (13)
γ-Aminobutyric acid	32 ± 6 (14)	44 ± 8 (18)	55 ± 5 (11)	54 ± 3 (8)
Glutamic acid	46 ± 9 (15)	66 ± 6 (13)	78 ± 12 (11)	87 ± 3 (6)
Pyruvic acid	83 ± 11 (6)	92 ± 6 (5)	109 ± 8 (5)	140 ± 7 (8)
Malic acid	50 (1)	53 (1)		47 ± 3 (2)
α-Ketoglutaric acid	38 ± 7 (3)	39 ± 8 (3)		41 ± 4 (3)
Glucose and γ-aminobutyric acid	77 ± 6 (10)	102 ± 9 (11)	121 ± 9 (4)	128 ± 9 (4)
Glucose and glutamic acid	66 ± 6 (16)	103 ± 7 (20)	118 ± 7 (5)	134 ± 3 (4)
Glucose and pyruvic acid	87 ± 5 (2)	105 ± 6 (5)	124 ± 7 (6)	142 ± 9 (5)
Glucose and malic acid	73 ± 0 (2)	104 ± 7 (5)		140 ± 6 (3)
Glucose and α-ketoglutaric acid	71 ± 3 (2)	101 ± 14 (4)		150 ± 9 (3)

* Glucose, 10 mMolar, was present in the medium used for the cold treatment.

uptake rate in the absence of potassium. In spite of the fact that the small rates of oxidation of malate and alpha-ketoglutarate are not stimulated by potassium the stimulatory influence of potassium on glucose oxidation is significantly increased when the intermediates of the tricarboxylic acid cycle are added with glucose. This increase in the stimulatory effect of high concentrations of potassium is not obtained when pyruvate, gamma-aminobyturate or glutamate are added with glucose.

The results presented so far were obtained with brain slices preincubated in the cold in a medium containing 10 mMolar glucose. In order to eliminate a possible interference due to the large amounts of glucose which had presumably diffused into the slices during the cold incubation, a second series of determinations were done using slices which had been preincubated in the cold in a glucose-free medium. The results are given in Table 7.

The respiratory rates are in general from 10 to 20 micromoles per gram per hour lower than in the previous series of determinations and in a few cases some effects which were not clear become apparent.

While the oxidation of glucose, pyruvic acid or gamma-aminobutyric acid is significantly increased by 3.6 mMolar potassium, a greater amount of potassium is required to stimulate the oxidation of glutamic acid.

When previously glucose-depleted slices respire in the presence of glucose, high concentrations of potassium do not stimulate oxygen uptake more than do lower concentrations. When such slices respire in the presence of pyruvate, stimulation continually increases with potassium concentration.

Table 7

Influence of various substrates of oxidation in relation to the effect of potassium on the oxygen uptake rate of brain slices.

Rates expressed as μmoles of oxygen consumed / g. fresh tissue / hour.

Substrates (*) (10 mMolar)	Potassium concentration of incubation medium (mMolar)			
	0	3.6	10.8	60
None	17 ± 4 (5)	19 ± 3 (5)	22 ± 1 (5)	20 ± 7 (5)
Glucose	59 ± 3 (7)	82 ± 3 (7)	106 ± 10 (11)	107 ± 6 (13)
γ-Aminobutyric acid	20 ± 3 (5)	32 ± 4 (6)	32 ± 7 (11)	31 ± 6 (8)
Glutamic acid	42 ± 6 (8)	45 ± 2 (5)	66 ± 10 (7)	74 ± 10 (6)
Pyruvic acid	69 ± 6 (9)	83 ± 8 (11)	106 ± 10 (11)	138 ± 9 (8)
Glucose and γ-aminobutyric acid	88 ± 7 (4)	115 ± 7 (5)	126 ± 9 (4)	132 ± 14 (4)
Glucose and glutamic acid	70 ± 3 (7)	88 ± 8 (9)	102 ± 5 (7)	124 ± 7 (8)
Glucose and pyruvic acid	73 ± 6 (4)	91 ± 5 (5)	113 ± 11 (5)	123 ± 17 (6)

* No glucose added to the medium of incubation used for the cold treatment.

2. Influence of Pyruvate, Malate and Malonate in Relation to the Effects of Potassium on Brain Aerobic Glycolysis.

The effects of pyruvate, malate and malonate on the aerobic glycolysis of potassium-depleted brain cortex slices incubated in presence of various potassium concentrations are shown in Table 8.

The rates of aerobic glycolysis are not markedly changed by the addition of pyruvate or malate, except a slight but significant increase due to pyruvate at a concentration of 3.6 mMolar potassium. Malonate markedly increases the lactic acid production at 3.6 and 60 mMolar potassium concentrations. In presence of small amounts of potassium, malonate antagonizes completely the large inhibitory effects on aerobic glycolysis.

3. Influence of Pyruvate and Glutamate in Relation to the Effects of Potassium on Anaerobic Glycolysis.

The effects of pyruvate and glutamate on the anaerobic glycolysis of potassium-depleted brain slices incubated in presence of various potassium concentrations are shown in Table 9.

Pyruvate significantly stimulates the rate of anaerobic glycolysis in the absence or presence of small amounts of potassium. In the absence of potassium, it brings the glycolytic activity to the level obtained in presence of 10 mMolar potassium. It stimulates to a greater extent the glycolytic activity in presence of 10 mMolar potassium but it does not overcome to a large extent the inhibitory effect of high concentrations of potassium (60 mMolar).

Table 8

Influence of pyruvate, malate and malonate in relation to the effects of potassium concentration on the rate of aerobic glycolysis of K-depleted brain slices.

Glucose, 10 mMolar, present in all cases.
Rates expressed as µmoles of lactic acid formed / g. fresh tissue / hour.

Substances added	Concentration (mMolar)	Potassium concentration of incubation media (mMolar)		
		0	3.6	60
None	-	122 ± 17 (12)	59 ± 13 (15)	105 ± 9 (9)
Pyruvate	10	113 ± 6 (2)	76 ± 7 (2)	107 ± 7 (2)
Malate	10	114 (1)	69 ± 6 (2)	95 ± 1 (2)
Malonate	10	122 (1)	111 ± 2 (2)	146 ± 1 (2)

Table 9

Influence of pyruvate and glutamate in relation to the effects of potassium concentration on the rate of anaerobic glycolysis of K-depleted brain slices.

Glucose, 10 mMolar, present in all cases.
Rates expressed as µmoles of CO_2 evolution / g. fresh tissue / hour.

Substances added	Concentration (mMolar)	Potassium concentration of incubation media (mMolar)		
		0	3.6	60
None	-	54 ± 2 (2)	74 ± 2 (2)	23 ± 9 (2)
Pyruvate	2	76 ± 8 (2)	122 ± 12 (2)	43 ± 6 (2)
L-(+)-glutamate	10	28 ± 6 (2)	43 ± 10 (2)	18 (1)
Pyruvate and L-(+)-glutamate	2 10	80 ± 14 (3)	80 ± 17 (3)	48 ±11 (3)

Glutamate inhibits the glycolytic activity obtained in the absence of potassium or in presence of 10 mMolar K. In the absence of potassium the inhibitory influence of glutamate is completely overcomed by pyruvate which conserves its stimulatory effect. In the presence of 10 mMolar K, pyruvate overcomes also the inhibitory influence of glutamate. Whether pyruvate is present or not glutamate completely antagonizes the stimulatory effect of 10 mMolar K.

DISCUSSION

Substrates of Oxidation

In the absence of potassium and of glucose, addition of gamma-aminobutyric acid exerts no effect on the rate of oxygen uptake. This suggests that this amino acid is dependant on potassium either to be taken up by the cells or to be metabolized. A similar remark may apply for glutamate which moreover requires greater concentrations of potassium to be oxidized at a maximal rate.

The fact that, in the absence of potassium, pyruvate is oxidized to a greater extent than glucose could suggest that the low rates of oxygen uptake obtained in presence of glucose in K-free conditions are due to a limitation in the rate of the initial breakdown of glucose. However as the rates of lactic formation are very high in the same system, another explanation is required. When added with glucose, pyruvate increases the oxygen uptake rate to the same extent as do small amounts of potassium. This is another indication supporting

the hypothesis previously put forward (see Chapter IV) according to which one of the rate-limiting points in the absence of potassium is the formation of acetyl-coenzyme A from pyruvate.

When added with glucose, gamma-aminobutyric acid also increases the oxygen uptake rate as does 3.6 mMolar K. This suggests that another rate-limiting point may be found in the glutamic acid-succinic semialdehyde oxidative pathway originally described by Roberts (331). The potassium-dependant enzyme of this pathway could be either the decarboxylase or the transaminase or both. This point will be discussed further in Chapters VIII and IX.

Intermediates of the tricarboxylic acid cycle like alpha-ketoglutarate or malate increase further the stimulated oxidation of glucose produced by high concentrations of potassium. This may indicate that, in this condition, low amounts of oxaloacetate (due to an activation of the "malic enzyme" (257) or to the increased formation of citrate (409) obtained in presence of high concentrations of potassium) are limiting the full stimulation by potassium of the oxidative reactions. The effects of alpha-ketoglutarate and of malate appear to be catalytic since the oxygen uptake rates obtained with these substrates in the absence of glucose are not stimulated by potassium.

Brain slices which have been depleted of their endogenous glucose content (by a cold incubation without glucose) do not oxidize glucose to a greater extent when the potassium concentrations are increased from 10 to 60 mMolar. On the other hand,

pyruvate is oxidized to a greater extent in this condition. This fact suggests that the initiation of the effects of high concentrations of potassium on the rates of oxidation of glucose is largely dependant on the presence of intracellular glucose. This may indicate that the high concentrations of potassium do not stimulate (or not more than small amounts of potassium) the hexokinase enzyme of the cellular membrane.

Changes in the Aerobic Glycolytic Activity.

Added pyruvate or malate exert no marked effect on the glycolytic rate. Moreover, as it was previously shown, potassium exerts no effect on the oxygen uptake obtained in presence of malate. However, when added with glucose, this substance increases further the stimulated rate of oxygen uptake obtained in presence of high concentrations of potassium. These facts do not support nor disprove the possibility of an activating action of K on the "malic enzyme" of brain tissues. This problem will be discussed further in Chapters VIII and IX. The effects of malonate on aerobic glycolysis are almost certainly not specific effects of malonate itself. They are undoubtedly due to inhibition of oxygen uptake (see next chapter) and the increased glycolysis that occurs is virtually anaerobic glycolysis.

Changes in the Anaerobic Glycolytic Activity.

Addition of 2 mMolar pyruvate stimulates the anaerobic glycolytic rate of brain slices in K-free conditions as does the addition of 10 mMolar K. This could indicate that potassium, like pyruvate (116), favours glycolysis by making available

large amounts of oxidized disphosphopyridine nucleotide. However, as the effects of small amounts of potassium and of pyruvate are additive, two different mechanisms of action are more probably involved. It appears very likely that the action of potassium is directed towards an enzyme site, either the hexokinase or the adenosine diphosphate-phosphopyruvic transphosphorylase of the cytoplasm.

It is very improbable that a direct enzymic inhibition is involved in the inhibitory effect apparent in presence of high concentrations of potassium since no enzyme of the glycolytic pathway has ever been shown to be inhibited by this ion. As pyruvate cannot overcome the inhibitory effect of high potassium concentrations it is likely that this phenomenon is not due to a shortage of oxidized diphosphopyridine nucleotide. The limiting factor may be in this case the shortage of the amounts of adenosine triphosphate required for the initial phosphorylation of glucose. This conclusion is supported by the results obtained with glutamate. This substance, like 60 mMolar K, completely blocks the activating effects of small amounts of potassium. Moreover, the inhibitory effects of 60 mMolar K and of glutamate are not additive. It was shown that addition of adenosine triphosphate abolishes the inhibitory effect of glutamate on the anaerobic glycolytic rate (377).

Pyruvate antagonizes the inhibitory effect of glutamate more successfully in the absence than in the presence of glutamate. This may indicate that pyruvate and glutamate compete for some sites responsible for their uptake into brain cells,

pyruvate being favoured in the absence of potassium and glutamate in presence of potassium. Results of Table 6 indicate that glutamate is poorly oxidized in the absence of potassium while pyruvate is very well oxidized.

Chapter VI

Drugs and Potassium-Stimulated Respiration.

Warburg (413) first demonstrated the inhibitory action of narcotics on tissue respiration *in vitro*. Later, Quastel and coworkers (289, 324) found that a variety of cerebral depressants can reduce the oxygen uptake rates of brain cortex slices. These early works have been extensively reviewed by Butler (50), Himwich (183) and Bain (18) in particular. The concentrations used to demonstrate significant inhibitory effects were, in most cases, many times higher than the amounts found or to be expected in brain after administration *in vivo* of therapeutic doses of these drugs (18, 180). However more recent works by McIlwain (273) and by Quastel (149) have demonstrated that stimulation by electrical pulses or by KCl or by 2,4-dinitrophenol (DNP) causes the respiration of brain slices to become more sensitive to the action of small quantities of drugs.

By using these stimulated systems, malonate (178, 395), barbiturates (149, 273), various other depressants (149, 280) and anticonvulsants (161), were shown to inhibit significantly the respiration at relatively small concentrations which have little or no effect on the unstimulated oxygen uptake rates. McIlwain claimed that the electrically stimulated respiration is more sensitive to the inhibitory action of some drugs than the KCl or DNP stimulated oxygen uptake rates.

These differences of sensitivity appear particularly marked in the cases of the effects of chlorpromazine (248, 280), procaine (39, 142) and atropine (267).

The sensitivity of the respiration of cold-treated slices in the absence or presence of potassium to the action of varying concentrations of these drugs was determined and compared to the results obtained with the usual method of potassium stimulation (addition of 100 mMolar KCl to untreated slices) or with electrical stimulation. The effects of some drugs like DNP, protoveratrine and protamine (also ganglioside preparations), which are known (275, 276, 427) to mimic the effects of potassium on metabolism or to interfere with the ionic transport across cellular membranes and therefore were likely to throw some light on the mechanisms of action of K, were also studied. Finally the time relationships of drug and potassium action on respiration were also given attention. Related data pertaining to the processes of potassium accumulation and sodium extrusion in brain slices will be reported and discussed in Chapter VII.

METHODS

In all the experiments described in this chapter, the calcium-free phosphate-buffered incubation medium described in chapter II was used for incubations in the cold and at 38°. Glucose, 10 mMolar, was present in all cases. When the drugs tested were added to media in the form of their sodium salts, corresponding amounts of sodium were omitted

from the media in order to keep constant the total sodium concentration. In some experiments, recorded in Table 14, either protamine or gangliosides were added to the media for incubation in the cold.

The gangliosides used were purified extracts from ox brain containing about 30% of N-acetyl neuraminic acid when assayed according to Long and Staples (254). The ganglioside preparations were kindly supplied to us by Dr. L.S. Wolfe.

In Tables 10 and 14 the underlined figures represent data which are statistically different, at the 1% level of probability, from the control data.

RESULTS

Malonate

Figure 14 illustrates the effects of varying concentrations of malonate on the oxygen uptake rates of cold-pretreated brain slices incubated in presence of various concentrations of potassium. Comparison of the three curves indicates that the respiration stimulated by high concentrations of K (60 mMolar) is very sensitive to the inhibitory effect of the drug, at all concentrations tested. At a concentration of 0.5 mMolar, malonate markedly inhibits the stimulated respiration while it has very little effect on the respiration of K-free slices. Higher concentrations of drug inhibit the respiration rates whether potassium is present or not, but malonate appears less effective in the latter condition. It is interesting to note that the effect of malonate in higher concentration is to reduce the metabolism in the

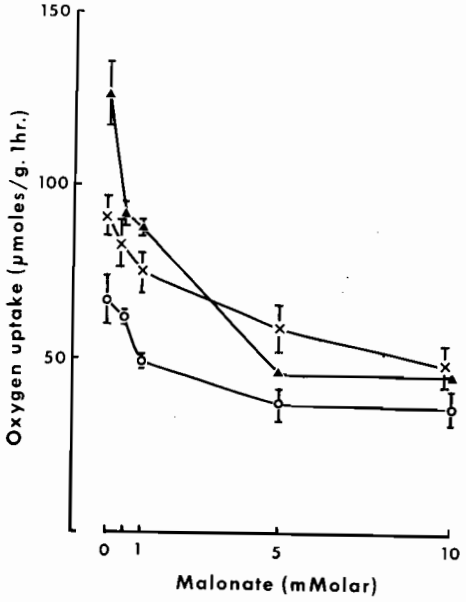

Figure 14.

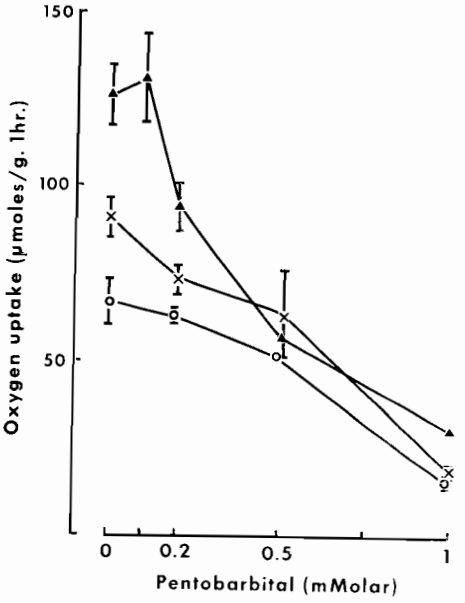

Figure 15.

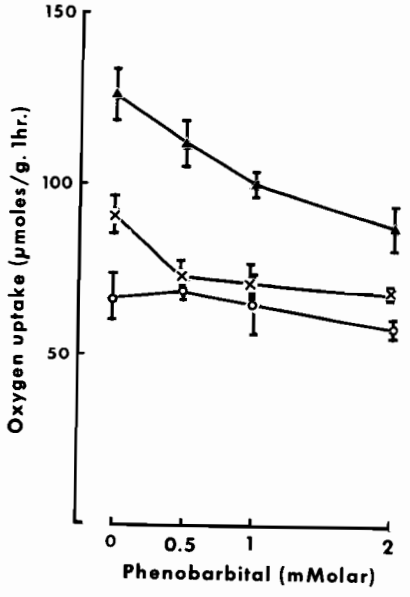

Figure 16.

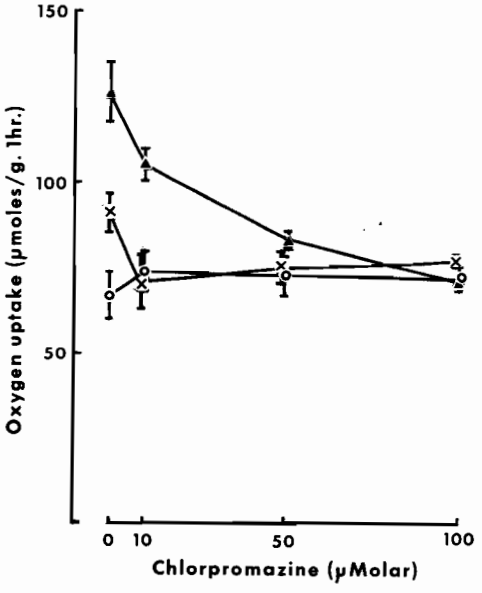

Figure 17.

Effects of drugs on the K-stimulated O_2 uptake rates of brain slices.

- ○ K-free saline media
- × saline media containing 3.6 mMolar K
- ▲ saline media containing 60 mMolar K

Conditions and number of determinations are given in Table 10.

presence or absence of potassium to about the same rate. Expressed otherwise, the extra respiration produced by potassium is preferentially inhibited by malonate.

Barbiturates

Figures 15 and 16 illustrate the effects of varying concentrations of pentobarbital and of phenobarbital, respectively, on the respiratory activity of pretreated brain slices incubated in presence of varying potassium concentrations.

The observations describing the effects of malonate apply equally well to the action of pentobarbital. Small concentrations of this drug (0.2 mMolar) inhibit markedly the potassium (3.6 or 60 mMolar) stimulated respiration without affecting significantly the rate obtained in the absence of potassium.

A concentration of 0.5 mMolar phenobarbital inhibits significantly the potassium (3.6 or 60 mMolar) stimulated oxygen uptake rates. Higher concentrations inhibit to a greater extent the respiratory rates obtained in presence of 60 mMolar K but not of 3.6 mMolar K. The respiration of slices incubated in the absence of potassium was unaffected by this drug, at all concentrations tested.

Chlorpromazine

Figure 17 illustrates the effects of chlorpromazine. (Note that the concentrations studied are considerably lower than those of the preceding drugs). Chlorpromazine exerts

rather uncommon effects on the respiration of potassium-depleted brain slices. In the absence of potassium, 10 micromolar chlorpromazine slightly but significantly increases the oxygen uptake rate. Increasing the concentration to 50 or 100 micromolar does not change the magnitude of this effect. A concentration of 10 micromolar inhibits significantly the potassium (3.6 or 60 mMolar) stimulated respiration. Concentrations of 50 and 100 micromolar cause greater inhibition of the respiratory activity in presence of 60 mMolar, but not with 3.6 mMolar K.

The experimental data illustrated in Figures 14 to 17, the standard deviations obtained and the number of determinations performed are summarized in Table 10.

Atropine, Procaine and Pentamethylenetetrazole.

Table 10 shows the effects of various other drugs on respiration. Atropine (1 mMolar) and the local anaesthetic procaine (100 micromolar) exert no effect on the low rates of oxygen uptake obtained in potassium-free conditions but inhibit significantly the K-stimulated respiration. The convulsant drug pentamethylenetetrazole exerts no effect.

Table 11 shows that all the drugs already mentioned are as effective as respiratory inhibitors when they are added 30 minutes after potassium as when they are present in the media of incubation from the beginning of the experiment.

Table 10

Influence of various drugs in relation to the effect of potassium on the rate of oxygen uptake of K-depleted brain cortex slices.

Glucose, 10 mMolar, present in all cases.
Rates expressed as µmoles of O_2 consumed / gram of fresh tissue / hour.

Drugs added to saline medium	Concentration (Molar)	Potassium concentration of medium (mMolar)		
		0	3.6	60
None	-	67 ± 7 (24)	91 ± 6 (20)	126 ± 9 (13)
Malonate	5×10^{-4}	62 ± 2 (2)	83 ± 7 (6)	92 ± 2 (2)
	1×10^{-3}	49 ± 2 (7)	75 ± 6 (11)	88 ± 2 (2)
	5×10^{-3}	37 ± 5 (5)	59 ± 7 (7)	46 (1)
	1×10^{-2}	36 ± 5 (7)	48 ± 6 (9)	45 (1)
Pentobarbital	1×10^{-4}	-	-	130 ± 13 (2)
	2×10^{-4}	63 ± 2 (2)	74 ± 5 (5)	94 ± 7 (2)
	5×10^{-4}	52 ± 3 (2)	64 ± 13 (4)	57 (1)
	1×10^{-3}	16 ± 2 (2)	19 ± 2 (4)	32 ± 1 (2)
Phenobarbital	5×10^{-4}	69 ± 3 (3)	73 ± 5 (4)	112 ± 7 (4)
	1×10^{-3}	65 ± 9 (4)	71 ± 6 (2)	100 ± 4 (5)
	2×10^{-3}	58 ± 3 (2)	68 ± 2 (2)	88 ± 6 (2)
Chlorpromazine	1×10^{-5}	74 ± 6 (9)	71 ± 8 (3)	105 ± 5 (5)
	5×10^{-5}	73 ± 6 (8)	75 ± 5 (7)	83 ± 3 (2)
	1×10^{-4}	72 ± 4 (5)	77 ± 2 (2)	71 (1)
2,4-Dinitrophenol	5×10^{-5}	68 ± 11 (8)	129 ± 9 (3)	137 ± 17 (3)
Atropine	1×10^{-3}	65 ± 9 (2)	74 ± 4 (6)	100 ± 2 (2)
Procaine	1×10^{-4}	62 ± 3 (3)	72 ± 1 (4)	105 ± 4 (9)
Pentamethylene-tetrazole	1×10^{-3}	65 ± 4 (2)	88 ± 3 (4)	129 ± 6 (3)

Table 11

Time relationship of the effects of potassium (3.6 mMolar) and drugs on the rate of oxygen uptake of K-depleted brain slices.

Glucose, 10 mMolar, present in all cases.
Rates expressed as μmoles of O_2 consumed / g. of fresh tissue / hour.

Drugs added to saline medium	Concentration (Molar)	Time of addition of drugs to medium after addition of potassium	
		0 minute	30 minutes
None	-	91 ± 4 (2)	89 ± 4 (2)
Malonate	1×10^{-3}	70 ± 5 (2)	75 ± 3 (2)
	5×10^{-3}	56 ± 2 (2)	55 (1)
Pentobarbital	2×10^{-4}	70 ± 4 (2)	65 ± 7 (2)
Phenobarbital	5×10^{-4}	74 ± 1 (2)	72 ± 3 (2)
Atropine	1×10^{-3}	77 ± 0 (2)	77 ± 1 (2)
Procaine	1×10^{-4}	71 ± 0 (2)	73 ± 3 (2)
Chlorpromazine	1×10^{-5}	68 ± 6 (2)	72 ± 3 (2)

2,4-Dinitrophenol

Table 10 shows the effect on respiration of 2,4-dinitrophenol (DNP). At a concentration of 50 mMolar, this drug does not change significantly the respiratory rates obtained in the absence of potassium or at 60 mMolar K. In presence of 3.6 mMolar K, DNP increases markedly the respiratory rate and to such an extent that this rate becomes equal to the fully stimulated activity.

Protoveratrine

Effects of protoveratrine on the respiration of brain slices incubated under various conditions are shown in Table 12 and illustrated in Figure 18.

The stimulatory effect of protoveratrine on the respiratory activity of brain slices incubated in presence of potassium, previously shown by Wollenberger (427), was confirmed. This drug conserves its stimulatory influence even when fresh slices are incubated in a potassium free medium. However potassium-depleted slices are not subject to the effect of protoveratrine if they are replaced in a potassium-free medium. The oxygen uptake rate of pretreated slices is stimulated by the drug if the tissue is incubated in presence of 3.6 mMolar K. The stimulation is obtained whether the drug is added to the medium at the same time as potassium or 30 minutes later. However, when potassium-depleted slices are first incubated with protoveratrine, addition after 30 minutes

Table 12

Time relationship of the effects of potassium and protoveratrine on the rate of oxygen uptake of fresh or potassium-depleted brain slices.

Glucose, 10 mMolar, present in all cases.
Rates expressed as µmoles of O_2 consumed / gram of fresh tissue / hour.

Drug added to saline medium	Concentration (Molar)	Time of addition of protoveratrine to saline medium * (minutes)	Potassium content of medium (mMolar)	
			0	3.6
			Non pretreated slices	
None	-	-	116 ± 9 (4)	122 ± 8 (4)
Protoveratrine	1×10^{-6}	0	141 ± 8 (3)	145 ± (1)
			Potassium-depleted slices	
None	-	-	67 ± 5 (4)	90 ± 4 (4)
Protoveratrine	1×10^{-6}	0	67 ± (1)	106 ± 4 (2)
	2×10^{-6}	0	65 (1)	110 ± (1)
	1×10^{-6}	+30	-	106 ± 2 (3)
	1×10^{-6}	-10	-	64 ± 5 (3)
	1×10^{-6}	-30	-	53 ± 5 (7)

* Addition of 3.6 mMolar K to saline medium is taken as zero time. Minus means "previous to" and plus, "after" addition of potassium.

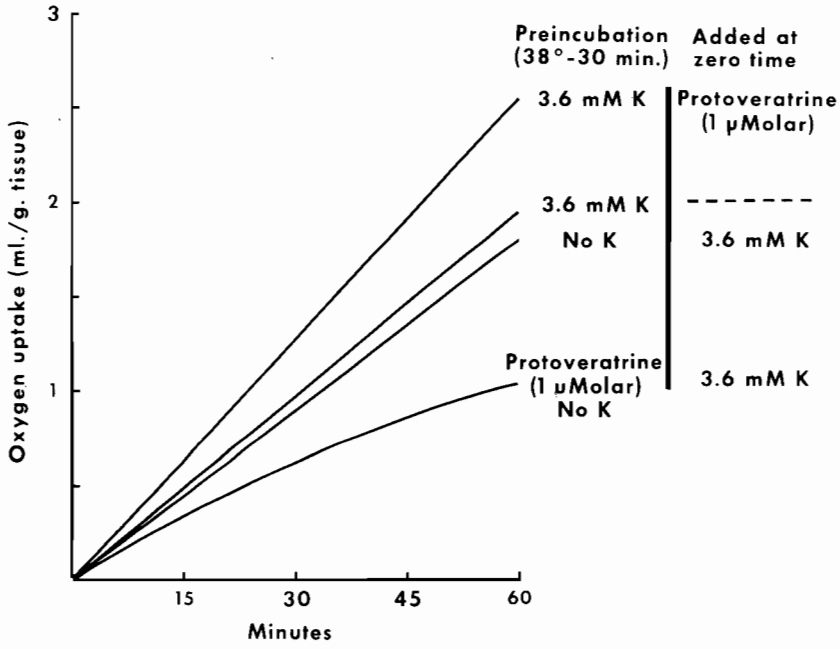

Figure 18. Effect of protoveratrine on the O_2 uptake rate of K-depleted brain slices incubated in the presence or absence of 3.6 mMolar K.

Conditions and number of determinations are given in Table 12.

of 3.6 mMolar potassium brings no stimulation of the oxygen uptake rate. In that case, both the specific stimulatory effect of the drug and that of potassium are lost.

Protamine

The effects of protamine are reported in Table 13 and illustrated in Figure 19. At a concentration of 100 micrograms per ml. protamine exerts no significant effect on the respiration of potassium-depleted brain slices incubated in the absence of potassium. Under the same condition, a concentration of 250 micrograms per ml. exerts a small inhibitory effect. The respiratory activity of pretreated brain slices replaced in presence of potassium is significantly inhibited by protamine concentrations of 100 and 250 micrograms per ml.

When potassium (3.6 mMolar) is added to the medium 30 minutes after protamine, the inhibition caused by the basic protein is still greater than previously found. In this condition the stimulatory effect of potassium is completely blocked. However 100 micrograms per ml. of protamine added 30 minutes after potassium does not affect the respiratory rates. Under the same condition, 250 micrograms per ml. of protamine depresses the respiration to a certain extent.

In presence of 60 mMolar potassium, the respiratory activity is also significantly inhibited by protamine.

Gangliosides

Table 14 shows the effects of gangliosides in relation

Table 13

Time relationship of the effects of potassium and protamine on the rate of oxygen uptake of K-depleted brain slices.

Glucose, 10 mMolar, present in all cases.
Rates expressed as μmoles of O_2 consumed / gram of fresh tissue / hour.

Substance added to medium	Concentration μgram / ml.	Time of addition of protamine to medium * (minutes)	Potassium content of medium (mMolar)		
			0	3.6	60
None	-	-	66 ± 6 (4)	92 ±4 (3)	130 ± 7 (2)
Protamine	100	0	63 ± 7 (2)	74 ±7 (9)	91 ± 11 (3)
	250	0	50 ± 3 (2)	69 ±2 (2)	-
	100	+30	-	87 ±5 (2)	-
	250	+30	-	76 (1)	-
	100	-30	-	63 ±4 (2)	-
	250	-30	-	40 ±2 (2)	-

* Addition of 3.6 mMolar K to saline medium is taken as zero time. Minus means "previous to" and plus, "after" addition of potassium.

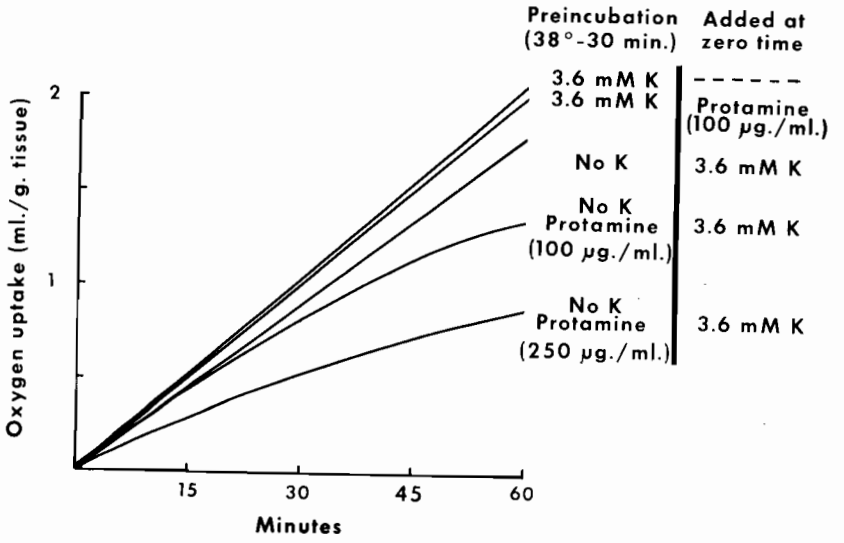

Figure 19. Effect of protamine on the O_2 uptake rate of K-depleted brain slices incubated in the presence or absence of 3.6 mMolar K.

Conditions and number of determinations are given in Table 13.

Table 14

Relationship of the effects of potassium, protamine and gangliosides on the rate of oxygen uptake of K-depleted brain slices.

Glucose, 10 mMolar, present in all cases.
Rates expressed as μmoles of O_2 consumed / gram of fresh tissue / hour.

Substances added to incubation media			Potassium content of medium (mMolar)	
At 0°C	At 38°C	Concentration μgram / ml.	0	3.6
-	-	-	65 (1)	89 ± 2 (4)
-	Gangliosides	300	66 (1)	87 ± 6 (3)
Protamine	-	250	58 (1)	72 ± 2 (4)
Protamine	Gangliosides	250 - 300	-	86 ± 6 (5)
Gangliosides	-	300	-	85 (1)
-	Protamine	250	-	70 ± 6 (2)
Gangliosides	Protamine	300 - 250	-	72 ± (1)

to the inhibitory influence of protamine on the respiration of brain slices. Protamine (250 micrograms per ml.) added to the cold pretreatment medium exerts no significant effect on the respiration of brain slices reincubated at 38°C in a potassium-free medium (and without protamine), but inhibits significantly the respiratory activity of slices reincubated in a medium containing 3.6 mMolar K. This inhibition following a pretreatment in the cold in presence of protamine is completely antagonized by gangliosides at a concentration of 300 micrograms per ml. (added to the medium of incubation at 38°C).

Gangliosides exert no effect on the respiration of slices which have not previously been incubated in presence of protamine. A pretreatment in the cold in presence of gangliosides does not antagonize the inhibitory effect of protamine added to the medium of incubation at 38°C.

DISCUSSION

The respiration of potassium-depleted slices when stimulated by addition of small (3.6 mM) or large amounts (60 mM) of potassium, was found to be very sensitive to small concentrations of a variety of drugs. In most cases statistically significant inhibitory effects were obtained with concentrations between 10 and 50 times smaller than those required for significant inhibition of the oxygen uptake rates obtained by the classical method of potassium stimulation (addition of 100 mM KCl to the medium with untreated alices).

Malonate, which is commonly used (178, 395) at a concentration of 10 mMolar, exerted a significant inhibitory effect (30%) at a concentration of 0.5 mMolar. Pentobarbital and phenobarbital exerted effects of a similar magnitude at concentrations of 0.2 and 0.5 mMolar, respectively, which correspond to the amounts found in brain in vivo after administration of therapeutic doses of these drugs (18,180). The respiration of brain slices when stimulated by the addition of 100 mMolar KCl, is decreased to around 75% of the control activity by 0.3 mMolar chlorpromazine or by 20 mM procaine (142,248). These concentrations are markedly higher than those (about 10 and 100 micromolar, respectively) required to inhibit to a similar extent the system discussed here or electrically stimulated slices (39,280). According to McIlwain (267) 1 mMolar atropine inhibits the response of brain slices to electrical stimulation but not to DNP or to KCl. We found that an identical concentration blocked to a considerable extent the stimulation of respiration obtained at 3.6 or 60 mMolar K. As had been shown in the case of electrically stimulated slices (280), pentamethylenetetrazole (1mMolar) exerted no effect on the respiration of cold-treated slices.

The increased sensitivity to drugs of the metabolic activity of potassium-depleted slices does not appear to be due, as might be thought, to impairment of the metabolic capacities of the tissue resulting from the cold-treatment itself. Such impairment seems unlikely since in most cases small concentrations of drugs could be found which specifically inhibited the K-stimulated respiration without exerting any significant

effect on the metabolic activity of slices incubated in K-free media. This phenomenon is best illustrated by the effects of phenobarbital and particularly by those of chlorpromazine which decreased markedly the K-stimulated respiration and increased slightly the oxygen uptake rate of K-free slices, at all concentrations tested (from 10 to 100 micromolar).

An important metabolic effect of chlorpromazine and of some barbiturates is thought to be an uncoupling of oxidative phosphorylation (17, 92, 180). Metabolic impairments of this type are often, but not always, associated with increased rates of oxygen uptake. Various drugs and the barbiturates in particular have been shown to exert a diphasic action on the oxygen uptake of brain slices: stimulation at low and inhibition at high concentrations (290). As far as we know however this has not been shown in the case of chlorpromazine. In order to determine if the apparently specific effects of this drug (inhibition in presence of K, slight activation in absence of K) could be related to disturbances of the phosphorylation processes, another well-known uncoupler of oxidative phosphorylation was tested: 2,4-dinitrophenol.

It was found that this drug has no effect on the respiration of slices kept in K-free conditions but, in presence of 3.6 mM K, it increased the oxygen uptake rate to the level normally obtained in presence of 60 mM K. From the lack of effect of DNP in the absence of K we may infer that the slight stimulatory action of chlorpromazine obtained under similar conditions is not likely to be due to the uncoupling properties

of this drug.

In view of the fact that, in presence of small amounts of K, DNP stimulates the respiration rate as well as does the addition of 60 mMolar K and also of the fact that the drug does not increase further the potassium (60 mMolar) stimulated rate, it is tempting to suggest that the action of high amounts of K, like that of DNP, is due to a disturbance of the processes of oxidative phosphorylation.

The lack of effect of DNP, in the absence of K, and its large stimulatory effect at 3.6 mMolar K are strikingly parallel to the effects of protoveratrine under similar conditions. This last drug had been shown by Wollenberger (427) to stimulate markedly the aerobic metabolism of brain slices provided that small amounts of potassium be present in the medium of incubation. Our study confirmed this finding and revealed new facts concerning the action of this drug. The presence of potassium in the medium is not required provided that the brain slices contain their normal amount of potassium when they are placed in presence of the drug. Moreover K-depleted slices which have been incubated in presence of 1 micromolar protoveratrine during a sufficiently long period of time (30 minutes) do not respond any more to the stimulatory action of external K nor to the action of the drug itself. It will be shown in the next chapter that under this last condition the amount of K taken up by the tissue is markedly reduced.

These facts suggest strongly that potassium increases the respiration of brain in as much as it is present in the

tissue and that its presence in the external medium is not sufficient to provoke the normal stimulatory effects. The fact that the inhibitory action of the drug increases with time suggests that this specific action is dependant on the amounts of protoveratrine accumulated by the slices and not on the external presence of the drug. This contrasts markedly with the stimulatory action of protoveratrine which is complete and appears immediately after addition of the drug to the medium of incubation (see Figure 18).

These observations give no information as to the site of action of K and of protoveratrine (at the membrane or at intracellular sites) but indicate merely that both agents have to be absorbed, accumulated or bound by the tissue in order to be effective. This question is studied further in the next chapter.

McIlwain (275) has recently shown that protamine and related basic proteins inhibit the electrically and KCl stimulated respiration of brain slices. Experiments reported in Table 13 and Figure 19 show that the inhibitory action of 100 micrograms per ml. of protamine is particularly efficient when the tissue is incubated in presence of protamine for 30 minutes before addition of potassium. This fact may be related to the time of penetration of the protein into the tissue or to differences in the relative affinity of some sites of the brain cells to potassium compared to protamine.

On the other hand, larger concentrations of protamine are required to inhibit significantly the respiration of slices which were previously incubated in presence of potassium. Moreover other experiments have shown (Table 14) that

addition of gangliosides to the medium of incubation at 38°C can reverse the inhibitory action of small amounts of protamine which had been added to the medium of preincubation in the cold. These antagonistic effects of protamine and of gangliosides on the system studied here are similar to the effects obtained by McIlwain (47) on the electrically stimulated respiration with identical concentrations of these substances.

The antagonistic metabolic effects of potassium and protamine which have been demonstrated and also the study of the time relationships of these phenomena indicate that the basic protein and potassium compete for attachment at unidentified cellular sites intimately related to the control of the respiration rates of the brain cells. Results obtained with gangliosides indicate that these substances remove the competitive action of protamine.

On the other hand results showing that protamine (like many other drugs tested) is more efficient as respiratory inhibitor in presence of high concentrations of K (60 mMolar) appear difficult to reconcile with the postulated competitive influence of this agent, except if it is postulated that the metabolic effects exerted by high external concentrations of K are qualitatively different from the influence of low K concentrations. The variations in the kind of effects on metabolism of potassium at different concentrations described in Chapters IV and V strongly suggest that potassium acts in two basically different ways.

Without prejudging the nature of the action of protamine it is obvious that this substance exerts its inhibitory influence on respiration through a mechanism which is completely different from that of all the other inhibitors studied. This fact is clearly shown when protamine and the other inhibitor drugs are compared for their metabolic action on brain slices preincubated in presence of potassium. A similar remark applies to protoveratrine for reasons already mentioned.

It is more difficult to find precise differences in the modes of action of the other inhibitory agents tested, in spite of the fact that drugs like the barbiturates, atropine and procaine, for example, are known to have very distinct physiological effects. This fact merely indicates that the test-system used is not specific enough to detect the individual metabolic properties underlying the specific physiological action of each one of these drugs.

Chapter VII

Factors Affecting Potassium Uptake and Sodium Extrusion in Brain Slices.

The experiments reported in this chapter were undertaken to determine the changes in the potassium and sodium content of brain slices produced by various agents which have been studied in the previous three chapters in regard to their metabolic influence: potassium, sodium, calcium, substrates of oxidation and drugs. A careful review of the literature pertaining to this subject reveals that much less attention had been given to the influence of these agents on the electrolyte exchanges occurring between brain slices and incubation medium than to their metabolic effects and very few studies considered the relationship existing between electrolyte exchanges and metabolic activity.

The influence of sodium and of potassium concentration on the potassium and sodium content of brain slices incubated aerobically or anaerobically was the subject of a series of studies by Pappius and Elliott (310,311,312,313). Krebs and coworkers (233,388) determined the turn-over rates of K exchanges *in vitro* and the influence of various substrates of oxidation, in particular of L-glutamate (see also 106), and Takagaki *et al.* studied the influence of D-glutamate on K uptake (384b). Concerning the study of the influence of drugs on K uptake in relation to metabolism, the major contributions were a remarkable study by Wollenberger (427)

on the action of protoveratrine, the works of McIlwain and coworkers (281) on protamine and gangliosides in relation to electrical stimulation, and a recent paper of Whittam (421) on the influence of ouabain (and of sodium-free saline media). Other studies related to this subject were mentioned in Chapter I.

Many of the studies mentioned demonstrated that respiration rates and K uptake of brain slices are affected simultaneously by the action of cations, substrates and drugs but gave little information regarding the relationship existing between the two processes. Cummins and McIlwain, in their recent study (83), Takagaki et al. (384b) and others, previously, were unable to show any correlation between potassium content and respiration rate of resting brain slices.

METHODS

The potassium and sodium content of slices was estimated by the method described in Chapter II. Results are given in microequivalents per gram of fresh tissue. This is calculated from the initial weight of slices, their final weight and their ionic content after incubation. It is assumed that an increase in weight up to 40% of the initial weight is due to uptake of medium with its K and Na content unchanged and that this fluid is not truly part of the tissue. A similar correction was applied and its validity examined by Pappius and Elliott (310,311). All results reported were obtained with cold-pretreated brain slices. According to data given in Chapter III,

these treated slices contained about 10 microequivalents of potassium and 160 microequivalents of sodium per gram of fresh weight at the beginning of the period of incubation at 38°C.

RESULTS AND DISCUSSION

1. Effect of Ionic Content of Medium of Incubation.

The time course of potassium uptake by K-depleted brain slices incubated at 38° in presence of oxygen, glucose and 3.6 or 18 mMolar K, is illustrated in Figure 20. The rate of uptake is rapid in the first minutes of incubation in presence of potassium: about 7 microequivalents per gram of tissue per minute in presence of 3.6 mMolar K and 14 microequivalents per gram per minute with 18 mMolar K in the medium. The rate of reaccumulation in presence of 3.6 mMolar K is higher than that obtained by Pappius and Elliott (311) with slices which had been depleted of potassium by anaerobic incubation at 38°. Cummins and McIlwain (83) found that brain slices reaccumulate from 7 to 10 microequivalents of K per gram of tissue after stoppage of electrical stimulation. This uptake occurred during the first minute.

The rate of potassium uptake was too rapid to determine whether the rate of metabolism changes with the potassium content of the slice or is constant at a rate determined by the K concentration in the medium (compare Figure 20 to Figure 4 which illustrates the rapid change in oxygen uptake rate

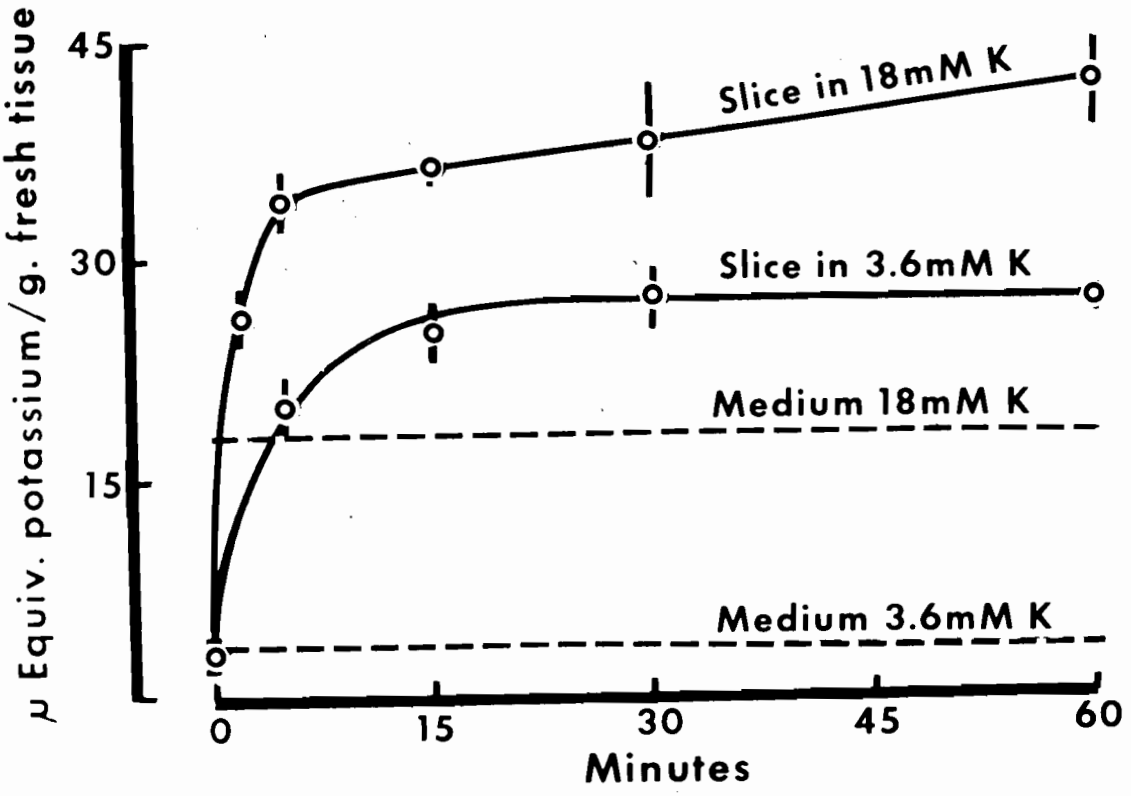

Figure 20. Time course of potassium accumulation in K-depleted brain slices incubated at 38°C in the presence of 10 mMolar glucose and 100% oxygen.

Each point is the average of 6 or more determinations and the vertical brackets indicate the standard deviations.

occurring after addition of 3.6 mMolar K to the medium of incubation). However the results illustrated in Figure 21 gave some indications regarding this question.

This figure shows that the maximum level of potassium reached in the slices increased with increasing potassium concentration in the medium. When this concentration was higher than 18 mMolar, the difference between the potassium content of the slice and that of the medium, that is the amount accumulated against the concentration gradient, progressively decreased.

As can be seen the increase in oxygen consumption rate is approximately paralleled by the increase in tissue potassium up to about 50 microequivalents per gram of tissue (20 mMolar in medium). As the potassium content of the medium is further increased the increase in the oxygen uptake rate is slightly less than proportional to the increase in tissue potassium. (With guinea-pig brain cortex slices preincubated for 1 or 2 hours in warm (38°C) potassium-free media and then placed in potassium-containing media, Cummins and McIlwain (83) observed little variation in respiration rate with the K content of the tissue unless electrical pulses were applied. In the latter case the respiration rate increased in proportion to the potassium content of the tissue up to 30 micromoles of K per gram of tissue).

The results shown in Figure 21 suggest that, under our condition, at moderate potassium concentrations (up to 20 mMolar) in the medium, it is the potassium content of the slice and not that in the medium that determines the rate of metabolism. Results obtained when studying the influence of

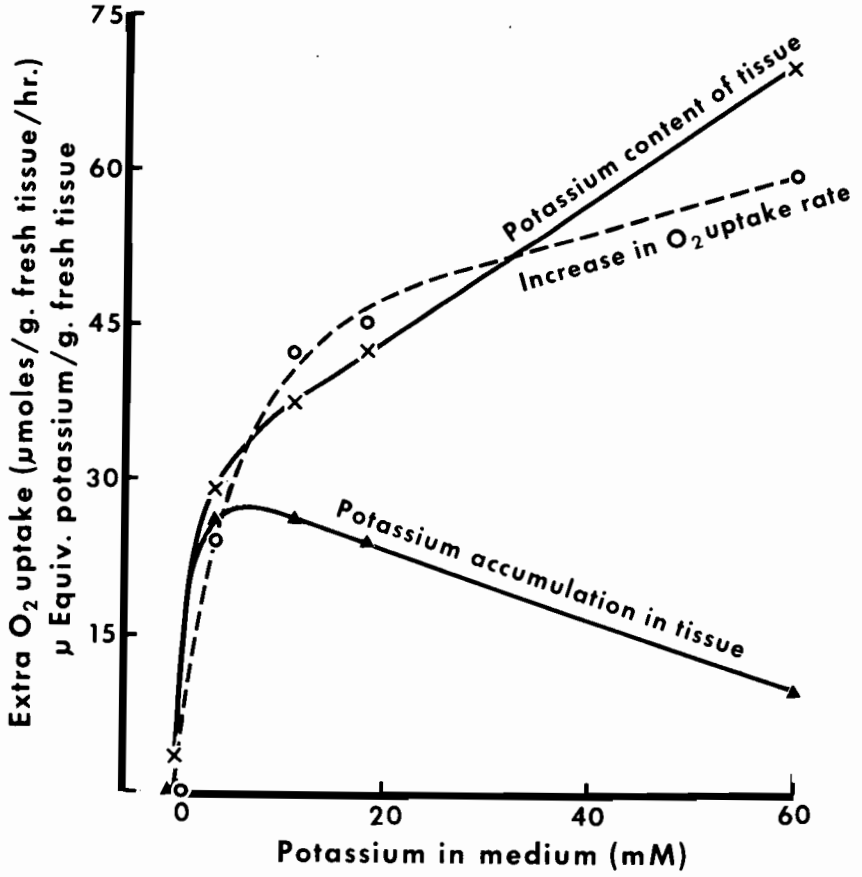

Figure 21. Relationship between potassium content of tissue, potassium concentration gradient between tissue and incubation medium and absolute increase of the O_2 uptake rate of K-depleted brain slices incubated in the presence of 10 mMolar glucose, 100% oxygen and varying concentrations of potassium.

protoveratrine and protamine (see later) also support this conclusion.

The fact that relatively small amounts of potassium are accumulated by the slices against the concentration gradient when high concentrations of potassium are present in the medium (even though the oxygen uptake rate is at its highest) is difficult to reconcile with the hypothesis put forward by McIlwain (271) suggesting that the metabolic changes observed under such conditions are caused by the increased energy expenditure necessary for maintenance of a high ratio of intracellular to extracellular potassium concentration. As shown in Table 15 the difference between the sodium content of slices and of medium also appears maximal at low external concentrations of potassium.

According to these observations and in view of the fact that these large amounts of potassium are known to produce marked falls of the phosphocreatine level in brain slices (156), it seems correct to conclude that the stimulated metabolic rates obtained under these conditions are more likely to be related to an impairment of the high energy phosphate production than to increased rates of energy utilization. High concentrations of potassium could either decrease the rate of synthesis of energy-rich phosphates (by uncoupling oxidation from phosphorylation) or increase the rate of their hydrolysis (by stimulation of ATPase).

As most of the experiments reported in this chapter were undertaken to study the relationship existing between potassium accumulation and metabolic activity, saline media containing

Table 15

Influence of the ionic content of the incubation medium on the potassium and sodium content of brain slices.

Magnesium, 1.7 mMolar, and glucose, 10 mMolar, present in all cases.

Electrolyte content of incubation medium		Electrolyte content of slices after one hour of incubation at 38°C μequivalents / g. fresh tissue			
		Potassium		Sodium	
Cations present in medium	Concentration (mMolar)	Tissue content	Difference tissue - medium	Tissue content	Difference tissue - medium
K Na	0 161	3 ± 1 (15)	+ 3	161 ± 9 (30)	0
K Na	3.6 157	29 ± 1 (30)	+ 26	125 ± 7 (40)	- 32
K Na Ca	3.6 157 3.4	28 ± 2 (12)	+ 25	134 ± 12 (5)	- 23
K Na*	3.6 36	28 ± 3 (4)	+ 25	-	-
K Na**	3.6 0	7 ± 1 (9)	+ 4	-	-
K Na	11 150	37 ± 2 (12)	+ 26	115 ± 11 (6)	- 35
K Na	18 143	42 ± 5 (15)	+ 24	114 ± 8 (4)	- 29
K Na	60 101	70 ± 7 (7)	+ 10	87 ± 4 (10)	- 14

* Most of the sodium was replaced by sucrose to maintain osmolarity.
** All the sodium was replaced by sucrose.

moderately low amounts of potassium (in which K uptake in brain slices is maximal) were used.

The results presented in Table 15 show that addition of 3.4 mMolar calcium to the medium of incubation does not alter the potassium uptake. It was previously shown (Chapter IV) that while the addition of this ion to saline media decreases the rate of respiration it does not change the magnitude of the stimulatory effect of low amounts of potassium.

Gardos (141) has shown that ethylenediaminetetraacetate inhibits the potassium uptake in brain slices (also in erythrocytes,140) presumably by chelating the divalent cations calcium and magnesium since addition of 1 mMolar $CaCl_2$ or of 5 mMolar $MgCl_2$ could reverse this inhibitory action. These observations are in keeping with an hypothesis put forward by Bacq (16) in particular, according to which displacement of calcium ions from biological membranes, where they are presumably bound, results in an impairment of the mechanism responsible for electrolyte transport. The lack of effect of calcium on K-uptake (Table 15) may be explained by the possibility that sufficient amounts of calcium remain bound to the membrane even in calcium-free media. Moreover small amounts of magnesium were present in the incubation medium used. This question will be discussed further in relation to the action of procaine and of protoveratrine, at the end of this chapter.

In accord with observations made under different experimental conditions by Pappius and Elliot (313) and by Whittam (421), accumulation of potassium does not occur in brain slices

incubated in a Na-free medium, as shown in Table 15. In this case, potassium exerts no stimulatory effect on the respiration of brain slices kept under otherwise optimal metabolic conditions, as was shown previously (Table 4). In presence of 36 mMolar Na, both stimulation of respiration and K uptake are fully recovered. These facts show the dependence of K uptake and K-stimulated oxygen uptake on sodium and also the close relationship existing between these two processes.

2. Effects of Substrates of Oxidation.

Table 16 shows the potassium and sodium contents of brain slices after an incubation of one hour in presence of various substrates of oxidation in a saline medium containing 3.6 mMolar K. The differences between electrolyte content of tissue and of saline medium are also shown and indicate the apparent concentration gradients maintained between tissue and medium in presence of each substrate of oxidation. Results representing the changes in sodium content are rather variable and their significance is difficult to assess. However it seems obvious that the amounts of K taken up by the slices are exchanged with approximatively proportional amounts of sodium extruded under most of the conditions studied (see also Tables 15 and 17).

Pappius and Elliott (311) have also shown reciprocal changes of potassium and sodium in brain slices kept under different experimental conditions.

Comparison of the results of Table 16 with those presented in Table 8, which reported the respiration rates obtained under

Table 16

Influence of substrates of oxidation on the potassium and sodium content of K-depleted brain cortex slices.

Potassium, 3.6 mMolar, present in all cases.

Substances added to medium (10 mMolar)	Electrolyte content of slices after one hour of incubation at 38°C µequivalents / g. fresh tissue			
	Potassium		Sodium	
	Tissue content	Difference tissue - medium	Tissue content	Difference tissue - medium
None	8 ± 1 (24)	+ 5	179 ± 16 (6)	+ 18
Glucose	29 ± 1 (30)	+ 26	125 ± 7 (40)	- 32
Pyruvate	21 ± 2 (18)	+ 18	121 ± 9 (5)	- 36
Glutamate	14 ± 1 (16)	+ 11	-	-
γ-Aminobutyrate	12 ± 1 (16)	+ 9	142 ± 5 (6)	- 15
α-Ketoglutarate	9 ± 1 (4)	+ 6	-	-
Glucose and pyruvate	31 ± 2 (12)	+ 28	-	-
Glucose and glutamate	37 ± 2 (15)	+ 34	105 ± (1)	- 52
Glucose and γ-aminobutyrate	31 ± 1 (17)	+ 28	130 ± 6 (5)	- 27
Glucose and α-ketoglutarate	30 ± 1 (5)	+ 27	127 ± 4 (6)	- 30
Glucose and malate	29 ± 2 (4)	+ 26	-	-

identical conditions, indicates that the amount of potassium taken up by slices in presence of most substrates of oxidation is roughly proportional to the corresponding increase in oxygen uptake rate caused by potassium. This relationship is best illustrated by the situation prevailing in the absence of substrate where little K is accumulated and no increase of respiration rate is obtained and also in presence of substrates like alpha-ketoglutarate and gamma-aminobutyrate where both effects are small.

3. Effects of Drugs.

The results presented in Table 17 show the effects of various drugs on the potassium and sodium content and on the fluid uptake of brain slices incubated aerobically in presence of 10 mMolar glucose and 3.6 mMolar potassium.

Increasing concentrations of malonate and of pentobarbital provoke increased inhibition of potassium uptake and of sodium extrusion. These effects on electrolyte content appear to be closely related to the inhibitory effects on the K-stimulated respiration of brain slices which were shown in Table 12. In the low concentration tested phenobarbital had little effect on the potassium uptake.

Dinitrophenol (DNP) in low concentration slightly but significantly reduced the amount of potassium taken up by the tissue. This influence of DNP could be expected as it is well known that this drug disturbs the mechanism responsible for

Table 17

Effects of drugs on potassium uptake and sodium extrusion by K-depleted brain cortex slices.

Potassium, 3.6 mMolar, and glucose, 10 mMolar, present in all cases.

Drugs added to medium	Concentration (Molar)	Electrolyte content of slices after one hour of incubation at 38°C μequivalents / g. fresh tissue			
		Potassium		Sodium	
		Tissue content	Difference tissue - medium	Tissue content	Difference tissue - medium
None	-	29 ± 1 (30)	+ 26	125 ± 7 (40)	- 32
Malonate	5×10^{-4}	27 ± 1 (24)	+ 24	121 ± 8 (2)	- 36
	1×10^{-3}	23 ± 2 (15)	+ 20	120 ± 8 (4)	- 37
	5×10^{-3}	19 ± 2 (9)	+ 16	159 ± 9 (6)	- 2
	1×10^{-2}	18 ± 2 (15)	+ 15	165 ± 10 (10)	+ 4
Pentobarbital	2×10^{-4}	25 ± 2 (7)	+ 22	126 ± 3 (3)	- 31
	5×10^{-4}	21 ± 3 (5)	+ 18	130 ± 8 (3)	- 27
	1×10^{-3}	17 ± 2 (4)	+ 14	151 ± 8 (2)	- 6
Phenobarbital	5×10^{-4}	27 ± 1 (4)	+ 24	140 ± 12 (3)	- 17
Procaine	1×10^{-4}	28 ± 1 (4)	+ 25	145 ± 10 (4)	- 12
Atropine	1×10^{-3}	28 ± 1 (8)	+ 25	145 ± 8 (10)	- 12
2,4-Dinitrophenol	5×10^{-5}	24 ± 1 (4)	+ 21	142 ± 8 (3)	- 15
Chlorpromazine	1×10^{-5}	28 ± 1 (4)	+ 25	104 ± 11 (4)	- 53
	5×10^{-5}	29 ± 0 (2)	+ 26	-	-
	1×10^{-4}	27 (1)	+ 24	-	-

the formation of energy-rich phosphate compounds which are likely to be the source of the energy required for the maintenance of electrolyte concentration gradients. It is interesting to note that the action of DNP resembles that of large amounts of KCl in as much as it reduces the potassium concentration gradient maintained by the slices while increasing the oxygen consumption rate.

The fact that potassium uptake is not completely blocked by concentrations of drugs like malonate and pentobarbital which markedly inhibit the respiration rate may be explained by increased rates of glycolysis which would prevail under these conditions and could perhaps constitute a source of energy sufficient to partially maintain the electrolyte concentration gradients.

DNP (279), malonate (396, see also Table 7), pentobarbital and various narcotics (414) are known to increase markedly the aerobic glycolytic rates of brain slices presumably as a result of inhibition of respiration and the Pasteur effect. Pappius and Elliott have demonstrated that glycolysis can to some extent support potassium uptake (311).

Other drugs, like procaine, atropine and chlorpromazine, which were shown previously to inhibit significantly the potassium stimulated oxygen uptake rates (see Table 12) had no apparent effect on the potassium content of brain slices incubated under identical conditions. This fact could indicate that these drugs, unlike malonate and the barbiturates, affect a part of the cellular metabolic activity which is not related to the

energy output required for maintenance of electrolyte concentration gradients. It is also possible that these agents influence the transport rate of electrolytes but not their steady-state equilibrium. Techniques making use of the radioactive isotopes of potassium could permit assessment of the validity of this suggestion. The effects of procaine will be discussed further in conjunction with the influence of protoveratrine at the end of this chapter.

The data presented in Table 18 show an unexpected effect of chlorpromazine on the sodium content of brain slices incubated in the absence of potassium. Concentrations of 10 to 100 micromolar chlorpromazine considerably reduce the sodium content of the tissue. The Fischer t test indicates that the difference between the concentrations of sodium in the tissue and in the medium obtained after addition of the drug to the incubation medium is significant ($p<0.005$). The effect on sodium content due to chlorpromazine appears comparable to that obtained after addition of 3.6 mMolar K. Under identical conditions chlorpromazine slightly stimulates the oxygen uptake rate of brain slices (Figure 17). Under similar conditions DNP, malonate and other drugs caused an <u>increase</u> in the total sodium content of the slices accompanied by an increase in fluid uptake.

Table 19 shows that malonate and pentobarbital reduce the potassium content of slices whether they are added to the medium of incubation at the same time as potassium or 30 minutes later. Unlike protamine, these drugs also conserve their inhibitory

Table 18

Effects of drugs on sodium content and fluid uptake of brain slices, in the absence of potassium.

Glucose, 10 mMolar, present in all cases.

Drugs added to medium	Concentration (Molar)	Na content of slices after one hour of incubation at 38°C µequivalents / g. fresh tissue		Fluid uptake
		Tissue content	Difference tissue - medium	% of fr. weight
None	-	161 ± 9 (30)	0	57 ± 9 (20)
Malonate	5×10^{-4}	163 (1)	+ 2	-
	1×10^{-3}	187 (1)	+ 26	67 (1)
	5×10^{-3}	181 (1)	+ 20	69 ± 2 (2)
	1×10^{-2}	179 (1)	+ 18	-
Procaine	1×10^{-4}	177 (1)	+ 16	-
Atropine	1×10^{-3}	160 ± 8 (3)	- 1	60 ± 2 (2)
2,4-Dinitrophenol	5×10^{-5}	180 ± 11 (11)	+ 19	70 ± 7 (11)
Chlorpromazine	1×10^{-5}	138 ± 3 (16)	- 23	-
	5×10^{-5}	126 ± 9 (13)	- 35	54 ± 6 (11)
	1×10^{-4}	131 ± 7 (3)	- 30	-

Table 19

Time relationship of the effects of drugs on the potassium uptake by K-depleted brain slices.

Potassium, 3.6 mMolar, and glucose, 10 mMolar, present in all cases.

Drugs added to saline medium	Concentration (Molar)	Potassium content of slices one hour after addition of drug μequivalents / g. fresh tissue	
Time of addition of drugs to medium after addition of potassium		0 minute	30 minutes
None	-	30 ± 1 (2)	29 ± 0 (2)
Malonate	1×10^{-3}	23 ± 2 (2)	23 ± 1 (2)
Pentobarbital	2×10^{-4}	24 ± 0 (2)	25 ± 1 (2)
Phenobarbital	5×10^{-4}	27 ± 0 (2)	27 ± 1 (2)
Procaine	1×10^{-4}	28 ± 1 (2)	28 ± 1 (2)
Chlorpromazine	1×10^{-5}	29 ± 2 (2)	27 ± 0 (2)

influence on respiration when added to the medium of incubation 30 minutes after potassium (Table 11).

Protamine is known to inhibit potassium uptake in kidney slices (4,133,294). Recently, McIlwain et al. (281) found that protamine inhibits the K uptake (7 to 10 microequivalents per gram of tissue) occurring in brain slices after stoppage of electrical pulses. Protamine was effective at a concentration of 100 micrograms per ml. Higher concentrations (200 to 400 micrograms per ml.) were required to diminish significantly (by about 17%) the potassium content of resting brain slices. They did not study the influence of this agent on potassium recovery after K-depletion of resting slices.

Results presented in Table 20 show that 100 microequivalents of protamine per ml. reduce markedly and more efficiently than any other agent so far mentioned the potassium uptake by cold-treated slices incubated aerobically at 38° in presence of glucose and 3.6 mMolar K. The inhibition appears greater when slices are first incubated with protamine and potassium is added 30 minutes later. On the other hand the effect of 100 micrograms of protamine per ml. on potassium accumulation is not apparent when slices are first incubated with potassium and the basic protein added 30 minutes later. In the latter case a greater concentration (250 micrograms per ml.) is required to cause a significant diminution of the K content found after final incubation. These data show clearly that the action of protamine, unlike that of the drugs previously studied, is more efficient in blocking K uptake than in releasing

Table 20

Time relationship of effect of protamine on the potassium uptake by K-depleted brain slices.

Potassium, 3.6 mMolar, and glucose, 10 mMolar, present in all cases.

Substance added to medium and concentration µgram / ml.		Time of addition of protamine to medium * (minutes)	potassium content of slices, one hour after last addition to medium µequivalents / g. fresh tissue	
			Tissue content	Difference tissue - medium
None	-	-	29 ± 1 (30)	+ 26
Protamine	100	0	18 ± 2 (9)	+ 15
	250	0	12 ± 2 (2)	+ 9
	100	- 30	16 ± 2 (2)	+ 13
	250	- 30	8 ± 0 (2)	+ 5
	100	+ 30	28 ± 1 (2)	+ 25
	250	+ 30	19 ± 1 (2)	+ 16

* Addition of 3.6 mMolar K to saline medium is taken as zero time. Minus means "previous to" and plus, "after" addition of potassium.

the potassium content of brain tissue. Comparison of these effects with results pertaining to the inhibitory influence of protamine on respiration indicates that this substance depresses the K-stimulated respiration only as it blocks K uptake. Protamine has no apparent influence on respiration when no effects on potassium uptake are observed. These observations support the view that protamine, unlike the other metabolic inhibitors studied, exerts its primary effect on the mechanism responsible for electrolyte transport and through this influence, alters the metabolism of brain slices. Studies on the action of gangliosides confirmed this hypothesis.

McIlwain et al. (281) have shown that when protamine and gangliosides are added together to the saline medium the small inhibitory effect of protamine on K reaccumulation after stoppage of electrical pulse does not take place. Their results indicate that a pretreatment of 15 minutes at 38° in presence of protamine (100 micrograms per ml.) had no significant inhibitory effect on K reaccumulation during a subsequent incubation in a protamine-free saline medium. Consequently these authors could not show that gangliosides reverse the inhibitory action of protamine on K uptake. This point appears particularly important since it is known (276) that protamine and gangliosides combine and form precipitates. The antagonistic action of these two substances on respiration and K uptake cannot be shown by adding the two substances together in the saline medium. In this case, the interaction occurs outside the tissue and obviously no conclusion can be drawn regarding the influence of these substances on tissue.

Our results (Table 21) indicate that preincubation in the cold with 250 micrograms of protamine per ml. (not with 100 micrograms per ml. however) causes a slight (20%) but significant inhibition of both K uptake and of oxygen uptake (Table 14) during subsequent incubation at 38° in a protamine-free medium. Both inhibitory effects are completely reversed by addition of 300 micrograms of gangliosides per ml. to the incubation medium used at 38°. Ganglioside alone exerts no influence on respiration or K uptake and a pretreatment in the cold with this substance does not reverse the inhibitory action obtained by adding protamine to the saline medium at 38°.

Wollenberger (427) claimed that the net uptake of K by guinea-pig brain slices was inhibited by protoveratrine in concentrations which stimulated respiration. He gave the results of only two determinations, determined the uptake by measuring the initial and final concentration of potassium in the medium and found differences no greater than 1 micro-equivalent per ml.

Table 22 shows the effects of protoveratrine on the potassium content of pretreated slices incubated in media to which 3.6 mMolar K was added either with or 10 or 30 minutes after the drug. Protoveratrine (1 micromolar) has no effect on K uptake but stimulates the oxygen uptake rate (Table 12) when it is added at the same time as potassium. Added 10 or 30 minutes before K, protoveratrine inhibits markedly the uptake of potassium and the respiration rate.

The inhibitory action of protoveratrine, like that of protamine appears primarily directed towards the mechanism of

Table 21

Relationship of the effects of protamine and gangliosides on potassium uptake by brain slices.

Potassium, 3.6 mMolar, and glucose, 10 mMolar, present in all cases.

Substances added to incubation media			Potassium content of slices after one hour of incubation at 38°C µequivalents / g. fresh tissue	
At 0°C	At 38°C	Concentration µgram / ml.	Tissue content	Difference tissue - medium
-	-	-	29 ± 1 (30)	+ 26
-	Gangliosides	300	29 (1)	+ 26
Protamine	-	100	30 ± 0 (2)	+ 27
Protamine	Gangliosides	100 - 300	30 ± 2 (2)	+ 27
Protamine	-	250	23 ± 2 (7)	+ 20
Protamine	Gangliosides	250 - 300	29 ± 1 (5)	+ 26
Gangliosides	-	300	32 (1)	+ 29
-	Protamine	250	12 ± 2 (2)	+ 9
Gangliosides	Protamine	300 - 250	14 (1)	+ 11

Table 22

Time relationship of the effect of protoveratrine on the potassium uptake by K-depleted brain slices.

Potassium, 3.6 mMolar, and glucose, 10 mMolar, present in all cases.

Substance added to medium	Concentration (Molar)	Time of addition of protoveratrine to medium * (minutes)	Potassium content of slices, one hour after last addition to medium µequivalents / g. fresh tissue	
			Tissue content	Difference tissue - medium
None	-	-	29 ± 1 (30)	+ 26
Protoveratrine	1×10^{-6}	0	30 ± 3 (5)	+ 27
	2×10^{-6}	0	28 ± 0 (2)	+ 25
	1×10^{-6}	- 10	19 ± 1 (3)	+ 16
	2×10^{-6}	- 10	16 ± 3 (3)	+ 13
	1×10^{-6}	- 30	16 ± 3 (3)	+ 13

* Addition of 3.6 mMolar K to saline medium is taken as zero time. Minus means "previous to" and plus, "after" addition of potassium.

uptake of potassium and the inhibitory influence on respiration is probably consequent. The stimulatory action on respiration which is accompanied by no apparent influence on K uptake is more difficult to understand. However even if there is no apparent change in the net uptake of potassium, there is little doubt that protoveratrine must interfere with the rates of K exchanges occurring between slices and medium of incubation.

Shanes (350,351) has shown that the veratrum alkaloids increase the permeability of the nerve membranes to potassium. These drugs, which are classified as labilizers (352), cause "interfacial dissolution" of monolayers of stearic acid (354). This influence is antagonized by calcium, procaine and other agents, which increase the surface pressure of the fatty acid film and are classified as stabilizers (352). According to Shanes, similar physical interactions are involved in natural membranes and control to some extent the electrolyte transport.

Wollenberger (427) has shown that protoveratrine, unlike dinitrophenol, does not change the rates of phosphorylation of brain homogenates. If the drug produces no impairment of the phosphorylation processes, the increased oxygen uptake rates obtained in its presence should be accompanied by increased rates of high energy phosphate production. The extra amounts of energy produced are perhaps required to maintain the concentration gradient of potassium at its maximal level in spite of the changes in the structure of the membranes brought by the drug. Conversely the action of other agents like calcium and procaine (shown to depress respiration without changing the net uptake of potassium) would be related

to their stabilization of the membrane structure. This would result in a situation where smaller amounts of energy are required to maintain the electrolyte concentration gradients at their maximal level and the rates of the oxidative processes would be reduced in consequence. Even if these suggestions appear as very speculative they give an idea of the mechanism by which potassium stimulates the respiration: through changes in the phosphorylation processes and apparently not through direct stimulation of the enzymes of glucose oxidation.

Chapter VIII

Influence of Potassium and of Other Factors on the Level and State of Amino Acids in Brain Tissue.

Elliott and coworkers (111,118) have shown that a large fraction of the Factor I present in unheated saline suspensions of brain is not active in inhibiting the spontaneous discharges of the crayfish stretch receptor neuron. They demonstrated that the fraction of Factor I which is occluded (not extracted into free solution with saline medium) is fairly constant (about 65% of the total). It can be completely released into the active form by heat, mild acid or alkali, or by homogenizing the tissue in hypotonic medium. In salt-free sucrose solutions it is also largely released.

It is well known that various amino acids which are closely related to intermediates of the tricarboxylic acid cycle can be formed from glucose in brain slices and that high concentrations of potassium increase this formation and influence the relative proportions of the amino acids formed (60,222,335). Various effects of potassium on the metabolism of amino acids in isolated tissues have been reviewed in Chapter I.

It has also been demonstrated that brain slices incubated under good metabolic conditions can accumulate against apparent concentration gradients various amino acids: glutamic acid and glutamine (343,377,384b,388), aspartic acid (229), gamma-aminobutyric acid (117) and tryptophan (399). In several instances, small amounts of potassium were found

to accompany and stimulate the uptake of these amino acids in brain slices (384b,388,399). Christensen and coworkers have shown that amino acid uptake is accompanied by potassium loss in ascites cells (66,308,328). They also demonstrated that high extracellular concentrations of potassium inhibit the uptake of glycine in ascites cells (65,328) and in erythrocytes (67). Elwin and coworkers (120) found a similar inhibitory effect of high concentrations of potassium on the uptake of serine in liver slices. Potassium also influences the uptake of amino acids in bacteria (8,91).

The experiments reported in this chapter were undertaken to determine if and in what proportion other major amino acids of brain, besides gamma-aminobutyric acid, are also occluded or bound (i.e. retained in tissue homogenates when extracted with saline media) and the influence of various factors (composition of the solutions used for extraction, successive extractions, techniques of homogenization and of centrifugation) on the state of these amino acids in brain homogenates.

The influence of external potassium concentration on the level and retention of amino acids in K-depleted brain slices incubated under aerobic or anaerobic conditions and in presence of 10 mMolar glucose was also studied. Finally the influence of potassium concentration on the activity of the glutamic decarboxylase and pyridoxal kinase enzymes of brain tissue homogenates was determined.

The amino acid content of brain was determined by chromatographic and colorimetric techniques modified from various methods previously described in the literature (70,331,433). The specificity and reproducibility of the modified method was determined.

METHODS

Preparation of Brain Extracts

Rats were decapitated and their brains quickly removed, weighed and homogenized at room temperature in 4 times their weight of either a) ethanol-water, 80:20 (v/v), b) phosphate-buffered (pH:7.8) potassium - and calcium-free saline medium, c) a solution of sucrose (320 mMolar) or d) water. In the case of experiment 5 of Table 26 a saline medium containing either 60 or 160 mMolar potassium was used and the sodium concentration was correspondingly diminished (to 100 and 0 mMolar, respectively) to keep the saline medium isotonic.

When experiments were done to compare the percentages of the amino acid content of brain extractable by various types of solution several brains were cut in halves and the hemispheres distributed in the homogenization tubes in such a way as to eliminate the variability due to differences in amino acid content between brain of different rats. In other cases the brains of two or more rats were pooled. Experiments reported in Tables 23 and 27 and in each subdivision of Table 26 were indepently done according to this method. Homogenization was done in Potter-Elvehjem tubes except in the case of experiment 3 of Table 26 where the Emanuel-Chaikoff apparatus was used.

After homogenization, aliquots of the suspensions were pipetted into centrifuge tubes. In most cases centrifugation was done in a Servall centrifuge (12,000 r.p.m. for 15 minutes at room temperature. In the case of experiment 5 of Table 26 an International centrifuge was used (20,000 r.p.m. for

20 minutes) and in the case of experiment 4 of Table 26, a Spinco ultracentrifuge was used (40,000 r.p.m. for 60 minutes). In these last two instances the centrifugation was done at a temperature close to 0°C.

To determine the percentages of the amino acid content of brain which could be extracted by 6 successive washings with saline medium (Table 27) the tissue was resuspended and homogenized in a fresh amount of saline after each centrifugation. An aliquot of the supernatant fraction obtained at each step was mixed with absolute ethanol (final ethanol concentration: 80% v/v) and centrifuged to discard the precipated proteins. The residue obtained after the 6th centrifugation was also treated with 80% ethanol. As a complete separation of the supernatant from the residue obtained after each centrifugation could not be achieved, the volume of solution left with the residue in the bottom of the tube was estimated (total original volume - volume of supernatant removed - volume of dry matter assumed to be 20% of the fresh weight of tissue) and a correction was applied to allow for the amount of amino acids left in supernatant (about 15% of the total volume of the suspension) which remained with the residue.

In the experiments of Table 26, two aliquots of each homogenate were centrifuged separately and ethanol was added to residue and supernatant fractions together, in one case (total), to supernatant alone, in the other. In experiments conducted to determine the total amino acid content of tissue, brain was immediately homogenized in 80% ethanol and centrifuged in a

clinical centrifuge and the precipitated proteins were discarded (Tables 23,26,28,30).

The results reported in Table 23 show that extraction with 80% ethanol is quantitative since the residue obtained after centrifugation contains only the small amounts of amino acids which could be expected to be found there due to the fact that the separation of the supernatant and residue fractions is never complete. The amounts of amino acids found in the supernatant fraction after extraction with 85% ethanol are slightly smaller than those obtained with 75 or 80% ethanol but the differences are probably not significant. As a rule 80%(v/v) ethanol was used as the extraction fluid.

Experiments on Slices

Tissue slices obtained from 4 or 5 brains were randomized according to the method previously described (Chapter II), weighed and 240 mgm. of tissue were either (a) immediately homogenized in 8 ml. of 80% ethanol or (b) incubated in the cold during 60 minutes in an erlenmeyer containing 3 ml. of phosphate-buffered calcium- and potassium-free saline medium containing 10 mMolar glucose and then isolated on a perforated disk and homogenized in ethanol or (c) preincubated in the cold and isolated as for (b) and then placed in a Warburg flask containing 2 ml. of fresh medium of varying potassium content (0,3.6,10 and 60 mMolar) and incubated for 60 minutes in presence of 10 mMolar glucose and 100% oxygen at 38°. After this incubation the slices were isolated, reweighed and homogenized in 8 ml. (final volume) of 80% ethanol (for determination of tissue amino acid content) or ethanol was added to the

Table 23

Influence of ethanol concentration on extraction of amino acids from rat brain.

Ethanol concentration in extracting solution	Amounts of amino acids extracted micrograms / g. of fresh brain					
	Glutamic acid	Glutamine	Aspartic acid	γ-Amino-butyric acid	Glycine and Serine	Alanine
75 %(v/v) Ethanol-water	1270 ± 150 (4)	480 ± 40 (4)	320 ± 40 (4)	225 ± 25 (4)	160 ± 30 (4)	120 ± 25 (4)
80% (v/v) Ethanol-water	1240	490	305	235	165	115
(Remaining in residue)	(120)	(65)	(40)	(45)	(20)	(20)
85% (v/v) Ethanol-water	1230 ± 100 (4)	460 ± 40 (3)	295 ± 20 (4)	220 ± 30 (4)	150 ± 30 (3)	125 ± 15 (3)

Warburg flasks and the whole content was homogenized (amino acid content of tissue plus medium). The homogenates were then centrifuged in a clinical centrifuge and the supernatant fractions were stored in the cold. In some cases the aerobic incubation in warm saline medium was done in presence of drugs. In other cases this incubation was done anaerobically in a bicarbonate-buffered saline containing 0, 10 or 60 mMolar potassium.

Chromatographic Techniques

Whatman no.4 papers were used in all cases. Four solvent systems were tested: (1) n-butanol-acetic acid-water (120:30:50 v/v), (2) phenol-water (80:20 w/v), (3) n-butanol-pyridine-water (60:60:60: v/v) and (4) tertiary-butanol-methylethyl ketone-water (40:40:20 v/v). Use of solvent (2) was discontinued because it frequently gave a background coloration on the paper which interfered with amino acid determination. The system consisting in a first run (of 14 hours) in solvent (1) followed by a second run (of 16 hours) in the other dimension in solvent (3) was found useful provided that the pyridine had been distilled to remove substances which leave a residue on the paper and cause interference in the determination of amino acids. This system was used in all experiments reported in the second part of this chapter (experiments on slices). The system consisting in a first run in solvent (4) (of 18 hours) followed by a run in solvent (1) (of 14 hours) gave very satisfying results. This solvent system can be used without previous

purification and gives a very good separation of the major amino acids of brain. It was used in all the experiments reported in the first part of this chapter (Tables 23 to 27). It is important to use this solvent-system in the order mentioned since the methylethyl ketone solvent interferes with ninhydrin coloration (less color being obtained). A long period of ventilation of the paper is required when this solvent is used last. However when the second run is done in n-butanol-acetic acid the traces of the first solvent left on the paper are apparently washed out and only a relatively short period of drying (4 hours) in a stream of air is required. Bidimensional descending chromatography was performed in "Chromatocab" cabinets.

Identification of the Amino Acids and Specificity of the Analytical Method.

The identification of the amino acids contained in the brain extracts was made (a) by consulting a table of Rf values reported in the literature (Smith 364), (b) by chromatographing solutions of the 16 amino acids found in highest concentration in brain (according to Tallan, Moore and Stein 387), and (c) by preparing a standard solution of these amino acids (mixed in the proportions found in brain) and comparing the chromatographic pattern of this synthetic mixture with that of brain extracts. The relative mobilities of these amino acids in the three solvent-systems used is shown in Table 24. It can be seen that the solvent-systems used gave a very good separation of all the amino acids tested (except glycine and serine) and consequently the specificity of the analytical

Table 24

Relation of mobility of amino acids to that of γ-aminobutyric acid in three solvent systems.

Amino acids chromatographed	$\frac{\text{Distance amino acid travels from origin}}{\text{distance γ-aminobutyric acid travels from origin}} \times 100$		
	n-butanol-acetic acid-water	t-butanol-methyl ethyl ketone-water	n-butanol-pyridine-water
Phenylalanine	125	280	-
Tryptophan	116	250	-
Methionine	113	240	-
Tyrosine	101	210	-
γ-Aminobutyric acid	100	100	100
Alanine	76	140	105
Threonine	70	130	-
Glutamine	70	82	25
Glutathione	67 - 26	70 - 0	-
Glycine	60	100	27
Serine	57	100	-
Aspartic acid	57	55	15
Glutamic acid	53	70	67
Taurine	49	170	-
Lysine	34	24	-
Cystine	14	12	-

method appears satisfactory.

Analytical Technique

As a rule, 500 microlitres of the ethanol extracts were applied as a spot on each paper. When the extracts were known to contain very small amounts of amino acids (e.g. the experiment of Table 27) larger volumes were applied. Various quantities of a standard solution containing all the amino acids to be determined were also applied on different papers for bidimensional chromatography in the same solvent-systems and the production of colorimetric standard curves. After chromatography and drying the papers were dipped in a solution of 0.25% ninhydrin in acetone and heated for 20 minutes in an oven at 65%. The stained spots were cut out and treated with 1 ml. of freshly prepared 0.5% ninhydrin in 80% (v/v) ethanol at room temperature for 15 minutes than 3 ml. of 70% (v/v) acetone were added and, after 15 minutes, the fluid was transfered to a centrifuge tube. The paper was reextracted for 15 minutes with a further 3 ml. of 70% acetone, in the case of the most concentrated amino acids (glutamic acid, glutamine, aspartic acid and gamma-aminobutyric acid). The combined fluids were centrifuged for 10 minutes in a clinical centrifuge. The absorption by these solutions was measured at 575 millimicra in a Beckman spectrophotometer against a similarly prepared ninhydrin-ethanol-acetone extract from a part of the paper which showed no colour.

The accuracy and reproducibility of this method is shown in Table 25 where the means and standard deviations of 7 experimental determinations are compared to the composition of the standard synthetic mixture of amino acids chromatographed. Only the values of glutamine are lower than expected. The same colorimetric method has previously been used for determination of gamma-aminobutyric acid (244).

Determination of Glutamic Decarboxylase and Pyridoxal Kinase Activity.

Potassium-depleted slices were homogenized (100 mgs. per ml.) in a medium containing 50 mMolar NaH_2PO_4 or KH_2PO_4 (pH:5.5), 40 mMolar sodium fluoride, 65 mMolar sodium glutamate and 0.4 mMolar pyridoxal-5 phosphate and incubated for 1 hour at 38° under anaerobic conditions in presence of 0, 3.6, 10 and 50 mMolar potassium. The CO_2 evolution was followed and gave the measure of the activity of the glutamic decarboxylase enzyme. As the activity of glutamic decarboxylase is dependent on the amount of pyridoxal-5 phosphate available, omission of this cofactor and addition of adenosine triphosphate and pyridoxal would provide a measurement of the activity of the enzyme pyridoxal kinase: any increase in carbon dioxide production by the homogenates incubated with ATP and pyridoxal as compared with homogenates incubated with ATP but without pyridoxal could be considered to be a measure of the amount of pyridoxal-5 phosphate synthesized. The influence of potassium concentration on this system was tested. The method described was previously used by Greenberg et al. (160).

Table 25

Accuracy and reproducibility of the analytical method.

	Concentrations of amino acids micrograms / ml. of solution					
	Glutamic acid	Glutamine	Aspartic acid	γ-Amino butyric acid	Glycine and Serine	Alanine
Composition of the standard solution	325	125	75	60	40	25
Experimental values	312	108	78	58	44	22
Standard deviations	± 20	± 9	± 4	± 6	± 4	± 3
Number of determinations	7	7	7	7	7	7

RESULTS AND DISCUSSION

Amino Acid Content of Brain Tissue.

The values reported in Table 23 expressing the amino acid content of brain are close to values obtained after extraction with picric acid, paper chromatography and ninhydrin coloration by DeRopp and Snedeker (96). However a lower concentration of glutamine was found and a higher concentration of alanine. The values of Table 23 also agree closely with the determinations of Tallan, Moore and Stein (on cat brain, 387) but are markedly lower than those of Porcellati and Thompson (318). An experiment done to determine the amount of amino acids left in the residue fraction after extraction with 80% ethanol (Table 23) showed that this extraction is nearly quantitative.

State of Amino Acids in Brain Tissue.

Table 26 shows the influence of various factors on the extraction of amino acids from brain homogenates. It can be seen that homogenization of brain tissue in K-free saline medium extracts only about 50% of the gamma-aminobutyric acid content and between 65 and 75% of the other amino acids determined. Homogenization in the Emanuel-Chaikoff apparatus, which presumably gives complete rupture of all brain cells, and centrifugation at 40,000 r.p.m. give the same results as homogenization in the Potter-Elvejhem apparatus and centrifugation at 12,000 or 20,000 r.p.m. These data support the

Table 26

Factors affecting extraction of amino acids from brain tissue.

Extraction media	Concentration of amino acids micrograms / g. fresh tissue (Percentage of total found in Snf)									
	Glutamic acid		Glutamine		Aspartic acid		γ-Amino-butyric acid		Alanine	
	Snf	Total	Snf	Total	Snf	Total	Snf	Total	Snf	Total
1. Ethanol-water (80%)		1265		482		289		241		102
2. K-free saline not heated	685 (64%)	1007	360 (75%)	480	255 (79%)	324	190 (57%)	332	80 (74%)	108
Heated at 100°C for 20 minutes	1105 (100%)	1080	407 (91%)	445	335 (100%)	297	340 (100%)	325		
3. K-free saline tissue homogenized in Emanuel-Chaikoff apparatus	798 (71%)	1124	342 (73%)	467	207 (69%)	300	157 (53%)	296	76 (83%)	92
4. K-free saline *	780 (69%)	1088	405 (80%)	509	244 (77%)	319	160 (47%)	338	83 (79%)	105
Sucrose (320mM) *	985 (84%)	1175	440 (90%)	487	318 (100%)	307	267 (81%)	328	92 (87%)	106
Water *	895 (86%)	1042	495 (98%)	506	286 (90%)	319	285 (96%)	298	95 (90%)	105
5. K-free saline **	780 (77%)	1015			238 (75%)	315	165 (53%)	312	66 (77%)	85
60 mM K saline **	1095 (94%)	1160			285 (85%)	335	218 (73%)	300	75 (79%)	95
160 mM K saline ** (Na-free)	1085 (89%)	1220			315 (100%)	298	270 (83%)	315	78 (93%)	84

* Centrifuged at 105,000 x g for 60 minutes.
** Centrifuged at 15,000 x g for 20 minutes.

view that the retention by the tissue of part of its amino acid content is not due to technical causes (such as incomplete rupture of cells). Elliott and Van Gelder (118) have shown that the occluded Factor I is not released by grinding the tissue with sand.

As was previously shown in the case of Factor I (111,118), it was found that heating brain homogenates or suspending them in water or salt-free sucrose solution release a considerable part of the occluded or bound amino acids. Homogenization in media containing high concentrations of potassium (particularly 160 mMolar K) also releases almost completely the fraction of amino acids content retained by the tissue. This may be an indication that the potassium ions can displace the amino acids from their sites of binding in disrupted cells. As previously mentioned (65,66,67) it is known that small amounts of potassium interchange with amino acids taken up in ascites cells and that high concentrations of potassium inhibit amino acid uptake in various cells. Some observations on the influence of potassium on amino acids retention in brain slices that will be reported at the end of this chapter are also in keeping with these data.

The results presented in Table 27 show that successive washings with a K-free saline medium gradually release the amino acids retained in the brain tissue. However, even after 6 successive extractions small amounts of amino acids are still found in the residue fraction. These data show the weakness of the attachment of the amino acids to the cell debris.

Little information can be obtained from these results concerning the nature of the postulated bonds or the site of

Table 27

Successive extractions of amino acids of brain tissue with potassium-free Ringer saline.

		Concentrations of amino acids micrograms / g. fresh tissue and % of calculated total					
		Glutamic acid		Aspartic acid		γ-Aminobutyric acid	
Successive extractions	1st	715	66%	183	56%	155	45%
	2nd	163	15%	55	17%	94	26%
	3rd	58	5%	17	5%	30	9%
	4th	41	4%	22	7%	19	6%
	5th	37	3%	12	4%	16	5%
	6th	19	2%	7	2%	9	3%
Residue		55	5%	31	9%	23	7%
Calculated total		(1088)	(100%)	(327)	(100%)	(346)	(100%)

occlusion of the amino acids. It seems improbable that the relatively large fractions of amino acids which were found to be retained by the tissue residue after extraction with saline media would be linked either to the "amino acids activating enzymes" (some of which were shown to be stimulated by potassium,196) or to the amino acid-specific ribonucleic acid involved in protein synthesis (251,434) since gamma-aminobutyric acid which appears to be occluded to a large extent is not known to be a protein constituent (114,387).

Various amino acids have recently been shown to be incorporated into lipoidal material of microsomal and supernatant fractions of rat liver (169) and other tissues (181). Cowie and coworkers (82,214), working on bacteria, have reported the existence of a "concentrating pool" involved in the intracellular accumulation of amino acids and independent of the mechanism responsible for amino acid interconversion and for protein synthesis (called "conversion pool"). They found that the amino acids of the "concentrating pool" are released when the cells are suspended in distilled water while the "metabolic pool" is unaffected by a similar treatment. These observations which were made on different types of cells support the view that the amino acid content of living cells may be divided into more than two fractions and that the state of the "free amino acids" may be more complex than has been previously assumed.

Levels of Amino Acids in Brain Cortex Slices.

The results presented in Table 28 show that an incubation of one hour in the cold markedly depletes the amino acid content

Table 28

Influence of potassium on levels of amino acids in brain cortex slices.

Metabolic conditions	Concentrations of amino acids micromoles / g. fresh tissue			
	Glutamic acid	Gamma-amino-butyric acid	Aspartic acid	Alanine
Fresh slices	10.6 ± 0.6 (5)	3.5 ± 0.4 (5)	2.3 ± 0.3 (5)	0.9 ± 0.1 (5)
Slices incubated one hour at 0°C (pretreatment)	3.0 ± 0.6 (5)	1.2 ± 0.1 (5)	0.9 ± 0.2 (4)	0.4 ± 0.1 (6)
Pretreatment and aerobic incubation for one hour at 38°C, in K-free saline Tissue + medium Tissue content % in tissue	6.6 ± 0.6 (7) 4.0 ± 0.4 (4) 60%	2.7 ± 0.2 (7) 1.6 ± 0.3 (3) 59%	1.7 ± 0.1 (6) 1.3 ± 0.2 (3) 74%	1.1 ± 0.3 (6) 0.6 ± 0.1 (2) 58%
in 3.6 mMolar K Tissue + medium	5.9 ± 0.6 (7)	1.9 ± 0.3 (6)	2.1 ± 0.2 (5)	1.6 ± 0.2 (7)
in 10 mMolar K Tissue + medium Tissue content % in tissue	6.0 ± 0.6 (6) 5.2 ± 0.5 (5) 88%	1.9 ± 0.3 (6) 1.6 ± 0.1 (2) 84%	2.3 ± 0.3 (7) 2.2 ± 0.3 (4) 96%	1.8 ± 0.2 (7) 1.1 ± 0.2 (3) 55%
in 60 mMolar K Tissue + medium Tissue content % in tissue	5.0 ± 0.4 (4) 4.8 ± 0.5 (4) 96%	3.0 ± 0.5 (7) 2.8 ± 0.2 (3) 93%	1.2 ± 0.2 (5) 1.1 ± 0.1 (2) 87%	1.7 ± 0.2 (3) 0.8 ± 0.2 (3) 50%
Pretreatment and anaerobic incubation for one hour at 38°C, in 10 mMolar K Tissue + medium Tissue content % in tissue	4.4 (1) 2.4 (1) 55%	2.0 (1) 0.8 (1) 34%	1.2 (1) 0.8 (1) 69%	
in 60 mMolar K Tissue + medium Tissue content % in tissue	2.9 (1) 1.0 (1) 33%	1.2 (1) 0.3 (1) 25%	1.0 (1) 1.0 (1) 35%	

of brain cortex slices. However the amounts of glutamic acid, gamma-aminobutyric acid, aspartic acid and alanine found in the tissue after this cold treatment (between 30 and 40% of the original content) are much greater than could be expected if complete equilibrium was obtained between tissue content and medium of incubation. This is an indication that a considerable part of the amino acid content of brain cortex slices is not free to diffuse.

After aerobic incubation for one hour at 38°C of cold-treated slices in a potassium-free medium containing 10 mMolar glucose, the amino acid content of tissue plus medium was found to be markedly increased. Incubation in presence of small amounts of K (3.6 or 10 mMolar) had no significant effect on the levels of glutamic and aspartic acids but decreased the level of the gamma-aminobutyric acid and increased the level of alanine. Comparison of the results obtained in presence of 10 mMolar K and those obtained after incubation in presence of 60 mMolar K shows that the higher concentration of potassium significantly decreases the levels of glutamic acid and of aspartic acid but increases the level of gamma-aminobutyric acid.

In the system studied here, the significance of the observed changes in the amino acid levels brought about by potassium is difficult to assess because the changes are probably the result of the influence of potassium on many distinct metabolic processes. From experiments performed with radioactive glucose (235,295) it is known that large amounts of amino acids can be formed from the intermediates of the tricarboxylic acid cycle.

These amino acids are apparently formed either through transamination from endogenous amino acids or through reaction of carbohydrates with free ammonia (59) which is known to be produced in relatively large amounts by brain slices incubated *in vitro* (260). In the system discussed here the absolute levels of amino acids were determined and the observed effects of potassium may represent influences of this ion on the rates of transformation of the carbohydrates, on the rates of amination of these substances, on the rates of amino acid interconversions (e.g. activity of the transaminases, of glutamic decarboxylase) and perhaps also on the rates of proteolysis occurring in the brain slices.

In spite of the great complexity of this system, some observations which had been made by others in experiments on the metabolism of radioactive glucose were confirmed. Chain *et al.* (235) have noted that high concentrations of potassium decrease the ratio of glutamic acid-gamma-aminobutyric acid. The results of Table 6 show that small amounts of potassium increase this ratio while 60 mMolar K markedly decreases it.

It is possible that a direct influence of potassium on the activity of the glutamic decarboxylase enzyme is involved in this effect. Happold has reported that potassium increases the activity of the bacterial enzyme (334) and results presented in Table 29 show that 60 mMolar K increases by about 50% the anaerobic decarboxylation of glutamate by brain homogenates. Other results in Table 29 indicate that the rate of formation of pyridoxal phosphate (coenzyme of the glutamic decarboxylase) from pyridoxal and adenosine triphosphate is

Table 29

Influence of potassium concentration on the glutamic decarboxylase and pyridoxal kinase of brain homogenates.

Activity expressed as micromoles of CO_2 evolution / g. fresh brain.

Substances added to medium *	Potassium concentration in homogenate **		
	0 mMolar	10 mMolar	60 mMolar
Pyridoxal-5-phosphate (0.4 mMolar)	8.1 ± 0.6 (2)	8.2 ± 0.6 (3)	12.3 ± 0.8 (3)
ATP (16 mMolar) and pyridoxal (6 mMolar)	5.8 (1)	7.3 ± 0.1 (2)	7.5 ± 0.3 (2)
ATP (16 mMolar)	6.0 (1)	5.9 ± 0.1 (2)	5.9 ± 0.2 (2)
Difference	0	1.4	1.6

* All media contained 65 mMolar sodium glutamate, 40 mMolar sodium fluoride and 50 mMolar sodium and potassium dihydrogen phosphates (pH:5.5); the proportions of potassium and sodium salts were varied according to the final concentration of potassium desired.

** Homogenates were prepared from K-depleted brain slices and were incubated under anaerobic conditions at 38°C.

apparently very low in the system studied here (and not markedly affected by potassium) since the rate of decarboxylation was not increased by the presence of ATP and pyridoxal by comparison with the rate obtained when pyridoxal alone was added.

Table 30 shows that incubation of brain slices in presence of 1 mMolar semicarbazide, a drug which has been shown to reduce the level of gamma-aminobutyric acid *in vivo* through inhibition of the glutamic decarboxylase (218b), results in a marked decrease of the level of gamma-aminobutyric acid. On the other hand other convulsant drugs tested, metrazol and picrotoxin, which have no effect on the levels of gamma-aminobutyric acid *in vivo* (118,218b), were found to have no significant effect on the amino acid content of the slices. The marked effect of semicarbazide on the system described here (increasing the glutamic acid-gamma-aminobutyric acid ratio while 60 mMolar K decreases it) support the view, previously expressed, that the observed effect of potassium on the levels of glutamic acid and of gamma-aminobutyric acid result from a direct influence of K at the enzymatic level.

As the activity of certain transaminases is also known to be influenced by potassium (334) it is possible that the complex changes observed in the glutamic acid-gamma aminobutyric ratio (increased at low concentrations of K and decreased in presence of 60 mMolar K) result from influences of potassium on both the rate of formation and the rate of removal of

Table 30

Influence of convulsive drugs on the levels of glutamic acid and γ-aminobutyric acid of brain cortex slices.

Substances added to incubation medium	Concentrations of amino acids in tissue plus medium micromoles / gram of fresh tissue		
	Glutamic acid	Gamma-amino-butyric acid	Ratio of glutamic acid / gamma- amino-butyric acid
Potassium, 10 mMolar	5.9	1.9	3.1
and semicarbazide (1mM)	5.6	0.6	9.3
and pentamethylene-tetrazole (1 mM)	5.9	2.0	3.0
and picrotoxin (1 mM)	5.9	1.7	2.9
Potassium, 60 mMolar	5.0	3.0	1.7
and semicarbazide (1mM)	7.0	1.3	5.5
and pentamethylene-tetrazole (1 mM)	5.1	3.4	1.5
and picrotoxin (1 mM)	4.8	2.5	1.9

gamma-aminobutyric acid. The metabolism of other amino acids like glutamine and glutathione which is known to be very active in brain (340) is also probably involved in these complex effects of potassium. This question will be discussed further in the next chapter.

High concentrations of potassium markedly decrease the levels of aspartic acid. A similar but less marked effect has been observed by Kini and Quastel (295) in their studies on the metabolism of glucose-C14. This effect may be due to an activation of the aspartic-alpha-ketoglutarate transaminase or to low levels of oxalo-acetic obtained under this condition. This lack could result either from greater rates of activity of the acetyl coenzyme A condensing enzyme (formation of citrate) or of the malic enzyme (production of pyruvic acid and CO_2 from malic acid). These two enzymes are known to be activated by potassium (257,410). The apparently increased formation of alanine in presence of potassium is probably dependent on a greater rate of formation from pyruvate since there is little if any glutamic-pyruvic transaminase activity in the brain (73).

Comparison of the levels of amino acids found in tissue plus medium to those found in tissue alone, under the different metabolic conditions used, show that, aerobically, a greater proportion of the amino acid content is retained in the tissue in presence of potassium than in K-free conditions. This is particularly evident at 60 mMolar K where about 90% of the amino acid content of the vessels is found in the tissue.

Only alanine seems unaffected by the presence of potassium. Under anaerobic conditions the percentage of amino acid content retained in the slices is considerably lower and increasing the potassium concentration to 60 mMolar K reduces this percentage further. These results were to be expected if it is assumed that the maintenance of a concentration gradient of amino acid by the slices is dependant on the metabolic activity and the rates of formation of high energy phosphate compounds by the tissue. Yet, even under the anaerobic condition and in presence of high concentration of potassium (where the anaerobic glycolytic rate is markedly depressed) about 30% of the amino acid content was found in the tissue. This may indicate that the binding or occlusion of amino acids is not completely dependent on metabolism.

Chapter IX

Discussion

The experimental work reported in the previous chapters was undertaken and carried out with the purpose of determining the metabolic effects on brain metabolism of the low concentrations of potassium (from 3 to 10 mMolar) normally present in extracellular biological fluids and in the standard saline media used for incubation of tissue slices in vitro, of comparing these metabolic effects with those occurring when brain slices are incubated in saline media containing high concentrations of potassium, and of studying the relationship existing between potassium uptake in, and metabolic influence of potassium on, brain slices.

Theoretically, potassium may act on (1) the plasma membrane of brain cells, possibly increasing the permeability to glucose and other substrates of oxidation, (2) the structure and permeability of intracellular particles, (3) specific enzyme sites of the glycolytic and / or oxidative pathways, (4) the complex mechanisms of control of the metabolism, by altering the ratio of ATP/ADP through an influence on either the formation or maintenance or utilization of the high energy phosphate compounds.

The experimental data which have been reported in the previous chapters did not provide direct evidence which would permit to describe precisely the mechanism of action of potassium. However, this investigation provided indications concerning some of the points of interference of potassium in

brain tissue metabolism. Amongst the experimental results which were obtained those which appear particularly significant in relation to this problem will be reviewed and discussed in this chapter.

Metabolic effects of low, physiological, concentrations of extracellular potassium.

The stimulatory action of low concentrations of potassium in the medium on the oxygen consumption rate of brain slices does not appear to be related to a stimulation of the rate of penetration of glucose into brain cells either by specific activation of the rates of phosphorylation by the phosphohexokinase enzyme of the membrane or by an unspecific alteration of the membrane permeability. This conclusion is suggested by the fact that, in the absence of potassium, the aerobic rate of formation of lactic acid from glucose is very rapid and by the fact that a calculation indicates that the amount of glucose utilized is greater in the absence of potassium (about 72 micromoles per gram of tissue per hour) than in the presence of 3.6 mMolar K (about 45 micromoles per gram per hour). (The calculated values were obtained from the rates of formation of lactic acid and from the rates of oxygen uptake and assuming that 6 micromoles of oxygen were consumed for each micromole of glucose oxidized). The same conclusion is reached even if only the increase of the O_2 uptake rate, above the autoxidation, that occurs when glucose is added is assumed to be due to the oxidation of glucose.

We obtained a good deal of evidence suggesting that the influence of low concentrations of potassium on metabolism is actually not primarily dependant on the potassium content of the medium of incubation and, consequently, that it is not related to an external action of potassium on the brain cells plasma membranes.

It was shown that, at small external concentrations of potassium (between 0 and 20 mMolar) the increase in oxygen uptake induced by the potassium is approximately proportional to the potassium content of the tissue and not to the external concentration of potassium. Moreover it was found that all metabolic conditions or agents which do not support, or which inhibit, the uptake and retention of potassium in brain slices also diminish or block (to an approximately proportional extent) the influence of potassium on the rate of oxygen consumption. This situation prevails in the absence of substrate of oxidation, in the presence of substrates which do not support to an appreciable extent the oxygen consumption or are not readily oxidized, in the absence of external sodium (replaced by sucrose), in the presence of various metabolic inhibitors, or in the presence of agents which are not known to affect primarily the metabolic activity but directly influence potassium uptake and retention in brain slices, like protoveratrine, protamine and ganglioside preparations.

These data strongly support the view that potassium, in low external concentrations, influences the metabolic activity to the extent that it is accumulated or retained by the tissue.

The lack of direct effect of external potassium on brain tissue metabolism contrasts markedly with the situation prevailing in yeast. Rothstein (332) has shown that the stimulation by potassium of fermentation by yeast cells is independant of the uptake of this ion by the cells and the same author suggests that the action of potassium is exerted at the external surface of the membrane, presumably activating the phosphohexokinase enzyme. The hexokinase of the liver (182) is also known to be activated by potassium but no information is available concerning the brain enzyme.

In spite of the fact that potassium is known to increase the activity of the ADP-phosphoenolpyruvic transphosphorylase (40), which transforms phosphoenolpyruvic acid into pyruvic acid, it is doubtful that the stimulatory action of small external concentrations of potassium is associated with the activation of any enzyme of the glycolytic pathway. The extremely active aerobic formation of lactic acid clearly shows that, in K-free conditions, the mechanisms responsible for the breakdown of glucose, far from being impaired, operate at maximal speed.

Addition to the saline medium of small concentrations of potassium provokes a marked fall of the glycolytic rate coinciding with increased rates of oxygen uptake (and apparently lower rates of glucose utilization, as previously mentioned). These phenomena are very probably the result of the operation of a mechanism of metabolic control which, under normal circumstances, limits the aerobic utilization of glucose,

namely the Pasteur effect. Balazs (21), and Balazs and Richter (22) have recently reviewed this complex problem and suggested that the Pasteur effect cannot be explained only by a direct action of oxygen on the activity of the triose phosphate dehydrogenase enzyme but involves also the phosphorylating reactions and the levels of high energy phosphate compounds. Our results indicate that the presence of small external concentrations of potassium is necessary for this mechanism to operate in brain slices.

Experimental data obtained in our study provide indirect evidence supporting the view that potassium, at low concentrations, may exert its stimulatory action on the oxygen consumption partly through a direct activation of some oxidative reactions: the formation of acetyl coenzyme A and the transamination of gamma-aminobutyric acid.

The possibility that the formation of acetyl coenzyme A may be a rate-limiting point of the oxidation of glucose in the absence of potassium is supported by the fact that, under this condition, provision of large quantities of exogenous pyruvate (added together with glucose to the saline media) significantly increases the low rate of oxygen uptake normally obtained when glucose alone is present as substrate of oxidation. Moreover, when added alone to the potassium-free saline medium, pyruvate increases to a greater extent than glucose the rate of oxygen uptake. A plausible explanation for these effects would be that the extra pyruvate suppresses to some extent the impairment due to the lack of potassium in the formation of

acetyl coenzyme A. Von Korff (409) has suggested that the formation of acetyl coenzyme A may be activated by potassium in cell-free systems. Kini and Quastel (222) obtained evidence suggesting that high concentrations of potassium increase the formation of acetyl coenzyme A from pyruvate in brain slices. The marked reduction by potassium of the rate of aerobic formation of lactic acid (see Figure 10) is perhaps related to the preferential oxidative removal of the pyruvate formed by glycolysis.

The possibility that the conversion of gamma-aminobutyric acid to succinic semialdehyde (and the subsequent oxidation of the latter) is also stimulated by potassium is suggested by the following data: (1) in the absence of potassium the level of gamma-aminobutyric acid was found to be high, (2) this level was significantly reduced in the presence of 3.6 mMolar K, (3) in the absence of potassium, addition of gamma-aminobutyrate to the saline medium had no effect on the rate of oxygen uptake, (4) it increased significantly this rate in the presence of 3.6 mMolar K. The activity of the serine transaminase enzyme (gamma-aminobutyric transaminase not tested) has been reported to be increased by potassium (167).

It is obvious that only a direct study on the influence of potassium concentration on the isolated enzyme systems responsible for formation of acetyl coenzyme A and for the conversion of gamma-aminobutyric acid into succinic semialdehyde would prove these assumptions. It is possible that the metabolic effects mentioned are not the results of a direct action of

potassium at the enzymatic level but are consequent to an influence of this ion on mechanisms controlling the oxidative activity of brain slices.

The acceleration of the rate of turn-over of the intermediates of the tricarboxylic acid cycle, and possibly changes in the levels of these compounds consequent to the effect of potassium on the respiration processes would result in marked changes in the levels of amino acids like glutamic, gamma-aminobutyric and aspartic acids.

The close relationship between rates of respiration and amounts of potassium accumulated and retained in brain slices and the mode of action on these two processes of various agents constitute particularly significant data concerning the mechanism of action of potassium on the metabolism of brain slices.

Previously in this discussion, it was mentioned that the metabolic action of potassium is dependant on the amounts of potassium taken up and retained in the slices rather than on the potassium concentration of the incubation medium. Under aerobic conditions and in presence of glucose an apparent potassium concentration gradient is built up (the ratio of the K concentration in tissue / K concentration in medium is approximately equal to 8.5 in presence of 3.6 mMolar K, in the saline medium). It appears as very likely that this K uptake in the tissue is energy-dependant. The presence, in the medium, of substrates of oxidation like glucose or pyruvate is required for this accumulation to occur. Several metabolic inhibitors were shown to inhibit this process. Moreover, there

is little doubt that not only the uptake of potassium but also the maintenance of the concentration gradient is energy-dependant since, even after the K uptake is completed and when a steady-state equilibrium is attained, metabolic inhibitors cause the release of an important fraction of the potassium tissue content.

These observations suggest that the energy expenditure required for the maintenance of the potassium concentration gradient may constitute the important factor which controls the metabolic activity of the tissue, rather than any direct action of potassium. It is well known that the metabolic activity of brain cells is markedly influenced by the intracellular levels of inorganic phosphate, of ADP and of ATP. An accelerated rate of hydrolysis of ATP, probably indirect, with production of ADP and inorganic phosphate may augment the rate of the phosphorylation reactions and increase the respiratory rate.

No information is available concerning the exact site of action of potassium. However, the results of experiments performed with protoveratrine, protamine and ganglioside preparations may provide indirect indications pertaining to this question. A careful analysis of the modes of interference of these agents with K uptake and K influence on metabolism strongly suggests that these interactions were taking place through displacements from common intracellular binding sites. The action of protamine and the reversal of its effect by ganglioside preparations, substances which are thought to have a structural rather than a metabolic role in brain tissue, support the view that sites of structural components are involved. The fact that higher concentrations of protamine were required

to produce potassium loss than for inhibition of potassium uptake is also in keeping with the hypothesis that the accumulated (and metabolically active) potassium is apparently bound in the tissue. In as much as the site of action of protoveratrine (352,354) and the sites of binding of protamine and of gangliosides (97,425) have been identified as membrane structures, cellular elements which are isolated by differential centrifugation techniques as "microsomal" fractions, it can be concluded that such are the sites of binding and of activity of potassium.

This conclusion is of considerable significance since it implies that the action of potassium does not occur at mitochondrial sites (where oxidative and phosphorylation processes are known to occur) and it is in keeping with the hypothesis previously expressed according to which the metabolic changes caused by potassium are the results of changes in the levels of high energy phosphate compounds rather than of a direct action on enzymic activity.

Recent works of Skou (362,363) Jarnefelt (204,205), Hokin (192,193), McIlwain (97) and others, which were reviewed in Chapter I (Part 2) have provided information concerning the importance of the microsomal subcellular particles in the processes of electrolyte transport and concerning the relationship of these processes with cellular metabolism.

It must be noted that under most experimental conditions tested, the increases in potassium content of brain slices were associated with roughly proportional decreases of the sodium content of the slices. No information was obtained

which would permit to assess the relative importance of each one of these two processes from the point of view of the metabolic changes simultaneously occurring. It is generally believed (see Part 2 of Chapter I) that the extrusion of sodium in many types of cells is an active process while the parallel potassium shifts are the result of a passive equilibrium secondary to the active extrusion of sodium. Our study and the previous work of Pappius and Elliott (313) indicate that sodium must be present in the system for potassium uptake and stimulation of the respiratory rate to occur.

Metabolic influence of high concentrations of potassium.

Increasing the potassium concentration in incubation media to 60 mMolar produces an increase of the rate of oxygen consumption and of the lactic acid formation. Under this condition about 73 micromoles of glucose per gram of tissue per hour are either transformed into lactic acid or oxidized. This rate is close to that obtained in the absence of potassium (72 micromoles/g. / hr.) and represents a 75% increase over the estimated rate obtained in 3.6 mMolar external potassium. These phenomena may be interpreted as a reversal of the Pasteur effect, since (as in the absence of potassium) the rate of anaerobic glycolysis is markedly inhibited by the high concentrations of potassium. These effects on the rates of anaerobic and aerobic glycolysis, brought by K-free conditions or by high concentrations of potassium are similar to those occurring in presence of 2,4-dinitrophenol and various other agents which have already been mentioned (Part 3 of Chapter I).

In the presence of high concentrations of potassium the oxidative reactions are presumably occurring at a very rapid rate. Addition of intermediates of the tricarboxylic acid cycle like malate and alpha-ketoglutarate further increases the rate of oxygen consumption. This may indicate that the level of some of the intermediates (like alpha-ketoglutarate and oxalo-acetate) is reduced and rate-limiting. Such situation could explain the fall in the levels of aspartic and glutamic acids observed under this condition.

The increased oxidative activity is possibly due to direct stimulation of the activity of some enzymes by potassium. Evidence has been presented by Kini and Quastel (222), supporting the view that the formation of acetyl coenzyme A is stimulated by high concentrations of potassium. Our study provided some experimental data indicating that the decarboxylation of glutamate and formation of gamma-aminobutyric acid is increased by high K concentrations. It was shown that 50 mMolar potassium increases by about 50% the rate of decarboxylation of glutamate by brain tissue homogenates. The possibility that high concentrations of potassium exert a similar effect in brain slices is suggested by the following data.

In the absence of glucose addition of glutamate to the saline medium, which has little effect on the rate of oxygen uptake in the absence of potassium, markedly increases this rate in presence of 60 mMolar K. In presence of 60 mMolar K, the level of glutamic acid is significantly decreased and that of gamma-aminobutyric acid, increased. Semicarbazide, which is known (218b) to inhibit the activity of the glutamic decarboxylase

enzyme *in vivo*, completely antagonizes this effect.

The fact that the stimulation of aerobic metabolism by high concentrations of potassium does not occur with homogenates is not necessarily an indication that the metabolic influence of high potassium concentration is directed towards the cellular plasma membrane and not towards specific oxidative enzymes. With disrupted cells, specific effects of potassium on individual oxidative reactions may not be reflected on the rates of general metabolism because of the loss of integration of the cellular unit.

Stimulation of the formation of acetyl coenzyme A from pyruvate, or of the formation of gamma-aminobutyric acid from glutamic acid, by high potassium concentrations could occur without affecting the overall metabolism if the integration of these processes with the remainder of the metabolic systems was disrupted. Both the formation of pyruvate and that of gamma-aminobutyric acid (255b) are known to occur mainly in the cell sap. The oxidation of pyruvate and that of gamma-aminobutyric acid occur in mitochondria. Hence these two systems require the integrity of the cellular organization in order that any changes in their activity be reflected in the general metabolic activity of the tissue. Verification of this hypothesis could be achieved by testing whether high concentrations of potassium can increase the oxygen uptake rates of mitochondria incubated in presence of pyruvate or of gamma-aminobutyric acid.

Some of the hypotheses which have been put forward as explanations of the Ashford and Dixon effect were invalidated.

Following an observation of Buchel (48) which showed that removal of calcium from the incubation medium brings an increase in the oxygen uptake rate of brain slices comparable in magnitude to that obtained by addition of high concentrations of potassium, it was suggested (323) that "the stimulating effect is due to increase in the K/Ca ratio rather than to the absolute magnitude of the potassium concentration". Our results indicate that the stimulatory action of high concentrations of potassium occurs as well in the presence as in the absence of calcium. The effect is proportionally greater in the presence of calcium. The influence of potassium on the rate of aerobic glycolysis is not affected either by calcium. However the inhibitory action of high K concentrations on anaerobic glycolysis does not occur in the absence of calcium.

McIlwain (271) has suggested that addition of high concentrations of potassium in the incubation medium would greatly increase the expenditure of energy necessary for the maintenance of a high ratio of intracellular to extracellular K concentrations and that the metabolic changes observed result from this greater demand for energy. We have previously mentioned that such a mechanism may explain the metabolic effects occurring at low, physiological, external concentrations of potassium since, under this condition, concentration gradients of potassium and of sodium are maintained. But when the external concentration of potassium is increased to 60 mMolar the potassium concentration gradient is markedly reduced. Moreover, as in the absence of potassium, in presence of 60 mMolar K the difference between the sodium concentration in the

saline medium and that in the tissue is very small or equal to zero. These facts do not support the hypothesis of McIlwain. It is interesting to note that electrical stimulation, which produces many metabolic changes ressembling those obtained in presence of high K concentrations, also markedly reduces the potassium content of brain slices (31).

The numerous differences which appear when comparing the metabolic effects of low and high external concentrations of potassium support the conclusion that potassium acts in two basically different ways. Consequently, the assumption, which is commonly made, that the effects of high concentrations of potassium are the result of a stimulation of metabolic reactions normally occurring at a lower rate in standard physiological saline media cannot be supported.

Several authors (106,126,271) have observed that the metabolic changes induced by high potassium concentrations are accompanied by a depletion of the stores of energy-rich phosphate compounds of brain slices. The fact that 2,4-dinitrophenol (DNP) can mimic many of the metabolic changes brought about by potassium (see 106) is in keeping with the hypothesis according to which the metabolic effects of high potassium concentrations are associated with and perhaps the result of an effect of uncoupling of the phosphorylation processes. Our results indicate that, in the presence of 3.6 mMolar K, DNP stimulates the respiratory rate as well as does the addition of 60 mMolar K, and this drug does not further increase the potassium (60 mMolar) stimulated rate.

These observations, the abnormality revealed by the reversal of the Pasteur effect, the reduced electrolyte concentration gradients, and various experimental findings reported in the literature (reviewed in Part 3 of Chapter I) which indicate that some energy-dependant or synthetic processes normally occurring in brain slices are impaired (decreased glycogen synthesis (226), decreased rate of phosphorus incorporation (126), increased swelling,310), suggest that the metabolic pattern occurring in the presence of a high external potassium concentration is more representative of an impaired metabolism than of the situation normally prevailing in brain <u>in vivo</u>.

Summary

An incubation of one hour at 0°C in an oxygenated K-free saline medium causes an almost complete depletion of the potassium content of brain cortex slices without seriously impairing the metabolic capacities of the tissue during subsequent re-incubation at 38°C under good metabolic conditions. This pretreatment permitted the study of the influence on metabolism of the low concentration of potassium normally present in extracellular fluids and in the standard saline media used for incubation of tissue slices in vitro and the relationship between potassium uptake in, and the metabolic influence of potassium on, brain slices.

In the presence of glucose, the low and falling respiratory rate of K-depleted brain slices incubated under K-free conditions is stabilized and stimulated by the addition of 3.6 mMolar potassium to the saline medium, while the rate of aerobic glycolysis is markedly reduced and the anaerobic breakdown of glucose is stimulated. The respiratory activity of brain slices incubated in the presence of 3.6 mMolar K was shown to be very sensitive to the stimulatory or inhibitory action of low concentrations of various drugs and chemical agents. By comparison with the levels obtained under K-free conditions, incubation of slices in the presence of low concentrations of potassium increases the total level of aspartic acid and alanine in tissue and medium and decreases that of gamma-aminobutyric acid. Potassium almost completely stops the diffusion into the incubation medium of the endogenous amino acids.

In the presence of 3.6 mMolar potassium, a rapid uptake of potassium occurs (also an approximately equal extrusion of sodium).

The increase in oxygen uptake induced by low external concentrations of potassium (from 3 to 20 mMolar) is closely proportional to the potassium content that develops in the tissue and not to the external concentration of potassium. Various metabolic conditions which do not support or inhibit the uptake and retention of potassium also diminish or block, to an approximately proportional extent, the influence of potassium on the rate of oxygen consumption. This situation prevails in the absence of substrate of oxidation or in the presence of substrates which are not readily oxidized, in the absence of external sodium and in the presence of various metabolic inhibitors or of agents which primarily influence the electrolyte transport processes, such as protoveratrine, protamine and gangliosides.

In view of these data it was concluded that potassium, in low external concentrations, influences the metabolic activity to the extent that it is accumulated or retained by the tissue and that the accumulated (and metabolically active) potassium is apparently bound in the tissue. It was suggested that the energy expenditure (utilization of high energy phosphate) required for the accumulation and retention of potassium in brain slices may constitute the important factor which controls the metabolic activity rather than a direct action of potassium at some enzyme sites.

High concentrations of potassium increase the respiratory rate further, stimulate the anaerobic glycolytic rate and, according to whether calcium is present or not, inhibit or further increase the anaerobic glycolytic rate. Calcium lowers the respiratory and the aerobic glycolytic activity but does

not alter the influence of potassium on these metabolic rates. In the presence of high external concentrations of potassium the apparent potassium and sodium concentration gradients maintained between tissue and saline medium are markedly reduced. High concentrations of potassium decrease the tissue level of glutamic and aspartic acids and increase the level of gamma-aminobutyric acid. Potassium (50 mMolar) stimulates the decarboxylation of glutamic acid by brain homogenates.

From these data it was concluded that potassium acts in two basically different ways, according to whether it is present in low or high external concentration, and the possibility that the effect of high K concentration is exerted at some enzyme sites or through an alteration of the levels of high energy phosphate compounds was discussed.

It was shown that between 25 and 50% of the gamma-aminobutyric, glutamic acid, glutamine, aspartic and other amino acids in brain is bound or occluded (i.e. not extracted by homogenization in Ringer-type saline media but released by heat or by extraction with ethanol). Homogenization of tissue in salt-free solution or in saline in which a fraction of the sodium is replaced by potassium causes the release of more than 75% of the bound amino acids. Other factors affecting this occlusion were studied.

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Claims to Original Research

1. Brain cortex slices incubated at 0°C in an oxygenated K-free saline medium lose more than 90% of their potassium content and gain an approximately equivalent amount of sodium.
2. When re-incubated at 38°C in a glucose-containing K-free saline medium, the K-depleted brain slices have a relatively low respiratory activity which falls with time. The aerobic rate of lactic acid formation is very high; the anaerobic glycolytic rate is low. After an hour of incubation under such conditions the sodium contents of tissue and of saline medium remain approximately equal. A considerable fraction of the endogenous amino acid content of the tissue is found in the incubation medium. The oxygen uptake rate of K-depleted slices incubated under K-free conditions is practically insensitive to the stimulatory or inhibitory action of low concentrations of a number of drugs and chemical agents (which are mentioned under no. 4).
3. Addition to the incubation medium of 3.6 mMolar K stimulates and stabilizes the respiratory rate at 38°C of K-depleted slices, inhibits to a large extent the aerobic formation of lactic acid from glucose and stimulates the anaerobic glycolytic rate. The total level of aspartic acid and alanine in tissue and medium is increased and that of gamma-aminobutyric acid decreased. Potassium almost completely stops the diffusion into the incubation medium of the endogenous amino acids.
4. The respiratory activity of K-depleted brain slices incubated in the presence of 3.6 mMolar potassium is very

sensitive to the stimulatory action of 2,4-dinitrophenol and of protoveratrine and to the inhibitory action of malonate, pentobarbital, phenobarbital, chlorpromazine, atropine, procaine, protamine and of protoveratrine (when this last drug is added to the medium before potassium). The inhibitory effect of protamine is reversed by gangliosides.

5. In the presence of glucose and of 3.6 mMolar potassium, a rapid uptake of potassium by K-depleted slices occurs and an approximately equal extrusion of sodium.
6. In the absence of substrate of oxidation, or in the presence of substrates which are not readily oxidized, or in the absence of external sodium, or in the presence of various metabolic inhibitors or of agents which primarily influence the electrolyte transport processes, namely protoveratrine, protamine and gangliosides, the stimulatory action of 3.6 mMolar K on respiration and the potassium uptake in the tissue are affected to approximately proportional extents.
7. The increase of the oxygen consumption rate induced by low external concentrations of potassium (from 3 to 20 mMolar) is closely proportional to the potassium concentration which develops in the tissue during the first few minutes.
8. Whether potassium is present or not calcium reduces the respiratory and aerobic glycolytic rates of K-depleted brain slices. Calcium does not alter the influence of potassium concentration on the aerobic metabolic rates. The classical inhibitory action of high concentrations of potassium on the rate of anaerobic glycolysis is not obtained in the absence of calcium.

9. High concentrations of potassium decrease the level of glutamic acid (also of aspartic acid) in brain slices and increase the level of gamma-aminobutyric acid. Semicarbazide exerts the opposite effect. Potassium (50 mMolar) was shown to increase the decarboxylation of glutamic acid by brain homogenates.
10. The apparent potassium and sodium concentration gradients between tissue and medium which are found at low external concentrations of potassium are markedly reduced in the presence of a high external concentration of potassium.
11. A considerable fraction of the chromatographically determined gamma-aminobutyric acid, glutamic acid, glutamine, aspartic and other amino acids of brain is not immediately extractable into free solution with cool Ringer-type saline media and remains bound or occluded in the residue fraction after homogenization and centrifugation at 15 or 105,000 x g for 30 minutes.
12. Homogenization of tissue in salt-free solutions, or in saline in which a fraction of the sodium is replaced by potassium, causes the release of more than 75% of the bound amino acids.