THE EFFECTS OF LITHIUM ON ADENOSINE TRIPHOSPHATASES AND ION TRANSPORT WITH REFERENCE TO AFFECTIVE ILLNESS

by

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"Therefore, go forth, companion: when you find
No highway more, no track, all being blind
The way to go shall glimmer in the mind."

(John Masefield)

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CONTENTS.

Statement in terms of Ph. D. regulation 2.4.11 of the University of Edinburgh.

Certain parts of the work described in this thesis have been published previously or are at present awaiting publication:

(1) A K^+ like effect of Li^+ on sodium transport from blood into cerebrospinal fluid. Glen, A.I.M. and Hesketh, J.E. (1975) J. Physiol. 252, 83-84P

(2) Effects of potassium and lithium on sodium transport from blood to cerebrospinal fluid. Hesketh, J.E. (1976) J. Neurochem., accepted for publication.

(3) Changes in membrane adenosine triphosphatases on administration of lithium salts in vivo. Hesketh, J.E. (1976) Biochem. Soc. Trans. 4, 328-330

(4) Effects of lithium on rat brain synaptosomal ATPases. Hesketh, J.E., Kinloch, N. and Reading, H.W. (1977) Proceedings of a symposium on The Synapse held in St. Andrews, April 1976. To be published.

(5) Determinations of lithium concentration in striatal and non-striatal dopamine systems by flameless atomic absorption spectrophotometry. McGovern, A.J., Arbuthnott, G.W., Hesketh, J.E. and Glen, A.I.M. (1977) Proceedings of a symposium on Striatal and Non-Striatal Dopamine held in Cagliari, Sardinia, May 1976. To be published.

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I declare that this thesis was totally composed by myself and that all the work described herein was initiated by myself. All the experimental work was carried out by myself with the following exceptions:

a) measurement of dopamine and tyrosine hydroxylase activity was done by Mrs. A. Wright.

b) measurement of HVA and DOPAC was done by Mr. N. Nicolaou.

c) subcellular fractions were processed for electron microscopy by Mr. A. Ross and co-workers.

d) measurement of blood pH, pCO_2 and pO_2 was done by Mr. P. Whelpdale.

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NOTES AND ABBREVIATIONS.

Note: throughout this thesis statistical probability values were calculated using two-tailed significance tests.

Abbreviations: Abbreviations used in this thesis are mostly those in common scientific usage and the more unusual are introduced in the text. A list of some of the abbreviations used is also given below:

C.S.F.	cerebrospinal fluid
ATP	adenosine 5'-triphosphate
ATPase	adenosine triphosphatase
Na/K ATPase	sodium and potassium stimulated, magnesium dependant ATPase
Mg ATPase	magnesium dependant ATPase
Ca + Mg ATPase	total magnesium dependant and calcium stimulated, magnesium dependant ATPase
Ca ATPase	calcium stimulated, magnesium dependant ATPase
C.P.M. or c.p.m.	counts per minute
HVA	homovanillic acid
DOPAC	3,4 dihydroxyphenylacetic acid
EDTA	ethylenediaminetetraacetic acid
Tris	2-Amino-2-hydroxymethylpropane-1,3diol
P_i	inorganic phosphate
B.D.H.	British Drug Houses Ltd.

ABSTRACT.

The thesis describes investigations concerning the effects of lithium on ATPases and ion transport. Effects of potassium and lithium on sodium transport from blood to cerebrospinal fluid and brain were studied using a ventriculo-cisternal perfusion technique. The fast component of sodium entry into cerebrospinal fluid was correlated with the cisternal potassium concentration. This together with results showing the potassium-dependance of choroid plexus Na/K ATPase activity was interpreted as showing choroid plexus sodium transport to be sensitive to cerebrospinal fluid potassium concentration and to be due to Na/K ATPase activity in the apical membranes of the plexuses. Effects of lithium on sodium transport were interpreted as a stimulation of the sodium-pump when presented to the potassium-sensitive side and an inhibition when presented to the sodium-sensitive side.

The effects of chronic lithium administration on ATPase activities were investigated in subcellular fractions prepared from rat cerebral cortex. Lithium was found to have no effect on synaptic plasma membrane Na/K ATPase activity but Mg ATPase specific activity was increased both in the membrane fraction and in mitochondria, after 21 and 7 days administration respectively. The changes in enzyme specific activity were not due to changes in fraction composition. Lithium administration for 21 days caused an increase in the concentration of homovanillic acid and

3,4dihydroxyphenylacetic acid in the striatum. This was interpreted as showing an increased release and turnover of dopamine.

ATPase activities were also studied in erythrocyte membrane preparations. Depressive patients were shown to have a reduced erythrocyte membrane Na/K ATPase specific activity compared to controls. It is suggested that such a reduced enzyme activity could explain many of the previously observed derangements of sodium metabolism in depression. Lithium-treated patients showed increased specific activities of both Mg ATPase and Na/K ATPase in erythrocyte membranes. It is suggested that the increase in Na/K ATPase activity was due to recovery of the patients but that the increase in Mg ATPase activity was due to lithium itself. Lithium administration had no effect on erythrocyte membrane ATPase activities in the rat. It is concluded that chronic lithium administration affects the specific activities of certain Mg ATPases but not the Na/K ATPase. It is speculated that the changes in membrane Mg ATPase activities reflect changes in actomyosin-like proteins which could lead to increases in the release of neurotransmitter in the brain.

SECTION I

INTRODUCTION.

In recent years lithium carbonate has become an important drug for the treatment of affective illness. Within the environs of Edinburgh for example, there are some 500 people out of a total population of approximately 500,000 receiving lithium carbonate treatment. The wide and increasing use of lithium salts in psychiatry has stimulated research for two main reasons. Firstly the ingestion of large quantities of an unnatural inorganic salt over long periods must be expected to produce marked metabolic disturbance and therefore research into the toxic effects of lithium treatment (Vacaflor, 1975) and the mechanisms involved may be of some value. Secondly, lithium salts have unique clinical properties and therefore an understanding of the mode of action of lithium in the treatment of affective disorders may produce some clues as to factors in the aetiology of these illnesses.

The first indication of the possible use of lithium salts in psychiatry is found in the writings of Soranus of Ephesus, who in the 5th century A.D. advocated alkaline mineral waters for the treatment of manic excitement (Johnson and Cade, 1975). Subsequently it has been noticed that several spa waters have a high lithium content (Johnson and Cade, 1975). The first medicinal use of lithium salts themselves was in the treatment of gout in the 19th century on account of the high solubility of lithium urate. In retrospect it has been pointed out that in certain cases of gout-associated depression lithium salts seemed to have

some beneficial effect on the mental symptoms (Schou, 1957; Johnson and Cade, 1975). Lithium bromide had a brief use as an hypnotic and anti-epileptic in the 1920's but this was not sustained. It was not until 1949 that Cade made the discovery that lithium salts had a tranquilizing effect when given to guinea-pigs and were efficacious in the treatment of mania (Cade, 1949). Cade's original observation has been followed by a series of clinical trials using lithium carbonate for the treatment of mania and depression. It is now generally accepted that lithium carbonate is an effective treatment for patients suffering from mania but its effectiveness in the treatment of depression is still equivocable (Coppen, 1973; Schou, 1973; Mendels, 1975; Peet, 1975).

The term affective illness includes both depressive and manic-depressive illness. The former is also referred to as unipolar depressive illness and the latter as bipolar depressive illness since it is characterised by both manic and depressive episodes. Both types of depressive illness are recurrent in that untreated episodes will remit spontaneously and then after a period of time the patient will relapse (Coppen, 1973). There is evidence (Coppen, 1973) that the interval between episodes decreases as the patient suffers successive episodes. Clinically, the most important effect of lithium salts is their ability, on prolonged administration, to reduce the morbidity of an affective illness either by reducing the

severity of an episode, by reducing the frequency of episodes or by abolishing future episodes (Coppen, 1973; Schou, 1973; Schou and Thomsen, 1975). Such a prophylactic action is unique in psychiatry.

Research into the mode of action of lithium salts is hampered by the lack of understanding of the biological processes leading to mania and depression. Theories of the aetiology of the affective disorders have centered on the involvement of disturbances in either electrolyte metabolism (Coppen, 1967; Baer, Platman and Fieve, 1970c) or the metabolism of the catecholamines and indolamines (Schildkraut, 1965; M.R.C. Brain Metabolism Unit, 1972).

Studies of potassium, calcium and magnesium metabolism in affective illness have not provided any consistent findings (Mullin, 1975). However there is considerable evidence for a disturbance of sodium metabolism in affective illness. Using salt loading techniques early workers found depressed patients to retain more sodium (Klein and Nunn, 1945; Klein, 1950). These studies also showed water and sodium balance to fluctuate with mood. Later studies showed reduced sodium excretion to be associated with depression in normal subjects and increased sodium excretion with manic phases of affective illness (Schottstaedt, Grace and Wolff, 1956a, b; Strom-Olsen and Weil-Maherbe, 1958). Contrary to these reports Russell (1960) found no change in total sodium balance. Further sophistication of experimental approach came with the use of tracer and isotope

dilution techniques. Although the interpretation of the results from such studies is fraught with problems (Shaw, 1971) they provide an approach to the study of electrolyte distribution in vivo. Using isotopic techniques Gibbons (1960) found a decreased exchangeable sodium in depressed patients, although no change was found on recovery of the patients. The lack of effect of recovery was confirmed by a later study but little can be deduced from the results since patients were examined only one day after receiving electroconvulsive therapy (Coppen, Shaw and Mangoni, 1962). A more extensive study examining patients five weeks after the beginning of treatment and using isotopic bromide to estimate extracellular space found recovery to be associated with an increase in total body water, a decrease in 24-hour exchangeable sodium and a decrease in the ~~non-exchangeable or~~ residual sodium (Coppen and Shaw, 1963). Residual sodium was considered to consist of two components, a component of slowly exchanging sodium in bone and sodium in the intracellular compartment of the body. On the basis of these results Coppen and Shaw suggested that depression was associated with an abnormality in sodium transport which resulted in an increase in the intracellular concentration of sodium. This hypothesis gained support with the finding that residual sodium was high both in depressed and manic patients (Coppen, Shaw, Malleson and Costain, 1966; Cox, Pearson and Speight, 1971). A further study found recovery to be associated with a decrease in residual sodium but the

effect did not reach statistical significance (Baer, Durell, Bunney, Levy, Murphy, Greenspan and Cardon, 1970a). Results from studies of exchangeable sodium have failed to find any consistent pattern (Jenner, Gjessing, Cox, Davies-Jones, Hullin and Hanna, 1967; Baer et al., 1970a; Cox et al., 1971). In conclusion balance and isotopic studies, although not entirely consistent, have shown depression to be associated with a retention of sodium and an increase in the non-exchangeable or residual sodium. Such results have been interpreted as showing an abnormality in sodium transport which causes an increase in intracellular sodium concentration (Coppen and Shaw, 1963; Coppen, 1967).

Various experimental approaches have been used to investigate sodium transport in depressed patients and to test the above hypothesis. Analysis of post-mortem brain from depressive suicides showed increased water content and increased sodium concentration compared to control brain tissue (Shaw, Frizel, Camps and White, 1969). By a most tentative argument these results were interpreted as showing an increased intracellular sodium concentration. However interpretation of such results in terms of intracellular and extracellular compartments is dubious and the results therefore do not provide strong evidence to support the abnormal sodium transport hypothesis. In further attempts to prove or disprove this hypothesis various workers have studied the transport of isotopic sodium from blood to lumbar cerebrospinal fluid. The results of such studies

have been contradictory. Coppen (1960) found depressed patients to have a decreased rate of sodium entry and to show an increase in transport on recovery. Later studies failed to repeat these findings (Fotherby, Ashcroft, Affleck and Forrest, 1963; Carroll, Steven, Pope and Davies, 1969) although Fotherby et al. did find reduced sodium transport in four cases of severe depression. More recently Baker (1971) found sodium entry into cerebrospinal fluid to be reduced in both manic and depressed patients compared to scizophrenics and other miscellaneous controls. Combining results from his own study and from all the previous studies on sodium entry into cerebrospinal fluid Carroll (1972) concluded that sodium entry into cerebrospinal fluid was significantly reduced in manic and depressed patients and that it increased significantly on recovery. In conclusion, there is considerable evidence that depression is associated with a decreased sodium transport into cerebrospinal fluid. Further evidence for abnormal sodium transport in depression came from a study of salivary sodium concentration in depressed patients (Glen, Ongley and Robinson, 1968). These workers found depressed patients to have an increased sodium activity in saliva compared to controls and they interpreted these results as showing a reduced active transport of sodium from saliva.

In recent years use has been made of the erythrocyte in order to study intracellular sodium concentration and

sodium transport in depressed patients. However once again the results have been contradictory. Erythrocyte sodium concentration was reported to be lower in depressed patients than in controls (Naylor, McNamee and Moody, 1970a; Mendels, Frazer and Secunda, 1972) but other studies have failed to confirm this (Naylor, McNamee and Moody, 1971). Naylor and colleagues found erythrocyte sodium concentration to decrease as patients recovered from depression (Naylor, McNamee and Moody, 1971; Naylor, Dick, Dick, Le Poidevin and Whyte, 1973) but this was not confirmed by Mendels (Mendels, Frazer, Secunda and Stokes, 1971; Mendels et al., 1972). Overall there is no consistent evidence for an abnormal erythrocyte sodium concentration associated with depression. Studies of active sodium transport in the erythrocyte showed no difference between depressed patients and controls (Naylor, McNamee and Moody, 1970b). These authors did find the passive permeability of the erythrocytes from depressed patients to be lower than that found in four control subjects. In addition Naylor found active sodium transport to increase as the patients recovered from depression. Other workers have found reduced active sodium transport in erythrocytes from manic-depressive patients during the manic phase of illness (Hokin-Neaverson, Spiegel and Lewis, 1974). Earlier studies showed changes in sodium metabolism in mania to be similar to those found in depression (Coppen et al., 1966; Glen et al., 1968). It is possible therefore that there is a common electrolyte

abnormality in mania and depression. There are many reports suggesting that a reduced active transport of sodium may be the abnormality in question but the evidence is far from unequivocable.

Since active sodium transport is considered to be brought about by the activity of the enzyme sodium plus potassium activated, magnesium dependant adenosine triphosphatase (Whittam and Wheeler, 1970) changes in active sodium transport might be expected to be reflected by changes in sodium plus potassium activated, magnesium dependant adenosine triphosphatase (Na/K ATPase) activity. Such considerations have led to a series of studies of Na/K ATPase activity in erythrocyte material from depressed patients. The results of such studies have shown the ATPase activity to increase as patients recovered from depression (Naylor et al., 1973) as expected if depression is associated with reduced sodium transport which increases on recovery. Naylor and colleagues (1973) suggested that depression was associated with a decrease in Na/K ATPase activity, a decrease in active sodium transport and an increase in intracellular sodium concentration. However, on their own admission, their results did not provide evidence for such a hypothesis. Hokin-Neaverson et al., (1974) suggested that manic-depression was associated with a genetic Na/K ATPase abnormality but there is no evidence for such a view. At present there is no evidence to suggest whether the putative sodium transport abnormality

in depression is an event in the aetiology of the illness or a result of hormonal and other metabolic changes occuring in depression.

With the advent of the use of lithium salts in depression further evidence has been put forward to support the concept of a membrane transport defect in depression. Several investigators have found lithium to be retained to a greater extent in mania and depression than in recovered or control states (Noack and Trautner, 1955; Greenspan, Green and Durell, 1968; Hullin, Swans, McDonald and Dransfield, 1968). As discussed by Greenspan (1975) this increased retention may reflect an increased intracellular concentration of lithium. Some evidence for such a view has come from erythrocyte studies. Mendels and Frazer (1973) found a high intracellular erythrocyte concentration to be associated with response to lithium therapy and they suggested there may be a difference in membrane transport between patients who respond to lithium and those that do not. Other studies have found higher and lower erythrocyte lithium concentrations in manic-depressive patients compared to controls (Elizur, Shopsin, Gershon and Ehlenberger, 1972; Lyttkens, Soderberg and Wetterberg, 1973) so the evidence concerning lithium distribution in mania and depression is not unequivocable. In order to study the effects of genetically-determined electrolyte distribution on lithium distribution Mendels and colleagues examined lithium distribution <u>in vitro</u> and <u>in vivo</u> in erythrocytes from sheep

known to differ in erythrocyte sodium and potassium distribution (Schless, Frazer, Mendels, Pandey and Theodorides, 1975). Lithium concentration was found to be higher in the low potassium, high sodium concentration erythrocytes. These results show that a genetically-determined difference in membrane properties can produce a difference in lithium distribution. A monozygotic-dizygotic twin study provided some evidence that lithium distribution in man was genetically determined (Dorus, Pandey and Frazer, 1974). On the basis of such results it has been suggested that some depressives have a genetically determined abnormality in the cation transport system and the erythrocyte membrane (Mendels and Frazer, 1974; Schless et al., 1975).

In conclusion there have been many studies suggesting a membrane or cation transport abnormality in depression but the experimental evidence is not totally in support of such hypotheses. However these ideas have generated much research into the effects of lithium on cation distribution and transport.

Observations on lithium-treated patients showed 24-hour exchangeable sodium to be increased (Baer et al., 1970a; Aronoff et al., 1971) although this was not confirmed by earlier studies (Coppen, Malleson and Shaw, 1965; Coppen and Shaw, 1967). Animal studies have subsequently showed that chronic lithium administration leads to a decrease in the brain sodium concentration (Baer, Kassir and Fieve, 1970b; Ho, Gershon and Pinckney, 1970; Birch and Jenner, 1973).

These animal and human studies led to suggestions that lithium might exert its therapeutic action through effects on sodium metabolism. In view of the role of the Na/K ATPase in the active transport of sodium out of the cell (Whittam and Wheeler, 1970) later hypotheses have centered around this enzyme. Early physiological and biochemical experiments used lithium either as a sodium substitute or as an additional alkali metal with which to study enzyme specificity. Such studies, although indicating possible pharmacological actions of lithium salts, used high lithium concentrations between ten and a hundred fold greater than the concentrations found in vivo when lithium is used therapeutically. These early studies showed lithium to possess a weak stimulatory effect on the Na/K ATPase in the absence of potassium (Bader and Sen, 1962; Whittam and Ager, 1964). Similarly in the absence of potassium lithium stimulated sodium efflux (Keynes and Swan, 1959; Beauge and Ortis, 1970). However in the presence of potassium replacement of sodium by lithium led to decreased sodium efflux (Keynes and Swan, 1959; Baker, Blaustein, Keynes, Manil, Shaw and Steinhardt, 1969). Willis and Fang (1970) found lithium (30 - 50 mM) to replace both sodium and potassium in Na/K ATPase activity in squirrel kidney cortex slices. In addition they described an ouabain-sensitive lithium dependant respiration in the absence of potassium. As the pharmacology of lithium gained interest lower concentrations of lithium (2 - 10 mM) were used in a series

of studies of ATPase activity and sodium transport. 3 mM lithium was shown to stimulate sodium efflux from the erythrocyte at sub-optimal (2 mM) potassium concentrations (Glen, Bradbury and Wilson, 1972) and these authors suggested such a stimulatory action on sodium transport might be involved in the therapeutic actions of lithium. In support of this suggestion low lithium concentrations were reported to stimulate Na/K ATPase activity at potassium concentrations below 2 mM (Tobin, Akera, Han and Brody, 1974). In addition Ploeger (1974a) has shown 10 mM lithium to have a weak stimulatory action on the sodium-pump (the active sodium transporting system associated with Na/K ATPase activity) in a rat vagal nerve preparation. There are also some reports of lithium, *in vitro*, having a weak stimulatory effect on brain and kidney Na/K ATPase activity in the presence of high, saturating concentrations of potassium (Gutman, Hochman and Wald, 1973b; Tobin *et al.*, 1974). Gutman *et al.* suggested lithium to uncouple the ATPase, that is to stimulate the enzyme activity whilst decreasing the transport of sodium and potassium. Overall these studies provide evidence that lithium can interact with the potassium-site of the Na/K ATPase and stimulate the enzyme. However there is little evidence to support the hypothesis that such an effect occurs *in vivo*, at therapeutic plasma concentrations of lithium (0.6 - 1.6 mM), or at physiological potassium concentrations.

Ploeger (1974a, b) has suggested that lithium may

exert its therapeutic effects through an inhibitory action on the sodium-pump at both the potassium and sodium sites. His evidence for such a hypothesis is however not convincing. The inhibitory effect at the potassium site was deduced from experiments in which pre-treatment of a rat vagal nerve preparation with lithium in the absence of potassium caused an inhibition of a subsequent potassium activated response. It is difficult to extrapolate such an effect to suggest an inhibition of the Na/K ATPase at the potassium site under physiological potassium concentrations. Applying lithium externally Ploeger claims to find an inhibition of the sodium-pump at the internal sodium site on the basis of lithium-sodium competition. It is difficult to be certain such an effect is occuring inside the cell. Stronger evidence for an inhibitory effect of lithium came from experiments in which Ploeger found superfusion with lithium to inhibit Na/K ATPase activity in a stimulated nerve. On the basis of these results Ploeger suggested lithium to reduce the reserve capacity of the sodium-pump. In further experiments he found chronic lithium treatment to reduce Na/K ATPase activity in rat vagal nerve homogenates. Such an inhibitory effect of chronic lithium treatment has also been reported by Reading and colleagues (Reading, Kinloch and Loose, 1975) in the rat cortex synaptic membrane. Gutman and colleagues found chronic lithium treatment to increase microsomal Na/K ATPase activity in the salivary gland, in the parotid gland and in the kidney medulla but

not in the brain (Gutman, Hochman and Strachman, 1973a; Gutman et al., 1973b). McNulty, O'Donovan and Leonard (1975) found Na/K ATPase activity in brain to be reduced after a single injection of lithium chloride. Although these studies are not all in agreement they provide some evidence for an inhibitory effect of lithium on the sodium-pump.

In interpreting and carrying out studies of the effects of lithium on Na/K ATPase it is important to consider two points. Firstly, effects of chronic treatment may be different from those of an acute application. Secondly, the Na/K ATPase is specifically orientated in the membrane in such a way that the internal and external enzyme sites have different properties (Whittam and Wheeler, 1970). The enzyme is activated externally by potassium and internally by sodium (Whittam, 1962; Whittam and Ager, 1964). In view of the differing cation specificity of the two opposing enzyme sites it is possible that lithium could exert different effects at the two sites. It is well established that lithium can stimulate the enzyme at the external site but its action at the internal site is unknown. As mentioned above Ploeger (1974b) has suggested lithium to inhibit the sodium-pump at the internal site and such an effect is feasible in view of the several reports that lithium is not actively transported out of the cell by the sodium-pump (Keynes and Swan, 1959; Hodgkin and Keynes, 1965; Maizels, 1968; Smith, 1974). The "sidedness" of the Na/K ATPase

and different effects of lithium on the two opposing enzyme sites have been suggested to be important in the therapeutic action of lithium (Glen and Reading, 1973). These authors suggested that in the depressive phase of a manic-depressive illness intracellular sodium concentration was high and lithium was predominantly extracellular. In this situation they suggested lithium to stimulate the Na/K ATPase at the potassium, external site. On the other hand they suggested that in mania intracellular sodium concentration was low and more lithium moved into the intracellular compartment where it had an inhibitory effect on the Na/K ATPase at the sodium site. The action of lithium in both cases was proposed to bring the intracellular sodium concentration back to within the normal range.

Whatever hypothesis is forwarded to describe the effects of lithium on the Na/K ATPase and sodium transport it must explain the observation that high concentrations of lithium inhibits sodium transport across the isolated frog skin epithelium (Zerahn, 1955; Leblanc, 1972; Biber and Curran 1970).

There is evidence that in HeLa cells cultured in the presence of lithium there is an increased Na/K ATPase activity and an increase in the number of ouabain-binding sites (Boardman, Hume, Lamb and Polson, 1975). These results suggest that in addition to activation effects on the enzyme lithium may also affect Na/K ATPase at the level of protein synthesis.

Recently it has been shown that lithium-treatment of patients leads to an increase in Na/K ATPase activity in erythrocyte membranes (Naylor, Dick, Dick and Moody, 1974; Dick, Naylor, Dick and Moody, 1974). It was not possible from these results to distinguish whether the effect was due to recovery of the patients or to lithium itself. It is possible however that lithium treatment affects Na/K ATPase activity in man.

Magnesium-dependant adenosine triphosphatase activity (Mg ATPase) has also been implicated in the therapeutic action of lithium (Glen and Reading, 1973). Termination of lithium administration to animals has been found to cause an increase in synaptic plasma membrane Mg ATPase activity (Reading, Dewar and Kinloch, 1974). These results were interpreted as suggesting lithium to cause a fusion between vesicles and the synaptic plasma membrane (Glen and Reading, 1973; Reading et al., 1974). Later results have shown lithium administration itself to increase synaptic membrane Mg ATPase activity (Reading et al., 1975). Considering the possible presence of an actomyosin-like ATPase in synaptosomal material and its proposed role in neurotransmitter release (Berl, Puszkin and Nicklas, 1973) it is possible that the Mg ATPase activity affected by lithium treatment could be associated with the actomyosin-like protein and neurotransmitter release. The advantage of such a hypothesis is that it would provide a connection between electrolyte and amine theories of affective illness.

Previous suggestions to connect these fields have involved interaction between sodium metabolism and amine re-uptake from the synaptic cleft (Singh, 1970; Mendels et al., 1971; Glen et al., 1972; Maas, 1972). There is some evidence for chronic lithium treatment of rats causing an increase in noradrenaline uptake by synaptosomes (Colburn, Goodwin, Bunney and Davis, 1967; Baldessarini and Yorke, 1970) and increased intraneuronal metabolism of noradrenaline (Schildkraut, Schanberg and Kopin, 1966). Overall however the effects of lithium on amine metabolism which have been described in the literature are very varied (Shaw, 1975).

Besides its effects on Mg ATPase, lithium has been reported to affect other magnesium dependant enzymes and magnesium distribution within the body. There is no consistent pattern in the reports of the effects of lithium on magnesium distribution in man (Hullin, 1975) but animal studies have shown an increase in plasma magnesium following lithium (Hullin, 1975; Andreoli, Villani and Brambilla, 1972; Mellerup, Plenge and Rafaelson, 1973; Haavaldson and Ingvaldson, 1973). Changes in magnesium distribution have been suggested to be important in the therapeutic action of lithium (Glen and Reading, 1973). It has also been suggested that lithium's ability to compete with magnesium in biological situations could explain its therapeutic actions (Birch, 1973; 1974). In support of this hypothesis Birch referred to lithium's chemical similarity to magnesium (Mellerup and Jorgenson, 1975) and the inhibition of the

magnesium dependant enzymes hexokinase, alkaline phosphatase and pyruvate kinase by lithium in vitro (Birch, Hullin, Inie and Leaf, 1974). However this hypothesis has been challenged because high lithium concentrations were needed for inhibition in some cases and because other workers have failed to find inhibition of these and other magnesium dependant enzymes (Agar, Gruca, Gupta and Harley, 1975; Gupta and Crollini, 1975; Pscheidt and Meltzer, 1975). Of some relevance to this thesis is the suggestion by Birch (1974) that lithium's therapeutic actions may be due to the formation and special properties of a lithium-ATP complex.

The previous pages have described the experimental results and hypotheses concerning the role of electrolytes and ATPases in affective illness and in the therapeutic action of lithium and have set out the experimental and theoretical basis of this thesis. Besides the literature discussed there are many other studies concerning the many pharmacological effects of lithium and there are several reviews which cover the various aspects which have not been considered in detail (Jenner, 1973; Schou, 1973; Steinberg, 1973; Johnson, 1975; Schou, 1976).

It is not possible to study the biochemistry of affective illness and the pharmacology of lithium by investigating human brain tissue except for the occasional post-mortem material. The most useful available human material is cerebrospinal fluid and blood but studies of these

materials suffer from giving only indirect information on brain function. As an alternative to human work one can study the effects of drugs on animal brain biochemistry. However this approach also has its limitation, primarily the lack of an adequate animal model for manic-depressive illness. Since lithium is reported to have little effect on the brain functions of normals (Schou, 1976) it is possible that effects of lithium on animal brain function are not at all related to its therapeutic action. Furthermore lithium is unique in that it has a prophylactic action. It is therefore important to study the effects of chronic lithium administration. When given therapeutically lithium is found in concentrations in the range 0.6 - 1.6 mM in plasma (Schou and Baastrup, 1967; Schou, 1976). Therefore in chronic administration experiments one should attempt to achieve such concentrations. It is also necessary to try to avoid toxic effects since these might affect the results obtained from the experiment. Plasma concentrations of 0.6 - 1.6 mM are accompanied by brain concentrations of the order of 0.5 mM/kg (Schou, 1976) and it is important therefore to use lithium at concentrations between 0.5 and 1.0 mM when doing acute in vivo or in vitro experiments. Only effects found with such a range of concentrations can be said to be of any possible therapeutic significance. In the work to be described acute in vitro and in vivo administration, chronic administration and lithium-treated patients have all been used in order to attempt to answer

certain questions. Throughout the work it was attempted to use lithium concentrations, *in vitro* and *in vivo*, comparable to those found in the therapeutic situation.

Schou (1973) has stated that "no comprehensive and experimentally testable hypothesis has been advanced for the biochemical mechanism underlying the mood-normalizing effects of lithium salts" and it is true that despite the wealth of experimental observations there is a dearth of specific, testable hypotheses. However where possible the work to be described was designed to answer certain specific questions raised by previous work described in this introduction. Several experimental approaches were used to study the effects of lithium on ATPase systems and ion transport and these approaches together with the experimental results are described in the following four sections.

Section II describes experiments designed to determine whether lithium (0.6 - 1.2 mM) could stimulate sodium transport when applied to the potassium-sensitive side of a sodium-transporting system and inhibit it when applied to the sodium-sensitive side. This was approached experimentally using the ventriculo-cisternal perfusion technique to study sodium transport from blood to cerebrospinal fluid. This system allowed study of the effects of lithium when presented to opposing sides of the sodium transporting system and so allowed testing of a premise basic to the hypothesis forwarded by Glen and Reading (1973). In the

process of this work experiments were carried out to examine whether sodium entry across the choroid plexus was sensitive to cerebrospinal fluid potassium concentration. This provided a basis for the lithium experiments and also contributed evidence for the implication of a sodium-pump in choroid plexus sodium transport. Finally, section II describes a study of lithium transport from cerebrospinal fluid.

Section III describes experiments which examined the effects of chronic lithium administration on ATPase activities in subcellular fraction prepared from rat brain in order to determine: (a) whether lithium affected Mg ATPase in all fractions or in synaptic plasma membranes alone. (b) whether changes in membrane Mg ATPase activity after lithium treatment could be due to vesicle-membrane fusion as suggested by Glen and Reading (1973) and (c) whether chronic lithium administration had any effect on synaptic plasma membrane Na/K ATPase activity. In addition an experiment is described in which lithium distribution in the brain was studied.

Section IV describes experiments designed to attempt to relate changes in Mg ATPase after lithium administration to changes in striatal dopamine metabolism.

Section V describes experiments on various ATPase activities in erythrocyte membranes from depressive patients. The aim of the experiments was to determine: (a) whether depression was associated with a decreased Na/K ATPase

activity since such an abnormality would explain a large number of previous observations, (b) the effects of lithium treatment on membrane ATPases in humans since in animals lithium was shown to affect Mg ATPase activity, (c) whether the changes observed were due to *in vivo* activation, *in vitro* activation, or effects via protein synthesis, and (d) whether ATPase changes were related to electrolyte changes. In addition an experiment is described in which the effect of chronic lithium administration on rat erythrocyte membrane ATPase activities was investigated.

SECTION II

INVESTIGATIONS OF SODIUM TRANSPORT FROM BLOOD TO BRAIN AND CEREBROSPINAL FLUID, THE EFFECTS OF LITHIUM ON SUCH SODIUM TRANSPORT AND THE TRANSPORT OF LITHIUM FROM CEREBROSPINAL FLUID.

INTRODUCTION.

In respect to its fluid-containing spaces the central nervous system is unique in that in addition to intracellular and extracellular fluid there is also the cerebrospinal fluid (C.S.F.) which provides a separate fluid compartment in and around the brain. C.S.F. is thought to resemble brain extracellular fluid in composition, the two fluids being in close contact with little or no restraint on the passage of substances between them (Davson, 1967, 1976). The close proximity of C.S.F. to nervous tissue and its ease of sampling both in man and animals has led to much experimental study. One approach has been to perfuse the C.S.F.-containing spaces with artificial fluid. In the case of the ventriclo-cisternal perfusion technique fluid is introduced into the lateral ventricles and withdrawn from the cisterna magna so allowing perfusion of the lateral, third and fourth ventricles. This section describes experiments in which the ventriculo-cisternal perfusion technique has been used to study the potassium dependance of sodium transport from blood to C.S.F. and the effects of lithium on sodium transport from blood to brain and C.S.F. and upon C.S.F. secretion rate.

C.S.F. is thought to be primarily secreted by the choroid plexuses (Davson, 1967). Dandy and Blackfan (1914) found that if the Aqueduct of Sylvius was plugged acutely a hydrocephalus developed, so showing that the ventricles were the source of C.S.F. Welch (1963) provided the first

strong evidence for the choroid plexuses as the source of fluid. He measured the haematocrit in the arterial and venous blood entering and leaving the rabbit choroid plexuses and concluded there was a loss of water from the blood during its passage through the choroid plexuses. From the amount of water lost he was able to calculate a theoretical C.S.F. secretion rate of 8µl/minute. Davson and his colleagues also using the rabbit measured C.S.F. secretion by gravimetric methods and by inulin and Dextran Blue dilution. Their values were 10.4, (Davson and Pollay, 1963), 10.6, 11.6 (Davson and Bradbury 1964) and 12.0 and 8.1 (Davson and Segal, 1970) comparing favourably with Welch's estimate and thus suggesting that most if not all C.S.F. may be produced by the choroid plexuses. Further evidence for C.S.F. secretion by the choroid plexuses came from the work of Ames and his colleagues (De Rougemont, Ames, Nesbett and Hofmann, 1960; Ames, Sakanoue and Endo, 1964) who collected, under oil, freshly secreted fluid from the choroid plexus of the lateral ventricles of the cat. The ionic composition of this fluid resembled that of cisternal C.S.F. and this suggested that C.S.F. is not altered to any great extent during its passage through the ventricles. There is still argument however over whether C.S.F. is produced exclusively by the choroid plexuses (Davson, 1967; Cserr, 1971).

As pointed out by Davson and Segal (1970) the turnover of ^{22}Na in C.S.F. (0.48% renewed per minute) is comparable

to the rate of fluid secretion (a secretion rate of 8.8 µl/minute and a total C.S.F. volume of 1.8 ml would give a renewal rate of 0.48% per minute) and this suggests that the major route for the transfer of isotopic sodium from blood to C.S.F. is via newly secreted fluid at the choroid plexuses. Davson and Pollay (1963) studied the transfer of isotopic sodium from blood to C.S.F. using the ventriculo-cisternal perfusion technique in the rabbit. They interpreted their results in terms of two components, a fast component due to transport across the choroid plexuses and a slow component due to transport from blood to brain and thence to C.S.F. The slow component is obvious in these experiments because perfusion at a high rate does not allow equilibration between C.S.F. and brain extracellular fluid and this results in diffusion of isotopic sodium from extracellular fluid into the perfusing fluid. Davson and Bradbury (1964), assuming isotopic sodium entered C.S.F. in secreted fluid at the same concentration as it was in plasma, calculated a theoretical curve for the entry of sodium into the perfusing fluid. Comparing this with the experimental curve they concluded, as did Davson and Pollay (1963), that isotopic sodium entry into C.S.F. was primarily in freshly secreted fluid.

It is now well established that both C.S.F. secretion and the entry of isotopic sodium into C.S.F. are inhibited by ouabain (Welch, 1963; Vates, Bonting and Oppelt, 1964; Ames, Higashi and Nesbett, 1965; Davson and Segal, 1970)

and several workers have therefore suggested that a Na/K ATPase is involved in the transport of sodium across the choroid plexuses and in the secretion of C.S.F. (Vates et al., 1964; Davson and Segal, 1970; Wright, 1972; Johansen, Reed and Woodbury, 1974). The choroid plexus Na/K ATPase has also been implicated in the removal of potassium from C.S.F. and in the control of C.S.F. potassium concentration (Bradbury and Stuclova, 1970; Husted and Reed, 1976). Bradbury and Stuclova perfused ^{42}K through the ventricles at various potassium concentrations and measured the loss of isotope from the perfusion fluid and the amount of isotope in the brain at the end of perfusion. It was then possible to calculate the "barrier-clearance" of potassium - that is the amount of isotope transported from C.S.F. into blood. The barrier-clearance was found to be sensitive to the C.S.F. potassium concentration and Bradbury and Stuclova interpreted this in terms of the choroid plexus Na/K ATPase being involved in the removal of potassium from C.S.F. Such a potassium-transporting system should occur at the apical (C.S.F.-facing) membrane of the choroid plexus epithelium (Bradbury and Kleeman, 1967) and so it was suggested that the Na/K ATPase was situated in the apical membrane and orientated so as to be sensitive to C.S.F. potassium (Bradbury and Stuclova, 1970; Johansen et al., 1974). In contrast, other workers (Pollay, Kaplan and Nelson, 1973) have suggested that transport of potassium from C.S.F. to blood across the sheep choroid plexus was due to passive diffusion and that

the Na/K ATPase was situated so as to be involved in the active transport of potassium from blood to C.S.F. However ouabain-binding studies (Quinton, Wright and Tormey, 1973) showed that in the frog ouabain-binding is predominant at the apical membrane of the choroid plexus epithelium so providing evidence for the apical siting of the Na/K ATPase. Furthermore Wright (1972) using isolated frog choroid plexus found ouabain to only inhibit net sodium flux if applied to the ventricular side.

In summary, the transport of sodium across the choroid plexuses is thought to be associated with C.S.F. secretion and to be due to Na/K ATPase activity. This Na/K ATPase is generally thought to be situated in the apical membrane and to be involved in the transport of potassium from C.S.F. On such a model the sodium transport into C.S.F. should be sensitive to the potassium concentration in C.S.F. In this section experiments are described in which ventriculo-cisternal perfusion was used to study the effect of reduced C.S.F. potassium concentration on sodium entry into C.S.F. and brain. Experiments are also described in which the effects of low concentrations of lithium on sodium entry into C.S.F. and brain were investigated.

Previous work has shown lithium at high concentrations (3 - 50 mM) to have a stimulatory action both on the efflux of sodium and on the Na/K ATPase. Thus lithium when presented extracellularly to the erythrocyte will stimulate both the Na/K ATPase (Whittam, 1962) and sodium efflux

(Glen et al., 1972). Similarly lithium will stimulate sodium efflux from muscle (Beaugé and Ortis, 1970) and stimulate Na/K ATPase activity in a rat vagus nerve preparation (Ploeger, 1974a), in rat kidney (Gutman et al., 1973a) and in rat brain preparation (Tobin et al., 1974). Amongst others Keynes and Swan (1959) have suggested lithium intracellularily might compete with sodium for the sodium-pump and Ploeger (1974b) although he applied lithium extracellularly interpreted his results in terms of lithium, intracellularly, inhibiting the sodium-pump. Glen and Reading (1973) suggested that an important aspect of lithium's therapeutic action might be its ability to stimulate the sodium-pump at the potassium sensitive side and inhibit it at the sodium sensitive side. It thus seemed important to study the effects of lithium on sodium transport when it was presented to two opposing sides of a membrane system actively transporting sodium. The ventriculocisternal perfusion technique was therefore used to study the effects of lithium in vivo on choroid plexus sodium transport. Ouabain-binding studies (Quinton et al., 1973) found no evidence for sodium-pumps present at the lateral membrane of the choroid plexus epithelium and it is probable that the transport of sodium and lithium from blood into the choroid plexus epithelium is by passive diffusion (Wright, 1972). Infusion of lithium into the blood should thus result in diffusion of lithium into the choroid plexus epithelium where it could interact with the apical membrane

Na/K ATPase from the intracellular side. As pointed out by Johansen et al., (1974) if the Na/K ATPase is orientated as described above then it should be sensitive to intracellular sodium. Thus presentation of lithium via the blood should allow lithium to interact with the sodium-pump at the sodium sensitive side. On the other hand perfusion of the ventricles with a lithium containing solution allows presentation of lithium to the opposite side of the apical membrane - the side which, as results to be presented will show, represents the potassium sensitive side of the sodium transporting system. In this section experiments will be described in which lithium was presented via the ventricles and via the bloodstream and the entry of ^{24}Na into C.S.F. and brain studied. As mentioned in Section I of this thesis when lithium is used clinically its concentration in plasma is of the order of 0.6 - 1.6 mM. Thus in the experiments to be described it was decided to use concentrations of lithium of the order of 1 mM.

During passage of a lithium-containing perfusion fluid through the ventricles lithium is lost from that fluid (Prockop and Marcus, 1972). The work of Prockop and Marcus was the only previous study of the transport of lithium between blood, brain and C.S.F. and it was therefore of interest to look at some features of lithium transport using ventriculo-cisternal perfusion. In addition to the fact that C.S.F. and extracellular fluid are in close proximity, the blood-brain and blood-C.S.F. barriers possess

certain features in common: the rate of transport of many substances (^{24}Na, for example) into brain and C.S.F. are very similar (Davson, 1967) and furthermore iodide, bromide and thiocyanate are all actively transported from C.S.F. across the choroid plexuses and also from extracellular fluid across the blood-brain barrier (Davson, 1976). Thus the transport of lithium into and out of C.S.F. may well reflect similar transport at the blood-brain barrier.

MATERIALS AND METHODS.

1. Chemicals.

All chemicals were British Drug Houses (B.D.H.) Analar grade except where otherwise specified.

^{24}Na was purchased as $^{24}NaCl$ in sterile isotonic saline from the Radiochemical Centre, Amersham, U.K. 100 μCi aliquots were made up to 10 ml with sterile isotonic saline. Approximately 30 - 40 μCi were used for each experiment.

Sterile, artificial C.S.F., pH 7.6, was made up by the Pharmacy, Royal Infirmary, Edinburgh. The composition was as follows: Na^+ 152 mM, K^+ 2.98 mM, Mg^{++} 1.0 mM, Ca^{++} 2.1 mM, Cl^- 131 mM, HCO_3^- 25.6 mM, $H_2PO_4^-$ 0.76 mM and dextrose 1 mg/ml. In addition, 100 mg/ml Dextran Blue 2000 (from Pharmacia Ltd., Sweden) was added immediately before the experiment. In certain experiments there was no potassium present in the perfusion fluid and in other specific experiments lithium chloride was added at a concentration of 1 mM. These alterations in composition would have changed the osmolarity and

chloride concentration by less than 2%.

2. Ventriculo-cisternal perfusion.

The perfusion technique used in this work was based on the technique developed by Davson and Pollay (1963) and used by Davson and his colleagues (Bradbury and Davson, 1964, 1965; Davson and Segal, 1970). All experiments involved bilateral perfusion of the ventricles as was used by Davson and Segal (1970). In the perfusion systems used by Davson and colleagues and by other workers (e.g. Pappenheimer, Heisey, Jordan and Downer, 1962) flow through the system was produced by a single pump situated on the inflow side of the system and the cisternal effluent drained away under hydrostatic pressure. In the present work, a second pump, matched to the inflow pump, was introduced into the outflow system between the cisterna magna and the fraction collector. Such a system has been used previously in the dog (Halliday and Moir, 1974). The inclusion of the outflow pump should result in perfusion with a C.S.F. pressure of zero assuming exact balancing of the inflow and outflow pumps. A zero pressure in the system should be sufficiently low to prevent any appreciable loss of material by bulk drainage.

The experimental arrangement is shown diagrammatically in Figure 1. On the inflow side of the system fluid passed through a T-piece manometer situated between the pump and the ventricles and the level of the fluid in the T-piece gave an indication of the fluid pressure in the inflow system. After passage through the ventricular system of

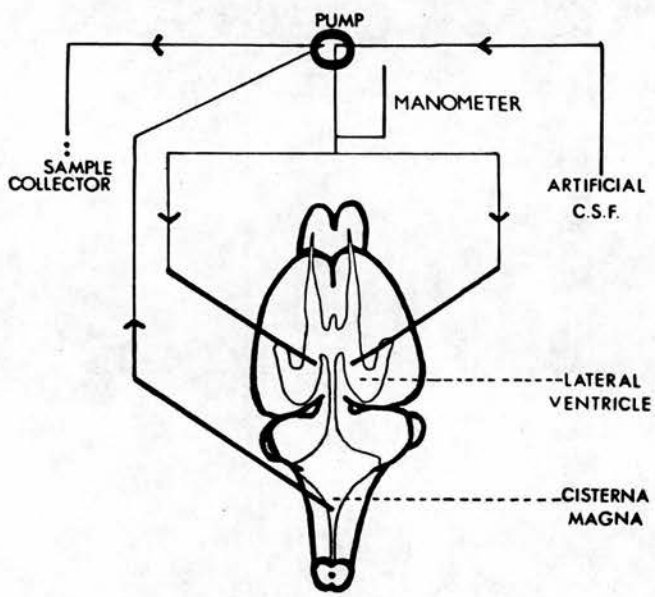

Figure 1. A diagrammatic representation of the experimental arrangement used in the ventriculo-cisternal perfusion experiments.

the animal, fluid was pumped out of the cisterna magna to a fraction collector.

Adult, male New Zealand White rabbits (weight 3 - 3.5 kg) were used throughout. All the experiments performed were of an acute nature and so no sterile procedures or conditions were used. The animals were anaesthetised with intravenous sodium thiopentone (40 mg/kg initially with subsequent doses as necessary) and were allowed to breathe naturally, no artificial respiration being used. Prior to surgery the top of the head was shaved and the subcutaneous tissue of the scalp and base of the neck infiltrated with 1 ml lignocaine solution (2%) as local anaesthetic. A midline incision was made from the front of the skull to the base of the neck and the periosteum and temporal muscles reflected laterally to expose the skull. The next stage was to drill two holes in the skull in order to place the inflow cannulae. These holes were located 10 mm behind the coronal suture and 10 mm lateral from the midline. After the holes had been bored using a hand twist-drill (2 mm), they were threaded using a 5BA tap and the two guide-tubes screwed firmly into position. The guide-tubes consisted of small screws with a cavity in the centre through which the inflow cannulae could pass (Moir and Dow, 1970). The firm positioning of the guide-tubes gave support to the cannulae and helped to prevent them moving during the experiment. 25G Yale Microlance needles were used as the inflow cannulae and they were connected with silicone rubber tubing to the

manometer and the reservoir of artificial C.S.F. Correct positioning of the inflow cannulae in the ventricles was achieved using the manometer present in the inflow system. The tubing leading to one of the cannulae was clamped so as to prevent flow of fluid and the other cannula then inserted through one of the guide-tubes into the brain. The fluid in the inflow system was pumped past the manometer to the cannula and so when flow out of the cannula was prevented there was a rise in pressure and the fluid level in the manometer rose. On inserting the cannula into the brain there was a rise in the level of fluid in the manometer. The cannula was slowly lowered further through the brain until a slow fall in the level of fluid in the manometer was seen. This fall in pressure indicated that the tip of the cannula was in the lateral ventricle. Once the cannula was positioned correctly it was clamped off and the procedure repeated for the second cannula. After the successful positioning of both cannulae articicial C.S.F. was infused into the ventricles for 15 minutes. During this infusion period the skin of the base of the neck was reflected laterally prior to cisternal cannulation. A 30 mm needle, bevelled, and with 2 holes drilled behind the bevel was used as the cisternal cannula. After the ventricles had been infused for 15 minutes the cisternal cannula was inserted through the nuchal musculature and between the axis vertebra and the base of the skull, into the cisterna magna. The animal's head was flexed slightly forward to

facilitate this operation. Successful placement of the cannula was evident from the rapid flow of the blue-coloured fluid through the outflow tubing connected to the cannula. The effluent fluid was pumped to an LKB automatic fraction collector. Inflow and outflow fluids were pumped using separate channels of a Watson-Marlow 1000 MHRE H.R. flow inducer which was calibrated so that inflow and outflow rates were equal. The speed selector on the flow inducer was not entirely satisfactory and this resulted in variation of pump rate between experiments. It was necessary therefore to measure the rate of perfusion at the end of each experiment. This was done by weighing the water pumped in a measured time period.

The perfusion system was allowed to equilibrate for 10 minutes after cisternal cannulation. After this time had elapsed the dead-space in the outflow system had filled with fluid and the perfusion system was then complete - fluid was being pumped into the ventricles, out of the cisterna and could be collected at the fraction collector. Fluid was perfused through the system at between 55 and 75 µl/minute.

Once the perfusion system was complete and there was a continuous flow of fluid, isotope was introduced into the bloodstream. ^{24}Na (as $^{24}NaCl$ in 0.9% saline) was infused into an ear vein using a Braun micro-injection pump with the following infusion schedule:

Time after beginning of infusion (minutes)	Rate of infusion (µl/minute)
0-1	1000
1-6	0.75
6-16	0.375
16-30	0.1
30-60	0.062
60 onwards	0.033

Such a schedule gave a constant (±5%) level of ^{24}Na in the plasma.

The time at which isotope infusion started was noted and this was used as the reference time to which isotope results were referred. ^{24}Na was infused for a period of one hour during which time 10 minute collections of the cisternal effluent were collected. There was a considerable dead-space in the outflow system and the time taken for fluid to flow from the cisternal cannula to the fraction collector was determined as 10 minutes. It was necessary therefore to correct all the effluent isotope results by subtracting 10 minutes from their noted collection time in order to obtain a true value of the time (with reference to the start of ^{24}Na infusion) at which the fluid left the cisterna.

During the infusion period arterial blood samples (1 ml) were taken every 15 minutes from the central ear artery. These were centrifuged at 1000 g for 5 minutes and aliquots of plasma taken for estimation of ^{24}Na. In a

few experiments arterial blood samples were also taken for blood gas analysis.

After infusion for one hour the venous infusion cannula was removed, the animal killed by thiopentone overdose and the brain excised. The lateral ventricle choroid plexus, which usually contained a considerable amount of blood, was dissected out and discarded. The brain was then blotted to remove residual traces of blood and homogenised by hand.

The cisternal effluent collections and the brain tissue were used for the following analyses:

a) 0.1 ml aliquots of cisternal effluent were taken for determination of ^{24}Na.

b) 0.1 ml aliquots of cisternal effluent together with samples of original perfusion fluid, were taken for determination of Dextran Blue concentrations.

c) The remaining cisternal effluent was stored at -20°C and later aliquots were taken for potassium and lithium determinations.

d) Weighed aliquots of brain (approximately 1 g) were taken for determination of ^{24}Na. In certain experiments an aliquot of brain was also taken for lithium determination.

3. Measurement of radioactivity.

^{24}Na is a strong γ-emitter with a short half-life of 15 hours. It was estimated using a Tracerlab γ-Guard 150 γ-ray spectrometer which had been calibrated specifically for the estimation of ^{24}Na. Aliquots (0.1 ml) of cisternal

effluent and plasma were diluted to a standard volume of 1 ml before counting. Approximately 1 g aliquots of brain were analysed. All samples were counted for 10 minutes and all results were corrected for background radiation. As there was only a small number of samples per experiment it was not necessary to correct the measured counts for isotope decay.

4. Measurement of potassium and lithium concentrations.

Potassium and lithium were measured in some of the cisternal effluent samples and in a few experiments lithium was also measured in brain. Samples were stored at -20°C, in plastic tubes, before analysis.

All potassium estimations were done by flame emission spectrophotometry using a Pye-Unicam SP90 atomic absorptiometer with an air-acetylene flame. An absorption filter was fitted between the flame and the monochromator in order to reduce interference from sodium and using this there was no interference from artificial C.S.F. As previously reported (Amidsen, 1967), there was no interference by lithium in the estimation of potassium. In view of the fact there was no interference from sample constituents, samples were compared with standards of known amounts of potassium chloride dissolved in distilled water. Samples and standards were diluted 1:70 with deionized water for analysis.

There are several reports concerning the effects of sodium, potassium and other ions on the estimation of

lithium. Amidsen (1967) reported lithium determination by flame emission spectrophotometry to be affected by the sodium concentration. However, the effect did vary between different machines. Other workers have found little or no consistent effects of sodium, potassium of other ions on lithium determination by flame emission spectrophotometry or atomic absorption spectrophotometry (Levy and Katz, 1970; Pybus and Bowers, 1970). Interference of artificial C.S.F. with lithium estimation was therefore investigated prior to the present study and standards were made up in deionized water or artificial C.S.F. as appropriate.

Initially lithium concentrations in the cisternal effluent were determined by flame emission spectrophotometry using a Pye-Unicam SP90 atomic absorptionmeter. Artificial C.S.F. was found to cause an enhancement of the signal. This enhancement was a constant percentage of the signal. Cisternal effluent samples were therefore compared to standards of lithium carbonate dissolved in artificial C.S.F. Both standards and samples were diluted 1:20 with deionized water for analysis.

During the course of the work presented in this thesis a Perkin-Elmer 360 atomic absorptiometer became available. This new machine allowed estimation of lithium by atomic absorption which was more satisfactory than using flame emission spectrophotometric techniques. Thus, thereafter lithium was measured by atomic absorption spectrophotometry. No interference by sodium, potassium or artificial C.S.F.

was found and therefore samples were compared with standards of lithium carbonate dissolved in deionized water.

In order to assay lithium in brain tissue the extraction procedure of Bradbury et al. was adopted (Bradbury, Kleeman, Bagdoyan and Berberian, 1968). The aliquot of brain (500-1000 mg) was placed in a weighed glass vial and the vial reweighed. The tissue was dried at 105°C for 48 hours and reweighed. After grinding the tissues to a fine powder with a glass rod, 0.75 N nitric acid (1 ml/mg wet tissue, Aristar grade) was added. The vials were then mixed with inversion for 24 hours and then stored at -20°C. After subsequently being thawed, they were centrifuged at 1500 g for 10 minutes and aliquots of the supernatant diluted 1:20 with deionized water for analysis. Recovery of lithium using this procedure was 90%.

5. Measurement of C.S.F. Secretion Rate.

The dilution of a non-diffusable substance on its passage through the ventricles during ventriculo-cisternal perfusion has been used by several workers to calculate C.S.F. secretion rates, (Pappenheimer et al., 1962; Davson and Segal, 1970). Early studies used inulin as the non-diffusable marker substance but work by Rall et al. (1962) showed that inulin diffused out of the perfusing fluid into the brain during ventriculo-cisternal perfusion. Such loss of inulin could give a spuriously high value for C.S.F. secretion and so later workers used Dextran Blue as the non-diffusable marker and this was found to give a lower value

for C.S.F. secretion than inulin (Davson and Segal, 1970). In the present work Dextran Blue 2000 was used. Its concentration in both the perfusion fluid and the cisternal effluent samples was estimated spectrophotometrically by its absorption at 625 nm. 0.1 ml aliquots of samples were diluted to 1 ml before estimation.

The derivation of the relationship between Dextran Blue concentrations and C.S.F. secretion rate is given below. It differs from that described by Davson (1967) since the latter applied to a situation where fluid was only pumped into the ventricular system whereas in the present experiments fluid was pumped both into and out of the ventricular system. However the final equation describing C.S.F. secretion in terms of Dextran Blue concentrations is the same as that derived by Davson.

Let K_p = rate of perfusion (μl/min)

Let K_f = rate of C.S.F. secretion (μl/min)

Let C_i, C_o be the concentrations of Dextran Blue in the inflowing and outflowing fluid respectively.

The rate at which Dextran Blue enters the ventricles = $K_p.C_i$.

Dextran Blue entering the ventricles is diluted by nascent C.S.F. Assuming that the system is in the steady-state and that the ventricles contain only nascent C.S.F. and perfusion fluid, the dilution of inflowing perfusion fluid is such that the concentration of Dextran Blue in the ventricles equals

$$\frac{K_p}{K_p + K_f} \cdot C_i$$

and the rate of removal of Dextran Blue equals

$$\frac{(K_p)^2}{K_p + K_f} \cdot C_i$$

The measured rate of appearance of Dextran Blue in the cisternal effluent equals $K_p.C_o$.

Assuming no Dextran Blue is lost from the perfusion fluid during passage through the ventricular system, the rate of removal derived above must equal the rate of appearance in the cisternal effluent.

Therefore, $K_p.C_o = \frac{(K_p)^2}{K_p + K_f} \cdot C_i$

Dividing both sides of the equation by K_p,

$$C_o = \frac{K_p}{K_f + K_p} \cdot C_i$$

Rearranging the equation,

$$K_f = \frac{C_i - C_o}{C_o} \cdot K_p \qquad (1)$$

Thus by measuring C_i, C_o and K_p it is possible to calculate K_f the rate of C.S.F. secretion. As shown in Figure 10 the concentration of Dextran Blue in the cisternal effluent increased during the early stages of perfusion but remained constant during the later stages when the system had reached a steady-state. An average value of C_o was calculated from the steady-state concentrations and this value was used in the calculation of C.S.F. secretion rates.

6. Measurement of Transfer constant, K_{out}, and clearance into brain.

Transport of a substance out of the ventricles during ventriculo-cisternal perfusion results in lower C_o/C_i ratio than that for the non-diffusable markers inulin or Dextran (Pollay and Davson, 1963; Bradbury and Davson, 1964, 1965). A transfer constant, K_{out}, can be calculated in order to give a numerical value to the rate of loss of a substance from the perfusing fluid. In addition it is possible to calculate the total clearance of a substance from the perfusion fluid and the clearance into brain. The calculations outlined below follow those of Bradbury and Davson (1964).

Let K_p, K_f, C_i and C_o denote rate of perfusion, rate of C.S.F. secretion and Dextran Blue concentrations as described above.

Let C_i^*, C_o^* be the concentrations of the substance under study in the inflowing and outflowing fluids respectively.

Let F equal the flux of substance under study from the perfusion fluid.

Let C_{pl}^* be the concentration of substance under study in plasma.

Let T be the total period of perfusion (min).

Let T_s be the period for which each cisternal sample is collected (min).

In the steady state,

$$F = K_p \cdot (C_i^* - C_o^*) - K_f \cdot C_o^*$$

Substituting for K_f from equation (1),

$$F = K_p \cdot C_i^* - C_o^* \cdot \frac{C_i}{C_o}$$

The transfer constant, K_{out}, equals the flux, F, divided by the mean concentration difference between perfusion fluid and plasma for the substance under study. The mean concentration of the perfusion fluid is uncertain but assuming it is the arithmetic mean $\frac{C_i^* - C_o^*}{2}$,

$$K_{out} = \frac{F}{\frac{C_i^* - C_o^*}{2} - C_{pl}^*}$$

In the present studies the concentration of the substance under study in plasma is approximately zero. Therefore, $K_{out} = \frac{F}{\frac{C_i^* - C_o^*}{2}}$ in units of ml/min. (2)

The total amount of substance to enter the ventricles during perfusion equals $C_i^* \cdot K_p \cdot T_s$

The total amount to be removed in the cisternal effluent equals the sum of $C_o^* \cdot K_p \cdot T_s$ for all cisternal samples collected.

The total clearance of the substance under study is the difference between the amount infused and the amount removed divided by the weighted mean of the concentration of the substance in the perfusion fluid. The clearance of the substance into brain is the total amount of substance in the brain at the end of perfusion divided by the weighted mean of the concentration of substance in the perfusion fluid. Expressing the brain clearance as a percentage of the total clearance the weighted means cancel out and

$$\% \text{ Clearance into brain} = \frac{\text{Amount of substance in brain}}{C_i^* . K_p . T - C_o^* . K_p . T_s} . 100$$

7. Measurement of Blood pCO_2, pO_2 and pH.

For certain experiments 1 ml arterial blood samples were taken from the central ear artery prior to and during perfusion. Samples were immediately sealed using parafilm and measurement of blood parameters was carried out within minutes using a Radiometer Blood Micro system.

8. Measurement of Choroid Plexus ATPase activity.

Adult, male New Zealand white rabbits were killed by cervical fracture and the brains removed. Lateral ventricle choroid plexuses were dissected out and homogenised in water (5 ml/plexus).

ATPase activity was measured in aliquots of the homogenate by measuring the liberation of inorganic phosphate from disodium ATP. Homogenate was incubated with 3 mM disodium ATP, 100 mM NaCl, 6 mM $MgCl_2$, 30 mM Tris buffer, pH 7.4, 0.1 mM EDTA and various concentrations of KCl. Inorganic phosphate was determined by the method of Martin and Doty as described by Lindberg and Ernster (1956).

RESULTS.

1. Analysis of ^{24}Na entry into the perfusing fluid.

In the majority of experiments carried out the time course of appearance of isotopic sodium in the cisternal effluent was qualitatively similar. The results for control experiments (in which the perfusion fluid had a potassium concentration of 2.98 mM) are shown in Figure 2. The curve

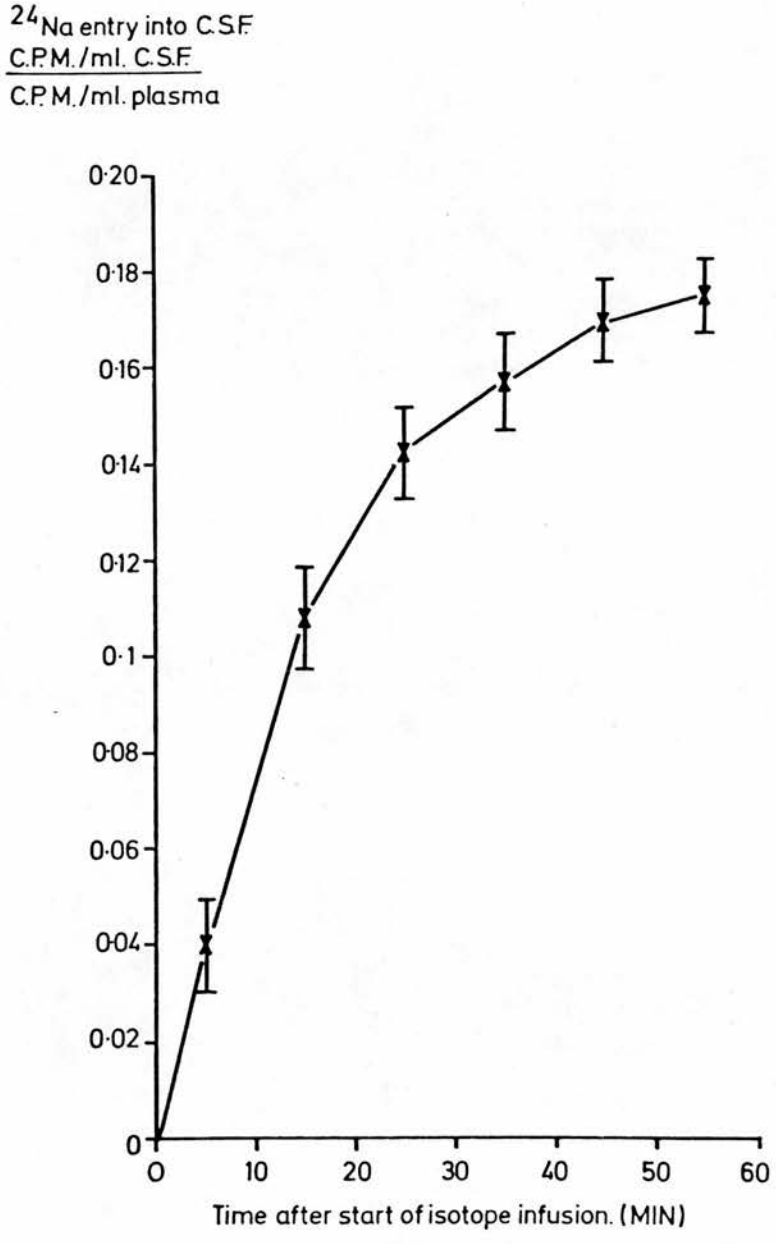

Figure 2. The entry of ^{24}Na from blood into ventriculo-cisternal perfusion fluid during perfusion with artificial C.S.F. containing normal ionic constituents. The points represent mean values from 11 experiments and the bars represent twice the standard errors of the means.

showing ^{24}Na entry into the perfusing fluid is comparable to the results of Davson and Pollay (1963) and Davson and Segal (1970). Davson and Pollay interpreted such a curve of entry in terms of two exponential components. As shown in Figure 3 plotting the results semilogarithmically allowed separation of the curve into two components. Graphical extrapolation of the slow component back to zero time allowed its estimation over the early period of entry. For each set of experiments the mean values of the slow component of entry were subtracted from the individual, experimental values of sodium entry to give values for the early fast component of sodium entry. The mean calculated values of the fast component for control experiments are shown in Figure 3. It can be seen that the fast component increases over the first twenty-five minutes after which it is constant and this was also true for potassium-free and lithium perfusions. The corrected value of the ratio of ^{24}Na in the perfusate to that in plasma ~~after~~ for the sample 20 - 30 minutes was therefore taken as an estimate of the sodium entry due to the fast component of entry. The slow component of entry was estimated graphically from semilogarithmic plots of data from each experiment.

2. Effects of potassium-free perfusion on ^{24}Na entry into the perfusing fluid and brain and upon C.S.F. secretion rate.

The entry of ^{24}Na into perfusing fluid during potassium-free perfusion is shown in Figure 4. It is qualitatively similar to the curve of entry in control experiments but

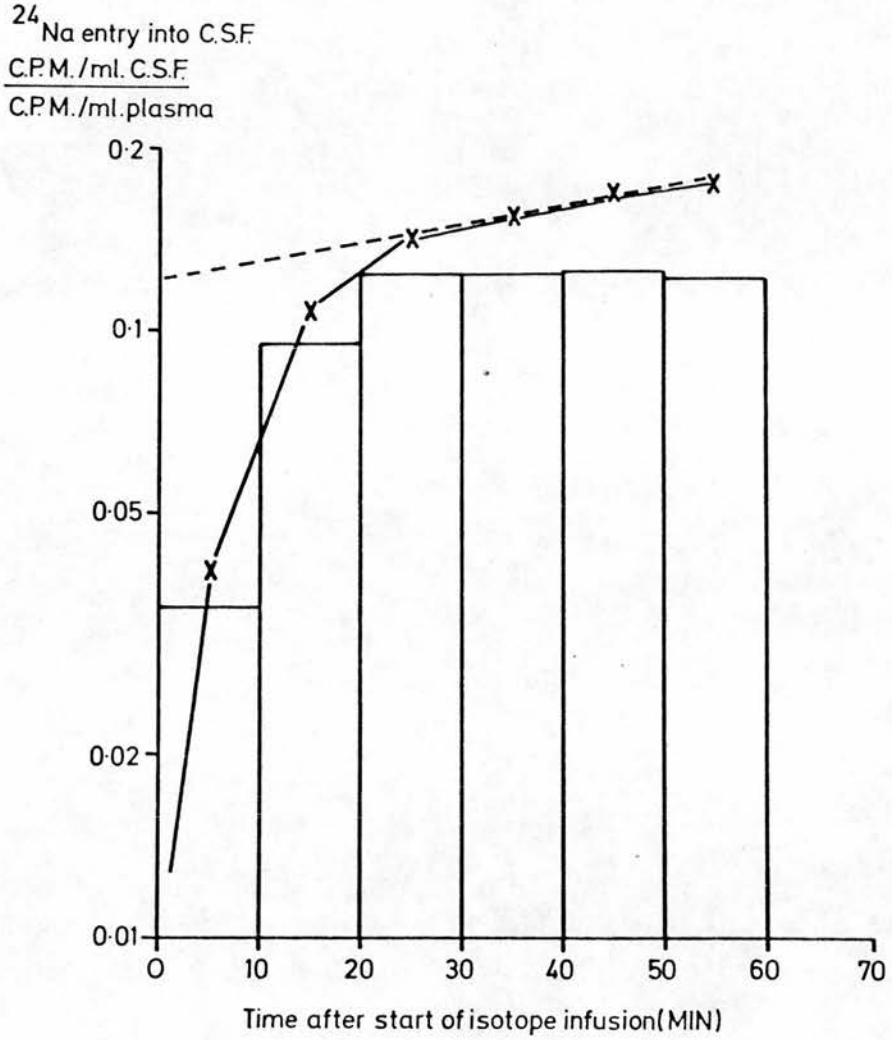

Figure 3. A semilogarithmic plot of the entry of ^{24}Na from blood into ventriculo-cisternal perfusion fluid during perfusion with an artificial C.S.F. containing normal ionic constituents. -X- represents the experimental data and --- shows extrapolation of the slow component of entry to zero time. The histograms represent values of the fast component obtained by subtraction of the slow component from the observed data.

there is a noticeable reduction in the sodium entry over the first 30 minutes. The results after analysis into fast and slow components of entry together with values for sodium entry into brain and C.S.F. secretion rate are shown in Table 1. Potassium-free perfusion had no effect on the slow component of sodium entry into C.S.F. or sodium entry into brain. There was however a small, but not significant, reduction in the mean values of the fast component of sodium entry and C.S.F. secretion rate.

Analysis of the cisternal effluent from potassium-free perfusions showed an appreciable concentration of potassium present (Figure 5). Potassium-free perfusion caused a rapid and variable influx of potassium into the perfusing fluid. It is likely, therefore, that there was a considerable concentration of potassium within the ventricles during these experiments and this may explain the small effect of potassium-free perfusion on sodium entry and C.S.F. secretion.

The cisternal effluent potassium concentration varied a great deal in these experiments and it was possible therefore to examine whether there was any relationship between the cisternal effluent potassium concentration and the sodium entry into C.S.F. and brain. As shown in Figure 6 the fast component of sodium entry into C.S.F. during potassium-free perfusion was significantly correlated ($r = 0.668$, $p < 0.02$) with the mean potassium concentration in the cisternal effluent collected during the first 30

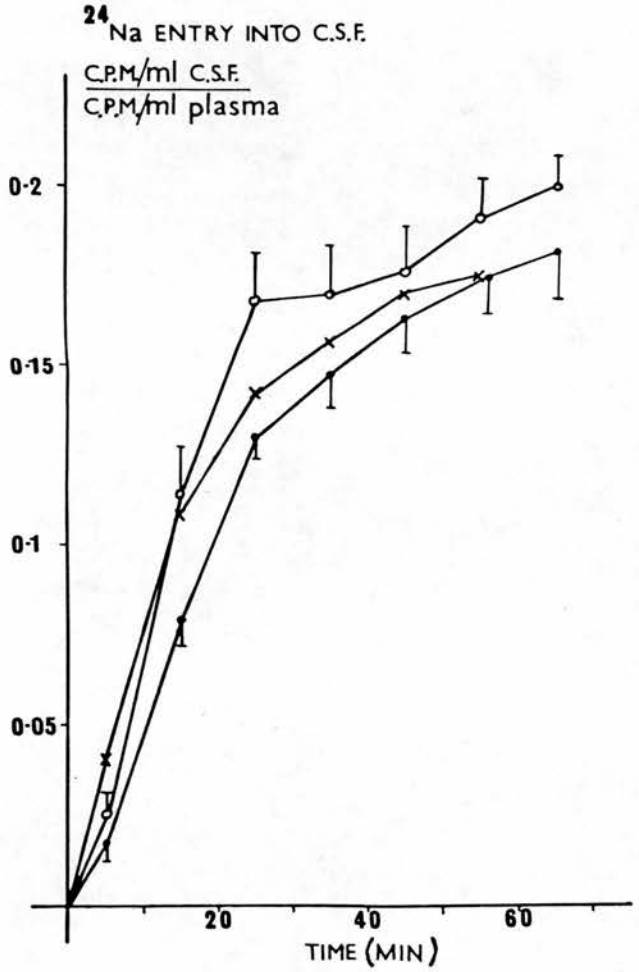

Figure 4. The entry of ^{24}Na into ventriculo-cisternal perfusion fluid during perfusion with artificial C.S.F. containing no potassium (-•-), 1 mM lithium and no potassium (-o-) and normal constituents (-x-).
Points represent mean values from 14 experiments in the case of potassium-free perfusions, from 10 experiments in the case of lithium perfusions and from 11 experiments in the case of control experiments. Bars represent the standard errors of the means.

Table 1. The effects of potassium-free perfusion and potassium-free perfusion in the presence of 1 mM lithium on ^{24}Na entry into ventriculo-cisternal perfusion fluid and brain and upon C.S.F. secretion rate. Values represent means ± standard deviations with the number of estimations in parentheses. Statistical comparisons used a Student's 't' test and the significances shown are for differences between potassium-free perfusion in the absence and presence of lithium; *$p<0.05$, **$p<0.005$

	C.S.F. Secretion rate (μl/min)	Fast component of ^{24}Na entry into C.S.F. $\left(\frac{\text{C.P.M./ml C.S.F.}}{\text{C.P.M./ml plasma}}\right)$	Slow component of ^{24}Na entry into C.S.F. $\left(\frac{\text{C.P.M./ml C.S.F.}}{\text{C.P.M./ml plasma}}/30 \text{ min}\right)$	^{24}Na entry into brain $\left(\frac{\text{C.P.M./g wet brain}}{\text{C.P.M./ml plasma}}\right)$
Controls (2.98 mM potassium in perfusion fluid)	10.8 ± 1.7 (6)	0.122 ± 0.032 (11)	0.039 ± 0.024 (11)	0.079 ± 0.006 (6)
Potassium-free perfusion	9.5 ± 2.3* (14)	0.109 ± 0.019** (13)	0.044 ± 0.029 (13)	0.088 ± 0.011 (10)
Potssium-free + lithium perfusion	12.2 ± 3.3* (10)	0.152 ± 0.039** (9)	0.033 ± 0.028 (9)	0.078 ± 0.010 (7)

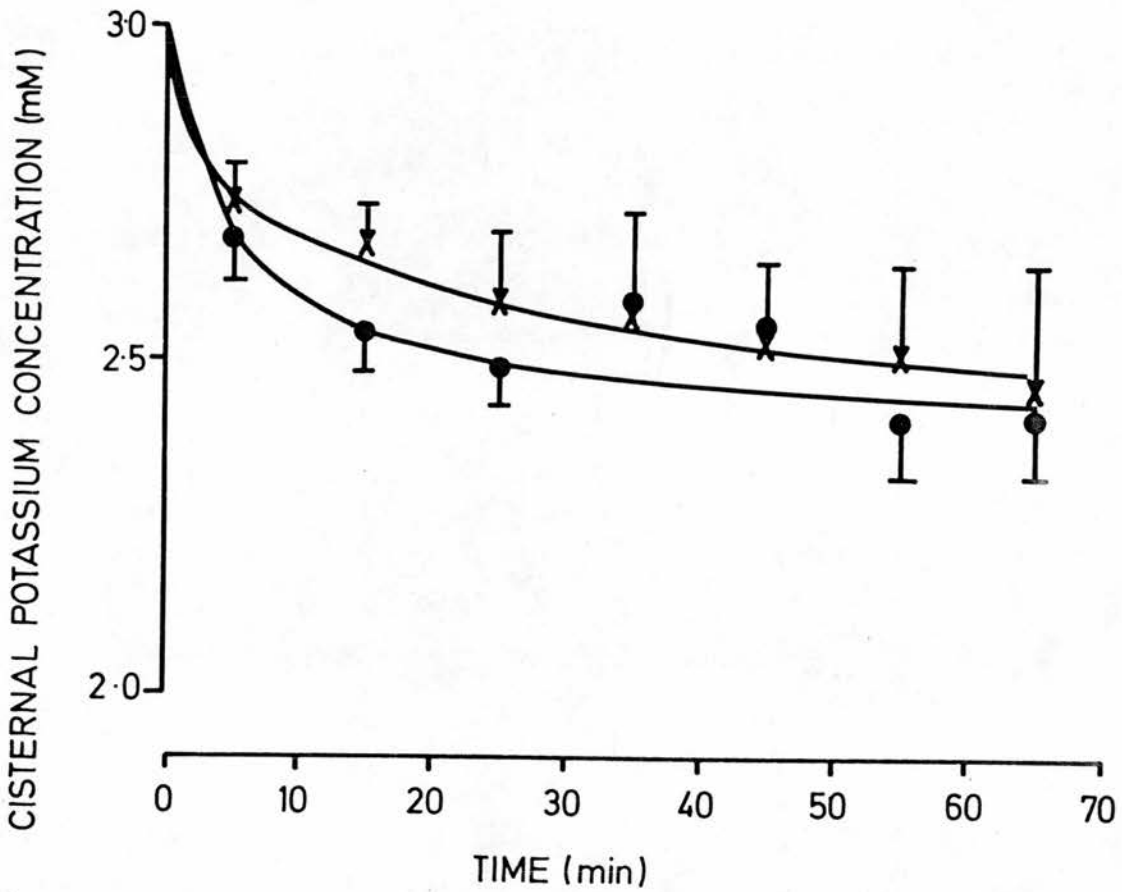

Figure 5. Potassium concentration in the cisternal effluent during perfusion with a potassium-free artificial C.S.F. (X) or a potassium-free artificial C.S.F. with 1 mM lithium added (•). Points represent mean values from 6 (lithium perfusions) and 11 (potassium-free perfusions) experiments. Bars represent the standard errors of the means. The bars for points 35 and 45 minutes represent the standard errors of the means for the potassium-free perfusion. It was not possible to show the small standard error for the lithium experiments as the bars co-incided with the other features of the diagram. Comparison of each pair of points using a Student's 't' test showed no significant difference between potassium-free and potassium-free + lithium perfusions.

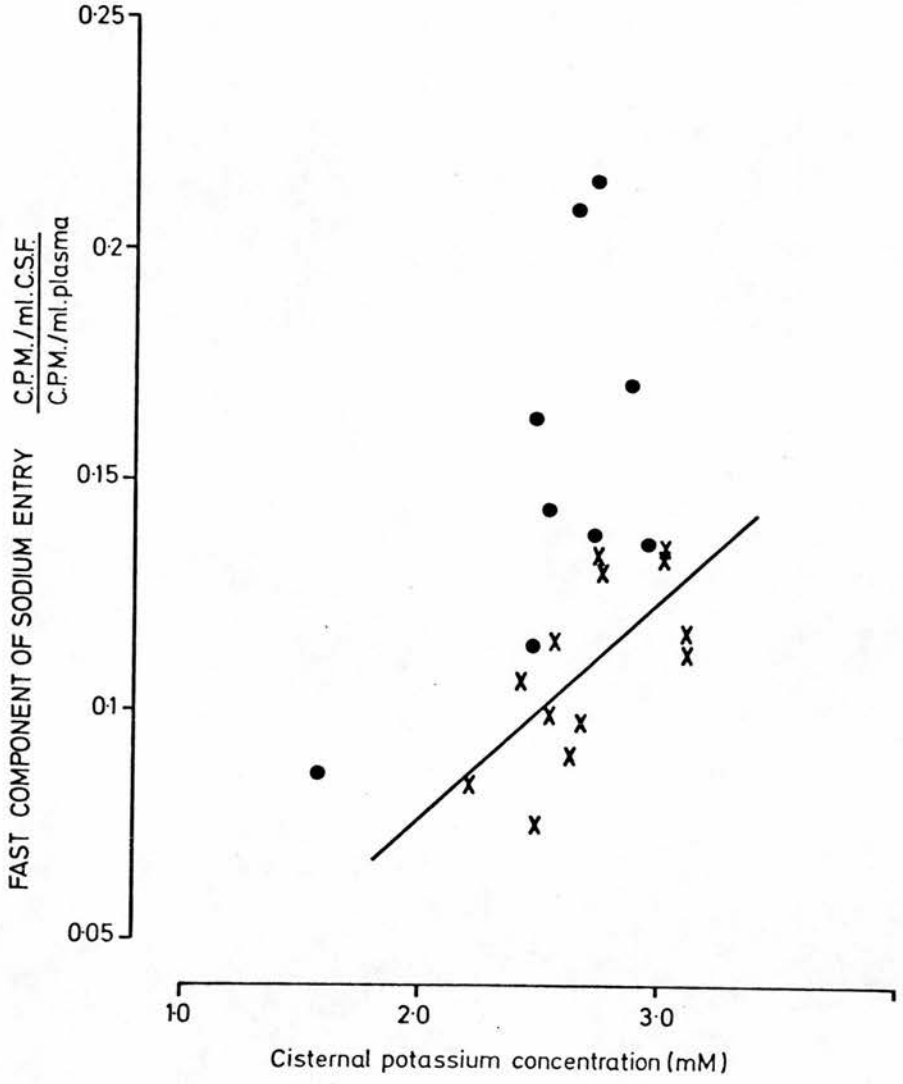

Figure 6. The relationship between the fast component of ^{24}Na entry into C.S.F. and the mean potassium concentration in the cisternal effluent collected during the first 30 minutes after ^{24}Na was introduced into the plasma. (X) represents results from potassium-free perfusions, (•) potassium-free + lithium perfusions. In the potassium-free perfusions the fast component of ^{24}Na entry is significantly correlated ($r=0.668$, $p<0.02$) with the cisternal potassium concentration. The line drawn is a regression line for the potassium-free results.

minutes of ^{24}Na infusion. There was no correlation of C.S.F. secretion, of slow component of sodium entry or of sodium entry into brain with the cisternal effluent potassium concentration.

The results of these experiments suggest that the fast component of sodium entry into C.S.F. is sensitive to the C.S.F. potassium concentration.

3. The effect of potassium concentration on choroid plexus ATPase activity.

The sensitivity of the fast component of sodium entry to C.S.F. potassium concentration suggests that such sodium entry is due to Na/K ATPase activity. To further test this hypothesis experiments were carried out to investigate if choroid plexus Na/K ATPase activity was sensitive to potassium concentrations comparable to those in the perfusion experiments. ATPase activity was measured in homogenised lateral ventricle choroid plexuses at various potassium concentrations in the incubation medium. The results, expressed as a percentage of the activity in the absence of potassium, are shown in Figure 7. The activity was sensitive to potassium concentrations up to 3 mM. Such results show that the perfusion results could be explained in terms of a potassium-sensitive sodium pump.

4. The effects of lithium, presented via the ventricles, on ^{24}Na entry into the perfusing fluid and brain and upon C.S.F. secretion rate.

In order to present lithium to the potassium sensitive side of the system transporting sodium into C.S.F., lithium

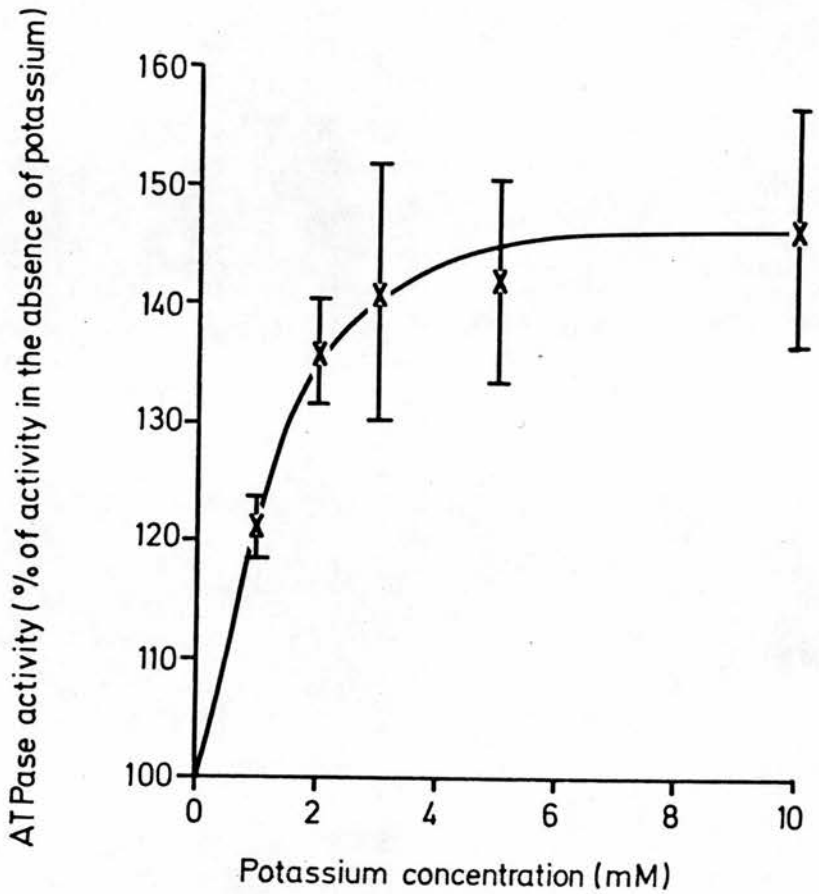

Figure 7. The potassium dependance of choroid plexus ATPase activity in vitro. The results are expressed as a % of the activity in the absence of potassium. Figures represent means of four experiments, bars represent twice the standard errors of the means.

chloride (1 mM) was added to the perfusing fluid in two series of experiments. The first series consisted of experiments in which lithium was perfused through the ventricles in a potassium-free artificial C.S.F. The entry of ^{24}Na into the perfusing fluid during these experiments is shown in Figure 4 and when compared with results from potassium-free perfusions there is increased early entry of ^{24}Na in the lithium experiments. Further analysis of lithium experiments showed an increased fast component of sodium entry into C.S.F. (Figure 6, Table 1. $p<0.005$) while there was no effect on the slow component of sodium entry into C.S.F. or sodium entry into brain (Table 1). As shown in Table 1 perfusion with lithium also caused an increase in C.S.F. secretion rate compared to potassium-free perfusions ($p<0.05$).

Inclusion of lithium in artificial C.S.F. during potassium-free perfusion had little effect on the cisternal potassium concentrations (Figure 5) and it is unlikely therefore that the effects of lithium perfusion on sodium entry and C.S.F. secretion could be due to lithium causing an increase in the ventricular potassium concentration.

The second set of experiments involved perfusion with artificial C.S.F. containing 1 mM lithium chloride in addition to the normal concentration of potassium (2.98 mM). The entry of sodium into perfusing fluid during these experiments is shown in Figure 8. Further analysis of the results (Table 2) shows lithium perfusion to cause a

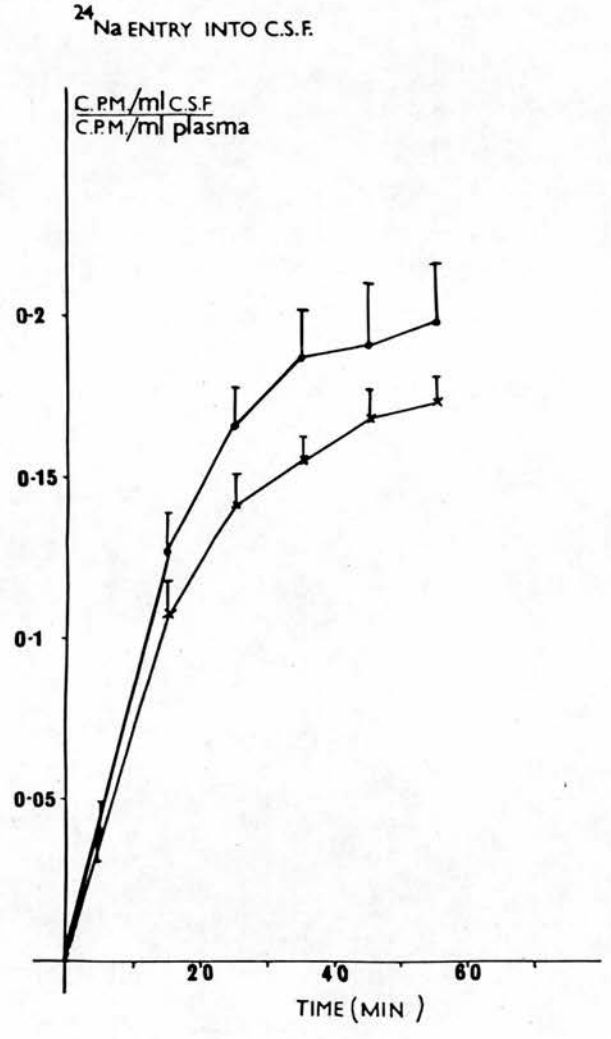

Figure 8. The effect of 1mM lithium chloride present in perfusion fluid of otherwise normal ionic constitution on the entry of ^{24}Na into ventriculo-cisternal perfusion fluid. Results are shown for control (-X-) and lithium (-•-) experiments. Points represent mean values from 11 control experiments and from 7 lithium experiments. Bars represent standard errors of the means.

Table 2. The effects of 1 mM lithium present in the perfusing fluid on ^{24}Na entry into ventriculo-cisternal perfusion fluid, into brain and upon C.S.F. secretion rate. Values represent mean ± standard deviations with the number of estimations in parentheses. Statistical comparisons used a Student 't' test; *$p<0.1$, **$p<0.05$

	C.S.F. Secretion rate (µl/min)	Fast component of ^{24}Na entry into C.S.F. $\frac{\text{C.P.M./ml C.S.F.}}{\text{C.P.M./ml plasma}}$	Slow component of ^{24}Na entry into C.S.F. $\frac{\text{C.P.M./ml C.S.F.}}{\text{C.P.M./ml plasma}}$/30 min	^{24}Na entry into brain $\frac{\text{C.P.M./g wet brain}}{\text{C.P.M./ml plasma}}$
Controls	10.8 ± 1.7 (6)	0.122 ± 0.032 (11)	0.039 ± 0.024 (11)	0.079 ± 0.006 (6)
+ 1 mM lithium in perfusion fluid	13.9 ± 2.9* (7)	0.158 ± 0.031** (7)	0.035 ± 0.028 (7)	0.084 ± 0.013 (7)

significant increase in the fast component of sodium entry ($p<0.05$) and a barely significant ($p<0.1$) increase in C.S.F. secretion rate. There was no effect on the slow component of sodium entry into perfusing fluid or the sodium entry into brain.

In both series of experiments the lithium concentration in the cisternal effluent was measured and in both cases found to be in the range 0.6-0.8 mM. Lithium was found to be barely detectable in plasma during these experiments, the concentration being approximately 10^{-6} M. It is most likely therefore that the effects on sodium transport and C.S.F. secretion described above were due to lithium present within the ventricular fluid and at concentrations in the range 0.6-1.0 mM.

5. The effects of intravenous infusion of lithium chloride on ^{24}Na entry into perfusing fluid and brain and upon C.S.F. secretion rate.

Infusion of 1 M lithium chloride following the schedule outlined for ^{24}Na gave plasma lithium concentrations in the range 0.6-1.2 mM. The lithium concentration in plasma during infusion was not constant, there being a steady decline over an hour. With plasma concentrations of lithium of 0.6-1.2 mM, the cisternal effluent was found to contain lithium concentrations of between 0.04 and 0.1 mM.

Two series of experiments were performed. In the first series, lithium and ^{24}Na infusions were started together. The curve of ^{24}Na entry into the perfusing fluid

is shown in Figure 9. In contrast to the results from previous experiments there was a flattening off in the curve over the final 30 minutes of perfusion. This is reflected in a reduced slow component of sodium entry but there was no effect on the fast component of sodium entry or upon sodium entry into brain (Table 3.). Lithium infusion also caused a marked decrease in C.S.F. secretion rate.

One explanation of these results was that lithium infusion caused a reduction in sodium transport across the choroid plexus and a reduction in C.S.F. secretion rate but that these effects were not immediate. Therefore a second series of experiments were performed in which lithium chloride was infused for 30 minutes prior to and also during ^{24}Na infusion. The results for these experiments are shown in Figure 9 and Table 3. There was again a marked reduction in C.S.F. secretion rate. The curve of entry of ^{24}Na into the perfusing fluid was qualitatively similar to controls showing no flattening off at later time periods. Further analysis showed a significant ($p < 0.05$) reduction in the fast component of sodium entry but no effect on the slow component of sodium entry into the perfusing fluid.

6. The effect of various experimental procedures on blood pCO_2, pH and pO_2.

The concentration of some ions in C.S.F. is known to affect respiration (Leusen, 1972) and blood pCO_2 and pH are known to affect C.S.F. secretion rate (Davson, 1967). It

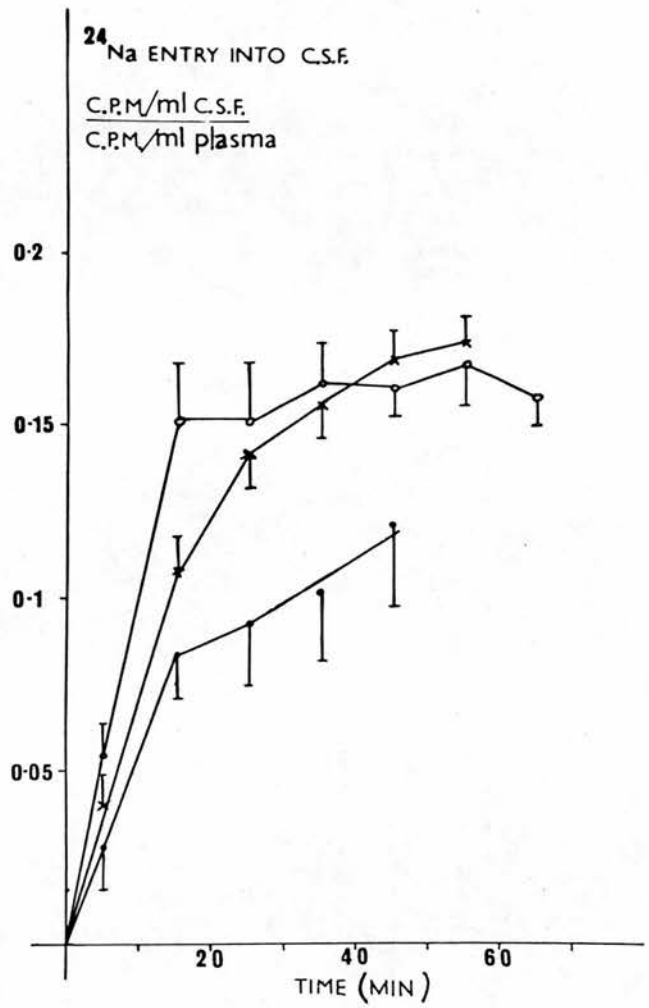

Figure 9. The effects of intravenous infusion of lithium chloride on the entry of ^{24}Na into ventriculo-cisternal perfusion fluid. Results are shown for control experiments (-X-), experiments in which lithium was infused concommitantly with ^{24}Na (-o-) and experiments in which lithium was infused 30 minutes prior to and during ^{24}Na infusion (-•-). Points represent mean values from 11 control experiments, 3 pre-infusion experiments and 4 concommitant infusion experiments. Bars represent the standard errors of the means.

Table 3. The effects of 0.6 - 1.2 mM lithium in plasma on C.S.F. secretion rate and on ^{24}Na entry into ventriculo-cisternal perfusion fluid and brain. Statistical comparisons used a Students 't' test; *$p<0.05$, **$p<0.025$, ***$p<0.005$, ****$p<0.001$. Values given are means ± standard deviations with number of estimations in parentheses.

	C.S.F. Secretion rate (µl/min)	Fast component of ^{24}Na entry into C.S.F. $\frac{\text{C.P.M./ml C.S.F.}}{\text{C.P.M./ml plasma}}$	Slow component of ^{24}Na entry into C.S.F. $\frac{\text{C.P.M./ml C.S.F.}}{\text{C.P.M./ml plasma}}$/30 min	^{24}Na entry into brain $\frac{\text{C.P.M./g wet brain}}{\text{C.P.M./ml plasma}}$
Control	10.8 ± 1.7 (6)	0.122 ± 0.032 (11)	0.039 ± 0.024 (11)	0.079 ± 0.006 (6)
0.6 - 1.2 mM lithium in plasma (pre-infusion)	5.6 ± 1.4*** (4)	0.069 ± 0.029* (3)	0.040 ± 0.012 (3)	0.080 ± 0.021 (4)
0.6 - 1.2 mM lithium in plasma (no pre-infusion)	7.4 ± 1.3** (4)	0.140 ± 0.029 (3)	0.005 ± 0.017	0.072 ± 0.008 (4)

Combining the results from all the lithium infusion experiments, the C.S.F. secretion rate was 6.5 ± 1.7**** (8).

was important therefore to eliminate changes in respiration as being indirectly responsible for the effects observed in the experiments described previously. In some of the experiments already described arterial blood pCO_2, pH and pO_2 were measured before and during perfusion with the various artificial cerebrospinal fluids and before and after intravenous infusion of lithium chloride. The results are shown in Table 4 and they show no evidence for the various experimental procedures changing blood pCO_2, pH or pO_2.

7. A study of the loss of lithium from the inflowing perfusion fluid.

When artificial C.S.F. containing 1.0 mM lithium was perfused through the ventricles there was a loss of lithium from the perfusion fluid. The results for a typical experiment are shown in Figure 10. The results are expressed as ratios of the inflowing to outflowing concentrations for Dextran Blue and lithium. The ratio for Dextran Blue represents dilution of the perfusion fluid with nascent C.S.F. Additional dilution of lithium is evident from the value of the C_o/C_i ratio being smaller than that of Dextran Blue and this represents transport of lithium from the perfusing fluid. Table 5 shows mean steady state values of C_o/C_i ratios for a series of experiments together with calculated values of the transfer constant, K_{out}, describing loss of lithium from the perfusing fluid and the percentage of lithium lost to brain.

Table 4. Arterial pCO_2, pH and pO_2 before and during various experimental procedures. Control values are those for samples taken prior to any experimental procedure. Values given are means of 2 or 3 estimations.

	Expt. No.	Control	Potassium free perfusion	1 mM Lithium in perfusion fluid	Lithium infusion into blood
pH	1	7.48	7.46	-	-
	2	7.31	7.31	-	-
	3	7.42	-	7.45	-
	4	7.40	-	7.39	-
	5	7.44	-	-	7.43
	6	7.46	-	-	7.42
pCO_2 (mm Hg)	1	30	32.5	-	-
	2	36	38	-	-
	3	27.5	-	25.5	-
	4	35	-	29.5	-
	5	29.5	-	-	26.5
	6	28	-	-	26
pO_2 (mm Hg)	1	54	65	-	-
	2	76	69	-	-
	3	81	-	79	-
	4	60	-	67	-
	5	70	-	-	67
	6	68	-	-	72.5

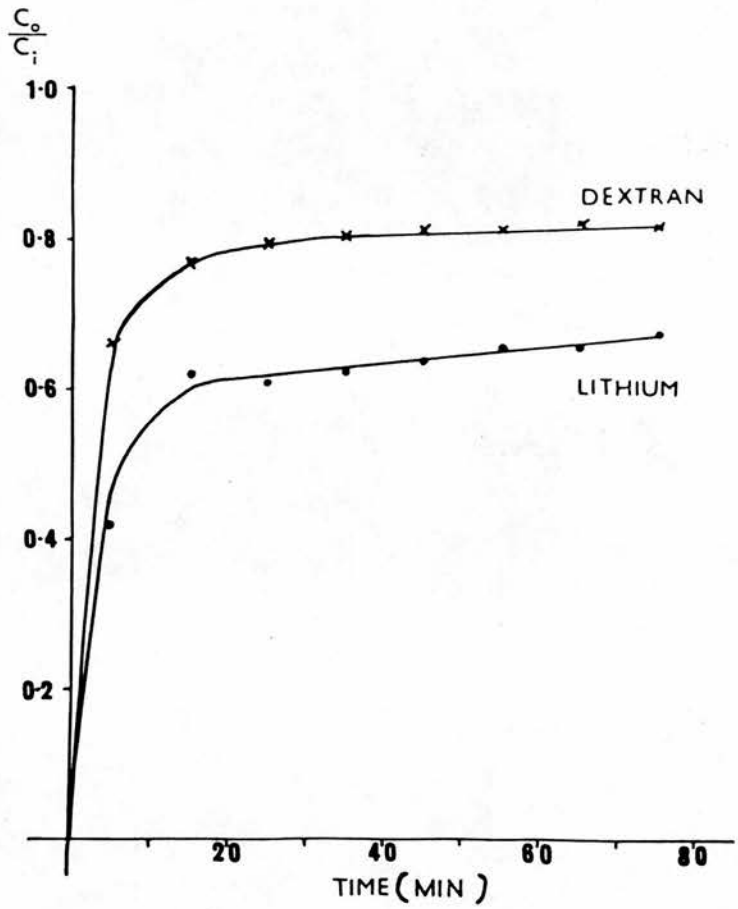

Figure 10. The ratios of lithium and Dextran Blue concentrations in inflowing fluid to those in cisternal effluent during ventriculo-cisternal perfusion. Results shown are from one experiment.

Table 5. Parameters describing the transport of lithium from perfusion fluid during ventriculo-cisternal perfusion. Figures given are mean values ± standard deviation with the number of estimations in parentheses. C_i and C_o represent inflowing and outflowing concentrations respectively.

Steady-state value of $\frac{C_o}{C_i}$ for Dextran Blue	Steady-state value of $\frac{C_o}{C_i}$ for lithium	Transfer constant K_{out} for lithium (µl/min)	Clearance of lithium into brain as a % of total clearance
0.83 ± 0.048 (6)	0.71 ± 0.073 (7)	10.2 ± 4.2 (7)	15.8 ± 1.7 (4)

DISCUSSION.

The purpose of these experiments was twofold. Firstly to investigate if sodium transport from blood to C.S.F. was sensitive to C.S.F. potassium concentration and secondly to investigate the effects of low concentrations of lithium on such sodium transport. In addition a brief study of the transport of lithium from the perfusing fluid was also carried out.

The analysis of ^{24}Na entry during ventriculo-cisternal perfusion in terms of two components follows the work of Davson and Pollay (1963). In the present work graphical analysis of the results allowed estimation of both the fast and slow components of entry. Davson and Pollay (1963) and Davson and Segal (1970) suggested that the fast component of sodium entry was due to the transport of sodium directly from blood to C.S.F. across the choroid plexuses and that the slow component was due to transport of sodium from blood to brain and thence to C.S.F. Assuming that all the ^{24}Na entered the perfusing fluid in nascent C.S.F. as a result of transport across the choroid plexuses, Davson (1967) calculated the steady-state ratio of ^{24}Na in the perfusing fluid to that in plasma to be 0.133. Using the method of analysis described in this section calculation of the steady-state value of the fast component of sodium entry for control experiments gave a value of 0.122. The good agreement between these two values supports the view that the fast component, as measured in this work represents

the transport of sodium across the choroid plexuses.

Perfusion with a potassium-free fluid caused a rapid and variable compensation for potassium which resulted in a high (2-3 mM) concentration of potassium in the cisternal effluent. Previous work (Bradbury and Davson, 1965; Bronsted, 1970) has shown similar effects of potassium-free perfusion and Bradbury and Davson (1965) interpreted their results in terms of a homeostatic mechanism controlling C.S.F. potassium concentration. The considerable potassium concentration in the cisternal effluent during potassium-free perfusion implies that ventricular fluid may contain potassium at concentrations of the same order in these experiments. Such a large compensation for potassium would explain why potassium-free perfusion had only a small effect on sodium entry into C.S.F. and C.S.F. secretion. Bronsted (1970) using an aqueductal perfusion technique found potassium-free perfsion to reduce net flux of sodium into C.S.F. but it was not possible to distinguish between sodium entering C.S.F. from blood and sodium entering from brain. The present experiments were able to show a correlation between C.S.F. cisternal potassium concentration and the fast component of sodium entering into C.S.F. Such a correlation could have been due to (a) sodium entry being a process sensitive to C.S.F. potassium (b) C.S.F. potassium affecting sodium entry indirectly or (c) the potassium homeostatic mechanism being dependant on sodium entry. Potassium-free perfusion had no effect on blood gases and so the

correlation is unlikely to be due to an indirect effect on respiration. When lithium was present during potassium-free perfusion there was an increase in the fast component of sodium entry but the cisternal potassium concentrations were unchanged from those in potassium-free perfusion experiments. This suggests that sodium entry did not affect potassium homeostasis. The most likely explanation of the correlation is that the sodium entry was directly sensitive to ventricular potassium concentration.

The fast component of sodium entry is considered to be due to a sodium-pump in the choroid plexus epithelium (Davson and Segal, 1970; Wright, 1972; Johansen et al., 1974). Bradbury and Stuclova (1970) suggested that the choroid plexus sodium-pump was situated at the apical (C.S.F. facing) membrane and this hypothesis was substantiated by the demonstration of ouabain-binding to the apical membrane of the frog choroid plexus epithelium (Quinton et al., 1973). On this hypothesis one would expect sodium entry into C.S.F. to be sensitive to C.S.F. potassium concentration. The present experiments have shown this to be the case. The in vitro studies on choroid plexus ATPase activity showed that the Na/K ATPase activity was sensitive to potassium concentrations comparable to those which affected sodium entry in the perfusion experiments. This supports the view that the potassium sensitive system seen in the perfusion experiments is the sodium-pump. Overall the experiments provide further evidence for the implication

of the choroid plexus sodium-pump in sodium transport across that tissue and they suggest the sodium-pump is orientated so as to be sensitive to C.S.F. potassium concentrations.

Despite the observations that both C.S.F. secretion and ^{24}Na entry into C.S.F. are inhibited by ouabain (Welch, 1963; Oppelt, Patlak and Rall, 1962; Vates et al., 1964; Davson and Segal, 1970) it cannot be categorically stated that all the sodium transport across the choroid plexuses is due to sodium-pump activity because the inhibitory effects of ouabain in vivo on ^{24}Na entry are not total and the effects of ouabain on C.S.F. secretion are variable, there only being a 55% inhibition by 10^{-6} M ouabain in the rabbit (Davson and Segal, 1970). The finding that 6.7×10^{-5} M ouabain, in vitro, totally abolished sodium flux across the choroid plexus (Wright, 1972) provides some evidence that the sodium-pump may be the sole mechanism for active sodium transport across the choroid plexus epithelium. However it is equivocable how great a proportion of choroid plexus sodium transport into C.S.F. is due to the activity of the sodium-pump and therefore the observed effects of lithium on sodium transport in the present experiments cannot be unequivocably attributed to effects on the sodium-pump.

Lithium chloride administered via the ventricles or by intravenous infusion did not affect the blood pCO_2, pH or pO_2 and it is unlikely therefore that the observed effects of lithium on sodium transport were due to indirect

effects of lithium on respiration.

If, as suggested, the choroid plexus Na/K ATPase is orientated so as to be sensitive to C.S.F. potassium concentration perfusion of the ventricles with a solution containing lithium will present lithium to the potassium sensitive side of the choroid plexus sodium-pump. Perfusion experiments of this nature showed a very low concentration of lithium in plasma (approximately 10^{-6} M) which suggests that the observed effects of such experiments were due to lithium acting at the potassium sensitive, ventricular side of the choroid plexus epithelium. It is unlikely the effects were due to changes in C.S.F. chloride concentration. The action of lithium presented in this manner was to cause an increase in C.S.F. secretion and the fast component of sodium entry both at reduced and normal C.S.F. potassium concentrations. In order to show that the effect of lithium was due to action on the sodium-pump, experiments were attempted which involved perfusion with an ouabain-containing solution. Perfusion with such a fluid caused death due to respiratory failure and so it was not possible to study the effects of ouabain in the present series of experiments. However it is most likely that lithium was affecting the sodium-pump in these experiments. The results of these experiments provide evidence for a stimulation of the sodium-pump by low concentration (0.6 - 1.0 mM) of lithium when presented to the potassium sensitive side of the system.

On the other hand results from experiments in which lithium was infused into the bloodstream suggest that lithium can also inhibit choroid plexus sodium transport. The low concentration of lithium in the cisternal effluent (0.04-0.1 mM) indicates that the effects observed during these experiments were due to lithium presented from the blood side rather than effects of lithium present on the ventricular side. Infusion of lithium and ^{24}Na concomittantly resulted in a large decrease in C.S.F. secretion rate together with an unusual curve of entry of ^{24}Na into the perfusing fluid which appeared to show an almost total abolition of the slow component of sodium entry. There were two inconsistencies in these results. Firstly the large decrease in C.S.F. secretion rate was not accompanied by a reduction in the fast component of sodium entry into C.S.F. Secondly the ^{24}Na entry into brain in these experiments was similar to that in controls. This, together with an almost non-existent slow component of sodium entry into C.S.F. would suggest that the ^{24}Na in brain had been unable to diffuse into the perfusing fluid. Such a barrier to diffusion is most unlikely. One explanation of both these inconsistencies is that lithium infusion caused a reduction in both C.S.F. secretion rate and the fast component of sodium entry but that these effects took some time to develop. If this was the case then there would be a delay before lithium inhibited the fast component of sodium entry and this could explain the unusual curve of entry without any change in

entry of ^{24}Na into C.S.F. from brain occuring. The present method of analysis would not have detected a delayed effect on the fast component. However C.S.F. secretion rate was calculated from steady-state values for samples collected some 40 minutes after the start of lithium infusion so that a delayed effect on C.S.F. secretion would be noticed. That a delayed inhibition of the fast component of sodium entry and C.S.F. secretion rate was the explanation of these results was substantiated by a second series of experiments in which lithium was infused 30 minutes before and during ^{24}Na infusion. The results of these experiments showed a marked decrease in C.S.F. secretion rate together with a normal curve of entry of ^{24}Na into C.S.F. and a significant decrease in the fast component of sodium entry.

Taken together the results of these two experiments show lithium presented via the bloodstream to cause a decrease in C.S.F. secretion and the transport of sodium across the choroid plexuses. Lithium infusion in the dog has been reported to have a positive inotropic action on the heart tending to raise blood pressure (Morgan, Procter, Ford, Velandia and Wasserman, 1974). If this had an effect on C.S.F. secretion it would tend to increase it. Changes in blood pressure have been reported to affect the entry of ^{24}Na into brain (Go and Pratt, 1975) and so the lack of effect of lithium infusion on sodium entry into brain suggests that any blood pressure changes in the present experiments were not sufficiently great to cause any effects

on sodium entry into C.S.F. or brain. It is unlikely therefore that the effects of lithium infusion were due to indirect effects of lithium on blood pressure.

The results of these experiments suggest therefore that lithium presented to the choroid plexuses from the blood causes an inhibition of sodium transport across the tissue. Transport of sodium from blood into the choroid plexus epithelium is thought to be due to diffusion (Wright, 1972) and it is unlikely that lithium would affect this step in the transport of sodium across the tissue. Thus the inhibitory effect of lithium must be on the transport of sodium out of the choroid plexus epithelium into C.S.F., a process which is thought to be due to the activity of the sodium-pump (Davson and Segal, 1970; Wright, 1972; Johansen et al., 1974). As pointed out by Johansen et al. (1974) such a sodium-pump should be sensitive to the concentration of sodium within the choroid plexus epithelium. The most likely explanation of the results of the lithium infusion experiments is therefore that lithium at low concentrations inhibits the sodium-pump when presented to the sodium sensitive side.

Amidsen and Schou (1968) showed lithium to have no effect on the transfer of ^{24}Na from blood to brain in the rat and the work described here in the rabbit also failed to find an effect of lithium on sodium entry into brain despite effects on the entry of sodium into C.S.F. In both studies the entry of sodium into brain was measured

as a ratio of ^{24}Na in brain to that in plasma. Such a ratio will depend on the equilibrium of several processes and so an effect of lithium on any one process could be hidden.

As shown in Figure 10 the dilution of lithium in the perfusing fluid during its passage through the ventricles was greater than the dilution of Dextran Blue and this shows that some lithium was lost from the perfusion fluid. The loss of lithium can be expressed mathematically by a transfer constant, K_{out}. The value of the transfer constant for lithium was similar to the published values for sodium, urea and creatinine but less than that for ^{42}K (Bradbury and Davson, 1964, 1965). The constant K_{out} depends on several factors including the permeability of the ependyma, whether or not the substance is actively transported and the ability of the brain cells to act as a sink for the substance in question((Bradbury and Davson, 1965; Cserr, 1965). The ability of brain cells to act as a sink for ^{42}K has been suggested as the reason for the large value of K_{out} for potassium and the fact that approximately 55% of potassium lost during perfusion is recovered from the brain whereas only 20% of sodium is recovered and 5% of calcium (Graziani, Escriva and Katzman, 1964; Bradbury and Davson, 1965; Cserr, 1965). The permeability of the ependyma to substances such as creatinine and inulin suggests that the permeability of the ependyma is not a limiting factor in the loss of small

molecules and ions from the perfsuing fluid.

The value of K_{out} for lithium is less than that for sugars which are removed by facilitated diffusion and it is similar to values for sodium, urea and creatinine which are thought to be lost by passive diffusion (Bradbury and Davson, 1964). It is likely therefore that the loss of lithium from the perfusing fluid is due to passive diffusion and suggests that lithium is free to diffuse between C.S.F. and brain extracellular fluid and therefore that C.S.F. concentrations of lithium will reflect the concentrations in extracellular fluid. Previous results have shown that in the dog lithium is also lost from perfusion fluid by passive diffusion (Prockop and Marcus, 1972). In contrast it has been suggested that rubidium is actively transported from C.S.F. in the rabbit (Bradbury, 1970). The present experiments found only 16% of the lithium lost during perfusion was recovered in the brain and this is comparable to results with sodium and calcium (Graziani et al., 1964; Cserr, 1965) but lower than that for potassium (Bradbury and Davson, 1965; Cserr, 1965). This together with the small value of K_{out} compared to potassium suggests that lithium is slow to penetrate brain intracellular space.

The conclusions from the experiments described in this section are that:

a) Lithium diffuses out of the perfusing fluid into brain but is slow to enter brain cells.

b) The fast component of sodium entry into C.S.F. is

sensitive to the potassium concentration in C.S.F. and this is compatible with choroid plexus sodium transport being due to a sodium-pump situated in the apical membrane.

c) Lithium, presented to the potassium sensitive side of the choroid plexus sodium-pump, caused a stimulation of sodium transport whereas if presented to the sodium sensitive side of the system lithium caused an inhibition of sodium transport.

SECTION III

AN INVESTIGATION OF THE EFFECTS OF LITHIUM ON ATPase ACTIVITIES IN SUBCELLULAR FRACTIONS PREPARED FROM RAT CEREBRAL CORTEX.

INTRODUCTION.

Changes in membrane ATPase activities have been suggested as important in the therapeutic action of lithium (Glen and Reading, 1973). Chronic lithium treatment was postulated to affect synaptic plasma membrane Mg ATPase activity, possibly due to fusion of the synaptic membrane and the synaptic vesicles. This section describes an investigation of the effects of chronic lithium treatment on ATPase activities in synaptosomal and mitochondrial fractions from rat brain.

Early studies of the distribution of ATPase activity in subcellular fractions derived from brain were difficult to interpret since the fractions were heterogeneous and not well defined in terms of morphology (Whittaker, 1965). Na/K ATPase activity was mainly associated with microsomal fractions but there was also considerable activity in the crude mitochondrial pellet (Schwartz, Bachelard and McIlwain, 1962; Albers, Rodriguez De Lores Arnaiz and De Robertis, 1965; Whittaker, 1965). Further analysis of ATPase distribution was possible with the advent of methods for subfractionation of the crude mitochondrial pellet and for the production of synaptosomes, synaptic vesicles, synaptic plasma membranes and synaptic mitochondria (Whittaker, Michaelson and Kirkland, 1964; Albers et al., 1965; Whittaker, 1965). Subsequent studies showed Na/K ATPase activity to be primarily associated with pinched-off nerve endings (synaptosomes) and synaptic plasma membranes

rather than microsomes (Hosie, 1965; Albers et al., 1965; Kurokawa, Sakamoto and Kato, 1965; Bradford, Brownlow and Gammack, 1966). Na/K ATPase activity has also been shown to be associated with neuronal plasma membranes by microdissection techniques (Cummins and Hyden, 1962). The occurrence of Na/K ATPase in synaptic plasma membranes is consistent with its proposed function in the active transport of sodium and potassium (Skou, 1965; Whittam and Wheeler, 1970; Abdel-Latif, 1973).

The distribution of Mg ATPase activity in subsynaptosomal fractions showed two distinct enzymes, a Mg ATPase activity stimulated by 2,4 dinitrophenol and a Mg ATPase activity insensitive to the phenol. The insensitive activity was associated with vesicular and synaptic membrane fractions while the mitochondrial Mg ATPase activity was stimulated by 2,4 dinitophenol (Hosie, 1965). Hosie found the Na/K ATPase activity to be located in the membrane fractions with none occurring in the vesicle fraction. She concluded that synaptic vesicles possessed an inherent Mg ATPase activity. Other workers have since confirmed the occurrence of Mg ATPase activity in synaptic vesicles but in addition they have reported a very low Na/K ATPase activity in vesicle fractions and they have attributed this to contamination with membranous material (Germain and Proulx, 1965; Kadota, Mori and Imaizumi, 1967; Morgan, Vincendon and Gombos, 1973). Mg ATPase activity has also been reported in catecholamine-containing granules in the

adrenal medulla and blood platelets (Banks, 1965; Poisner and Trifaro, 1967; Heinrich, Da Prada and Pletscher, 1972; Agostini and Taugner, 1973). The occurrence of Mg ATPase activity in synaptic plasma membrane fractions has been confirmed (Kurokawa et al., 1965; Reading, Dewar and Kinloch, 1974) but its function remains unknown. The function of the synaptic vesicle Mg ATPase is also obscure but it has been suggested to be involved in the release and storage of acetylcholine and catecholamines (Germain and Proulx, 1965; Kadota et al., 1967; Poisner and Taugner, 1973; Pletscher, Da Prada, Steffen, Lutold and Berneis, 1973). Recently evidence has started to accumulate suggesting the occurrence of actomyosin-like Mg ATPase in mammalian brain which might be involved in the exocytotic release of neurotransmitter (Berl, Puszkin and Nicklas, 1973).

Bowler and Duncan (1966) reported ATP-induced absorbancy changes in a supernatant fraction from crayfish nerve and they interpreted this as showing the presence of actomyosin-like protein in the nerve membrane. They suggested the Mg ATPase activity of this protein to control passive permeability. More recently, proteins with various properties similar to muscle actomyosin have been isolated from rat, cat and bovine brain (Puszkin, Berl, Puszkin and Clarke, 1968; Berl and Puszkin, 1970). These properties included superprecipitation by ATP, ATPase activity stimulated by both magnesium and calcium seperately, solubility

properties in potassium iodide (Puszkin et al., 1968), polyethylenesulphonate inhibition of Mg ATPase activity and Mg ATPase kinetics with respect to ATP and magnesium (Berl and Puszkin, 1970). Further studies have shown the presence of actin-like and myosin-like proteins in brain (Berl and Puszkin, 1970; Fine and Bray, 1971; Puszkin and Berl, 1972). The properties of these proteins resembled their muscle counterparts; the brain myosin-like protein (stenin) showed a much greater ATPase activity with calcium than magnesium but this situation was reversed by the addition of actin-like protein from brain (neurin). In addition neurin stimulated muscle myosin Mg ATPase activity, possessed 3-methylhistidine as does actin, polymerised in 0.1 M KCl in the presence of magnesium and underwent viscosity changes after interaction with muscle myosin. Overall, there is considerable evidence for the presence of a contractile protein in brain which possesses Mg ATPase activity.

More recent work has shown this contractile protein to occur in the synaptosomal fraction of rat and bovine brain (Puszkin, Nicklas, Berl, 1972). The protein was not found in any other subcellular fraction of brain and accounted for 8-10% of synaptosomal protein. This protein extracted from synaptosomal material showed properties similar to the protein extracted from whole brain. In a further study (Berl et al., 1973) synaptic vesicle and synaptic plasma membrane fractions were examined for

actin-like and myosin-like proteins. Vesicles were found to contain mostly stenin (myosin-like) and only a little neurin (actin-like). In contrast plasma membrane fractions contained mostly neurin with only a little stenin. Vesicular stenin possessed no Mg ATPase activity unless actin or neurin were added. Interpretation of these results is equivocable in view of the heterogeneity of the fractions concerned but the results suggest myosin-like protein to be associated with synaptic vesicles and actin with synaptic membranes. On the basis of these results Berl and his colleagues suggested that neurin and stenin are involved in the mechanism of exocytosis in a manner comparable to the contractile events in muscle; when calcium enters the cell it causes an interaction between neurin and stenin so causing a contractile event to occur which gives rise to release of neurotransmitter. Burridge and Philips (1975) have shown actin and myosin to interact with chromaffin granule membranes. Further evidence for a muscle-like contractile system in brain has come from the extraction of tropomyosin-like complexes from brain, from neurones in culture and from synaptic plasma membranes (Fine, Blitz, Hitchcock and Kaminer, 1973; Mahendran, Nicklas and Berl, 1974; Puszkin and Kochwa, 1974).

In summary, Na/K ATPase activity has been shown to be primarily associated with external membrane fractions such as synaptosomes and synaptic plasma membranes. Vesicles possess an inherent Mg ATPase activity as do synaptic plasma

membranes. The relationship between these two activities and their function is obscure. However in view of the reports of actomyosin-like Mg ATPase in synaptosomal fractions one must entertain the possibility that either synaptic plasma membrane or synaptic vesicle Mg ATPase activity is associated with actomyosin-like protein. The Mg ATPase activity in mitochondria is distinct from vesicular and membrane activity in that it is stimulated by 2,4 dinitophenol.

Despite the problem of possible glial contamination of synaptosomal fractions (Morgan, Wolfe, Mandel and Gombos, 1971; Henn, Anderson and Rustad, 1976), preparation of such fractions from lithium-treated animals is a useful approach to the study of the effects of chronic lithium treatment on ATPase activities associated with synaptic vesicles and membranes. The experiments to be described in this section examined the effects of chronic lithium treatment on synaptic membrane, synaptic vesicle and brain mitochondrial ATPase activities. It was suggested in the previous section that the distribution of lithium would determine the chronic effect of the drug on the Na/K ATPase activity associated with the sodium-pump. Using synaptic membrane material it was possible to investigate the effect of chronic lithium treatment on Na/K ATPase activity. Previous work on the effects of chronic lithium administration on Na/K ATPase activity in nervous tissue has been contradictory. Ploeger (1974a) found chronic lithium administration to decrease

Na/K ATPase activity in rat vagal nerve homogenate. Using the same dose and administration time Gutman and colleagues were unable to show any effect of lithium administration on rat brain microsomal Na/K ATPase activity although Na/K ATPase activity was increased in parotid gland and kidney medulla microsomes and decreased in microsomes prepared from colonic mucosa (Gutman et al., 1973a; 1973b). In agreement with the findings of Ploeger, Reading and his colleagues have reported chronic lithium administration to decrease rat synaptic plasma membrane Na/K ATPase activity (Reading, Kinloch and Loose, 1975). Furthermore Na/K ATPase activity was found to increase 1 week after the termination of lithium administration (Reading et al., 1974).

In view of these contradictory results it was of interest to study the effect of chronic diet administration of lithium on rat brain Na/K ATPase activity and this was done in the present experiments using rat cerebral cortex synaptic plasma membranes. Chronic lithium administration has been reported to increase rat synaptic plasma membrane Mg ATPase activity (Reading et al., 1975). However previous work had shown rat synaptic plasma membrane Mg ATPase activity to increase 3 weeks after termination of lithium administration (Reading et al., 1974). In view of this confusion experiments were carried out to further examine the effects of chronic lithium administration on rat synaptic plasma membrane Mg ATPase. The work was extended to examine the possible effects of lithium on Mg ATPase activities in

mitochondrial and synaptic vesicle fractions in order to determine whether the effect of lithium on Mg ATPase activity was restricted to plasma membrane fractions. Previous work has failed to show an effect of chronic lithium administration on rat brain microsomal Mg ATPase activity (Gutman et al., 1973a). Changes in subcellular fraction specific enzyme activities are open to equivocal interpretation in that they can be explained either in terms of changes in enzyme specific activities associated with a distinct subcellular structure or in terms of changes in the quantitative composition of the fraction. In an attempt to test whether changes in enzyme activities were due to changes in fraction composition, enzyme markers for membranes and mitochondria were also measured in the present experiments.

Christensen (1974) has reported that lithium is associated with the particulate fraction of rat brain following the long-term administration of lithium to rats although it is not possible from his results to determine the structures with which lithium could be associated. If lithium was strongly bound to subcellular structures it could be present in subcellular preparations. It was important therefore to determine whether lithium, added in vitro, had any effects on ATPase activity in the various fractions studied. In addition a preliminary attempt to measure lithium in subcellular fractions was made.

Finally, lithium distribution between various brain

areas was studied following chronic lithium administration in order to examine whether in future experiments it would be useful to study any particular area of the brain in relation to effects of lithium. At the time of carrying out these experiments there were three contradictory reports concerning lithium distribution. Ho et al. (1970) reported no regional variation in lithium concentration whereas both Edelfors and Gothgen (1971) and Ebadi, Simmons, Hendrickson and Lacy (1974) reported regional differences. Following the present work, other reports have come to press (Bond, Brooks and Judd, 1975; Edelfors, 1975).

MATERIALS AND METHODS.

All chemicals used were B.D.H. Analar grade except where otherwise stated.

1. Animals and Lithium administration.

Male, Wistar rats (200-250g) were used throughout. They were purchased from Edinburgh University Animal Breeding Station, Bush Farm, Edinburgh and housed three animals per cage under a regular, normal cycle of 12 hours light and 12 hours dark. All animals used for experiments were killed between 9.30 and 10.30 a.m.

Reading et al. (1974, 1975) used intraperitoneal injection (injection at 4-5 p.m., daily) to administer lithium chloride (30 mg/rat/day) to rats over periods of three to six weeks. However such treatment of the animals gave rise to a massive diuresis necessitating a daily change of cage. In addition the animals were irritable and in a

general unhealthy condition. This situation was partially improved by offering the animals a salt-lick in their cage. Although the animals' general condition improved, lithium administration still caused diuresis. As shown by Morrison et al. (Morrison, Pritchard, Braude and D'Aguanno, 1971) intraperitoneal administration of lithium produces a high and short-lived peak of lithium concentration in plasma followed by a rapid fall to almost zero after 24 hours. Chronic administration of lithium by intraperitoneal injection would be expected therefore to give rise to wide variation in plasma lithium concentration. The high concentrations attained may exascerbate the problem of toxicity (Thomsen and Oleson, 1974). It was decided therefore to attempt administration of lithium chlorde to rats by giving animals ground food to which lithium chloride had been added. Animals were also offered 0.9% saline and water to drink as this had been previously shown to reduce toxic effects of lithium (Thomsen and Oleson, 1974).

Initially lithium chloride was given in the food at a concentration of 100 mmol/kg food but this regimen caused the animals to lose weight. Subsequently a dose of 60 mmol lithium chloride/kg food was used together with saline and water to drink. This lower dose was similar to that used by other workers (Thomsen and Oleson, 1974; Bond et al., 1975) and was found satisfactory from the point of view of animal health; the animals maintained weight, appeared healthy and had little or no diuresis.

Although the animals receiving lithium in the diet did not gain as much weight as those receiving intraperitoneal injection (Figure 11) their general health was much superior. The lower weight gain in the diet-administered group may be due to the animals eating less.

Results shown in Figures 12 and 13 give a comparison of plasma and brain concentrations of lithium following diet and intraperitoneal injection administration of lithium chloride. They show considerably higher ($p<0.001$) plasma and brain concentrations of lithium following diet administration (100 mM/kg food). Using the lower diet dosage of lithium, plasma lithium concentrations were also considerably higher than those achieved by injection administration. Comparison of lithium concentrations between injected and diet administered groups is difficult since animals were killed 16 hours after injection and an unknown but probably much shorter time after eating food containing lithium. Morrison et al. (1971) showed oral administration of lithium to produce stable lithium concentrations in both plasma and brain but there was no peak value as found after injection administration. It is probable that in the present experiments intraperitoneal injection gave periodically higher brain lithium concentrations than diet administration but that diet administration produced more stable concentrations. It was decided that diet administration was superior to injection administration since it probably produced pharmacokinetics

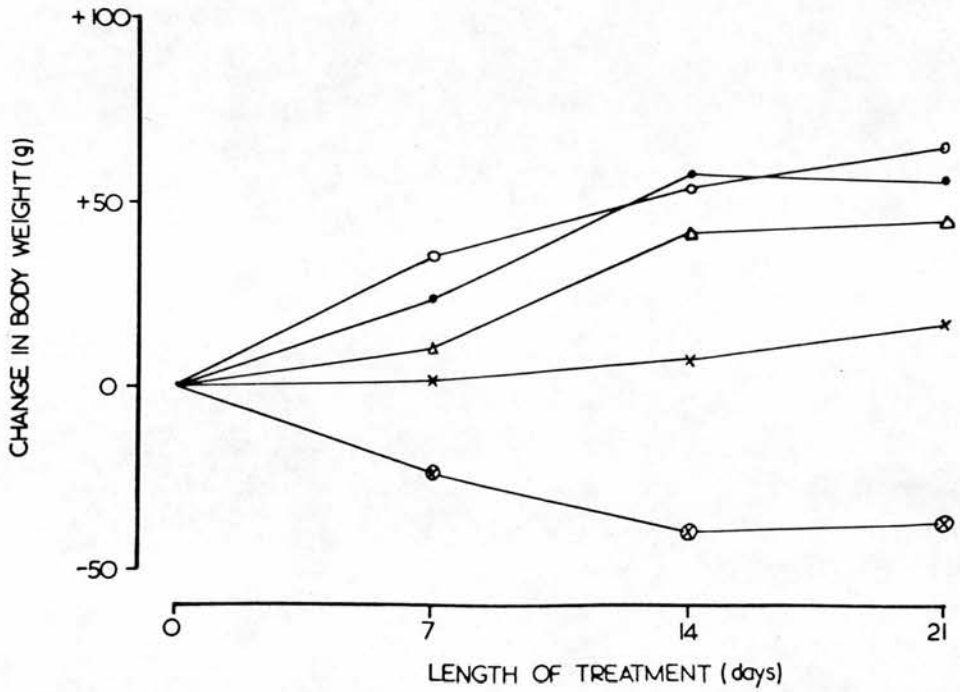

Figure 11. Change in body weight following administration of lithium chloride to rats. Results are shown from experiments in which lithium chloride was given by diet administration and by intraperitoneal injection: -Δ- intraperitoneal injection (30 mg LiCl/rat/day), -●- intraperitoneal injection (30 mg LiCl/rat/day) together with a salt-lick as supplementation, -x- diet administration (60 mmol LiCl/kg food) and -⊗- diet administration (100 mmol LiCl/kg food). Animals receiving lithium chloride in the diet were offered a choice of water and 0.9% saline to drink. Results are also shown from control animals (-o-) which received ground food, a salt-lick and a choice of 0.9% saline and water to drink. Values given represent the change in mean body weight since start of treatment. Data from 6 animals in each group.

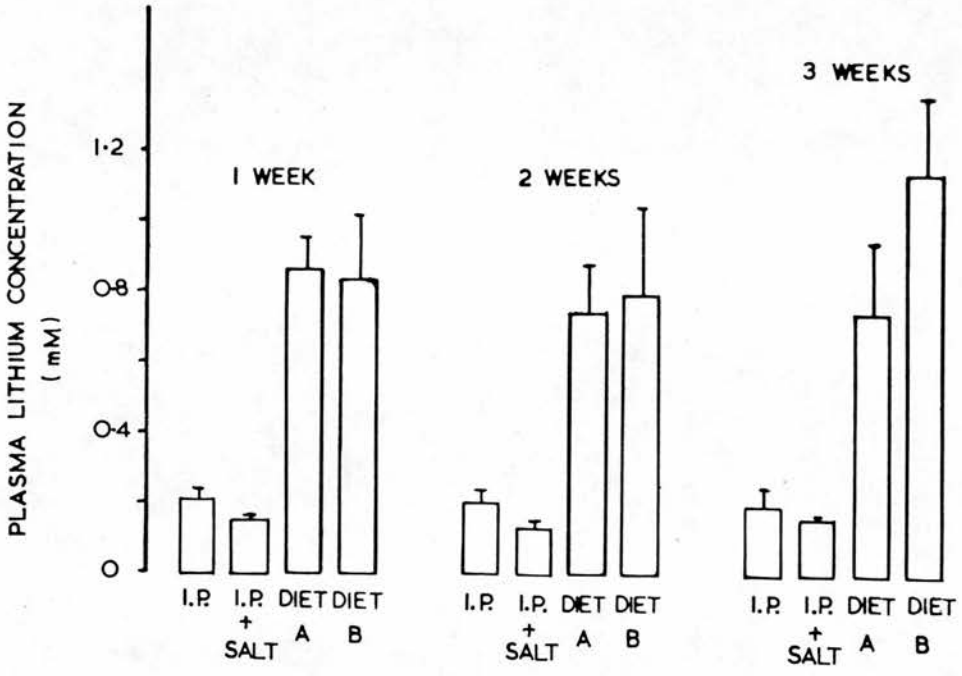

Figure 12. Plasma lithium concentration following diet and intraperitoneal injection (i.p.) administration of lithium chloride to rats for 1, 2 and 3 weeks. Results are shown from animals which received lithium chloride by daily intraperitoneal injection (30 mg LiCl/rat/day), with and without salt-supplementation in the form of a salt-lick, and by diet administration with a choice of water and 0.9% saline to drink. Diet administration was given at two doses, Diet A (60 mmol LiCl/kg food) and Diet B (100 mmol LiCl/kg food). Values represent means $\pm$ standard deviations from 6 (Diet B and i.p. groups) and 14 (Diet A group) animals. Groups were compared using a Students 't' test, both diet groups different from i.p. groups ($p<0.001$) at each week, i.p. group significantly different from i.p. + salt group at week 1 ($p<0.025$) and week 2 ($p<0.005$).

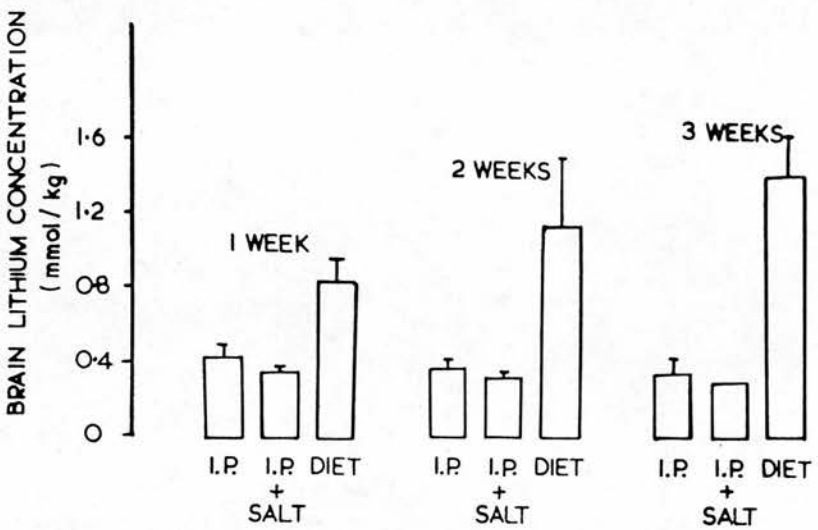

Figure 13. Brain lithium concentration following diet and intraperitoneal injection (i.p.) administration of lithium chloride to rats for 1, 2 and 3 weeks. Results are shown from animals which received lithium chloride by diet administration (100 mmol LiCl/kg food) and by daily intraperitoneal injection (30 mg LiCl/rat/day) with and without salt supplementation in the form of a salt-lick. Animals given lithium chloride in the food had a choice of water and 0.9% saline to drink. Values represent means $\pm$ standard deviations from six animals. Groups were compared using a Students 't' test, diet group significantly different ($p<0.001$) from i.p. groups at each week. There was no significant difference between i.p. and i.p. + salt groups.

which were closer to those found in the clinical situation (Schou, 1976). Use of 60 mmol LiCl/kg food produced plasma lithium concentrations comparable to those achieved clinically. Thus in all experiments to be described lithium was administered in the diet using ground food containing 60 mmol lithium chloride/kg food. Control animals received ground food (standard rat diet, Oxoid Modified 45B) and a choice of 0.9% saline and water to drink.

As shown in Figures 12 and 13 access of the animals to a salt-lick reduced their plasma lithium concentrations when lithium was administered by intraperitoneal injection (significantly reduced at 1 and 2 weeks treatment, $p < 0.05$ and $p < 0.005$). This may represent a difference in the excretion of lithium since a sodium-enriched diet has been shown to increase lithium excretion in humans (Thomsen and Schou, 1968).

2. Preparation of subcellular fractions from rat cerebral cortex.

Mitochondrial, synaptic membrane and synaptic vesicle fractions were prepared from rat cerebral cortex using methods described by Whittaker and Barker (1972). A summary of the preparation of membrane and vesicle fractions is shown in Figure 14. All procedures were carried out at 0 - 4°C.

Animals were killed by cervical fracture and decapitated. After removing the top of the skull, a slice of cortical tissue (approximately 0.5g per animal) was taken

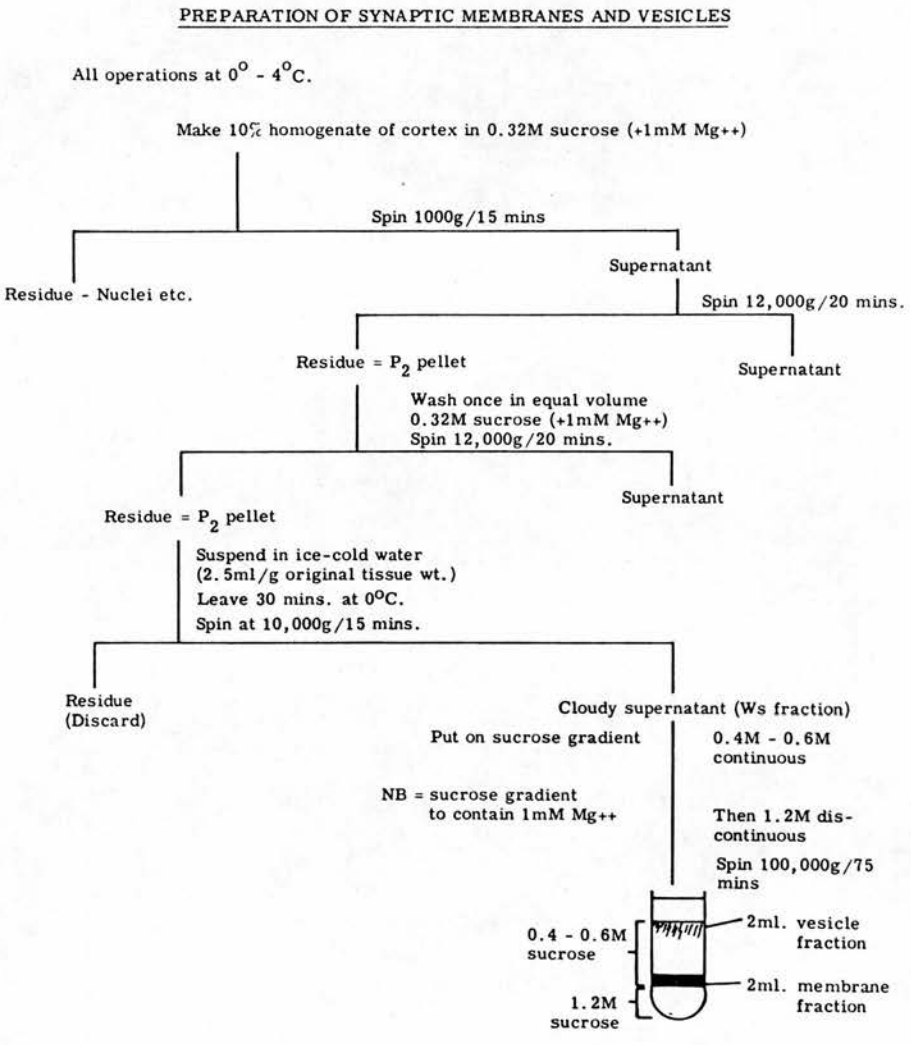

Figure 14. Preparation of synaptic vesicle and synaptic plasma membrane fractions from rat cerebral cortex.

and kept on ice. Tissue from two animals was pooled and homogenised in 10 volumes of 0.32 M sucrose (+1 mM $MgCl_2$). Homogenisation was at a speed of 1061 revolutions/minute for two periods of one minute with a 30 second interval between. The mortar was kept in ice the whole time. A glass mortar with a fitted teflon pestle (clearance 7 thousands of an inch) was used throughout. The homogenate was centrifuged at 1000 g for 15 minutes to remove nuclei and cell debris and the resulting supernatant centrifuged at 12,000 g for 20 minutes to remove microsomes, so producing P_2 the crude mitochondrial pellet. This pellet was washed once in sucrose by resuspension and centrifugation at 12,000 g for 20 minutes. The crude mitochondrial pellet consists mostly of synaptosomes and mitochondria. The synaptosomes were lysed in ice-cold water (2.5 ml/g original tissue) and left at 0-4°C for 30 minutes. The suspension was then centrifuged at 10,000 g for 15 minutes and the resulting supernatant (W_s) layered on to a sucrose gradient, 0.4-0.6 M continuous, 1.2 M discontinuous. After centrifugation at 100,000 g for 75 minutes two layers of material could be seen. A faint band of material was visible some 1-2 cm below the fluid meniscus. 2 ml of this material were taken and this is described as the vesicle fraction. The synaptic membrane fraction consisted of 2 ml of white, fluffy membranous material taken from just above the 0.6 M:1.2 M sucrose interface. These fractions were stored at 4°C before assay.

The residue from the post-lysis centrifugation of the crude mitochondrial pellet was discarded in early experiments. Latterly however it was used for the preparation of a mitochondrial fraction. The pellet was resuspended in 0.32 M sucrose and layered on to a 0.8 M:1.2 M discontinuous sucrose gradient and centrifuged at 53,000 g for 2 hours. Mitochondria appeared as a pellet at the bottom of the tube with the synaptosomal fraction at the 0.8 M: 1.2 M sucrose interface. The mitochondrial pellet was yellow or brown in colour and there was a white layer of synaptosomal material just above it. This white layer was removed by gentle agitation in 0.32 M sucrose (two washings required). The mitochondrial pellet was then resuspended in 3 ml water and stored at $4^{o}C$.

In order to achieve good separation of synaptosomes and mitochondria and thus to produce reasonably pure mitochondrial fractions it was necessary to omit magnesium from the sucrose used for the discontinuous gradient. Other workers have often used 1 mM EDTA in media used for mitochondrial preparation (Biesold, 1974) but this was not found necessary in the present work.

Subcellular fractions produced by the above procedures were characterised using both electron microscopy and enzyme markers. Both electron microscopy and enzyme content showed the fractions to be of satisfactory purity.

3. Measurement of ATPase activity.

ATPase activity was measured by estimation of the

inorganic phosphate liberated during incubation with disodium-ATP (B.D.H., Poole). Incubation was at 37.5°C for various periods of time: 30 minutes for synaptic plasma membranes, 1 hour for synaptic vesicles and 15 minutes for mitochondria. ATPase activity was linear with respect to length of incubation. The incubation conditions for the estimation of ATPase activities in synaptic plasma membranes are shown in Table 6. and were based on those used by Donting and Caravaggio (1963), Kurokawa et al (1965) and Reading et al. (1974). ATPase activities were defined in terms of cation activation. Mg ATPase activity was defined as the activity found in the presence of magnesium as the only cation and Na/K ATPase was defined as the additional activity in the presence of sodium and potassium. For the assay of vesicular Na/K ATPase it was found necessary to use Tris buffer, not water, to make up the volume of the incubation medium and this gave a final concentration of 90 mM Tris. Tris was used to make up volume for all assays of vesicle and mitochondrial fractions. All assays were done in triplicate and run with enzyme and reaction blanks. The reaction was stopped with 1 ml 24% trichloracetic acid and inorganic phosphate estimated by the method of Martin and Doty as described by Lindberg and Ernster (1956). Samples were compared for phosphate content with standards of KH_2PO_4. Specific activities were calculated as μmol P_i liberated /hr/mg protein.

Table 6. Incubation conditions for assay of ATPase activities in synaptic plasma membranes. The table shows final concentrations (mM) of chemicals included in the incubation medium. The total volume of the incubation medium was 2.5 ml. Na/K ATPase activity was defined as the difference between total and magnesium dependant activity.

	Total ATPase activity	Magnesium dependant ATPase activity
Disodium ATP	3	3
$MgCl_2$	.6	6
KCl	5	-
NaCl	100	-
Tris-HCl buffer, pH 7.4	30	30
Tris-EDTA pH 7.4	0.1	0.1

4. Measurement of Succinic dehydrogenase activity.

Succinic dehydrogenase activity was measured spectrophotometrically by following the succinate dependant reduction of potassium ferricyanide. The procedure followed that described by Singer (1974) and is given below.

Reagents: 0.3 M K_3PO_4 buffer, pH 7.5
0.01 M KCN
0.01 M $KFeCN_6$
0.2 M sodium succinate, pH 7.5

Procedure: Phosphate buffer (0.5 ml), cyanide (0.3 ml) and succinate (0.3 ml) together with enzyme and water (combined volume 1.4 ml) was pipetted into a cuvette and left at 38°C for 7 minutes. The reaction was started by adding 0.5 ml ferricyanide and the optical density at 420 nm was followed for 5 - 15 minutes, the cuvette being kept at 38°C. The blank consisted of all the above constituents except enzyme. In the absence of succinate there was no measurable reduction of ferricyanide. Results are expressed as change in optical density/10 minutes/mg protein.

5. Measurement of p-nitrophenylphosphatase activity.

p-nitrophenylphosphatase activity was measured using previously described methods (Lowry, Roberts, Wu, Hixon and Crawford, 1954; Albers et al., 1965) as modified by Dewar (1972). The method measures the p-nitrophenol liberated by its optical density in alkaline solution.

Reagents: a) 0.1 M glycine in 1 mM $MgCl_2$ and 85 mM NaOH

b) 0.4% p-nitrophenylphosphate (Sigma Chemical Co. Ltd.) in 1 mM HCl, pH 6.5 - 8

c) A mixture of reagent a) and b) in equal proportions.

d) 0.02 M NaOH

Procedure: 0.03 ml sample was added to 0.3 ml of reagent c) and incubated at 38°C for 30 minutes. The reaction was stopped by cooling to 0°C and 3 ml of 0.02 M NaOH added. The optical density at 420 nm was measured and measured again after additon of 2 drops concentrated hydrochloric acid. The difference in optical densities was a measure of the p-nitrophenol present. Results are expressed as change in optical density/20 minutes/mg protein.

6. Electron Microscopy.

The morphological characteristics of various subcellular fractions were studied by electron microscopy using thin section techniques. Fractions were prepared as described earlier and then resuspended in 5 ml 0.32 M sucrose. The suspensions were then centrifuged at 60,000 g for 15 minutes to pellet the subcellular material and the supernatant fluid aspirated off. The pellet was fixed in 1 ml 5% glutaraldehyde in 0.5 M sodium cacodylate buffer, pH 7.3. The material was left in fixative for 18 hours at 0-4°C and then washed twice in 10% sucrose in cacodylate buffer (each wash was for ten minutes). Tissue was post-fixed in osmium tetroxide, 1% in cacodylate buffer, for 1 hour. After dehydrating the tissue in alcohol (4 ten minute washes in 10% ethanol then 4 ten minute washes in absolute alcohol) the tissue was

cleared in epoxypropane (2 ten minute washes) and embedded in araldite. Tissue was left in araldite for 3 days before cutting. 500 Å thick sections were cut through the embedded material using a LKB III ultra microtome and the sections stained in saturated uranyl acetate (15 minutes) and lead citrate (10 minutes). The sections were finally rinsed in 5 mM NaOH and then distilled water. Sections were examined on an A.E.I. EM6M electron microscope using copper grids.

7. Protein was estimated by the method of Lowry et al. (1951) as given in Appendix 2.

8. Measurement of lithium in whole brain and plasma.

All estimations of lithium in plasma and in digests of whole brain were done by flame atomic absorption spectrophotometry at 670 nm using a Perkin-Elmer 360 atomic absorptiometer.

Blood was taken from the severed cervical blood vessels at the time of death and centrifuged at 1000 g for 5 minutes to separate plasma and erythrocytes. Plasma was stored at -20°C before analysis. Samples and standards were diluted 1:10 for analysis. Brain was digested using the procedure of Bradbury et al. (1968) as described in section II. The digest was diluted 1:10 for analysis. All standards used consisted of known amounts of lithium chloride dissolved in distilled water (Baker Chemical Co. Ltd., New Jersey).

9. Measurement of lithium in discrete areas of brain and in subcellular fractions.

Measurement of lithium in small amounts of various

kinds of tissue was carried out using a flameless method of atomic absorption spectrophotometric analysis. This method was more sensitive and used less material than the usual flame methods of atomic absorption analysis and therefore allowed analysis of very low concentrations of lithium in small amounts of tissue. It was first applied to the measurement of lithium distribution in the brain and later to the measurement of lithium in rat brain subcellular fractions and human erythrocyte membranes (see section V).

a) Digestion of brain tissue: Weighed aliquots (10 - 100 mg) of fresh brain were dried at 105°C for 48 hours and reweighed. The dried samples were then ground to a powder and a weighed aliquot transferred to a plastic tube (Eppendorf) using a Cahn electrobalance fitted with a tantalum boat. The weighed powder was digested in 1.5 M nitric acid (500 µl) at 80°C for 48 hours. The contents were evaporated to dryness and the residue redissolved in 0.75 M nitric acid. Nitric acid did not interfere with the subsequent analysis.

b) Measurement of lithium in subcellular fractions: It was difficult to measure lithium directly in subcellular fractions since sucrose present in the fractions charred in the furnace and interfered with the estimation. One solution to this problem was to add ammonium hydroxide to the fraction so reducing the interference. However this also reduced the sensitivity of the estimation.

c) Flameless atomic absorption spectrophotometric

analysis: Lithium analysis was carried out at 670 nm using a Perkin-Elmer HGA-74 heated graphite furnace. Blanks consisted of relevant material (brain digests or brain subcellular fractions) prepared from rats which had not received lithium treatment and standards consisted of similar material with known amounts of lithium chloride (Baker Chemical Co. Ltd.,) added. Standards, samples and blanks were diluted 1:5 with deionized water for analysis of lithium in brain areas whereas subcellular fractions were analysed both without dilution and at 1:5 dilution. 50 µl of solution was pipetted into the graphite furnace and the analysis carried out using the working programme summarised in Figure 15. After a short pre-drying period to reduce spraying, the sample was dried at 240°C, thermally destroyed by steadily increasing the temperature to 1360°C and atomised rapidly at 2500°C. The furnace was operated using uninterrupted gas flow (200 ml/min) with Argon as the inert gas.

The sensitivity of the analysis was 8 µg/litre for 1% absorption. Using 50 µl samples it was possible to detect amounts of lithium in the order of 1 ng. Absorption was linear over a range of lithium concentrations up to 700 µg Li/litre (0.1 mM).

10. Dissection of brain areas.

The brain was removed, washed in chilled saline and dissected over ice as quickly as possible. The following areas were taken for lithium analysis: cerebellum,

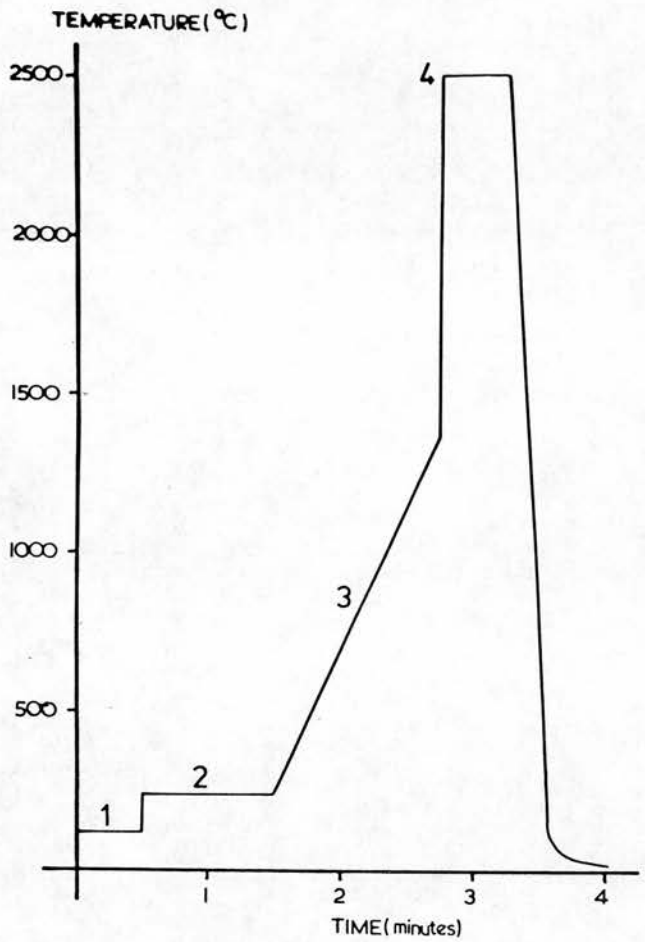

Figure 15. A diagrammatic representation of the working programme used for analysis of lithium by flameless atomic absorption spectrophotometry. Analysis was carried out as described in the text using a Perkin Elmer HGA-74 graphite furnace. The figure shows the temperature and duration of the predrying (1), drying (2), thermal destruction (3) and atomization (4) stages of the analysis.

hypothalamus, striatum, lower frontal cortex, cortex and brain stem. The procedure for separating the various areas is described below.

The cerebellum was removed by cutting the cerebellar peduncles and the remaining tissue sectioned into three by a series of three complete coronal knife-cuts - one at the level of the optic chiasma, another behind the mamillary bodies and a third behind the superior colliculi. The tissue anterior to the optical chiasma section will be referred to as anterior tissue, that tissue posterior to the optic chiasma section but anterior to the section behind the mamillary bodies will be referred to as central tissue and finally the tissue posterior to the section behind the mamillary bodies but anterior to the section behind the superior colliculi was brain stem. A horizontal cut was made along the line of the anterior commissure through the anterior and central tissue. The central tissue ventral to the horizontal cut with the cortical tissue on either side removed was hypothalamus. Lower frontal cortex was the anterior tissue which was ventral to the horizontal cut. Striata were removed from the dorsal anterior tissue with curved forceps. Cortical tissue was peeled off from the dorsal anterior tissue and pooled with the cortical parts of the central tissue block and was termed cortex.

RESULTS.

1. Composition of subcellular fraction.

A series of experiments was performed in order to

study the morphology and biochemical characteristics of mitochondrial, synaptic vesicle and synaptic plasma membrane fractions produced by the procedure used for later experiments. Fractions prepared from control animals were examined biochemically by studying enzymes which are considered to be markers for certain subcellular structures and by electron microscopy. Succinic dehydrogenase was used as a marker for mitochondria (Rodriguez De Lores Arnaiz et al., 1967; Morgan et al., 1973; Gurd, Jones, Mahler and Moore, 1974; Marchbanks, 1974), Na/K ATPase as a marker for plasma membranes (Whittaker and Barker, 1972) and p-nitrophenylphosphatase as a marker for membrane material (Rodriguez De Lores Arnaiz et al., 1967; Reading et al., 1974). Mg ATPase was also studied as this enzyme is associated with synaptic vesicles (Hosie, 1965; Kurokawa et al., 1965). Assuming that a marker enzyme is specific to a particular structure, then by comparison of specific enzyme activities in the fractions to those in the supposed specific locus of that marker it was possible to calculate an estimation of the contamination of one fraction with another. Contamination therefore can be defined as the percentage of protein in a fraction which is due to a specific contaminating structure.

Electron micrographs of the various fractions are shown in Figures 16 - 18. The three fractions examined had distinct morphological characteristics. The distinct nature of the fractions was also shown by their enzyme

content as is shown in Figure 19. Morphologically the synaptic vesicle fraction comprised mostly of small synaptic vesicle structures together with a little membrane contamination. There was no evidence for the presence of mitochondria. The membrane contamination of the vesicle fraction was also reflected in the biochemical studies which showed some Na/K ATPase and p-nitrophenylphosphatase activity in the vesicle fraction. However the activities of such markers were very low, especially in the case of Na/K ATPase which in many instances was not detectable. Estimates of the membrane contamination in the vesicle fraction were 10% (using Na/K ATPase) and 25% using p-nitrophenylphosphatase. The synaptic vesicle fraction showed considerable enrichment in Mg ATPase activity relative to Na/K ATPase compared to the synaptic membrane fraction. The ratio of the specific activity of Mg ATPase to Na/K ATPase was 3.5 in the vesicle fraction compared to 0.57 in the membranes.

The electron micrographs of the mitochondrial fraction showed typical mitochondrial structure such as cristae together with many ill-defined membranous structures. In some cases the latter showed the remains of cristal structure suggesting that much of ill-defined membranous structure was lysed mitochondrial material. Biochemically the mitochondrial fraction showed characteristically high Mg ATPase and succinic dehydrogenase activities together with some Na/K ATPase and p-nitrophenylphosphatase activity. Using

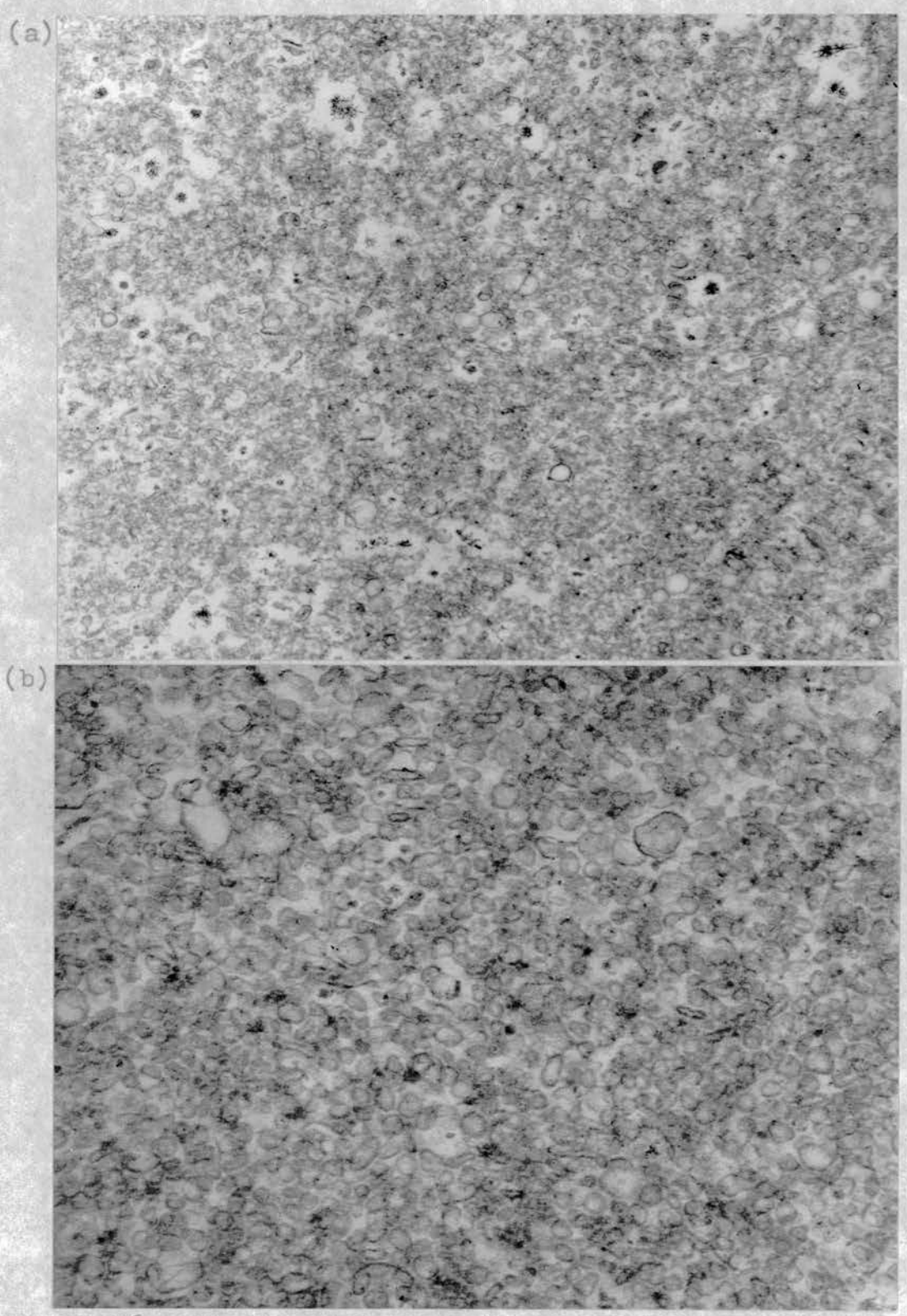

Figure 16. Electron micrographs of synaptic vesicle fractions prepared from cerebral cortex of control rats. Both pictures show predominantly synaptic vesicle structures with a small amount of larger membranous material. Magnification: (a) x 40,000, (b) x 100,000

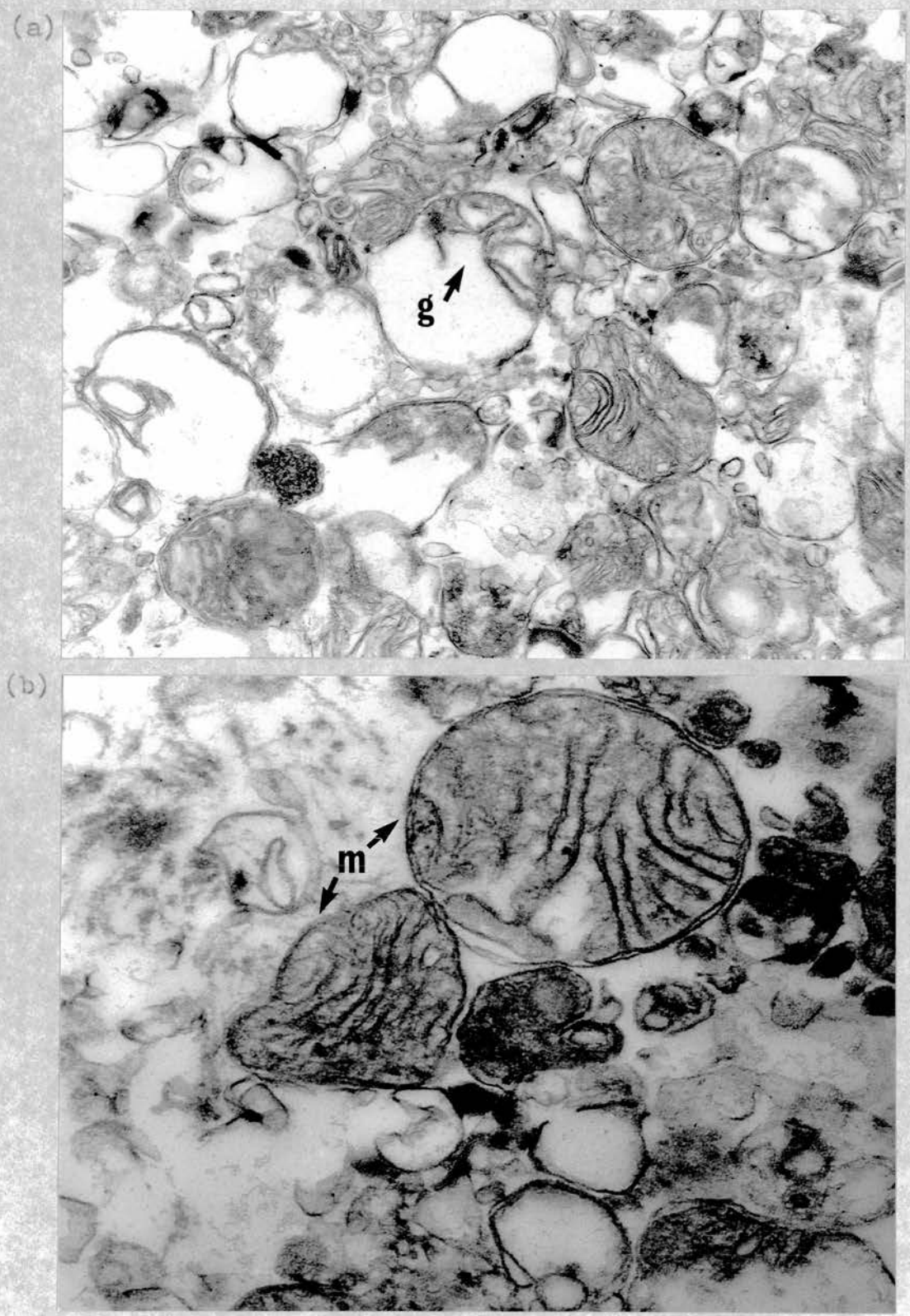

Figure 17. Electron micrographs of mitochondrial fractions prepared from cerebral cortex of control rats. Both pictures show intact mitochondria with typical cristal structure (M) and membranous structures with possible remains of cristae (G). Magnification: (a) x 40,000, (b) x 100,000.

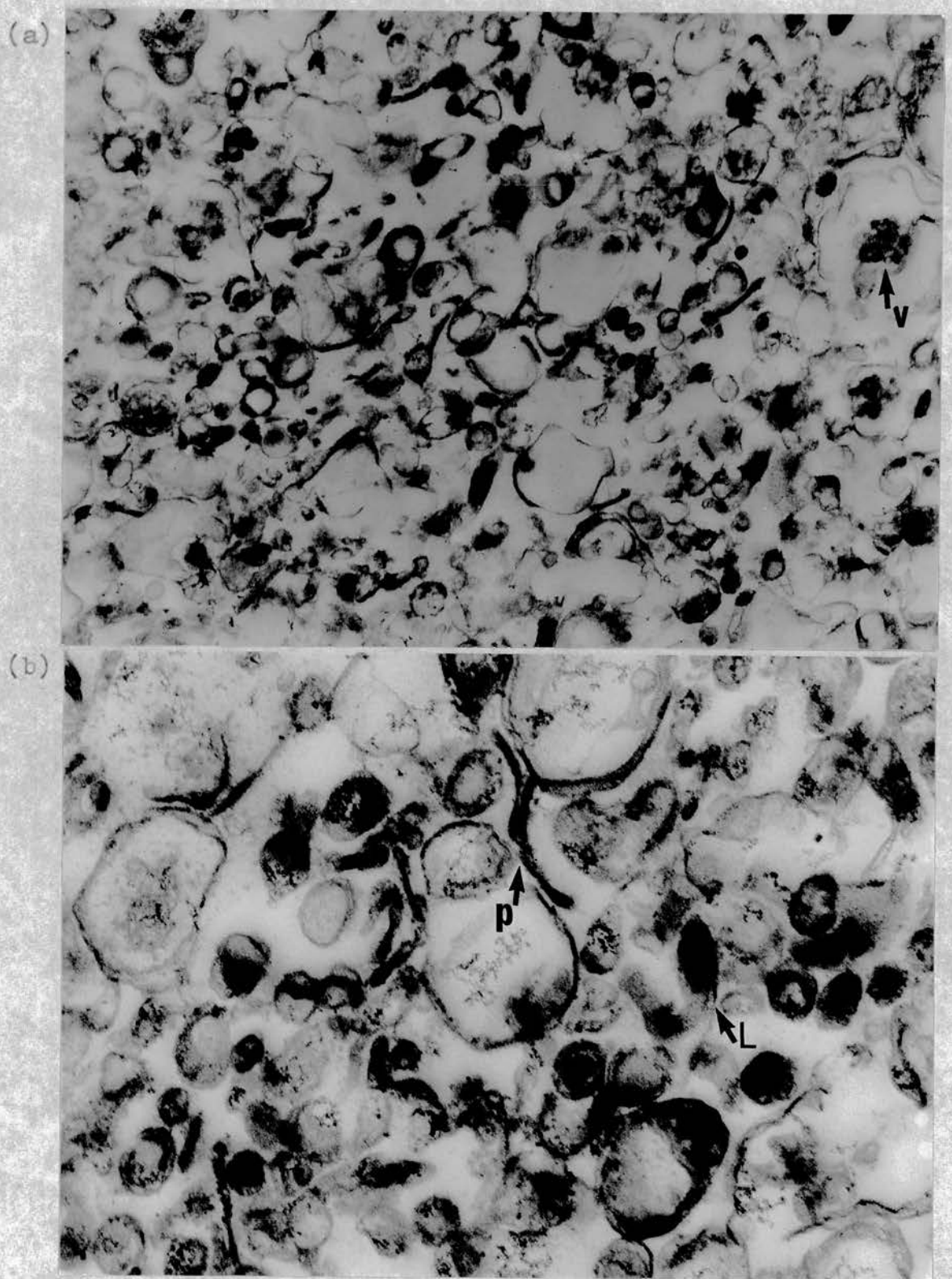

Figure 18. Electron micrographs of synaptic plasma membrane fractions prepared from cerebral cortex of control rats. Both pictures show predominantly empty membranous structures and membrane fragments. Spherical densely-stained bodies, possibly lysosomes (L), vesicles (V) and post-synaptic junction complexes (P) are also present. Magnification: (a) x 40,000, (b) x 80,000.

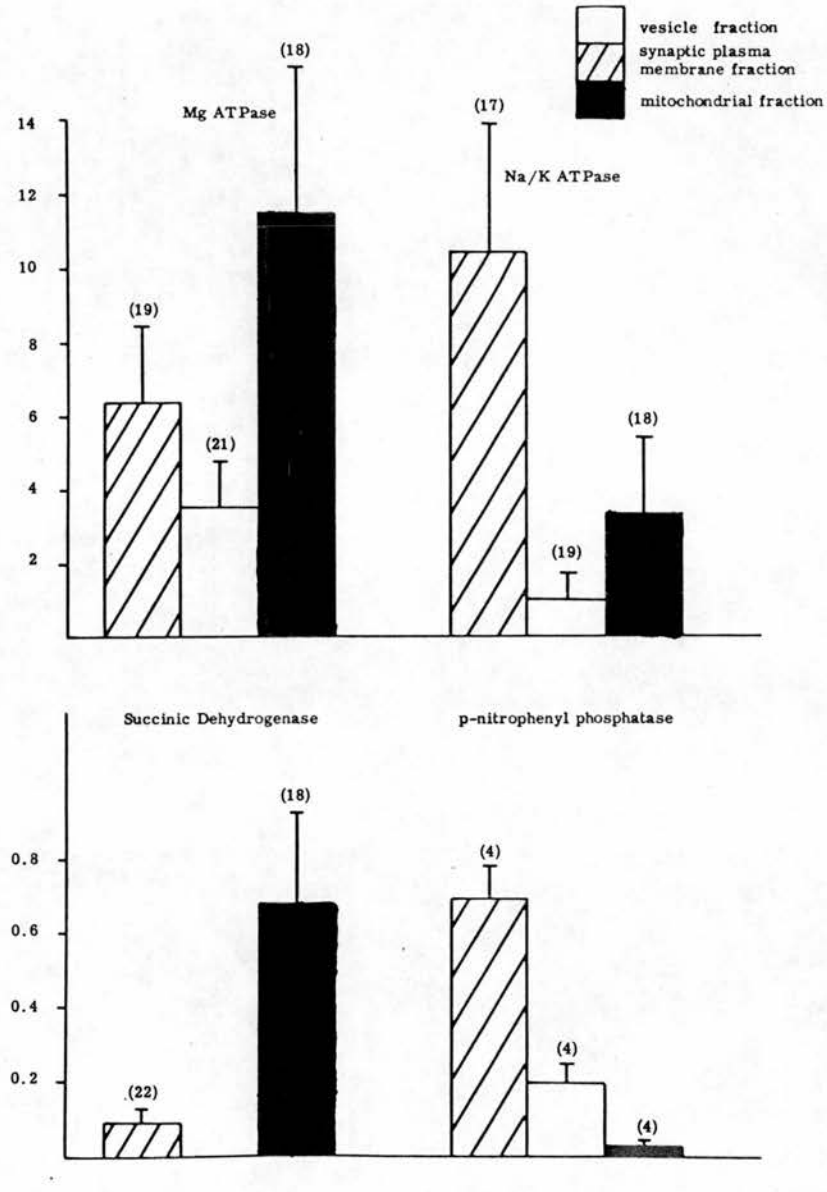

Figure 19. Biochemical characteristics of subcellular fractions prepared from cerebral cortex of control rats. ATPase activities are expressed as μmol P_i liberated /hr/ mg protein, succinic dehydrogenase as change in optical density/10 min/mg protein and p-nitrophenylphosphatase activity as change in optical density/20 min/mg protein. Values given are means $\pm$ standard deviations with the number of preparations studied in parentheses.

these membrane marker enzymes, membrane contamination of the mitochondrial fraction was estimated at between 10% (p-nitrophenylphosphatase) and 30% (Na/K ATPase).

Measurement of succinic dehydrogenase activity in the synaptic plasma membrane fraction showed little (13%) mitochondrial contamination. This was supported by the electron microscopy which failed to show any considerable mitochondrial contamination. The plasma membrane fraction had characteristically high Na/K ATPase and p-nitrophenylphosphatase activities. Morphologically the fraction consisted mostly of membranous material, 'ghost'-like structures which were most probably intact synaptosomes and broken-up, lysed membrane structures. Some myelin contamination was evident together with some densely staining bodies, possibly lysosomes. Possible post synaptic thickenings and synaptic junctions can be seen in Figure 18.

In conclusion, both the biochemical and morphological studies suggest that the fractions produced were clearly distinct and of considerable purity with only limited contamination. In the experiments to be described ATPase activities were compared between control and drug-treated preparations and in such a situation it was essential not simply to assume certain contamination of fractions, but to measure marker enzymes to investigate whether changes in contamination did occur.

2. The effect of chronic lithium administration on ATPase activities in various subcellular fractions.

Rats were given lithium chloride in their diet (60 mmol/kg food) for periods of 1, 2 and 3 weeks. Plasma lithium concentrations were in the range 0.5-1.0 mM. Following lithium treatment, animals were killed and subcellular fractions prepared from the cerebral cortices. Three control and three drug-treated preparations were attempted on each occasion. The results presented in the following paragraphs represent data from two or three chronic lithium treatment experiments. In order to combine the results from all the experiments the data were expressed as percentages of the mean control enzyme activity for each batch of preparations. Statistical analysis of results expressed as percentages of mean control enzyme activity was carried out using a Wilcoxon ranking test.

The results shown in Table 7. show the effect of lithium administration on rat cerebral cortex synaptic plasma membrane ATPase activities. Lithium treatment had no significant effect on Na/K ATPase activity but after three weeks of lithium treatment there was a significant increase (p 0.01) in synaptic plasma membrane Mg ATPase specific activity. Mitochondrial contamination of the fractions was monitored by measuring succinic dehydrogenase activity. As shown in Table 7. there was no effect of lithium treatment on the specific activity of synaptic plasma membrane succinic dehydrogenase so suggesting no changes in the mitochondrial contamination of the fraction. The increased Mg ATPase

Table 7. The effect of chronic lithium chloride administration on the specific activity of Na/K ATPase, Mg ATPase and succinic dehydrogenase in synaptic plasma membrane fractions. Synaptic plasma membranes were prepared from rats given lithium chloride in the diet (60 mmol LiCl/kg food) and control animals. Results are expressed as percentages of the mean control value of each batch of preparations. Values are means ± standard deviations with the number of preparations in parentheses. Groups were compared using a Wilcoxon ranking test, *p 0.01 compared to controls.

	duration of lithium treatment (weeks)	Na/K ATPase	Mg ATPase	succinic dehydrogenase
controls		100 ± 24 (19)	100 ± 13 (19)	100 ± 21 (19)
lithium-treated	1	117 ± 17 (6)	97 ± 21 (6)	113 ± 41 (6)
	2	84 ± 17 (7)	94 ± 23 (5)	113 ± 67 (6)
	3	94 ± 21 (5)	125 ± 18* (8)	102 ± 35 (8)

activity after 3 weeks was not paralleled by a change in mitochondrial contamination or membrane fraction purity as judged by Na/K ATPase activity and so it is unlikely that changes in fraction composition could account for the increased specific activity of the Mg ATPase.

In view of this increased Mg ATPase in the membrane fraction further experiments were carried out to examine the effects of lithium on mitochondrial and synaptic vesicle Mg ATPase activity. As shown in Table 8. lithium treatment, although tending to increase vesicle Mg ATPase activity, failed to produce a significant effect. There was however a marked increase ($p<0.01$) in vesicle fraction Na/K ATPase activity after 3 weeks of lithium treatment. This indicates increased membrane contamination of the fraction. Further experiments are needed to establish whether this is a true effect of lithium changing the fraction characteristics so that there is a redistribution of material during fractionation.

Lithium treatment of animals for one week caused a significant increase ($p<0.01$) in the specific activity of mitochondrial Mg ATPase (Table 9.). This was not accompanied by any change in succinic dehydrogenase or Na/K ATPase activity thus showing that accompanying changes in fraction composition could not explain the change in Mg ATPase activity. Longer treatment with lithium (3 weeks) was associated with a return of the mitochondrial Mg ATPase activity to control levels. There was a trend for lithium

Table 8. The effect of chronic lithium chloride administration on the specific activity of Na/K ATPase and Mg ATPase in synaptic vesicle fractions. Synaptic vesicles were prepared from rats given lithium chloride in the diet (60 mmol LiCl/kg food) and control animals. Results are expressed as percentages of the mean control value of each batch of preparations. Values are means ± standard deviations with the number of preparations in parentheses. Groups were compared using a Wilcoxon ranking test, *p 0.01 compared to controls.

	duration of lithium treatment (weeks)	Na/K ATPase	Mg ATPase
controls		100 ± 30 (14)	100 ± 20 (18)
lithium-treated	1	87 ± 39 (6)	115 ± 29 (6)
	2	94 ± 50 (5)	115 ± 23 (6)
	3	187 ± 78* (5)	117 ± 21 (8)

Table 9. The effect of chronic lithium chloride administration on the specific activity of Na/K ATPase, Mg ATPase and succinic dehydrogenase in mitochondrial fractions. Mitochondria were prepared from rats given lithium chloride in the diet (60 mmol/kg food) and control animals. Results are expressed as percentages of the mean control value of each batch of preparations. Values are given as means ± standard deviations with the number of preparations in parentheses. Groups were compared using a Wilcoxon ranking test, *p 0.01 compared to controls.

	duration of lithium treatment (weeks)	Na/K ATPase	Mg ATPase	succinic dehydrogenase
controls		100 ± 42 (15)	100 ± 10 (18)	100 ± 16 (18)
lithium-treated	1	98 ± 25 (6)	115 ± 9* (6)	105 ± 15 (6)
	2	-	112 ± 12 (6)	84 ± 14 (6)
	3	101 ± 96 (4)	104 ± 17 (6)	84 ± 18 (6)

treatment to reduce mitochondrial succinic dehydrogenase activity but the effect was not of statistical significance.

3. The lithium concentration in subcellular fractions.

Mitochondrial, synaptic plasma membrane and synaptic vesicle fractions were analysed for their lithium content. No lithium was detectable in the fractions using flame atomic absorption spectrophotometric analysis. However, using flameless atomic absorption methods with a graphite furnace it was possible to detect very low levels of lithium in the fractions. The amount of lithium present was so small that it could not be accurately determined but the approximate lithium concentration in all three types of fraction was 2 uM. Such a concentration of lithium could result in a concentration of 0.1 - 0.2 uM in the ATPase incubation medium.

4. The effect of lithium, in vitro, on subcellular fraction ATPase activities.

Experiments were carried out to investigate the effect of lithium, in vitro, on Na/K ATPase and Mg ATPase activities in the same types of subcellular fraction as were studied in the chronic treatment experiments. Lithium concentrations used were chosen to span the range from the low concentrations expected when subcellular fractions from lithium-treated rats were assayed to the higher concentrations found in the plasma of both animals and humans treated with lithium salts. Subcellular fractions were incubated with lithium for 10 minutes prior to ATPase assay. Lithium was added

to the incubation medium as lithium chloride. The results are shown in Table 10. They show no evidence of any effect of lithium, at any concentration, on Na/K ATPase or Mg ATPase activity in any of the fractions studied.

5. The distribution of lithium in various areas of rat brain.

Rats were given lithium in the diet (60 mmol/kg diet) for three weeks. Such a drug regimen produced plasma lithium concentrations of 0.5 - 1.0 mM. After 3 weeks treatment the animals were killed by cervical fracture and the brain removed. Various brain areas were dissected out and their lithium content measured. The results are shown in Table 11. An analysis of variance showed there were significant differences in the lithium content of the various areas ($p < 0.05$). Comparisons of the individual groups showed the striatum to have a significantly higher lithium content than the cerebellum ($p < 0.025$). The lithium content of the other areas studied was not significantly different from that of cerebellum.

DISCUSSION.

The subcellular fractions produced in the present work had not only to be of high purity but also to be reproducable in order to allow comparison between control and drug-treated preparations. Previous workers have used a variety of techniques to produce pure fractions of synaptosomal material; extensive washing of the crude mitochondrial pellet to remove microsomes (Morgan et al., 1971; Gurd et al., 1974), lysis of synaptosomes rather than lysis of

Table 10. The effect of lithium chloride, *in vitro*, on ATPase activities in synaptic plasma membrane, synaptic vesicle and mitochondrial fractions prepared from cerebral cortex of control rats. Preparations were pre-incubated with lithium chloride for 10 minutes prior to assay. Results are expressed as μmol P_i/hr/ml fraction. Values are means $\pm$ standard deviations with the number of estimations in parentheses.

lithium concentration	synaptic plasma membrane Na/K ATPase	synaptic plasma membrane Mg ATPase	synaptic vesicle Mg ATPase	mitochondrial Mg ATPase
zero	6.95 $\pm$ 0.83 (3)	5.55 $\pm$ 0.35 (3)	1.38 $\pm$ 0.43 (5)	6.14 $\pm$ 0.81 (5)
10^{-8} M	7.50 $\pm$ 0.33 (3)	5.38 $\pm$ 0.13 (3)	1.26 $\pm$ 0.18 (5)	5.66 $\pm$ 0.73 (5)
10^{-7} M	7.45 $\pm$ 0.66 (3)	5.42 $\pm$ 0.34 (3)	1.44 $\pm$ 0.12 (5)	5.62 $\pm$ 0.80 (5)
10^{-6} M	5.85 $\pm$ 0.62 (3)	5.22 $\pm$ 0.83 (3)	1.19 $\pm$ 0.25 (5)	6.36 $\pm$ 1.09 (5)
10^{-4} M	7.00 $\pm$ 1.08 (3)	6.00 $\pm$ 0.04 (3)	1.24 $\pm$ 0.26 (5)	6.54 $\pm$ 0.33 (5)
10^{-3} M	7.08 $\pm$ 0.51 (3)	5.92 $\pm$ 0.47 (3)	1.32 $\pm$ 0.26 (5)	6.91 $\pm$ 0.62 (5)

Table 11. Lithium concentration in various areas of rat brain following chronic lithium chloride administration. Rats were given lithium chloride in the diet (60 mmol LiCl/kg food) for 3 weeks. Results are expressed as mmol/kg wet tissue. Values given are means ± standard deviations from five animals. An analysis of variance showed the groups to be significantly different, p 0.05. Comparison of areas with the cerebellum using a Students 't' test showed the striatum to be significantly different (p 0.025) from cerebellum.

Brain area	Lithium concentration (mmol/kg)
cerebellum	0.35 ± 0.08
hypothalamus	0.43 ± 0.08
cortex	0.36 ± 0.10
lower frontal cortex	0.33 ± 0.06
brain stem	0.37 ± 0.05
striatum	0.55 ± 0.10

the crude mitochondrial pellet (Cotman and Matthews, 1971; Gurd et al., 1974) and filtration of vesicular material to remove membranes (Morgan et al., 1973). All these procedures extend both the preparation time and the number of operative procedures involved and are thus not well suited to comparative enzymological studies. Therefore a fractionation procedure was chosen which had a short preparation time and a minimum of operative procedures. An additional washing of the crude mitochondrial pellet was introduced to minimise microsomal contamination of the synaptic fractions.

The procedure adopted produced fractions which were distinct in their morphological and biochemical properties. Morphologically the fractions appeared to be of considerable purity and were of similar appearance to those presented in the literature (Lapetina, Soto and De Robertis, 1967; Rodriguez De Lores Arnaiz et al., 1967; Morgan et al., 1971; 1973). Both morphologically and biochemically the synaptic vesicle fraction seemed to possess little contamination. Estimates of membrane contamination as judged by Na/K ATPase and p-nitrophenylphosphate activity were 10 - 25%. Such values compare favourably with those reported in the literature (Whittaker et al., 1964; Morgan et al., 1973). Mitochondrial contamination of the synaptic membrane fraction was very low (13%) and comparable to that achieved by similar and more complex preparative procedures (Whittaker et al., 1964; Cotman and Matthews, 1971; Morgan et al., 1971;

Gurd et al., 1974). When examined electron microscopically the synaptic membrane fractions appeared to consist of intact synaptosomal 'ghosts' and broken-up lysed membranous material. Membranes were also present in the mitochondrial fraction and were estimated to give rise to some 15 - 30% contamination.

The characteristics of the various fractions were such that it was possible to consider the fractions as being distinct in their biochemical and structural contents. The separation of material achieved allows one to allot, with some confidence, changes in fractions to particular subcellular structures.

The distribution of ATPase activities between the various fractions studied was similar to that reported by earlier workers (Hosie, 1965; Kurokawa et al., 1965; Reading et al., 1974) in that Mg ATPase activity was associated with all fractions but particularly vesicles and mitochondria whereas Na/K ATPase was found predominantly in the synaptic plasma membrane fraction.

Lithium treatment for three weeks was found to significantly increase the specific activity of synaptic plasma membrane Mg ATPase. This result confirms the earlier finding of Reading et al. (1975) that synaptic plasma membrane Mg ATPase activity is increased during lithium administration. There are three possible explanations of the observed rise in specific enzyme activity. Firstly, lithium present in the fractions could have activated the

enzyme *in vitro*. This, however, can be discounted since lithium over a wide concentration range showed no effect *in vitro* on synaptic plasma membrane Mg ATPase activity. Secondly, the change in Mg ATPase activity could have arisen as a result of a change in the composition of the membrane fraction. Reading *et al*. (1974) have interpreted changes in subcellular fraction ATPases in terms of a redistribution of synaptosomal material during preparation. However, such an explanation of the present results is untenable in view of the unchanged mitochondrial contamination and the unchanged membrane content of the fraction as judged by enzyme markers. Glen and Reading (1973) suggested that changes in Mg ATPase activity in synaptic membrane fractions might reflect effects of lithium on vesicle-membrane fusion and neurotransmitter release. The present results provide no evidence for such a hypothesis.

The most probable explanation of the observed increase in synaptic membrane Mg ATPase activity is that it is due to a real increase in the specific activity of the enzyme present in the synaptic membrane material. In view of the possible glial contamination of synaptosomal material (Morgan *et al*., 1971; Henn *et al*., 1976) it cannot be unequivocally stated whether the increase in Mg ATPase activity occurred in neuronal or glial cells. There is immunohistochemical evidence (Matus, 1976) for synaptic membrane fraction containing material present in the synaptic membrane itself. It is possible therefore that

the increased Mg ATPase observed in synaptic membrane fractions is a lithium-induced change in a synaptic component.

In contrast to the earlier work of Reading et al. (1975) the present work failed to show an effect of lithium treatment on synaptic plasma membrane Na/K ATPase. The observed increase in Mg ATPase activity (25%) in the present studies was also less than the 50 - 150% increase observed by Reading. The reason for the difference in the two sets of results is unknown but it may lie in the different mode of lithium administration used in the two studies. Oral administration of lithium does not produce the short-lived, high lithium concentrations found after intraperitoneal injection (Morrison et al., 1971). It may be that such short-lived, high concentrations of lithium are effective in changing ATPase activity and in particular it is possible that only high, toxic lithium concentrations are effective in altering Na/K ATPase activity. Reading et al. (1975) showed that animals offered a salt-lick in addition to lithium showed less change in ATPase activity than animals with no salt supplementation. Results presented here showed the salt supplementation to decrease the plasma lithium concentration. It would seem therefore that the changes in synaptic membrane ATPases observed during lithium treatment are sensitive to the lithium levels achieved and that mode of administration is important in determining the effects of lithium. The present experiments, in which

plasma lithium concentrations of 0.5 - 1.0 mM were achieved and in which the levels of drug were probably stable (Schou, 1976) provided no evidence that chronic lithium treatment affects brain Na/K ATPase activity.

The specific activity of mitochondrial Mg ATPase was increased after 1 week of lithium treatment. The succinic dehydrogenase and Na/K ATPase activities were unchanged suggesting that changes in fraction composition could not explain the increased Mg ATPase specific activity. Lithium, *in vitro*, had no effect on mitochondrial Mg ATPase activity. It is most likely that the increase in Mg ATPase activity reflects an increase in enzyme specific activity in the mitochondria. The return of mitochondrial Mg ATPase specific activity to control level after further lithium treatment (3 weeks) is a similar effect to that described for synaptic membrane Mg ATPase (Reading *et al.*, 1975). It is evident therefore that the increases in both mitochondrial and membrane enzymes are only transient. Further experimental evidence is required to be able to state whether such changes represent an *in vivo* activation of the enzymes or the presence of more catalytic protein. The functional significance of the changes is unclear. The function and nature of the membrane Mg ATPase is unknown so the significance of changes in its activity are particularly obscure. It is unlikely that the Mg ATPase activity affected by lithium treatment is due to the activity of adenyl cyclase since chronic lithium treatment has been shown to decrease

rat brain synaptic plasma membrane adenyl cyclase activity (Reading et al., 1975). It is possible that the change may reflect an alteration in synaptosomal contractile protein Mg ATPase activity functioning in neurotransmitter release. The mitochondrial ATPase is associated with ion transport and ATP synthesis (Racker, 1975) and its increased activity may be reflected in increased ATP synthesis in the mitochondria.

There was no evidence of lithium affecting vesicle Mg ATPase at the treatment times studied. It is possible that such a change was missed since the changes in the other fractions were only transient. Previous workers have studied microsomal fractions with contradictory results. Using chronic treatment Gutman et al. (1973a) found no effect of lithium on microsomal ATPases in brain but McNulty et al. (1975) found a single dose of lithium to decrease Mg ATPase activity in rat brain subcellular fractions which although not defined were probably microsomal. Ploeger (1974a) reported chronic lithium treatment to decrease rat vagal nerve homogenate Mg ATPase. It is not possible to give a rationale for these varying effects of lithium on Mg ATPases in different tissues and different subcellular fractions. Differences in lithium administration, lithium distribution and the possibility of different enzymes in different physiological systems are some of the possible explanations.

The results of analysis of subcellular fractions

prepared from lithium-treated animals showed very little lithium present in mitochondria, synaptic membrane of synaptic vesicle fractions. This would suggest that lithium is not tightly bound to these particular sub-cellular structures. Chronic treatment of rats with lithium gave rise to an uneven distribution of lithium in the brain. Results showed a particular concentration of lithium in the striatum with little difference in distribution in the other areas studied although hypothalamus and cortex tended to have a higher lithium content than brain stem and cerebellum. Previous work on lithium distribution has given varied results. Ho et al. (1970) failed to show differences in lithium content between different brain areas. However later workers did find differences after both acute and chronic administration. The striatum was reported to have a high lithium content by some workers (Ebadi et al., 1974; Bond et al., 1975) and hypothalamus by others (Edelfors, 1975). Possible explanations of the uneven distribution of lithium include differences in cell type, cell size, cell number and the size of extracellular space in the various regions of the brain. Although the significance of the regional distribution of lithium is unclear it is evident from the present work and that of others that the effects of lithium may not be uniform throughout the brain.

The work described in this section has led to the following conclusions:

1. Lithium, *in vitro*, does not affect Mg ATPase or Na/K ATPase activity in mitochondrial, synaptic plasma membrane or vesicle fractions.

2. Chronic lithium administration in the diet leads to a transient increase in the specific activity of Mg ATPase in synaptic plasma membrane and in mitochondrial fractions prepared from rat cerebral cortex. The effect of lithium treatment on Mg ATPase activity is not restricted to synaptic plasma membranes.

3. Chronic lithium treatment has no effect on the specific activity of synaptic vesicle Mg ATPase.

4. Chronic lithium treatment, using diet administration, has no effect on synaptic membrane Na/K ATPase.

5. The results provided no evidence for chronic lithium treatment leading to a change in composition of the synaptic membrane fraction. The lithium-induced changes in Mg ATPase activity were not related to vesicle-membrane fusion.

6. The effects of chronic lithium treatment on synaptic membrane Mg ATPase and Na/K ATPase activities may depend on the mode of administration of the drug.

7. Lithium is not evenly distributed throughout the brain. Of the areas studied, striatum had the highest lithium concentration.

SECTION IV

AN INVESTIGATION OF THE EFFECTS OF CHRONIC LITHIUM TREATMENT ON DOPAMINE METABOLISM IN THE RAT STRIATUM.

INTRODUCTION.

Results presented in the previous section showed chronic lithium treatment to increase the specific activity of synaptic plasma membrane Mg ATPase. The functional significance of such a change is uncertain. However, if the Mg ATPase activity is associated with an actomyosin-like protein involved in neurotransmitter release (Berl et al., 1973) then changes in Mg ATPase would be expected to be associated with changes in the release and the metabolism of neurotransmitters. The aim of the work to be described in this section was to establish whether or not the regimen of lithium treatment which produced changes in synaptic plasma membrane Mg ATPase also produced changes in neurotransmitter metabolism. Neurotransmitter metabolism was studied in animals chronically treated with lithium, using the same dose of lithium and the same length of treatment as was found to cause changes in Mg ATPase activity. The approach used was to measure the concentration of dopamine and its metabolites, homovanillic acid (HVA) and 3,4 dihydroxyphenylacetic acid (DOPAC) in striatal material from lithium-treated rats. Although this approach suffered from the disadvantage that neurotransmitter metabolism was being studied in a different brain area to that in which ATPase changes had been found, there were advantages gained from studying the striatum: the striatum has been shown to have a high concentration of lithium (Ebadi et al., 1974; Bond et al., 1975; this thesis) and in addition it is associated with a

well-characterised dopaminergic neuronal system which has its nerve terminals in the striatum (Hornykiewicz, 1973).

Previous studies of the effects of lithium on catecholamine metabolism in the brain have provided few consistent results. The variation in the effects found may be due to the differences in length of treatment, mode of administration and dose of lithium used in the various experiments (Shaw, 1975). Such differences in drug treatment make it difficult to compare results from the various experiments. In the case of noradrenaline metabolism, in general lithium seems to cause an increase in intraneuronal metabolism together with increased re-uptake and possibly decreased release of noradrenaline (Shaw, 1975). Less work has been done on the effects of lithium on dopamine metabolism. However, the available evidence suggests lithium treatment causes a decrease in dopamine turnover. Using chronic diet administration for 3 weeks, lithium treatment was found to have no effect on whole brain dopamine concentrations although it did cause a reduction in dopamine turnover as judged by the depletion of brain dopamine after inhibition of tyrosine hydroxylase (Corrodi, Fuxe and Schou, 1969). Using histochemical techniques, these workers demonstrated increased dopamine depletion in the tubero-infundibular system and decreased depletion in the mesotencephalic area of the brain. Later workers showed intraperitoneal injection of lithium chloride for 14 or 15 days to cause decreases in both striatal and whole brain dopamine synthesis (Friedman

and Gershon, 1973; Poitou and Bohuon, 1975), findings consistent with decreased dopamine turnover. A further report however failed to observe any significant effects of lithium on dopamine turnover in various areas of rat brain (Ho, Loh, Graves, Hitzemann and Gershon, 1970). Generally lithium treatment has been found to have no effect on steady-state dopamine concentrations (Corrodi et al., 1969; Ho et al., 1970; Friedman and Gershon, 1973) although Leonard (1975) showed a decrease in whole brain dopamine content following a single lithium injection. Studies of striatal HVA content following chronic lithium administration have failed to show any effect of lithium on dopamine turnover or catabolism (Perez-Cruet, Tagliamonte, Tagliamonte and Gessa, 1971; Schubert, 1973).

The present work used a different approach from the previous studies in that rather than measure dopamine turnover and synthesis by using metabolic inhibitors and radioactive substrates, metabolism has been studied by measuring striatal concentrations of dopamine and its major metabolites, HVA and DOPAC (Sharman, 1973). The reason for using metabolite studies was in order to try and distinguish between release and re-uptake processes. The hypothesis that DOPAC is the product of intraneuronal metabolism and HVA the product of extraneuronal metabolism (Roffler-Tarlov, Sharman and Tegerdine, 1971; Wilk, Watson and Travis, 1975; Roth, Murrin and Walters, 1976) allows analysis of changes in metabolite concentrations in terms of intraneuronal and

extraneuronal compartmentation and release and re-uptake of neurotransmitter. Measurement of dopamine concentrations allows distinction between effects on turnover of the amine and effects on release alone.

MATERIALS AND METHODS.

1. General.

All chemicals were of Analar Grade from British Drug Houses Co. Ltd. unless otherwise stated. S-Adenosyl-(methyl-^{3}H)methionine and (side chain-2,3-^{3}H)tyrosine were purchased from The Radiochemical Centre, Amersham, U.K.

Male, Wistar rats (200 - 250 g) were given lithium chloride in the diet (60 mmol LiCl/kg food) for a period of up to 3 weeks. In experiments involving DOPAC, HVA and dopamine estimations animals were anaesthetised with Nembutal (70 mg/kg, intraperitoneally) and blood taken by cardiac puncture. Animals were then killed by decapitation and the brain removed. In experiments involving tyrosine hydroxylase estimations animals were not anaesthetised and were killed by cervical fracture. Striata were dissected out as described in the previous section and stored in liquid nitrogen until analysis. Plasma lithium analysis was carried out as described in the previous section.

2. Estimation of homovanillic acid (HVA) and 3,4dihydroxyphenylacetic acid (DOPAC).

HVA and DOPAC were measured in striata by gas-liquid chromatography using an electron capture detector (Pearson and Sharman, 1975 modified by Nikolaou). The procedure for

assay is outlined below.

Tissue (20 - 40 mg) was homogenised in 0.1 N perchloric acid (300 µl/10 mg tissue) and centrifuged at 10,000 g for 15 minutes. 675 µl of supernatant was transferred to a plastic (Eppendorf) tube and 0.5 ml toluene added. Following vortexing for 30 seconds the mixture was centrifuged at 1000 g for 2 minutes. The aqueous layer was then extracted with 0.5 ml ethyl acetate (Reeve Angel Scientific Ltd.) for 1 minute. Following vortexing, the tube was centrifuged (1000 g, 2 minutes) and the ethyl acetate layer transferred to a reaction vial. The aqueous layer was extracted twice more with ethyl acetate and the organic extracts combined and evaporated to dryness under a stream of nitrogen. The dried extracts were then reacted with 0.2 ml twice redistilled trifluoroacetic anhydride (Aldrich Chemical Co. Ltd.) and 0.1 ml redistilled hexafluoroisopropanol containing 5% v/v freshly prepared boron trifluoride etherate. The reaction was carried out at 100°C for 1 hour. After cooling, the vial contents were evaporated to small volume at room temperature under a stream of nitrogen. The oily residue was dissolved in 1 ml of dry ethyl acetate containing 100 ng/ml pentafluorophenylbenzoate as internal standard. 2 µl of this solution was injected into the gas chromatogram. Standards of HVA and DOPAC were run with all assays.

Gas-liquid chromatography of the trifluoroacetyl-hexafluoroisopropyl derivatives was carried out using a Hewlett-Packard 5710 Gas Chromatograph fitted with ^{63}Ni

electron capture detectors maintained at 250°C. Carrier gas was argon with 5% methane and this was delivered at a flow rate of 50 ml/minute (corresponding to a gas pressure of 40 p.s.i.). The column used was a 2% SE52 liquid phase coated on Chromosorb 2 (Hewlett-Packard Ltd.) and the working temperature was 115°C.

Retention times of the HVA and DOPAC derivatives compared to the internal standard were 0.35 and 0.52 respectively. Areas of sample peaks were calculated and the metabolite contents calculated from comparison with areas of peaks derived from known standards of HVA and DOPAC. Recoveries of HVA and DOPAC were 90% and 87% respectively. Results were not corrected for recoveries.

3. Estimation of dopamine.

The estimation of striatal dopamine was done using a radiometric assay employing methylation of dopamine with S-Adenosyl-(methyl-^{3}H)methionine followed by extraction and measurement of the labelled methoxytyramine formed. The method used was derived from those described by Coyle and Hendry (1973) and Palkovits, Brownstein, Saavedra and Axelrod (1974). The assay procedure is outlined below: Striata (20 mg) were homogenised in 0.1 N perchloric acid (300 µl/10 mg tissue). The homogenate was centrifuged at 10,000 g for 15 minutes and 300 µl of the supernatant fluid used for the subsequent assay. 300 µl aliquots of 0.1 N perchloric acid were used as blanks and standards of dopamine added to brain extracts were run with all assays.

Standards, samples and blanks were reacted with labelled S-Adenosyl methionine in the presence of catechol-o-methyl transferase. 300 µl of samples were added to a mixture containing 500 µg of dithiothreitol, 0.5 µmol $MgCl_2$, 140 µmol Tris-HCl buffer, pH 9.6, 2.5 µl of rat liver catechol-o-methyl transferase (prepared as described by Axelrod and Tamchick, 1958) and 2.5 µCi of S-Adenosyl-(methyl-^{3}H)methionine. After incubation at 37.5°C for 1 hour the reaction was stopped by the addition of 500 µl 0.5 M borate buffer, pH 10. After addition of non-radioactive carrier (7 µg methoxytyramine) and 1 mg EDTA in a total volume of 50 µl, the o-methylated reaction products were extracted into 9 ml of water saturated ethyl acetate-methanol (10:1 v/v) by shaking for 30 seconds. The phases were separated by centrifugation (1000 g, 5 minutes) and 8.5 ml of the organic layer transferred to another tube containing 0.5 ml 0.5 M borate buffer, pH 10. 8 ml of the subsequent organic phase was mixed for 30 seconds with 0.5 ml 0.1 N hydrochloric acid. After separation of the two phases by centrifugation the organic phase was discarded. The acid aqueous phase was washed in 8 ml water-saturated ethyl acetate by mixing and centrifugation.

The procedure so far described results in the methylation of both noradrenaline and dopamine and the extraction of their methylated derivatives. In order to separate normetanephrine (noradrenaline derivative) from methoxytyramine (dopamine derivative) the normetanephrine side-chain

is cleaved by periodate to form vanillin which is then separated from methoxytyramine. The acid aqueous phase described above was reacted with 50 µl of 3% (w/v) sodium metaperiodate and 0.5 ml 0.5 M sodium phosphate buffer, pH 7.5 for 3 minutes at $0^{o}C$. The reaction was stopped by the addition of 50 µl 10% (v/v) glycerol and 10 ml toluene added. After mixing and separation of the two phases, the toluene layer could be used for noradrenaline estimation as it contained the labelled vanillin. The aqueous phase contained labelled methoxytyramine and was used for dopamine estimation. 5 ml toluene was added to the aqueous layer and the mixture shaken and centrifuged to separate the two phases. 0.5 ml 1 M borate buffer, pH 11 and 6 ml toluene-isoamyl alcohol (3:2) was added to the aqueous layer. After mixing and separation of the two phases 5 ml of the organic layer was taken and added to 10 ml NE260 liquid scintillant (Nuclear Enterprises Ltd.).

4. Measurement of tyrosine hydroxylase activity.

Tyrosine hydroxylase was measured in striatal material by measuring the labelled dopa produced by incubation with (side chain-2,3-^{3}H)tyrosine exactly as described by Hendry and Iverson (1971). Results are expressed as nmol dopa formed/hr/g wet tissue.

5. Efficiency of radioactive counting.

Efficiency of the counting of the tritiated products of both dopamine and tyrosine hydroxylase assays was 30%. All samples were corrected for quenching by the external

standard channels ratio method. All samples were counted for 10 minutes.

RESULTS.

1. The effect of chronic lithium treatment on striatal HVA, DOPAC and dopamine concentrations.

Rats were treated with lithium chloride in the diet (60 mmol/kg food) for 3 weeks. Plasma lithium concentrations in samples taken prior to decapitation were 0.48 - 0.63 mM. Analyses of HVA, DOPAC and dopamine in the striata from lithium-treated and from control rats are shown in Table 12. The results show the lithium-treated animals to have significantly increased HVA (52%) and DOPAC (31%) concentrations in the striatum. Lithium treatment had no significant effect on the dopamine concentration in striatum.

2. The effect of chronic lithium treatment on striatal tyrosine hydroxylase activity.

An experiment was carried out to investigate whether the increased HVA and DOPAC concentrations found in lithium-treated animals were associated with changes in tyrosine hydroxylase activity. Rats were given lithium chloride in the diet, for 1, 2 and 3 weeks. Plasma lithium concentrations in these animals were in the range 0.5 - 1.0 mM. Striatal tyrosine hydroxylase activity was measured in lithium-treated animals and controls. The results (Table 13.) show no effect of lithium treatment on the enzyme activity.

Table 12. The effect of chronic lithium administration on concentrations of homovanillic acid (HVA), 3,4dihydroxyphenylacetic acid (DOPAC) and dopamine in the striatum. Results are shown from rats given lithium chloride in the diet (60 mmol/kg food) for 3 weeks and control animals. Values given are means ± standard deviations with number of animals in parentheses. Groups were compared using a Students 't' test, *$p<0.025$, **$p<0.005$ compared to controls.

	controls	lithium-treated
HVA (μg/g wet tissue)	1.03 ± 0.20 (8)	1.57 ± 0.38** (9)
DOPAC (μg/g wet tissue)	0.83 ± 0.10 (8)	1.09 ± 0.25* (9)
Dopamine (μg/g wet tissue)	9.3 ± 1.9 (7)	11.5 ± 2.1 (8)

Table 13. The effect of chronic lithium administration on striatal tyrosine hydroxylase activity. Results are shown from rats given lithium chloride in the diet (60 mmol/kg food) for 1, 2 and 3 weeks and control animals. Enzyme activity is expressed as nmol dopa formed/hr/g wet tissue. Values given are means ± standard deviation with the number of animals in parentheses.

	Duration of lithium treatment (weeks)	Tyrosine hydroxylase activity (nmol dopa/hr/g)
controls		22.52 ± 4.79 (5)
lithium-treated	1	25.85 ± 10.57 (6)
	2	27.40 ± 12.08 (4)
	3	23.09 ± 5.1 (5)

DISCUSSION.

The concentrations of HVA and DOPAC in striata from control rats are in the range of values reported by other workers (Wilk et al., 1975; Roth et al., 1976; Korf, Zieleman and Westernik, 1976) although the ratio of HVA: DOPAC is greater than that previously reported (Pearson and Sharman, 1975; Wilk et al., 1975; Korf et al., 1976). Chronic lithium chloride administration for 3 weeks caused a significant increase in rat striatal HVA and DOPAC concentrations while the dopamine concentration ~~remained unchanged~~ was not significantly changed by lithium administration.

Changes in HVA and DOPAC concentrations could be caused by interference with transport of the metabolites from the brain, interference with release of neurotransmitter or interference with neurotransmitter re-uptake. Administration of probenecid has been shown to reduce transport of HVA from the brain and this is reflected in increased striatal HVA concentrations (Guldberg and Brock, 1971; Roffler-Tarlov et al., 1971; Wilk et al., 1975). However, probenecid administration was not accompanied by increased DOPAC concentrations. Considering these findings, the increase in both DOPAC and HVA in the present experiments suggests that the effects of lithium are not due to interference with transport processes for removal of dopamine metabolites from the brain. Analysis of metabolite concentrations in terms of release and re-uptake can be made assuming DOPAC reflects intraneuronal metabolism and HVA extraneuronal

metabolism (Roffler-Tarlov et al., 1971; Sharman, 1973; Roth et al., 1976). However such interpretation is equivocable since the above hypothesis concerning the compartmentation of dopamine metabolites has recently been challenged on the evidence that a putative dopamine uptake blocker, nomifensine, does not affect DOPAC metabolism in the striatum (Korf et al., 1976). However, assuming DOPAC concentrations do reflect intraneuronal dopamine metabolism, the increase in striatal DOPAC following lithium administration suggests that lithium is not affecting dopamine metabolite concentrations through an inhibition of dopamine re-uptake. If the increased HVA and DOPAC concentrations are not due to interference with re-uptake or transport from the brain, then they must reflect increased release of dopamine and increased biochemical activity of the striatal dopaminergic neurons. Since the present evidence favours the hypothesis that DOPAC reflects intraneuronal metabolism, the most likely interpretation of the present results is that lithium treatment causes an increased release of dopamine from striatal nerve terminals. However such an increase in release was not associated with a change in steady-state dopamine concentration and this would suggest that there was an increased turnover of dopamine in the lithium-treated striata. It cannot be decided from the present evidence by what mechanism lithium treatment causes increased dopamine turnover.

Since lithium-treatment caused an increase in dopamine

turnover and since both changes in dopamine turnover and nerve activity are thought to be associated with changes in tyrosine hydroxylase activity (Roth, Walters, Murrin and Morgenroth, 1975) it was of interest to investigate whether lithium treatment caused any change in striatal tyrosine hydroxylase activity. However lithium treatment for 1, 2 and 3 weeks was found to have no effect on tyrosine hydroxylase activity in rat striatum. Since assay conditions were optimal for enzyme activity it is possible that activation effects were missed. The results show that there was no induction of enzyme associated with the increased dopamine turnover.

The finding that lithium treatment increased dopamine turnover is in conflict with previous reports. Lithium administration by intraperitoneal injection for 14 days was shown to reduce striatal dopamine synthesis (Friedman and Gershon, 1973) and other studies have shown 2 - 3 weeks of lithium treatment to reduce dopamine turnover and synthesis in whole brain (Corrodi et al., 1969; Poitou and Bohuon, 1975). However the picture is not entirely clear since Corrodi et al., showed the effects of lithium treatment on dopamine turnover to vary with the area of the brain studied. Furthermore other workers have failed to find any changes in dopamine turnover following chronic lithium treatment (Ho et al., 1970; Bliss and Ailion, 1970). Diet administration of lithium has been reported to both have no effect and to decrease dopamine turnover (Corrodi et al., 1969; Bliss and

Ailion, 1970). Similar discrepancy was found with intraperitoneal administration (Ho et al., 1970; Friedman and Gershon, 1973; Poitou and Bohuon, 1975). The differences in various results reported cannot therefore be readily explained by different modes of drug administration. The present results also differ from previous reports of striatal HVA concentration following lithium administration (Persson, 1970; Perez-Cruet et al., 1971). However the earlier studies involved acute and short-term (5 day) lithium administration and this might explain the difference between those results and the present ones. Whether differences in the stress to which the animals were subject could account for the variation in results is purely speculative. The different effect of lithium treatment on dopamine metabolism to that described by earlier workers cannot be at present explained.

The object of the experiment examining the effects of lithium on dopamine metabolism was to investigate whether changes in amine metabolism and release occurred after the same drug treatment which caused the changes observed in synaptic plasma membrane Mg ATPase activity. In conclusion it can be said that 3 weeks of diet administration of lithium both increases synaptic plasma membrane Mg ATPase activity and affects dopamine metabolism. The mechanism by which lithium affects dopamine metabolism is not clear from the experiments but there is probably an increase in dopamine release and turnover in the striatum. Further

studies, perhaps using synaptosomal preparations, are needed to test further whether this effect of lithium on amine metabolism is due to it affecting the release of neurotransmitter. The results although not showing a link between the effects of lithium on Mg ATPase activity and amine metabolism suggest that such a hypothesis may be worth consideration.

SECTION V

AN INVESTIGATION OF ERYTHROCYTE MEMBRANE ATPase ACTIVITIES IN DEPRESSIVE PATIENTS AND THE EFFECTS OF LITHIUM TREATMENT ON SUCH ACTIVITIES.

INTRODUCTION.

Erythrocytes provide material which is readily available from human subjects and from which membrane preparations can be made in order to measure enzyme activities. Erythrocyte membranes can be prepared relatively easily, in large yield and without contamination from subcellular organelles. In the present studies ATPase activities were measured in membrane preparations derived from erythrocytes of untreated and lithium-treated depressive patients in order to study ATPase activities in depression and the effects of lithium treatment.

Two major kinds of membrane preparations have been used over the past twenty years: the resealed ghost preparation (Hoffman, Tosteson, Whittam, 1960; Hoffman, 1962; Bodeman and Passow, 1972) and the so-called erythrocyte membrane preparation (Hanahan, 1973). The term membranes is used throughout this thesis to refer to the latter type of preparation. The majority of membrane preparation methods involve hypotonic haemolysis of the cells followed by subsequent washing of the membranes in hypotonic buffer and by using such methods membranes free from haemoglobin can be prepared. In the experiments to be described a method involving hypotonic haemolysis and subsequent washing in 10 mM Tris buffer (20 mosm, ideal osmolarity) pH 7.4 was used (Dick, Dick and Tosteson, 1969).

Membrane preparations are artifacts of their own preparation and must be regarded as only derivatives of

the true erythrocyte membrane (Hanahan, 1973). In three illuminating studies it has been shown that the properties of membrane preparations depend markedly on the conditions used during preparation (Bramley, Coleman and Finean, 1971; Hanahan and Ekholm, 1972; Hanahan, Ekholm and Hildenbrandt, 1973). As discussed by Hanahan (1973) this causes difficulties in comparing results from membrane preparations derived under different conditions. Membranes prepared by hypotonic haemolysis possess a limiting plasma membrane and permeability properties which vary according to the preparative conditions (Bramley et al., 1971). Measurement of ATPase activity in membrane preparations requires ATP, sodium and calcium to be present at the inner side of the membrane (Whittam and Ager, 1962; Schatzmann and Vincenzi, 1969; Bramley et al., 1971) and because of this ATPase estimations are particularly susceptible to variations in the permeability of membrane preparations (Bramley et al., 1971; Hanahan et al., 1973). Both intact erythrocytes and membranes prepared by centrifuging a hypotonic haemolysate without subsequent hypotonic washing show no ATPase activity unless broken up by freezing and thawing. Freeze-thaw treatment and sonication also increase ATPase activity in some membrane preparations exposed to several hypotonic washes. Such an increase in activity is thought to be due to increased permeability of the preparation to ATP or to activators (Bramley et al., 1971; Hanahan et al., 1973). Cryptic activity of enzymes

other than ATPases have also been reported (Duchan and Collier, 1971). By suitable manipulation of the osmolarity and pH of the haemolysing buffer it is possible to control the ATPase activity of the final membrane preparation (Bramley et al., 1971; Hanahan et al., 1973). Using hyptonic bicarbonate buffers, Bramley and colleagues were able to show minimum retention of haemoglobin in membrane preparations when 10 - 20 mosm buffer was used. Little non-haem protein was lost using buffers of 20 - 80 mosm but below 20 mosm there was a marked increase in the loss of protein. This was accompanied by a loss of acetylcholinesterase and Ca + Mg ATPase activity. Membranes prepared at 80 mosm buffer had low Na/K and Ca + Mg ATPase activity but it was increased by sonication or Triton-X-100 treatment. In contrast membranes prepared at 10 mosm buffer had high ATPase activities but these were not increased by disruptive treatments. Experiments using radioactively labelled ATP showed membranes prepared with 10 mosm buffer to have a high permeability. Bramley et al., (1971) interpreted these results as showing a variation in the permeability of the membranes depending on preparative conditions together with fragmentation and inactivation of enzymes when very low (<10mosm) osmolarity buffers were used. Hanahan et al., (1973) showed that using Tris buffer, pH 7.4, 20 mosm as haemolysing buffer the resulting membranes possessed no cryptic ATPase activity and were therefore permeable to ATP. These

workers also investigated the effects of the pH of the haemolysing buffer and found that using a pH 5.8 buffer resulted in a preparation with high haemoglobin retention together with low ATPase activities which were increased by freeze-thaw treatment. Acetylcholinesterase activity was little affected by buffer pH (Hanahan et al., 1973) or by buffer osmolarity between 20 and 80 mosm (Bramley et al., 1971). Considering acetylcholinesterase is thought to be situated on the outside of the erythrocyte membrane (Juliano, 1973) this supports the hypothesis that the differences in ATPase activity between membranes prepared under different conditions are due to differences in permeability properties. Hanahan et al., (1973) also reported that the chemical nature of the buffer was immaterial in determining the membrane preperties at a given buffer pH and osmolarity.

It is well established that the Na/K ATPase activity present in the erythrocyte membrane is associated with active transport of sodium and potassium (Dunham and Glynn, 1961; Skou, 1965; Whittam and Wheeler, 1970). In addition to this ATPase activity deperdant on Mg, stimulated by sodium and potassium and sensitive to ouabain Dunham and Glynn (1961) also detected an activity dependant on magnesium only and which was insensitive to ouabain. In addition they found calcium to cause a stimulation of the ouabain-insensitive activity. Such magnesium dependant (Mg ATPase) and calcium stimulated magnesium dependant

activities (Ca + Mg ATPase) were also reported by Wins and Schoffeniels (1966). As pointed out by Wins and Schoffeniels the physiological function of the Mg ATPase is unknown. It could possibly reflect adenyl cyclase activity since adenyl cyclase produces pyrophosphate which might be detected in the analysis of inorganic phosphate. Adenyl cyclase has been reported present in the human erythrocyte membrane (Sheppard and Burghardt, 1968; Loose, R., unpublished observations) but the activity is very low.

Nakao et al., (Nakao, Nagano, Adachi and Nakao, 1963) were able, by potassium iodide fractionation, to obtain two preparations from erythrocyte membranes. One possessed Na/K ATPase activity which was sensitive to ouabain and the other contained an ouabain-insensitive Mg ATPase which was not stimulated by sodium or potassium. These workers suggested therefore that the Na/K ATPase and Mg ATPase were separate enzymes. Bowler and Duncan (1969) studying the heat sensitivity of ATPases in crayfish muscle membranes also concluded that Na/K ATPase and Mg ATPase were separate enzymes. Recently, Drickamer (1975) studied ATPases by studying erythrocyte membrane protein phosphorylation in the presence of magnesium alone, calcium and magnesium and finally sodium, potassium and magnesium. Using gel electrophoresis he was able to separate three different phosphorylated proteins corresponding to the different ionic conditions. He concluded

that the three ATPases were distinct enzymes. The available evidence suggests therefore that the Mg ATPase and Na/K ATPase are distinct enzymes.

The function of the calcium stimulated ATPase (Ca ATPase) and Ca + Mg ATPase has been a subject of controversy and confusion. Wins and Schoffeniels (1966) suggested they might be associated with actomyosin-like protein in erythrocyte membranes (Ohnishi, 1962). In contrast Ca ATPase has also been suggested to be involved in the active transport of calcium out of the erythrocyte (Schatzmann and Vincenzi, 1969). ATP has been shown to be necessary for active transport of calcium from the erythrocyte (Schatzmann, 1966; Schatzmann and Vincenzi, 1969). In addition erythrocyte ghost ATPase activity has been shown to be stimulated by internal, but not external, calcium (Schatzmann and Vincenzi, 1969) and more recently Schatzmann (1973) has shown that the intracellular calcium concentration for half maximal stimulation of calcium transport to be in close agreement with the value of the dissociation constant for Ca and the Ca ATPase. The situation was complicated however by the finding of two Ca ATPase activities in erythrocyte membranes - a high affinity and low affinity activity (Schatzmann and Rossi, 1971). These workers also pointed out that the low affinity activity alone or the total activity, but not the high affinity activity alone, could account for active calcium transport. Wolf (1972) and

Schatzmann (1973) failed to repeat the finding of two separate activities and Schatzmann was of the opinion that the occurrence of two activities was an artifact due to exposure of the membranes to EDTA. Recently, however, Quist and Roufougalis (1975) have found two separate activities in membrane preparations some of which were exposed to EDTA and some not. Furthermore these workers showed that on exposing the erythrocyte membranes to low ionic strength the high affinity Ca ATPase (or an activator) was lost from the membrane whereas the low affinity was not. Other studies of the relationship between calcium transport and Ca ATPase have used inhibitory substances such as lanthanum chloride and ruthenium red. Using inside-out vesicular erythrocyte membranes Weiner and Lee (1969) showed 0.1 mM La^{+++} to completely inhibit both active transport of calcium into the vesicles and Ca ATPase activity while having no effect on the Mg ATPase or Na/K ATPase activity. Ruthenium red was found to specifically inhibit the Ca ATPase (Watson, Vincenzi and Davis, 1971). Subsequent work using La^{+++} and ruthenium red has not been conclusive as to whether all or part of the Ca ATPase activity is involved in transport but this may be due to the problem of impermeability of some of the ghost preparations used (Quist and Roufougalis, 1973; 1975a; 1975b). In conclusion there is considerable experimental evidence to support the hypothesis that at least part of the Ca ATPase

activity in the erythrocyte membrane is associated with the active transport of calcium.

There is however the possiblity of a second Ca ATPase enzyme. There is also evidence supporting the hypothesis that part of the Ca ATPase activity is due to a contractile protein. Exposure of erythrocyte membranes to low ionic strength causes a loss of protein from the membrane (some 25% of total membrane protein). The major components lost are two polypeptides called spectrin (Marchesi, Steers, Marchesi and Tillack, 1970; Juliano, 1973). In addition such extraction procedures also cause a loss in Ca ATPase activity from the membrane and this has been associated with fibrillar material (Rosenthal, Kregenow and Moses, 1970; Avissar, De Vries, Ben-Shaul and Cohen, 1975). Such activity could be the high affinity Ca ATPase which Quist and Roufougalis (1975a) found was lost from erythrocyte membranes on exposure to low ionic strength. The relationship between spectrin and actomyosin-like proteins extracted from erythrocyte membranes (Ohnishi, 1962) is uncertain but recent work has suggested actin-like protein to be present in low ionic strength extracts but to be a minor band separate from spectrin (Tiltney and Detmers, 1975). These workers were able to show the characteristically decorated arrow head formation of actin in the presence of heavy meromyosin. Spectrin filaments were also seen but were not decorated or of double helical structure. Other workers studied erythrocyte

membrane phosphorylation and showed that on exposure to low strength solutions the membranes showed a decrease in Ca + Mg dependant phosphorylation in the proteins running in the spectrin region on gel electrophoresis, (Weidermann and Brdczka, 1975). They also showed Ca ATPase activity to be present in a small fraction (10%) of spectrin and they suggested Ca ATPase was not associated with spectrin. Guidotti (1972) has reported low ionic strength exposure to remove a myosin-like protein from erythrocyte membranes.

Despite the confusion evident from these studies, it can be concluded that erythrocyte membranes probably contain actin-like and myosin-like proteins which possess Ca + Mg ATPase activity. The interaction of actin and myosin in the intact membrane could possibly be responsible for Mg ATPase activity since extracted fibrillar material possesses Mg ATPase activity in the presence of rabbit muscle actin (Avisser et al., 1975) and interaction of muscle actin and myosin is known to give rise to Mg ATPase activity (Bendall, 1969).

Results presented in section III showed chronic lithium administration to rats to increase synaptic plasma membrane Mg ATPase specific activity. In order to investigate whether lithium administration to humans had a similar effect on membrane Mg ATPase, Mg ATPase activity was studied in erythrocyte membranes prepared from psychiatric patients receiving lithium treatment.

Previous work has implicated Na/K ATPase activity and

sodium transport in erythrocytes as altering during the course of depressive illness. Naylor et al., (1973) studied Na/K ATPase in erythrocyte membranes from patients suffering from depressive illness and showed a correlation between amelioration of mood and increased Na/K ATPase activity. Hokin-Neaverson et al. (1974) showed bipolar manic depressives to have a lower ouabain-sensitive sodium efflux from erythrocytes than control subjects. In view of the implication of the Na/K ATPase with active sodium transport (Whittam and Wheeler, 1970) and in view of Naylor's finding that amelioration of mood is correlated with Na/K ATPase one would expect a decreased specific activity of Na/K ATPase in erythrocyte membranes prepared from depressive patients. This was investigated in the present study. Experiments were also designed to examine the effect of lithium treatment on Na/K ATPase activity and to try and elucidate whether the reported effect of lithium treatment on Na/K ATPase (Dick et al., 1974) was due to an effect of the drug or a secondary effect due to a change in the state of the patient.

Dick et al., (1974) reported a correlation between mood and erythrocyte membrane Na/K ATPase activity in a single patient. Since the mature human erythrocyte carries out no protein synthesis (Harris and Kellermeyer, 1970) such a correlation must reflect in vivo activation of the enzymes. In view of the extensive washing of the membranes this was thought to be unlikely. Experiments were carried

out to study ATPase activities in membranes prepared from three control individuals over a period of time in order to study the extent of variation and to investigate if this could be accounted for methodologically. In addition, experiments were carried out to investigate whether the effects of lithium on erythrocyte ATPases were dependant on the production of new cells. In an attempt to relate changes in ATPase activities caused by lithium treatment to possible changes in ion distribution the effect of lithium treatment on plasma and erythrocyte sodium, potassium and magnesium concentrations was studied.

Calcium ions have been implicated in many cellular processes (Cuthbert, 1970) and it was therefore of interest to study Ca ATPase in the present experiments since this may reflect the activity of the calcium pump.

The data, relating to human material, to be described in this section is derived from studies of Na/K, Mg and Ca ATPases in erythrocyte membranes from untreated depressive patients and both long and short term lithium treated patients. The ion distribution data is derived from a random selection of these same patients.

In order to connect the human erythrocyte membrane work with the effects of lithium treatment on rat synaptic membranes an additional series of animal experiments were performed in which the effects of chronic lithium treatment on rat erythrocyte membrane ATPases and rat plasma and erythrocyte ion distribution were studied.

MATERIALS AND METHODS.

Most chemicals used were Analar grade from British Drug Houses, Ltd., Poole, and may be assumed to be so unless stated otherwise. Tris was purchased from Sigma Chemical Co., Ltd. Animals and the administration of lithium to them in the diet was as described in Section III.

1. Preparation of human erythrocyte membranes.

The method of preparation used in the present series of experiments was that of Dick et al., (1969) with certain slight modifications. A diagram outlining the method is shown in Figure 20. Blood (10 - 20 ml) was collected by venipuncture and placed in sodium heparin tubes. After centrifugation at 1000 g for 4 minutes the plasma was discarded and the cell pellet then washed three times in ice-cold isotonic sodium chloride (156mM) buffered with 5 mM Tris, pH 7.4. Washing was by repeated centrifugations at 1000 g for 4 minutes at room temperature. After one or two washes the buffy coat was removed by aspiration and discarded. The washed cell pellet was resuspended in the cell wash solution to an approximate 40% haematocrit. An aliquot of the cell suspension was taken for accurate determination of the haematocrit and a known volume of the remainder haemolysed by addition of 10 mM Tris buffer, pH 7.4 (34 ml buffer:6 ml cell suspension) and vortexed briefly. The haemolysate was left at 0°C for 10 - 15 minutes and then centrifuged at 28,000 g for 105 minutes

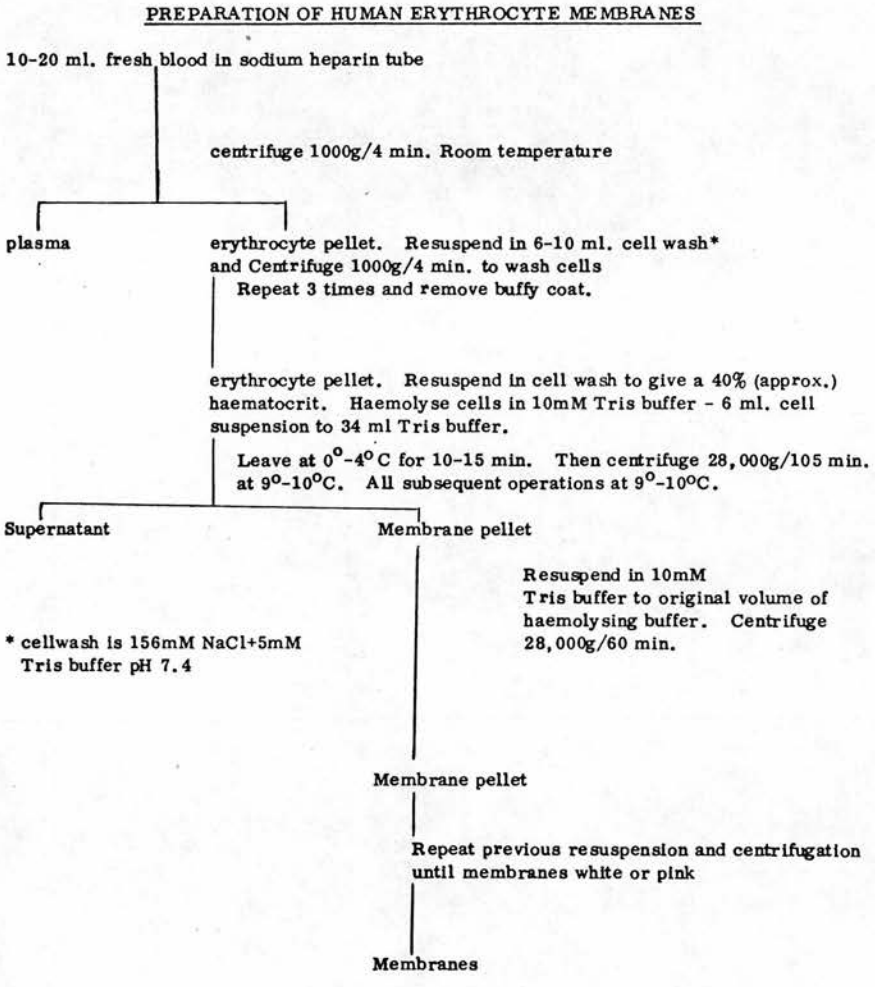

Figure 20. A diagram illustrating the method used for preparation of human erythrocyte membranes.

using a Beckman L2-65B ultracentrifuge with a swing-out rotor. The speed of this and subsequent centrifugations was increased from that originally used by Dick et al. in order to achieve adequate pelleting of the membranes. In order to obtain reproducible preparations it was also necessary for all the post-lysis centrifugations to be carried out between 9 and 10°C. Centrifugation at 28,000 g for 105 minutes produced a red membrane pellet. The membranes were then washed three times by resuspension in 10 mM Tris buffer and centrifugation at 28,000 g for 1 hour. At the end of the final centrifugation there was a layer of fluffy, white or faintly pinkish membranes at the bottom of the tube together with a pellet of cell debris and haemoglobin. The membranes were dispersed by brief vortexing and decanted into a fresh tube for storage. The pellet of debris remained in the centrifuge tube and it was then washed once in 10 mM Tris buffer by vortexing and the wash added to the membranes. 10 mM Tris buffer was added to the membranes to give a final volume equal to the original volume of cell suspension and the membranes were well mixed and left overnight at 4°C before assaying for ATPase activity.

The above method produced adequate membrane preparations which possessed ATPase activity but the pelleting of the membranes was poor. Use of an angle-head rotor (together with an MSE Superspeed 65 ultracentrifuge) for all post-lysis centrifugations gave much improved pelleting

and a much higher yield of membranes in terms of protein but the specific activity of the ATPases in the membranes was essentially unchanged (Table 14.). The fact that increased yield is accompanied by no changes in specific activity of the various ATPases indicates that the suspensions of membranes during preparation are homogenous and therefore the efficiency of pelleting of the membranes should not be critical in determining the ATPase activities of the final membrane pellet. The Beckman ultracentrifuge together with the swing-out rotor was used throughout this study on account of it allowing a faster preparation time.

Using the Beckman ultracentrifuge, membranes prepared from a single sample of blood were reproducable in their ATPase activities as judged by enzyme activity in five preparations made from a single sample of cell haemolysate (Table 15.). The preparative procedure outlined above seems therefore to give reproducible membrane preparations from a given cell haemolysate.

2. Preparation of rat erythrocyte membranes.

Animals were anaesthetised with nembutal (70 mg/kg) and the chest opened. 5 ml blood was removed by cardiac puncture and placed in a sodium heparin tube. Blood from two animals was pooled for each membrane preparation. Membrane preparation was as described above for human material except that an angle rotor was used together with an MSE Superspeed 65 ultracentrifuge.

Table 14. Characteristics of human erythrocyte membrane prepared using swing-out and angle-head rotors. Membranes were prepared from a single haemolysate using either a swing-out rotor (with Beckman L2-65B ultracentrifuge) or an angle-head rotor (with M.S.E. 65 Superspeed ultracentrifuge) for all post-lysis centrifugations. ATPase specific activities are expressed as nmol P_i/hr/mg protein. Protein yield is expressed as mg protein/litre cells. Values given are means ± standard deviations from three preparations in each case..

	Swing-out rotor, Beckman L2-65B ultracentrifuge	Angle-head rotor, MSE Superspeed 65 centrifuge
Na/K ATPase activity	113 ± 7	132 ± 23
Mg ATPase activity	303 ± 19	303 ± 24
Ca + Mg ATPase activity	767 ± 40	688 ± 84
Protein yield	3.17 ± 0.08	7.31 ± 0.46

Table 15. The reproducibility of erythrocyte membrane ATPase specific activity in membranes prepared from a single haemolysate. A single haemolysate was divided into 5 aliquots and 5 separate membrane preparations produced (using swing-out rotor). The mean ATPase specific activities ± standard deviations for these five preparations are shown below. Activities are expressed as nmol P_i/hr/mg protein.

Na/K ATPase activity	Mg ATPase activity	Ca + Mg ATPase activity
245 ± 11	240 ± 28	730 ± 91

3. Estimation of ATPase activity.

a. Incubation conditions.

ATPase activity was assayed by measuring the inorganic phosphate produced during incubation of the membrane preparation with 3 mM disodium-ATP (from B.D.H., Poole or Boehringer Ltd.). Incubation was for 30 minutes at 37.5°C. The incubation conditions were based on those of Kurokawa, et al., (1965), Bonting and Caravaggio (1963) and Wins and Schoffeniels (1966). For the estimation of Na/K ATPase and Mg ATPase activities the incubation conditions were as described in Section III, with water being used to make up the volume of the Mg ATPase incubation medium. ATPase activity in the presence of Ca^{++} was estimated using an assay medium containing 0.15 mM $CaCl_2$ in addition to EDTA, Tris and $MgCl_2$ in the same concentrations as was used for the assay of Mg ATPase activities. Reaction and enzyme blanks were run with all assays and inorganic phosphate liberated estimated by comparison with standard phosphate solutions. All estimations were done in triplicate.

Enzyme activity was measured under three different sets of ionic conditions: in the presence of magnesium as the sole cation, in the presence of calcium and magnesium and in the presence of sodium, potassium and magnesium. The activity in the presence of magnesium alone was defined as the Mg^{++} dependant ATPase activity and this was insensitive to the presence of ouabain. The difference between activity in the presence of sodium, potassium and magnesium

and activity in the presence of magnesium alone was defined as the Na/K stimulated Mg^{++} dependant ATPase activity and this was completely abolished by ouabain. The activity in the presence of both calcium and magnesium was defined as the Ca^{++} + Mg^{++} dependant ATPase activity (Ca + Mg ATPase) and the difference between that and the Mg^{++} dependant activity was defined as the Ca^{++} stimulated Mg^{++} dependant activity (to be hereafter referred to as the Ca stimulated or Ca ATPase activity). During the routine estimation of ATPases all activities were defined in terms of differential cation activation without the use of ouabain.

b. Estimation of inorganic phosphate.

Initially, during the development of the preparation and assay of erythrocyte membranes, inorganic phosphate produced during incubation with ATP was determined by the method of Martin and Doty as described by Lindberg and Ernster (1956). This method involves precipitation of protein with trichloroacetic acid, reaction of the phosphate to form a phosphomolybdate complex and then extraction of the complex into an isobutanol-benzene mixture. An aliquot of the complex-containing organic layer is then reduced with stannous chloride and the resulting blue colour measured spectrophotometrically. Although this method had proved adequate in the determination of ATPase activities in subcellular fractions of brain it was found not to be satisfactory for ATPase assays involving erythrocyte

membranes on account of the large variability between replicate samples and general lack of reproducibility.

Mozersky and his colleagues (Mozersky, Pettinati and Kolman, 1966) reported the Martin and Doty method to have low reproducibility and low sensitivity. They pointed out three problems associated with the method: the reduction of the phosphomolybdate complex was not reproducible, silicotungstic acid caused interference and the presence of protein reduced the recovery of phosphate. In contrast Martin and Doty (1949) added silicotungstic acid in order to prevent protein chelating phosphate and so preventing its reaction with molybdate. In view of these previous studies it was possible that the poor reproducibility of the Martin and Doty method in the present experiments was due to protein interference. Therefore an experiment was carried out to investigate the effects of albumin and silicotungstic acid (prepared as described by Martin and Doty, 1949) on the estimation of phosphate in a standard KH_2PO_4 solution. The results are shown in Table 16. and they show that neither the presence of protein in an amount comparable to that in ATPase assay samples nor the use of silicotungstic acid affected the reproducibility of the estimation of phosphate. This suggests that protein interference is an unlikely explanation of the poor reproducibility of the method for phosphate estimation. In the presence of albumin, addition of silicotungstic acid increased the absorbance obtained with a given

Table 16. The effects of albumin and silicotungstic acid on the estimation of inorganic phosphate by the method of Martin and Doty. A standard phosphate solution (KH_2PO_4) was assayed by the method of Martin and Doty as described by Lindberg and Ernster (1956), (a) in the presence and absence of bovine serum albumin (0.1 mg/ml) and (b) in the presence of albumin and silicotungstic acid (1 ml, prepared as described by Martin and Doty, 1949). Results are shown as absorbance readings (standard units). Values are means ± standard deviations with the number of estimations in parentheses. Groups were compared using a Students 't' test, *p 0.005 between groups with and without silicotungstic acid.

	Absorbance
control (no albumin, no silicotungstic acid)	0.166 ± 0.007 (12)
Albumin added	0.161 ± 0.006* (12)
Albumin and silicotungstic acid added	0.172 ± 0.009* (12)

phosphate standard and this may be due to the prevention of occlusion of phosphate by silicotungstic acid. The effect, although statistically significant (p 0.005) is only small (6.8% increase in mean value).

ATPase activities in erythrocyte membranes are low compared to other tissues (Bonting and Caravaggio, 1963). This low activity exascerbates the poor reproducibility in the analysis method since it is necessary to attempt to measure a small difference in absorbance between the reaction blank and the sample with enzyme present. Thus a standard deviation of 5 - 6% in the measured absorbance produced a 15 - 30% standard deviation in enzyme activity. An alternative approach to the problem of reproducibility was therefore to use a more sensitive method for the analysis of inorganic phosphate rather than attempting to improve the Martin and Doty method. Mozersky et al. (1966) reported that estimation of phosphate by measurement of the absorbance of the unreduced, yellow phosphomolybdate complex after extraction into isobutanol-benzene gave increased sensitivity compared to the conventional Martin and Doty method. Similar methods, also measuring the formation of the unreduced phosphomolybdate complex but using different solvents for extraction, have been devised (Wahler and Wollenberger, 1958; Naylor et al., 1973) and one of these together with the method of Lecocq and Inesi (1966) were investigated as possible alternative methods of phosphate analysis.

The method described by Lecocq and Inesi involves formation of a phosphomolybdovanadate comples which is estimated spectrophotometrically by its absorbance at 350 or 400 nm. The absorption spectrum of the complex was studied by reacting a standard phosphate solution with the necessary reagents as described by Lecocq and Inesi (1966) and the results are shown in Figure 21. The spectrum shows a broad peak at 350 nm but at 400 nm the absorbance changes rapidly with wavelength. Therefore only the absorbance at 350 nm is suitable for spectrophotometric analysis. As shown in Figure 22 estimation of a standard phosphate solution by measurement of the absorbance of the phosphomolybdovanadate complex at 350 nm gave a doubling of sensitivity compared to the method due to Martin and Doty. However use of Stowards method of phosphate analysis (described by Naylor et al., 1973) gave a ten-fold increase in sensitivity as shown in Figure 22. This method involves extraction of the phosphomolybdate complex into ethyl acetate and measurement of the absorbance at 310 nm. In principle it is comparable to the methods of Mozersky et al. (1966) and Wahler and Wollenberger (1958). The latter used extraction into isopropyl acetate and were able to detect 10^{-9} mol of phosphate. As shown in Figure 21 the absorption of the phosphomolybdate complex in ethyl acetate has a broad peak around 310 nm making the method perfectly adequate for spectrophotometric analysis. The spectrum of the phosphomolybdate

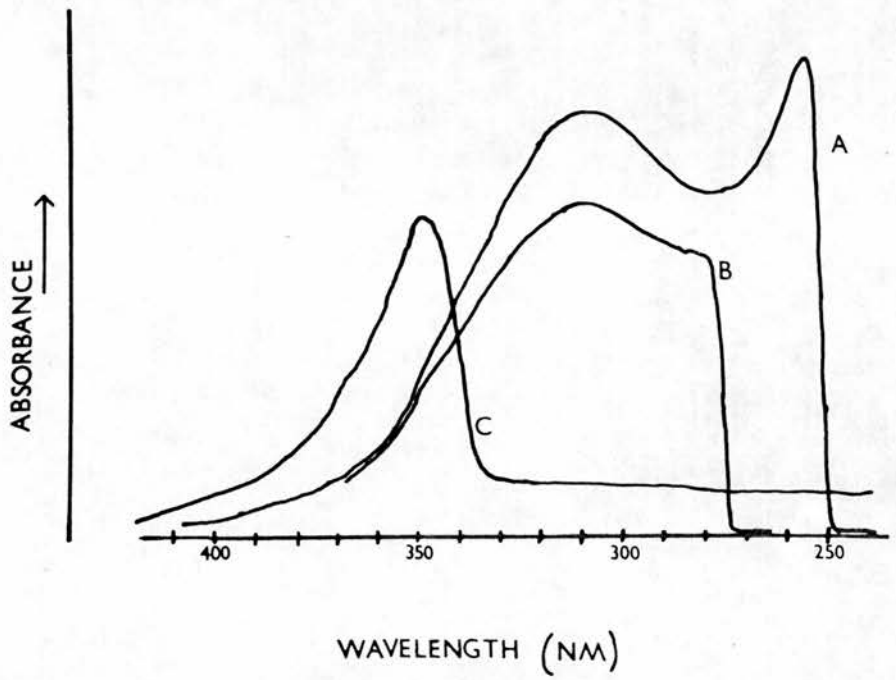

Figure 21. Absorption spectra of derivatives used in the estimation of inorganic phosphate. A standard phosphate solution (KH_2PO_4) was reacted (a) as per the method of Stoward as described by Naylor et al. (1973), (b) as per the method of Martin and Doty as described by Lindberg and Ernster (1956) and (c) as per the method of Lecocq and Inesi (1966). Spectra shown are for the ethyl acetate extract used in the method of Stoward (A), the isobutanol-benzene extract obtained by the method of Martin and Doty and used by Mozersky et al. (1966) (B) and the reaction mixture obtained using the procedure of Lecocq and Inesi (1966) (C). Absorbance is given in arbitary units.

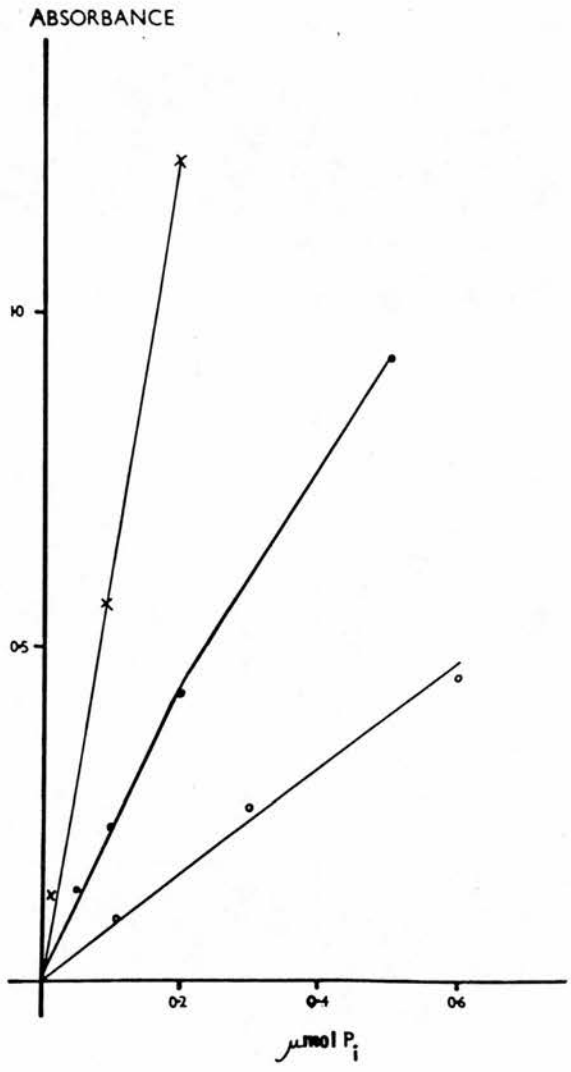

Figure 22. Sensitivity of different methods for estimation of inorganic phosphate. Standard curves are shown for estimation of phosphate in a solution of KH_2PO_4 by (a) the method of Stoward as described by Naylor et al. (1973),-X-, (b) the method of Martin and Doty as described by Lindberg and Ernster (1956), -o-, and (c) the method of Lecocq and Inesi (1966) using absorbance at 350 nm as the measure of phosphate, -•-. Absorbance is given in standard absorbance units. Values are means of three estimations.

complex in isobutanol-benzene also shows a peak at 310 nm (Figure 21) and is comparable to the spectrum of the complex extracted into ethyl acetate. The high sensitivity in the method of Stoward may be due to the omission of the reduction reaction used by Martin and Doty and the choice of solvent for extraction.

In view of the high sensitivity of Stowards method it was subsequently used for all inorganic phosphate estimations. The estimation was not affected by the ionic constituents of the incubation medium and using the method for ATPase estimations ATPase activity was shown to be linear with respect to time (over 1 hour) and amount of membrane material added (0.02 - 0.16 mg protein). The only problem encountered was that occasionally a batch of ATP could not be used on account of a high blank value.

The details of the method of phosphate analysis are given below:

Reagents:

Perchloric acid-tungstosilicic acid reagent (PATSA):

48.5 - 50 ml perchloric acid (Analar, 72%) was added to 300 ml water, 40 g dodecatungstosilicic acid (Analar, from B.D.H.,) added, dissolved and the solution made up to 500 ml.

Molybdate reagent:

14.5 g sodium molybdate dihydrate and 131.3 g sodium chloride were dissolved in 1 litre of water.

Standard phosphate solution:

453.6 mg KH_2PO_4/100 ml. This was diluted by between 1/50 and 1/500 and 2.5 ml taken for analysis. This gave standards of between 16.6 and 166 nmol.

Procedure:

The ATPase reaction was stopped by placing the tubes in ice and the tubes left in ice for 5 - 10 minutes. Each tube was subsequently reacted in turn. 0.5 ml PATSA and then 1.5 ml molybdate reagent were added and the sample vortexed briefly. 4 ml ethyl acetate was added, the mixture vortexed for 30 seconds and the tubes then returned to ice. After separation of the two layers, a 1 ml aliquot of the upper organic layer was placed in a clean tube. Whilst sampling the upper layer care was taken not to disturb the precipitate of protein at the interface between the layers otherwise the aliquots of the organic layer became turbid and it was not possible to measure the optical density accurately. The absorbance at 310 nm was measured in the aliquots of the upper layer and the amount of phosphate in the original samples calculated by comparison of the optical densities with those from standard phosphate solutions. Standards were run with each assay.

c. Expression of results.

Most ATPase activities are given as specific activities in terms of nanomoles of inorganic phosphate liberated per hour per milligram of protein (nmol P_i/hr/mg protein). Haematocrits of each cell suspension were measured prior

to haemolysis so that results could also be expressed as nmol P_i/hr/litre of original cells if required.

Protein was measured by the method of Lowry et al., (1951) as given in Appendix 2. Haematocrits were measured using an MSE microhaematocrit centrifuge. Samples were centrifuged at 16,000 g for 5 minutes.

4. Estimation of Haemoglobin.

Haemoglobin was estimated in a certain number of preparations. The method of analysis used was that described by Bodemann and Passow (1972).

Samples, standards (bovine haemoglobin, Sigma Chemical Co.) and blanks were diluted 1 + 4 with glacial acetic acid and left at room temperature for 15 minutes. The absorbance of the solutions was then measured at 400 nm.

5. Standard ATPase.

In certain experiments a standard ATPase preparation was assayed in addition to the membrane preparations. A brain microsomal Na/K ATPase preparation (Sigma Chemical Co.) was used. The purchased preparation was dissolved in water and aliquots placed in separate tubes. The solutions were then freeze-dried overnight and stored at -20°C. When required, tubes were thawed and incubation medium added.

6. Estimation of lithium in membrane preparations.

Attempts were made to estimate the concentrations of lithium in membranes prepared from people receiving lithium treatment. Such preparations were stored at -20°C until

analysed.

Lithium was estimated by atomic absorption spectrophotometry using both flame and flameless techniques. A Perkin-Elmer 360 atomic absorptiometer was used throughout, together with a HGA-74 heated graphite furnace for the flameless technique. For estimation by standard flame methods the membranes were not diluted and they were compared to standards of lithium chloride in deionized distilled water. The method of analysis using the graphite furnace was that described in section III. 50 µl of membrane suspension was injected into the furnace and compared with 50 µl aliquots of standards. Standards consisted of membrane preparations from people not receiving lithium treatment with known amounts of lithium chloride (Baker Chemical Co. Ltd.) added.

7. Measurement of plasma and intracellular erythrocyte ion concentrations.

The procedure adopted for separation of plasma and subsequent washing of the cell pellet followed methods used by Smith and Samuel (1970) and Glen and Bellinger (1973).

Measurement of ion concentrations in erythrocytes separated from plasma, but not washed, is subject to error due to plasma contamination (Beilin, Knight, Munro-Faure and Anderson, 1966). Such error is particularly evident in the case of sodium because the erythrocyte contains little sodium whereas plasma has a high sodium concentration.

Plasma contamination of erythrocytes separated by centrifugation has been termed 'trapped plasma'. The problem of trapped plasma may be overcome either by estimating the plasma contamination or by washing the cells in suitable ion-free solutions (Beilin et al., 1966).

Glen and Bellinger (1973) used ^{51}Cr-EDTA in order to measure the extent of plasma trapping. Isotope was added to whole blood and the plasma and erythrocytes separated and ^{51}Cr estimated in both. The contamination of the erythrocytes with plasma as judged by the amount of ^{51}Cr in the erythrocyte pellet was less than 1%. However in the present work ^{51}Cr-EDTA was found unsatisfactory for the estimation of trapped plasma for two reasons. Firstly sodium content of erythrocytes was more consistent when uncorrected. Secondly in some cases the sodium content corrected for trapped plasma (Beilin et al., 1966) had a negative value. Similar problems have been found previously (Loose, R., personal communication). The negative sodium content found when using trapped plasma corrections suggested that there was some binding of ^{51}Cr to erythrocytes. This was investigated in two ways.

Firstly the ^{51}Cr content of rat erythrocytes was studied as a function of the number of washes in isotonic choline chloride to which the erythrocytes had been exposed. 1 g samples of erythrocyte pellets were counted using a Gamma guard γ-ray spectrometer set specifically for ^{51}Cr. All samples were counted for 20 minutes. ^{51}Cr-EDTA (from

the Radiochemical Centre, Amersham) was added to pooled rat blood so as to give approximately 2 μCi/ml blood. After mixing, aliquots were washed in isosmotic (260 mosm) choline chloride by mixing and centrifugation as described below. As shown in Figure 23 it was not possible to remove all the ^{51}Cr from the erythrocytes suggesting some binding did occur. The curve shown in the figure also suggests that two washes is sufficient to remove all the ^{51}Cr present due to plasma contamination of the erythrocyte pellet.

A second experiment was performed in which aliquots of rat erythrocytes (separated from plasma but not washed) were suspended in a solution of isosmotic choline chloride containing approximately 5 μCi/ml of ^{51}Cr-EDTA and then separated by centrifugation as described below. ^{51}Cr was estimated in 1 ml aliquots of suspension fluid before erythrocytes were added and after they had been removed by centrifugation. The results (Table 17.) show a 7.7% reduction ($p<0.025$) in the ^{51}Cr concentration of the suspension fluid after contact with erythrocytes. This suggests rat erythrocytes bind isotopic chromium. ^{51}Cr, as free chromium ions, is used to tag erythrocytes (Harris and Kellermayer, 1970) and it is possible that free ^{51}Cr in the isotope preparation used could account for the apparent binding of ^{51}Cr-EDTA to erythrocytes. The ^{51}Cr-EDTA preparation is only 99% complexed when prepared (The Radiochemical Centre, personal communication) and this

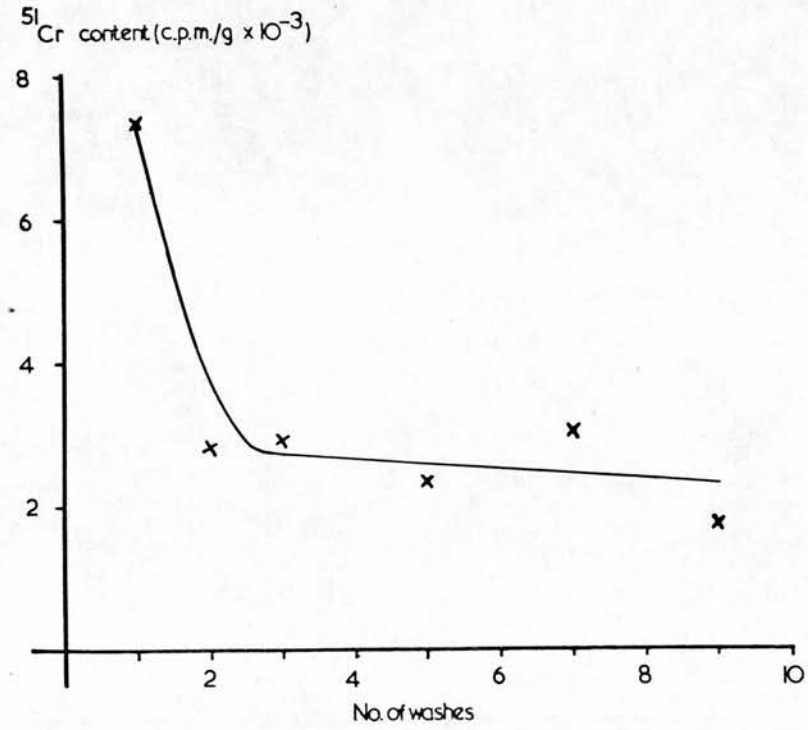

Figure 23. ^{51}Cr content of rat erythrocytes as a function of number of washes in 260 mosm choline chloride. ^{51}Cr-EDTA was added to rat blood (2 μCi/ml blood) and the erythrocytes separated by centrifugation to 16,000 g. Erythrocytes were subsequently washed in 260 mosm choline chloride as described in the text. Aliquots of erythrocytes were taken for estimation of ^{51}Cr. The figure shows ^{51}Cr content of erythrocytes (expressed as C.P.M./kg erythrocytes, all counts corrected for background) after various numbers of washes. Results are from a single experiment.

Table 17. Concentration of ^{51}Cr-EDTA in 260 mosm choline chloride before and after contact with rat erythrocytes. ^{51}Cr-EDTA (5 μCi/ml) was added to 260 mosm choline chloride and 1 ml aliquots taken for ^{51}Cr estimation. Rat erythrocytes were added to the remaining solution (1 ml erythrocytes/4 ml choline chloride), mixed and erythrocytes removed immediately by centrifugation (the solutions were brought momentarily up to 16,000 g). 1 ml aliquots of the choline chloride solution were taken for ^{51}Cr estimation. Results show the ^{51}Cr concentration in the choline chloride as C.P.M./ml (corrected for background). Values are means ± standard deviations with number of estimations in parentheses. Groups were compared using a Wilcoxon ranking test, $p<0.01$.

	^{51}Cr-EDTA concentration (C.P.M./ml)
Original choline chloride solution	16625 ± 206 (5)
Choline chloride after contact with rat erythrocytes	15344 ± 810 (5)

could perhaps be reduced during storage. The results of these experiments suggest the ^{51}Cr-EDTA preparation used by Glen and Bellinger (1973) is not satisfactory for estimation of trapped plasma in the case of the rat and they put doubt on its value for use with human material.

Further experiments studied the effect of successive washing of rat erythrocytes on their sodium content (Figure 24). Washing of erythrocytes and estimation of sodium was as described below. The results show two or three washes to cause a drastic reduction in sodium content concomittant with the reduction in ^{51}Cr labelling (Figure 23). This reduction in sodium content probably represents the removal of plasma contamination. The small, steady loss of sodium with subsequent successive washes is probably due to leakage of sodium from the erythrocytes. Beauge and Ortis (1971) showed sodium to leak out from rat erythrocytes suspended in sodium-free solution. If leakage is the correct explanation of this slow loss of sodium, extrapolation of the sodium content back to 'zero wash' (see Figure 24) should allow estimation of the true intracellular sodium concentration of the rat erythrocyte. This was found to have a value of 2.7 mmol/kg wet cells which compares well with the value of 4.4 mM reported by Beauge and Ortis (1971). It is evident from these results that three washes in sodium-free solution is sufficient to reduce trapped plasma to negligable proportions. Beilin et al. (1966) pointed out firstly that the use of high

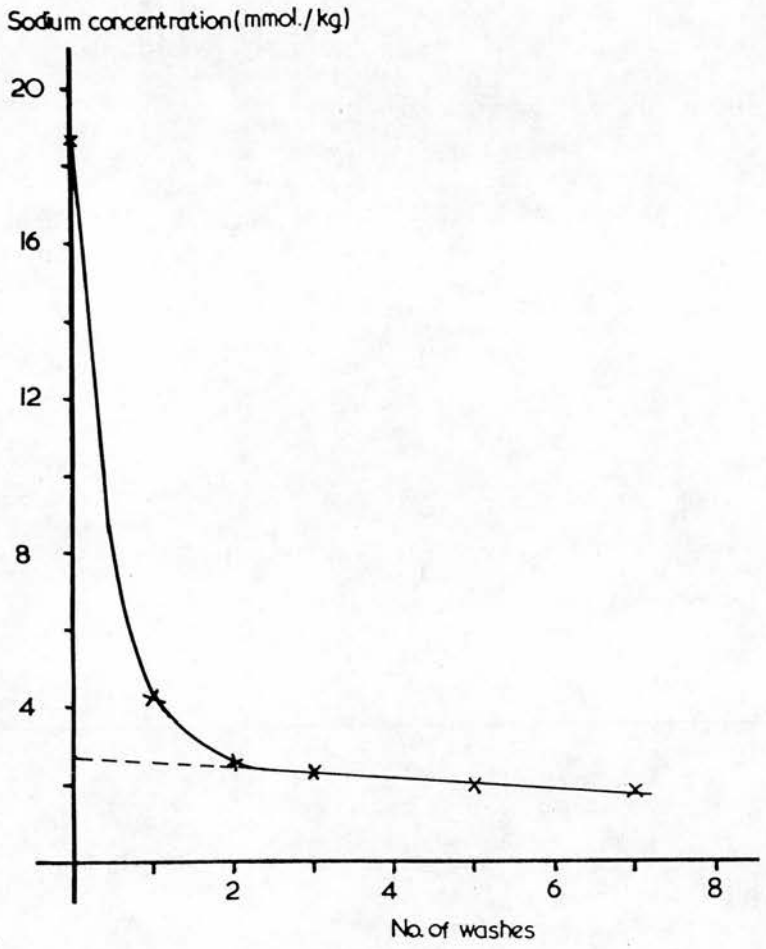

Figure 24. Sodium concentration in rat erythrocytes after washes in 260 mosm choline chloride. Rat erythrocytes were washed and sodium estimated in them as described in the text. The figure shows mean values from three experiments and these experimental values are described by the solid curve shown. Extrapolation of the sodium concentration to 'zero-wash' is also shown (----).

centrifugal force (approximately 20,000 g) reduced trapped plasma to a small constant value and secondly that the washing of erythrocytes did away with the need for estimating trapped plasma. In the present experiments which used a centrifugal force of 16,000 g and three washes of erythrocytes it was not necessary therefore to routinely estimate trapped plasma.

The procedure for preparation of plasma and of cell haemolysate was as follows: 5 - 10 ml fresh blood was placed in a heparinised tube (approximately 200 units mucous heparin) and centrifuged by bringing momentarily up to 16,000 g. This and all subsequent centrifugations were done at 10°C using an MSE Mistral 2L centrifuge fitted with a high speed angle-head. After aspirating off the plasma, the cell pellet was resuspended in 285 mosm sucrose (human material) or 260 mosm choline chloride (rat material) and the centrifugation repeated. After three such washes the buffy coat and top layer of cells was removed from the cell pellet. A weighed aliquot of erythrocytes was then haemolysed in a weighed quantity of deionized water (1 ml cells:10 ml water). Haemolysate and plasma samples were stored at -20°C until analysis.

Analysis of Na, K, Mg and Li was by atomic absorption spectrophotometry using a Perkin-Elmer 360 atomic absorptiometer set at the following wavelengths: lithium (670 nm), sodium (589 nm), potassium (766 nm) and magnesium (285 nm). All samples and standards were diluted 1:30 for sodium,

potassium and magnesium analysis and 1:10 for lithium analysis. Lanthanum chloride (0.1% w/v) in deionized water was used for all dilutions. Standards consisted of the respective metallic chlorides dissolved in deionized water. Ion concentrations were expressed per litre for plasma and per kilogram of wet cells for the erythrocytes.

In the case of the animal work, the rats were anaesthetised with nembutal (70 mg/kg) and 5 ml blood removed by cardiac puncture.

8. Information regarding patients studied.

a. General: All blood samples were taken between 9 and 10.30 a.m. and were delivered to the laboratory within 45 minutes. Patients took only a light breakfast on the morning of sampling but otherwise there was no dietary control. Those patients on lithium took no medication on the morning of sampling. The small group of untreated epileptics studied were outpatients at the Northern General Hospital, Edinburgh. The patients in the ill, untreated depressed group were all inpatients at the Metabolic Ward, Craig House, Royal Edinburgh Hospital while of the lithium treated group some were inpatients in the same ward but the majority were outpatients.

Besides the group of epileptic patients, all the patients studied were suffering or had suffered from some kind of depressive illness. Two major types of patients were studied: a group of patients who were ill and on no drug treatment (except sodium amytal as a night sedative)

and a group of patients who had received lithium treatment. In addition some of the untreated group were re-examined after a period of receiving lithium therapy.

b. Diagnosis: Patients were diagnosed by two independant psychiatrists, the referring clinical psychiatrist and a research psychiatrist. Criteria for the diagnosis of depression were those recommended by the M.R.C. Clinical Psychiatry Committee (1965) and the criteria for the diagnosis of mania were those described by Feighner, Robins, Guze, Woodruff, Winokur and Munoz (1972). Unipolar and bipolar depressives were diagnosed using the criteria discussed by Perris (1966). Patients receiving lithium treatment were assessed as to whether they were ill or recovered.

RESULTS.

1. Variation in erythrocyte membrane ATPase activities in specific individuals.

An experiment was performed to investigate the variation in ATPase activities of membranes prepared from three individuals on several occasions over a period of weeks. Three healthy males aged 24 (J.H.), 28 (A.C.) and 32 (A.V.) were studied. Blood samples were taken between 9 and 10 a.m. once a week for five weeks. Each week, aliquots of a standard ATPase preparation were assayed along with the membrane preparations.

Haematocrits were measured at the cell suspension stage of each membrane preparation and ATPase activities

were expressed both as nmol P_i/hr/litre of cells and nmol P_i/hr/mg protein. Haemoglobin and total protein were estimated in each preparation and protein yield per litre of original cells calculated. The results for each individual are shown in Figures 25, 26 and 27.

The results show a large variation in the ATPase activity of consecutive membrane preparations from a given individual. There was however little variation in the activity of the standard ATPase preparation. It is unlikely therefore that the variation in the membrane ATPase activities was due to methodological error in the ATPase assay itself.

As shown in Figure 28 the enzyme activities expressed in terms of protein are significantly correlated ($r = 0.833$, $p<0.001$) with activities expressed per litre of cells. It is unlikely therefore that the variation in activity was due to variation in the protein composition of the preparation. This is further substantiated by the lack of correlation between either protein yield or membrane haemoglobin content and enzyme activity (Figures 25, 26 and 27). Variation in enzyme activity, if caused by variation in preparative techniques, must therefore be due to qualitatively different preparations. There is no consistent pattern in the variation of enzyme activity between the three individuals studied and this also suggests that there was no consistent methodological error.

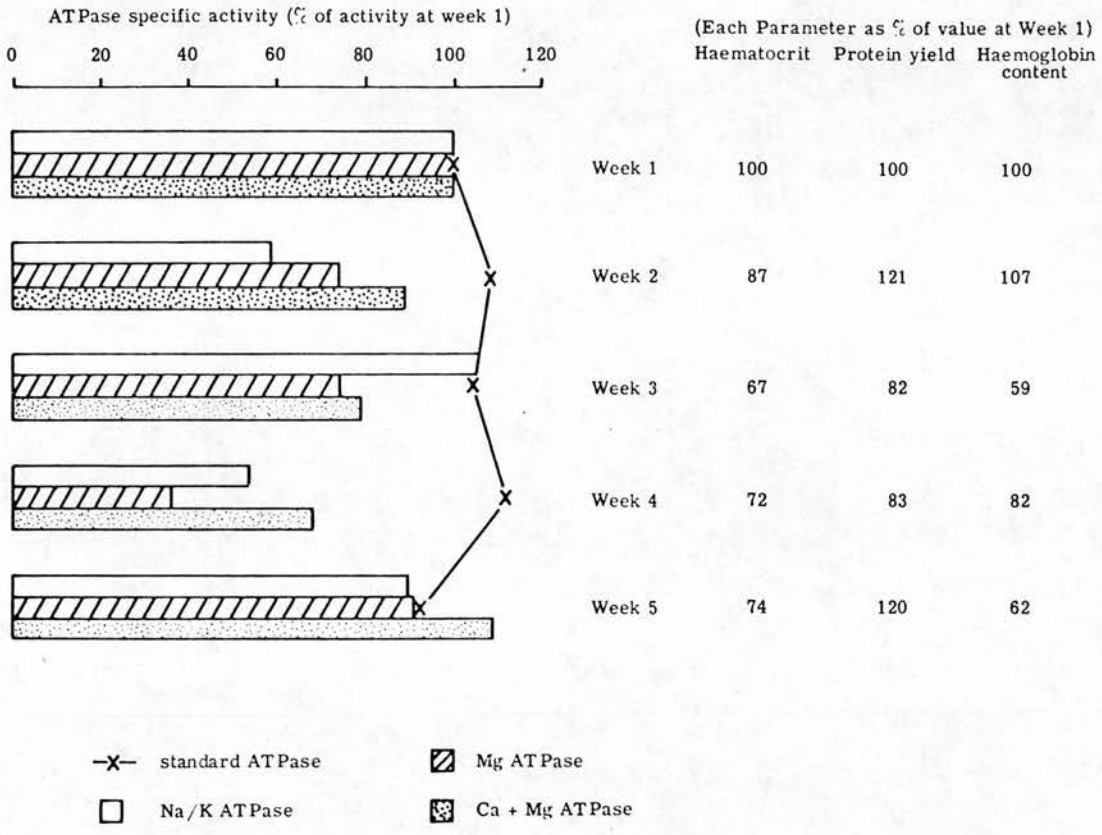

Figure 25. Characteristics of erythrocyte membranes prepared from a single subject on five separate occasions. Results given are for subject J.H. (male, 24). Specific activities of membrane ATPases, activity of a standard ATPase preparation, protein yield and haemoglobin content of the membranes and haematocrit of cell suspensions used for preparation of membranes are shown for each preparation. All parameters are expressed as a percentage of the value for the preparation at week 1. All parameters were measured in duplicate or triplicate and results shown are mean values.

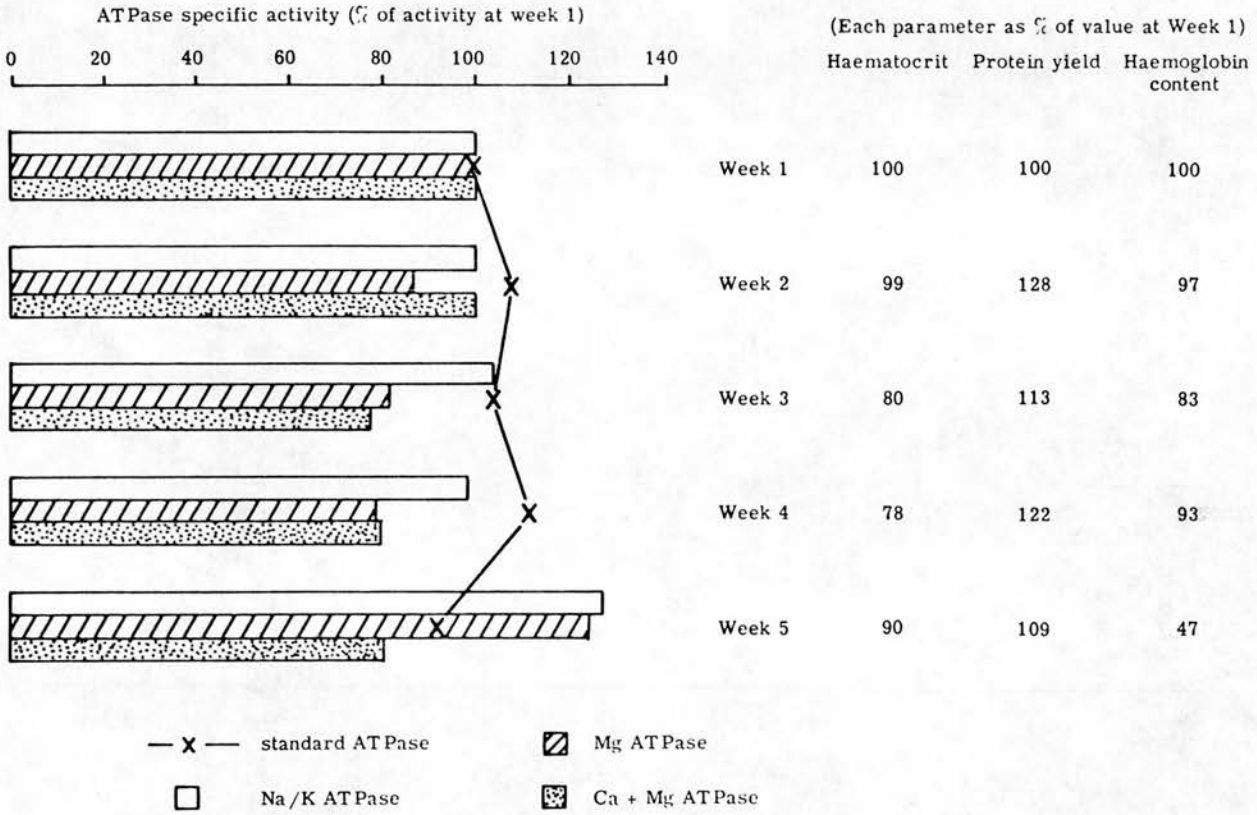

Figure 26. Characteristics of erythrocyte membranes prepared from a single subject on five separate occasions. Results given are for subject A.C. (male, 28). Specific activities of membrane ATPases, activity of a standard ATPase preparation, protein yield and haemoglobin content of the membranes and haematocrit of cell suspensions used for preparation of membranes are shown for each preparation. All parameters are expressed as a percentage of the value for the preparation at week 1. All parameters were measured in duplicate or triplicate and results shown are mean values.

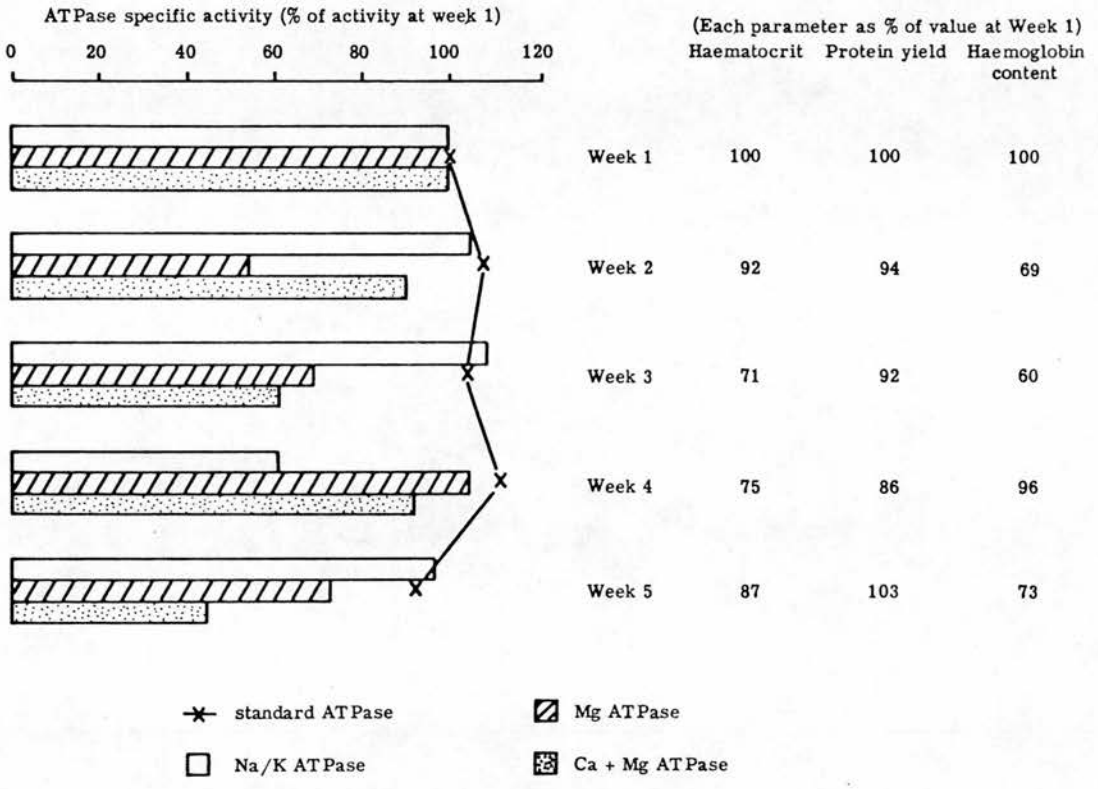

Figure 27. Characteristics of erythrocyte membranes prepared from a single subject on five separate occasions. Results given are for subject A.V. (male, 32). Specific activities of membrane ATPases, activity of a standard ATPase preparation, protein yield and haemoglobin content of the membranes and haematocrit of cell suspensions used for preparation of membranes are shown for each preparation. All parameters are expressed as a percentage of the value for the preparation at week 1. All parameters were measured in duplicate or triplicate and results shown are mean values.

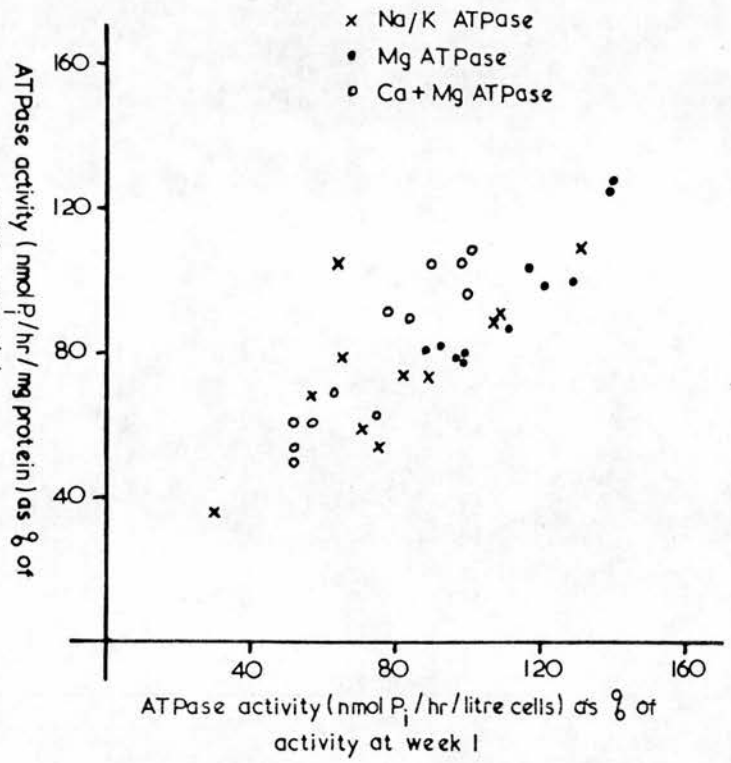

Figure 28. Correlation between erythrocyte membrane ATPase activity related to membrane protein and activity related to volume of cells from which membranes were prepared. Results are shown for membranes prepared from three control subjects (J.H., A.C. and A.V.) on five occasions. Activities were calculated both as nmol P_i/hr/mg protein and nmol P_i/hr/l cells and then expressed as percentages of the activities (per mg protein and per l cells) of the membranes prepared at week 1. The figure shows the relationship between the activity per mg protein as a percentage of the value at week 1 and the activity per l cells as a percentage of the value at week 1. There was a significant correlation ($r = 0.833$, $p<0.001$) between the two expressions of enzyme activities.

There was only one step in the membrane preparation which was variable from week to week and that was the haemolysis stage. The cell suspension was haemolysed at a constant ratio of cell suspension to haemolysing buffer but the haematocrit of the cell suspension varied and so the ratio of volume of cells to volume of haemolysing buffer also varied from week to week. Inspection of Figures 25 - 27 shows no consistent pattern between the haematocrit of the cell suspension before haemolysis and the various ATPase activities in the membrane preparations. There was no correlation between enzyme activity and haematocrit value. It is unlikely therefore that variations in the cell:buffer ratio caused the variation in ATPase activity.

2. Age and Sex effects.

Table 18 shows the age and sex composition of the various groups of patients studied. There was no significant difference between the age composition of the various groups of depressive patients or the controls. The epileptic group however consisted of considerably younger people. Only the unipolar untreated group was unbalanced in terms of sex composition. Comparison of male and female control subjects showed no significant difference in ATPase activities between the sexes but there was a trend towards high Ca ATPase and Ca + Mg ATPase activity in males and high Na/K ATPase in females (Table 19). None of the ATPase activities were correlated

Table 18. Age and sex composition of the groups of subjects used in the study of human erythrocyte membrane ATPase activities. Ages are given as mean values ± standard deviations with number of subjects in parentheses. Numbers of males and females in each group are given.

Subject group	Age	Sex
Ill untreated bipolar	42 ± 16 (12)	7 male, 5 female
Ill untreated unipolar	50 ± 15 (9)	1 male, 8 female
Lithium-recovered	47 ± 17 (9)	3 male, 6 female
Lithium-failed	54 ± 11 (5)	3 male, 2 female
Total lithium-treated group	50 ± 16 (14)	6 male, 8 female
Epileptic	18 ± 9 (6)	2 male, 4 female
Controls	41 ± 14 (10)	4 male, 6 female

Table 19. Specific activities of ATPases in erythrocyte membranes prepared from male and female control subjects. Results are expressed as nmol P_i/hr/mg protein and grouped according to sex of subject. Values given are means ± standard deviations with number of subjects in parentheses.

	Male subjects	Female subjects
Na/K ATPase activity	286 ± 67 (4)	377 ± 151 (6)
Mg ATPase activity	376 ± 61 (4)	323 ± 156 (6)
Ca + Mg ATPase activity	1104 ± 136 (4)	809 ± 331 (6)
Ca ATPase activity	728 ± 84 (4)	487 ± 264 (6)

with age. It is unlikely therefore that age or sex factors contributed to differences in ATPase activities between the groups. They may however have increased the variance of the parameters studied and so where possible groups have been compared on an age and sex matched basis.

3. ATPase activities in erythrocyte membranes from ill, untreated depressive and untreated epileptic patients.

Results given in Table 20 are a comparison of the ATPase activities in membranes prepared from a group of controls and activities in groups of ill, untreated, depressive patients. All patients were depressed at the date of sampling. Regardless of diagnosis the ill, untreated patients showed a reduced Na/K ATPase activity compared to controls ($p<0.05$). Comparison of controls with an age and sex matched group of these patients (Table 21.) also showed a reduced Na/K ATPase activity in the ill, depressed patient group ($p<0.025$).

On dividing the group of patients into bipolar and unipolar groups (Table 20.) the unipolar group shows a marked reduction in Na/K ATPase compared to controls ($p<0.005$) whilst the bipolar group showed only a slight reduction in Na/K ATPase activity with respect to controls. Mg dependant, Ca + Mg dependant and Ca stimulated ATPase activities were not significantly altered in ill patients as compared to controls.

A small group of untreated epileptics were also studied and these showed a Na/K ATPase activity very similar

Table 20. Specific activities of ATPases in erythrocyte membranes prepared from blood of ill, untreated depressive patients and from control subjects. Results are shown for all ill untreated patients regardless of diagnosis and the same patients divided into unipolar and bipolar depressive groups according to diagnosis. Specific activities are expressed as nmol P_i/hr/mg protein. Values given are means ± standard deviations with number of subjects in parentheses. Groups were compared using a students 't' test, *p 0.05, **p 0.005 compared to controls.

	controls	ill untreated depressive patients (total group)	Unipolar patients	Bipolar patients
Na/K ATPase activity	342 ± 132 (10)	217 ± 137* (21)	156 ± 94** (9)	263 ± 145 (12)
Mg ATPase activity	347 ± 130 (10)	279 ± 107 (21)	256 ± 135 (9)	294 ± 80 (12)
Ca + Mg ATPase activity	940 ± 314 (10)	894 ± 280 (19)	819 ± 303 (7)	937 ± 255 (12)
Ca ATPase activity	591 ± 248 (10)	618 ± 211 (19)	576 ± 203 (7)	643 ± 211 (12)

Table 21. Specific activities of ATPases in erythrocyte membranes prepared from blood of a group of ill, untreated depressive patients and from a group of age and sex matched control subjects. Activities are expressed as nmol P_i/hr/mg protein. Individual values are shown for each matched pair and in addition means ± standard deviations are shown. Groups were compared using a paired 't' test, *p 0.025.

Na/K ATPase activity		Mg ATPase activity		Ca + Mg ATPase activity		Ca ATPase activity	
controls	depressive patients	controls	depressive patients	controls	depressive patients	controls	depressive patients
201	106	420	322	1114	1260	677	938
388	308	284	428	926	1204	642	776
256	195	615	142	961	469	346	327
248	319	248	384	358	918	110	780
294	180	183	427	551	900	368	473
315	70	185	243	696	698	511	455
484	103	269	270	893	793	623	523
667	133	435	266	1395	903	960	637
290	402	394	305	1197	1099	802	794
268	172	439	338	1305	771	866	433
341 ± 132	199 ± 104*	347 ± 130	313 ± 84	940 ± 314	902 ± 227	591 ± 248	614 ± 190

to that of controls (Table 22.).

4. The effects of long-term lithium administration on ATPase activities in erythrocyte membranes from depressive patients.

The first part of this study consisted of a comparison between a group of untreated, depressed patients and a group of patients (including some ill and some recovered) who had received lithium treatment for at least one year. The two groups were age and sex matched. The results (Table 23.) show both an increased Na/K ATPase activity and an increased Mg dependant ATPase activity in the lithium-treated group ($p<0.025$, $p<0.05$ respectively). There was no difference in the Ca + Mg ATPase or Ca ATPase activities between the two groups.

Subsequent to this initial study further patients were investigated and the results of the total study are shown in Table 24. The groups were not age and sex matched but the composition in terms of age and sex was not significantly different. Comparison of untreated patients with the total group of all lithium treated patients showed a barely significant ($p<0.1$) increase in Mg^{++} ATPase activity. Such an increase was also seen in the lithium-failed group. The Mg ATPase specific activity of the lithium-recovered group was greater than that of the untreated group but the difference was not statistically significant. There was also a decreased Ca ATPase activity in the lithium recovered and total lithium

Table 22. Na/K ATPase specific activity in erythrocyte membranes prepared from blood of a group of untreated epileptic patients and from a group of control subjects. Results are expressed as nmol P_i/hr/mg protein. Values given are means ± standard deviations with the number of patients in parentheses.

	Na/K ATPase activity
controls	342 ± 132 (10)
epileptic patients	309 ± 137 (6)

Table 23. Specific activities of ATPases in erythrocyte membranes prepared from blood of a group of ill, untreated depressive patients and an age and sex matched group of depressive patients who had received lithium treatment for at least one year. Activities are expressed as nmol P_i/hr/mg protein. Individual values are given for each matched pair together with means ± standard deviations. Groups were compared using a paired 't' test, *p 0.05, **p 0.025.

Na/K ATPase activity		Mg ATPase activity		Ca + Mg ATPase activity		Ca ATPase activity	
untreated patients	lithium-treated patients	untreated patients	lithium-treated patients	untreated patients	lithium-treated patients	untreated patients	lithium-treated patients
172	162	338	450	771	724	433	274
150	300	177	533	916	1100	739	567
86	240	212	473	504	1030	292	557
106	73	322	259	618	900	296	641
133	181	266	330	1260	690	994	360
88	187	113	143	903	1180	790	1037
195	267	332	400	899	945	567	545
70	195	142	117	576	625	434	478
180	265	243	352	469	464	226	112
131 ± 43	208 ± 65**	238 ± 79	343 ± 130*	768 ± 240	851 ± 225	530 ± 247	507 ± 244

Table 24. Specific activities of ATPases in erythrocyte membranes prepared from blood of a group of ill, untreated depressive patients and a group of patients who had received lithium treatment for at least 1 year. The lithium-treated group was also divided into those patients who had recovered and those who were ill (lithium-failed). Values given are means ± standard deviations with the number of subjects in parentheses. Groups were compared using a Students 't' test, *p 0.1, ** p 0.05 compared to the untreated group.

	untreated patients	lithium-treated patients (total group)	lithium-recovered patients	lithium-failed patients
Na/K ATPase activity	217 ± 137 (21)	223 ± 91 (14)	248 ± 95 (9)	179 ± 65 (5)
Mg ATPase activity	279 ± 107 (21)	350 ± 113* (14)	331 ± 130 (9)	384 ± 66* (5)
Ca + Mg ATPase activity	894 ± 280 (19)	841 ± 281 (14)	768 ± 298 (9)	975 ± 183 (5)
Ca ATPase activity	618 ± 211 (19)	490 ± 190* (14)	437 ± 189** (9)	585 ± 151 (5)

groups ($p<0.05$, $p<0.1$ respectively) but no change in the Ca + Mg ATPase activity. None of the groups showed significant differences in Na/K ATPase activity when compared to controls. However there were trends towards an increased Na/K ATPase activity in the lithium-recovered group and a trend towards a decreased activity in the lithium-failed group. The lithium failed group showed a higher Mg ATPase and Ca + Mg ATPase activity than the recovered group but these differences were not statistically significant.

A study was also carried out in which samples were taken from certain of the patients while ill and untreated and from the same patients after they had received lithium treatment for between 9 months and 1 year. All these patients except one had recovered and had had no episode of illness since starting lithium. The results from this experiment are shown in Table 25. and they show an increased activity of all the ATPase activities measured after lithium treatment.

5. The effects of short-term lithium treatment on ATPase activities in erythrocyte membranes from depressive patients.

ATPase activities were measured in membranes prepared from blood of a group of the depressed patients while ill and untreated and again after 2 - 4 weeks of receiving lithium therapy. The results are shown in Table 26. They show an increased Mg ATPase specific activity ($p<0.05$)

Table 25. Specific activities of ATPases in erythrocyte membranes prepared from blood of a group of depressive patients while ill and untreated and after 9 - 12 months lithium treatment. Activities are expressed as nmol P_i/hr/mg protein. Individual values, before and after lithium treatment, are given together with means ± standard deviations. Groups were compared using a paired 't' test, *p 0.1, **p 0.005.

Na/K ATPase activity		Mg ATPase activity		Ca + Mg ATPase activity		Ca ATPase activity	
untreated	lithium-treated	untreated	lithium-treated	untreated	lithium-treated	untreated	lithium-treated
106	225	322	925	938	1490	616	565
308	362	428	591	1204	1642	776	1051
195	230	142	370	369	1220	227	850
312	380	332	361	584	907	252	546
180	179	427	326	900	984	473	658
86	213	212	190	620	743	408	553
216	334	410	461	1105	1391	695	930
88	172	113	275	576	939	463	664
172	363	338	455	771	1400	433	945
185 ± 80	273 ± 80**	303 ± 113	439 ± 203*	785 ± 258	1191 ± 292**	483 ± 175	751 ± 183**

Table 26. Specific activities of ATPase in erythrocyte membranes prepared from blood of a group of depressive patients while ill and untreated and again after 2 - 4 weeks lithium treatment. Two patients (marker ECT in table below) also received electroconvulsive shock treatment. Activities are expressed as nmol P_i/hr/mg protein. Individual values, before and after treatment, are given together with means ± standard deviations. Groups were compared using a paired 't' test, *p 0.05.

	Na/K ATPase activity		Mg ATPase activity		Ca + Mg ATPase activity		Ca ATPase activity	
	untreated	lithium-treated	untreated	lithium-treated	untreated	lithium-treated	untreated	lithium-treated
ECT	97	247	101	367	-	-	-	-
	301	242	342	379	1193	805	851	426
	290	251	290	407	1135	980	845	573
	487	442	243	425	698	983	455	558
ECT	570	436	182	178	794	566	612	338
	161	295	373	425	1025	1275	652	850
	318 ± 167	319 ± 86	255 ± 93	364 ± 86*	969 ± 192	888 ± 226	683 ± 150	559 ± 162

after lithium treatment but there was no difference in the activities of the other ATPases. Two of the patients studied had also received electroconvulsive shock treatment during the period between sampling.

6. Estimation of lithium in erythrocyte membranes.

It was important to investigate the possibility that the observed differences in ATPase activities in membranes from people receiving lithium were due to activation of the enzymes by lithium present in the membranes.

Membranes from six lithium-treated patients were analysed for lithium by both flame and flameless atomic absorption spectrophotometry. No lithium could be detected by either method despite a detection limit of 1 nmol.

Therefore it is possible to state with some confidence that there is no lithium present in the membrane preparations from lithium-treated patients.

7. The effects of long-term lithium treatment on plasma and erythrocyte ion concentrations.

Plasma and erythrocyte ion concentrations were measured in a group of ill, untreated patients and a group of patients who had received lithium treatment for at least 1 year. The results are shown in Table 27. The only significant difference between the two groups is the increased intracellular sodium in erythrocytes from lithium-treated patients ($p<0.05$).

Table 27. Plasma and erythrocyte ion concentrations for a group of ill, untreated depressive patients and a group of patients who had received lithium treatment for at least one year. Plasma ion concentrations are expressed as mM, erythrocyte ion concentrations as mmol/kg wet cells. Values given are means ± standard deviations, with the number of subjects in parentheses. Groups were compared using a Students 't' test, *p 0.05.

	untreated patients	lithium-treated patients
Plasma magnesium concentration	0.81 ± 0.11 (13)	0.82 ± 0.07 (13)
Plasma sodium concentration	134.2 ± 10.4 (13)	137.5 ± 9.5 (13)
Plasma potassium concentration	4.36 ± 0.73 (13)	4.36 ± 0.46 (13)
Plasma lithium concentration	-	0.83 ± 0.18 (13)
Erythrocyte magnesium concentration	2.11 ± 0.34 (13)	2.17 ± 0.23 (12)
Erythrocyte sodium concentration	7.64 ± 1.66 * (13)	8.80 ± 0.86* (12)
Erythrocyte potassium concentration	93.8 ± 6.5 (13)	92.1 ± 9.0 (12)
Erythrocyte lithium concentration	-	0.39 ± 0.13 (12)

8. The effects of chronic lithium administration to rats on erythrocyte membrane ATPase activities and ion concentrations in erythrocytes and plasma.

ATPases were measured in erythrocyte membranes prepared from control rats and from rats given lithium chloride in their diet for 3 weeks (60 mmol LiCl/kg food). Plasma lithium concentrations at the time of blood sampling were between 0.48 and 0.63 mM. No effect of lithium on the ATPases was evident (Table 28.).

In a separate experiment administration of lithium chloride in the diet (100 mmol LiCl/kg food) caused a reduction in intracellular erythrocyte magnesium concentration ($p<0.01$). The lithium-treated group also showed an increase in plasma magnesium but this effect was not statistically significant. There was no effect of lithium on plasma or erythrocyte sodium concentration. These results are shown in Table 29.

DISCUSSION.

The specific activities of the ATPases in membranes prepared from human erythrocytes were of similar order to those described by Dick et al., (1974) and Feig and Guidotti (1974). In view of the studies of Hanahan et al. (1973) and Bramley et al. (1971) the preparative procedure used in the present experiments (20 mosm haemolysing buffer, pH 7.4) would be expected to produce membranes exhibiting their total ATPase capacity with no cryptic activity.

Table 28. The effect of chronic lithium administration on rat erythrocyte membrane ATPase specific activities. Rats were given lithium chloride in the diet (60 mmol LiCl/kg food) for 3 weeks. Activities are expressed as nmol P_i/hr/mg protein. Values given are means ± standard deviations, number of animals in parentheses.

	controls	lithium-treated
Na/K ATPase activity	371 ± 163 (4)	408 ± 152 (4)
Mg ATPase activity	497 ± 82 (4)	533 ± 131 (4)
Ca + Mg ATPase activity	1009 ± 143 (4)	992 ± 75 (4)
Ca ATPase activity	512 ± 199 (4)	460 ± 90 (4)

Table 29. The effect of chronic lithium administration on rat erythrocyte and plasma ion concentrations. Rats were given lithium chloride in the diet (100 mmol LiCl/kg food) for 1, 2 and 3 weeks. Plasma ion concentrations are expressed as mM, erythrocyte ion concentrations as mmol/kg wet cells. Values given are means ± standard deviations with the number of animals in parentheses. Groups were compared using a Students 't' test, *p 0.01 compared to contols.

	controls	lithium-treated		
Length of lithium treatment	-	1 week	2 weeks	3 weeks
Plasma lithium concentration	-	0.83 ± 0.19 (6)	0.79 ± 0.25 (6)	1.13 ± 0.22 (6)
Erythrocyte lithium concentration	-	1.00 ± 0.09 (5)	1.20 ± 0.16 (5)	1.29 ± 0.21 (6)
Plasma sodium concentration	141.2 ± 4.4 (17)	138 ± 4.4 (6)	144.5 ± 4.5 (6)	142.8 ± 3.3 (6)
Erythrocyte sodium concentration	2.33 ± 0.37 (17)	2.12 ± 0.18 (5)	2.52 ± 0.23 (6)	2.28 ± 0.28 (6)
Plasma magnesium concentration	0.91 ± 0.11 (17)	1.01 ± 0.09 (6)	1.02 ± 0.17 (6)	0.99 ± 0.12 (6)
Erythrocyte magnesium concentration	1.64 ± 0.17 (17)	1.91 ± 0.12* (5)	1.62 ± 0.17 (6)	1.91 ± 0.15* (6)

There was considerable variation in ATPase activities in erythrocyte membranes prepared from a single control subject on successive occasions. Such variation was found in all three ATPase activities studied and in all three individual subjects. There was no consistent pattern of variation either between the various enzymes or between the three individuals and this suggests that the variation in activity was not due to any consistent methodological error. A standard ATPase was assayed together with the membrane preparations and this showed little variation in enzyme activity so providing evidence that the variation in membrane ATPase activities was not due to methodological error in the ATPase assay. The variation in activity must therefore have been due either to *in vivo* variation, to variation in the sample of blood cells taken or to variation produced during preparation of the membranes.

Sampling variation could have occured if successive samples contained different populations of erythrocytes in respect to cell age. Hanahan (1973) has pointed out that the age distribution of erythrocytes could be a cause of variation in membrane preparations. Cohen *et al*. (Cohen, Ekholm, Luthra and Hanahan, 1975) were unable to show any consistent relationship between erythrocyte cell age as judged by cell density and ATPase activities in freeze-thawed cells. Other workers have shown Ca ATPase activity in membrane preparations to depend on cell age and in addition Mg ATPase was found to be increased in

people with high (10 - 30%) reticulocyte counts (Feig and Guidotti, 1974). However the number of cells present in each blood sample is so large that it is unlikely that weekly samples contained different populations of cells.

Variation in specific enzyme activity could have arisen from variation in the enzyme, protein or haemoglobin content of the membranes. Hanahan et al. (1972) showed that under preparative conditions similar to those used in these experiments, Mg ATPase and Ca ATPase activity was lost during preparation but there was no loss of Na/K ATPase activity. Variation in such loss of activity could lead to variation in enzyme activity in the final preparation. On a given day membrane preparations from a single haemolysate were reproducible in terms of protein yield and ATPase activities. Thus the pelleting of the membranes would seem to be reproducible during a given preparation. In addition, when the yield of membranes was drastically increased by using an angle rotor during preparation the specific activities of the ATPases were unchanged. There would therefore not seem to be any heterogeneity in the membrane suspensions during preparation. When ATPase activities in membranes from three individuals were studied over several weeks there was a marked correlation between the ATPase activities expressed per litre of original cells and ATPase activities expressed per milligram of protein. This correlation between activity referred to the original cellular material and activity

referred to the final preparation indicates that the variation in ATPase activities within the individuals was not due either to variation in the quantitative yield of membranes in terms of protein or to variation in the yield of enzyme in the membranes. This is supported by the lack of consistent pattern between either protein yield or membrane haemoglobin content with ATPase activities in successive preparations. The variation in ATPase specific activity cannot be explained therefore by variation in haemoglobin or total protein content of the membranes, variation in the recovery of enzyme activity in the membranes or by heterogeneity of the membrane suspensions together with variation in the efficiency of centrifugation. The quantitative aspects of the preparation are reproducible. Variation could have occured in the qualitative nature of the preparation - for example in the degree of crypticity of the enzymes involved. Enzyme crypticity, however, is an unlikely explanation in view of the lack of pattern between variation in the three ATPases studied.

The only step in the preparation which varied from week to week was the haemolysis stage. Variation in cell to haemolysing buffer ratio was a possible cause of alterations in the membrane properties which might be reflected in altered ATPase activities. It would be interesting to study membranes prepared from a single blood sample using cell suspensions of different haematocrits. Such an

experiment would be informative on whether variation in cell suspension haematocrit could have been a possible source of the observed variation in membrane ATPase activities. However, such a source of variation is unlikely since there was no consistent pattern between the haemolysis conditions as judged by the haematocrit of the cell suspension to be lysed and the final membrane ATPase activities.

In conclusion, no methodological or preparative rationale can be given to explain the variation in ATPase activities found within individuals. It is also unlikely that the variation was due to differences in the populations of erythrocytes samples. Therefore one must consider the possibility that the variation reflects alterations of activity, in vivo.

The mature human erythrocyte synthesises no protein (Harris and Kellermeyer, 1970) and so a changed amount of enzyme measured in a red cell population can only occur in association with new cells. As the life of the mature human erythrocyte is approximately 120 days (Harris and Kellermeyer, 1970) the changes in ATPase activities found over periods of two or three weeks could not have been due to different amounts of enzyme present in cells sampled. Thus the changes in activity must have been due to alterations in the activation and inhibition of enzyme in vivo. It would seem unlikely that the membrane preparation, after extensive washing, could reflect in vivo activation.

However, the results of experiments in which patients were studied after only 2-4 weeks of lithium treatment showed an increased erythrocyte membrane Mg ATPase activity after this period. Such an effect could not have been due to new enzyme and it further suggests that erythrocyte membrane ATPase activities may reflect *in vivo* activation of these enzymes.

Measurement of ATPase activities in erythrocyte membranes from depressed patients showed no difference in Mg ATPase and Ca ATPase specific activities compared to a group of controls. However groups of depressive patients of mixed diagnosis (both unipolar and bipolar) showed a significantly decreased Na/K ATPase specific activity compared to controls, both matched and unmatched with regard to age and sex. Separation of patients into unipolar and bipolar groups showed a decreased specific activity of Na/K ATPase in the unipolar group compared to controls but only a slight, non-significant difference between the bipolar group and controls. This is in contrast to the work of Hokin-Neaverson *et al.* (1974) who found decreased erythrocyte sodium efflux in bipolar, manic patients. The reason for the difference between unipolar and bipolar patients is unclear but it may be related to severity of symptoms. However, this remains to be investigated.

Investigation of a small group of untreated epileptics showed no difference in Na/K ATPase specific activity

compared to controls. These patients were outpatients and so do not act as hospitalisation controls. However the results suggest that the changes in Na/K ATPase activity seen in depressives is not a general effect associated with central nervous system dysfunction.

The possibility that the decreased Na/K ATPase specific activity found in depressives was due to some effect of hospitalisation cannot be unequivocally ruled out. However Naylor et al. (1973) showed that in hospitalised depressive patients amelioration of mood was associated with increased erythrocyte membrane Na/K ATPase activity. This showed that recovery from depression was associated with increased Na/K ATPase activity despite the patients being hospitalised both when ill and recovered so suggesting a relationship between enzyme activity and clinical state. Hokin-Neaverson et al. (1974) found a group of bipolar patients, including both inpatients and outpatients to have reduced sodium efflux. These studies make it unlikely that the observed decreased specific activity of erythrocyte membrane Na/K ATPase in depressed patients was due to hospitalisation.

It cannot be decided from the present experiments whether the decreased specific activity of Na/K ATPase is due to a reduced amount of enzyme, to changes in activation or to inhibition of the enzyme. The results of Naylor et al. (1973) showed a change in erythrocyte membrane Na/K ATPase over a 42 day period and this period is long

enough for the change to be accounted for in terms of new cells. Results discussed earlier, however, suggest erythrocyte membrane ATPase activities may reflect *in vivo* activation and inhibition.

A further question posed by the present work is whether the reduced specific activity of Na/K ATPase in depressive patients is the result of metabolic disturbances due to depression or an event in the aetiology of depression itself. The present results do not allow this question to be answered.

Comparison of untreated depressive patients with patients who had received lithium treatment for at least one year showed the lithium-treated group had a significantly higher erythrocyte membrane Na/K ATPase specific activity. Similarly, patients studied both untreated and after 9 - 12 months lithium treatment showed increased Na/K ATPase specific activity after lithium treatment. No lithium could be detected in membranes prepared from erythrocytes of lithium-treated patients and therefore it is unlikely that the increased ATPase activity was due to *in vitro* activation by lithium present in the membrane preparations. The increased Na/K ATPase specific activity in erythrocyte membranes following lithium treatment confirms the findings of Dick *et al*. (1974). These workers were unable to determine whether this effect was due to the drug itself or due to a change in clinical state of the patient. In the present work it was possible to

examine this question. Splitting the group of lithium-treated patients into those who had recovered and were well and those who were ill despite receiving lithium showed a trend towards increased membrane Na/K ATPase in the recovered group compared to untreated patients whereas the lithium-failed group showed a trend towards decreased membrane Na/K ATPase activity. These results suggest therefore that lithium treatment only causes an increase in Na/K ATPase activity when it is associated with recovery of the patients. This is supported by the finding that amelioration in mood is correlated with increased Na/K ATPase activity (Naylor et al., 1973). Lithium itself would seem therefore not to cause any change in erythrocyte membrane Na/K ATPase specific activity.

Lithium-treated patients showed an increased intracellular erythrocyte sodium concentration compared to untreated patients. The majority of these patients had recovered so it was not possible to separate drug and recovery effects in this case. Erythrocyte sodium concentration has been shown to decrease on recovery from depressive illness (Naylor et al., 1971; Naylor et al., 1973). Recovery from depression during lithium treatment has been found to be associated with an increase in erythrocyte sodium concentration (Mendels et al., 1971; Mendels and Frazer, 1974) although this increase has not been confirmed by a third study (Mendels et al., 1972). It may be that the increase in sodium concentration found

during recovery on lithium is an effect of lithium rather than an effect due to change in clinical state. It is anomalous that in the present work the increased erythrocyte sodium concentration was associated with increased Na/K ATPase specific activity in the erythrocyte membrane. Previously Naylor et al. (1973) showed increased Na/K ATPase activity to be associated with decreased erythrocyte sodium concentration. It is possible the anomaly in the present work was caused by patients receiving lithium treatment. Perhaps lithium prevented an increased activity in the sodium pump being reflected in a decreased intracellular sodium concentration. It is possible lithium increased the intracellular sodium concentration by competing with sodium for the extrusion mechanism so inhibiting sodium efflux (see Section II of this thesis).

In view of the association of Na/K ATPase activity with the sodium pump (Whittam and Wheeler, 1970) the changes in Na/K ATPase activity observed in depression would be expected to be reflected by changes in the active transport of sodium and potassium. The present results provide evidence therefore that there is decreased active sodium efflux in depressive patients. Earlier workers showed decreased sodium efflux from erythrocytes of bipolar, manic patients compared to controls (Hokin-Neaverson et al., 1974). It is probable therefore that both depressive and manic phases of affective disorders are associated with decreased sodium transport.

Both short-term (2 - 4 weeks) and long-term (9 - 12 months) lithium treatment caused an increased specific activity of erythrocyte membrane Mg ATPase. This effect is seen with short-term treatment where recovery was at the most only partial and with long-term treatment regardless of clinical state and this suggests the increased activity is due to an action of lithium and not due to a change in clinical state of the patients.

It has been reported that people with high reticulocyte counts (10 - 30%) have an increased specific activity of erythrocyte membrane Mg ATPase compared to control subjects (Feig and Guidotti, 1974). It was possible therefore that the effect of lithium treatment observed in the present work was due to an effect on erythrocyte production. This is unlikely however since lithium treated patients have reticulocyte counts within the normal range (Bille, Jensen, Jenson and Poulsen, 1975).

The increase in erythrocyte membrane Mg ATPase activity observed after only 2 - 4 weeks of lithium treatment suggests that the effect of lithium on specific enzyme activity is not dependant on the production of new cells and thus is not dependant on synthesis of more enzyme. The effect of lithium is most likely due to activation by some unknown factor. Examination of erythrocyte membranes from lithium-treated patients failed to detect any lithium in the preparations. Lingsch and Martin (1976) failed to detect lithium in the plasma membranes of

erythrocytes from lithium-treated patients but the sensitivity of their technique was such that it was equivocable whether there was any lithium present. The high sensitivity of the present analytical method used to detect lithium allows the unequivocable statement that there was no significant amount of lithium present in the erythrocyte membrane preparations. Therefore increased ATPase activity in erythrocyte membranes from lithium-treated patients was not due to lithium present in the membranes.

Other workers have shown that erythrocytes from lithium-treated patients possess decreased choline transport compared to control subjects and that this decreased transport is evident both when the cells have been extensively washed and in ghosts when both intracellular and extracellular lithium has been removed (Lee, Lingsch, Lyle and Martin, 1974; Lingsch and Martin, 1976). Results from a patient who had stopped receiving lithium showed a slow increase in erythrocyte choline transport after termination of lithium treatment, consistent with an increase associated with new erythrocytes. This was interpreted as lithium affecting choline transport via a mechanism which could operate only in the developing erythrocyte and not by activation such as suggested to explain its effect on Mg ATPase specific activity.

The physiological significance of the increased Mg ATPase activity as a result of lithium treatment is uncertain as the function of this enzyme in the erythrocyte is

unknown. One might speculate however that the enzyme affected is associated with actomyosin-like protein in the membranes.

Lithium treatment had no consistent effect on Ca ATPase activity. This activity is thought to reflect, in part at least, the active calcium transport system and the present results suggest therefore that chronic administration of lithium does not affect the active transport of calcium. This is of some interest since calcium is of such importance to many aspects of cell function (Cuthbert, 1970).

There have been several conflicting reports concerning the effects of lithium administration to humans on plasma magnesium concentrations. Lithium has been reported to increase (Nielsen, 1964a; Bunney, Goodwin, Davis and Fawcett, 1968; Aronoff, Evans and Durell, 1971) decrease (Frizel, Coppen and Marks, 1968) and have no effect upon (Dunner, Meltzer, Schriener and Feigelson, 1975) plasma magnesium. The present results show no effect of lithium on plasma magnesium. Length of time receiving lithium or diagnosis cannot explain these discrepencies when all these studies are compared. Dunner et al. (1975) claimed that previous workers' results showing increased plasma magnesium were due to the short length of time of lithium administration but careful consideration of the literature does not support this view.

In agreement with previous workers (Nielsen, 1964a;

Dunner et al., 1975) the present results show no effect of lithium treatment on human erythrocyte magnesium concentrations. Administration of lithium to rats caused a trend towards reduced erythrocyte magnesium concentrations in support of the findings of Haavaldsen and Ingvaldsen (1973). Lithium also raised plasma magnesium in rats as previously shown by other workers (Nielsen, 1964b; Andreoli, Villani and Brambilla, 1972; Birch and Jenner, 1973; Haavaldsen and Ingvaldsen, 1973). As discussed by Hullin (1975) the mechanism involved in lithium's action on magnesium metabolism is unknown. However the evidence for decreased erythrocyte magnesium and increased plasma magnesium suggests the possibility of ionic displacement.

The lack of effect of lithium treatment on human erythrocyte and plasma magnesium concentrations compared to its effect of increasing erythrocyte membrane Mg ATPase activity suggests there is no relationship between magnesium concentrations and Mg ATPase activity. This is supported by the results from the animal work in which 3 week treatment with lithium affected magnesium distribution but not (although, this was using a lower dose) the Mg ATPase. It has been suggested that lithium might exert its action on magnesium dependant enzymes through its effects on magnesium distribution (Glen, 1976). This does not seem to be the case for the Mg ATPase of the erythrocyte membrane. The mechanism by which this enzyme is

affected by lithium remains to be investigated. Likewise the reason for the lack of effect of lithium on rat erythrocyte membrane Mg ATPase also remains to be investigated. The difference in lithium distribution between plasma and erythrocyte in the two species could perhaps explain the difference in the effects of lithium. Alternatively, the difference could be in the nature of the erythrocyte membrane preparations.

The experiments described in this section have led to the following conclusions:

1. There is considerable variation in erythrocyte membrane ATPase activities when a single person is sampled over successive weeks. No methodological rationale was found to explain this, so suggesting the possibility that the enzyme activities reflected <u>in vivo</u> variation.
2. Depressive patients had a lower erythrocyte membrane Na/K ATPase specific activity compared to controls. This was particularly evident in unipolar patients.
3. Lithium-treated patients possessed an increased erythrocyte membrane Na/K ATPase activity compared to ill, untreated patients. This however was probably due to change in illness state and not to lithium itself.
4. Lithium-treated patients showed an increased erythrocyte sodium concentration compared to untreated patients.
5. Lithium itself caused an increase in erythrocyte membrane Mg ATPase activity and this was not dependant on the production of new erythrocytes. The change did not

seem related to lithium effects on magnesium distribution.

6. Lithium administration for 3 weeks had no effect on rat erythrocyte membrane ATPases.

7. Lithium increased rat plasma magnesium concentration and tended to decrease rat erythrocyte magnesium concentration but had no effect on magnesium distribution in humans.

SECTION VI

DISCUSSION.

Results presented in section V showed erythrocyte membrane Na/K ATPase activity to be reduced in depressive patients and this was particularly evident in unipolar patients. Na/K ATPase activity was not significantly reduced in a group of bipolar patients. It was argued that these effects were unlikely to be due to an effect of hospitalisation and it was concluded that depression was associated with a reduction in Na/K ATPase activity. A reduced Na/K ATPase activity in depression has been postulated previously (Naylor et al., 1973) but the present results provide the first direct evidence for such a hypothesis. In view of the association between active sodium transport and Na/K ATPase activity (Whittam and Wheeler, 1970), a reduction in Na/K ATPase activity would be expected to result in a reduction in active sodium transport. This would possibly give rise to further changes in sodium metabolism. If the observed reduction in Na/K ATPase activity in erythrocytes occured in all cells of the body during depression such a change in enzyme function could explain the previously reported changes in sodium metabolism including reduced sodium transport out of saliva, reduced sodium transport into lumbar cerebrospinal fluid and increased residual ~~non-exchangeable~~ sodium (Coppen, 1967; Glen et al., 1968; Carroll, 1972). It is possible that the previously observed abnormalities in sodium metabolism during depression are all the result of a single cause, namely a reduction in Na/K ATPase activity

associated with the sodium-pump. Mendels and colleagues (Mendels and Frazer, 1974; Schless et al., 1975) have suggested there is a defect in erythrocyte membrane cation transport in depression such that it causes an increase in the intracellular erythrocyte lithium concentration. Lithium transport in the erythrocyte is not thought to involve the sodium-pump (Maizels, 1968; Mendels and Frazer, 1974; Haas, Schooler and Tosteson, 1975; Duhm, Eisenried, Becker and Greil, 1976) and therefore changes in sodium-pump activity are unlikely to directly affect the intracellular lithium concentration. However there is evidence that the erythrocyte intracellular sodium concentration influences the intracellular concentration of lithium. Duhm et al. (1976) found the ratio of intracellular to extracellular lithium concentrations to be proportional to the ratio of sodium concentrations. In addition, the ratio of intracellular to extracellular lithium concentrations has been shown to be related to the intracellular sodium concentration (Mendels and Frazer, 1974). Thus it is possible that an increase in erythrocyte intracellular sodium concentration could lead to an increase in erythrocyte intracellular lithium concentration. Thus if a reduction in Na/K ATPase activity was associated with a reduction in active sodium efflux and an increase in intracellular sodium concentration, a reduction in Na/K ATPase activity in depression could explain the increased intracellular lithium concentration reported in depressive patients (Mendels and Frazer,

1973, 1974; Lyttkens et al., 1973) and therefore the erythrocyte membrane cation transport defect postulated by Mendels and Frazer (1974) could be a reduction in Na/K ATPase activity. As yet however there is no consistent evidence for an increased intracellular sodium concentration in depression. In summary, it is possible to embrace the majority of reported changes in sodium and lithium metabolism in depression in one hypothesis by postulating a reduction in membrane Na/K ATPase activity in depressive patients.

It cannot be determined from the present results whether the reduction in Na/K ATPase activity was due to a reduction in the amount of catalytic protein or a change in the state of activation of the enzyme. Enzyme activity was measured in an extensively washed preparation and this would suggest activation effects to be an unlikely explanation. However for reasons discussed in section V the possibility of the Na/K ATPase activity reflecting changes in activation of the enzyme must be considered. Dick et al. (1974) have postulated a correlation between mood and erythrocyte membrane Na/K ATPase activity in a single subject over a period of weeks. In view of the lifespan of the human erythrocyte (Harris and Kellermeyer, 1970) such an effect would most probably reflect changes in activation of the enzyme. If the results do reflect changes in activation of the enzyme, the nature of the activators and inhibitors involved remains obscure. 3-5-cyclic adenosine-

monophosphate, thyroid hormones and adrenocorticosteroids have all been shown to have effects on Na/K ATPase activity (Edelman, 1973; Schwartz, Lindenmeyer and Allen, 1975) and various endocrine changes have been reported in depression (Coppen, 1967). However a link between changes in ATPase activity in depression and hormonal influences is purely speculative.

The finding that there is reduced Na/K ATPase activity in depression is in contradiction to the results of Naylor and colleagues who failed to show any difference in erythrocyte active sodium transport between depressed patients and controls (Naylor et al., 1970b). However these authors only studied four control subjects and so they may have obtained a false impression of the control population. Other workers found erythrocyte active sodium transport to be reduced in a group of manic-depressive patients during the manic phase of illness and on the basis of these results suggested there to be a genetic abnormality in active sodium transport in manic-depressive illness (Hokin-Neaverson et al., 1974). The present work was unable to support such a hypothesis since bipolar (manic-depressive) patients did not show a significant reduction in erythrocyte membrane Na/K ATPase activity compared to controls. It would be useful to study more bipolar patients and in particular patients in the manic phase of illness in order to see if there is a difference between unipolar and bipolar patients.

Several workers have suggested there to be a genetic abnormality in cation t ansport in depression (Hokin-Neaverson et al., 1974; Mendels and Frazer, 1974) and others suggested an abnormality in sodium transport to be important in the aetiology of depression (Coppen, 1967; Baer et al., 1970c; Singh, 1970). The present work suggests that a reduced Na/K ATPase activity could be the abnormality involved but there is no evidence concerning whether the change in activity is genetically derived and whether the change is an event in the aetiology of depression of the result of metabolic and hormonal disturbances occuring in depression. It remains to be seen whether a reduction in Na/K ATPase activity and active sodium transport is an important event in depression. Despite its importance being equivocable, the reduction in Na/K ATPase activity in depression suggests this enzyme as a possible site of action of lithium salts.

Lithium has been reported to affect a large number of biochemical processes and it is difficult to decide which, if any, are of relevance to its therapeutic action. One important criterion is the concentration of lithium employed. When used therapeutically concentrations of 0.6 - 1.6 mM are achieved in plasma (Schou and Baastrup, 1967; Schou, 1976). This plasma concentration is accompanied by concentrations of approximately 0.2 mM in lumbar cerebrospinal fluid (Appendix I). There is thought to be little or no restriction on ion movement between C.S.F. and brain

extracellular fluid (Davson, 1967) and results presented in section II together with findings of Prockop and Marcus (1972) show lithium can diffuse out of the C.S.F.-containing spaces into brain. It is likely therefore that in patients receiving lithium treatment the brain extracellular fluid lithium concentration is similar to that in C.S.F., that is approximately 0.2 mM. In rats the erythrocyte:plasma ratio of lithium concentrations is approximately 1.3 (section V). This value is in good agreement with rat cerebral slices tissue:medium ratio for lithium (1.29 - 1.66, Wraae, Hillman and Round, 1976). This suggests the intracellular:extracellular lithium concentration ratio in the brain is similar to the erythrocyte:plasma ratio. If this is the case, taking the erythrocyte:plasma lithium ratio in the human as 0.5 (section V) and the brain extracellular lithium concentration as 0.2 mM one would predict an intracellular lithium concentration of approximately 0.1 mM in the brain of patients receiving lithium therapy. These calculations suggest that only effects of lithium found with concentrations of 0.1 - 1.0 mM can be of any relevance to the therapeutic actions of the drug. A second criterion, important in chronic treatment experiments, is the state of the animals. If the animals are particularly stressed or they show signs of toxicity then it is possible that changes found during lithium treatment are not relevant to the therapeutic action of lithium but to its toxic effects or to general stress of the animals. Daily intraperitoneal

injection of lithium chloride is both stressful and unpleasant to the animals. In addition it leads to high, toxic concentrations of lithium in the body (Morrison et al., 1971; Schou, 1976). Diet administration, although resulting in reduced gain in body weight, is probably a better method of lithium administration since the lithium concentrations are lower and more stable (Morrison et al., 1971; Schou, 1976) and the animals are less stressed.

Using therapeutically relevant concentrations (0.6 - 1.2 mM), lithium was found to have significant effects on sodium transport from blood to C.S.F. (section II). These results were interpreted as showing a stimulation of the sodium-pump by lithium applied to the potassium sensitive side of the system and an inhibition when applied to the sodium-sensitive side. Previous work has shown lithium to stimulate Na/K ATPase activity and sodium transport when presented to the potassium sensitive side of the system in a variety of tissues but these effects were obtained with high concentrations of lithium and mostly either in the absence of potassium or in the presence of a low (2 mM) potassium concentration (Whittam and Ager, 1964; Glen et al., 1972; Tobin et al., 1974; Ploeger, 1974a; Robinson, 1975). The present results suggest therapeutically relevant concentrations of lithium to have an effect at physiological concentrations of potassium. Lithium might stimulate sodium transport from the potassium sensitive side of the system through an action on the potassium transporting

site (Whittam and Ager, 1964), the external sodium allosteric site (Cavieres and Ellory, 1974) or a combination of both (Robinson, 1975). The present results do not distinguish between such possibilities. However it is possible to glean a little information on the nature of the effect. Since the removal of lithium from ventricular perfusion fluid is probably by diffusion (Prockop and Marcus, 1972; this thesis, section II) it is unlikely that lithium was actively transported across the choroid plexuses in the present experiments. Thus the effect of lithium on stimulating sodium transport across the choroid plexus was not dependant on lithium itself being transported by the sodium-pump. It has been suggested that lithium inhibits the sodium-pump when present intracellularly (Ploeger, 1974b) and the present results support this hypothesis and suggest such an effect could occur at therapeutic concentrations of lithium. Overall the results suggest that both the stimulatory and inhibitory effects of lithium on the sodium-pump could occur when lithium is given clinically. These studies provide experimental evidence to support the basic premise of the hypothesis forwarded by Glen and Reading (1973) to explain the action of lithium: that lithium stimulates the sodium-pump at the potassium sensitive side and inhibits it at the sodium sensitive side. However other premises of their hypothesis, such as the intracellular sodium concentration in depression and mania, have yet to be supported by experimental evidence.

The hypothesis that the orientation of the sodium-pump will determine the effect of lithium upon it and that lithium will stimulate at the potassium sensitive side and inhibit at the sodium sensitive side may explain and be compatible with some of the previous contradictory reports of the effects of lithium on the sodium-pump. Ploeger's finding (1974b) that lithium given during a potassium free pre-incubation period inhibited the sodium-pump may be due to lithium acting at the intracellular site of the pump rather than at the extracellular site as he suggested. There are several reports of lithium inhibiting sodium transport across epithelial tissue (Zerahn, 1955; Biber abd Curran, 1970; Herrera Egea and Herrera, 1971; Leblanc, 1972; Dolman and Edmonds, 1976). The exact mechanism of sodium transport across epithelia is equivocable (Koeford-Johnsen and Ussing, 1958; Finn, 1976) and this makes interpretation of the effect of lithium difficult. In some cases lithium has been shown to inhibit the saturable component of transport across epithelia (Biber and Curran, 1970; Leblanc, 1972) and it is possible this effect reflects an inhibition of the sodium-pump by lithium interacting with the sodium sensitive side of a pump system. Lithium has been reported to reduce the potential difference and short circuit current across the isolated frog skin and also to reduce the ouabain-sensitive oxygen consumption (Candia and Chiarandini, 1973). The authors of this report suggested on the basis of these results that the effects

of lithium on epithelial sodium transport might be due, in part, to an inhibition of the sodium-pump. The picture is complicated since lithium has been shown to have inhibitory effects when presented from both sides of the colonic epithelium (Dolman and Edmonds, 1976). Further clarification of lithium's effect in these systems awaits a clearer understanding of epithelial sodium transport. Low concentrations of lithium presented in vitro to the optimally stimulated Na/K ATPase have been shown to have no effect on activity in some cases (Ploeger, 1974a; this thesis, section III). However these results were obtained using nerve homogenates and synaptic plasma membranes and both such preparations would have lost the orientation of the Na/K ATPase in that lithium would have been able to interact with both sides of the system simultaneously. Opposing stimulation and inhibition may explain the lack of effect actually observed. As discussed in section V an intracellular inhibitory effect of lithium on the sodium-pump can explain the anomaly that lithium-treated patients were found to have an increased erythrocyte sodium concentration and an increased erythrocyte membrane Na/K ATPase activity.

The hypothesis that lithium has opposite effects on the sodium-pump depending on the side of the system with which it interacts suggests that when lithium is given chronically its distribution inside and outside cells will determine the net effect on the sodium-pump. Results

presented in sections III and V examined the effect of chronic lithium administration on erythrocyte membrane and synaptic plasma membrane Na/K ATPase activity. Lithium-treated patients were shown to have an increased Na/K ATPase specific activity compared with untreated patients but as discussed in section V this was probably due to the recovery of the lithium-treated group rather than an effect of lithium itself on the enzyme. The animal experiments investigated the effect of lithium on synaptic plasma membrane Na/K ATPase activity but failed to find an effect. The present results therefore do not provide any evidence for chronic lithium treatment causing any change in Na/K ATPase activity. Although the chronic treatment experiments were unlikely to have shown any direct effects of lithium on ATPase activities, such effects would have been expected to lead to homeostatic changes in activation or amount of enzyme present and such changes would have been detected. Maybe the distribution of lithium during chronic administration is such that there is no net effect on the sodium-pump. Such a suggestion however needs verification by experiment.

The observation that chronic lithium administration to rats did not affect the Na/K ATPase activity in the synaptic plasma membrane is in contrast to the findings of Reading et al. (1975) and in disagreement with the finding of reduced vagal nerve homogenate Na/K ATPase after chronic lithium administration (Ploeger, 1974a). One explanation

of this difference is that in the present work lithium was administered in the diet as opposed to intraperitoneal injection as used by the previous workers mentioned above. Intraperitoneal administration would be expected to lead to peaks of high lithium concentration in plasma and tissues whereas diet administration would be expected to lead to stable lithium concentrations (Morrison et al., 1971; Schou, 1976). It may be that the reduced Na/K ATPase activity reported by the previous workers was due to very high, toxic concentrations of lithium in the body. It is possible that only high, toxic lithium concentrations lead to chronic changes in Na/K ATPase activity. If this is the case then the reduction in Na/K ATPase observed by some workers may be related to toxic effects of lithium rather than its therapeutic action. Alternatively different modes of administration might lead to different pharmacokinetics and distribution of lithium. The results presented in sections III and V contrast with those of Boardman et al. (1975) who found lithium to cause an increase in Na/K ATPase activity and ouabain-binding in HeLa cells. These workers interpreted their results as showing lithium to interact with the mechanism of protein synthesis to increase Na/K ATPase production. However the concentrations of lithium used were higher than those found in the therapeutic situation.

In conclusion, the situation with regards to the implication of the sodium-pump in the therapeutic action of

lithium is confused. The results presented in this thesis, found with lithium concentrations and administration similar to the therapeutic situation, suggest that although lithium acutely affects the sodium-pump lithium given over long periods to patients does not. The results provide no evidence to suggest that chronic lithium administration itself affects Na/K ATPase activity.

In contrast the results provide considerable evidence to suggest that chronic lithium administration to patients changes the specific activity of Mg ATPase enzymes. Both long and short-term lithium administration to patients was found to increase the erythrocyte membrane Mg ATPase specific activity. In addition lithium administration to rats led to an increase in synaptic plasma membrane and mitochondrial Mg ATPase specific activity. These results suggest that changes in Mg ATPase enzymes might occur in the brain of patients receiving lithium. The effects in brain were found using diet administration of lithium and may therefore be of clinical relevance since the lithium concentrations in the tissues are probably of a similar magnitude to those found in the therapeutic situation.

Lithium administration to rats failed to produce any change in erythrocyte membrane Mg ATPase activity. The reason for the species difference between man and the rat in the effect of lithium on Mg ATPase is unknown. One possibility is the difference in erythrocyte:plasma lithium ratio. It is of interest to note that chronic lithium

administration has been shown to inhibit erythrocyte choline transport in man but does not have such an effect in the rat (Lee et al., 1974; Lingsch and Martin, 1976). It would be of interest to know the reasons for the different behaviour of rat and human erythrocytes.

Lithium treatment for 2 - 4 weeks produced an increase in human erythrocyte Mg ATPase activity. In view of the lifespan of the erythrocyte and its inability to synthesise protein for most of its life (Harris and Kellermeyer, 1970) this effect probably reflects in vivo activation of the enzyme. The nature of the activator is unknown.

Lithium in vitro had no effect on synaptic plasma membrane or mitochondrial Mg ATPase (section III). A similar lack of effect of lithium in vitro has been reported for Mg ATPase in other tissues (Gupta and Crollini, 1975). Such results do not support the hypothesis that lithium exerts its effects by means of competition with magnesium for magnesium-dependant enzymes (Birch, 1973, 1974).

The function of the Mg ATPase activity found in erythrocyte and synaptic plasma membranes is unknown and therefore the physiological significance of the changes in these activities following administration of lithium is obscure. However it is possible to speculate: chronic lithium administration to rats for three weeks gave rise to an increase in cerebral cortex synaptic plasma membrane Mg ATPase specific activity (section III) and to changes in dopamine metabolism in the striatum (section IV). One

may postulate that there is significance in the fact that these events occurred after the same regimen of lithium treatment. Evidence has been presented earlier in this thesis that suggests there is an actomyosin-like protein in erythrocyte membranes and in synaptosomal material which possesses Mg ATPase activity. It is possible that lithium treatment increased the activity of such Mg ATPase activities and that this led to an increased release of neurotransmitter. Such a hypothesis is highly speculative. Until the subcellular distribution of actin-like and myosin-like proteins is well established it must remain equivocable whether or not the synaptic membrane fraction studied was associated with actomyosin-like protein Mg ATPase activity. There is little evidence for chronic lithium treatment increasing the release of neurotransmitter. The available evidence concerning amine metabolism following lithium treatment is contradictory (Shaw, 1975) although there is some consistent evidence of increased noradrenaline uptake and increased intraneuronal metabolism. The latter would suggest a possible decrease in release. In vitro, lithium increases the resting release of acetylcholine and inhibits the stimulated release (Vizi, 1975). In order to test the hypothesis that lithium treatment increases release of neurotransmitter through an effect on synaptic plasma Mg ATPase activity it is necessary to establish that an increase in release occurs after the regimen of lithium treatment which caused an increase in synaptic plasma

membrane Mg ATPase activity and in the same brain area.

In conclusion, this thesis has presented an argument and evidence to suggest that there is a reduction in Na/K ATPase activity in depression. Evidence was presented to suggest lithium can stimulate the sodium-pump when presented to the potassium sensitive side of the system and inhibit it when presented to the sodium sensitive side. Experiments failed to provide evidence for chronic lithium administration affecting Na/K ATPase. It was suggested that when given therapeutically lithium has no long-term effect on Na/K ATPase activity. Results presented suggest therapeutic lithium treatment may increase the specific activities of certain Mg ATPases in the brain. The physiological significance of such changes are unknown but it is speculated they could be related to the release of neurotransmitter. Whether or not the changes described are of relevance to the therapeutic action of lithium salts remains to be investigated.

APPENDIX 1.

Lithium concentration in human lumbar cerebrospinal fluid.

Lithium was measured in lumbar C.S.F. samples from 5 depressive patients who had received lithium treatment for at least 6 weeks. Following lumbar puncture, C.S.F. samples were stored at -20°C before analysis. Lithium was estimated by atomic absorption spectrophotometry at 670 nm using an SP 90 Pye Unicam atomic absorptiometer. Samples were compared with standards of aqueous lithium carbonate.

The lithium concentration in the samples was 0.21 ± 0.04 (Mean value ± standard deviation).

APPENDIX 2.

Method of estimation of protein.

Total protein was estimated throughout the work described in this thesis by the method of Lowry et al. (1951) following the procedure outlined below:

Reagents: A) 2% (w/v) Na_2CO_3 in 0.1 M NaOH.

B) 1% (w/v) $CuSO_4.5H_2O$.

C) 2% (w/v) sodium potassium tartrate.

D) 50 parts of A added to 1 part B and 1 part C.

E) Folin-Ciocalateau reagent (B.D.H., Poole) diluted 1 + 1.5 with water.

F) Bovine serum albumin (Sigma Ltd.) as standard. 15 - 75 µg in 0.3 ml.

Procedure: 3 ml solution C was added to 0.3 ml of samples, standards and blanks (water). After mixing, the solutions were left at room temperature for 15 minutes. 0.3 ml solution D was then added to each tube and the solutions mixed by vortexing. After standing at room temperature for 30 minutes, the absorbance of the solutions was measured at 750 nm. Protein content of samples was calculated by comparison of absorbances with those of standard solutions.

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