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STUDIES OF FACTORS WHICH AFFECT THE CATION-PERMEABILITY

AND ELECTROLYTE DISTRIBUTION IN MAMMALIAN CELLS

by

M. A. Radcliffe, B.Sc. (Dunelm)

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St. Cuthbert's Society,

DURHAM.



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GLOSSARY

ACTH	adrenocorticotrophic hormone
ADP	adenosine 5'-diphosphate
adx	adrenalectomized
ATP	adenosine 5'-triphosphate
ATPase	adenosine 5'-triphosphatase (E.C. 3.6.1.3)
°C	degrees Celsius
Ci	curie
CNS	central nervous system
CPK	creatine phosphokinase; ATP-creatine phosphotransferase (E.C. 2.7.3.2)
cps	counts per second
CRF	corticotrophin releasing factor
CSF	cerebro-spinal fluid
DNA	deoxyribonucleic acid
e	extra-cellular (as in $[Na^+]_e$)
ECF	extra-cellular fluid
EDTA	ethylenediaminetetraacetic acid
EHT	extra-high tension
E_K	potassium equilibrium potential
E_m	resting membrane potential
EST	electroshock seizure threshold
g	unit of centrifugal force (as in 2 000g)
α -GPDH	alpha-glycerophosphate dehydrogenase (E.C. 1.1.2.1)
H_{50}	$[NaCl]$ in which 50% haemolysis occurs
i	intra-cellular (as in $[K^+]_i$)
ICF	intra-cellular fluid

Mg^{2+} -ATPase	magnesium-dependent adenosine 5'-triphosphatase (E.C. 3.6.1.3)
$Na^+ + K^+ - Mg^{2+}$ -ATPase	sodium- and potassium-stimulated, magnesium- dependent adenosine 5'-triphosphatase (E.C. 3.6.1.3)
pcv	packed cell volume
P_i	inorganic phosphate; orthophosphate
R	residual (as in $[Na^+]_R$)
RNA	ribonucleic acid
rpm	revolutions per minute
SE	standard error of the mean
SPF	specific pathogen free
Tris	Tris(hydroxymethyl)-aminomethane
TSH	thyroid stimulating hormone

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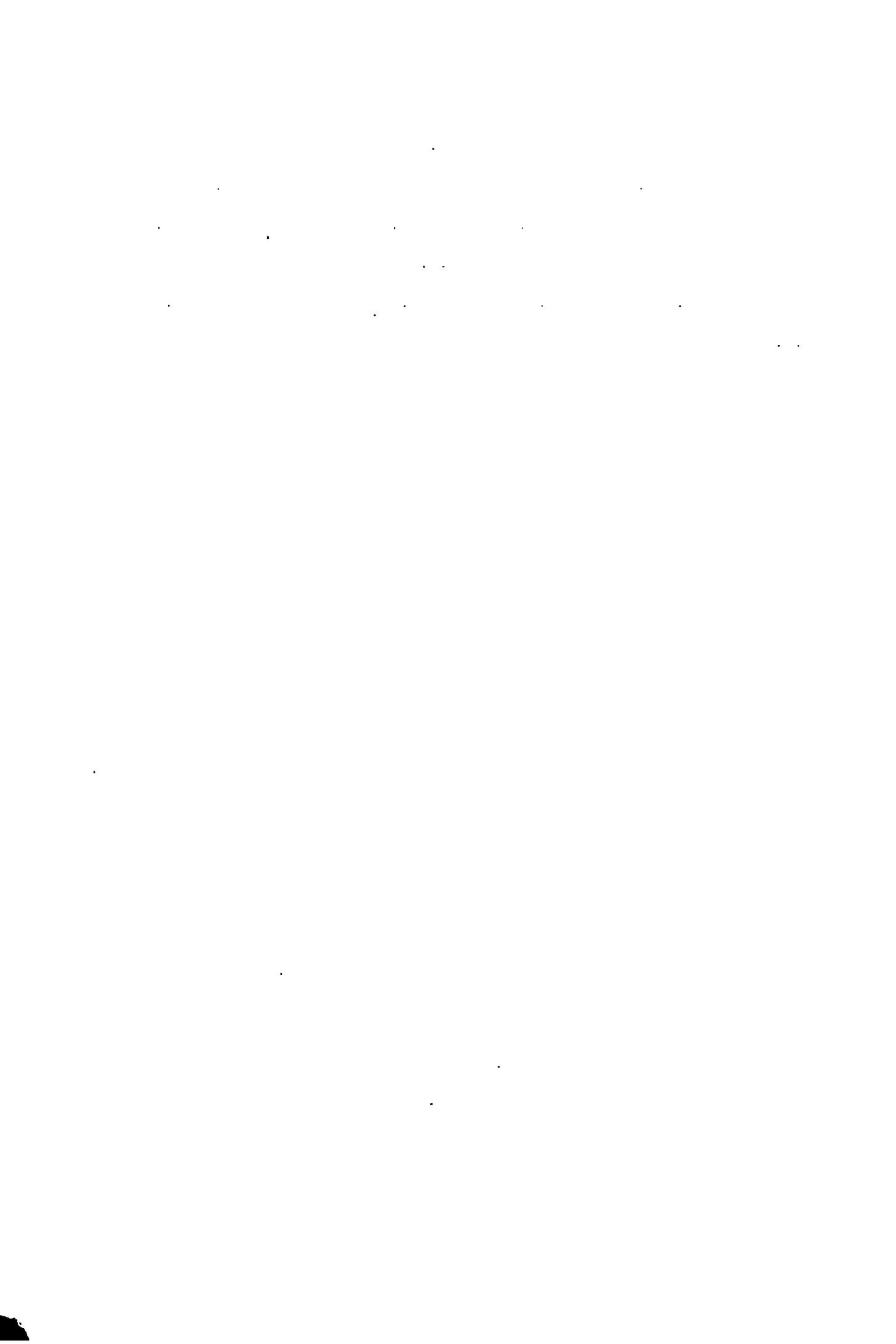
ABSTRACT

This thesis initially proposes an hypothesis which relates clinical findings of abnormal body monovalent cation distribution and adrenocorticosteroid secretion in subjects suffering affective disorders to the regulation of plasma membrane cation permeability. The hypothesis is partially examined by direct investigation of the sodium and potassium content and membrane magnesium-dependent adenosinetriphosphatase activities of erythrocytes from normal and affectively disordered human subjects. Thus, whilst erythrocyte sodium content remains normal, potassium content and monovalent cation-stimulated, magnesium-dependent adenosinetriphosphatase activity are elevated in manic depression.

Further investigations are concerned with determining the influence of adrenocorticosteroids upon these and related factors in rat skeletal muscle, kidney, erythrocytes and brain. They reveal that, whilst monovalent cation-stimulated, magnesium-dependent adenosinetriphosphatase activity remains uninfluenced by adrenocorticosteroid depletion in rat brain, it is reduced in skeletal muscle and kidney, and elevated in erythrocytes. Despite evidence for an associated significant decrease in the sodium content of rat erythrocytes, the rate of sodium efflux is reduced, and the activity of another membrane-associated enzyme (acetylcholinesterase) remains uninfluenced. Although the dietary administration of isotonic sodium chloride solution is shown to counteract a number of the effects of adrenocorticosteroid depletion upon rat tissues in a manner which suggests that renal sodium reabsorption is the principal hormone-sensitive target process, certain results obtained in skeletal muscle and erythrocyte studies are interpreted in terms of an extra-renal regulatory influence of adrenocorticosteroids upon plasma membrane characteristics.

Experimental observations are discussed throughout in association with those

established by previous workers, and in connexion with the extent to which adrenocorticosteroidal control of monovalent cation distribution may be implicated in the aetiology of manic-depression. This finally leads to a reconsideration of the clinical evidence for abnormal monovalent cation distribution in mania and depression, and of the hypothesis initially proposed.



SECTION I

INTRODUCTION TO THE SYSTEM UNDER STUDY



The differential distributions of inorganic ions in mammalian (and other vertebrate) body fluids is of great physiological significance. The major functional division of body fluid is into extra-cellular fluid (E.C.F.) and intra-cellular fluid (I.C.F.) compartments, and these are regarded as being physically separated by the bounding or 'plasma' membranes of cells. Amongst other ways, I.C.F. is differentiated from E.C.F. by its relatively low Na^+ content and high K^+ content, and there is now much evidence to show that the control of the differential distribution of these (and other) cations may be attributed to plasma membrane properties.

It is widely appreciated that the maintenance of differential Na^+ and K^+ distributions is essential to the functional integrity of virtually all cells, and particularly to the electrical properties of excitable cells. Studies with Na^+ and K^+ radioisotopes reveal that considerable trans-membrane fluxes of these ions do, in fact, occur. Membrane ion sorption and transport theories have been propounded to account for their net differential distributions. With regard to ion transport, the transfer of ions across cell membranes has broadly been resolved into passive and active components. Passive transfer is characterized by ion species movement down its chemical activity gradient or an electrical potential gradient, and active transfer by the converse. The rate of transfer is thus dependent upon the gradient characteristic, the permselectivity and magnitude of permeability of a membrane, and the mobilities of permeating ion species.

One of the most widely studied active transport systems is that responsible for the active transfer of Na^+ and K^+ across plasma membranes against their chemical activity gradients, and this system is thought to play a very significant role in determining the Na^+ and K^+ content of I.C.F. and E.C.F. compartments. Studies in giant nerve axons of cephalopods indicate that Na^+ extrusion is closely coupled to the metabolic supply of ATP, in these cells at least (Hodgkin & Keynes, 1955; Caldwell, 1956, 1960; Caldwell,

Hodgkin & Shaw, 1959; Caldwell, Hodgkin, Keynes & Shaw, 1960). Of great relevance to this finding is the discovery by Skou (1957) of an ATP-hydrolysing enzyme system (ATPase; E.C. 3.6.1.3) in a particulate fraction of Carcinus nerve. The enzyme system was found to be Mg^{2+} -dependent, and stimulated by varying proportions of Na^+ and K^+ . Skou (1957) suggested that this $Na^+K^+Mg^{2+}$ -ATPase is involved in the active extrusion of Na^+ from the nerve fibre.

It is of great interest, therefore, that Post, Merritt, Kinsolving & Albright (1960) subsequently showed that the $[Mg^{2+}]$, $[Na^+]$, and $[K^+]$ which gave half-maximal activation of this enzyme in human erythrocyte membrane fragments were similar to those mediating half-maximal transport of Na^+ and K^+ in intact erythrocytes, and that both mechanisms were inhibited by similar concentrations of the cardiac glycoside ouabain. $Na^+K^+Mg^{2+}$ -ATPase has subsequently been studied in membrane preparations from many other tissues, and its activity has been found to correlate with active Na^+K^+ flux over a wide range (see, for instance, Bonting & Caravaggio, 1963). Furthermore, by suitably adjusting the intra- and extra-cellular $[Na^+]$ and $[K^+]$ of resealed human erythrocyte ghosts, it has been claimed that it is thermodynamically possible to reverse the normal course of ATPase function, and thereby synthesize ATP from ADP and P_i (Garrahan & Glynn, 1967d).

In addition to the ouabain-sensitive $Na^+K^+Mg^{2+}$ -ATPase, all tissues so far investigated exhibit a Mg^{2+} -ATPase component which is ouabain-insensitive and not monovalent cation stimulated. This component has often been disregarded or considered as a contaminant of $Na^+K^+Mg^{2+}$ -ATPase preparations, although the important question arises whether Mg^{2+} - and $Na^+K^+Mg^{2+}$ -ATPase activities are properties of a single enzyme system or two distinct enzymes. The evidence may be taken to favour the latter, particularly because their activity ratio varies widely from tissue to tissue, because they possess different temperature sensitivities and activation energies in such widely

divergent tissues as rat brain (Bowler & Duncan, 1968) and liver (Bakkeren & Bonting, 1968), and because their activities have been shown to alter differentially in $[\text{NaCl}]$ -sensitive tissues such as freshwater- and saline-adapted duck nasal secretory glands (Fletcher, Stainer & Holmes, 1967; Ernst, Goertemiller & Ellis, 1967). The enzymes are therefore differentiated in incubations by employing Mg^{2+} - and $\text{Mg}^{2+}+\text{Na}^++\text{K}^+$ -containing incubation media, or by the extent to which ouabain inhibits ATP hydrolysis in the presence of $\text{Mg}^{2+}+\text{Na}^++\text{K}^+$. Since these Mg^{2+} -dependent ATPases have been found to hydrolyse ATP to ADP and orthophosphate (P_i), their activities are usually expressed as the number of moles of P_i released from ATP on unit weight of plasma membrane protein and unit time bases.

The fact that $\text{Na}^++\text{K}^+-\text{Mg}^{2+}$ -ATPase activity adapts to the demands made upon $[\text{NaCl}]$ -sensitive tissues such as duck nasal glands (see above) shows that Na^+ availability is at least one factor determining its activity. However, since plasma membranes may be regarded as good candidates for the structural targets of a number of hormones, it is not surprising that some evidence for hormonal regulation of Na^+-K^+ transport and $\text{Na}^++\text{K}^+-\text{Mg}^{2+}$ -ATPase is also available. Examples include the stimulation of the enzyme in rat ventral prostatic microsomal preparations by testosterone (Farnsworth, 1968), in pig thyroid gland by T.S.H. and certain oestrogens (Takagi, 1968), in rat uterus by oestradiol-17 β (Karmakar, 1969), and in rat liver and kidney by tri-iodothyronine (Ismail-Beigi & Edelman, 1971). There is also evidence showing that the Na^+ reabsorptive capacity of mammalian kidney is regulated by adrenocorticosteroids in a manner which involves altered $\text{Na}^++\text{K}^+-\text{Mg}^{2+}$ -ATPase activity (see, for instance, Chignell & Titus, 1966): this is particularly interesting in view of the general influence of adrenocorticosteroids upon electrolyte metabolism (see, for instance, Lipsett, Schwartz & Thorn, 1961).

In view of the strong evidence implicating $\text{Na}^++\text{K}^+-\text{Mg}^{2+}$ -ATPase in the control of Na^+ and K^+ distribution between E.C.F. and I.C.F., it is clear that alter-

ations in its enzymic characteristics could constitute the primary lesion in diseases involving abnormal electrolyte distribution. No evidence for this was available when the work reported in this thesis began, but one very interesting instance of apparently systemic Na^+ redistribution had received recent attention in the literature.

The association of feelings of mental depression and attitudes of futility or hopelessness in human subjects was found to be associated with decreased rates of urinary water, Na^+ and K^+ excretion by Schottstaedt, Grace & Wolff (1956). Subsequent investigators applied isotope dilution techniques in studies of Na^+ and K^+ distributions in subjects regarded as suffering the affective disorders depression and mania. Evidence was found for Na^+ retention during the course of depression (Gibbons, 1960; Coppen & Shaw, 1963; Shaw, 1966) and mania (Coppen, Shaw, Malleon & Costain, 1966; Shaw, 1966), and this evidence implicated a body $[\text{Na}^+]$ component which included the I.C.F. compartment in particular. Similar studies of K^+ distribution in depressed subjects revealed no detectable abnormality (Coppen & Shaw, 1963; Shaw, 1966; Shaw & Coppen, 1966). However, the change in Na^+ distribution was very marked in both depression and mania: the respective data showed mean 50% and 200% increases in the Na^+ content of the component involved (Shaw, 1966). Since this component was regarded as especially including the I.C.F. compartment, it appeared that the change was systemic.

The apparently very marked alteration of body Na^+ distribution in depression and mania is of interest, partly because there is evidence for abnormal active Na^+ transport in depression. Thus, the rate of transfer of Na^+ from blood to C.S.F. is reported to be reduced in depressive subjects (Coppen, 1960), and that of parotid salivary duct Na^+ reabsorption lowered in manic-depressives (Glen, Ongley & Robinson, 1968). The question therefore arises whether reduced active Na^+ transport is related to altered $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}$ characteristics. It is indeed known that this enzyme is involved in C.S.F. formation in the

cat (Vates, Bonting & Oppelt, 1964), and in Na^+ reabsorption in dog and sheep parotid glands (Schwartz, Laseter & Kraintz, 1963; Filsell & Jarrett, 1965). Altered C.N.S. cation distributions would be the basis of the behavioural manifestations of mania and depression.

Of additional interest in this context is evidence for altered adrenocorticosteroid secretion in human subjects suffering affective disorders. This evidence is widely scattered and difficult to assess, although it is relevant because clinical studies show that significant proportions of patients with Cushing's syndrome and Addison's disease do suffer symptoms of euphoria and depression (Bleuler & Stoll, 1962). Thus, increased plasma and urinary 11-hydroxycorticosteroid and 17-hydroxycorticosteroid levels have been generally observed in depression, returning to normal upon recovery (see, for instance, Dewhurst, El Kabir, Exley, Harris & Mandelbrote, 1968; Rubin, Young & Clark, 1968; Bunney, Fawcett, Davis & Gifford, 1969; Sclare & Grant, 1969). There is also evidence for altered adrenocorticosteroid secretion patterns in patients suffering affective disorders (Conroy, Hughes & Mills, 1968; Fullerton, Wenzel, Lohrenz & Fahs, 1968; Lohrenz, Fullerton, Fahs & Wenzel, 1968; Ellman & Blacker, 1969). Indeed, it has been shown that increased $^{22}\text{Na}^+$ retention is a correlate of elevated 17-hydroxycorticosteroid excretion in depression (Baer, Durell, Bunney, Levy & Cardon, 1969), although Moody & Allsopp (1969) failed to distinguish such a pattern.

It is most frequently believed that the elevated adrenocorticosteroid secretion which accompanies depression is a special example of the adrenocortical activation which occurs in suitably aroused healthy subjects. However, there is evidence of pituitary-adrenocortical resistance to dexamethasone feedback inhibition in severe depression (Butler & Besser, 1968), and this has been observed in association with hypothalamo-pituitary insensitivity to hypoglycaemia in depressive subjects (Carroll, 1969). There are also reports which suggest that such patients suffer from relative adrenocorticosteroid

deficiency (Cumming & Kort, 1956; Kurland, 1965; McClure & Cleghorn, 1968). It is possible, therefore, that altered adrenocorticosteroidal output is not secondary to the affective changes characterizing mania and depression, but instrumental in their aetiology.

It seems possible that the observations set out above may be considered together with regard to the aetiology of certain affective disorders. It is therefore proposed that the Na^+ redistribution which occurs in mania and depression is related to changes in the mechanism of plasma membrane permeability control, most probably of the enzyme $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}$; furthermore, that these changes are related to the degree of adrenocorticosteroid availability and interaction with plasma membranes.

For the purpose of the investigations reported in this thesis, it has been decided to examine this hypothesis in terms of the electrolyte content and plasma membrane ATP-hydrolysing characteristics of tissues from human subjects suffering affective disorders, and animals undergoing adrenocorticosteroid depletion.

Initial studies involved skeletal muscle, which was selected because it is a bulk tissue, and therefore one most likely to reflect the Na^+ redistribution apparent in subjects suffering mania and depression (see above). For reasons given in the text, these studies were extended to kidney, erythrocytes and brain. With tissue specialization in mind, detailed evidence for and against the involvement of adrenocorticosteroids and ATP hydrolysis in the control of Na^+ and K^+ distribution is considered for each tissue in turn. Finally, this evidence is reconsidered in association with that giving rise to the above hypothesis.

S E C T I O N I I

GENERAL MATERIALS AND METHODS

This Section contains a description of materials and methods common to the experimental work considered in Sections III - VI. The techniques used in studies of specific tissues are given in the relevant Sections.

MATERIALS

- a) Animals: ♂ CFE (of remote Sprague-Dawley origin) and CFHB (of remote Wistar origin) rats were bred from S.P.F. breeding nuclei supplied by Carworth Europe, Alconbury, Huntingdon. Animals of 150-200g body weight were used.
- b) Reagents: All reagents used were of the purest available grade; inorganic salts were usually of AnalaR grade. Solutions were made up in glass-distilled or deionized water, except where otherwise stated.

METHODS

- a) Treatment of glassware: All glassware was soaked in a 2% solution of 'Quadralene' laboratory detergent (Fisons Scientific Apparatus Ltd.) or in 'Decon 75' (I.C.I. Ltd.), rinsed 6 times in tapwater and 6 times in distilled water. It was then oven dried. When appropriate, 6 deionized water rinses followed the distilled water rinses.
- b) Technique of adrenalectomy:
- (i) Anaesthetic: 5mg sodium pentobarbitone (Boots Pure Drug Co, Ltd.)/100g body weight in NaCl injection, B.P. (Evans Medical Ltd.), administered intraperitoneally, or anaesthetic ether (Macfarlan Smith Ltd.) by vapour inhalation.
- (ii) Adrenal ablation: Hair was shaved on both sides of each animal from the area in the region of the last rib. Prior to operating, this area was swabbed with an antiseptic solution. Adrenalectomy proceeded on a heated operating table. Incisions were made in the skin and body wall

musculature consecutively, those on the animal's right side being made high in the angle formed by the last rib and vertebral column, and those on the left slightly further latero-chordal. After incisions had been made on either side, a glass tube of suitable diameter was placed under the animal in order to display the adrenal gland. Retractors were introduced, when necessary, to displace the kidney and liver so that the connective tissue associated with the gland could be grasped with curved forceps. The adrenal gland was then detached, without noticeable haemorrhage, by introduction of a second pair of curved forceps to grasp and tear the connective tissue between the kidney and the initial pair of forceps. Special care was taken to avoid rupture of the kidney capsule or of the adrenal gland itself. The excised gland was inspected for entirety. (If the success of the operation was doubted at this stage, the animal was discarded.) The body wall incision was closed with a single thread suture, and the skin incision with two 12mm silver wound clips (Messrs. H. Hauptner, Solingen, Germany). The wound was then treated with a transparent 'Nobecutane' spray dressing (B.D.H. Pharmaceuticals Ltd.). Newly adrenalectomized animals were placed in a chamber at $26^{\circ} \pm 2^{\circ} \text{C}$ to recover from anaesthesia. There was no evidence of subsequent wound infection in any case.

(iii) Post-mortem examination: Since the presence of accessory adrenocortical tissue has been noted in rats (Deane, 1962), animals were examined for evidence of this. Peri-adrenal fat, not removed during the course of adrenalectomy, was examined in particular. No evidence of accessory tissue or adrenal rest formation were found.

c) Maintenance of rats: All animals were exposed to a 12hr light:12hr dark artificial photoperiod, the period of illumination commencing at 0700 hrs.

(i) Stock rats: Rats were maintained in cages containing not more than

6 individuals, and were placed in stock rooms at $23^{\circ} \pm 2^{\circ}\text{C}$. They received a diet consisting of Laboratory Small Animals Diet (Spillers Ltd.) in pellet form and tapwater, both ad lib.

(ii) Adrenalectomized rats: These were individually caged at $25^{\circ} \pm 2^{\circ}\text{C}$. Their diet consisted of Laboratory Small Animals Diet (as above) and distilled water or 0.9% NaCl, all ad lib.

(iii) Maintenance and sacrifice of rats in chronic experimental series: Control animals, whether intact or sham-adrenalectomized, were kept under the same conditions as adrenalectomized rats during the course of an experimental series. Animals were sacrificed, whenever possible, between 0900 and 1000hrs.

d) Tissue homogenization and microsomal resuspension technique: Both processes were carried out in Potter-Elvehjem pyrex glass tube mortars with Teflon pestles, having a clearance of 0.10-0.15mm. For tissue homogenization the pestle was rotated at 1000rpm by use of an MSE Ltd. Homogenizer motor, and a suitable number of passes was made through the entire tissue suspension (for details, see Sections III - VI). In the case of microsomal resuspension, a similar procedure was adopted, but the pestle was not rotated.

e) Estimation of inorganic phosphate: This procedure was adopted in the assay of Mg^{2+} -dependent ATP hydrolysis, in which ADP and P_i were formed. P_i was estimated by the method of Fiske and Subbarow (1925), using a proprietary phosphorus standard solution (Sigma Chemical Co.). Microsomal protein was precipitated by the addition of 1ml of 12% (w/v) trichloroacetic acid (BDH Ltd. AnalaR grade) at the completion of incubation periods, and the tubes immediately shaken and transferred onto crushed ice. The protein precipitate was then sedimented by centrifugation in an MSE Ltd 'Mistral' 2L centrifuge, rotor no. 6885 at 2000xg for 15min, and at 0° - 4°C . Duplicate 1ml aliquots of each supernatant, kept on ice, were then dispensed into 1ml aliquots of a freshly made up solution of

1 volume 5% (w/v) $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$: 1 volume 5N H_2SO_4 . This mixture was allowed to thermoequilibrate at $18^\circ\text{-}21^\circ\text{C}$ for 10min, when 0.25ml aliquots of Fiske & Subbarow reducer (Sigma Chemical Co.), freshly dissolved in distilled water, were added. After 15min at $18^\circ\text{-}21^\circ\text{C}$, the optical density of the blue coloured solution was determined versus a distilled water blank at 660nm in a Hilger & Watts Ltd. spectrophotometer, using a tungsten filament lamp operating through a 1cm light path. The P_i content was determined by reference to calibration lines drawn from values obtained by applying the above technique to serially diluted stock phosphorus standard solution, containing 20 μg P (as KH_2PO_4) / ml.

f) Estimation of microsomal protein: The method of Lowry, Rosebrough, Farr & Randall (1951) was used, and bovine serum albumin (Fraction V) (Sigma Chemical Co.) taken as the standard.

Aliquots of microsomal resuspensions were sedimented in an MSE Ltd. 'Automatic Superspeed 40' ultracentrifuge, rotor 2409, at 107 000xg for 1hr, and at $0^\circ\text{-}4^\circ\text{C}$. After discarding the supernatant, the pellets were washed in deionized water by alternate withdrawal into and expulsion from a Pasteur pipette. The pipette was further washed out with deionized water, and the washings added to the microsomal resuspension. The suspension was then re-centrifuged (see above), the supernatant discarded and pellet resuspended in a known volume of deionized water by means of a small-volume Potter-Elvehjem apparatus (see above). This resuspension was scrutinized for homogeneity. Duplicate or triplicate 0.2ml aliquots of such resuspensions were added to 3ml aliquots of a reagent consisting of 50 volumes 2% (w/v) Na_2CO_3 in 0.1N NaOH: 1 volume 0.5% (w/v) $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$ in 1% (w/v) K-Na tartrate. After 30min at $18^\circ\text{-}21^\circ\text{C}$, 0.3ml aliquots of a 33% (v/v) solution of Folin & Ciocalteu's phenol reagent (BDH Ltd.) were added to each tube. The tubes were agitated and left for 1hr at $18^\circ\text{-}21^\circ\text{C}$. The optical density of the resultant solution was then determined versus a

distilled water blank in a Hilger & Watts Ltd. spectrophotometer, using a tungsten filament lamp operating through a 1cm light path. Calibration lines were drawn from values obtained from serially diluted stock 1mg/ml bovine serum albumin (Fraction V) in deionized water. Under these conditions, optical density was a linear function of protein concentration. It was arranged that the protein concentration of the microsomal resuspensions taken for analysis fell within this range. The protein content of stock solutions of bovine serum albumin was periodically checked by determining their absorbancy at 280nm in the spectrophotometer, using a deuterium arc lamp and silica cuvettes.

g) Statistical treatment of results: The methods employed are given in the text of Sections III - VI. Where appropriate, reference was made to the statistical tables of Fisher & Yates (1963).

S E C T I O N I I I

S K E L E T A L M U S C L E S T U D I E S

CHAPTER 1The Control of Monovalent Cation Distribution in Skeletal Muscle

The structure of skeletal muscle is reviewed by Davson (1970), and will not be discussed here, except in the case of certain ultrastructural elements thought to be directly involved in the control of ionic permeability. Fenn (1936) and Conway (1957) have reviewed electrolyte distributions in this tissue. However, accurate determination of intra- and extra-cellular $[Na^+]$ and $[K^+]$ has been fraught with difficulties - based, as it must be, upon estimates of compartmental volume, water content and ion sequestering capacity. Determinations of total tissue water and monovalent cation content have been comparatively simple.

Compartmental volume was initially calculated from tissue $[Cl^-]$ values (Fenn, 1936), on the basis that extra-cellular fluid is a plasma ultrafiltrate and the assumption that Cl^- was entirely extra-cellular. The assumption has proved incorrect (Conway, 1957), and it is also likely that a fraction of muscle $[Cl^-]$ is associated with connective tissue. (Cotlove & Hogben, 1962). Experimental estimation of extra-cellular fluid space has come to depend upon in vivo or in vitro tissue equilibration with high molecular weight compounds such as sucrose, mannose and inulin. Even then, as Ling & Kromash (1967) have shown, estimates are larger the lower the compound's molecular weight, the greater its concentration, and the longer the exposure period. Their analysis of marker uptakes revealed an initial rapid and subsequent slow component; the latter was considered to represent cell uptake. In the case of polyglutamate it was possible to recognize and compensate for cell membrane penetration and tissue adsorption. However, the possible osmotic influence of such compounds is not accounted for. Adamič (1968) derived compartmental $[Na^+]$ values in rat diaphragm muscle by analysis of wash-out curves for ²⁴Na-loaded strips, though difficulty in estimating extra-cellular space

again arose because of suspected leakage from damaged fibres. In these circumstances it is clear that no single technique can be favoured in estimations of muscle monovalent cation distributions, and it is disturbing that the availability of precise figures cannot be expected. With appropriate reservations, the accompanying table (TABLE III-1) is presented as a summary of Na^+ and K^+ distributions in mammalian skeletal muscle, according to several authors.

The table shows that Sréter & Woo (1963) have reported two groups of values in a series of rat muscles treated by their standard procedure. The data strongly suggest that the intra-cellular ionic composition is a correlate of a muscle's complement of the histologically identifiable slow (red) and fast (white) fibre types. The ratio ($[\text{K}^+]_i / [\text{Na}^+]_i$) is proportionately greater the larger the complement of white fibres. Sréter (1963) also observed large changes in $[\text{Na}^+]_i$ and $[\text{K}^+]_i$ upon indirect low-frequency stimulation of muscles with predominantly white fibres. Prolonged stimulation (6hr) resulted in extreme cationic debt to the extent of a 1204% increase in $[\text{Na}^+]_i$ and 37.8% decrease in $[\text{K}^+]_i$ for white gastrocnemius fibres as compared with respective values of 395% and 8.9% for the red fibres. Further work upon these fibre types in rat (Locke & Solomon, 1967) has revealed that topical or intra-venous ouabain administration simulates the effects of denervation in reducing E_m from -83.5 mV in gastrocnemius, and -68.8 mV in soleus to the -60 mV level. (These authors do not note whether their recordings were from surface gastrocnemius fibres.) Locke & Solomon concluded that denervation and ouabain effects involved a common mechanism, probably that of active monovalent cation transport. Thus, the E_m increment above -60 mV would be attributed to this component. In addition, Williams, Withrow & Woodbury (1971), working with rat gracilis muscle, report that intraperitoneal ouabain administration results in an E_m fall from -95 mV to -65 mV,

MAMMAL	MUSCLE	[ELECTROLYTE], mM				REFERENCE	
		Intracellular fluid		Extracellular fluid (plasma)			
		[Na ⁺] _i	[K ⁺] _i	[Na ⁺] _e	[K ⁺] _e		
rat	diaphragm	23.0	152.7	144.4	4.4	Sréter & Woo (1963)	
	soleus	22.6	153.8	141.0	4.2		
	extensor digitorum longus	14.0	168.9	139.7	4.3		
	vastus lateralis	12.3	166.2	138.7	4.2		
	gastrocnemius (deep)	16.26	160.33	148.45	4.15		
	(surface)	9.69	168.81	148.55	4.15		
	extensor digitorum longus	14.0	165.0	-	-		Dockry, <u>et al.</u> (1966)
	diaphragm	6.22	-	-	-		Greese, <u>et al.</u> (1968)
gracilis	26.3	166.8	147.5	3.8	Williams, <u>et al.</u> (1971)		
man	gastrocnemius	13.0	153.0	-	-	Mokotoff, <u>et al.</u> (1952)	
	-	16.9	152.7	-	-	Talao, <u>et al.</u> (1953)	
	-	5.0	169.0	-	-	Cort & Matthews (1954)	
	-	13.1	165.7	-	-	Wilson (1955)	
	-	26.1	150.0	-	-	Barnes, <u>et al.</u> (1957)	
	quadriceps femoris	10.8	164.3	-	-	Litchfield & Gaddie (1958)	
	vastus lateralis	19.0	167.0	141.0	4.55	Graham, <u>et al.</u> (1967)	

TABLE III-1. VALUES FOR MAMMALIAN SKELETAL MUSCLE [Na⁺] AND [K⁺] DERIVED FROM DATA IN THE LITERATURE.

concomitant with Na^+ and K^+ redistributions.

In this context, Dockry, Kernan & Tangney (1966) had shown that the extent of redistribution of Na^+ and K^+ during recovery in Na^+ -loaded rat extensor digitorum longus and soleus muscles was enhanced if the muscles remained innervated throughout loading and recovery periods; further, that the addition of acetylcholine to the recovery fluid resulted in a similar response in denervated extensor. This points to the possibility that an active transport component is the adaptive feature through which fibre types are differentiated, and that this may be influenced by their types of innervation, Guth & Brown (1965) showed that decrease and subsequent regeneration of rat sternomastoid cholinesterase activity occurred upon denervation and reinnervation, and it is known that a general surface sensitivity to acetylcholine develops after motor nerve section (Axelsson & Thesleff, 1959). These aspects of nerve/muscle inter-relationship are reviewed by Thesleff (1960) and Guth (1968). However, it is interesting to note that Kernan (1966) showed that denervation of frog sartorius resulted in an increased E_m , despite a decreased rate of Na^+ extrusion in Na^+ -loaded muscles; he thought this could be due to a concomitant decrease in K^+ uptake.

Before considering possible definitions of the active transport component in muscle, it is appropriate to mention other components thought to govern monovalent cation availability within intra- and extra-cellular compartments. By using special Na^+ and K^+ glasses, some workers have devised ion-selective micro-electrodes with which actual ion activities may be measured. Whilst Ling (1955, 1962) has proposed a fixed charge or sorption hypothesis explaining differential Na^+ and K^+ distributions in terms of preferential K^+ -binding by non-diffusible intra-cellular anions of low field strength, the experimental evidence available for K^+ mobility indicates that it is not restrained.

Lev (1964) essentially repeated Hinke's (1959) findings for Homarus muscle fibres when he found that the K^+ activity coefficient in frog fibres was in the range 0.751 - 0.795, close to that determined in KCl solutions of similar molarity and to theoretical predictions by Nanninga (1961). Unfortunately, values are not available for mammalian muscle. As regards Na^+ activity, Hinke (1959) found unexpectedly low values in Homarus; Lev (1964) estimated that 70% of $[Na^+]_i$ was unavailable to the electrode in frog fibres. These observations point either to a sorption phenomenon, e.g. that incorporated in Ling's proposal, or to the existence of a secondary intra-cellular compartment. Using radio-isotopes of supposedly inert solutes and certain ions, Kushmerick & Podolsky (1969) demonstrated that the rates of migration of all but Ca^{2+} were similar after micro-injection into Rana pipiens semitendinosus fibres. The ratio (diffusivity in muscle/diffusivity in water) was about 0.5, although for Ca^{2+} it was 0.01. Hence it seemed likely that Ca^{2+} is chemically bound in the fibre, whilst the small reduction in diffusivity of other substances could be explained in physical terms of the viscosity of intra-cellular fluid. From monovalent and divalent cation binding studies in a microsomal preparation of rabbit sarcoplasmic reticulum and transverse tubular system, Carvalho (1966) concluded that there was a considerable Ca^{2+} binding capacity, and that other ions could compete for the sites concerned. However, Na^+ and K^+ affinities were lowest of all. The author considered that the imidazole group of histidine and phosphoryl groups of lecithin and phosphatidyl serine were likely candidates for involvement in the cation exchange mechanism.

By histochemical use of the Na^+ -specific reagent pyroantimonate, Zadunaisky (1966) revealed that Na^+ could be located in high concentration inside the transverse tubules of Rana pipiens sartorius T-system. This result might be accepted with reservation in that other structures appeared to be Na^+ -free:

it is known, for instance, that intra-cellular organelles such as mitochondria do contain Na^+ . However, it does indicate that the T-system is an important Na^+ compartment, likely to be involved in restricting its availability to microelectrodes in the studies of Hinke (1959) and Lev (1964). It is relevant to note that the volume of the T-system is thought to vary: Foulks, Pacey & Perry (1965) noted an increase in frog muscle under K^+ and Cl^- depletion (Na^+ loading), and similar observations have been made for other isolated muscles. Akaike (1971), for instance, has found that Na^+ gain and K^+ loss occur during saline storage of rat soleus and extensor digitorum longus, and may be counteracted by the addition of rat, cat, cow or rabbit serum to the solution. It is possible that additional extra-cellular Na^+ could be retained in the intermediate T-system compartment and assayed as if it were intra-cellular. If this were so, it might resolve the problem of the Na^+ activity coefficient without resort to a sequestration hypothesis.

On the assumption that intra-cellular Na^+ and K^+ are not substantially bound, it is appropriate to consider cell membrane permeability mechanisms for an explanation of their distributions. Of indirect relevance here is the work of Caldwell, Hodgkin, Keynes & Shaw (1960a,b) on giant axons of Loligo. They demonstrated that active Na^+ and K^+ transport could be restored in CN^- -poisoned preparations by intra-cellular injection of ATP or a phosphagen - e.g. phosphoenolpyruvate, ADP. Conway (1960) pointed out that the effectiveness of metabolic inhibitors in reducing muscle monovalent cation fluxes was dependent upon the tissue's metabolic state, i.e. upon the extent of its ATP and phosphagen reservoirs. This work has shown that monovalent cation fluxes are indeed associated with an ATP-requiring mechanism.

Concerning the identity of the mechanism, several workers have investigated the ATPase characteristics of skeletal muscle in the attempt to distinguish

an enzyme with monovalent cation requirements similar to those of Skou's (1957) crab nerve microsomal enzyme. The presence of an enzyme of this type that could be readily demonstrated is essential for its investigation in the context of adrenocorticosteroid regulation and affective disorders. The subject of skeletal muscle Na^+K^+ -stimulated Mg^{2+} -ATPase has been reviewed by Bonting (1970), though he makes little mention of mammalian muscle. Bonting, Simon & Hawkins (1961) first demonstrated a Na^+K^+ -stimulated, ouabain-inhibited enzyme in the reconstituted aqueous lyophilized homogenate of cat muscle, and reported a very low activity of about 1.8% of that in a similar preparation of grey matter. Further studies by Bonting, Caravaggio & Hawkins (1962) and Bonting & Caravaggio (1963) reported the enzyme in frog sartorius and extensor digitorum longus IV preparations. Duggan (1964, 1965), however, repeated this type of experiment on frog sartorius and could not demonstrate synergistic monovalent cation stimulation or ouabain-sensitivity. Corrie & Bonting (1966) then reported the ouabain and erythrophthleine reduction of E_m in frog sartorius and claimed that the concomitant decrease in $[\text{K}^+]_i$ and increase in $[\text{Na}^+]_i$ were consistent with the presence of the classical enzyme. In subsequent publications (Duggan, Ryan & Harrington 1967; Heffron & Duggan, 1967; Duggan, 1968a, b), Duggan and co-workers have reported the absence of this Skou-type enzyme, and Manery, Riordan & Dryden (1968) reported the failure to distinguish such a surface ATPase in Rana pipiens muscle. Giacomelli, Bibbiani, Bergamini & Pellegrino (1967), using a histochemical lead phosphate deposition technique, meanwhile reported that the enzyme was present in triad lateral sacs and sarcolemma of frog ideofibularis and rat extensor digitorum longus.

As regards mammalian muscle, Samaha & Gergely (1965, 1966) reported the presence of $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+}$ -ATPase in human cadaver muscle samples, whilst Lee Tanaka & Yu (1965), Fratantoni & Askari (1965), and Engel & Tice (1966)

reported the absence of a synergistically stimulated enzyme in rabbit muscles. Buckley, Evans & Nowell (1968) found no evidence for the ATPase in a rat diaphragm preparation. It seemed that the solution to this dilemma might well lie in the preparative techniques applied to muscle, a matter which receives subsequent attention.

CHAPTER 2

Experiments upon the Enzymatic Control of Skeletal Muscle Monovalent Cation Distribution

The presence of a number of membrane systems in skeletal muscle fibres, in addition to the myofibrillar component, presents a considerable problem in the investigation of tissue homogenates. The plasma membrane proper appears to be the innermost, osmiophilic component of a composite bounding sarcolemma (Andersson-Cedergren, 1959; Mauro & Adams, 1961). Sarcolemmal invaginations, the transverse tubules of the T-system, may be regarded as extensions of the plasma membrane and therefore of the extra-cellular compartment (Franzini-Armstrong & Porter, 1964). They are closely associated with the endoplasmic reticulum (sarcoplasmic reticulum, L-system) at characteristic triad regions (Andersson-Cedergren, 1959). In addition to fragments of these two membrane systems, a muscle fibre homogenate would contain organelles (e.g. sarcosomes) and membrane fragments derived from them. Thus the quest for a plasma membrane bound $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+}$ -ATPase, using the conventional homogenate technique, might be expected to prove difficult. It is not surprising that the limited volume of literature concerned with this problem presents a good deal of conflicting evidence.

TABLE III-2 summarizes the literature on mammalian and frog muscle with regard to three factors:

- a) mode of extraction,
- b) the cellular fraction considered to be under investigation, and
- c) the effects of monovalent cations upon Mg^{2+} -ATPase.

These are now briefly outlined.

a) Mode of extraction. At the time of the experiments reported in this thesis several techniques had been used. Histochemical ones apart, these generally involved homogenization of tissue. Though Bonting, et al. (1961) demonstrated a classical synergistic $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+}$ ATPase in a whole

FRACTION	METHOD		DIFFERENTIAL CERTIFICATION OF HOMOGENATE CONTAINING							LYOPHILIZED WHOLE HOMOGENATE	MICROSOMAL AGEING	SARCOLEMMA SEGMENTS	HISTOCHEMICAL DEMONSTRATION
	CATION EFFECT		250mM sucrose	880mM sucrose	detergent	sucrose + dialysis	.250M 2-mercaptoethanol	ECI-LAB treatment					
MICROSOMAL	STIMULATION	COUPLED	Duggan, et al. (1967) Rubin & Krets (1967)*		Suzuka & Gergely (1965, 1966)*			Ash & Schwartz (1970)*	Roeting, et al. (1961) " (1962) Roeting & Cas- stvedt (1963)	Suzuka & Gergely (1965, 1966)*			
		NON- COUPLED	Frelund & Aakari (1965)* Duggan, et al. (1967)										
	INHIBITION			Duggan, et al. (1967)									
	NONE OBSERVED							Suzuka, et al. (1971)*					
SARCOPLASMIC RETICULUM	STIMULATION	COUPLED			Duggan, et al. (1965)*			Duggan, et al. (1970)				Giacomelli, et al. (1967)*	
		NON- COUPLED	Duggan (1964)	Dugal & Rice (1966)*			Dugal & Rice (1966)*						
	INHIBITION			Duggan (1964) Duggan, et al. (1970)						Duggan, et al. (1965)*			
	NONE OBSERVED		Duggan, et al. (1965)*	Duggan (1968)									
SARCOLEMA	STIMULATION	COUPLED						Suzuka, et al. (1971)*			Peter (1970)* Seiler (1971)*	Giacomelli, et al. (1967)* Morita, et al. (1969)*	
		NON- COUPLED											

TABLE III-2. SUMMARY OF METHODS ADOPTED IN THE QUEST FOR SKELETAL MUSCLE Ca^{2+} - Mg^{2+} -ATPase ACTIVITY.

Sources are more fully discussed in the text. They refer to frog and mammalian (•) preparations.

homogenate of cat muscle (unspecified), subsequent workers adopted differential centrifugation techniques in attempts to identify the fractional location of the enzyme. Of the preparative procedures available, those used most frequently were:

- i) simple homogenization in buffered isotonic sucrose containing EDTA, a method widely used for other tissues;
- ii) an extension of this to include detergents (notably deoxycholate), which had proved successful in rabbit cardiac muscle (Auditore, 1962).

As the table indicates, each has enjoyed partial success.

b) Membrane fraction under study. Despite electron microscopic scrutiny of the fractions obtained by differential centrifugation, no worker has presented evidence showing which membrane components were tested for ATPase activity, though some have referred to their microsomal preparations as derived from sarcoplasmic reticulum. Using histochemical techniques, Giacomelli et al. (1967) reported $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+}$ -ATPase activity in the lateral sacs of triads (i.e. the sarcoplasmic reticulum) and sarcolemma (presumably plasma membrane) in frog ileofibularis and rat extensor digitorum longus; Morita, Sugimoto, Ueda & Mayahara (1969) reported a sarcolemmal ATPase in rat tibialis anterior, using an incubation medium which suggests that it could have been the $\text{Na}^+ + \text{K}^+$ -stimulated one.

c) Monovalent cation stimulated Mg^{2+} -ATPases observed. Whilst the 'classical' enzyme of Skou (1957) and subsequent workers (concerned with a variety of tissues) is, by definition, a synergistically stimulated ATPase, this criterion has not always been observed in skeletal muscle preparations. Indeed, some workers have not distinguished between synergistic and merely additive monovalent cation stimulation. This matter will receive later attention. In the tabulated summary (TABLE III-2) it has been convenient to present the monovalent cation effects upon Mg^{2+} -ATPase activity as:

i) coupled Na^+ and K^+ stimulation, which may be synergistic or additive;

ii) non-coupled stimulation, by Na^+ or K^+ alone;

iii) inhibition by Na^+ and K^+ , alone and in combination;

iv) none, irrespective of $[\text{Na}^+]$ and/or $[\text{K}^+]$.

Since the task of this research was to define a bulk tissue $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+}$ -ATPase that could control Na^+ and K^+ distribution, the quest for a technique suitable for its exposure in skeletal muscle was the initial line of attack.

MATERIALS AND METHODS

A. ENZYME ACTIVITY STUDIES

MATERIALS.

a) Animals: ♂ CFE albino, barrier-maintained rats, as described in Section II.

b) Skeletal muscle: Thigh muscles from both hind limbs were used. Attempts were made to sample specific muscles, but insufficient experimental material could be provided by single animals; frequently, the entire thigh musculature was employed.

c) Reagents: These are listed in Section II, or in appropriate 'Methods' and 'Results' paragraphs.

METHODS

a) Tissue sampling: Animals were sacrificed by cervical dislocation, the muscles quickly excised and placed into small aliquots of the extraction medium at $0-4^\circ\text{C}$. They were substantially freed of connective tissue, blotted on Whatman No. 1 filter paper and quickly weighed.

b) Preparation of microsomal fraction. The preparative sequence is summarized in FIG.III-1. The compositions of extraction media are given in 'Results'. All procedures were carried out at 0° - 4° C. Great care was taken in the resuspension of microsomes in order to obtain an homogeneous preparation.

c) Enzyme assays:

(i) Mg²⁺-ATPases

Incubations of 0.5ml aliquots of fresh or aged membrane resuspensions were made in duplicate at $37^{\circ}\pm 0.1^{\circ}$ C in the presence of 0.5ml Tris-ATP (usually of 4mM final concentration) and 1.0ml Mg²⁺, Na⁺, K⁺ in various proportions (as chlorides), made up in 50 mM Tris-HCl or L-histidine-HCl, pH 7.3 at $37^{\circ}\pm 0.5^{\circ}$ C. Orthophosphate determinations were made in duplicate as described in Section II.

Protein estimations were made for duplicate aliquots of deionized water-washed and -resuspended fresh membrane fraction, or on thawed, resuspended samples which had been stored at -20° C for not longer than 2 weeks. The technique is described in Section II.

(ii) Succinate dehydrogenase: E.C. 1.3.99.1.

Incubations of fractions obtained in the above extraction procedure were made in 10ml (nominal) Warburg flasks by the manometric technique given in Umbreit, Burris & Stauffer (1964) after Schneider & Potter (1943). Aliquots of raw homogenate or 0.1M phosphate buffer resuspensions of each pellet were assayed for their O₂ consumption in a Townson & Mercer Ltd. Warburg apparatus at shaking speed 3 and $25^{\circ}\pm 0.1^{\circ}$ C after 10min thermoequilibration. The reaction was started by addition of substrate from the side-arm.

TABLE III-3 summarizes the flask contents.

Protein estimations were made as described in Section II.

(iii) L- α -glycerophosphate dehydrogenase (α -GPDH): E.C. 1.1.2.1.

Incubation procedures were as for succinate dehydrogenase, using 0.5M DL- α -glycerophosphate (Sigma Chemical Co.) in place of disodium succinate.

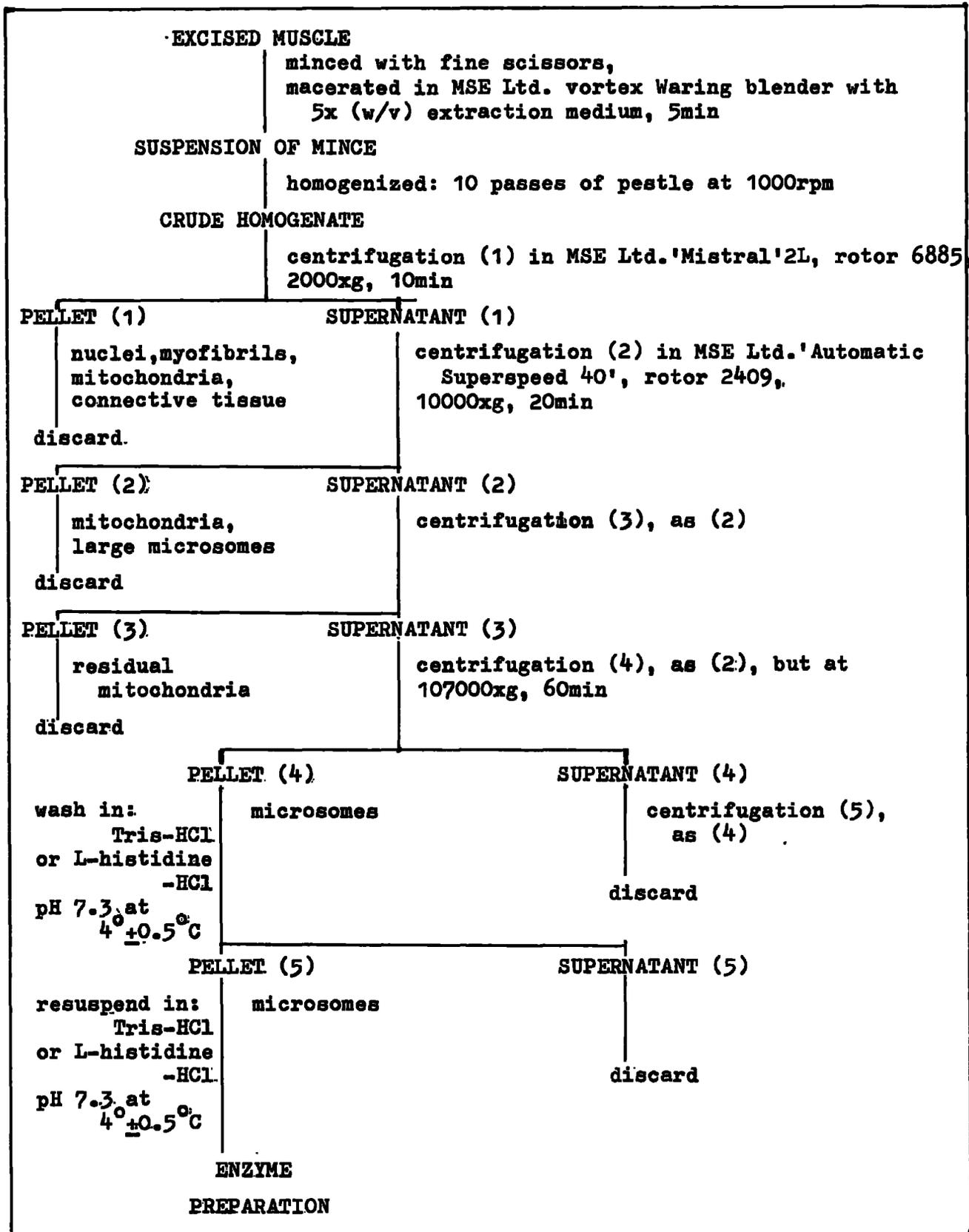


FIG. III-1. PROCEDURE FOR EXTRACTION OF SKELETAL MUSCLE MICROSOMES.

All steps were performed at 0^o-4^o C.

REAGENT	SUPPLIER	VOLUMES (ml)	
		CONTROL	EXPERIMENTAL
distilled water		3.0	0.5
0.1M phosphate buffer, pH 7.4	BDH (AnalaR)	-	1.0
0.5M disodium succinate.6H ₂ O	Sigma (grade IX)	-	0.3
1x10 ⁻⁴ M cytochrome c	Sigma (type IV)	-	0.4
4x10 ⁻³ M CaCl ₂ .6H ₂ O	BDH (AnalaR)	-	0.3
4x10 ⁻³ M AlCl ₃ .6H ₂ O	BDH (lab.)	-	0.3
pellet resuspension		-	0.2
2N NaOH (centre well)	BDH (AnalaR)	0.1	0.1

TABLE III-3. WARBURG FLASK CONTENTS FOR SUCCINATE DEHYDROGENASE AND L- α -GLYCERO-
PHOSPHATE DEHYDROGENASE ASSAYS.

Protein estimations were made as described in Section II.

B. ELECTRONMICROSCOPIC STUDY OF MEMBRANE FRACTIONS

a) Skeletal muscle fraction: These were prepared as described above.

(See FIG.III-1).

(i) 2 000-10 000xg fraction, pellet (3);

(ii) 10 000-107 000xg fraction, pellet (5).

b) Reagents: All were the purest available.

(i) 0.1M sodium cacodylate (BDH Ltd.) buffered with HCl to pH 7.4.

(ii) 5% w/v glutaraldehyde-p-aldehyde (TAAB Labs) in (i), as fixative.

(iii) 2% w/v osmic acid (BDH Ltd.), for post-fixing.

(iv) 50% - absolute ethanol (BP Chemicals Ltd.) series.

(v) propylene oxide (BDH Ltd.).

(vi) 1 part absolute ethanol/1 part propylene oxide.

(vii) Araldite: 26ml Araldite added to 24ml hardener and stirred for 10min in acetone-washed glassware.

(viii) 1 part propylene oxide/1 part Araldite.

(ix) lead citrate solution (BDH Ltd.).

METHOD

All steps were performed at 0°-4°C, unless otherwise stated. Pellets were:

(i) gently resuspended in cacodylate buffer by alternate withdrawal into and release from a Pasteur pipette;

(ii) recentrifuged at 107 000xg, and the supernatants discarded;

(iii) resuspended in fixative (as in (i)) and left for 3hr;

(iv) treated as in (ii);

(v) treated as in (iii), but left for 14hr;

(vi) washed in cacodylate buffer by gentle agitation for 10min;

(vii) post-fixed 3hr;

(viii) washed in distilled water 10 times;

(ix) dehydrated through the ethanol series, with 2 absolute ethanol changes (10min each step);

(x) placed in absolute ethanol/propylene oxide, 10min;

(xi) transferred to propylene oxide, 10min;

(xii) transferred to propylene oxide/Araldite, 1hr, 20°C;

(xiii) transferred to Araldite (1), 1hr, 20°C;

(xiv) transferred to Araldite (2), 14hr, 20°C;

(xv) embedded in Araldite (3), 48hr, 60°C;

(xvi) sectioned on a Cambridge Huxley Ultramicrotome;

(xvii) stained with 2% uranyl acetate and Reynold's lead citrate just prior to examination in an AEI EM6 electron microscope.

RESULTS

A. ENZYME ACTIVITY STUDIES

1. Mg²⁺-ATPases

a) Experiments involving different extraction media

(i) Medium consisting of:

250mM sucrose (BDH AnalaR) + 5mM disodium ethylenediaminetetraacetic acid (Na₂EDTA.2H₂O) (BDH AnalaR) + 50mM Tris(hydroxymethyl)-aminomethane - HCl (Tris - HCl) (from Sigma Chemical Co. 'Trizma' base, reagent grade) OR L-histidine - HCl (from Sigma Chemical Co. free base, Σ grade), pH 7.3 at 4_±0.5°C.

Extraction characteristics

It was found necessary to coarsely mince the muscle samples with fine scissors to provide segments that could be adequately processed in the Waring blender and homogenization steps. Pellet (1) was always large and was considered to consist of nuclear, myofibrillar, connective tissue and inadequately processed material. Pellet (2) and, where it occurred, pellet (3) were small and red-pink coloured. The final pellets (4) and (5) were

translucent and were carefully resuspended in the buffer component which was appropriate to the extraction medium used at the outset. The final supernatant was faintly straw-coloured.

Time course of ATP hydrolysis

FIG.III-2 is a plot of hydrolysis by a Tris-HCl - extracted preparation in the presence of 4mM Mg^{2+} , and its stimulation by $100\text{mM Na}^+ + 20\text{mM K}^+$. The Mg^{2+} - dependent activity did not remain linear after 9min at $37^{\circ} \pm 0.1^{\circ}\text{C}$. On the basis of this result, an incubation period of 5min was adopted for subsequent experiments.

Effects of equimolar Na^+ and K^+ in the presence of 4mM Mg^{2+}

TABLE III-4 shows the effects of 50mM Na^+ and 50mM K^+ in this context. In all instances hydrolysis was stimulated, and the values given for Na^+ and K^+ represent the extent of stimulation above the Mg^{2+} activation level. It can be seen that extraction in the presence of 50mM Tris-HCl (see preparations T1-T6) yielded the more active preparations (cf. preparations H1-H4), although the extent of stimulation was not consistent for either buffer.

Effects of Na^+ and K^+ in the absence of Mg^{2+}

In certain experiments there was sufficient microsomal material for control incubations of this kind to be made, and some ATP hydrolysis did indeed occur. When 50mM Na^+ or K^+ were tested alone or in combination, an activation occurred of the order of 10% that elicited by 4mM Mg^{2+} . The values obtained, and the respective compensations (in parentheses) for hydrolyses occurring in the presence of $\text{Mg}^{2+} + \text{Na}^+$, $\text{Mg}^{2+} + \text{K}^+$, and $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ are shown for preparations T5 and T6 in TABLE III-4.

Effects of varying $[\text{Na}^+]$ and $[\text{K}^+]$ in the presence of 4mM Mg^{2+}

In the likelihood that the effects of Na^+ or K^+ might be concentration-dependent, four experiments included incubations in which their stimulation

FIG. III-2



**FIG. III-2. TIME COURSE OF ATP HYDROLYSIS BY A RAT SKELETAL
MUSCLE MICROSOMAL PREPARATION.**

Extraction medium:

250mM sucrose + 5mM Na₂EDTA.2H₂O + 50mM Tris-HCl, pH 7.3 at 4° ± 0.5°C.

Incubation conditions:

50mM Tris-HCl + 4mM Tris-ATP, pH 7.3, 37° ± 0.1°C.

● = hydrolysis in the presence of 4mM Mg²⁺;

■ = 100mM Na⁺ + 20mM K⁺ - stimulation of hydrolysis in the presence of 4mM Mg²⁺ (shown as the increment in hydrolysis above that obtained in the presence of 4mM Mg²⁺ alone).

Ordinate:

nmoles P_i liberated/mg protein.

Values plotted are the means of those obtained in duplicate incubations.

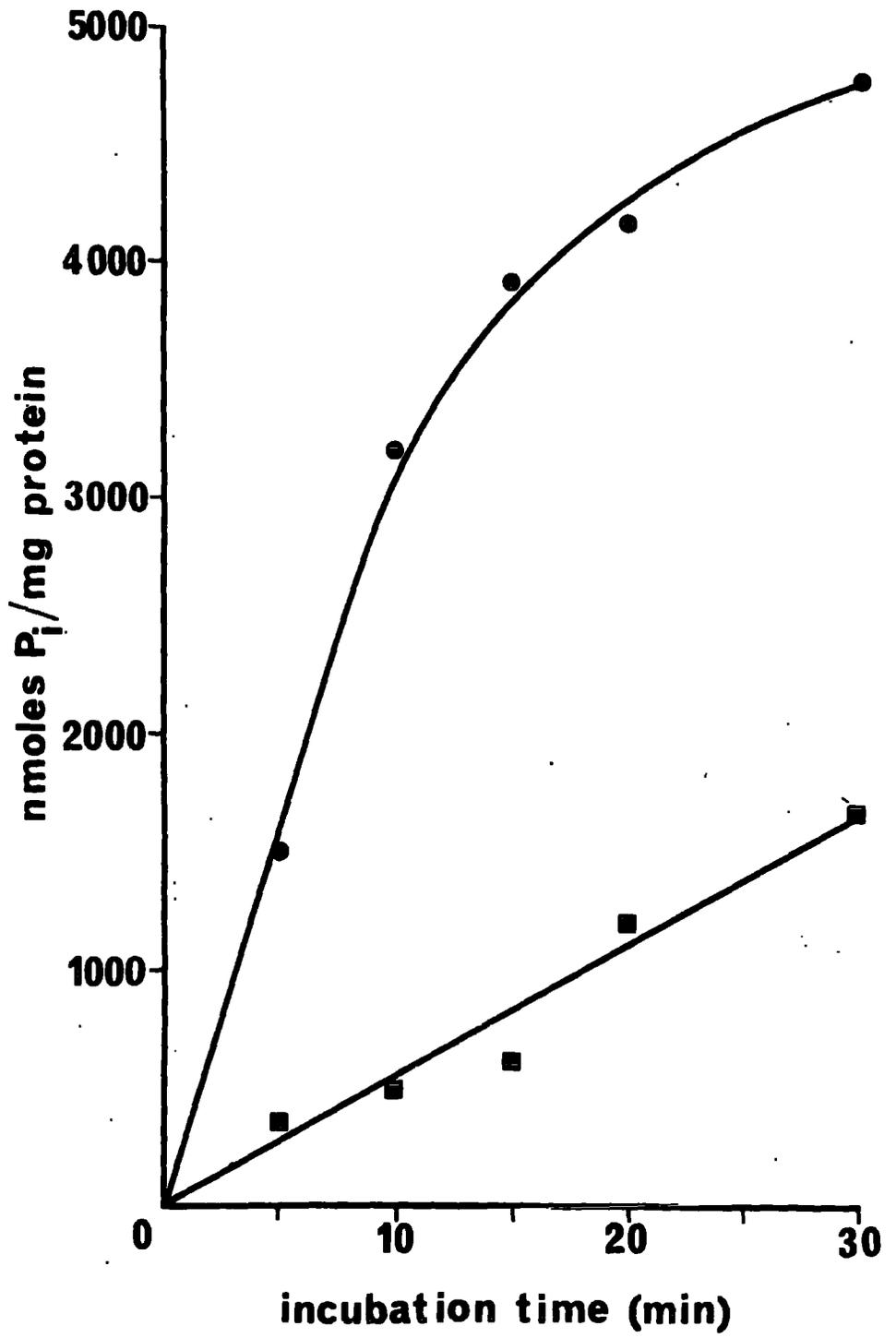


TABLE III-4

TABLE III-4. ATP HYDROLYSIS BY MEDIUM (1) EXTRACTED MICROSOMAL PREPARATIONS OF RAT SKELETAL MUSCLE.

Extraction medium:

250mM sucrose + 5mM Na₂EDTA.2H₂O + 50mM Tris-HCl (preparations T1 - T6)

or 50mM L-histidine-HCl (preparations H1 - H4), pH 7.3 at 4° ± 0.5°C.

Incubation conditions:

50mM Tris-HCl + 4mM Tris-ATP, pH 7.3, 37° ± 0.1°C.

Mg²⁺ (4mM), Na⁺ (50mM), and K⁺ (50mM) were included as indicated.

Values given are the means of those obtained in duplicate incubations. Those shown for incubations containing Na⁺ and/or K⁺ in addition to Mg²⁺ represent the increment in hydrolysis above that obtained in the presence of Mg²⁺ alone.

Values given in parentheses are derived by compensation for hydrolyses occurring in the absence of Mg²⁺.

PREPARATION	EXTRACTION METHOD	nmoles P_i liberated/mg protein/min						
		Na^+	K^+	$Na^+ + K^+$	Mg^{2+}	$Mg^{2+} + Na^+$	$Mg^{2+} + K^+$	$Mg^{2+} + Na^+ + K^+$
T1	Tris-HCl-buffered	-	-	-	5852.0	1299.0	1702.0	-
T2		-	-	-	4064.8	727.5	1191.9	1131.6
T3		-	-	-	4780.6	943.4	1106.0	1141.2
T4		-	-	-	4804.0	665.5	1275.0	1218.0
T5		497.8	583.7	622.6	5940.3	700.4	1335.9	1958.5
T6		344.0	532.2	376.4	4620.9	694.4 (202.6) (350.4)	1044.9 (752.2) (512.7)	1408.3 (1335.9) (1031.9)
H1	histidine-HCl- buffered	-	-	-	2216.8	121.8	316.7	280.1
H2		-	-	-	1608.0	99.2	268.0	228.3
H3		-	-	-	1280.0	104.0	216.0	248.0
H4		-	-	-	2257.0	0.0	503.6	503.6

TABLE III-4. ATP HYDROLYSIS BY MEDIUM (i) EXTRACTED MICROSOMAL PREPARATIONS OF RAT SKELETAL MUSCLE.

of Mg^{2+} -ATPase was more fully studied. Although the magnitude of stimulation was never consistent within the group (see above), its pattern was always the same. FIG.III-3 demonstrates this pattern as found in the Tris-HCl-extracted preparation T1, and shows that equimolar $[Na^+]$ and $[K^+]$ stimulated the Mg^{2+} -ATPase differentially: K^+ was greater than Na^+ -stimulation throughout the range tested.

When Na^+ and K^+ were present simultaneously there was no combination of concentrations at which even an additive stimulation was elicited.

FIG.III-4 summarizes the results obtained with preparation T4, in which a range of $[Na^+]$ and $[K^+]$ from 0 - 100mM was tested in a number of combinations; it shows observed and predicted (additive) values. This again was the pattern generally obtained; in no instance was there evidence of a synergistically stimulated $Na^+ + K^+ - Mg^{2+}$ -ATPase.

Effects of incubation in the presence of ouabain

Ouabain (Sigma Chemical Co.) was dissolved in 50mM Tris-HCl (pH 7.3) and included in incubations to give a final concentration of $5 \times 10^{-4} M$. It had no effect upon ATPase activity in any of the conditions reported above. Ouabain at $1 \times 10^{-3} M$ was used in two experiments and found to slightly reduce the Mg^{2+} -ATPase activity to an extent sufficient to account for the changes seen in other Mg^{2+} -containing incubations.

Effects of ageing microsomes at $-20^\circ C$

Quadruplicate samples of freshly-obtained microsomal resuspensions were stored at $-20^\circ C$. After various periods of time they were thawed in pairs at $8^\circ \pm 0.5^\circ C$ for up to 45min, resuspended by gentle homogenization at $0^\circ - 4^\circ C$, and their ATPase activities assayed in the usual manner. There was no evidence for alterations of specific activity in control samples which were frozen and immediately thawed. FIG.III-5 shows the means of replicate estimations obtained for preparation T4, so that they may be compared with those shown above in FIG.III-4. Though less extensive series of incubation

FIG. III-3



FIG. III-3. EFFECT OF [Na⁺] AND [K⁺] UPON Mg²⁺-ATPase IN RAT SKELETAL MUSCLE MICROSOMAL PREPARATION T1.

Extraction medium:

250mM sucrose + 5mM Na₂EDTA.2H₂O + 50mM Tris-HCl, pH 7.3 at 4° ± 0.5°C.

Incubation conditions:

50mM Tris-HCl + 4mM Tris-ATP + 4mM Mg²⁺, pH 7.3, 37° ± 0.1°C.

● = Na⁺ - stimulation;

■ = K⁺ - stimulation.

Ordinate:

nmoles P_i liberated/mg protein/min.

Values plotted are the means of those obtained in duplicate incubations, and represent the increment in hydrolysis above that obtained in the presence of 4mM Mg²⁺ alone.

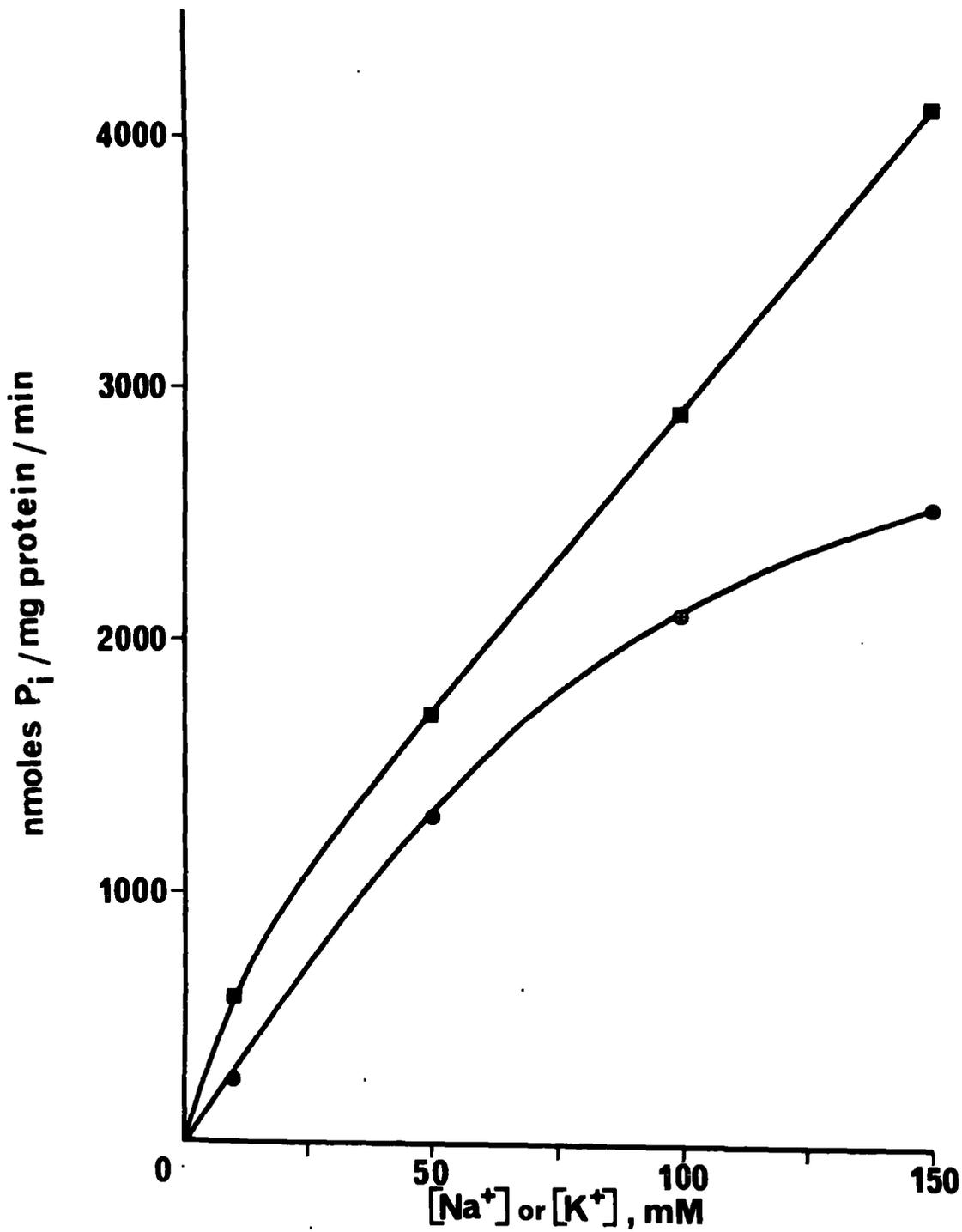


FIG. III-4



FIG. III-4. EFFECT OF Na⁺ UPON K⁺-STIMULATION OF Mg²⁺-ATPase IN RAT SKELETAL MUSCLE
MICROSOMAL PREPARATION T4.

Extraction medium:

250mM sucrose + 5mM Na₂EDTA.2H₂O + 50mM Tris-HCl, pH 7.3 at 4° ± 0.5°C.

Incubation conditions:

50mM Tris-HCl + 4mM Tris-ATP + 4mM Mg²⁺, pH 7.3, 37° ± 0.1°C.

● = observed effect;

■ = predicted effect.

Ordinate:

Values plotted are the means of those obtained in duplicate incubations, and represent the increment in hydrolysis above that obtained in the presence of 4mM Mg²⁺ alone.

FIG. III-5



FIG. III-5. EFFECT OF AGEING AT -20°C UPON Mg²⁺-ATPase AND MONO-VALENT CATION - AFFECTED Mg²⁺-ATPases IN RAT SKELETAL MUSCLE MICROSOMAL PREPARATION T₄.

Extraction medium:

250mM sucrose + 5mM Na₂EDTA.2H₂O + 50mM Tris-HCl, pH 7.3 at 4° ± 0.5°C.

Incubation conditions:

50mM Tris-HCl + 4mM Tris-ATP + 4mM Mg²⁺, pH 7.3, 37° ± 0.1°C.

A = Mg²⁺-ATPase;

B = 20mM K⁺ - stimulation

C = 50mM K⁺ - stimulation

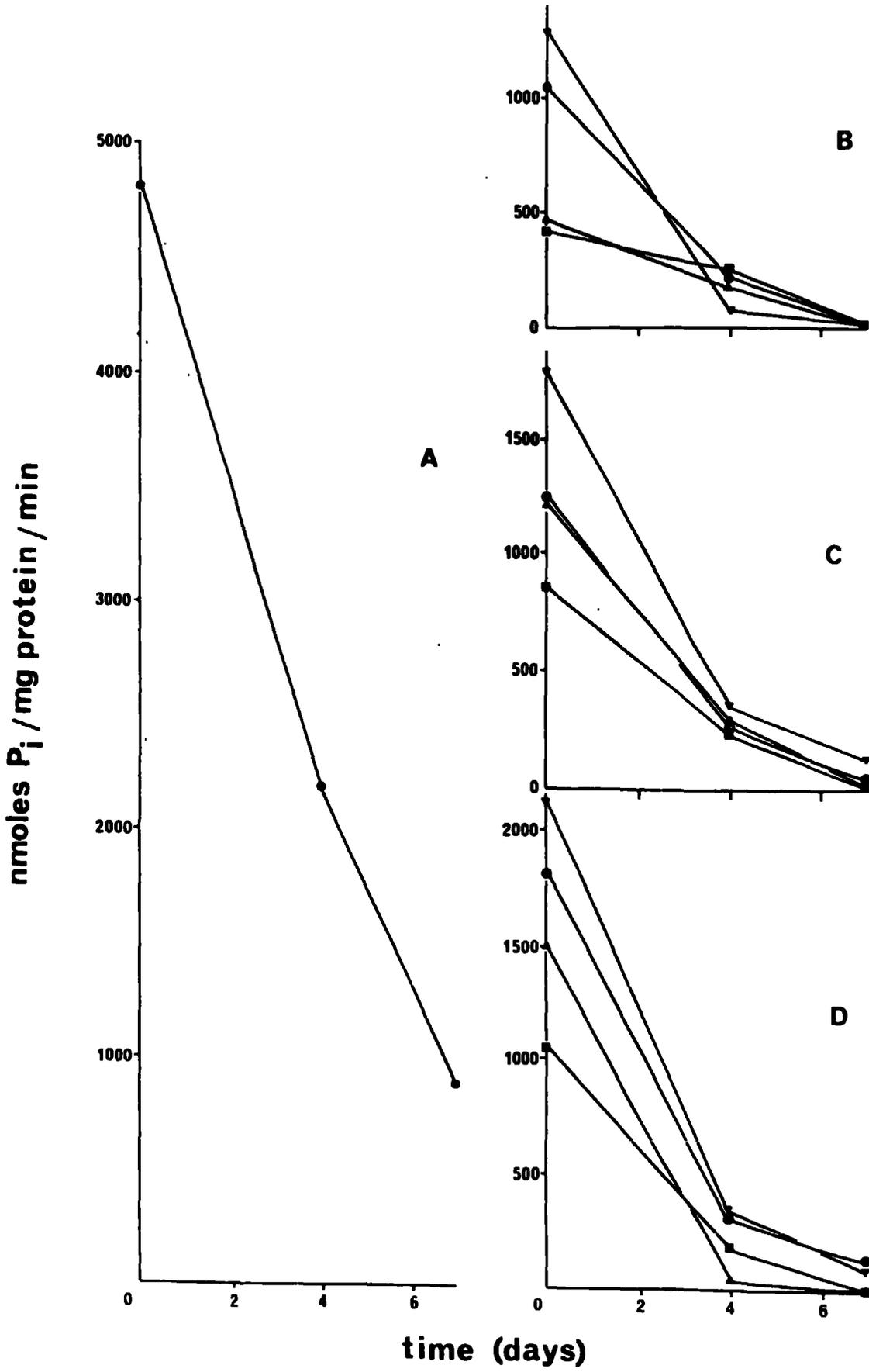
D = 100mM K⁺ - stimulation

● = 0mM Na⁺
■ = 20mM Na⁺
▲ = 50mM Na⁺
▼ = 100mM Na⁺

Ordinate:

nmoles P_i liberated/mg protein/min.

Values plotted are the means of those obtained in duplicate incubations. Those shown in conditions B - D represent the increment in hydrolysis above that obtained in the presence of 4mM Mg²⁺ alone.



conditions were employed in three other ageing studies, the trend of results was clearly similar: namely a general loss of ATPase activity, with a greater rate of decay of Mg^{2+} -dependent as compared with Na^+ and/or K^+ -stimulated Mg^{2+} -dependent activity.

Effect of incubation in the presence of sodium deoxycholate

Duplicate aliquots of the L-histidine-HCl - extracted microsomal preparation H4 (see TABLE III-4) were exposed to a very low concentration - 0.05% (1.2mM) - of Na deoxycholate (Sigma Chemical Co.). The effect was an inactivation of Mg^{2+} -ATPase by 34.1% to 1487.8 nmoles P_i liberated/mg protein/min, and a 100% reduction in monovalent cation stimulation. The experiment was preliminary to testing the reputed effectiveness of this detergent in exposing a classical $Na^+ + K^+ - Mg^{2+}$ -ATPase in muscle, a problem which is further treated below.

Effects of preincubation and subsequent incubation in the presence of phosphatidyl choline

L- α -lecithin (Sigma Chemical Co., type II E) was dispersed in aliquots of incubation media (without ATP) as described by Bachelard & Silva (1966), yielding a final concentration of 50 μ g/ml upon addition of the microsomal preparation and ATP. Aliquots of microsomal suspension from the L-histidine-HCl extraction H4 (see TABLE III-4) were exposed to the phospholipid for 30min at $0-4^\circ C$ in the presence of appropriate ions. There followed the normal thermopreincubation period of 10min at $37 \pm 0.1^\circ C$ and subsequent addition of Tris-ATP. This treatment resulted in a 4% reduction of Mg^{2+} -ATPase activity, no alteration in $Mg^{2+} + Na^+$ - or $Mg^{2+} + K^+$ -stimulation, and a 10.9% reduction in $Mg^{2+} + Na^+ + K^+$ -stimulation.

(ii) Medium consisting of:

250mM sucrose + 5mM $Na_2EDTA \cdot 2H_2O$ + 0.1% (2.4mM) sodium deoxycholate + 50mM Tris-HCl, pH 7.3 at $4 \pm 0.5^\circ C$.

Extraction characteristics and efficiency

Some difficulties presented themselves in initial experiments as a result of the frothing tendency of the deoxycholate medium during maceration and homogenization, and it was feared that some material may have been lost or poorly processed. Early experiments are therefore not considered, and the results set out here refer to those in which the extraction had been satisfactorily standardised. The final pellets (4) and (5) were not translucent, but red-pink in colour.

The extraction efficiency discussed here is a measure of the microsomal protein yield per unit wet weight of muscle employed in an extraction. It is calculated for a group of 12 extractions using medium (i) and compared with that derived for 4 medium (ii) type preparations. From the known wet weight of fresh muscle used for an extraction and the estimated weight of protein finally obtained (by analysis of the final microsomal suspension), values for protein yield in μg microsomal protein/mg wet weight of fresh tissue were derived and compared. The calculated extraction efficiencies are set out in TABLE III-5. It is clear from the application of Student's 't' test that significantly more microsomal protein is yielded by extraction in medium (ii).

Effects of equimolar Na^+ and K^+ in the presence and absence of Mg^{2+}

TABLE III-6 summarizes the results obtained for 4 preparations D1-D4. Mg^{2+} -dependent hydrolysis was stimulated by Na^+ and K^+ in all instances, although, for preparation D2, the apparent Na^+ -stimulation could be entirely accounted for by Na^+ -activated hydrolysis in the absence of Mg^{2+} . Indeed, in this case there was a depression of Mg^{2+} -activity. However, this effect was not obtained in the remaining preparations, and was not further investigated. As implied above, and previously discovered in the preparations T1 - T6 extracted in medium (i), some 50mM Na^+ - and/or 50mM K^+ -stimulated hydrolysis occurred in the absence of Mg^{2+} . The levels were of the order of 12% of the 4mM Mg^{2+} activation level.

EXTRACTION MEDIA	EXTRACTION EFFICIENCY (µg/mg)	
	(i)	(ii)
\bar{x}	1.309	4.570
n	12	4
t.	8.76	
P	< 0.001	

TABLE III-5. COMPARISON OF EXTRACTION EFFICIENCIES IN MEDIA (i) AND (ii) FOR RAT SKELETAL MUSCLE.

Extraction media:

(i) 250mM sucrose + 5mM $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ + 50mM Tris-HCl, pH 7.3 at $4^\circ \pm 0.5^\circ\text{C}$.

(ii) 250mM sucrose + 5mM $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ + 0.1% (2.4mM) sodium deoxycholate + 50mM Tris-HCl, pH 7.3 at $4^\circ \pm 0.5^\circ\text{C}$.

Extraction efficiency:

µg microsomal protein obtained/mg wet weight of fresh muscle.

TABLE III-6. ATP HYDROLYSIS BY MEDIUM (ii) EXTRACTED PREPARATIONS OF RAT SKELETAL MUSCLE.

Extraction medium:

250mM sucrose + 5mM Na₂EDTA.2H₂O + 0.1% Na deoxycholate + 50mM Tris-HCl, pH 7.3 at 4° ± 0.5°C.

(Preparations D1. - D4.)

Incubation conditions:

50mM Tris-HCl + 4mM Tris-ATP, pH 7.3, 37° ± 0.1°C.

Mg²⁺ (4mM), Na⁺ (50mM) and K⁺ (50mM) were included as indicated.

Values given are the means of those obtained in duplicate incubations. Those shown for incubations containing Na⁺ and/or K⁺ in addition to Mg²⁺ represent the increment in hydrolysis above that obtained in the presence of Mg²⁺ alone.

Values given in parentheses are derived by compensation for hydrolysis occurring in the absence of Mg²⁺.

PREPARATION.	nmoles P_i liberated/mg protein/min						
	Na^+	K^+	$Na^+ + K^+$	Mg^{2+}	$Mg^{2+} + Na^+$	$Mg^{2+} + K^+$	$Mg^{2+} + Na^+ + K^+$
D1	-	-	-	3449.0	667.4	1094.0	898.6
D2	396.0	528.0	386.0	3200.0	302.0	676.0	678.0
					(-94.0)	(148.0)	(292.0)
D3	394.2	471.2	425.0	3884.8	643.7	1044.1	1074.9
					(249.5)	(572.9)	(649.9)
D4	279.4	344.1	264.6	2425.4	460.7	693.8	771.5
					(181.3)	(349.7)	(506.9)

TABLE III-6. ATP HYDROLYSIS BY MEDIUM (i) EXTRACTED PREPARATIONS OF RAT SKELETAL MUSCLE.

In comparing the activities obtained under standard incubation conditions for preparations extracted in media (i) and (ii), application of Student's 't' test to data from TABLE III-4 and TABLE III-6 suggests that the monovalent cation affected -Mg^{2+} -ATPase activities are significantly different. TABLE III-7 is a summary of the compared values for Tris-HCl - buffered extractions.

$[\text{Na}^+]$ and $[\text{K}^+]$ ranges from 0-100mM were tested singly and in combination in the presence of 4mM Mg^{2+} for preparation D1, and the ATP hydrolyses obtained are plotted in FIG.III-6 together with predicted (additive) values. There was no evidence of a synergistically - stimulated $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+}$ -ATPase; the pattern obtained resembles that for preparations extracted in medium (i) (c.f.FIG.III-4).

Effects of incubation in the presence of ouabain

Ouabain was added to incubation media (as before) in all experiments to give a final concentration of 5×10^{-4} M. It had a slight inhibitory effect upon K^+ -, $\text{Na}^+ + \text{K}^+$ - and $\text{Mg}^{2+} + \text{Na}^+$ -mediated hydrolyses. However, a considerable effect was noted under $\text{Mg}^{2+} + \text{K}^+$ and $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ conditions, with reductions of the order of 60-70% and 40-50% respectively. FIG.III-7 illustrates this effect in preparation D2. This result is in marked contrast with that obtained for medium (i) extracts.

Effects of ageing of microsomes at -20°C

Quadruplicate samples of freshly-prepared microsomal fraction D1 were treated as for the ageing procedure involving extraction medium (i) type preparations, and ATP hydrolysis assayed in duplicate thawed samples after 1 and 3 days. FIG.III-8 shows the mean results obtained. On day 1, Mg^{2+} -ATPase activity had fallen to 16.5%, the $\text{Mg}^{2+} + 100\text{mM } \text{K}^+ + 50\text{mM } \text{Na}^+$ -stimulated increment to 15.3%, and all other ionic conditions showed a fall to within the range 21.2 - 24.5% as compared with day 0 activities. Preparations thawed on day 3 were devoid of ATPase activity. This result may be compared

TABLE III-7. COMPARISON OF ATP HYDROLYSIS BY RAT SKELETAL MUSCLE MICROSOMAL PREPARATIONS,
USING EXTRACTION MEDIA (i) AND (ii).

Extraction media:

(i) 250mM sucrose + 5mM Na₂EDTA.2H₂O + 50mM Tris-HCl, pH 7.3 at 4° ± 0.5°C;

(ii) 250mM sucrose + 5mM Na₂EDTA.2H₂O + 0.1% Na deoxycholate + 50mM Tris-HCl, pH 7.3 at 4° ± 0.5°C.

Incubation conditions:

50mM Tris-HCl + 4mM Tris-ATP, pH 7.3, 37° ± 0.1°C.

Mg²⁺ (4mM), Na⁺ (50mM) and K⁺ (50mM) were included as indicated.

Values given are derived from data given in TABLES III-4 and III-6 for preparations T1 - T6 and D1 - D4 respectively.

IONS	nmoles P_i liberated/mg protein/min							
	Mg^{2+}		$Mg^{2+}+Na^+$		$Mg^{2+}+K^+$		$Mg^{2+}+Na^++K^+$	
EXTRACTION MEDIA.	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)
\bar{x}	5011.0	3240.0	838.3	518.7	1276.0	887.0	1411.6	856.0
n	6	4	6	4	6	4	5	4
t	2.12		20.6		27.9		20.2	
P	> 0.05		< 0.001		< 0.001		< 0.001	

TABLE III-7. COMPARISON OF ATP HYDROLYSIS BY RAT SKELETAL MUSCLE MICROSOMAL PREPARATIONS, USING EXTRACTION MEDIA (i) AND (ii).

FIG. III-6

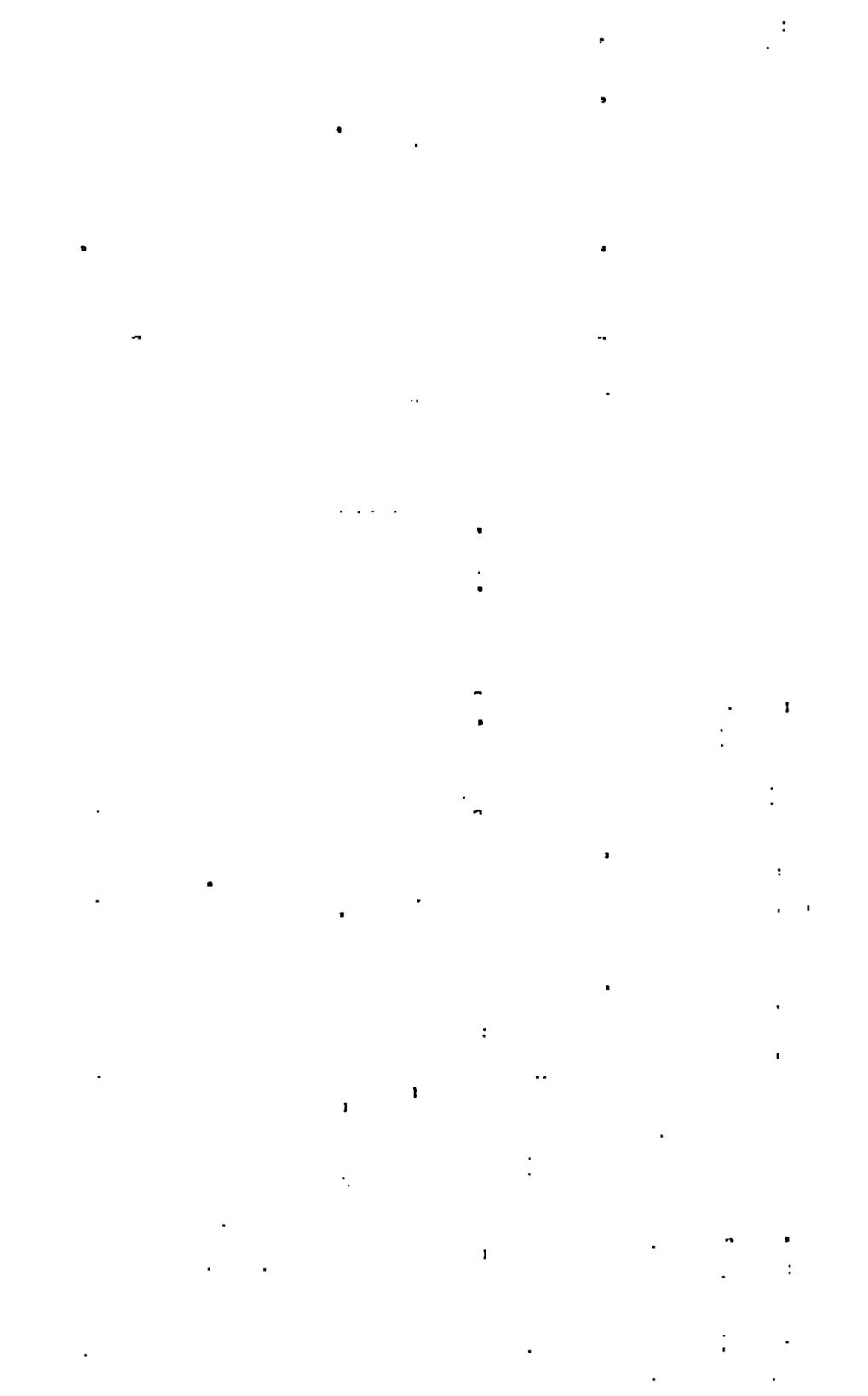


FIG. III-6. EFFECT OF $[Na^+]$ AND $[K^+]$ UPON Mg^{2+} -ATPase IN RAT SKELETAL MUSCLE MICROSOMAL PREPARATION D1, (I) SINGLY AND (II) IN COMBINATION.

Extraction medium:

250mM sucrose + 5mM $Na_2EDTA \cdot 2H_2O$ + 0.1% Na deoxycholate + 50mM Tris-HCl, pH 7.3 at $4^{\circ} \pm 0.5^{\circ}C$.

Incubation conditions:

50mM Tris-HCl + 4mM Tris-ATP + 4mM Mg^{2+} , pH 7.3, $37^{\circ} \pm 0.1^{\circ}C$.

I [$\bullet = Na^+$ - stimulation;
 $\blacksquare = K^+$ - stimulation.

II [$\bullet =$ observed effect;
 $\blacksquare =$ predicted effect.

Ordinate:

nmoles P_i liberated/mg protein/min.

Values plotted are the means of those obtained in duplicate incubations, and represent the increment in hydrolysis above that obtained in the presence of 4mM Mg^{2+} alone.

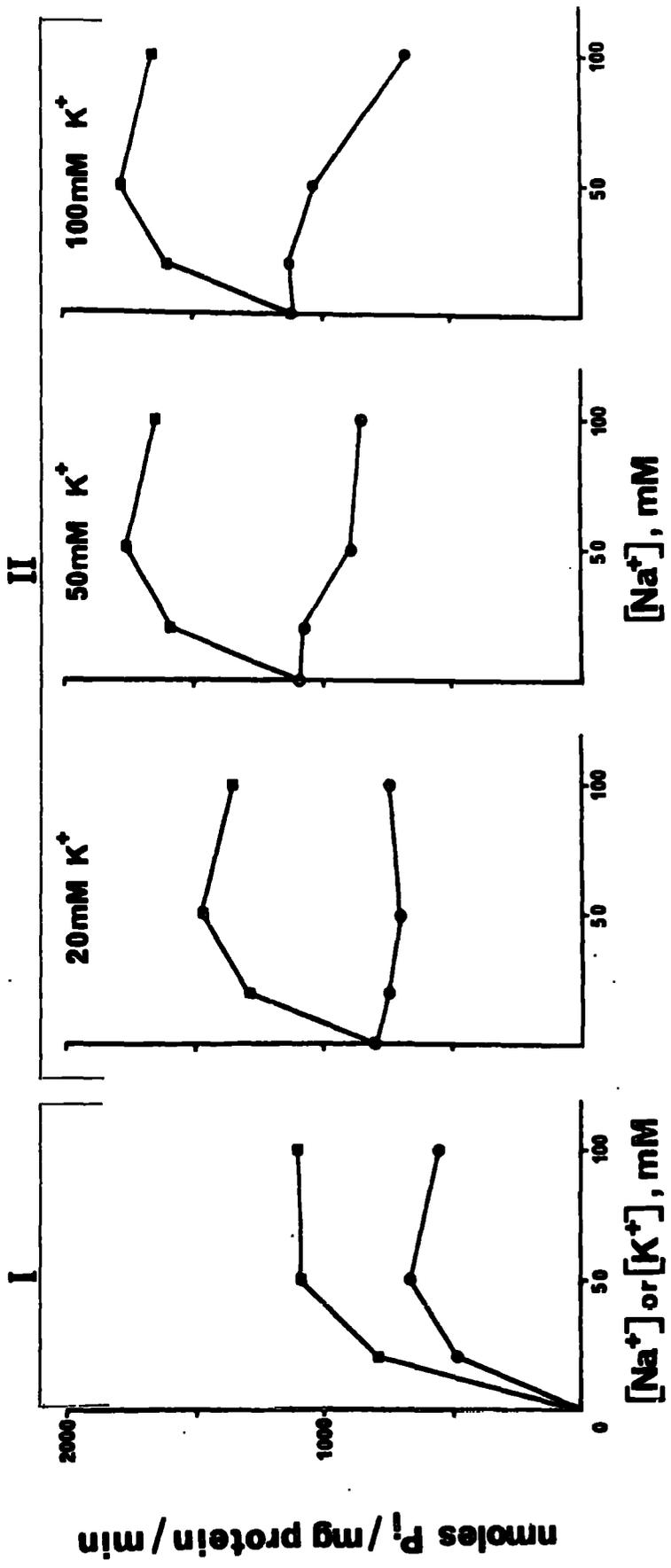


FIG. III-7



FIG. III-7. EFFECT OF 5×10^{-4} M OUABAIN UPON ATP HYDROLYSIS IN THE PRESENCE OF Mg^{2+} , Na^+ AND K^+ FOR RAT SKELETAL MUSCLE MICROSOMAL PREPARATION D2.

Extraction medium:

250mM sucrose + 5mM $Na_2EDTA \cdot 2H_2O$ + 0.1% Na deoxycholate + 50mM Tris-HCl, pH 7.3 at $4 \pm 0.5^\circ C$.

Incubation conditions:

50mM Tris-HCl + 4mM Tris-ATP, pH 7.3, $37 \pm 0.1^\circ C$.

 = control;

 = + ouabain.

Ordinate:

nmoles P_i liberated/mg protein/min.

Values plotted are the means of those obtained in duplicate incubations. Those shown for incubations containing Na^+ and/or K^+ represent the increment in hydrolysis above that obtained in the presence of Mg^{2+} alone.

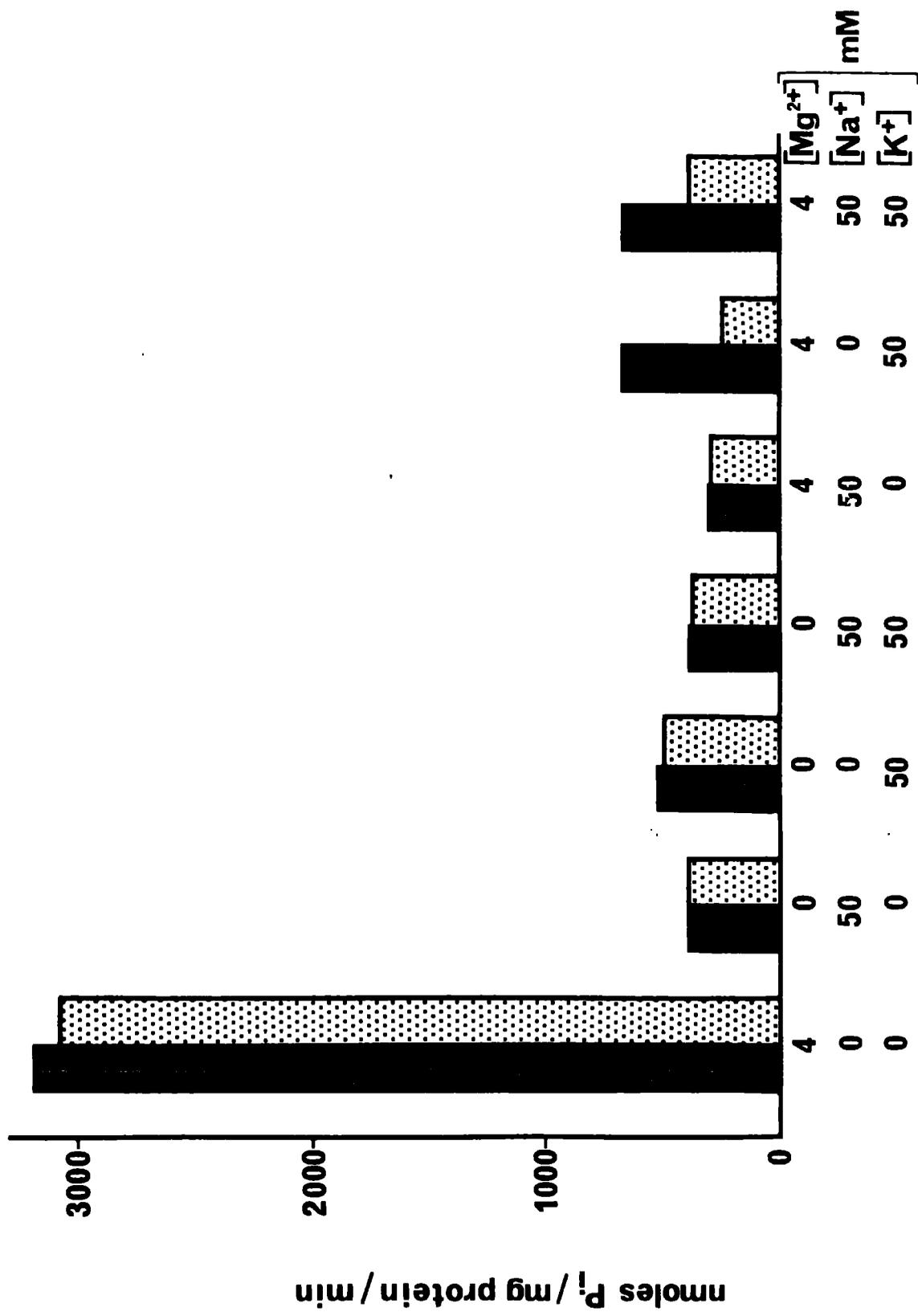


FIG. III-8



FIG. III-8. EFFECT OF AGEING AT -20°C UPON Mg^{2+} -ATPase AND MONO-
VALENT CATION - AFFECTED Mg^{2+} -ATPases IN RAT SKELETAL MUSCLE
MICROSOMAL PREPARATION D1.

Extraction medium:

250mM sucrose + 5mM $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ + 0.1% Na deoxycholate +
50mM Tris-HCl, pH 7.3 at $4^{\circ}\pm 0.5^{\circ}\text{C}$.

Incubation conditions:

50mM Tris-HCl + 4mM Tris-ATP + 4mM Mg^{2+} , pH 7.3, $37^{\circ}\pm 0.1^{\circ}\text{C}$.

A = Mg^{2+} -ATPase;

B = 50mM K^{+} - stimulation
C = 100mM K^{+} - stimulation

+

● = 0mM Na^{+}

▲ = 50mM Na^{+}

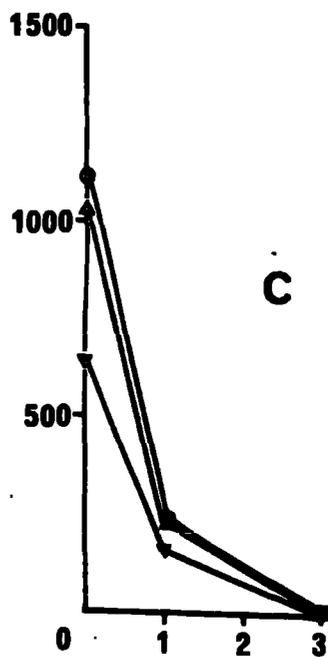
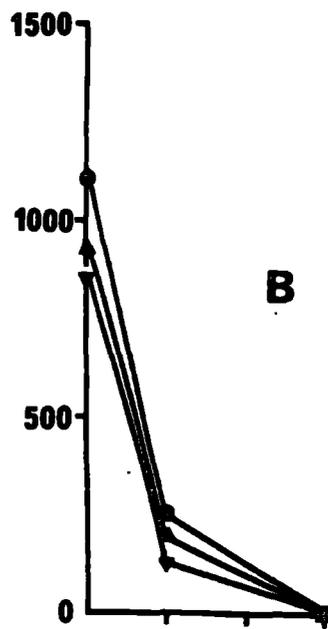
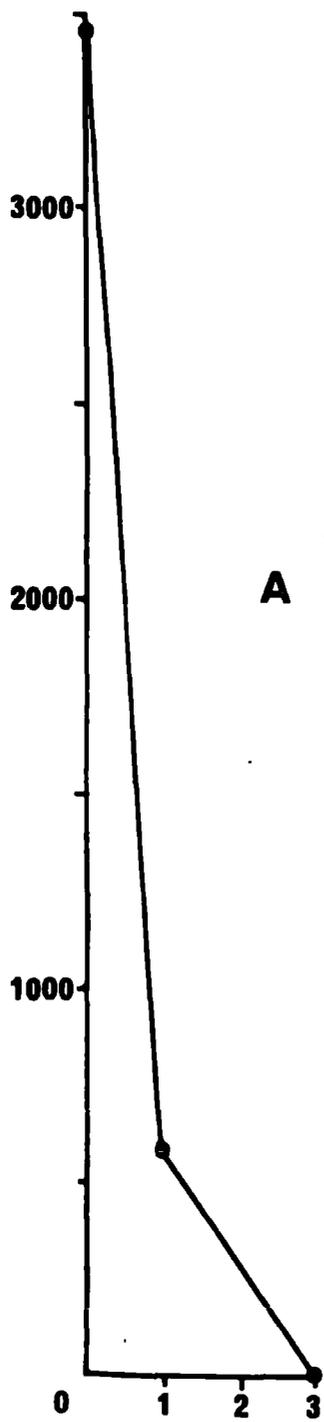
▼ = 100mM Na^{+}

Ordinate:

nmoles P_i liberated/mg protein/min.

Values plotted are the means of those obtained in duplicate incubations. Those shown in conditions B and C represent the increment in hydrolysis above that obtained in the presence of 4mM Mg^{2+} alone.

nmoles P_i / mg protein / min



time (days)

with the slower rate of decay with preparations extracted in medium (i) (see FIG.III-5).

Effects of incubation in the presence of sodium azide

Duplicate aliquots of the microsomal preparations D3 and D4 were incubated in the presence of 5mM NaN_3 (BDH Lab. grade). The effect of this treatment, as compared with control hydrolyses, is summarized in TABLE III-8 for 4mM Mg^{2+} , 50mM Na^+ , and 50mM K^+ alone and in combination. For each preparation there was a marked stimulation of Na^+ -, K^+ -, and Na^+K^+ -mediated hydrolyses and fall in Mg^{2+}K^+ -mediated hydrolysis. Values given in parentheses are derived by compensation for hydrolyses in the presence of Na^+ , K^+ , or Na^+K^+ . The Mg^{2+} -ATPase was comparatively unaffected.

(iii) Medium consisting of:

250mM D-mannitol (BDH biochemical) + 5mM $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ + 50mM Tris-HCl, pH 7.3 at $4^{\circ}\pm 0.5^{\circ}\text{C}$.

Extraction characteristics

These were as for extractions in medium (i).

Effects of equimolar Na^+ and K^+ in the presence of 4mM Mg^{2+} , and the influence of incubation with ouabain

The levels of ATP hydrolysis obtained in these circumstances are set out in FIG.III-9, and are the means of duplicate assays of samples from a single extraction. 50mM Na^+ , 50mM K^+ , and $5 \times 10^{-4}\text{M}$ ouabain were tested throughout. The pattern of stimulation resembled that seen in preparations extracted in media (i) and (ii), but the specific activity was considerably lowered. Though a synergistically - stimulated $\text{Na}^+\text{K}^+\text{Mg}^{2+}$ -ATPase was not revealed, ouabain did inhibit Mg^{2+} -, $\text{Mg}^{2+}\text{Na}^+$ -, Mg^{2+}K^+ -, and $\text{Mg}^{2+}\text{Na}^+\text{K}^+$ -stimulated activity by 12.5%, 47.7%, 6.0% and 49.1% respectively.

TABLE III-8

TABLE III-8. THE EFFECT OF SODIUM AZIDE UPON ATP HYDROLYSIS BY TWO MEDIUM (ii) EXTRACTED RAT SKELETAL MUSCLE MICROSOMAL PREPARATIONS (D3 AND D4).

Extraction medium:

250mM sucrose + 5mM Na₂EDTA.2H₂O + 0.1% Na deoxycholate + 50mM Tris-HCl, pH 7.3 at 4^o±0.5^oC.

Incubation conditions:

50mM Tris-HCl + 4mM Tris-ATP, pH 7.3, 37^o±0.1^oC.

Mg²⁺ (4mM), Na⁺ (50mM), K⁺ (50mM) and NaN₃ (5mM) were included as indicated.

Values given are the means of those obtained in duplicate incubations. Those shown for incubations containing Na⁺ and/or K⁺ in addition to Mg²⁺ represent the increment in hydrolysis above that obtained in the presence of Mg²⁺ alone.

Values given in parentheses are derived by compensation for hydrolysis occurring in the absence of Mg²⁺.

PREPARATION	INCUBATION CONDITIONS	nmoles P _i liberated/mg protein/min						
		Na ⁺	K ⁺	Na ⁺ +K ⁺	Mg ²⁺	Mg ²⁺ +Na ⁺	Mg ²⁺ +K ⁺	Mg ²⁺ +Na ⁺ +K ⁺
D3	CONTROL	394.2	471.2	425.0	3884.8	643.7 (249.5)	1044.1 (572.9)	1074.9 (649.9)
	5mM. NaN ₃	474.3	548.2	498.1	3751.4	528.1 (107.8)	807.0 (258.8)	1056.4 (558.3)
	% CHANGE	+20.3	+16.3	+17.2	-3.5	-9.6 (-56.8)	-22.8 (-54.9)	-1.8 (-10.1)
D4	CONTROL	279.4	344.1	264.6	2425.4	460.7 (181.3)	693.8 (349.7)	771.5 (506.9)
	5mM NaN ₃	343.6	403.3	323.8	2580.7	462.5 (118.9)	589.8 (186.5)	767.8 (444.0)
	% CHANGE	+22.9	+17.2	+22.3	+6.4	+0.3 (-34.5)	-15.0 (-46.7)	-0.5 (-12.5)

TABLE III-8. THE EFFECT OF SODIUM AZIDE UPON ATP HYDROLYSIS BY TWO MEDIUM (i) EXTRACTED RAT SKELETAL MUSCLE MICROSOMAL PREPARATIONS (D3 AND D4).

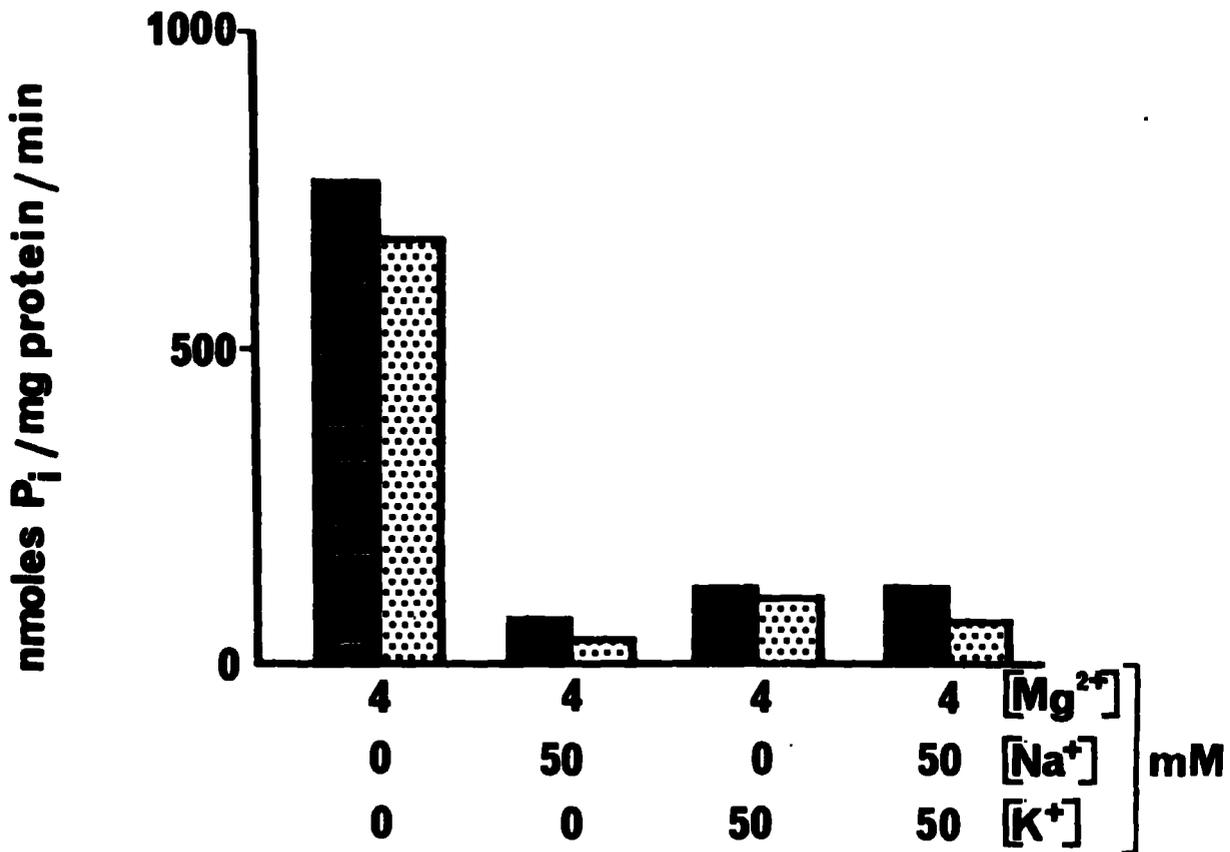


FIG. III-9. EFFECT OF 5×10^{-4} M OUABAIN UPON ATP HYDROLYSIS IN THE PRESENCE OF Mg^{2+} , Na^+ AND K^+ FOR A D-MANNITOL-EXTRACTED RAT SKELETAL MUSCLE MICROSOMAL PREPARATION.

Extraction medium:

250mM D-mannitol + 5mM $Na_2EDTA \cdot 2H_2O$ + 50mM Tris-HCl, pH 7.3 at $4^\circ \pm 0.5^\circ C$.

Incubation conditions:

50mM Tris-HCl + 4mM Tris-ATP, pH 7.3, $37^\circ \pm 0.1^\circ C$.

Mg^{2+} , Na^+ and K^+ were included, as indicated.

Ordinate:

nmoles P_i liberated/mg protein/min. = control; = + ouabain

Values plotted are the means of those obtained in duplicate incubations. Those shown for incubations containing Na^+ and/or K^+ represent the increment in hydrolysis above that obtained in the presence of Mg^{2+} alone.

2. Succinate dehydrogenase

As a test for the presence of mitochondrion-derived material, fractions from a routine preparative sequence in 50mM Tris-HCl - medium (i) were incubated in the presence and absence of 0.5M disodium succinate and 1×10^{-4} M cytochrome c, and their O_2 consumption measured as described in 'Materials and Methods'. The extraction proceeded to the pellet (4) stage, omitting the final wash. Protein estimates were made for the crude homogenate and for the pellet resuspensions.

Negligible endogenous gas uptake was observed, and the levels of succinate-stimulated uptake for each fraction are shown in FIG.III-10, being expressed as $\mu l O_2$ uptake/mg protein/hr (except for the supernatant (4) fraction, for which specific quantification cannot be made on this basis). A residual mitochondrial pellet (3) was not formed. Gas uptake occurred in all fractions tested, a result which suggested that (i) some mitochondria had been disrupted in the extraction procedure, the resultant fragments having contaminated final fractions, or (ii) succinate dehydrogenase was vulnerable to solubilization under these conditions. In the circumstances, a second test for the presence of mitochondria was considered appropriate.

3. L- α -glycerophosphate dehydrogenase (α -GPDH)

Assays were made with fractions from two preparations G1 and G2. There was no endogenous gas uptake, and the levels of stimulation by 0.5M DL- α -glycerophosphate are shown in FIG.III-11. Residual mitochondrial pellets (3) were not formed. In the case of this enzyme, activity was not found in post-mitochondrial fractions, and this was taken to suggest that (i) mitochondria, or their derivatives, were absent in pellet (4) and supernatant (4), or (ii) if mitochondrial derivatives were present, they were not sufficiently integrated for α -GPDH activity to be seen under the prevailing incubation conditions.

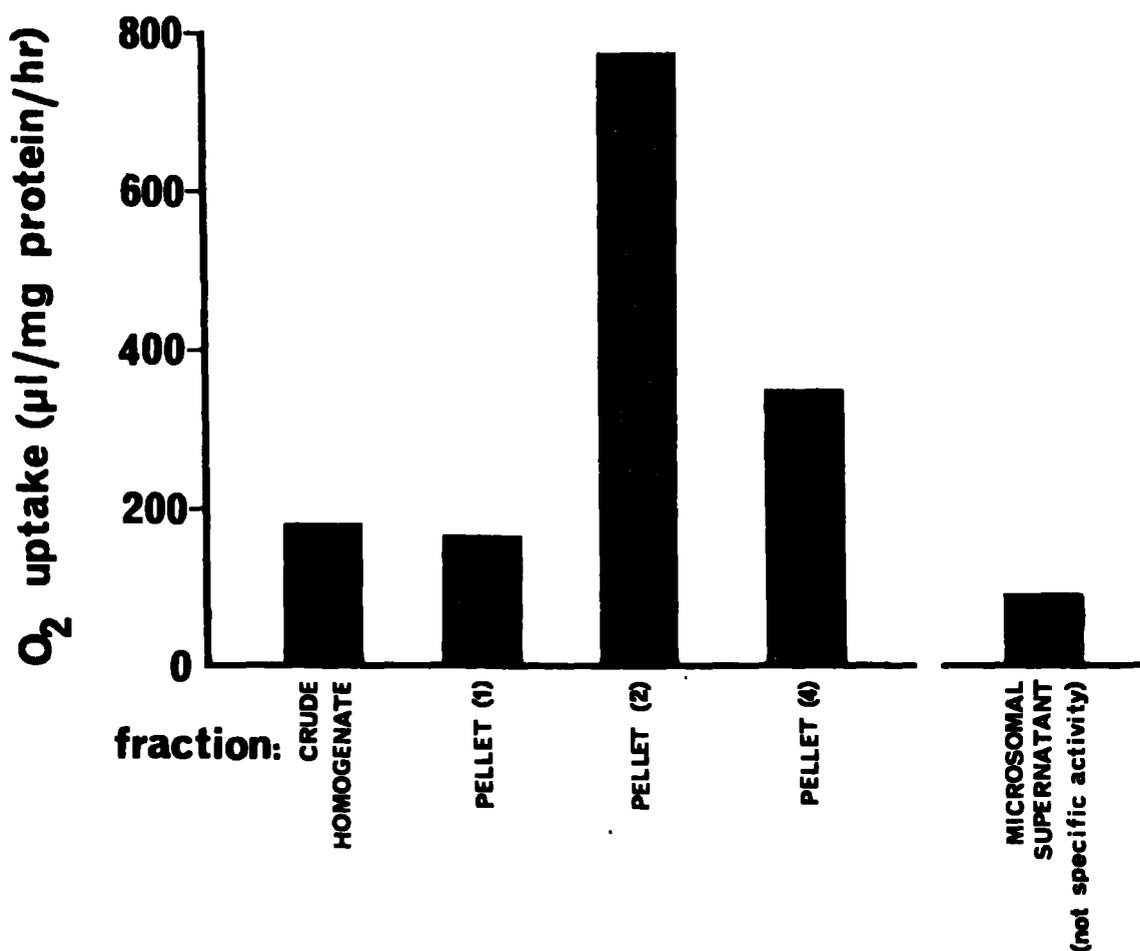


FIG. III-10. SUCCINATE DEHYDROGENASE ACTIVITY IN RAT SKELETAL MUSCLE FRACTIONS.

Extraction medium:

250mM sucrose + 5mM Na₂EDTA.2H₂O + 50mM Tris-HCl, pH 7.3 at 4^o ± 0.5^oC.

Incubation conditions:

See 'Materials and Methods', FIG. III-1, and TABLE III-3.

Ordinate:

O₂ uptake in µl/mg protein/hr.

Values plotted are those obtained in single incubations.

FIG. III-11

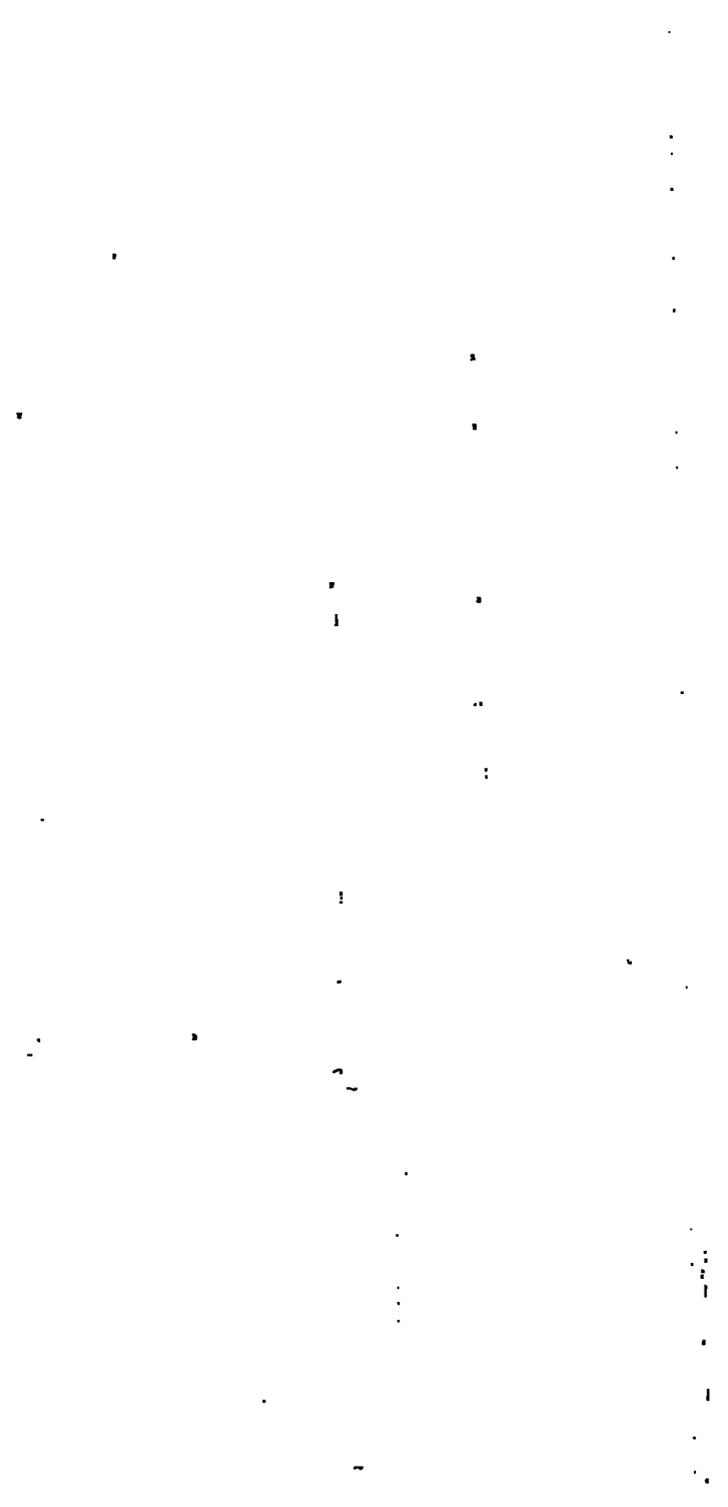


FIG. III-11. L- α -GLYCEROPHOSPHATE DEHYDROGENASE ACTIVITY IN RAT SKELETAL MUSCLE FRACTIONS OBTAINED FROM TWO PREPARATIONS (G1 AND G2).

Extraction medium:

250mM sucrose + 5mM Na₂EDTA.2H₂O + 50mM Tris-HCl, pH 7.3 at 4^o ± 0.5^oC.

Incubation conditions:

See 'Materials and Methods', FIG. III-1: and TABLE III-3.

Ordinate:

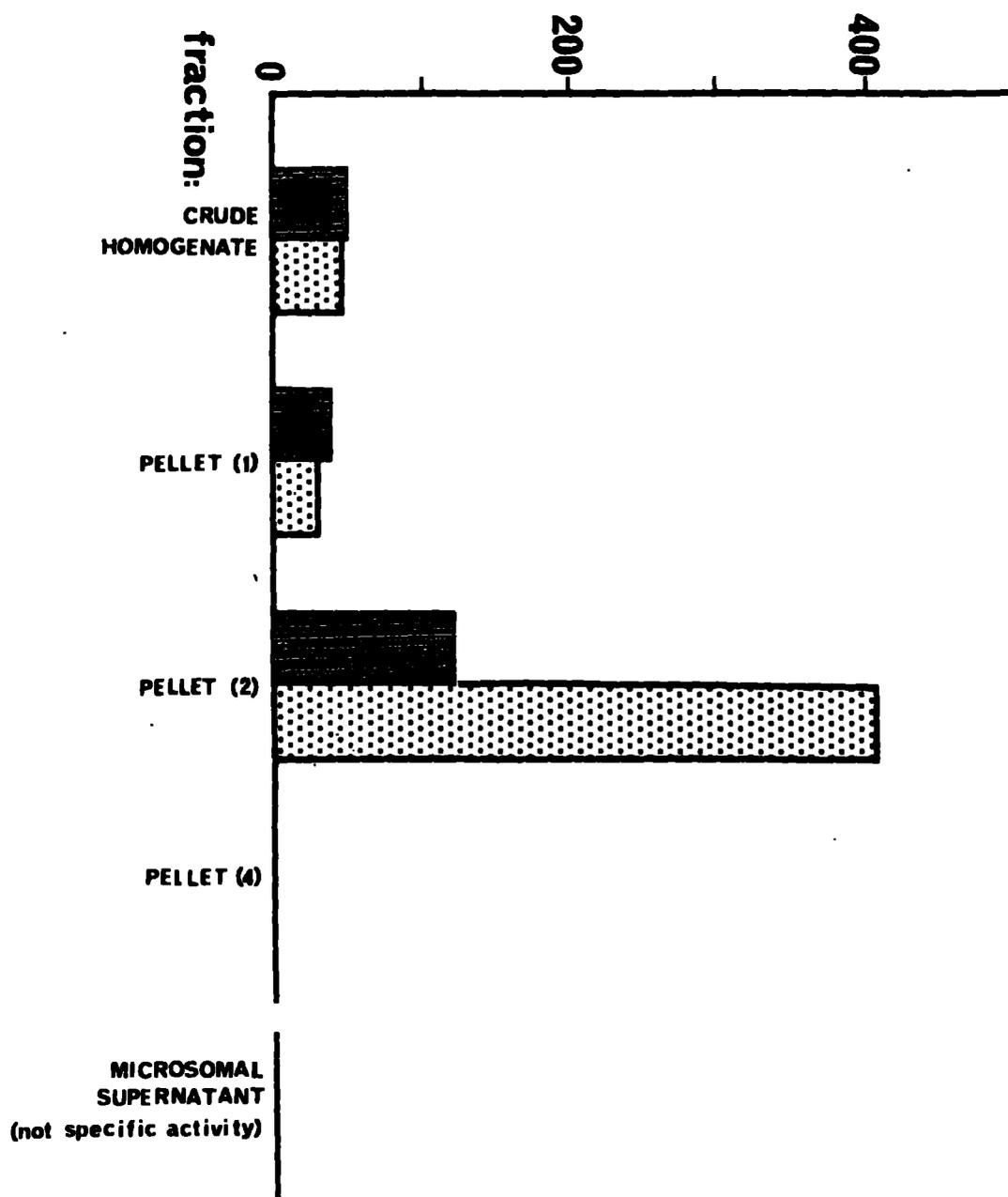
O₂ uptake in ul/mg protein/hr.

Values obtained in single incubations are plotted for the two preparations.

 = preparation G1;

 = preparation G2.

O₂ uptake (μl/mg protein/hr)



It was evident from these results, and those for succinate dehydrogenase, that a non-enzymatic (e.g. histological) test for contamination of final fractions by mitochondrial material would be advisable.

B. ELECTRONMICROSCOPIC STUDY OF MEMBRANE FRACTIONS

1. 2 000-10 000xg Fraction; Pellet (3) (see FIG.III-1)

FIG.III-12 is an electronmicrograph showing the general appearance of this material. The fraction consists of:

(a) mitochondria with diameters in the approximate range 0.25-0.70 μ

(i) the majority of which appear to be intact, with outer and inner bounding membranes

(ii) some of which (labelled D) show indications of disruption through rupture of the outer membrane;

(b) vesicles with diameters

(i) $< 0.25\mu$, devoid of inclusions, and occasionally forming aggregates (A)

(ii) of mitochondrial dimensions (labelled V), and occasionally containing what appear to be the remnants of cristae and matrix, but devoid of outer membrane.

FIG.III-13 shows intact mitochondria at higher magnifications, and may be compared with FIG.III-14, which shows a type (ii) vesicle with contents (C). A feature which is highlighted in this figure is the almost complete loss of the outer, probably mitochondrial, membrane.

FIG.III-15 shows an aggregation of small vesicles at high magnifications: these bear resemblance to mitochondrial cristae and the area is indeed partially bounded by a single membrane (m).

There is thus evidence for the presence of membrane vesicles in this fraction, and that a proportion of these are fragments of mitochondrial

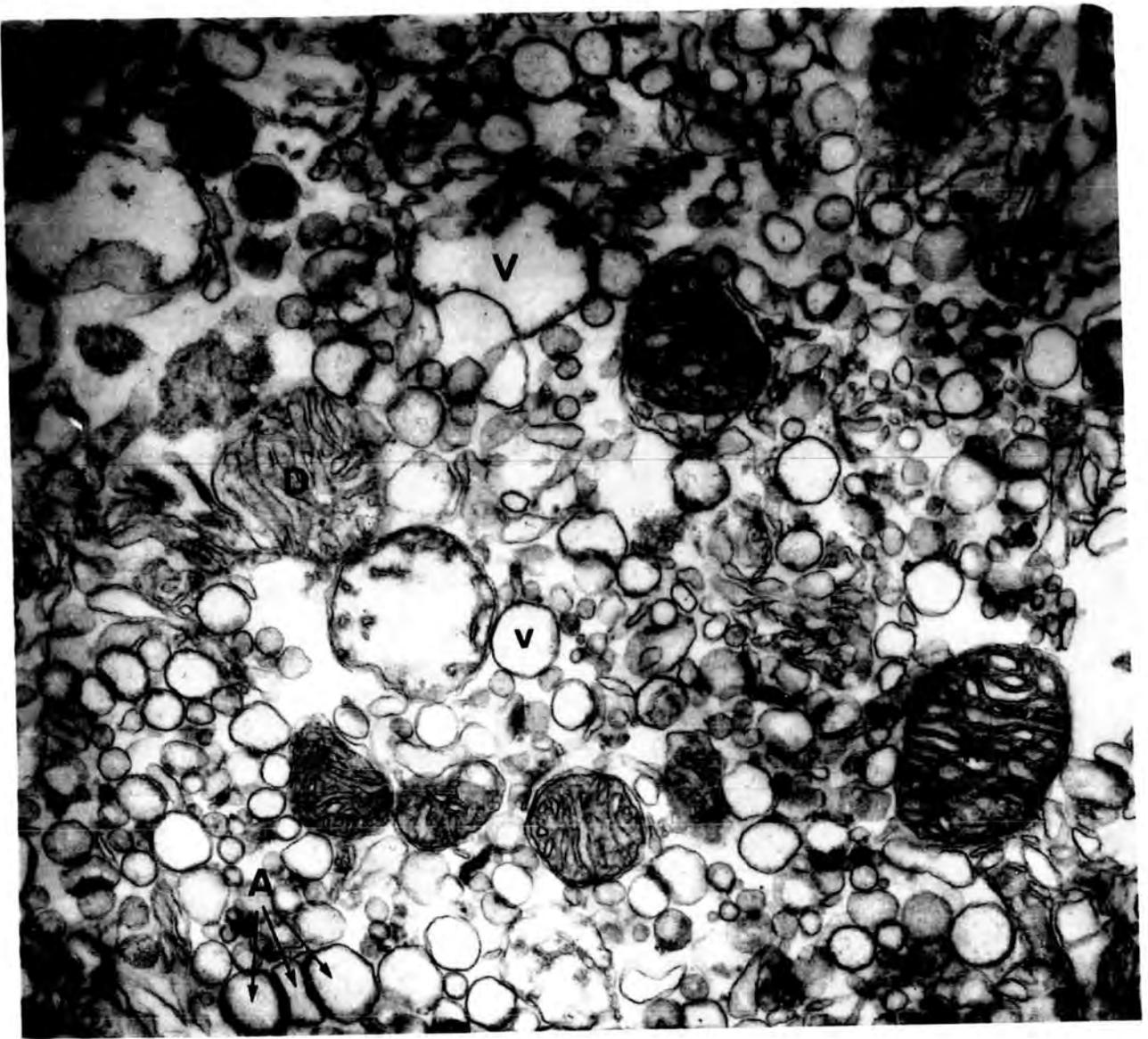


FIG. III-12. ELECTRONMICROGRAPH SHOWING THE GENERAL APPEARANCE OF THE 2 000-10 000xg FRACTION OF A RAT SKELETAL MUSCLE HOMOGENATE.

Preparative procedure:

See 'Materials and Methods'.

Scale:

— = 0.5 μ .

Key:

M = intact mitochondrion; D = disrupted mitochondrion;

V = large vesicle; v = small vesicle; A = vesicular aggregate.



FIG. III-13. ELECTRONMICROGRAPH SHOWING THE DETAILED APPEARANCE OF THE 2 000-10 000xg FRACTION OF A RAT SKELETAL MUSCLE HOMOGENATE.

Preparative procedure:

See 'Materials and Methods'.

Scale:

————— = 0.2 μ .

Key:

M = intact mitochondrion;

v = small vesicle.

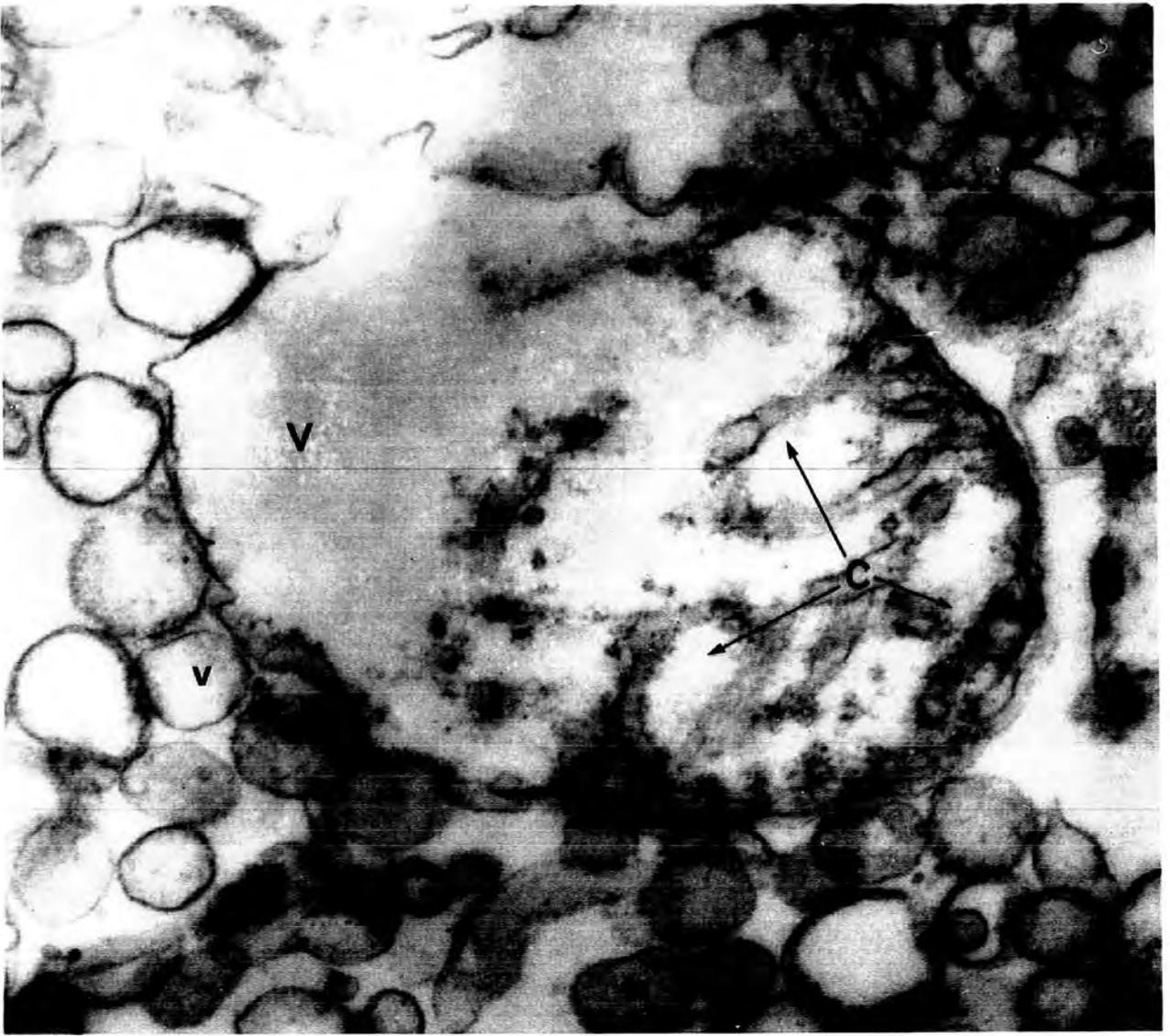


FIG. III-14. ELECTRONMICROGRAPH SHOWING THE DETAILED APPEARANCE OF THE 2 000-10 000xg FRACTION OF A RAT SKELETAL MUSCLE HOMOGENATE.

Preparative procedure:

See 'Materials and Methods'.

Scale:

————— = 0.2 μ .

Key:

V = large vesicle with contents (C);

v = small vesicle.

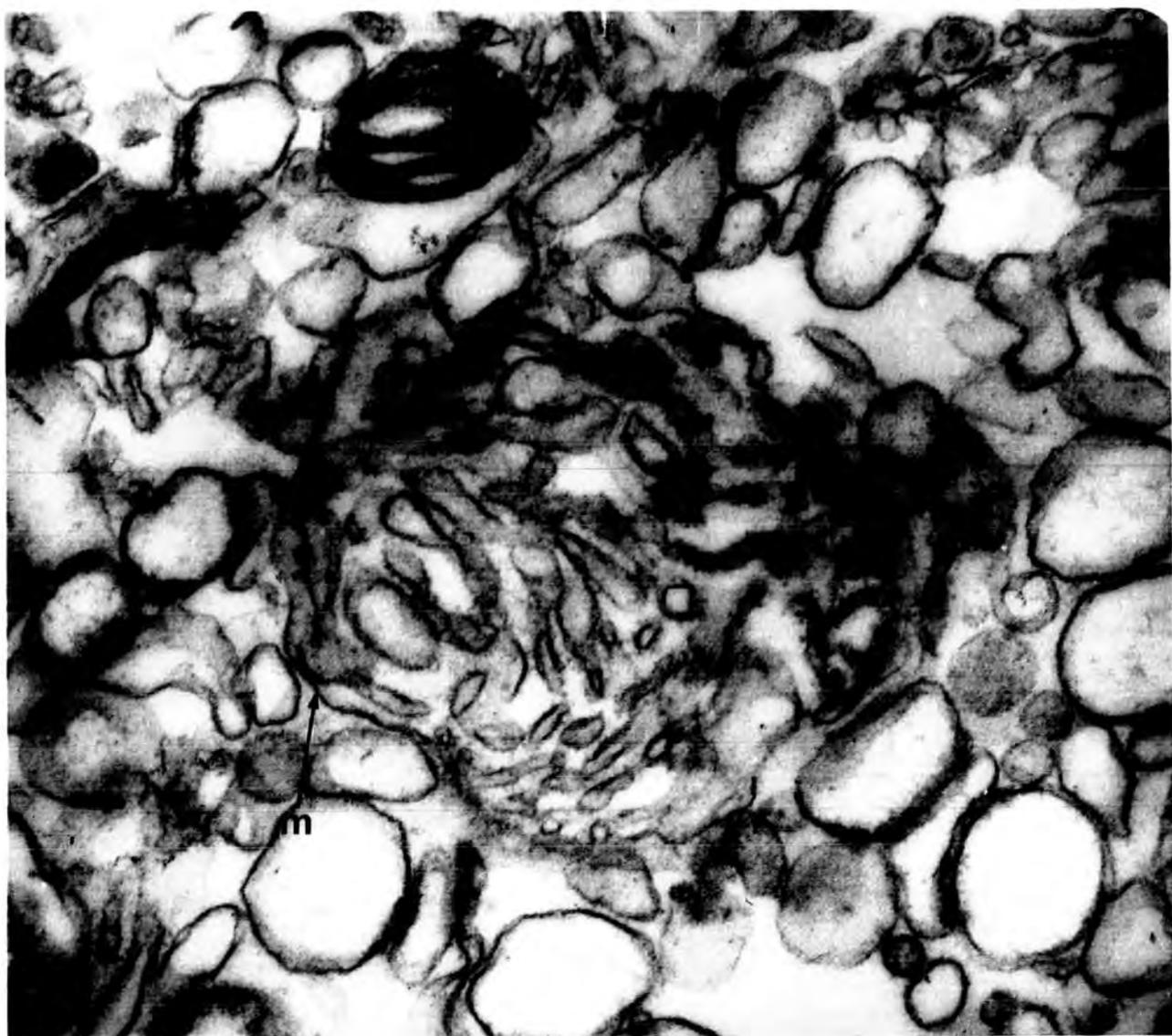


FIG. III-15. ELECTRONMICROGRAPH SHOWING THE DETAILED APPEARANCE
OF THE 2 000-10 000xg FRACTION OF A RAT SKELETAL MUSCLE HOMOGENATE.

Preparative procedure:

See 'Materials and Methods'.

Scale:

————— = 0.2 μ .

Key:

Certain small vesicles form an aggregate which is partially
bounded by a membrane (m).

origin. Furthermore, the size of such fragments may well encompass the entire range of vesicular diameter observed.

2. 10 000 - 107 000xg Fraction; Pellet (5) (see FIG.III-1)

FIG.III-16 shows the general appearance of this fraction. There was no evidence of mitochondrial contamination, although it is clear that mitochondrial fragments would, in any case, be difficult to distinguish from membrane vesicles having their origins in other cell organelles. An interesting feature of this fraction was the fairly frequent occurrence of triad-like vesicular aggregations (A). The significance of these associations is unknown, and other types of aggregate are also present. The fraction is rich in ribosomal material, though it is not possible to distinguish whether it is structurally associated with the membrane component.

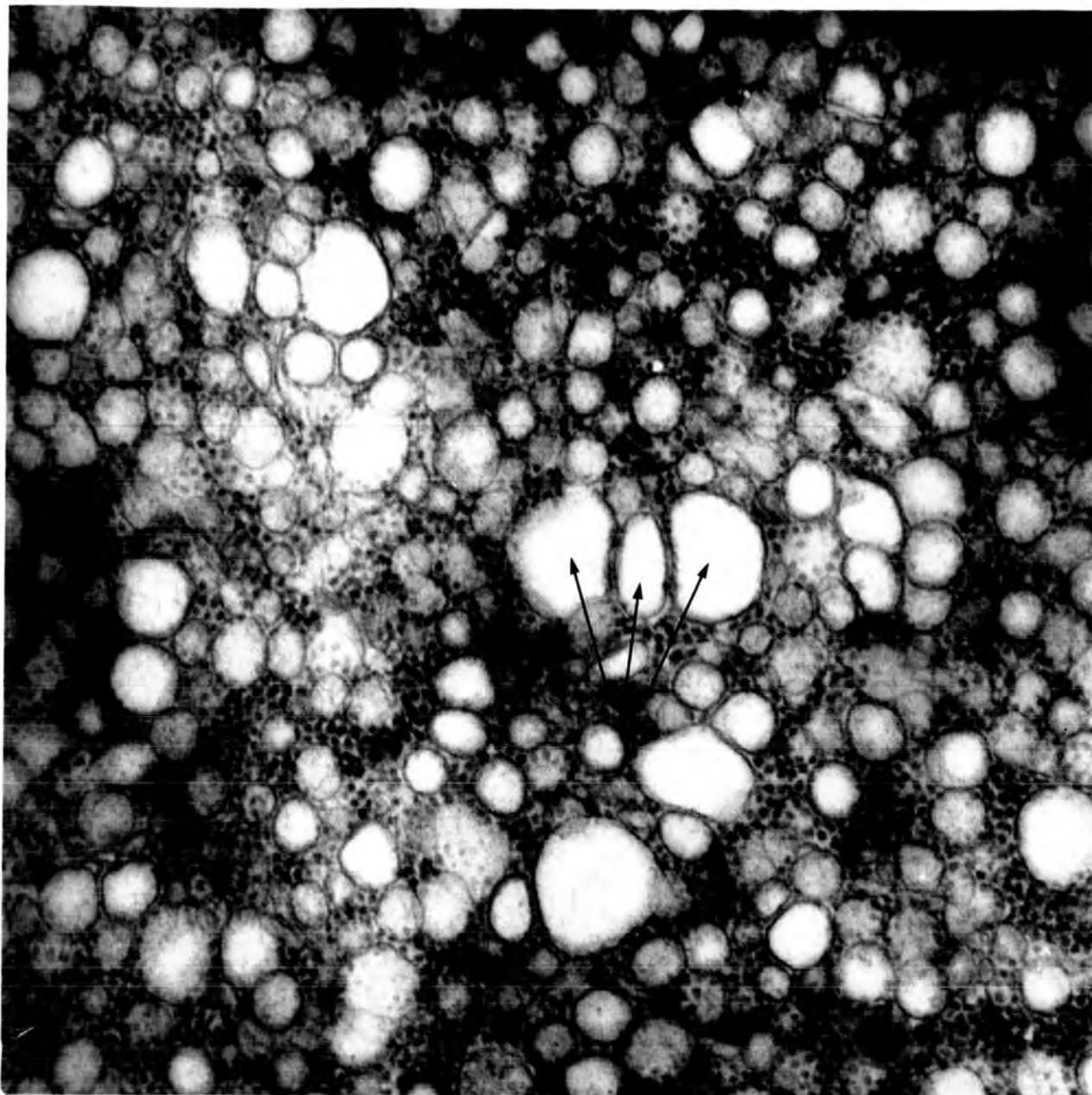


FIG. III-16. ELECTRONMICROGRAPH SHOWING THE GENERAL APPEARANCE OF THE 10 000-107 000xg FRACTION OF A RAT SKELETAL MUSCLE HOMOGENATE.

Preparative procedure:

See 'Materials and Methods'.

Scale:

— = 0.2 μ .

Key:

Ribosomal (granular) and vesicular material is present, the latter occasionally forming triad-like associations (A).

CHAPTER 3The Influence of Adrenalectomy upon Skeletal Muscle Mg^{2+} -ATPases

The effects of adrenocorticosteroids upon tissues in general are wide-ranging. Most of the hormones have been implicated in the reabsorption of Na^+ by renal tubules, sweat glands, salivary glands and the gastrointestinal tract, and there are species-specific spectra of potency. In general, whereas Na^+ reabsorption (and, where appropriate, concomitant changes in K^+ and water distribution) is markedly stimulated by aldosterone, hydrocortisone is reported to have a very weak mineralocorticoid action, and 11-deoxycorticosterone an intermediate potency. Some authors appear to consider that the action of aldosterone is solely upon renal active transport, and in this respect may secondarily mediate overall changes in an animal's electrolyte distribution status. Indeed, there is limited evidence for an effect of adrenocorticosteroids upon this aspect of skeletal muscle function.

Conway & Hingerty (1946) presented evidence for Na^+ loss and K^+ gain in rat muscle 3-6 days post-adrenalectomy, their calculation revealing a fall in $[Na^+]_i$ by 5.4 mmoles/kg dry weight with an associated $[K^+]_i$ rise by 21 mmoles/kg dry weight. Later these authors (Conway & Hingerty, 1953) reported the effects of intra-muscular deoxycorticosterone or cortisone administration to adrenalectomized rats maintained on high or low $[Na^+]$ diets. Animals given a low $[Na^+]$ diet lost Na^+ from skeletal muscle and plasma and gained K^+ in these compartments, both to a greater extent than rats fed the alternative diet. Both steroids reduced the magnitude of this exchange, cortisone being the more potent in animals on the low $[Na^+]$ diet. Thus, it was deduced that muscle cation content changes were purely secondary to those of plasma, the latter being determined by a primary action of the hormones upon renal tubules. This view was supported by the finding that $^{24}Na^+$ and $^{42}K^+$ entry and exit rates were apparently normal

in sartorius muscles from frogs which had received cortisone injections.

In contrast with this finding, Flückiger & Verzar (1954) reported that aldosterone altered isolated rat diaphragm $^{24}\text{Na}^+$ and $^{42}\text{K}^+$ permeability, and Woodbury & Koch (1957) reported that a fall in $[\text{Na}^+]$ and a rise in $[\text{K}^+]$ occurred in mouse muscle and brain after a 4-day aldosterone treatment.

In the latter study, mice which received deoxycorticosterone treatment for a similar period showed a rise in muscle $[\text{Na}^+]$ with no $[\text{K}^+]$ change, and a rise in brain $[\text{K}^+]$ with no change in $[\text{Na}^+]$. In the light of Conway & Hingerty's (1953) findings in rat, this is an instance of species and tissue difference in steroid response. Although it is tempting to interpret Woodbury & Koch's (1957) aldosterone data as a demonstration of direct action of the hormone on muscle, it is conceivable that such an effect may have arisen as a result of tissue Na^+ loading, which could follow abnormally high Na^+ reabsorption.

In the light of the very extensive actions of steroid hormones, it is understandable that the interpretation of experimental data proves difficult, especially since hormone effects are normally demonstrable only after administration to whole animal preparations. For instance, Finn & Welt (1963) found that aldosterone administration did not alter urinary or muscle $[\text{Na}^+]$ or $[\text{K}^+]$ in rats fed a low $[\text{Na}^+]$ diet: here it is possible that the endogenous aldosterone secretory response resulting from the level of dietary Na^+ intake may have been large, so that maximal target organ response to aldosterone may have already been elicited.

Most evidence favours the conclusion that muscle monovalent cation permeability changes may in some way be attributed to an influence of adrenocorticosteroids. Although little can be said as regards the actual mechanisms which mediate such effects, there is certainly evidence to implicate a microsomal $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}$ in the steroidal control of renal tubule Na^+

reabsorption (see Section IV). Thus, it was decided to examine whether ATPase activities, which could be associated with the control of monovalent cation transport in skeletal muscle, altered following adrenalectomy. The experiments described below were carried out in parallel with those reported in Chapter 2.

MATERIALS AND METHODS

MATERIALS

- a) Animals: ♂ CFE albino rats, as described in Section II.
- b) Skeletal muscle: Hind limb muscles were used, as described in Chapter 2.
- c) Reagents: These are given in the text and in legends to tables.

METHODS

- a) Bilateral adrenalectomy: The technique is given in Section II. Control animals were sham-adrenalectomized; i.e. their adrenal glands were exposed in the standard manner, but not ablated.
- b) Post-operative maintenance: The method is outlined in Section II. For the experimental group which is the subject of this Chapter, comparisons were made between sham-operated animals given normal solid diet and distilled water fluid diet ad lib., and operated ones which had received the same diet or 0.9% NaCl in place of distilled water.
- c) Enzyme preparation: The 10 000-107 000xg microsomal fraction (pellet (5); see FIG.III-1) was obtained from homogenates prepared according to the sequence described in Chapter 2, using an extraction medium of the medium (i) type consisting of:
 250mM sucrose + 5mM Na₂EDTA.2H₂O + 50mM Tris-HCl, pH 7.3 at 4^o±0.5^oC.
 The pellet was resuspended in 50mM Tris-HCl, pH 7.3 at 4^o±0.5^oC.
- d) Enzyme assay: The compositions of incubation media are given in legends to tables. Media were buffered with 50mM Tris-HCl, pH 7.3 at 37^o±0.5^oC.

Orthophosphate and protein determinations were made as outlined in Chapter 2.

RESULTS

Extraction characteristics

These were substantially as found for medium (i) extracted preparations described in Chapter 2., and, indeed, no in situ morphological difference between muscle of adrenalectomized, sham-adrenalectomized or intact rats was observed.

Effects of equimolar Na^+ and K^+ in the presence of 4mM Mg^{2+}

TABLE III-9 summarizes the effects of 50mM Na^+ and 50mM K^+ upon Mg^{2+} -dependent ATP hydrolysis. Values given for monovalent cation stimulation represent the increment in hydrolysis above that obtained in the presence of 4mM Mg^{2+} alone. The calculated values for activity ratios are additionally provided as an index of the differential relationships apparent in sham-operated and operated animals receiving distilled water or saline.

TABLE III-10 lists the percentage activities obtained in preparations from adrenalectomized as compared with sham-adrenalectomized animals under each incubation condition.

These data reveal a post-operative decrease in ATPase activity under all incubation conditions, the initial decrement (days 0-2) being very considerable in cases where animals did not receive 0.9% NaCl. Mg^{2+} -ATPase activity subsequently fell gradually to 32.4% of control in those animals surviving 9 days' adrenalectomy without saline compensation. Administration of such a diet prevented this fall, and slightly reduced the magnitude of initial ATPase depression.

The magnitude of monovalent cation stimulation in preparations from sham adrenalectomized rats followed a pattern resembling that seen in Tris-HCl medium (i) extracted preparations from intact rats, as reported earlier (see TABLE III-4), and there were marked changes with adrenalectomy. The

TABLE III-9

TABLE III-9. ATP HYDROLYSIS BY SKELETAL MUSCLE MICROSOMAL PREPARATIONS FROM SHAM-ADRENALECTOMIZED AND ADRENALECTOMIZED RATS, AND THE EFFECT OF FLUID DIET.

Extraction medium:

250mM sucrose + 5mM Na₂EDTA.2H₂O = 50mM Tris-HCl, pH 7.3 at 4^o ± 0.5^oC.

Incubation conditions:

50mM Tris-HCl ± 4mM Tris-ATP, pH 7.3, 37^o ± 0.1^oC.

Mg²⁺ (4mM), Na⁺ (50mM) and K⁺ (50mM) were included as indicated.

Post-operative fluid diet:

Distilled water or 0.9% NaCl were given ad lib., as indicated.

Adx. = adrenalectomized.

Control (0 days adx.) animals were sham-adrenalectomized.

n = number of animals.

Values given are the means of those obtained in duplicate incubations.

FLUID DIET	DAYS ADX.	n	nmoles P _i liberated/mg protein/min (\bar{x})				Mg ²⁺ +Na ⁺	Mg ²⁺ +K ⁺	Mg ²⁺ +Na ⁺ +K ⁺	Mg ²⁺ +Na ⁺ +K ⁺
			Mg ²⁺	Mg ²⁺ +Na ⁺	Mg ²⁺ +K ⁺	Mg ²⁺ +Na ⁺ +K ⁺	Mg ²⁺	Mg ²⁺	Mg ²⁺	Mg ²⁺ +K ⁺
DISTILLED WATER	0	4	4731.9	752.3	1258.6	1301.3	0.159	0.266	0.275	1.033
	2	2	2202.0	76.9	379.7	368.7	0.035	0.172	0.167	0.970
	4	2	1943.0	37.2	255.4	217.5	0.019	0.131	0.111	0.847
	6	2	2526.0	-158.4	366.8	316.5	-	0.145	0.125	0.862
	9	2	1537.0	-254.1	296.6	181.6	-	0.192	0.118	0.674
0.9% NaCl	3	2	2622.1	573.6	804.9	1156.8	0.218	0.306	0.441	1.441
	8	2	2835.6	1010.2	1211.1	1544.1	0.356	0.427	0.544	1.274

TABLE III-9. ATP HYDROLYSIS BY SKELETAL MUSCLE MICROSOMAL PREPARATIONS FROM SHAM-ADRENALECTOMIZED AND ADRENALECTOMIZED RATS, AND THE EFFECT OF FLUID DIET.

FLUID DIET	DAYS ADX.	% ATPase ACTIVITY			
		Mg ²⁺	Mg ²⁺ +Na ⁺	Mg ²⁺ +K ⁺	Mg ²⁺ +Na ⁺ +K ⁺
DISTILLED WATER.	0	100.0	100.0	100.0	100.0
	2	46.5	10.2	30.1	28.3
	4	41.0	4.9	20.2	16.7
	6	53.3	(-21.0)	29.1	24.3
	9	32.4	(-33.7)	23.5	13.9
0.9% NaCl	3	55.4	76.2	63.9	88.8
	8	59.9	134.2	96.2	118.6

TABLE III-10. COMPARISON OF SKELETAL MUSCLE MICROSOMAL ATPase ACTIVITIES IN ADRENALECTOMIZED AND SHAM-ADRENALECTOMIZED RATS.

% ATPase activity:

The values given in this TABLE are derived from the data given in TABLE III-9.

extent of Na^+ stimulation of Mg^{2+} -ATPase fell dramatically within 4 days, and preparations investigated after 6 and 9 days revealed 21.0% and 33.7% inhibitions of Mg^{2+} -ATPase respectively. The initial fall was markedly less in 0.9% NaCl maintained animals, in which the Na^+ -stimulated increment was 34.2% above control level at 8 days after operation.

K^+ -stimulated Mg^{2+} -ATPase activity showed a less dramatic, though still considerable, reduction following adrenalectomy: it stabilized at between 20 and 30% of the control level during the experimental period for animals not receiving saline. Again, the initial decrement was less in saline-treated animals, in which normal activity was restored after 8 days.

A gradual loss of ATPase activity in the presence of $\text{Mg}^{2+}+\text{Na}^++\text{K}^+$ occurred in animals receiving distilled water fluid diet, and followed an initial decrement close to that recorded for $\text{Mg}^{2+}+\text{K}^+$ incubations. Administration of 0.9% NaCl once again lessened this initial change, and by 8 days activity was above the control level.

It is clear from these results that the effects of adrenalectomy upon ATPase activity are to some extent dependent upon the post-operative fluid diet. Thus, the ratio $\text{Mg}^{2+}+\text{Na}^+/\text{Mg}^{2+}$ activity showed a dramatic fall in distilled water maintained rats, but an increase and - after 8 days - a doubling in saline maintained ones. Likewise, a slight fall in the $\text{Mg}^{2+}+\text{K}^+/\text{Mg}^{2+}$ activity ratio in those not receiving saline was prevented by its administration and thereby converted to an increase by 8 days. A similar change occurred in the $\text{Mg}^{2+}+\text{Na}^++\text{K}^+/\text{Mg}^{2+}$ activity ratio, with an eventual 8 day doubling under saline maintenance.

Calculation of the $\text{Mg}^{2+}+\text{Na}^++\text{K}^+/\text{Mg}^{2+}+\text{K}^+$ activity ratio reveals that by far the greater component of $\text{Mg}^{2+}+\text{Na}^++\text{K}^+$ -activated ATP hydrolysis could be accounted for by $\text{Mg}^{2+}+\text{K}^+$ -stimulated activity in sham operated rats, and that, in operated animals not receiving saline, in vitro incubation with 50mM Na^+ resulted in its progressive inhibition. Administration of saline resulted

in an elevated ratio, suggesting that a loss of the Na^+ -stimulated component is somehow prevented. However, in no case was there evidence of a synergistic $\text{Na}^+\text{K}^+\text{Mg}^{2+}$ -ATPase.

Effect of incubation in the presence of ouabain

5×10^{-4} M ouabain had no effect upon any of the ATPase activities found in these preparations, a result which is in accord with that obtained in previously described medium (i) extracted preparations (see Chapter 2).

CHAPTER 4Discussion of Experimental Results and the Control of Monovalent Cation Distribution in Skeletal MuscleSource of Microsomal Mg^{2+} -ATPase

The specific activity of Mg^{2+} -ATPase in the preparations described in Chapters 2 and 3 of this Section was high. Preparations from extractions in medium (i) yielded mean specific activities of 5011.0 nmoles P_i /mg protein/min when the medium was Tris-HCl-buffered, and 1840.4 nmoles P_i /mg protein/min when it was histidine-HCl-buffered (as derived from data in TABLE III-4). These values are comparable with those calculated from the data of Fratantoni & Askari (1965) and Engel & Tice (1966), who obtained levels of 5300-7500 and 1760 nmoles P_i /mg protein/min respectively. However, they are considerably higher than those obtained for rat brain. Using a histidine-HCl-buffered extraction medium essentially similar to medium (i), Bowler & Duncan (1968) obtained a value of 268.7 nmoles P_i /mg protein/min for such a preparation in the presence of 4mM Mg^{2+} . The possibilities arise that this discrepancy is a genuine reflection of different tissue characteristics, or that it was due to contamination of the muscle fraction with sub-cellular material - such as mitochondria and sarcoplasmic reticulum - exhibiting Mg^{2+} ATPase activity.

Contamination of the muscle preparations investigated above with myofibrillar material is unlikely, and there was no electronmicrographic evidence for it. Samaha & Gergely (1966) considered that their deoxycholate-extracted preparations contained mitochondrial material; however, the specific activities of Mg^{2+} -ATPase obtained in fresh 10 000-30 000xg fractions used by them were reported as 260 nmoles P_i /mg protein/min (Samaha & Gergely, 1965) and 150 nmoles P_i /mg protein/min (Samaha & Gergely, 1966). These values are comparatively very low, and contrast with the insignificant loss in mean specific activity observed in deoxycholate-extracted preparations here reported

in Chapter 2 (see TABLE III-7). A differential loss of Mg^{2+} -ATPase activity by sodium azide or ageing treatments was reported by Samaha & Gergely (1965, 1966), and resulted in an apparent 'exposure' of monovalent cation stimulation. This effect could not be repeated in the investigations of Chapter 2: in particular, though azide is a known inhibitor of mitochondrial Mg^{2+} -ATPase (see below), data presented in TABLE III-8 reveal a negligible effect upon the microsomal enzyme under investigation. However, as will be further discussed, enzymatic and electronmicroscopic evidence indicated a probable degree of fragmented mitochondrial contamination of medium (i)-extracted pellets (see FIGS. III-10 and III-12 - 16). In addition, the equivalent microsomal pellets obtained from medium (ii) type extractions had the red-pink colour otherwise associated with mitochondrial pellets from the medium (i) type (see FIG. III-1), and they contained significantly more protein (see TABLE III-5).

Thus, enhanced mitochondrial fragmentation would be expected in deoxycholate-containing extraction media, and indeed Ulrich (1963) and Auditore & Wade (1964) presented evidence of this for liver and kidney mitochondria respectively. Conversely, Muscatello & Andersson-Cedergren (1962) had shown that extraction in the presence of 0.27% deoxycholate resulted in protein dissolution of the so-called sarcotubular fraction from a frog skeletal muscle homogenate, a result consistent with the low specific activities of Samaha & Gergely's 0.3% deoxycholate-extracted preparation, and with the slight effect of 0.1% deoxycholate reported here in Chapter 2. It is therefore feasible that the relative contributions of sarcotubular and mitochondrial enzymes to microsomal Mg^{2+} -ATPase activity can depend upon the conditions of tissue exposure to detergent-containing media.

Upon this basis, Samaha & Gergely's preparations would be expected to show Mg^{2+} -ATPase activity largely attributable to mitochondrial origin, and the effect of 5mM azide in causing 97% and 98% inhibition of the enzyme in their

10 000-30 000xg and 600-8 000xg (mitochondrial) fractions respectively (see Samaha & Gergely, 1966) do confirm this. Azide inhibition of mitochondrial Mg^{2+} -ATPase was reported by Robertson & Boyer (1955), later suggested by Schwartz & Laseter (1963), and implied in the contraction of glycerinated mitochondria reported by Nakazawa (1964). As pointed out above, the effect of azide was negligible in preparations investigated and reported in TABLE III-8, and this may be taken to indicate a negligible mitochondrial contribution to the enzyme activity which was measured. Consequently, the possibility that mitochondrial Mg^{2+} -ATPase may have masked a $Na^+K^+Mg^{2+}$ -ATPase due to a preferential inhibitory interaction of Na^+ with the mitochondrial enzyme (as suggested by Ulrich, 1963., and later proposed by Samaha & Gergely, 1966) is unlikely; indeed, Ash & Schwartz (1970) have recently reported rat muscle $Na^+K^+Mg^{2+}$ -ATPase activity in a preparation in which they claimed evidence for mitochondrial contamination.

Slater (1960) commented that the fragility of sarcosomes is greater than that of liver mitochondria, and that a degree of rupturing may even occur in isotonic (250mM) sucrose. However, he further pointed out that EDTA stabilizes sarcosomes, and it was always incorporated in the extraction media used in the work reported here. Despite this, some evidence for mitochondrial disruption became available through succinate dehydrogenase assays (see FIG.III-10), in which activity was traced in all fractions: indeed, Caplan & Greenawalt (1966) have shown that a number of multi-enzyme pathways are preserved in lysed mitochondria. In addition, electronmicrographic evidence reported in Chapter 2 is consistent with a degree of fragmentation in medium (i)-extracted preparations. FIGS.III-12, III-14 and III-15 reveal evidence for this in pellet (3), and it is noteworthy that Caplan & Greenawalt (1966) obtained a morphologically similar fraction upon osmotic lysis of isolated rat liver mitochondria. From their work it is possible to conclude that a proportion of the small vesicles of $<0.25\mu$

diameter in pellet (3) are mitochondrial fragments, and that material of this nature may have contaminated the microsomal pellet (4). However, it is impossible to distinguish such material from microsomes of other origin in that pellet (see FIG.III-16).

The microsomal pellet investigated for ATPase activity in the work reported in this Section bears morphological resemblance (see FIG.III-16) to the sarcotubular fraction described by Muscatello & Andersson-Cedergren (1962) and Muscatello, Andersson-Cedergren & Azzone (1962), and to the similarly-prepared sarcoplasmic reticulum fraction described by Engel & Tice (1966). It is thus likely that a significant proportion of the vesicles obtained by the procedures adopted here was of sarcotubular origin. We may conclude that the Mg^{2+} -ATPase activity found in preparations reported in Chapters 2 and 3 is unlikely to have incorporated a significant mitochondrial component. It is recalled that Engel & Tice (1966) indeed obtained values for the specific activity of the microsomal enzyme close to those reported in the Chapters above. In addition, although some recent work by Peter (1970), Seiler (1971) and Sulakhe, Fedelesova, McNamara & Dhalla (1971) provides evidence for a $Na^{+}+K^{+}-Mg^{2+}$ -ATPase in preparations of reputed sarcolemmal origin, the earlier histochemical study by Giacomelli et al.(1967) revealed that this activity is also associated with intracellular membrane components which evidently predominate in the microsomal fraction which is the subject of the present study.

Absence of ouabain-sensitive $Na^{+}+K^{+}-Mg^{2+}$ -ATPase

All the preparations reported here are characterized by the absence of a synergistically activated active transport ATPase of the type reported by Bonting et al. (1961), Samaha & Gergely (1965, 1966), Rubin & Katz (1967) and Rogus, Price & Zierler (1969). In this respect there is again close resemblance to preparations reported by Fratantoni & Askari (1965) and Engel & Tice (1966). As revealed in Chapter 2, the use of extraction media

essentially similar to those recommended by Samaha & Gergely (1965, 1966), and Rogus et al. (1969) - containing deoxycholate and D-mannitol respectively - yielded ATPases which resembled those from the equivalent isotonic sucrose homogenate fraction. Extraction in 0.1% deoxycholate in fact resulted in a significant fall in the specific activities of monovalent cation stimulated Mg^{2+} -ATPase components (see TABLE III-7), an effect resembling that of 0.05% deoxycholate present in incubation media. The low specific activity of D-mannitol preparations was as predicted from the work of Rogus et al. (1969).

Ouabain at $5 \times 10^{-4} M$ had no effect upon isotonic sucrose extracted ATPase preparations, but considerable inhibition of $Mg^{2+}+K^{+}$ - and non-synergistic $Mg^{2+}+Na^{+}+K^{+}$ -mediated hydrolyses occurred in deoxycholate extracted ones, and of $Mg^{2+}+Na^{+}$ - and $Mg^{2+}+Na^{+}+K^{+}$ - mediated hydrolyses in D-mannitol extracted preparations. Such inhibition was unexpected in the apparent absence of a clearly synergistic, ouabain-sensitive $Na^{+}+K^{+}-Mg^{2+}$ -ATPase, as revealed by incubation in media of appropriate ionic composition (see Section I). The origins and possible roles of other monovalent cation stimulated activities are now considered.

Presence of Mg^{2+} -ATPases which are stimulated by single monovalent cations

Certain authors have noted the occurrence of $Mg^{2+}+Na^{+}$ - and $Mg^{2+}+K^{+}$ - mediated hydrolyses in amphibian and mammalian skeletal muscle fractions, and the findings reported in this Section are largely in accord with theirs. The work of Fratantoni & Askari (1965) and Engel & Tice (1966) has already been brought to attention in this context.

The occurrence of such enzyme activity has, interestingly, been noted in other tissues. Skou (1957) reported a $Mg^{2+}+Na^{+}$ -stimulated ATPase component in Garcinus nerve, but did not detect a $Mg^{2+}+K^{+}$ -stimulated one; he regarded the $Na^{+}-Mg^{2+}$ -ATPase as a fragment of the synergistic enzyme investigated in the same experimental series. Skou (1962) reported a similar activation

pattern by low concentrations of monovalent cation in mammalian brain and kidney microsomal preparations. Fujita, Nagano, Mizuno, Tashima, Nakao & Nakao (1968) detected several ouabain-inhibited ATPase components in pig brain microsomes: these were Mg^{2+} -, $Mg^{2+}+Na^{+}$ -, $Mg^{2+}+K^{+}$ - and $Mg^{2+}+Na^{+}+K^{+}$ -mediated activities, and in addition there was a further Mg^{2+} -dependent, but ouabain-insensitive, component. Evidence for ouabain-sensitive $Na^{+}+K^{+}-Mg^{2+}$ - and $Na^{+}-Mg^{2+}$ -ATPases with different substrate affinities and K^{+} sensitivities has been presented by Neufeld & Levy (1969), who considered that it pointed to the presence of two distinct enzymatic sites in calf brain microsomes. In addition, ouabain-insensitive Na^{+} stimulation of rat brain mitochondrial Mg^{2+} -ATPase was reported by Beattie & Basford (1968), and valinomycin-induced exposure of such an enzyme by Cereijo-Santalô (1968). These findings interestingly contrast with Ulrich's (1963) demonstration of monovalent cation inhibition of liver mitochondrial Mg^{2+} -ATPase.

From these instances it is clear that the interactions of monovalent cations with Mg^{2+} -ATPases are potentially very complex, even in vitro in the case of fractionated tissue homogenates. The occurrence of Na^{+} stimulation of Mg^{2+} -ATPase in the absence of K^{+} in preparations in which a coupled $Na^{+}+K^{+}-Mg^{2+}$ -ATPase is also demonstrable leads us to ask: is such non-coupled activity artifactual, and, if not, what is its function? The answers to these questions are clearly important in gaining an understanding of mammalian muscle, from which the evidence for a tightly coupled enzyme is equivocal, in the sense that its activation by the simultaneous presence of Na^{+} and K^{+} is not obligatory.

Degree of linkage of Na^{+} and K^{+} movements and $Na^{+}+K^{+}-Mg^{2+}$ -ATPase

In contrast with our knowledge of red blood cells, the stoichiometry of the ' Na^{+} pump' of muscle does not appear to have been closely defined, presumably because of the difficulty in distinguishing between K^{+} which may have been actively transported in exchange for Na^{+} and that which may have moved passively

down the electrical gradient generated by active Na^+ extrusion. There has arisen the possibility that an active Na^+ transport component (an electrogenic Na^+ pump) may contribute to E_m , and some of the evidence for such an ouabain-sensitive component in mammalian skeletal muscle has been mentioned in Chapter 1 of the present Section.

Since Kernan's (1962) demonstration of an apparently electrogenic Na^+ pump in frog muscle through studies of Na^+ efflux during recovery of Na^+ -loaded tissue, several authors have confirmed that E_m may exceed E_K in such circumstances (Keynes & Rybová, 1963; Cross, Keynes & Rybová, 1965; Frumento, 1965; Mullins & Awad, 1965; Adrian & Slayman, 1966; Harris & Ochs, 1966). Their measurements indicate that either a fraction of such Na^+ efflux is independent of K^+ influx, or that it is coupled to it in other than a neutral 1:1 manner. By replacing K^+ with Rb^+ in their recovery medium, Adrian & Slayman (1966) obtained evidence suggesting only partial coupling; further, by the use of cocaine to reduce membrane K^+ conductance, these authors were able to decrease the short-circuiting effect of K^+ influx and demonstrate an augmented hyperpolarization.

Further evidence for the existence of an electrogenic Na^+ pump in muscle will not be pursued here, but it seems that there is at least evidence that the extent of coupling of Na^+ - K^+ exchange may vary as circumstances dictate. Thus, if a monovalent cation stimulated Mg^{2+} -ATPase is involved in the control of active transport, it may be expected to exist as a tightly-coupled Na^+ + K^+ - Mg^{2+} -ATPase or as non-coupled Na^+ - Mg^{2+} -ATPase (and K^+ - Mg^{2+} -ATPase). The data presented in FIGS.III-4 and III-6 would further suggest the possibility of competition for non-specifically-oriented ATPase sites in vitro. It is suggested that the non-coupled enzymes are subunits of a Na^+ + K^+ - Mg^{2+} -ATPase: such enzymic fragmentation has been artificially achieved in human red blood cell preparations by sonication (Askari & Fratantoni, 1964), in which ouabain-sensitivity was not retained. The possible existence of in situ

physiological mechanisms whereby varying degrees of coupling may be achieved should not be disregarded.

In skeletal muscle, therefore, it may be considered that the existence of non-linked active transport mechanisms for single monovalent cations is correlated with the presence of non-synergistic or even non-coupled monovalent cation stimulated Mg^{2+} -ATPases. Such enzymes may also be present in other tissues, and there is already some evidence of this (see above). However, a general clue as to the degree of linkage of Na^+ and K^+ movements has been provided by Jain, Strickholm & Cordes (1969) in their study of the behaviour of a synaptic vesicular $Na^+K^+Mg^{2+}$ -ATPase preparation in association with a black lipid membrane torus separating buffered media containing Mg^{2+} , Na^+ and K^+ . Upon addition of ATP to their system they observed a fall in membrane resistance associated with a current flow. This effect was Na^+ -dependent and ouabain-inhibited. Since a 1:1 Na^+K^+ exchange could not have occurred, this was interpreted as a case for electrogenic Na^+ transport. Although the ATPase preparation used by these investigators was that of Cotman, Mahler & Anderson (1968), in which the enzyme was regarded as a preparative index, some information about its monovalent cation sensitivity is available from the earlier work of Hosie (1965). She found that, in addition to classical synergistic Na^+K^+ activation, some was elicited by Na^+ and K^+ separately. This observation is again consistent with the accounts of non-coupled, monovalent cation stimulated Mg^{2+} -ATPase discussed above, and it is disappointing that many authors appear not to have attempted to distinguish such activities in their preparations.

Influence of adrenalectomy upon Mg^{2+} -ATPases

It is clear from the evidence presented in Chapter 3 that marked alterations in all types of Mg^{2+} -ATPase activity under investigation did occur within two days of operation. A very marked loss of $Mg^{2+}Na^+$ -mediated ATP hydrolysis occurred, and, in animals not receiving saline compensation, the addition of Na^+ to incubation media later resulted in an inhibition of Mg^{2+} -ATPase.

This remarkable effect is reminiscent of the inhibition of mitochondrial Mg^{2+} -ATPase by Na^+ (Ulrich, 1963), and once again suggests the possibility of mitochondrial contamination of the membrane fraction under study. However, similar effects were also noted in brain microsomal preparations subjected to low temperature incubations in which Mg^{2+} - and Na^+K^+ - Mg^{2+} -ATPases prove to be differentially inactivated (Bowler & Duncan 1968). The post-adrenalectomy fall in $Mg^{2+}Na^+$ -stimulated hydrolysis may thus equally involve the inactivation or loss of the Na^+ -stimulated enzyme and a change in the characteristics of Mg^{2+} -ATPase which renders it Na^+ -sensitive.

That $Mg^{2+}Na^+$ - and $Mg^{2+}K^+$ -stimulated enzyme activities are distinct is suggested by their differential rates of decay after operation in non-saline-compensated animals. However, the inhibitory interaction of Na^+ with Mg^{2+} -ATPase described above also appears to be reflected in $Mg^{2+}Na^+K^+$ -mediated hydrolyses in these circumstances: this is seen in the gradual fall in $Mg^{2+}Na^+K^+/Mg^{2+}K^+$ activity ratio (see TABLE III-9). Thus, there is evidence, not only for a differential loss of cation stimulated ATPase activity during adrenalectomy, but also for the development of unusual monovalent cation sensitivity, presumably associated with steroid-controlled ATPase molecular characteristics.

Quite apart from its apparent development of Na^+ sensitivity, the fall in skeletal muscle Mg^{2+} -ATPase activity is of interest in the light of reported changes in myocardial function after adrenalectomy. The hypotension and eventual circulatory failure associated with adrenal insufficiency have been shown to involve impaired cardiac function even in the absence of blood volume changes (Verrier, Rovetto & Lefer, 1969), and have been correlated with subnormal cardiac weight and ventricular myofibrillar degeneration (Glen-Bott, Imms, Jones & Papadaki, 1970). This latter morphological change was unaccompanied by noticeable alterations in the appearance of mitochondria, transverse tubules or sarcoplasmic reticulum. It is, however, conceivable

that less specific changes than those evident from morphological scrutiny may in fact occur, and that the easy fatigability and skeletal muscle weakness widely associated with hyponatremia in adrenalectomy are linked with primary changes in muscle permeability control mechanisms. Reid, O'Neal & Lewin (1956), in their study of Mg^{2+} -ATPase distribution in rat liver fractions with adrenalectomy, found that mitochondrial and microsomal enzymes were distinct entities with differential hormone sensitivities: microsomal Mg^{2+} -ATPase activity was lost after operation. This finding is in accord with that reported in Chapter 3 (see TABLES III-9 and III-10).

It is notable from the experiments reported in Chapter 3 that ATPase activities are affected by dietary NaCl intake. In cases in which operated animals received isotonic NaCl, monovalent cation stimulated activities were markedly protected from post-operative decay, though the loss of Mg^{2+} -ATPase was less affected. The 8 day increase in specific activity of Na^+ - Mg^{2+} -ATPase further suggests the ability of this enzymic component to adapt to alterations in Na^+ availability, and presumably to meet Na^+ transport requirements. Such observations complement those outlined earlier in association with the demonstration of an electrogenic Na^+ pump in Na^+ -loaded frog muscle, and with similar work by Akaike & Kowa (1970) on soleus and extensor digitorum longus muscles from intact and K^+ -deficient rats. These authors report that active extrusion of Na^+ by Na^+ -loaded muscles taken from K^+ -deficient animals was greater than that from normal rat muscles, and greater by soleus than extensor. Furthermore, the $[Na^+]_i$ of fresh muscles was increased by 153% and 273% in K^+ -deficient animals for soleus and extensor respectively, whilst $[K^+]_i$ decreased by 34.8% and 30.6% by comparison with muscles from normal rats. The $[Na^+]_i$ and $[K^+]_i$ of fresh muscles from normal animals were in agreement with those quoted in Chapter 1 from Sréter & Woo (1963) (see TABLE III-1).

Studies of skeletal muscle in affective disorders

It will be recalled from Section I that the aim of this work was to establish parameters relating to the enzymic control of monovalent cation distribution upon which might be based a study of bulk tissue characteristics in subjects with affective disorders.

In the case of skeletal muscle, this achievement has not been realized because of the general disparity concerning the definition of active transport mechanisms in skeletal muscle - especially as regards the existence and roles of the spectrum of monovalent cation stimulated ATPases. However, it is relevant to note that there is recent evidence for skeletal muscle abnormality in acute psychosis. From a series of papers by Meltzer and associates (Engel & Meltzer, 1970; Meltzer & Moline, 1970; Meltzer & Engel, 1970; Fischman, Meltzer & Poppei, 1970) it may be appreciated that patients with acute psychoses have been shown to exhibit elevated serum levels of the creatine phosphokinase (CPK) isoenzyme associated with muscle; also that a parallel histochemical examination revealed myofibrillar disruption which could not be attributed to tranquillizer administration. It was estimated that the greatest intensity of myopathic change coincided with peak serum CPK levels, at least in males (Meltzer & Engel, 1970). These findings resemble those of Glen-Bott et al. (1970) in connexion with the effects of adrenalectomy, in that myofibrillar changes were apparently unaccompanied by mitochondrial or sarcotubular ones. In this context, the reputed involvement of adrenocorticosteroid imbalance, or target tissue insensitivity, in the aetiology of manic-depressive psychosis (see Section I) is of possible relevance.

Main conclusions

1. Experimental evidence for the presence of a synergistic $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}$ in mammalian skeletal muscle is equivocal: there is evidence for the existence of non-linked, monovalent cation stimulated $\text{Mg}^{2+} - \text{ATPases}$.
2. Although it is conceivable that these non-linked enzymes are preparative

artifacts, they have been discovered in a variety of tissues. It is suggested that they may, however, be naturally derived from the linked (parent) enzyme.

3. Non-linked enzymes are possible candidates for the control of electrogenic Na^+ transport in skeletal muscle.

4. Studies of the effect of adrenalectomy and Na^+ availability upon the ATPases show that they adapt to demands for Na^+ transport, and that the absence of adrenocorticosteroids renders the Mg^{2+} -ATPase Na^+ -inhibited.

5. Since, at this stage, it was not possible to determine with certainty how monovalent cation stimulated Mg^{2+} -ATPase(s) contribute(s) to the control of monovalent cation distribution in skeletal muscle, it was decided not to adopt this as an index tissue in investigations of permeability control in subjects suffering affective disorders.

S E C T I O N I V

KIDNEY STUDIES

CHAPTER 1.The Control of Active Reabsorption of Na^+ in the Kidney

The kidney is an organ of paramount importance in the maintenance of the stable composition of mammalian body fluids, and the passive tubular reabsorption of many non-electrolytes and electrolytes from tubular lumen (glomerular ultrafiltrate) into peritubular fluid (blood) is considered to depend predominantly upon the work performed and concentration gradient established by active Na^+ reabsorption into the latter compartment. Active transport has therefore been studied as a property of tubular epithelial cells, and $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}$ considered as a component of the ion transfer mechanism.

Working with the proximal tubules of Necturus maculosus kidney in situ, Schatzmann, Windhager & Solomon (1958) demonstrated that intravenous ouabain administration reduced water reabsorption by about 50%, and it was not long before Orloff & Burg (1960) reported that the introduction of strophanthidin via the renal portal circulation of chickens resulted in an increased urinary Na^+ and decreased urinary K^+ and H^+ excretion. These authors (Orloff & Burg, 1960) proposed that the excretion of K^+ occurred in conjunction with Na^+ reabsorption, and from such studies of the renal influence of cardiac glycosides it became evident that the composition of body fluids is mediated by active Na^+ transport. Later work with mammalian kidney cortex slices by Whittam & Willis (1963), Whittembury (1965) and Willis (1966) revealed a 50% inhibition of their K^+ uptake by ouabain, and a 50% dependence of Na^+ transport upon the presence of K^+ in bathing media. These results were again consistent with active, linked $\text{Na}^+ - \text{K}^+$ transport, and, indeed, the presence of ouabain-sensitive $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}$ had by then been demonstrated in tissue extracts by Bonting et al. (1961, 1962), Skou (1962) and Wheeler & Whittam (1962).

It had been made clear some time before by Roemmelt, Sartorius & Pitts (1949) that the reabsorption of a small (2%), but highly significant, fraction of

filtered Na^+ was under adrenocorticosteroidal control. Their experiments with adrenalectomized dogs showed that Na^+ reabsorption was 98% complete with no substitution treatment, and that intravenous administration of adrenocortical extract fully restored it after a latent period of about 40min.. From this it is evident that Na^+ reabsorption is very largely non-adrenocorticosteroid mediated, and also that instantaneous adjustments in reabsorption are unlikely to be regulated by alterations in steroidal output. Nevertheless, the maintenance of 2% decrement in Na^+ reclamation over prolonged periods would severely deplete $[\text{Na}^+]_e$, so that steroidal regulation could indeed be of considerable functional significance.

In this context, several reports are available of alterations in renal $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}$ activity following adrenalectomy and steroid administration. Chignell & Titus (1966), Landon, Jazab, & Forte (1966), Katz & Epstein (1967), and Jørgensen (1968, 1969) are all in agreement that adrenalectomy leads to about 50% reduction in enzyme activity after approximately 7 days in the rat. Although they appear to have measured total ATP hydrolysis in the presence of $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$, Suzuki & Ogawa (1969) noted a similar effect in the rat (but a slight increase in activity of the mouse enzyme). However, the above authors are not in agreement as regards the effects of in vivo administration of steroids upon ATPase activity in adrenalectomized animals. Chignell & Titus (1966) reported that chronic administration of corticosterone was far more effective than that of aldosterone in restoring activity; Landon et al. (1966) observed that very high concentrations of aldosterone or deoxycorticosterone restored it; Jørgensen (1969) claimed that the acute administration of aldosterone was more effective than that of corticosterone, whilst chronic aldosterone substitution would partially, and that of corticosterone fully restore activity; finally, Suzuki & Ogawa (1969) found that activity was restored by chronic administration of aldosterone, deoxycorticosterone or cortisol in rats. In addition, Suzuki & Ogawa (1968) had shown that chronic administration of aldosterone and corticosterone respectively

increased and decreased total ATPase activity in kidneys from intact rats.

As regards in vitro effects of steroids upon renal $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}$, Landon et al. (1966) discovered that the treatment of preparations from adrenalectomized rats with aldosterone did not restore activity, but that the presence of aldosterone or deoxycorticosterone did to some extent protect from ouabain inhibition. (It may be noted that steroids and cardiac glycosides are structurally similar). In this context, the in vivo infusion of aldosterone had already been shown to reverse the effect of ouabain infusion on dog kidney (Greene & Wilde, 1962). This constitutes the very tentative evidence for direct plasma membrane interaction of enzyme and steroid molecules, and the current view of aldosterone action is that it is indirect - possibly stimulating specific protein synthesis via its known interaction with cell nuclei (Edelman, 1965). In the case of rat kidney, Edelman & Fimognari (1968) have noted a rapid and specific binding of aldosterone to nuclei, and Forte & Landon (1968) an aldosterone mediated stimulation of RNA synthesis. Conversely, Liew & Gornall (1969) traced an aldosterone mediated increase in ^{32}P incorporation into ATP prior to its incorporation into RNA in heart muscle from adrenalectomized rats, so that there is a possibility that extra-nuclear effects may be primary. However, it is relevant that the onset of aldosterone effects upon Na^+ reabsorption is known to be delayed (see, for instance, Roemmelt et al. (1949), as discussed earlier), and this is held to be consistent with a primary effect upon protein synthesis.

The finding that administration of 0.9% NaCl to adrenalectomized rats prevented the loss of monovalent cation stimulated $\text{Mg}^{2+} - \text{ATPase}$ activities in skeletal muscle (see Section III, Chapter 3) may here be recalled: similar effects have been reported for kidney $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}$, and are relevant to the present exposition. The operated animals used by Landon et al. (1966) and Chignell & Titus (1966) were reported to have received isotonic saline as their fluid diet; although some received tapwater for 1-2 days prior to

sacrifice, the degree of change in renal $\text{Na}^+\text{K}^+\text{Mg}^{2+}\text{-ATPase}$ activity was apparently uninfluenced. In contrast with these reports, it is of great interest that Jørgensen (1968) found a distinct difference in the rate and magnitude of fall of enzyme activity and the parallel plasma Na^+/K^+ ratio in rats after adrenalectomy, with and without saline compensation: in particular, a dramatic fall occurred during the 1-2 day period in those receiving tapwater instead of 0.9% NaCl. It thus appeared that, not only were the plasma Na^+/K^+ ratio and renal enzyme activity closely related, but that the enzyme was not extensively under the primary control of adrenocorticosteroids. Such a conclusion may also be drawn from the data of Suzuki & Ogawa (1971), from which it is calculated that 5-day adrenalectomized rats showed a 32% decrease in enzyme activity unless saline maintained, in which case there was an 11% decrement - uninfluenced by aldosterone. It may thus be concluded that an important determinant in the maintenance of $\text{Na}^+\text{K}^+\text{Mg}^{2+}\text{-ATPase}$ activity is the continued availability of Na^+ to the enzyme in situ.

It follows from the above observations that, contrary to what was earlier proposed by a number of workers, aldosterone does not directly influence $\text{Na}^+\text{K}^+\text{Mg}^{2+}\text{-ATPase}$ activity. A current view is that the action of this steroid more probably involves the maintenance of Na^+ availability to the enzyme, and, by analogy with the model proposed by Sharp & Leaf (1966) for the urinary bladder of Bufo marinus, would thereby entail an increased passive permeability of the mucosal (tubular lumen) membrane of renal tubular epithelial cells to Na^+ . As suggested by these authors, the delayed onset of aldosterone-mediated Na^+ conservation would be related to the synthesis of protein (a 'permease'), which may in turn influence passive permeability, and thereby enhance availability of Na^+ to the active transport mechanism situated in the serosal (peritubular) membrane. Thus, as suggested by the experiments of Jørgensen (1968) and Suzuki & Ogawa (1971), alterations in $\text{Na}^+\text{K}^+\text{Mg}^{2+}\text{-ATPase}$ activity in kidney are secondary to Na^+ availability. This is further supported by the fact that, although Na^+ deprivation may lead to a reduction in activity

of this enzyme (Gutman & Beyth, 1969), aldosterone will not enhance Na^+ retention during the period of high renal Na^+ output associated with Na^+ loading (Blair-West, Coghlan, Denton, Scott & Wright, 1968).

As intimated above, the reabsorption of water in kidney is (osmotically) linked to active Na^+ reabsorption, so that both are ouabain sensitive (see Schatzmann et al., 1958; Orloff & Burg, 1960). It also transpires that the diuretic effect of ethacrynic acid may be associated with the inhibition of active Na^+ transport. In this connexion, it is of considerable interest that, although Duggan & Noll (1965), Banerjee, Khanna & Sen (1970) and Davis (1970) have reported inhibition of renal $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+}$ -ATPase by this agent, Davis (1970) noted that the effect was non-specific (Mg^{2+} -ATPase was also inhibited) and Proverbio, Robinson & Whittembury (1970) saw no alteration in $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+}$ -ATPase, but an inhibition of Mg^{2+} -ATPase. Indeed, in their studies of microsomal preparations and of Na^+ -loaded kidney slices, Proverbio et al. (1970) were able to correlate ouabain inhibition of $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+}$ -ATPase with its prevention of $\text{Na}^+ - \text{K}^+$ exchange; ethacrynic acid inhibition of Mg^{2+} -ATPase may have been associated with its readier prevention of a non-linked $\text{Na}^+ + \text{Cl}^-$ extrusion component. It was suggested that the latter would be effective in cell volume regulation, although it seems that Mg^{2+} -ATPase may not be involved, since its inhibition only occurred at high ethacrynic acid concentration. Despite this, the observations are relevant in the context of Kleinzeller & Knotková's (1964) work, from which it may be concluded that the recovery of volume in swollen, Na^+ -loaded rabbit kidney cortex slices is not substantially K^+ -dependent: they considered that the loss of NaCl solution from swollen cells may have involved a contractile filtering system, and mentioned having observed ouabain-insensitive conformational changes when ATP was added to kidney microsomes. In the light of observations made by Bowler & Duncan (1967a), it is conceivable that Mg^{2+} -ATPase may be involved in the mechanism of NaCl efflux. Work with ethacrynic acid has therefore resulted in the suggestion that two active Na^+ transport pumps are present in kidney, and it

is possible that their relative importance may be correlated with the functional divisions of nephrons: Schmidt & Dubach (1969) have already considered the distribution of $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}$ in these units.

It is clear that, although its precise role in Na^+ transport is difficult to define, the presence of a $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}$ in kidney is not disputed, and that its fall in activity after adrenalectomy is well documented for the rat. With these points in mind, a small group of experiments was performed to complement those reported previously in Chapter 3 of Section III. The main objects were to confirm that a synergistically stimulated $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}$ could be demonstrated in the rat - using techniques similar to those for skeletal muscle experiments, and that the enzyme was indeed affected by adrenalectomy - thereby providing an index of operative success.

CHAPTER 2Experiments upon $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}$ in Rat Kidney Microsomal Preparations

The presence and function of this enzyme in kidney has been discussed in outline in Chapter 1, and we are now more specifically concerned with the methods employed in demonstrating its activity in homogenates.

Bonting et al. (1961, 1962) succeeded in demonstrating the enzyme in distilled water reconstituted, lyophilized whole homogenates, and a full study of the particulate distribution and characteristics was initiated by Skou (1962). An important observation made by Skou (1962) was that homogenization in buffered isotonic sucrose containing 0.1% deoxycholate and 5mM EDTA appeared to yield a more active $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}$ component than preparation in a medium consisting of buffered isotonic sucrose alone: this occurred as the result of a decrease in $\text{Mg}^{2+} - \text{ATPase}$ and readier separation of the ATPase components. Unfortunately, Skou (1962) has not reported the specific activities of the enzymes when extracted in deoxycholate-free media, but he does say that the best obtained $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+ / \text{Mg}^{2+}$ activity ratio was not more than 2 in these circumstances, and that they were elevated to the 4-6 range when deoxycholate was used. It should be noted that, in Skou's (1962) ratio (see above), the dividend is the total ATPase activity in the presence of 6mM $\text{Mg}^{2+} + 100\text{mM Na}^+ + 20\text{mM K}^+$, and the divisor is the activity in the presence of 3mM Mg^{2+} : hence, this expression must not be interpreted as synonymous with the $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase} / \text{Mg}^{2+} - \text{ATPase}$ activity ratio which is used by a number of other workers and in this thesis.

Subsequent workers have largely adopted preparative methods essentially similar to Skou's (see Chignell & Titus, 1966; Katz & Epstein, 1967; Jørgensen, 1968, 1969; Suzuki & Ogawa, 1968, 1969, 1971; Jørgensen & Skou, 1969, 1971; Davis, 1970), although Landon et al. (1966) dialysed the endoplasmic reticulum fraction and, like Duggan & Noll (1965), Paul & Gonick (1968), Gutman & Beyth (1969) and Proverbio et al. (1970), did not adopt a deoxycholate-containing homogenizing medium.

Despite the fact that specific enzyme activities have not been uniformly expressed in the literature, it is possible to summarize most mammalian kidney work in terms of the above $\text{Na}^+\text{K}^+\text{Mg}^{2+}\text{-ATPase}/\text{Mg}^{2+}\text{-ATPase}$ activity ratio in relation to the tissue region investigated and its mode of extraction. This approach has been made here, and is presented in TABLE IV.-1. From this it will be seen that, although a $\text{Na}^+\text{K}^+\text{Mg}^{2+}\text{-ATPase}$ was certainly obtained by homogenization in isotonic sucrose based media, the activity ratio was consistently elevated when detergent (0.1% deoxycholate in most instances, as recommended by Skou (1962)) was incorporated in extraction media, or microsomes preincubated with it.

That the $\text{Na}^+\text{K}^+\text{Mg}^{2+}\text{-ATPase}$ in this tissue is a genuinely synergistic enzyme was initially suggested by Skou (1962). Low concentrations of Na^+ alone in the presence of Mg^{2+} were found to slightly elevate ATP hydrolysis, but K^+ and other monovalent cations had no detectable effect when added in the absence of Na^+ . Again, Landon & Norris (1963) detected no activation by Na^+ or K^+ alone in the presence of Mg^{2+} . However, in each case there was considerable synergistic activation in the presence of $\text{Mg}^{2+}\text{Na}^+\text{K}^+$.

As pointed out in Chapter 1, the aim of the group of experiments now reported was to confirm that $\text{Na}^+\text{K}^+\text{Mg}^{2+}\text{-ATPase}$ could be demonstrated in a tissue in which its activity would also be expected to alter after adrenalectomy.

MATERIALS AND METHODS

MATERIALS

- a) Animals: ♂ CFE albino rats, as described in Section II.
- b) Kidney: Decapsulated kidneys were used.
- c) Reagents: These are given in the text.

METHODS

- a) Bilateral adrenalectomy: The technique is given in Section II.

TISSUE SOURCE METHOD OF EXTRACTION		ENTIRE KIDNEY		KIDNEY CORTEX		KIDNEY MEDULLA	
		$\frac{\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}}{\text{Mg}^{2+} - \text{ATPase}}$	Reference	$\frac{\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}}{\text{Mg}^{2+} - \text{ATPase}}$	Reference	$\frac{\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}}{\text{Mg}^{2+} - \text{ATPase}}$	Reference
		WHOLE HOMOGENATE IN	distilled water + lyophilization	0.2	Bonting, et al (1962)	0.6 0.3 0.7 0.2	Bonting, et al (1961) Bonting, et al (1962) " " Paul & Gonick (1968)
250mM sucrose detergent	1.1		Chignell & Titus (1966)				
MICROSOMES FROM HOMOGENATE IN	250mM sucrose		0.4	Jørgensen (1968)	0.5 0.5 1.0	Duggan & Noll (1965) Gutman & Beyth (1969) Proverbio, et al. (1970)	1.1
	250mM sucrose + detergent	1.3	Skou (1962)	1.8	Katz & Epstein (1967)		
		1.0	Jørgensen (1968)				
	250mM sucrose + detergent	1.9	Davis (1970)				
250mM sucrose + dialysis	1.4	Landon, et al. (1966)					

TABLE IV-1. SUMMARY OF $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}$ ACTIVITY RELATIVE TO $\text{Mg}^{2+} - \text{ATPase}$ ACTIVITY IN CONJUNCTION WITH MODE OF ENZYME PREPARATION FROM MAMMALIAN KIDNEY, AS DERIVED FROM DATA IN THE LITERATURE.

b) Post-operative maintenance: The method is outlined in Section II.

The animals here reported were given normal solid diet and either distilled water or 0.9% NaCl fluid diet until their sacrifice 6 days post-operatively.

c) Tissue sampling: Intact and operated animals were sacrificed by cervical dislocation, the kidneys rapidly excised, placed in small aliquots of appropriate extraction medium at 0° - 4°C , decapsulated and freed of connective tissue. They were then quickly blotted on Whatman No. 1 filter paper and weighed.

d) Preparation of microsomal fraction: The preparative sequence is summarized in FIG.IV-1. Extraction media were of two types:

(i) 250mM sucrose + 5mM $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ + 30mM L-histidine-HCl, pH 6.8
at $4^{\circ}\pm 0.5^{\circ}\text{C}$;

(ii) as medium (i), but incorporating 0.1% sodium deoxycholate.

The enzyme preparation consisted of a carefully resuspended 10 000-107 000xg fraction (pellet(5), see FIG.IV-1), this range of sedimentation having been selected because it encompassed those generally adopted by other workers.

e) Enzyme assay: Incubations of 0.5ml aliquots of fresh resuspensions were made in duplicate at $37^{\circ}\pm 0.1^{\circ}\text{C}$ in the presence of 0.5ml of 4mM Tris-ATP and 1.0ml of 4mM Mg^{2+} , 100mM Na^{+} and 20mM K^{+} (as chlorides).

Enzyme resuspension and ions were made up in 30mM L-histidine-HCl, pH 7.3 at $37^{\circ}\pm 0.5^{\circ}\text{C}$. Orthophosphate and protein determinations were made in duplicate as previously described in Section II.

f) Treatment of data: Data were compared by the application of Student's 't' test.

RESULTS

Extraction characteristics

The tissue was readily disrupted in either extraction medium by homogenization alone. Pellet (1) was large, and the final pellets (4) and (5) of intermediate size. The latter consisted of translucent microsomal material.

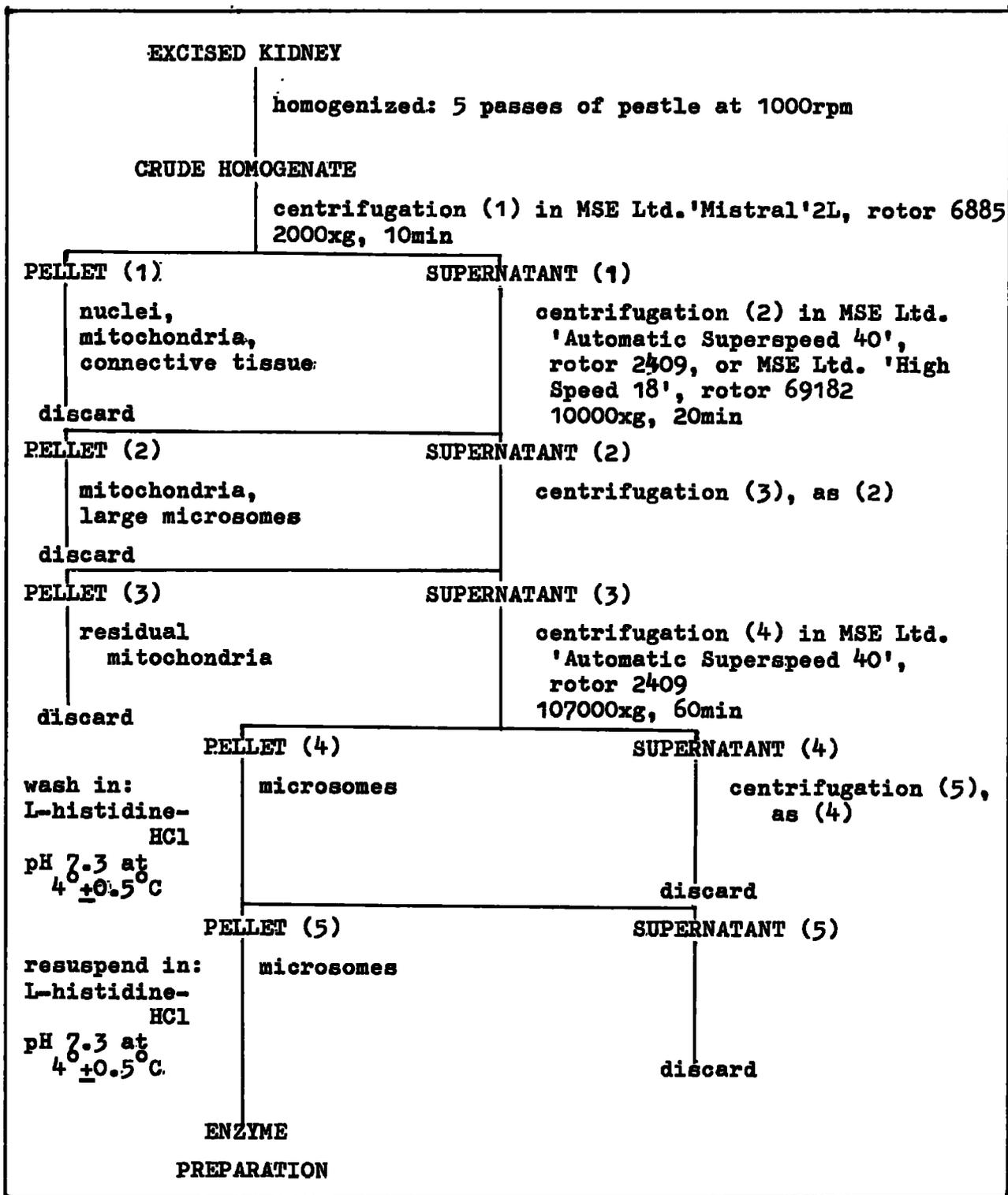


FIG. IV-1. PROCEDURE FOR EXTRACTION OF KIDNEY MICROSOMES.

All steps were performed at 0°-4°C

Time course of ATP hydrolysis

A preliminary investigation of medium (ii)-extracted preparations from intact rats revealed that ATP hydrolysis in the presence of Mg^{2+} or $Mg^{2+}+Na^{+}+K^{+}$ was linear over a 15min assay period (as reported by Jørgensen, 1968). A 10min period was adopted.

Influence of extraction in the presence of deoxycholate upon ATPases

It is clear from the data presented in TABLE IV-2 (see conditions A and B) that the effect of deoxycholate was to cause a significant decrease of 24.7% in mean Mg^{2+} -ATPase activity and an increase of 509.4% in mean $Na^{+}+K^{+}+Mg^{2+}$ -ATPase activity. This resulted in an 8-fold increase in mean activity ratio.

Effects of adrenalectomy and saline compensation upon ATPases

Mg^{2+} -ATPase activity was uninfluenced by adrenalectomy or fluid diet (compare conditions B with C and B with D in TABLE IV-2). However, dramatic effects were observed in the case of $Na^{+}+K^{+}+Mg^{2+}$ -ATPase. Maintenance of operated animals on a distilled water fluid diet resulted in an almost complete (94.1%) loss of mean activity, and a concomitant 16-fold decrease in mean activity ratio (see conditions B and C in TABLE IV-2). This loss was prevented by saline compensation, the decrease in mean activity having been reduced to 20% and the mean activity ratio being of the same order as that obtained for intact animals (compare conditions B with D in TABLE IV-2). Indeed, under these circumstances, neither ATPase changed significantly, as revealed by the calculated P values given in TABLE IV-2.

TABLE IV-2

TABLE IV-2. THE EFFECT OF EXTRACTION TECHNIQUE AND ADRENALECTOMY , WITH AND WITHOUT SALINE COMPENSATION, UPON RAT KIDNEY MICROSOMAL ATPase ACTIVITY.

Extraction media:

- (i) 250mM sucrose + 5mM Na₂EDTA.2H₂O + 30mM L-histidine-HCl, pH 6.8 at 4°±0.5°C;
(ii) as medium (i), but incorporating 0.1% Na deoxycholate.

Incubation conditions:

30mM L-histidine-HCl + 4mM Tris-ATP, pH 7.3, 37°±0.1°C.

Mg²⁺ (4mM), Na⁺ (100mM) and K⁺ (20mM) were included as indicated.

Na⁺+K⁺-Mg²⁺-ATPase activity is the increment in enzyme activity obtained by the inclusion of Na⁺ and K⁺ with Mg²⁺.

$$\frac{\text{Na}^{\pm}+\text{K}^{\pm}-\text{Mg}^{2+}\text{-ATPase}}{\text{Mg}^{2+}\text{-ATPase}}$$
 is the activity ratio described in the text.

Post-operative fluid diet:

Distilled water or 0.9% NaCl were given ad lib., as indicated.

Adx. = adrenalectomized.

Values (1 and 2) given for each preparation are the means obtained in duplicate incubations of microsomal material from different animals.

FLUID DIET	EXTRACTION MEDIUM	DAYS ADX.	CONDITION	VALUES	ATPase ACTIVITY								
					nmoles: P _i liberated/mg protein/min		Na ⁺ +K ⁺ -Mg ²⁺ -	% CHANGE					
					Mg ²⁺ -		Na ⁺ +K ⁺ -Mg ²⁺ -	Mg ²⁺ -	COMPARE. CONDITIONS	Mg ²⁺ -	Na ⁺ +K ⁺ -Mg ²⁺ -		
					t	p	A	B	t	p	A	B	
DISTILLED WATER	(i)	0	A	1		871.6		127.4		0.15			
				2		859.0		123.1		0.14			
				M		865.30		125.25					
	(ii)	0	B	1		632.0		615.5		0.97			
				2		671.9		911.0		1.36			
				M		651.95		763.25		B with A	-24.7	+509.4	
				t	A	10.19	A	4.32					
				p	B	< 0.01	B	< 0.05					
	(ii)	6	C	1		631.1		42.4		0.07			
				2		681.4		48.1		0.07			
				M		656.25		45.25		C with B	+0.7	-94.1	
t				B	1.34	B	4.86						
p				C	> 0.30	C	< 0.05						
0.9% NaCl		6	D	1		693.3		692.3		1.00			
				2		603.5		525.1		0.87			
				M		648.40		608.70		D with B	-1.0	-20.3	
				t	B	0.23	B	0.91					
				p	D	> 0.80	D	> 0.40					
				t	C	0.15	C	6.73					

TABLE IV-2. THE EFFECT OF EXTRACTION TECHNIQUE AND ADRENALECTOMY, WITH AND WITHOUT SALINE COMPENSATION, UPON RAT KIDNEY MICROSOMAL ATPase ACTIVITY.

CHAPTER 3Discussion of Experimental Results and the Control of Renal Na⁺
ReabsorptionInfluence of extraction in the presence of deoxycholate upon ATPase
activities

The 24.7% decrease in mean specific activity of Mg²⁺-ATPase, reported in Chapter 2, is in accord with the direction - though not the magnitude - of change reported by Skou (1962). From Skou's (1962) activity data it is calculated for one pair of preparations that the effect of homogenizing with 0.1% deoxycholate was to reduce Mg²⁺-ATPase activity by 85%. On the other hand, the data of Jørgensen (1968) reveal little change in this ATPase component; this is perhaps explicable in that he adopted a method involving preincubation of isotonic sucrose extracted subcellular fractions with the above concentration of deoxycholate, although a similar procedure revealed a 28% decrement according to Skou (1962). These differences are difficult to resolve, as experimental conditions were closely similar.

Again, although Skou (1962) detected no significant change in Na⁺+K⁺-Mg²⁺-ATPase activity after extraction in a deoxycholate-containing medium (but obtained better activity ratios by so reducing the Mg²⁺-ATPase component), Jørgensen (1968) showed a clear change in detergent treated fractions sedimenting at 25 000xg and 105 000xg. These were, respectively, 177% and 450% increases, and the latter value is in agreement with the 509.4% increase here reported in Chapter 2.

With regard to values obtained for Na⁺+K⁺-Mg²⁺-ATPase/Mg²⁺-ATPase activity ratios, it may be noted that those reported in Chapter 2 for medium (i) and medium (ii)-extracted preparations (see TABLE IV-2, conditions A and B) are distinctly different and in broad agreement with the values calculated from equivalent data in the literature (see TABLE IV-1).

Influence of adrenalectomy upon ATPase activities

It is widely agreed that the microsomal Mg^{2+} -ATPase in this tissue is uninfluenced or insignificantly altered by adrenalectomy (Chignell & Titus, 1966; Landon, et al., 1966; Katz & Epstein, 1967; Jørgensen, 1968), and the observations reported in Chapter 2 are in full accordance with this. As pointed out in Chapter 1, there is also general agreement that $Na^+K^+Mg^{2+}$ -ATPase activity is reduced post-operatively, and in this respect the results presented above (see Chapter 2) are no exception. However, the magnitude of the decrement has been variously reported as 50% (Chignell & Titus, 1966), 24% (Landon et al., 1966), 38% (Katz & Epstein, 1967; Jørgensen, 1968) and 43% (Jørgensen, 1969) in animals with access to 0.9% NaCl, and as 46% in those not receiving saline compensation (Jørgensen, 1968). The reductions in mean specific activity reported above (see TABLE IV-2) are comparatively well differentiated, there having been an almost total loss of $Na^+K^+Mg^{2+}$ -ATPase in rats not receiving saline and an insignificant loss in those which were. It is possible that this difference may be a reflection of varying susceptibility of rat strains to adrenalectomy, and the results of some supplementary work in connexion with this thesis suggested that σ^1 CFE rats were more susceptible than σ^1 CFHB rats.

In situ control of $Na^+K^+Mg^{2+}$ -ATPase activity

It is evident from work executed by others (and previously discussed in Chapter 1), and from the experimental results obtained here (see Chapter 2), that the continual availability of Na^+ to this enzyme is the decisive factor in the maintenance of its activity. It would therefore follow that the action of adrenocorticosteroids (especially aldosterone) might be expected to centre upon the control of all or a part of this Na^+ availability. Such a proposal has already received consideration (see Chapter 1). Alterations in $Na^+K^+Mg^{2+}$ -ATPase activity do appear to correlate with changes in plasma $[Na^+]$ and $[K^+]$, and this was made clear in adrenalectomized rats by Jørgensen (1968).

Control of renal Na⁺ reabsorption

Recent work - for instance, that of Proverbio et al. (1970) - suggests that, in addition to Na⁺+K⁺-Mg²⁺-ATPase, a further (? enzymic) Na⁺-reabsorption component is operative in kidney. Presumably the activity of this component would also be determined by Na⁺ availability. It is clear that the functioning of these components is ultimately dependent upon the passive permeability properties of the luminal membrane of tubular epithelial cells, and that the mechanism governing these properties is itself regulated by adrenocorticosteroids. The nature of this mechanism remains unknown.

Main conclusions

1. Na⁺+K⁺-Mg²⁺-ATPase is present in kidney microsomal preparations.
2. This enzyme is a component of the renal Na⁺ reabsorption mechanism.
3. The maintenance of this enzyme activity is dependent upon the availability of Na⁺ to it.
4. The effect of adrenalectomy is to reduce Na⁺+K⁺-Mg²⁺-ATPase activity, and it may be reversed by making Na⁺ available in excess.
5. It follows that adrenocorticosteroids act by regulating the availability of Na⁺ to the micro-environment of this enzyme, within renal tubular epithelial cells. This probably involves their interaction with the mechanism which controls passive Na⁺ permeability of the luminal membrane.

S E C T I O N V

ERYTHROCYTE STUDIES

CHAPTER 1Erythrocyte Cation Permeability and its Bearing upon Cell Function

Erythrocytes are amongst the most accessible cells, particularly in human subjects, and they have the additional advantage of yielding plasma membrane preparations free of subcellular organelles, though slightly contaminated with haemoglobin. They have consequently received much attention from clinical and scientific viewpoints. This Section is concerned with the influence of adrenocorticosteroids upon properties held to be common to the plasma membranes of erythrocytes and other cells (e.g. neurons). A direct assessment is made of erythrocyte characteristics in human subjects suffering affective disorders.

Relationship of cation distribution and permeability to erythrocyte survival1) Cation distribution

Amongst the mammals there is considerable variability in erythrocyte $[Na^+]_i$ and $[K^+]_i$ (Ponder, 1948; Bernstein, 1954): those of man, rat, rabbit, guinea-pig, pig and horse contain relatively low $[Na^+]_i$ and high $[K^+]_i$, whereas those of dog, cat and ox show the reverse. In addition, there are strains of sheep (Tosteson & Hoffman, 1960) and opossum (Baker & Simmonds, 1966) with cells having each type of distribution. Despite these marked species and strain differences, $[Na^+]_i$ is always lower, and $[K^+]_i$ always higher, than would be expected were they to obey Gibbs-Donnan equilibrium distributions with respect to plasma, and this discrepancy has been the basis for an intensive investigation of active Na^+ and K^+ transport in erythrocytes. Such a transport mechanism is essential in order to counteract the passive movements of these cations towards their equilibria.

2) Control of cell volume

The implications of uncontrolled passive Na^+ and K^+ movements are considerable, since, if the cell membrane retained its impermeability to haemoglobin, the resultant osmotic pressure inside the cell would lead to water influx, increased

cell volume, and increased susceptibility to membrane disruption. This is, in fact, considered to be the state of affairs in hypotonic haemolysis (see below), and it is clear that the control of Na^+ and K^+ distribution is linked to that of water flux, and hence to that of cell volume (Tosteson & Hoffman, 1960). Furthermore, the incubation of erythrocytes in the absence of glucose results in depletion of $[\text{ATP}]_i$ and the assumption of spheroidity (Nakao, Nakao & Yamazoe, 1960; Nakao Nakao, Yamazoe & Yoshikawa, 1961), and depletion of $[\text{ATP}]_i$ is paralleled by decreased K^+ influx (Whittam, 1958). Other work has shown that cold storage or incubation of erythrocytes in glucose-free media results in $[\text{Na}^+]_i$ gain and $[\text{K}^+]_i$ loss, and that this change may be reversed by returning cold stored blood to 37°C (Harris, 1940) or by the provision of glucose, mannose or fructose in the incubation medium (Maizels, 1949, 1951). Although an osmotic influence of extracellular glucose is not denied, Lindemann & Passow (1960) have shown that inhibition of glycolysis by F^- results in eventual $[\text{K}^+]_i$ loss, the delay in onset of this process being related to the $[\text{ATP}]_i$. The dependence of active transmembrane Na^+ and K^+ fluxes upon the glycolytic supply of ATP (and, to some degree, vice versa: see, for instance, Whittam & Ager, 1965; Parker & Hoffman, 1967) has been the subject of much recent research.

Monovalent cation flux components

The Na^+ and K^+ movements across erythrocyte membranes have been resolved into passive leak and active pump components, mainly as a result of Schatzmann's (1953) finding that cardiac glycosides specifically inhibit their active transport in 4°C stored blood when subsequently incubated at 37°C in the presence of glucose. No attempt will be made to summarize the now considerable literature in this field, other than to say that the recent findings of Post, Albright & Dayani (1967) for human erythrocytes are largely representative. These authors confirmed the specificity of ouabain as a pump inhibitor by demonstrating its ineffectiveness in the absence of Na^+ , K^+ or ATP. They also

noted that the rates of passive Na^+ and K^+ movement were closely related to $[\text{Na}^+]_e$ and $[\text{K}^+]_e$. Net transport was accounted for by the sum of the rates of active and passive ion fluxes when differentiated upon the basis of their ouabain sensitivity.

With regard to active transport, there is now much evidence to suggest that K^+ influx is not an absolute requisite of active Na^+ extrusion in erythrocytes. Glynn (1956) had distinguished a Na^+ efflux component which existed in the absence of $[\text{K}^+]_e$ and which was apparently glucose-independent, although incapable of mediating net Na^+ loss. He subsequently showed (Glynn, 1957) that this, apparently passive, component was glycoside sensitive. The current interpretation of this component is that it is due to the conversion of the Na^+-K^+ pump mechanism to a 1:1 Na^+ exchange system, of which both influx and efflux components are ouabain sensitive (Garrahan & Glynn, 1967a; Levin, Rector & Seldin, 1968; Villamil & Kleeman, 1969; Sachs, 1970). It is ATP-dependent, but not associated with ouabain sensitive ATP hydrolysis (Garrahan & Glynn, 1967c), and the relative magnitudes of Na^+-K^+ and Na^+-Na^+ exchange are dependent upon $[\text{Na}^+]_i$, $[\text{ATP}]_i$ and $[\text{P}_i]_i$ (Garrahan & Glynn, 1967b). In the circumstances, it is difficult to decide whether Na^+-Na^+ exchange is an active process. The work of Hoffman (1962) and Maizels (1968) indicates that no other ion will substitute for Na^+_i in the transport mechanisms, although it is clear from knowledge of the Na^+-Na^+ exchange mechanism that the (external) K^+ site is less selective.

Certain workers have been led to question the supposition that active Na^+ efflux in erythrocytes is entirely mediated by an ouabain-sensitive mechanism. Post, *et al.* (1967) themselves observed that ouabain treated cells contained slightly more K^+ than plasma, and attributed this to a residual pump activity equivalent to about 2% of normal. Glynn's (1957) data reveal that, irrespective of $[\text{K}^+]_e$, Na^+ efflux was not entirely abolished by cardiac glycosides. A value of about 66% inhibition by ouabain may be derived, and subsequent

results are consistent with this estimate (Garrahan & Glynn, 1967a). Thus, about 33% of Na^+ efflux is cardiac glycoside-insensitive in human erythrocytes, as used by these authors, and as much as 50% in rabbit cells according to Villamil & Kleeman (1969). Hoffman & Kregenow (1966) were able to distinguish at least a part of this ouabain insensitive component by its sensitivity to the diuretic ethacrynic acid. They found that ouabain inhibition of Na^+ efflux occurring in the presence of $[\text{K}^+]_e$ and enhanced upon addition of $[\text{Na}^+]_e$ may be regarded as consistent with the presence of Na^+-K^+ and Na^+-Na^+ exchange components, as described above: overall Na^+ efflux was indeed reduced by 66%. The residual efflux component was reduced by 62% in the presence of 1mM ethacrynic acid, and this fraction was thought to represent a second pump mechanism ('pump II'). The small remaining Na^+ efflux component was attributed to exchange diffusion, i.e. the bidirectional transmembrane carriage of Na^+ by a mechanism functionally dependent upon formation of a Na^+ -carrier complex, but not mediating a net Na^+ transfer.

A further complication is posed by Sachs' (1971) finding that, although net Na^+ efflux in the presence of ouabain was inhibited by the diuretic furosemide, this agent additionally inhibited the ouabain sensitive Na^+-Na^+ exchange occurring in the absence of $[\text{K}^+]_e$. It may thus be that the ouabain sensitive Na^+-K^+ exchange mechanism can be converted through an ouabain sensitive Na^+-Na^+ to an ouabain insensitive Na^+-Na^+ exchange system.

Correlation of cation distribution with $\text{Na}^++\text{K}^+-\text{Mg}^{2+}$ -ATPase activity

It has been pointed out that the differential distributions of Na^+ and K^+ across erythrocyte membranes exhibits species specificity (see Ponder, 1948; Bernstein, 1954), and, with regard to active transport mechanism, it is of great interest that quantitative correlations have been found to exist between $[\text{Na}^+]_i : [\text{K}^+]_i$ and $\text{Na}^++\text{K}^+-\text{Mg}^{2+}$ -ATPase activity. It has been generally concluded that the maintenance of a low $[\text{Na}^+]_i : [\text{K}^+]_i$ ratio is associated with relatively high enzyme activity (Tosteson, Moulton & Blaustein, 1960; Chan, Calabrese & Theil, 1964; Baker & Simmonds, 1966; Greeff, Grobecker & Piechowski, 1964;

Debenedetti & Lucaroni, 1965; Brewer, Eaton, Beck, Feitler & Schreffler, 1968), and only the data of Duggan, ~~Beer~~ & Noll (1965) appear to refute this. In addition, Vigliocco, Rega & Garrahan (1970) have found that the activity of a K^+Mg^{2+} -activated, ouabain sensitive phosphatase, currently thought to represent the functional external moiety of the membrane bound $Na^+K^+Mg^{2+}$ -ATPase complex (Garrahan, Pouchan & Rega, 1969; Rega, Garrahan & Pouchan, 1970; Askari & Rao, 1971), also varies in this manner.

A thorough investigation of the relationship between active Na^+K^+ transport and $Na^+K^+Mg^{2+}$ -ATPase in human erythrocytes was initiated by Post, Merritt, Kinsolving & Albright (1960) and Dunham & Glynn (1961), who found that the phenomena shared many important features. They bore close resemblance in their being plasma membrane characteristics, in their requirement for ATP and for the simultaneous presence of Na^+ and K^+ , in the magnitude of their cardiac glycoside sensitivity, and in their activation pattern as a function of relative cation concentrations. Subsequent investigations have amplified these findings, and have largely confirmed that the ATP hydrolysis associated with active Na^+K^+ fluxes may be due to $Na^+K^+Mg^{2+}$ -ATPase activity: inhibition of enzyme activity by X-irradiation is closely related to that of cation transport (Bresciani, Auricchio & Fiore, 1964); gain of $[Na^+]_i$ and loss of $[K^+]_i$ associated with storage at $4^\circ C$ is related to the very high temperature coefficient of the enzyme (Wood & Beutler, 1967); the irreversible photooxidative inactivation of $Na^+K^+Mg^{2+}$ -ATPase is a component associated with the initiation of haemolysis by rose bengal (Duncan & Bowler, 1969). However, it is not claimed that this relationship has always proved consistent.

Evidence which is available from studies of the control of relative magnitudes of Na^+K^+ and Na^+Na^+ exchange fluxes, and permeability characteristics in disease, suggests that erythrocyte $Na^+K^+Mg^{2+}$ -ATPase may be a highly adaptable membrane component. Its specific activity and ouabain sensitivity have frequently been reported as rather variable. Thus, the ethacrynic acid sensitive

pump II of Hoffman & Kregenow (1966) was re-investigated by Lubowitz & Whittam (1969), who found that this diuretic markedly inhibited both $\text{Na}^+\text{K}^+\text{Mg}^{2+}$ - and Mg^{2+} -ATPase activities in membrane preparations. Although this is clearly a non-specific effect, it is of interest that Sachs (1971) reported inhibition of the Na^+Na^+ exchange by furosemide (see above); this may be seen as additional evidence in favour of the mediation of Na^+Na^+ exchange by a $\text{Na}^+\text{K}^+\text{Mg}^{2+}$ -ATPase system which is altered by $[\text{K}^+]_e$ depletion. Nevertheless, it may also be that ethacrynic acid is peculiar amongst diuretics in that it actually protects erythrocytes against digitonin- and deoxycholate-induced haemolysis, whereas furosemide slightly enhances hypotonic haemolysis (Sheppard, Tsien & Burghardt, 1969).

The influence of psychoactive drugs and steroid hormones upon monovalent cation permeability and distribution

A good deal of information about this aspect of erythrocyte function is available from studies involving the assessment of susceptibility to hypotonic haemolysis (fragility). This technique has yielded very useful indices of plasma membrane functioning. Investigations which are the subject of Chapters 2-4 of this Section sprang from the question whether adrenocorticosteroids directly influence ionic permeability in cells, and, if so, whether this might be a factor determining the electrolyte redistributions associated with manic-depression (see Section I): it is therefore appropriate to classify present evidence with this question in mind.

1) Effects of drugs used in the treatment of affective disorders

Whereas high concentrations of phenothiazine tranquillizers and of certain antidepressants are haemolytic, in low concentrations (10^{-8} - 10^{-4} M) they reversibly protect or stabilize human erythrocytes against hypotonic haemolysis; i.e. the cells are rendered less fragile, and therefore less susceptible to disruption (Seeman & Weinstein, 1966; van Steveninck, Gjörsund & Booiij, 1967; Kwant & van Steveninck, 1968; Sheppard, Tsien & Burghardt, 1969; Despopoulos, 1970). This effect is associated with an increased critical haemolytic volume (van Steveninck, et al., 1967), the assumption of spheroidity and increased

mean cell volume in isotonic media (Kwant & van Steveninck, 1968), the prevention of K^+ efflux (Seeman & Weinstein, 1966; Kwant & van Steveninck, 1968) despite an elevated Na^+ influx (Kwant & van Steveninck, 1968), and an increase in membrane surface area caused by membrane expansion in intact cells (Seeman, & Kwant, 1969; Seeman, Kwant, Sauks & Argent, 1969) and erythrocyte ghosts (Seeman, Kwant & Sauks, 1969).

It is interesting that certain of the local anaesthetics which protect erythrocytes from hypotonic haemolysis (Seeman, 1966b) have been found to enhance passive Na^+ influx and K^+ efflux (Andersen & Gravenstein, 1965; Andersen, 1968). However, similar changes were elicited by the general anaesthetics chloroform and ether only at concentrations exceeding those present in arterial blood during clinical anaesthesia (Halsey, Smith & Wood, 1970). In contrast with the brain enzyme (see Section VI), there appear to be no reports of phenothiazine or antidepressant drug effects upon erythrocyte $Na^+K^+Mg^{2+}$ -ATPase, and this is one of the subjects considered in Chapters 3 and 4 of the present Section. However, in the light of the sequence for mammalian species-specific susceptibility to hypotonic haemolysis derived by Goldman, Gent & Good (1969, 1970), this phenomenon appears to bear no direct relationship to $[Na^+]_i : [K^+]_i$ ratio (and, it is suspected, none to $Na^+K^+Mg^{2+}$ -ATPase activity). It also seems unlikely that the stimulation of both Mg^{2+} - and $Na^+K^+Mg^{2+}$ -ATPase by lysolecithin, saponin and digitonin (Mirčevová, Šimonová, Michalec & Kolman, 1968) bears direct relationship to their haemolytic effect; it would probably be a repercussion of widespread alteration in membrane lipid content or structural association.

2) Effects of adrenocorticosteroids and related substances

In the course of investigating the regulation of erythropoiesis and erythrocyte destruction, Gordon & Piliero (1950) noticed that cells taken from adrenalectomized animals possessed a decreased susceptibility to hypotonic haemolysis. Nigel & Gordon (1951) subsequently demonstrated that this resulted from the presence of an unidentified plasma factor which could be removed from the cells

by saline washes, so that they regained normal fragility. It seems conceivable that this factor may have been cholesterol, of which elevated circulatory availability may be expected as a concomitant of suppressed adrenocorticosteroid biosynthesis.

Seeman (1966a) and Sheppard, Tsien & Burghardt (1969) have since presented evidence that steroid hormones (e.g. cortisone, hydrocortisone, deoxycorticosterone, testosterone and progesterone) are themselves capable of protecting against hypotonic haemolysis within the in vitro concentration range of 10^{-6} - 10^{-3} M. Progesterone additionally retards the onset of spontaneous lysis in cold-stored whole blood (DeVenuto, 1968), and hydrocortisone against phospholipase C-induced haemolysis of sheep erythrocytes (Eagelstein, Reason & Hsia, 1969). It thus seems that steroids may affect plasmamembranes by becoming structurally incorporated into them (Ohtsuka & Koide, 1969), and this is borne out in certain cases by the ability of steroids to simultaneously exchange with membrane cholesterol and maintain normal osmotic fragility (see, for instance, Bruckdorfer, Demel, de Gier & van Deenen, 1969).

The data of Streeten and Solomon (1954) suggested that intravenous infusion of cortisone and hydrocortisone in man may be related to elevated erythrocyte $[K^+]_i$ and decreased $^{42}K^+$ efflux rates. Kessler, Nelson, Elder, Rosano & Nelson (1957) later showed that a rapid elevation of human erythrocyte $[Na^+]_i$ could be induced by a single intravenous injection of hydrocortisone. Experiments with canine erythrocytes reveal that adrenalectomy results in an increased Na^+ influx rate, which may be partially counteracted by aldosterone in vitro (Spach & Streeten, 1964), and that hydrocortisone in vitro enhances $^{22}Na^+$ influx, but does not affect its efflux from these cells (Streeten & Moses, 1968). Streeten & Moses (1968) further discovered that the anti-aldosterone effect of hydrocortisone appeared to be ATP-dependent, but did not involve altered ATPase activity. However, it is known that $Na^+ + K^+ - Mg^{2+}$ -ATPase activity is very low in dog erythrocytes (see above), and it may be relevant that these authors did not distinguish it from the

Mg^{2+} -ATPase component. Spach & Streeten's (1964) work is in accord with that of Losert, Senft & Senft (1964), who found that adrenalectomy in rats resulted in an elevated $[Na^+]_i$ in certain tissues, including erythrocytes, and that it was counteracted by aldosterone administration; reciprocal redistribution of K^+ also occurred. It is, therefore, disappointing to find that, according to Sulser & Wilbrandt (1957), Llaurodo & Brito (1967) and Bauer & Rathschlag-Schaefer (1968), aldosterone and hydrocortisone have no effect upon erythrocyte $[Na^+]_i$ or $[K^+]_i$ in vivo or in vitro.

To counterbalance the paucity of evidence for or against a steroidal influence upon erythrocyte membrane $Na^+K^+Mg^{2+}$ -ATPase, it has been claimed by Vaccarezza & Willson (1964a) that ACTH administration leads to an elevated blood cell cholinesterase activity in rats, and that this was probably due to an increased corticosteroid output, since a similar effect was observed upon corticosterone administration (Vaccarezza & Willson, 1964b). Furthermore, these authors (Vaccarezza & Willson, 1965) discovered that the enzyme activity was depressed by adrenalectomy, and was recovered upon corticosterone administration. Their results are of great interest in the light of the evidence presented by Duncan (1967) in favour of a role for cholinesterase in connexion with the control of cation permeability at the post-synaptic membrane. However, Whittaker, Charlier & Ramaswamy (1971) observed that human erythrocyte acetylcholinesterase is uninfluenced by the sex steroid components of oral contraceptives.

Erythrocyte characteristics in disease

In view of the ready accessibility of erythrocytes to the experimenter, it is not surprising that a wealth of information has accumulated about their characteristics in disease. It is known that erythrocytes may reflect alterations in the control of body composition: for instance, it is known that the maintenance of rats on a fat free diet is associated with changes in Na^+ and K^+ interaction with ATPase which may spring from altered membrane fatty acid composition (Fariás, Goldemberg & Trucco, 1968), and that their

maintenance on a low $[K^+]$ diet results in increased Mg^{2+} - and $Na^+ + K^+ - Mg^{2+}$ -ATPase activities with no change in that of membrane cholinesterase (Chan & Sanslone, 1969).

Although hereditary spherocytotic erythrocytes possess normal $[Na^+]_i$ (Bertles, 1957), they are distinguished by abnormally high rates of Na^+ influx (Bertles, 1957; Jacob & Jandl, 1964) and efflux (Jacob & Jandl, 1964), which correlate with their increased susceptibility to osmotic haemolysis (Emerson, Shen, Ham, Fleming & Castle, 1956; Hendley & Porter, 1969), decreased Mg^{2+} -ATPase activity (Nakao, Kurashina & Nakao, 1967) and elevated $Na^+ + K^+ - Mg^{2+}$ -ATPase activity (Nakao, et al., 1967; Wiley, 1969). The erythrocytes of subjects who suffer paroxysmal nocturnal haemoglobinuria have apparently normal fragility (Lewis & Vincent, 1968), though elevated Mg^{2+} - and $Na^+ + K^+ - Mg^{2+}$ -ATPase activities (Brabec, Mircevova & Palek, 1969) and depressed membrane cholinesterase activity (Brabec, et al., 1969; Sirchia, Ferrone & Nocca, 1966; Kunstling & Rosse, 1969). Normal total ATPase activity is associated with depressed membrane cholinesterase activity in primary refractory anaemia (Palek, Brabec, Vopatova, Michalec & Mirčevová, 1969), although total ATPase activity is depressed in cases of non-spherocytic haemolytic anaemia (Harvald, Hanel, Squires & Trap-Jensen, 1964). A low Mg^{2+} -ATPase activity was found in the case of an infant suffering hypochromic anaemia (Cotte, Kissin, Mathieu, Poncet, Monnet, Salle & Germain, 1968), and low $Na^+ + K^+ - Mg^{2+}$ -ATPase activity associated with increased $[Na^+]_i$ and decreased $[K^+]_i$ in uraemia (Welt, 1967). Decreased membrane cholinesterase activity was reported by Kaplan, Herz & Hsu (1964) in ABO haemolytic disease of the newborn human.

As regards diseases which are not specifically erythrocyte-centred, it is interesting that a postulated Ca^{2+} transport defect in cystic fibrosis should be associated with normal Mg^{2+} - and $Na^+ + K^+ - Mg^{2+}$ -ATPase, but depressed $Mg^{2+} - Ca^{2+}$ -ATPase activity in erythrocyte membrane preparations (Horton, Cole & Bader, 1970); also that the enhanced osmotic fragility of cells in bovine anaplasmosis is correlated with elevated membrane cholinesterase activity

(Wallace, 1967). Increased $[\text{Na}^+]_i$ has been reported in hyperthyroidism (Goolden, Bateman & Torr, 1971) and decreased $[\text{K}^+]_i$ in aldosteronism (Boyd, 1970).

Some assessment of haematological parameters has also been made in schizophrenic and mentally depressed subjects. Abnormally elevated erythrocyte Mg^{2+} - and $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+}$ -ATPase activities were reported for schizophrenics by Seeman & O'Brien (1963), although that of $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+}$ -ATPase was later refuted by Parker & Hoffman (1964). Normal membrane cholinesterase was reported in schizophrenia and mental depression by Ellman & Callaway (1961).

Although it is not claimed that a consistent overall pattern may be derived from the above observations of blood parameters in disease, it seems not unreasonable to resort to erythrocytes as monitors of cellular alterations in systemic disease. This approach has indeed been adopted in the studies of mentally depressed patients which are reported in Chapter 3 of this Section.

CHAPTER 2Determination of Erythrocyte Cation Content and Flux, and Susceptibility to Hypotonic Haemolysis

The experiments reported in this Chapter were concerned with the determination of $[Na^+]_i$ and $[K^+]_i$ and of certain $^{22}Na^+$ flux characteristics for human and rat erythrocytes. Tests of the susceptibility of rat erythrocytes to hypotonic haemolysis were also made. The effects of adrenalectomy, with and without saline compensation, are given for rats, whilst those of the affective disorders manic-depression, endogenous and reactive depressions are given for human subjects. The effects of tannic acid upon rat erythrocyte $^{22}Na^+$ fluxes are additionally described: part of these results has been previously published (Radcliffe, Duncan & Bowler, 1971).

The results are discussed in conjunction with those reported in Chapter 3.

MATERIALS AND METHODSMATERIALS

- a) Animals: ♂ CFHB albino rats, as described in Section II.
- b) Human subjects:
 - (i) Normal: ♂ and ♀ members of the staff of Durham University Zoology Department.
 - (ii) Inpatients suffering affective disorders: adult ♂ and ♀ patients of Winterton Hospital, Sedgfield, Co. Durham, and of the Psychiatric Ward of North Tees General Hospital, Stockton-on-Tees, Co. Durham.
- c) Reagents: These are given in the text.

METHODSa) Calibrations

- (i) Calibration of Pye Unicam SP 90 Series 2 Atomic Absorption Spectrophotometer for $[Na^+]$ and $[K^+]$ determinations

NaCl and KCl (BDH, Ltd., AnalaR grade) were dried, weighed and dissolved

in deionized water to yield 0.3mM stock solutions. Standard solutions were prepared by serial dilution.

The Na^+ and K^+ solutions were burned in an air-acetylene flame. At a slit width of 0.1mm, maximum Na^+ and K^+ sensitivities occurred at wavelengths of 578nm and 758nm respectively. Readings of % transmission were taken for each solution during 30sec. burning periods; these were stable within $\pm 1\%$. 1min. flushes with deionized water were made between determinations. Calibration lines of $[\text{Na}^+]$ and $[\text{K}^+]$ against % transmission were drawn and the standard solutions checked before and after each run. As Na^+ and K^+ were present together in unknowns, a check was made to ensure that cross interference was precluded: there was none in the concentration ranges under study.

(ii) Calibration of Isotope Developments Ltd. Model 7000 scaler for $^{22}\text{Na}^+$ determinations

An aliquot of stock $^{22}\text{NaCl}$ solution (Radiochemical Centre, Amersham, Berks.), diluted to 4 $\mu\text{Ci/ml}$ with deionized water was pipetted onto a Whatman No. 1 filter paper disc in a flat Al planchet and dried under an infra-red lamp. The planchet was inserted into a Pantax Pb castle directly below a Mullard end-window Geiger-Müller tube with a 2.3 mg/cm^2 mica window, and connected to the scaler. The instrument was switched on at 0 volts E.H.T. for 30min prior to calibration.

The E.H.T. was raised stepwise, and the number of counts recorded over 5min. periods at each interval for background alone and background + sample. The counts/sec due to sample alone were computed and plotted against E.H.T. applied. A characteristic plateau was obtained, and 700 V selected as the operating E.H.T. for subsequent experiments.

b) Blood sampling

(i) Rats: Blood was obtained by cardiac puncture under light ether anaesthesia, a sterile Luer 21g x 1.5in hypodermic needle being inserted into the left ventricle ventro-dorsally via the thorax wall (Burhoe, 1940), or axially via

the exposed diaphragm. The sample was steadily withdrawn into a sterile 10ml syringe containing approx. 100 i.u. heparin (Evans Medical Ltd., Liverpool) in a small volume of 154mM NaCl. 5-7ml of blood were obtained within 2min. After removal of the needle from the syringe, samples were gently evacuated into polypropylene tubes at 18°-21°C and the tubes sealed with Parafilm.

(ii) Human subjects: 10ml blood samples were withdrawn from a forearm vein. The subjects under study were not regularly sampled for other purposes, and their diagnoses were not given by the Consultant Psychiatrist until the estimation of erythrocyte characteristics was complete.

c) Bilateral adrenalectomy and maintenance of rats

The techniques are given in Section II. Animals were operated upon under ether anaesthesia. Control (intact) animals were maintained upon normal solid diet and distilled water fluid diet ad lib., whilst operated ones were given the same diet or 0.9% NaCl in place of distilled water.

d) Estimation of erythrocyte $[Na^+]_i$ and $[K^+]_i$

Duplicate aliquots of freshly obtained blood were treated as summarized in FIG.V-1. $[Na^+]$ and $[K^+]$ were determined by duplicate or triplicate flame photometric analyses of suitably diluted aliquots of the final solution.

Supernatants (2) and (3) were checked for ion leakage. A Pye Unicam SP 90 Atomic Absorption Spectrophotometer was used as described in (a) (i) above.

$[Na^+]_i$ and $[K^+]_i$ were expressed as mmoles/l p.c.v..

e) Estimation of $^{22}Na^+$ efflux from loaded erythrocytes

Erythrocytes from freshly drawn blood were isolated and $^{22}Na^+$ -loaded as summarized in FIG.V-2. The final pellet was immediately resuspended to 3x its p.c.v. in 145mM NaCl + 6mM KCl + 3mM NaH_2PO_4 + 20mM D-glucose, which had been adjusted to pH 7.4 with Tris and thermoequilibrated at 37°±0.1°C. 1ml aliquots of this resuspension were quickly dispensed into 15ml glass centrifuge tubes and incubated in a shaking metabolic incubator at 100 x 25mm strokes/min and

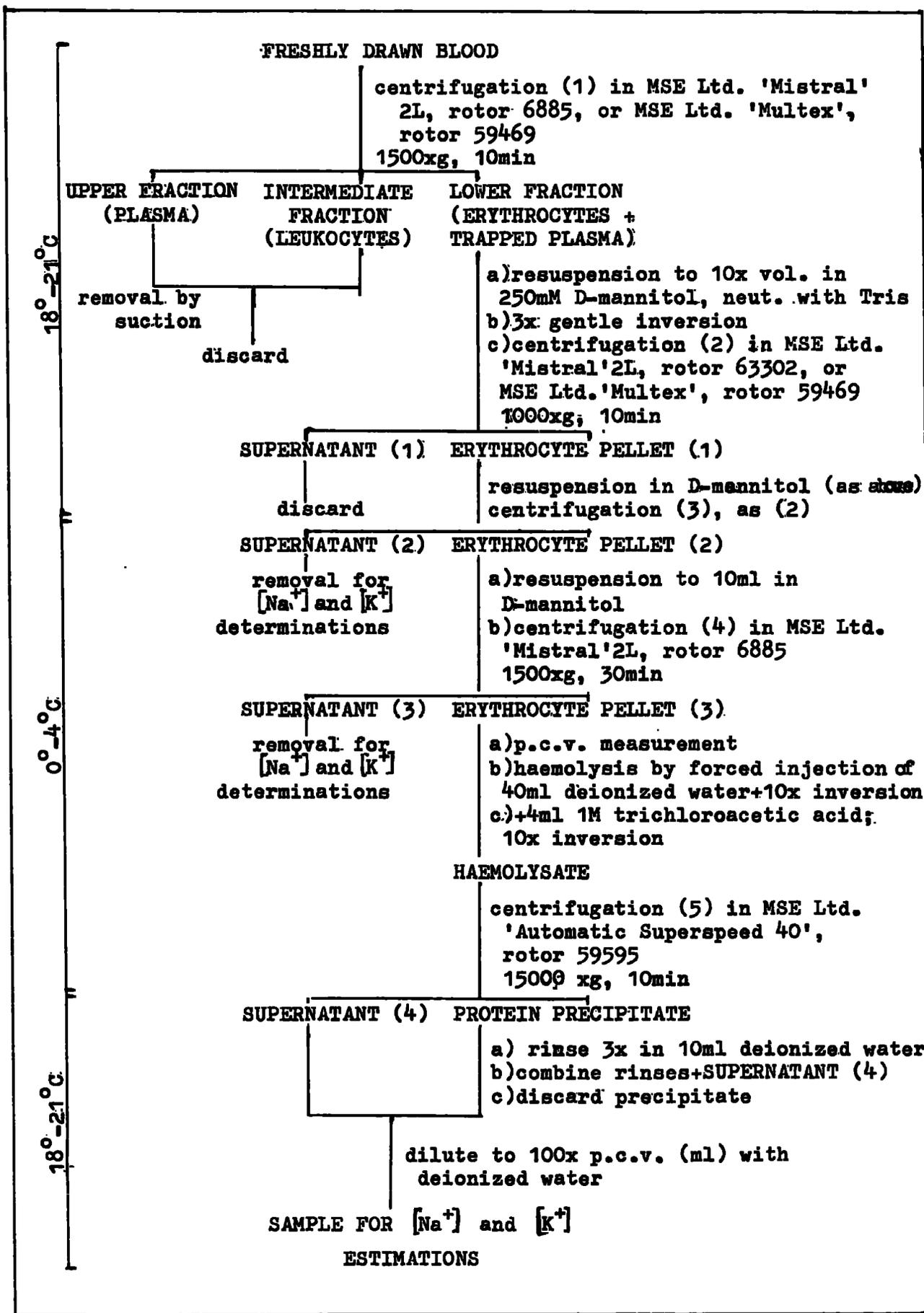


FIG. V-1. PREPARATION OF ERYTHROCYTES FOR [Na⁺]_i AND [K⁺]_i ESTIMATIONS.

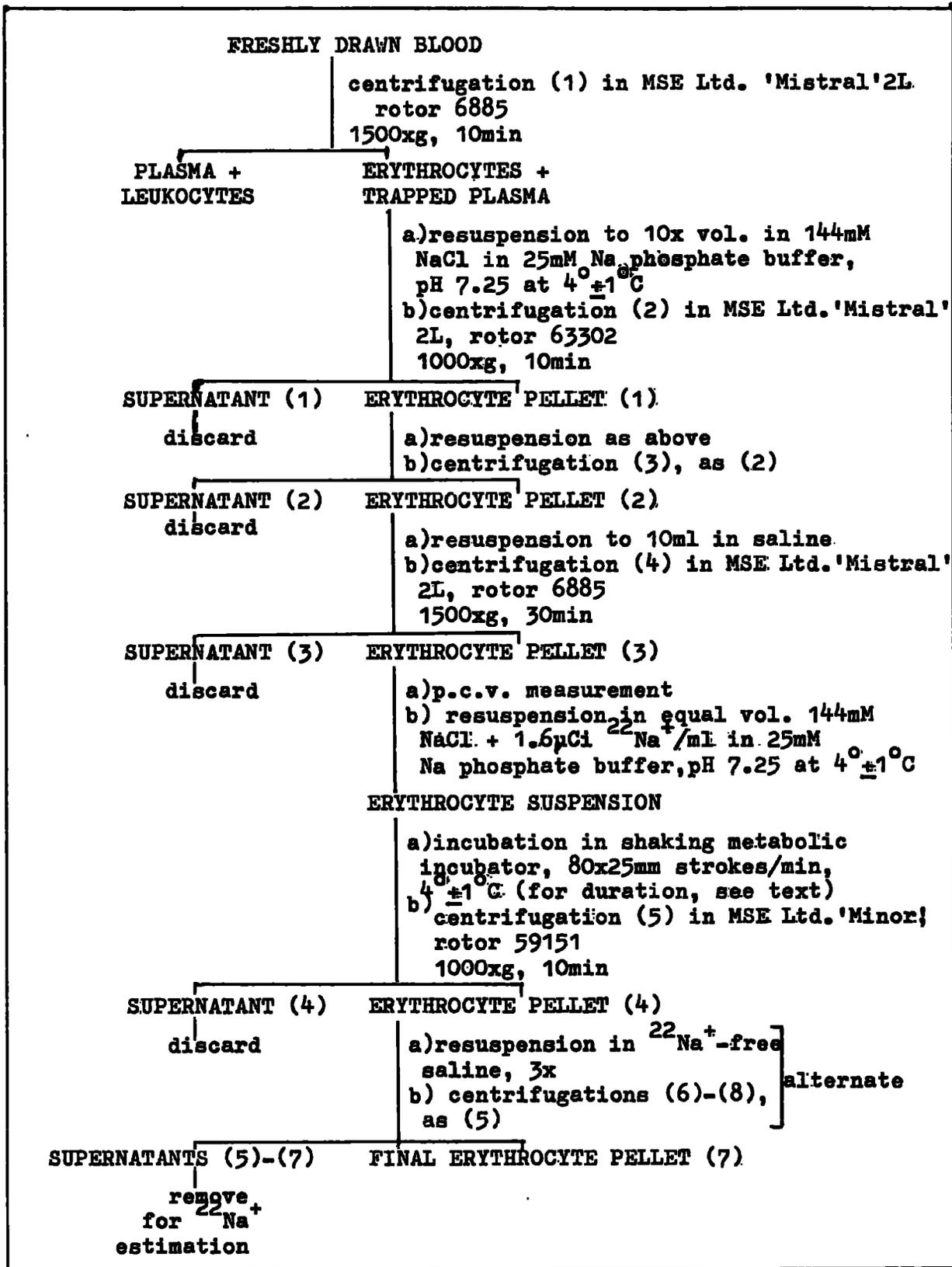


FIG. V-2. PREPARATION OF ²²Na⁺-LOADED ERYTHROCYTES.

All procedures at 0°-4°C, unless otherwise stated.

$37^{\circ} \pm 1^{\circ}\text{C}$. One sample was retained for immediate $^{22}\text{Na}^{+}$ determination, the remainder being removed at suitable time intervals. 0.3ml aliquots of the whole suspension and of the supernatant (obtained after centrifugation in an M.S.E. Ltd. 'Minor' centrifuge, rotor 59151, at 1000xg for 3min at 18° - 21°C) were pipetted onto Whatman No. 1 filter paper discs in flat Al planchets, dried and counted as described in (a)(ii) above. The activity due to intracellular $^{22}\text{Na}^{+}$ was computed and expressed as c.p.s./ml p.c.v.. Taking the activity at 0min as 100%, the relative intracellular activities at subsequent time intervals were plotted logarithmically against incubation time in mins. The line of best fit was found by the method of least squares, and the rate constant for $^{22}\text{Na}^{+}$ efflux was derived from it, using the equation :

$$\text{- rate constant} = \ln \frac{\text{c.p.s./ ml p.c.v. at 60min}}{\text{c.p.s./ ml p.c.v. at 0min}} \text{ hr}^{-1} .$$

f) Treatment of rat erythrocytes with tannic acid

(i) Preparation of tannic acid solutions: Since tannic acid has been reported to have a high affinity for clean glass (Edelberg, 1952), all glassware was siliconized with Sigmacote (Sigma Chemical Co.) and equilibrated before use with tannic acid (B.D.H. Ltd., Lab. Reagent grade) of appropriate concentration in distilled water. For the experiments reported in this Chapter, tannic acid solutions were prepared at pH 7.2 in 145mM NaCl in 15mM Na phosphate buffer, so as to yield a final concentration of $1 \times 10^{-5}\text{M}$ tannic acid upon addition of the erythrocyte suspension.

(ii) Exposure to $1 \times 10^{-5}\text{M}$ tannic acid prior to $^{22}\text{Na}^{+}$ loading

Erythrocytes were separated and washed as summarized in FIG.V-2 as far as the pellet (3) stage. One volume of a 50% resuspension of cells in saline was then introduced into 8 volumes of tannic acid ($1 \times 10^{-5}\text{M}$ final concentration) by gentle alternate uptake into and evacuation from a Pasteur pipette. Resuspension was repeated several times during a 30min incubation at 0°C . Control cells were treated in the absence of tannic acid. The cells were separated

by centrifugation in an M.S.E. Ltd. 'Mistral' 2L centrifuge, rotor 63302, at 1000xg for 10min and at 0°-4°C. They were then washed in an equal volume of saline, recentrifuged and $^{22}\text{Na}^+$ loaded as outlined in FIG.V-2. The loading period is given in Results.

(iii) Exposure to $1 \times 10^{-5}\text{M}$ tannic acid following $^{22}\text{Na}^+$ loading:

Erythrocytes were separated, washed and $^{22}\text{Na}^+$ loaded as summarized in FIG.V-2. Experimental cells were then incubated with tannic acid of final concentration $1 \times 10^{-5}\text{M}$ for 30min as described above. Control cells were treated in saline alone. The cells were then separated by centrifugation in an M.S.E. Ltd. 'Minor' centrifuge, rotor 59151, at 1000xg for 10min and at 0°-4°C. They were then washed in an equal volume of saline, recentrifuged, and $^{22}\text{Na}^+$ efflux was traced in the usual manner (see (e) above).

g) Measurement of rat erythrocyte susceptibility to hypotonic haemolysis

Cells were isolated from freshly drawn blood as summarized in FIG.V-2 as far as the pellet (3) stage. All procedures were carried out and solutions buffered at 18°-21°C. The washed cells were then resuspended to 15x their p.c.v. in 144mM NaCl in 10mM Na phosphate buffer, pH 7.4. Duplicate 0.2 ml aliquots of this suspension were pipetted into 3ml samples of test solutions in centrifuge tubes, and the contents gently mixed by whirling. Test solutions consisted of a [NaCl] series in 10mM Na phosphate buffer, pH 7.4. Tubes were incubated at 18°-21°C for 5min from the time of erythrocyte addition, and then centrifuged in an M.S.E. Ltd. 'Mistral' 2L centrifuge, rotor 6885, at 2000xg for 1min. Aliquots of supernatant were immediately transferred into glass cuvettes, and their haemoglobin content measured colorimetrically versus a distilled water blank. Optical density was recorded at 545nm, using a Hilger & Watts, Ltd. spectrophotometer (for description, see Section II). There was no haemolysis in 154mM NaCl, and the optical density recorded for haemolysis in deionized water was taken as 100%. % haemolysis was plotted as a function of [NaCl] in the customary manner.

RESULTSA. Determinations of $[Na^+]_i$ and $[K^+]_i$ 1. Influence of adrenalectomy and post-operative fluid diet upon rat erythrocytes

Erythrocytes from control (intact) rats receiving distilled water were compared with those from adrenalectomized animals receiving the same diet or 0.9% NaCl. Rats from all groups were sacrificed at given time intervals after the day upon which operations were performed. The $[Na^+]_i$ and $[K^+]_i$ values obtained in these experiments are set out in TABLE V-1; P values, calculated by application of Student's *t*-test, are also given.

Examination of TABLE V-1 shows that mean $[Na^+]_i$ and $[K^+]_i$ were both below control values in cells from adrenalectomized rats receiving no saline compensation. The initial fall was abrupt for both cations, as assessed by estimations made on day 1 post-adrenalectomy; the new mean value was significantly different from control for $[Na^+]_i$ ($P < 0.001$), but not for $[K^+]_i$ ($P > 0.10$). Mean $[Na^+]_i$ was further reduced on day 3, although mean $[K^+]_i$ remained unaltered with respect to day 1. A further slight reduction in mean $[K^+]_i$ was evident on day 10, but this value remained not significantly different from control ($P > 0.10$). Mean $[Na^+]_i$ showed evidence of returning towards the control value at this stage, although it was still significantly different ($P < 0.01$).

These extensive changes were not seen in rats receiving saline compensation. Examination of TABLE V-1 shows that in no case did mean cation content prove to be statistically different from control. Nevertheless, initial falls in mean $[Na^+]_i$ and $[K^+]_i$ were evident 1 day post-operatively. This fall continued into day 2 in the case of $[K^+]_i$, although mean $[Na^+]_i$ was above control on that day. However, this change in $[Na^+]_i$ was transitory, and the range of values obtained was great (4.4 - 9.4 mmoles Na^+ /1 p.c.v.). On days 3 and 8 the mean $[Na^+]_i$ value was virtually the same as that obtained on day 1. Mean $[K^+]_i$ rose to a value slightly above control on day 3, and thereafter showed

FLUID. DIET		DISTILLED WATER				0.9% NaCl				
[CATION] (mmoles/l. pcv)	DAYS POST- ADX.	0 (control)	1	3	10	1	2	3	8	15
	[Na ⁺] _i	n	44	6	6	4	4	8	8	4
\bar{x}		5.69	3.38	2.91	3.77	5.00	6.21	5.02	5.05	6.50
S.E.		0.18	0.09	0.08	0.07	0.51	0.70	0.22	0.08	0.57
P		-	< 0.001	< 0.001	< 0.01	> 0.20	> 0.40	> 0.10	> 0.20	> 0.10
[K ⁺] _i	n	44	6	6	4	4	8	8	4	4
	\bar{x}	95.07	91.16	91.16	90.25	94.00	92.37	95.62	94.75	92.50
	S.E.	0.86	0.60	0.70	0.64	0.51	0.84	0.76	1.11	0.28
	P	-	> 0.10	> 0.10	> 0.10	> 0.60	> 0.20	> 0.70	> 0.90	> 0.30

TABLE V-1. INFLUENCE OF ADRENALECTOMY AND POST-OPERATIVE FLUID DIET UPON RAT ERYTHROCYTE [Na⁺]_i AND [K⁺]_i.

a fall, by day 15 having reapproached the day 2 value. On day 15, mean $[Na^+]_i$ was again slightly above control.

Thus, the overall effect of saline compensation was to lessen the degree of reduction in $[Na^+]_i$ and $[K^+]_i$ observed in erythrocytes from adrenalectomized rats otherwise receiving distilled water. The fall in erythrocyte $[Na^+]_i$ in such animals proved to be statistically significant, whilst that in $[K^+]_i$ did not.

2. Influence of affective disorders and psychoactive drug therapy upon human erythrocytes

Subjects were classified according to their affective status at the time of blood sampling, viz. normal, manic-depression (manic or depressive phase), endogenous depression and reactive depression. Each category of disordered subjects was grouped with respect to drug therapy as follows:

- (A) subjects not differentiated upon a drug therapy basis; i.e. all subjects within a given affective category,
- (B) subjects not receiving psychoactive drugs,
- (C) subjects receiving chlorpromazine as 'Largactil' (May & Baker, Ltd.),
- (D) subjects receiving haloperidol as 'Serenace' (G.D. Searle & Co, Ltd.).

Subjects of normal affect and not receiving psychoactive drugs were regarded as controls, and are designated by the code (N) in TABLE V-2.

TABLE V-2 is a record of the mean $[Na^+]_i$ and $[K^+]_i$ values and their standard errors. It shows the P values derived by applying Student's 't' test in comparing the following groups of values:

- (i) normal subjects (N) with those suffering affective disorders and undergoing the drug therapies designated (A) - (D) above,
- (ii) subjects receiving no psychoactive drugs (B) with those receiving chlorpromazine (C) or haloperidol (D).

When values for erythrocyte cation content are considered as factors of affective status alone (group (A)), the P values reveal that mean $[Na^+]_i$ was apparently not significantly different from normal in any affective disease category,

TABLE V-2

TABLE V-2. INFLUENCE OF AFFECTIVE STATUS AND PSYCHOACTIVE DRUG THERAPY UPON HUMAN ERYTHROCYTE $[Na^+]_i$ AND $[K^+]_i$.

Analysis of cation content:

See 'Materials and Methods'.

Classification of affective status:

See text.

Drug therapy:

See text, but note:

(A) N.D. = subjects not differentiated upon a drug therapy basis;

(B) NONE = those not receiving psychoactive drugs;

(C) CPZ = those receiving chlorpromazine;

(D) HALOP = those receiving haloperidol.

(N) = normal subjects, not receiving psychoactive drugs.

P values shown are those derived by comparing data given for the groups designated, e.g. N+A), B+D).

[CATION] (mmoles/ l pcv)	DRUG THERAPY	VALUE	AFFECTIVE STATUS				
			NORMAL (N)	MANIC-DEPRESSION		ENDOGENOUS DEPRESSION	REACTIVE DEPRESSION
				MANIC PHASE	DEPRESSIVE PHASE		
[Na ⁺] _i	N.D. (A)	n $\bar{x} \pm 1 \times S.E.$ P	- - -	5 11.36 \pm 0.83 N+A) > 0.20	13 11.15 \pm 0.57 N+A) > 0.70	19 12.93 \pm 0.50 N+A) > 0.50	20 12.33 \pm 0.46 N+A) > 0.80
	NONE (B)	n $\bar{x} \pm 1 \times S.E.$ P	9 12.51 \pm 0.45 -	2 12.65 \pm 1.85 N+B) > 0.90	7 11.42 \pm 0.75 N+B) > 0.20	- - -	18 12.42 \pm 0.51 N+B) > 0.90
	CPZ (C)	n $\bar{x} \pm 1 \times S.E.$ P	- - -	- - -	6 10.83 \pm 0.85 N+C) > 0.05 B+C) > 0.80	- - -	2 11.58 \pm 1.00 N+C) > 0.40 B+C) > 0.60
	HALOP (D)	n $\bar{x} \pm 1 \times S.E.$ P	- - -	3 10.50 \pm 0.49 N+D) < 0.05 B+D) > 0.20	- - -	- - -	- - -
[K ⁺] _i	N.D. (A)	n $\bar{x} \pm 1 \times S.E.$ P	- - -	5 114.4 \pm 5.52 N+A) < 0.05	13 116.46 \pm 3.49 N+A) < 0.05	19 104.31 \pm 1.77 N+A) > 0.70	20 105.10 \pm 1.18 N+A) > 0.10
	NONE (B)	n $\bar{x} \pm 1 \times S.E.$ P	9 101.4 \pm 2.05 -	2 124.00 \pm 2.00 N+B) < 0.001	7 117.28 \pm 5.79 N+B) < 0.02	- - -	18 104.77 \pm 1.12 N+B) > 0.10
	CPZ (C)	n $\bar{x} \pm 1 \times S.E.$ P	- - -	- - -	6 115.50 \pm 3.36 N+C) < 0.01 B+C) > 0.70	- - -	2 108.00 \pm 8.00 N+C) > 0.20 B+C) > 0.10
	HALOP (D)	n $\bar{x} \pm 1 \times S.E.$ P	- - -	3 108.00 \pm 7.02 N+D) > 0.20 B+D) > 0.10	- - -	- - -	- - -

TABLE V-2. INFLUENCE OF AFFECTIVE STATUS AND PSYCHOACTIVE DRUG THERAPY UPON HUMAN ERYTHROCYTE [Na⁺]_i AND [K⁺]_i.

but that mean $[K^+]_i$ was significantly greater than normal in manic phase ($P \leq 0.05$) and depressive phase ($P \leq 0.05$) manic-depressives. It was, however, possible to subdivide manic-depressives and reactive depressives upon the basis of the drug therapies (B)-(D) (see below, and TABLE V-2). Endogenous depressives could not be re-grouped upon this basis because they received a wide variety of drugs, but the P values suggest that neither mean $[Na^+]_i$ ($P > 0.50$) nor mean $[K^+]_i$ ($P > 0.70$) were abnormal in this category.

A consideration of categories of disease which could be subdivided into groups (B)-(D) is more illuminating. Mean erythrocyte $[K^+]_i$ was markedly elevated above normal in manic ($P \leq 0.001$) and depressive ($P \leq 0.02$) phases of manic-depression, and uninfluenced in reactive depression ($P > 0.10$) when subjects were not receiving psychoactive drugs (group B). Administration of haloperidol (group B) to manic-depressives (manic phase) was associated with reduction of mean $[K^+]_i$ to a level not significantly different from normal ($P > 0.20$). Chlorpromazine administration (group C) had virtually no effect upon mean $[K^+]_i$ in manic-depressives (depressive phase) ($P > 0.70$) or upon the value for reactive depressives ($P > 0.10$), as judged from the limited data available.

There was no conclusive evidence for altered erythrocyte $[Na^+]_i$ in the subjects under study. Two manic-depressive (manic phase) subjects who were not receiving psychoactive drugs, and who were available for this study, presented widely divergent values of 14.5 and 10.8 mmoles Na^+ /l p.c.v., and a mean value not significantly different from normal ($P > 0.90$). However, those receiving haloperidol gave a mean value significantly below normal ($P < 0.05$). The mean $[Na^+]_i$ value was lowered, but not significantly different from normal, in manic-depressives (depressive phase) not treated with psychoactive drugs ($P > 0.20$); the administration of chlorpromazine resulted in a slight, but not significant lowering of this mean value ($P > 0.80$). Mean $[Na^+]_i$ remained normal in reactive depressives not receiving psychoactive drugs ($P > 0.90$), and results available from two patients receiving chlorpromazine suggest that the drug did not influence this value ($P > 0.60$).

B. Determinations of $^{22}\text{Na}^+$ influx and efflux characteristics

1. Influx

Influence of adrenalectomy upon $^{22}\text{Na}^+$ influx into isolated rat erythrocytes

Erythrocytes from control (intact) and adrenalectomized rats which had received distilled water fluid diet were $^{22}\text{Na}^+$ -loaded for 10hr, and their radioactive content assayed in the normal manner after initial separation and washing procedures (see Methods (e)). Adrenalectomized rats were sacrificed 1, 3 and 5 days post-operatively, and their mean erythrocyte $^{22}\text{Na}^+$ content was not different from that of control animals (see FIG.V-3). These results show that adrenalectomy does not influence the extent to which rat erythrocytes may become $^{22}\text{Na}^+$ -loaded, even when animals are not saline compensated.

Influence of exposure of rat erythrocytes to $1 \times 10^{-5}\text{M}$ tannic acid upon subsequent $^{22}\text{Na}^+$ loading

The time course of $^{22}\text{Na}^+$ loading was followed in control and tannic acid pre-treated erythrocytes from normal rats, and the result obtained in a representative 36hr experiment is shown in FIG.V-4. Control cells showed an increased loading rate after 6hr, but this did not occur in those treated with tannic acid: the radioisotope contents continued to diverge over 24hr.

2. Efflux

a) Comparison of normal human and rat erythrocytes

FIGS. V-5 and V-6 are plots of the results obtained in representative experiments using normal human and rat cells respectively, and illustrate that the $^{22}\text{Na}^+$ efflux rate from loaded rat cells is greater than that from similarly treated human erythrocytes. The lines of best fit are calculated by the method of least squares, and efflux rate constants are derived by the formula given above (see Methods (e)). In the case of the experiments shown in FIGS. V-5 and V-6, the rate constants for $^{22}\text{Na}^+$ efflux were -0.24hr^{-1} for human, and -2.16hr^{-1} for rat erythrocytes.

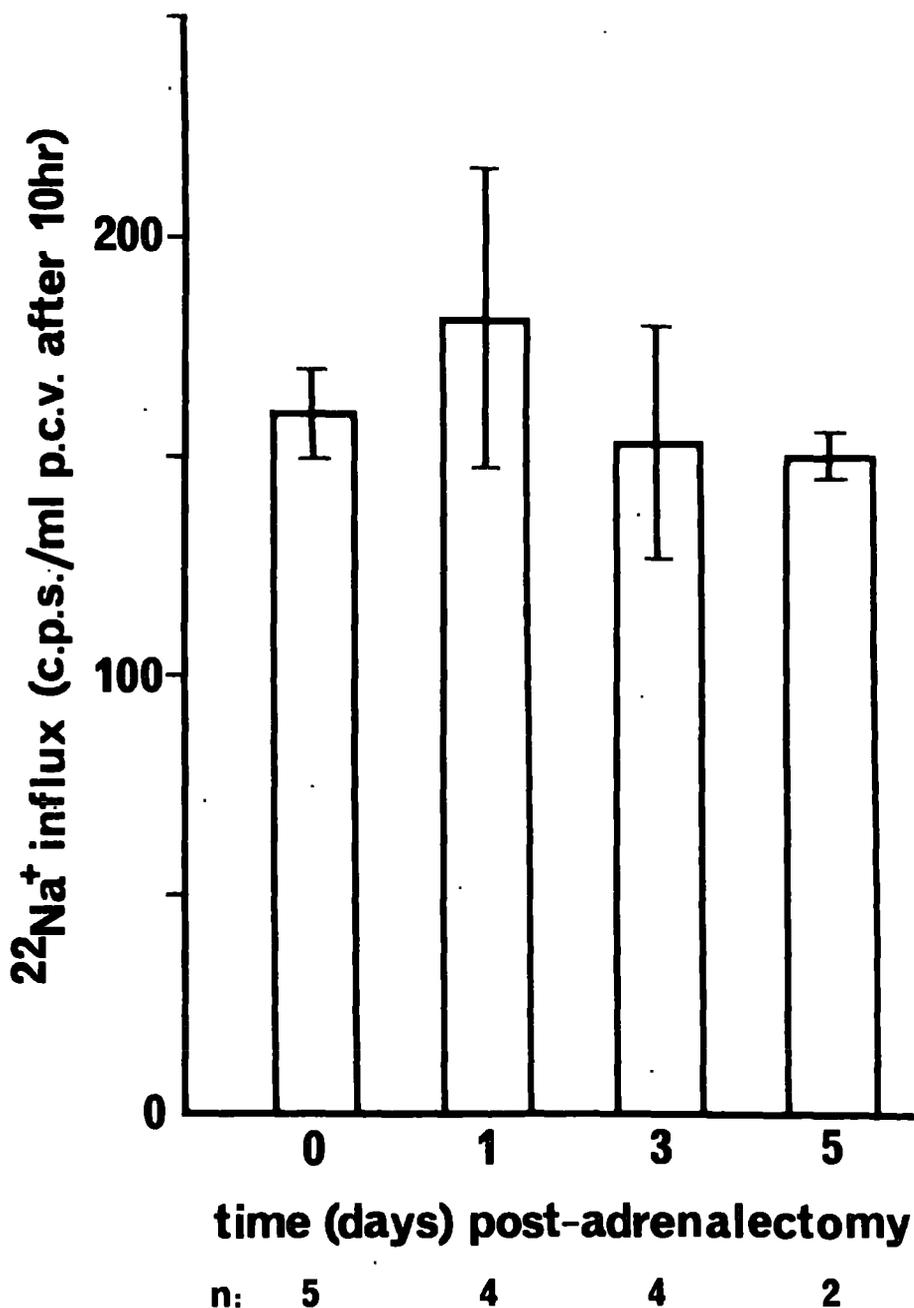


FIG. V-3. INFLUENCE OF ADRENALECTOMY UPON $^{22}\text{Na}^+$ LOADING OF RAT ERYTHROCYTE

Methods of loading and analysis: See 'Materials and Methods'.

Post-operative fluid diet: All animals received distilled water ad lib.

Ordinate: $^{22}\text{Na}^+$ influx in c.p.s./ml p.c.v. after 10hr loading period.

n = number of animals tested.

| = \pm S.E..

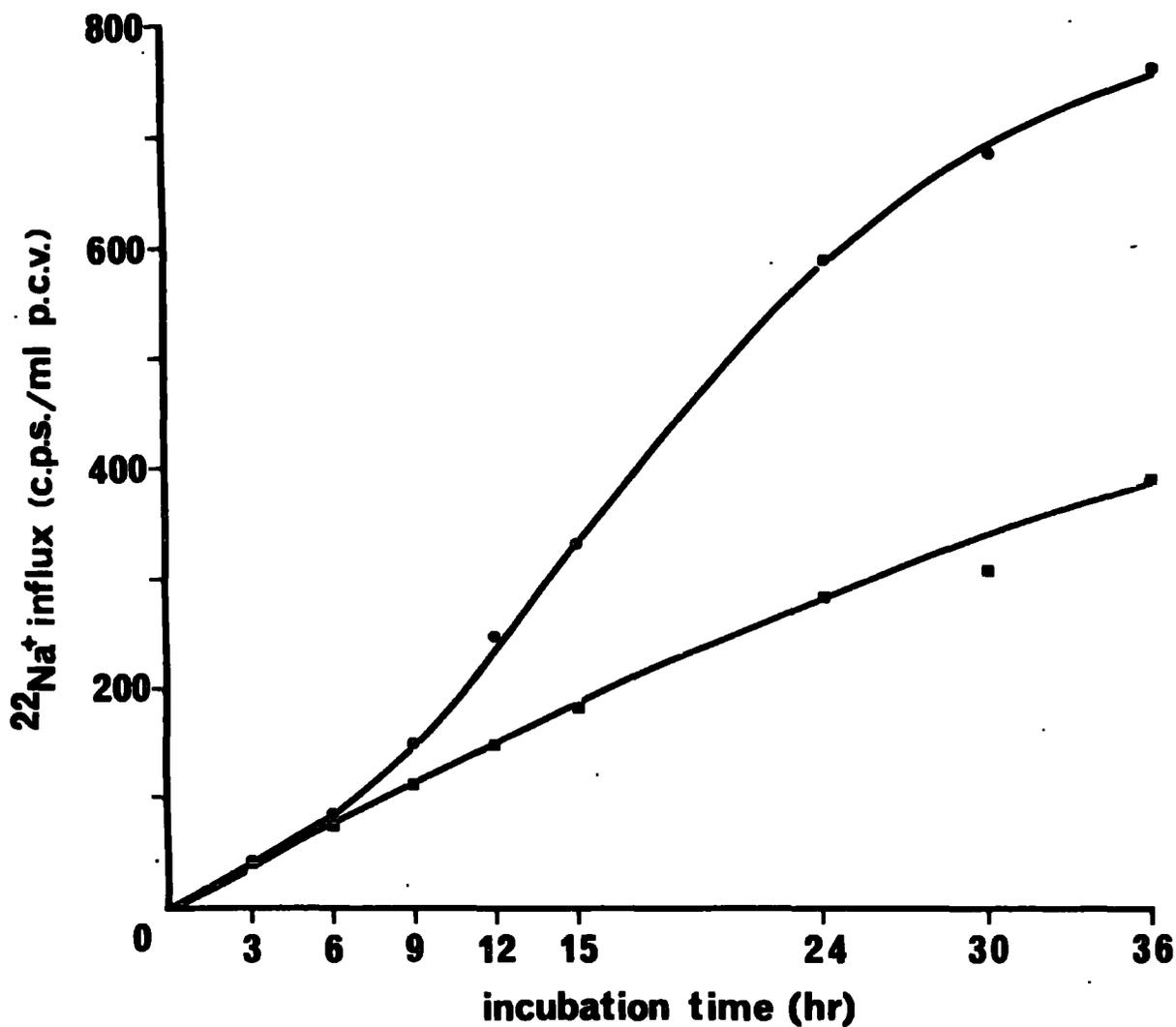


FIG. V-4. INFLUENCE OF 1×10^{-5} M TANNIC ACID UPON $^{22}\text{Na}^+$ LOADING OF RAT ERYTHROCYTES.

Treatment with tannic acid:

Cells were incubated in the presence of 1×10^{-5} M tannic acid for 30min at 0°C prior to $^{22}\text{Na}^+$ loading.

Methods of loading and analysis: See 'Materials and Methods'.

Ordinate: $^{22}\text{Na}^+$ influx in c.p.s./ml p.c.v..

● = control cells; ■ = experimental cells.

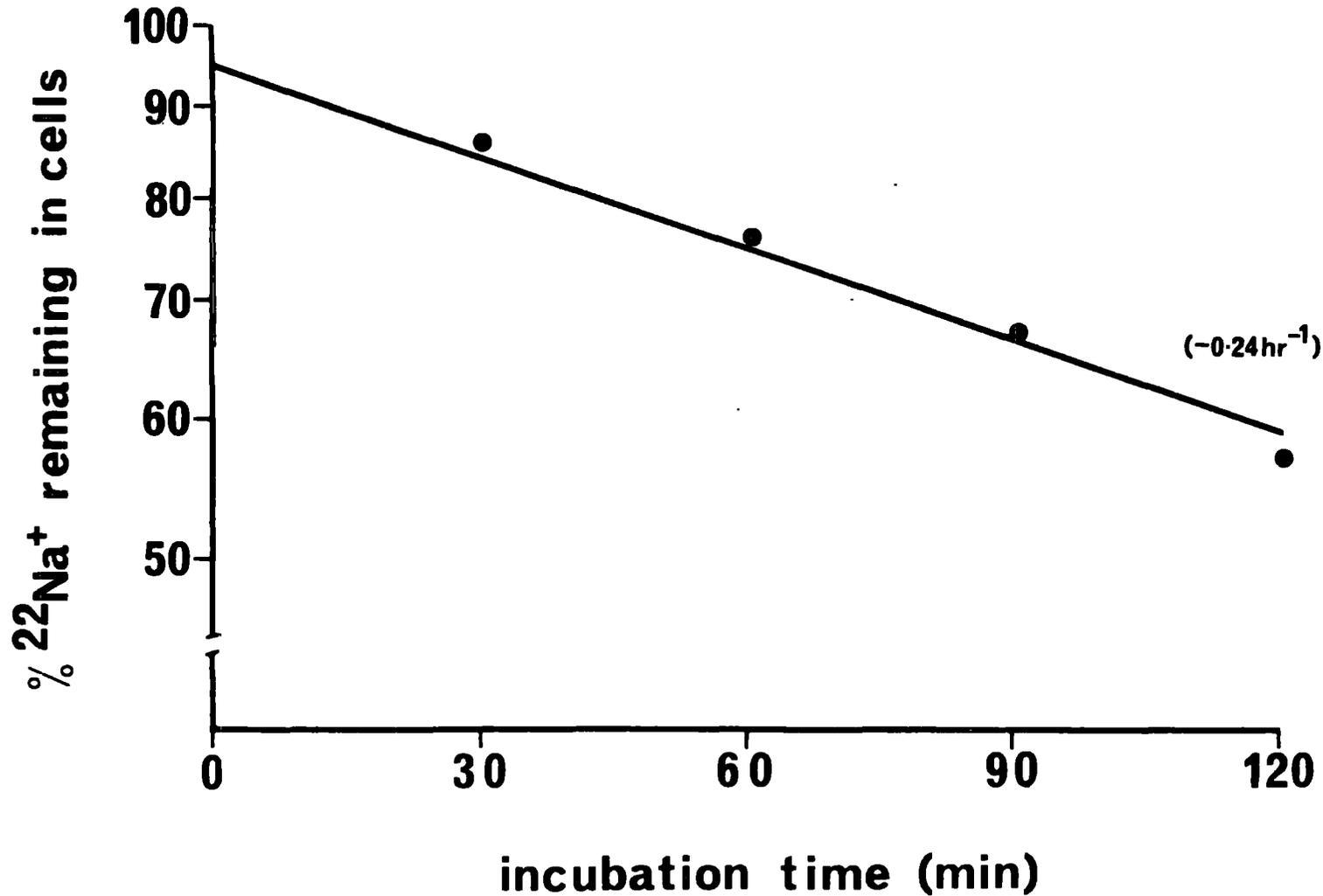


FIG. V-5. $^{22}\text{Na}^+$ EFFLUX FROM NORMAL HUMAN ERYTHROCYTES.

Methods of loading, efflux and analysis: See 'Materials and Methods'.

Ordinate: % $^{22}\text{Na}^+$ remaining in cells. The rate constant for efflux is given in parentheses.

FIG. V-6

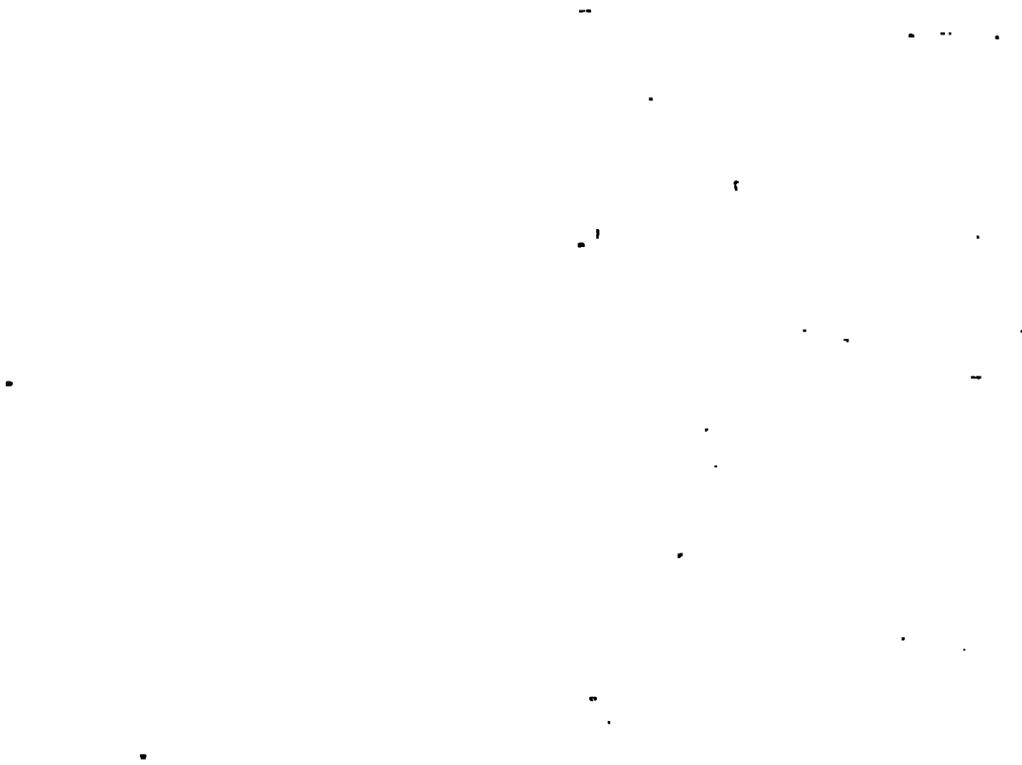


FIG. V-6. INFLUENCE OF 1×10^{-4} M OUABAIN UPON $^{22}\text{Na}^+$ EFFLUX FROM
NORMAL RAT ERYTHROCYTES.

Methods of loading, efflux and analysis:

See 'Materials and Methods'.

Drug inclusion:

1×10^{-4} M ouabain was present in the final resuspension medium.

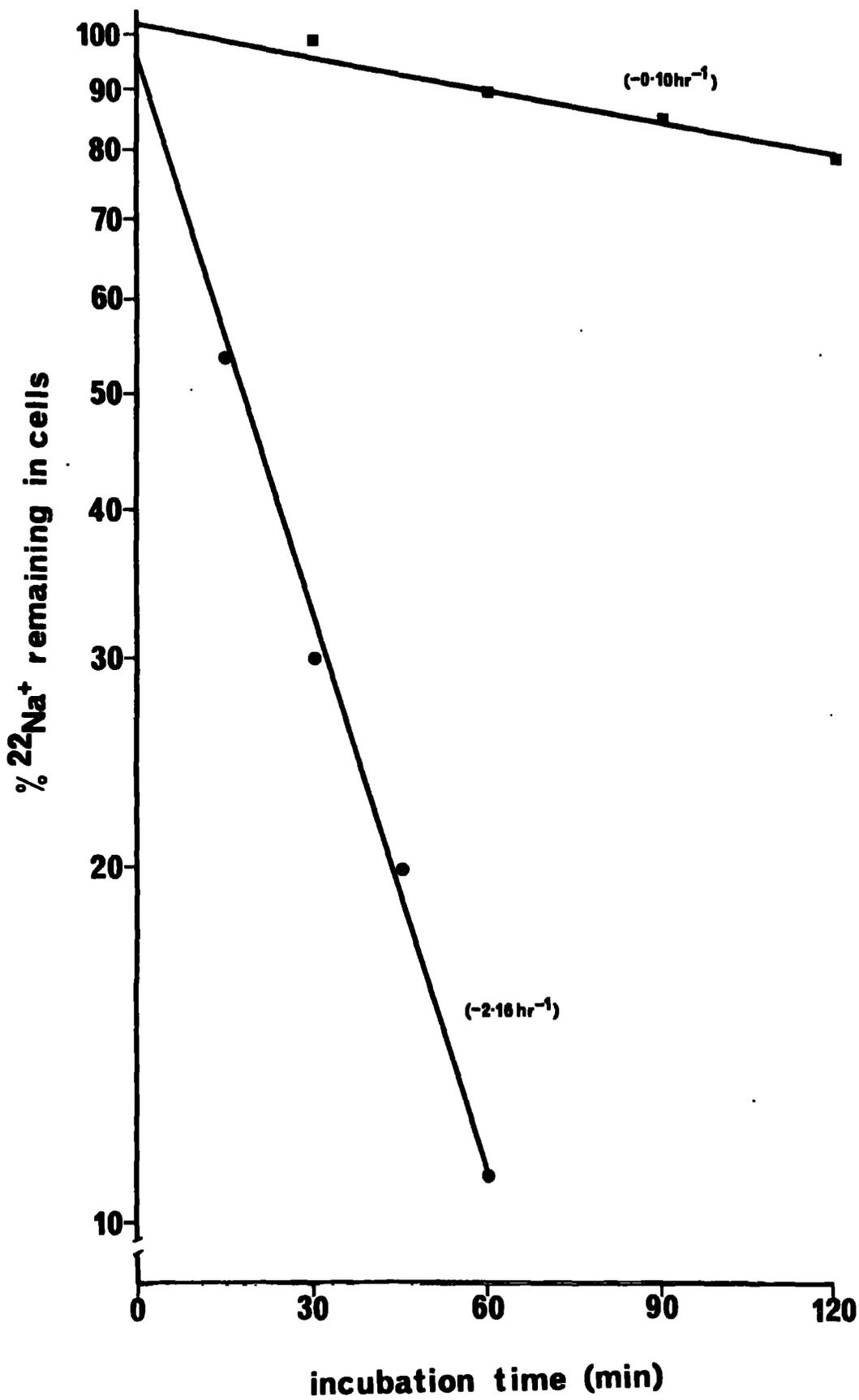
● = control;

■ = + ouabain.

Ordinate:

% $^{22}\text{Na}^+$ remaining in cells.

The rate constant for efflux is given in parentheses.



b) Rat erythrocytesInfluence of 1×10^{-4} M ouabain upon $^{22}\text{Na}^+$ efflux from normal rat erythrocytes

FIG.V-6 illustrates a representative experiment which showed that ouabain almost completely inhibits $^{22}\text{Na}^+$ efflux from loaded erythrocytes when the drug is present in the final incubation medium (see Methods (e)). The rate constant for efflux was markedly reduced from -2.16hr^{-1} to -0.10hr^{-1} , ouabain-insensitive (residual) $^{22}\text{Na}^+$ efflux representing approximately 10% of total efflux.

Influence of adrenalectomy upon $^{22}\text{Na}^+$ efflux from rat erythrocytes

Two series ((A) and (B)) of adrenalectomized rats were maintained post-operatively upon a distilled water fluid diet. Individual rats were sacrificed 1, 3 and 5 days post-operatively, their erythrocytes separated and $^{22}\text{Na}^+$ -loaded in the usual manner. $^{22}\text{Na}^+$ efflux was then studied as a function of time during 60min incubation periods. Erythrocytes from such animals do not $^{22}\text{Na}^+$ load differentially (see B 1. above), and this study was made to discover whether their $^{22}\text{Na}^+$ efflux characteristics are abnormal.

A fall in the $^{22}\text{Na}^+$ efflux rate constant occurred as a function of time post-adrenalectomy (see FIG. V-7, (A) and (B)). In rats of series (A), the rate constant value diminished from -1.96hr^{-1} for the control (intact) animal, via -1.46hr^{-1} to -0.95hr^{-1} for rats sacrificed 1 and 3 days after adrenalectomy. The same pattern was evident in the replicate, group (B), experimental series, in which the control value of -2.16hr^{-1} diminished through -1.55hr^{-1} and -0.83hr^{-1} to -0.74hr^{-1} for animals investigated 1, 3 and 5 days post-operatively. These results suggest that a part of the $^{22}\text{Na}^+$ efflux from rat erythrocytes is attributable to a component which is subject to adrenocorticosteroidal regulation.

Influence of saline compensation upon the change in $^{22}\text{Na}^+$ efflux rate which results from adrenalectomy

Some investigations were made of the effect of administration of a 0.9% NaCl

FIG. V-7



FIG. V-7. INFLUENCE OF ADRENALECTOMY UPON $^{22}\text{Na}^+$ EFFLUX FROM
RAT ERYTHROCYTES. IN TWO EXPERIMENTAL SERIES (A AND B).

Methods of loading, efflux and analysis:

See 'Materials and Methods'.

Post-operative fluid diet:

Control (day 0) rats received distilled water.

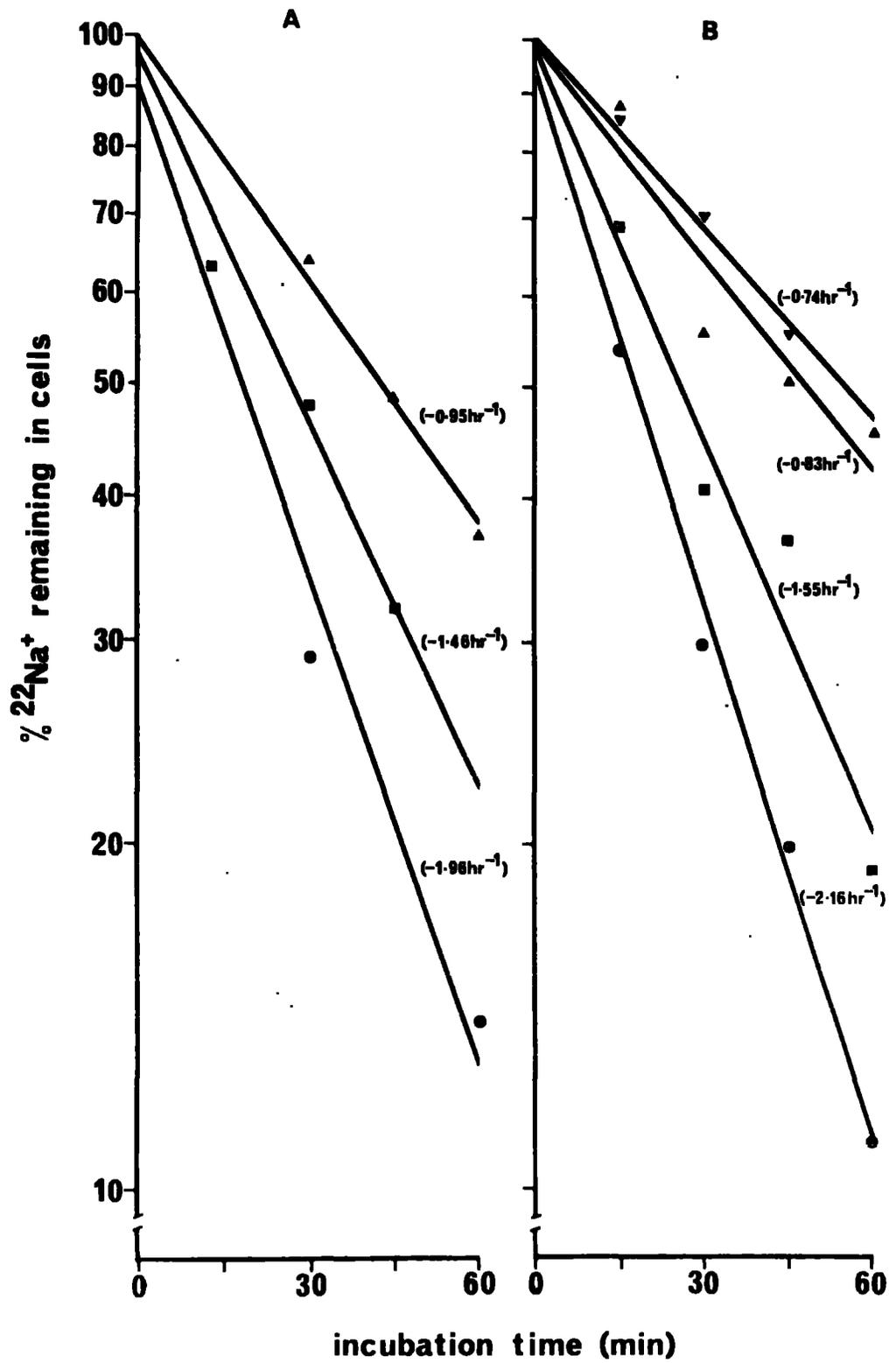
Adrenalectomized rats received distilled water.

- = control (day0) rats;
- = 1 day post-adrenalectomy;
- ▲ = 3 days post-adrenalectomy;
- ▼ = 5 days post-adrenalectomy.

Ordinate:

% $^{22}\text{Na}^+$ remaining in cells.

The rate constant for efflux is given in parentheses.



fluid diet to adrenalectomized rats. FIG.V-8 shows the result of a representative experiment, in which it was found that saline compensation prevented the reduction in $^{22}\text{Na}^+$ efflux rate constant associated with the fifth post-operative day. Cells taken from a saline-maintained, adrenalectomized animal 6 days post-operatively gave a rate constant for efflux of -1.47hr^{-1} . In the case of the example illustrated in FIG.V-8, the amount of $^{22}\text{Na}^+$ loss from cells after 60min incubation was reduced by 41% in cells from the adrenalectomized animal receiving water as compared with 13% in those from the rat maintained on 0.9% NaCl.

Influence of exposure of rat erythrocytes to $1 \times 10^{-5}\text{M}$ tannic acid upon $^{22}\text{Na}^+$ efflux

(i) Effect of exposure prior to $^{22}\text{Na}^+$ loading

$^{22}\text{Na}^+$ efflux was traced for cells which had been loaded for 16.5hr, after which period the radioisotopic content of control and experimental cells was markedly dissimilar, as pointed out earlier (see FIG.V-4). The results are plotted in FIG.V-9, which shows that the efflux rate is uninfluenced by tannic acid in these circumstances.

(ii) Effect of exposure to tannic acid following $^{22}\text{Na}^+$ loading

The cells were loaded for 16.5hr, as in the case of (i) above. They were subsequently exposed to the reagent in the standard manner (see Methods (f) (iii)), and $^{22}\text{Na}^+$ efflux was studied immediately or after an 8hr storage period in saline at 0° - 4°C . This experiment was designed to test whether the tannic acid treatment resulted in any marked change after 6hr, as it did in the case of $^{22}\text{Na}^+$ influx (see B 1. above). The results obtained are plotted for one preparation in FIG.V-10, (A) and (B) respectively. Neither form of tannic acid treatment affected the $^{22}\text{Na}^+$ efflux, which was the same as that for untreated erythrocytes.

C. Influence of adrenalectomy and post-operative fluid diet upon susceptibility of rat erythrocytes to hypotonic haemolysis

Erythrocytes were taken from intact and adrenalectomized rats receiving a

FIG. V-8



FIG. V-8. INFLUENCE OF POST-OPERATIVE FLUID DIET UPON $^{22}\text{Na}^+$ EFFLUX FROM RAT ERYTHROCYTES.

Methods of loading, efflux and analysis:

See 'Materials and Methods'.

Post-operative fluid diet:

Control (day 0) and 5 day adrenalectomized rats received distilled water. (Data from FIG. V-7, series B.)

6 day adrenalectomized rat received 0.9% NaCl.

● = control (day 0) rat;

▼ = 5 days post-adrenalectomy;

∇ = 6 days post-adrenalectomy.

Ordinate:

% $^{22}\text{Na}^+$ remaining in cells.

The rate constant for efflux is given in parentheses.

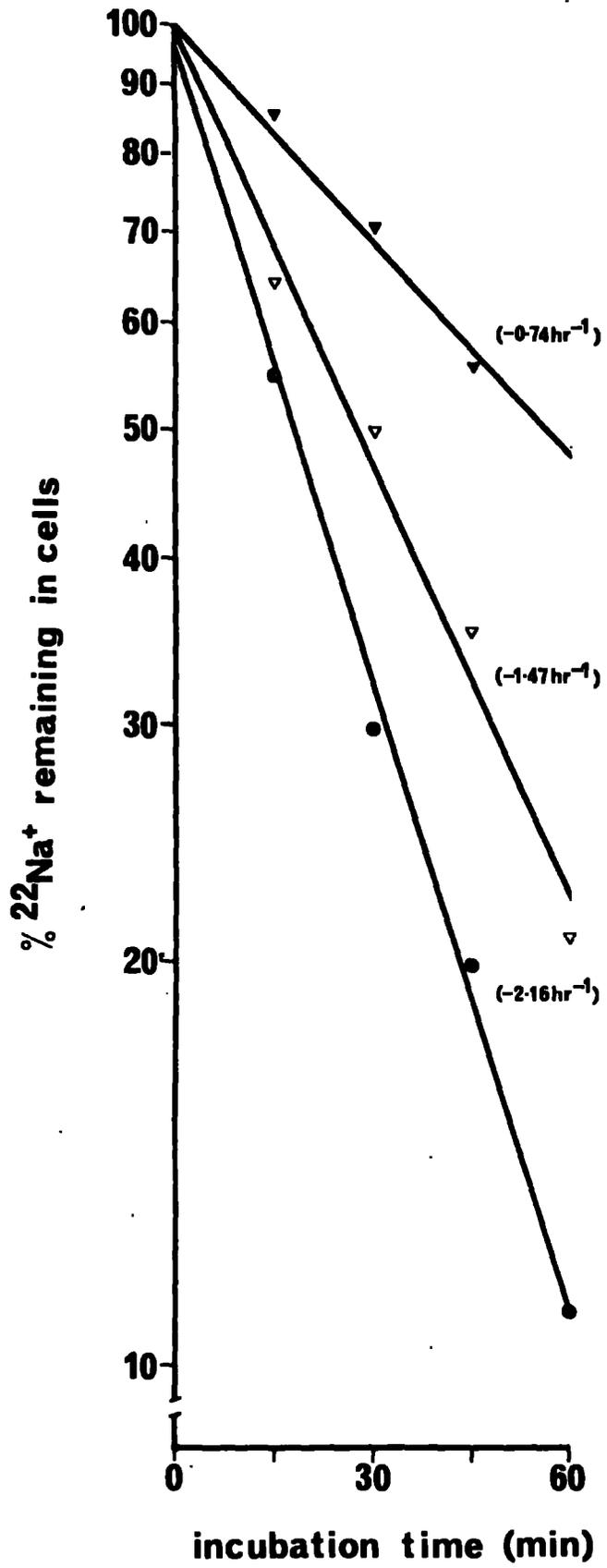


FIG. V-9

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FIG. V-9. INFLUENCE OF 1×10^{-5} M TANNIC ACID UPON $^{22}\text{Na}^+$ EFFLUX FROM NORMAL RAT ERYTHROCYTES.

Tannic acid exposure:

Cells were incubated in the presence of 1×10^{-5} M tannic acid for 30min at 0°C prior to $^{22}\text{Na}^+$ loading.

Methods of loading, efflux and analysis:

See 'Materials and Methods'.

Ordinate:

$^{22}\text{Na}^+$ efflux in c.p.s./ml p.c.v..

● = control cells;

■ = tannic acid treated cells.

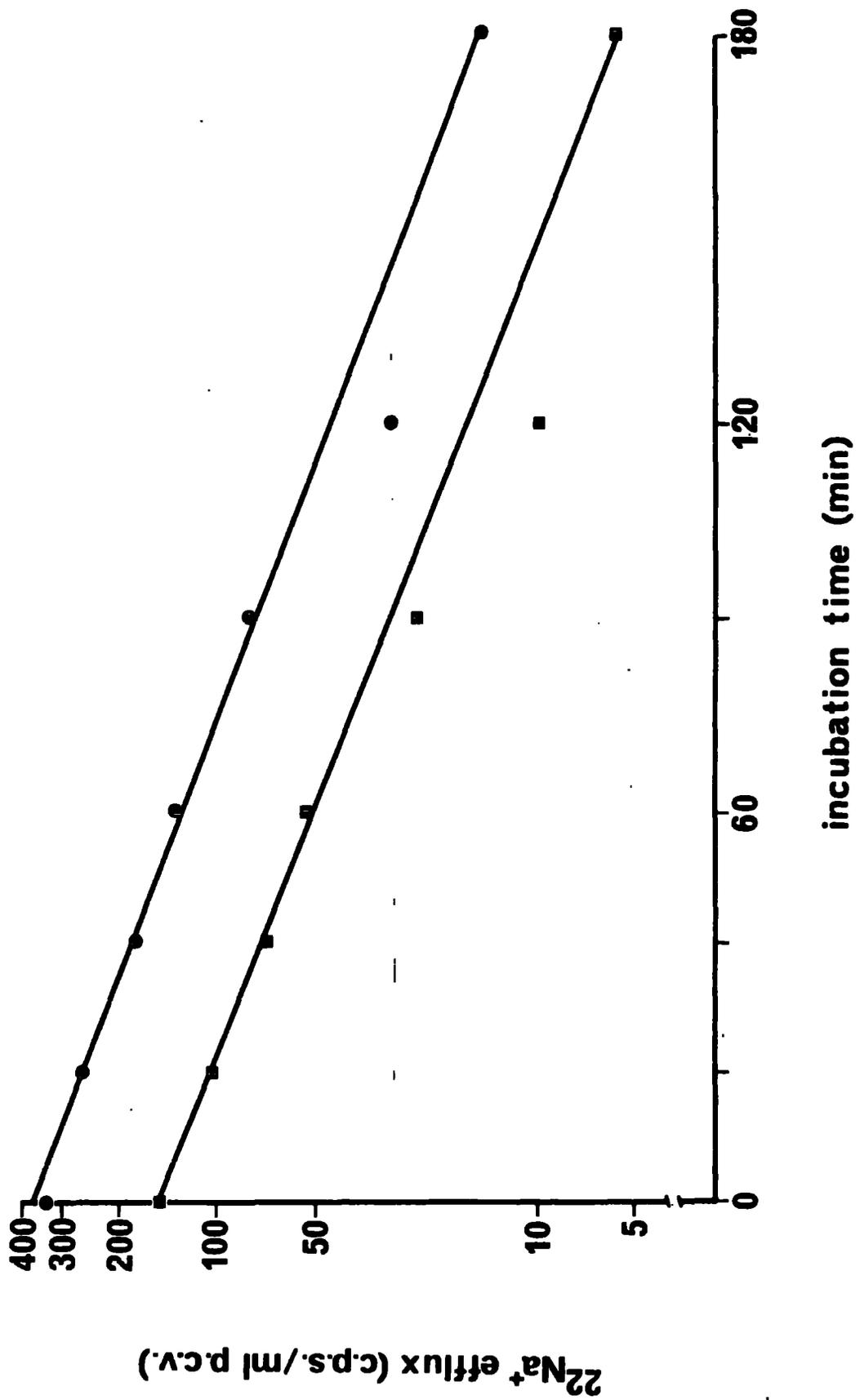


FIG. V-10



FIG. V-10. INFLUENCE OF 1×10^{-5} M TANNIC ACID UPON $^{22}\text{Na}^+$ EFFLUX FROM NORMAL RAT ERYTHROCYTES.

Methods of loading, efflux and analysis:

See 'Materials and Methods'.

Tannic acid exposure:

Cells were treated in the presence of 1×10^{-5} M tannic acid for 30min after $^{22}\text{Na}^+$ loading.

$^{22}\text{Na}^+$ efflux was subsequently traced:

A - immediately; or

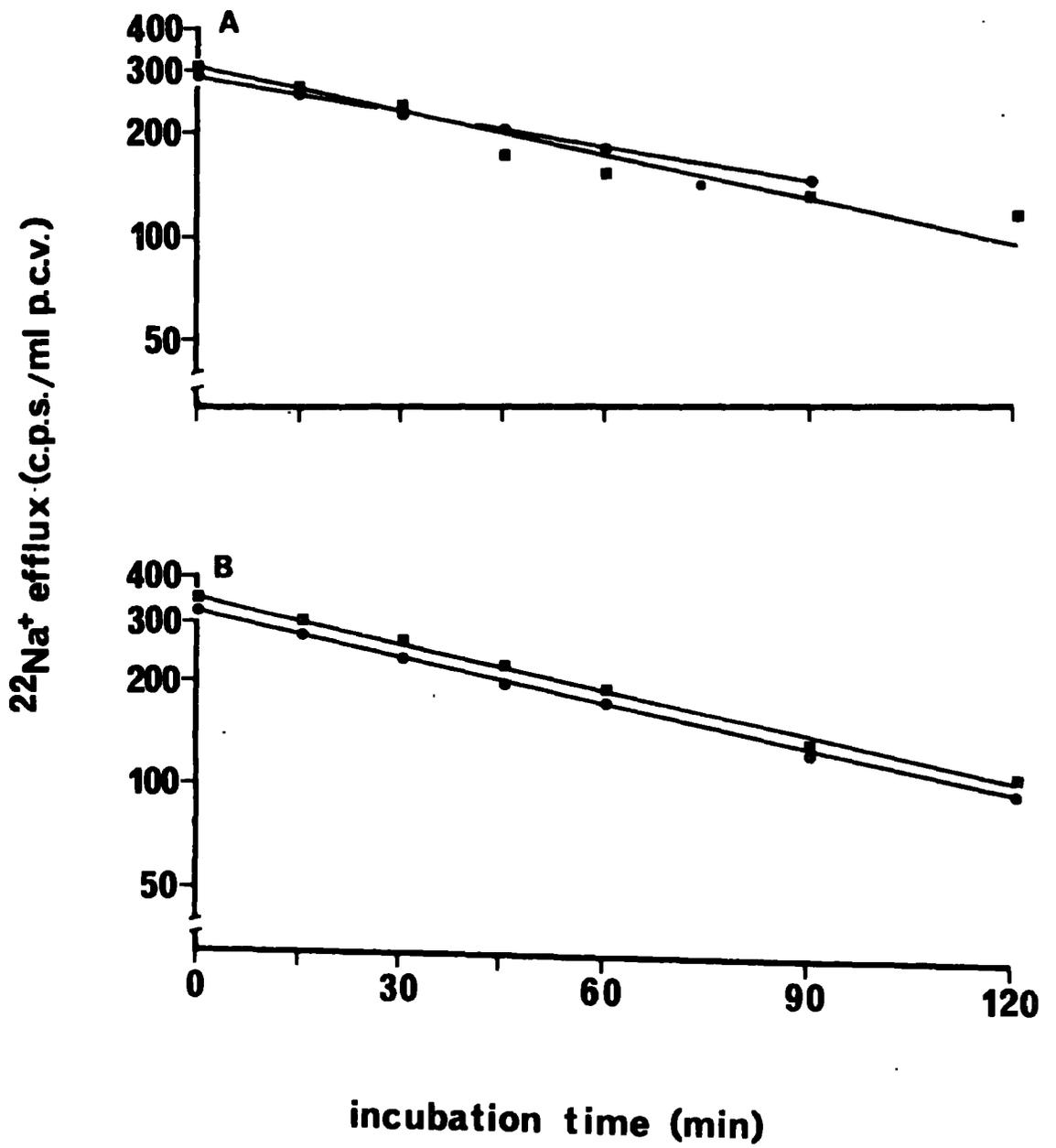
B - after 8hr at 0° - 4°C .

Ordinate:

$^{22}\text{Na}^+$ efflux in c.p.s./ml p.c.v..

● = control cells;

■ = tannic acid treated cells.



distilled water fluid diet, and from adrenalectomized rats receiving 0.9% NaCl. They were treated as outlined in Methods (g) (see above), and haemolysis curves plotted of % haemolysis against $[\text{NaCl}]$. These curves are shown in FIG.V-11, (A), (B) and (C). Curve (A) was obtained from erythrocytes of normal rats; the $[\text{NaCl}]$ in which 50% haemolysis occurred was 73.5mM (this value may be called the H_{50}).

The effect of adrenalectomy, followed by distilled water fluid diet for up to 3 and 5 days post-operatively, was a shift of the normal haemolysis curve to the left (compare FIG.V-11, (A) and (B)). Under these circumstances, therefore, the cells became resistant to hypotonic lysis. The H_{50} values were reduced to 72mM and 68mM on days 3 and 5 respectively.

When adrenalectomized rats received a 0.9% NaCl fluid diet, this effect was reversed: namely, the cells were rendered more susceptible to lysis. The haemolysis curve was shifted to the right (compare FIG.V-11, (A) and (C)) and the H_{50} was increased to 81.5mM on the fifth post-operative day.

FIG. V-11



FIG. V-11. INFLUENCE OF ADRENALECTOMY AND POST-OPERATIVE FLUID DIET UPON THE SUSCEPTIBILITY OF RAT ERYTHROCYTES TO HYPOTONIC HAEMOLYSIS.

Method of estimation of degree of haemolysis:

See 'Materials and Methods'.

Post-operative fluid diet:

A - control rats receiving distilled water.

B - adrenalectomized rats receiving distilled water-

C -- 5 day adrenalectomized rats receiving 0.9% NaCl.

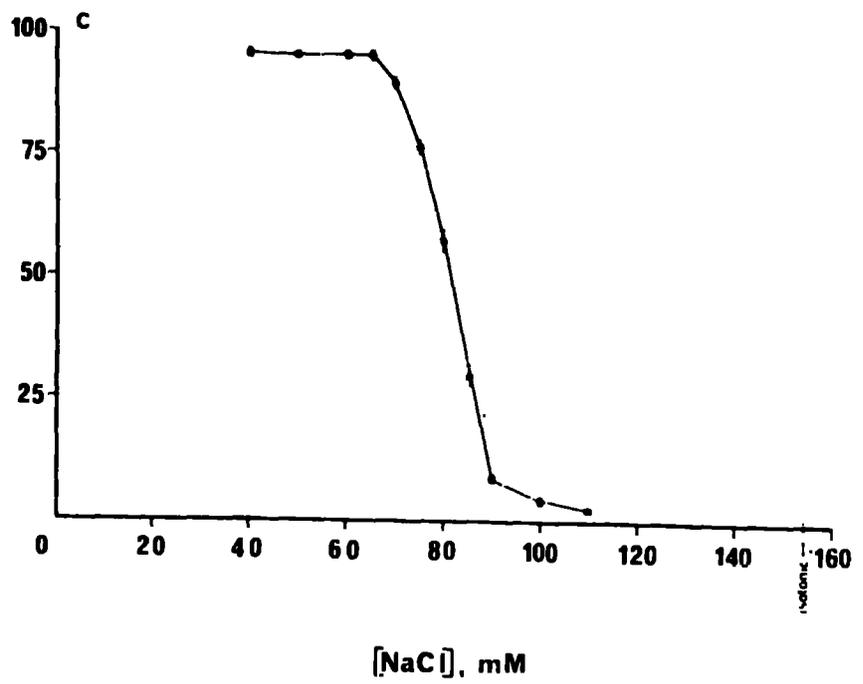
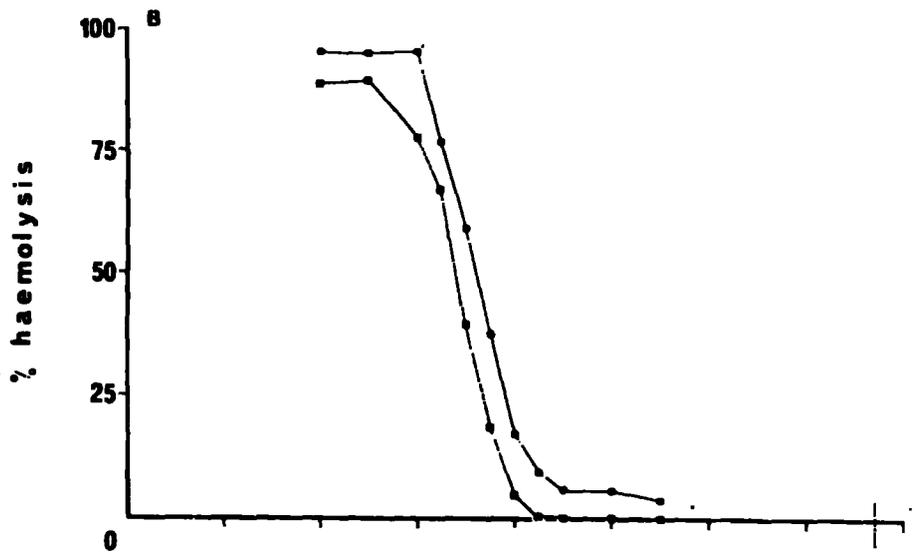
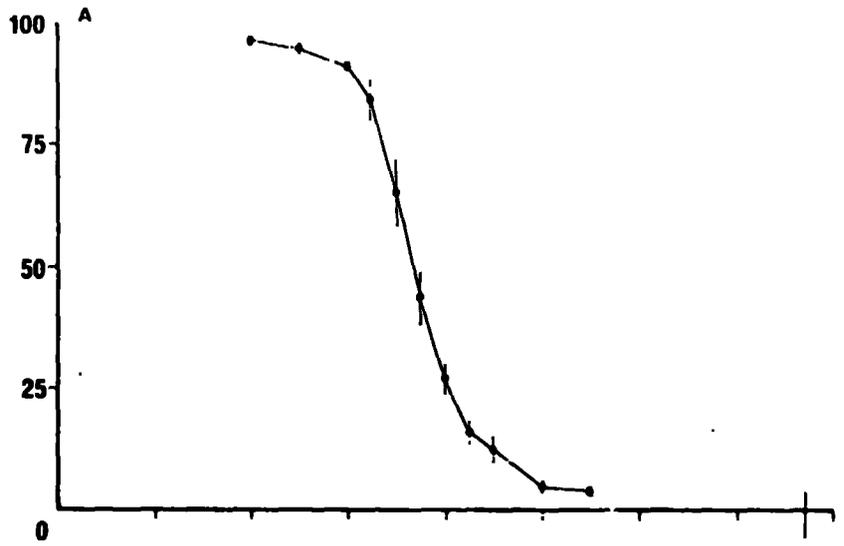
[● = 3 days;
■ = 5 days.

n = $\left[\begin{array}{l} 5 \text{ in A;} \\ 2 \text{ in B;} \\ 5 \text{ in C.} \end{array} \right.$

Ordinate:

% haemolysis.

$\left[\begin{array}{l} \bullet \\ \bullet \\ \bullet \end{array} \right] = \pm 1 \times S.E..$



CHAPTER 3Determination of Erythrocyte Mg^{2+} - and $Na^{+}+K^{+}$ - Mg^{2+} -ATPase, and Acetylcholinesterase Activities

The investigations reported in this Chapter were of Mg^{2+} - and $Na^{+}+K^{+}$ - Mg^{2+} -ATPase activities in erythrocytes from normal and adrenalectomized rats, and from human subjects of normal and abnormal affective status. The results obtained for rat cells prompted a reinvestigation of acetylcholinesterase (E.C. 3.1.1.7). This enzyme is located on the outer surface of erythrocyte membranes (Shinagawa & Ogura, 1961; Herz, Kaplan & Stevenson, 1963), and had been previously investigated in erythrocytes from adrenalectomized rats by Vaccarezza & Willson (1965) (see Chapter 1).

The results are discussed in conjunction with those of Chapter 2.

MATERIALS AND METHODSMATERIALS

- a) Animals: ♂ CFHB albino rats, as described in Section II.
- b) Human subjects: These were as described in Chapter 2 of this Section.
- c) Reagents: These are given in the text.

METHODS

- a) Blood sampling: Samples were obtained as described in Chapter 2, except that they were immediately transferred to glass tubes at 0° - $4^{\circ}C$.
- b) Bilateral adrenalectomy and maintenance of rats: The techniques are outlined in Section II. Control (intact) and operated animals were maintained as described in Chapter 2 of this Section.
- c) Preparation of erythrocyte membrane fragments for ATPase assays:
The preparation sequence is summarized in FIG.V-12. Duplicate aliquots of freshly obtained blood were used whenever possible. Especial care was taken to obtain homogeneous microsomal resuspensions.

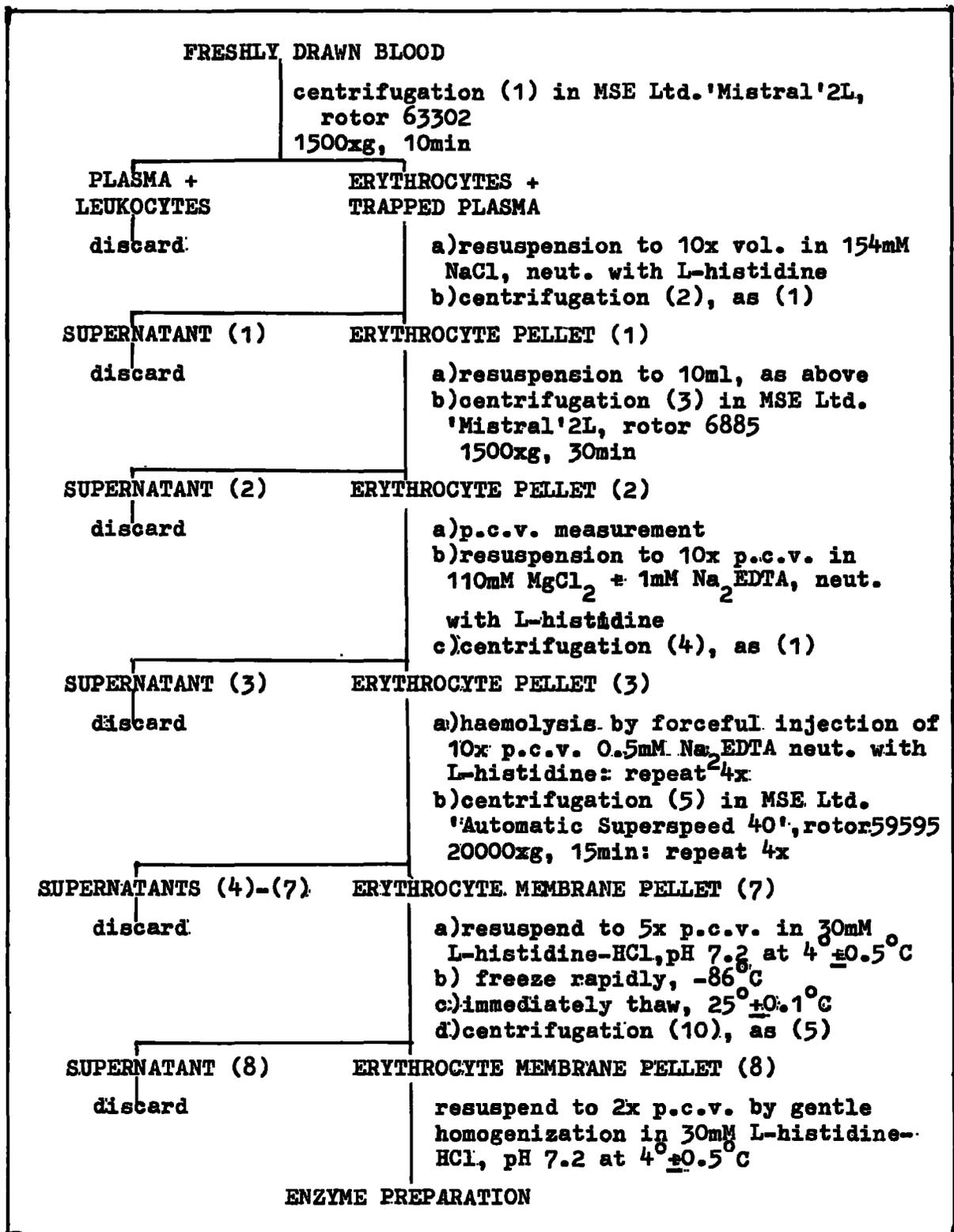


FIG. V-12. PREPARATION OF ERYTHROCYTE MEMBRANE FRAGMENTS.

All procedures at 0°-4°C, unless otherwise stated.

d) Preparation of erythrocytes for acetylcholinesterase assays:

Duplicate aliquots of freshly obtained blood were treated as shown in FIG.V-12 as far as the pellet (3) stage. They were then suspended to 4x their p.c.v. in the saline recommended by Augustinsson (1957). This was freshly made up at 18°-21°C from stock solutions stored at 4°-8°C, being composed of 100 parts 0.9% NaCl: 30 parts 1.26% NaHCO₃: 2 parts 1.76% MgCl₂·6H₂O by volume, and saturated with a 95% N₂: 5% CO₂ gas mixture to yield a pH of 7.4. Aliquots of erythrocyte suspensions were immediately dispensed into Warburg vessels (see below), the stock suspensions being gently inverted twice before each pipetting in order to minimize variation in sample size.

e) Enzyme assays

(i) Mg²⁺ - and Na⁺+K⁺-Mg²⁺-ATPases: Incubations of 0.5ml aliquots of freshly obtained membrane resuspensions were made in duplicate for 15-20min at 37°±0.1°C in the presence of 0.5ml Tris-ATP (2mM final concentration) and 1ml Mg²⁺ (4mM final concentration); Na⁺ (100mM final concentration) and K⁺ (20mM final concentration) were included in the Mg²⁺ solution for Na⁺+K⁺-Mg²⁺-ATPase assays. The chloride salts of cations were used and were dissolved in 30mM L-histidine-HCl, pH 7.2 at 37°±0.5°C.

Orthophosphate determinations were made in duplicate as described in Section II.

Enzyme activities were expressed as nmoles P_i released / ml p.c.v. /min.

(ii) Acetylcholinesterase: Incubations of 1.6ml aliquots of erythrocyte suspensions were made at 25°±0.1°C in 20ml (nominal capacity) Warburg flasks of known volume by the manometric technique of Augustinsson (1957).

Acetylcholine chloride (10mM final concentration) was used as substrate, being prepared from concentrated stock solution (stored at 8°-10°C in HCl at pH 4.0) by dilution with saline. 0.4ml aliquots of substrate were placed in the side arms of flasks, the flasks and manometers flushed with 95% N₂:5% CO₂ gas mixture and thermoequilibrated for 10min prior to mixing of substrate and cell suspensions in the main flask chambers. Gas evolution was monitored at 5min

intervals for 30min in a Townson & Mercer Ltd. Warburg apparatus at shaking speed 4. TABLE V-3 summarizes the flask contents.

After taking into account the small degree of CO_2 evolution due to spontaneous substrate hydrolysis, the volume of gas evolved was computed as $\mu\text{l CO}_2/\text{ml p.c.v.}/30\text{min}$. This value was re-expressed as $\mu\text{moles substrate hydrolysed}/\text{ml p.c.v.}/30\text{min}$.

RESULTS

A. Mg^{2+} - and $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+}$ -ATPase activities

1. General

Preparative procedure and extraction characteristics

The procedure summarized in FIG.V-12 was adopted as the result of trials with a variety of methods. In reviewing the biochemical aspects of active transport, Albers (1967) aptly commented that 'studies of erythrocyte ATPases must be undertaken with particular care because of their heterogeneity and low activity'. In the work reported here, therefore, every effort was made to evolve a preparative procedure which would yield reliable estimations of enzyme activity, whilst not dissociating the ATPase molecules from membranes. Na_2EDTA - and Mg^{2+} -containing washes were adopted in an attempt to reduce Ca^{2+} contamination of the membrane fraction, and cells were disrupted by hypotonic haemolysis in order to render enzymatic sites accessible to ATP in vitro (Marchesi & Palade, 1967, having concluded that both ATPases are internally located, and Hoffman, 1962, that ATP must be present inside resealed erythrocyte ghosts for ATPase activity to occur). It was noted that pellets (3) - (7) consisted of a large upper, virtually haemoglobin-free layer, and a lower, more compact layer. The latter was possibly identical with that consisting of 'cell debris and fibrin clot', reported by Nakao et al. (1967). In order to avoid possible loss of active material, this layer was not discarded.

Even when these precautions were taken, activity was often very low. It was

REAGENT	SUPPLIER	VOLUMES (ml)		
		CONTROLS		EXPERIMENTAL
		1	2	
saline (see text)	from BDH Ltd. (AnalaR) salts	2.0	1.6	-
acetylcholine chloride (50mM) in saline	from Sigma Chemical Co. (crystalline, approx. 99%)	-	0.4	0.4
erythrocyte suspension in saline		-	-	1.6
95% N ₂ :5% CO ₂	British Oxygen Co.	s a t u r a t e d		

TABLE V-3. WARBURG FLASK CONTENTS FOR ACETYLCHOLINESTERASE ASSAYS.

thought that this may have been the result of resealing of ghost membranes, and their failure to disrupt when finally suspended by homogenization. The washed membrane suspension was therefore subjected to freezing and thawing prior to final suspension, in order to render any resealed ghosts ATP-permeable. The sequential use of these techniques (see FIG.V-12) afforded preparations from normal rats which had similar enzyme activities. The procedure was therefore adopted as standard in the studies reported below.

2. Rat erythrocytes

Influence of adrenalectomy and sodium pentobarbitone anaesthesia, with a subsequent fluid diet of distilled water ad lib.

Initial experiments (not reported) suggested that erythrocyte ATPase activities were elevated in adrenalectomized rats, and a systematic appraisal of this phenomenon was therefore made. Three series of experiments were performed, using erythrocytes from adrenalectomized and non-operated rats, all of which had received equivalent doses of the anaesthetic (see Section II) when adrenalectomies were performed. Animals were sacrificed 1, 3, 5 and 7 days after that date, having received a distilled water fluid diet throughout the experimental period. The Mg^{2+} - and $Na^+K^+Mg^{2+}$ -ATPase activities of the erythrocyte preparations were compared with those of control (intact) rats which had not been anaesthetized, and which had received distilled water fluid diet for equal periods. The results of these experiments are given in TABLE V-4 which shows mean values for enzyme activity and the P values obtained by application of Student's 't' test in comparing control (intact, unanaesthetized) with experimental animals.

The data reveal a slight depression of both Mg^{2+} - and $Na^+K^+Mg^{2+}$ -ATPase activities 1, 3 and 5 days after anaesthetic administration to intact rats. However, the P values show that this depression was not statistically significant, and after 7 days the mean values were remarkably similar to those obtained from the erythrocytes of control (intact, unanaesthetized) rats.

TABLE V-4

TABLE V-4. INFLUENCE OF ADRENALECTOMY AND SODIUM PENTOBARBITONE ANAESTHESIA UPON RAT ERYTHROCYTE
Mg²⁺ - AND Na⁺+K⁺-Mg²⁺-ATPase ACTIVITIES.

Preparative procedure:

See 'Materials and Methods'.

Incubation conditions:

30mM L-histidine-HCl + 2mM Tris-ATP, pH 7.2, 37° ± 0.1°C.

Mg²⁺ (4mM), Na⁺ (100mM) and K⁺ (20mM) were included as indicated.

Na⁺+K⁺-Mg²⁺-ATPase activity is the increment in enzyme activity obtained by the inclusion of Na⁺ and K⁺ with Mg²⁺.

Post-operative fluid diet:

Distilled water ad lib.

Anaesthetic dose:

5mg sodium pentobarbitone/100g body weight, administered intraperitoneally.

Adx. = adrenalectomized. None = no treatment.

PRE-TREATMENT		NONE	ANAESTHETIC ALONE				ANAESTHETIC + ADRENALECTOMY				
DAYS POST-ADX.		0 (control)	1	3	5	7	1	3	5	7	
ATPase ACTIVITY (nmoles P _i liberated/ml p.c.v./min)	Mg ²⁺ -ATPase	n	9	3	3	3	3	3	3	3	3
		M	113.40	104.17	98.20	102.56	112.40	251.17	413.83	295.80	224.30
		S.E.	5.95	11.94	14.57	5.97	4.69	17.18	9.19	14.92	11.02
		P	-	> 0.40	> 0.20	> 0.30	> 0.90	< 0.001	< 0.001	< 0.001	< 0.001
	Na ⁺ +K ⁺ -Mg ²⁺ -ATPase	n	9	3	3	3	3	3	3	3	3
		M	73.30	63.70	56.83	65.20	73.43	227.96	292.13	254.10	123.16
		S.E.	4.68	13.98	7.37	8.03	4.28	4.38	9.17	10.66	6.09
		P	-	> 0.50	> 0.10	> 0.40	> 0.90	< 0.001	< 0.001	< 0.001	< 0.001

TABLE V-4. INFLUENCE OF ADRENALECTOMY AND SODIUM PENTOBARBITONE ANAESTHESIA UPON RAT ERYTHROCYTE Mg²⁺- AND Na⁺+K⁺-Mg²⁺-ATPase ACTIVITIES.

In contrast to this result, there was a marked change in Mg^{2+} - and Na^+K^+ - Mg^{2+} -ATPase activities of erythrocytes from adrenalectomized rats. Both enzyme activities were increased with reference to control ($P < 0.001$ in all cases), and the increase was time dependent (see TABLE V-4). In addition, the activation pattern was similar for both Mg^{2+} - and Na^+K^+ - Mg^{2+} -ATPases. Maximal activation occurred on the third day post-adrenalectomy, reaching 3.6x and 3.9x the control value respectively. Thereafter, activities decreased to yield day 7 values near control.

3. Human erythrocytes

Influence of affective disorders and psychoactive drug therapy

Normal, manic-depressive (manic and depressive phases) and endogenous depressive subjects were studied. They were classified according to drug therapy as follows:

- (A) not differentiated; i.e. all subjects within a given affective category,
- (B) subjects not receiving psychoactive drugs,
- (C) subjects receiving chlorpromazine as 'Largactil' (May & Baker Ltd.).

Normal subjects (N) received no psychoactive drugs and were used as controls. Manic-depressive subjects receiving haloperidol were not available for this study.

TABLE V-5 gives mean Mg^{2+} - and Na^+K^+ - Mg^{2+} -ATPase activities and P values obtained by application of Student's 't' test in comparing:

- (i) normal subjects (N) with disordered patients in drug therapy groups (A)-(C) (see above),
- (ii) patients not receiving psychoactive drugs (B) with those given chlorpromazine (C).

The calculated P values (see TABLE V-5) reveal that manic-depressive (depressive phase) and endogenous depressive patients were indistinguishable from normals (N) when not differentiated upon the basis of drug therapy

TABLE V-5

TABLE V-5. INFLUENCE OF AFFECTIVE STATUS AND PSYCHOACTIVE DRUG THERAPY UPON HUMAN ERYTHROCYTE
Mg²⁺ - AND Na⁺ +K⁺-Mg²⁺-ATPase ACTIVITIES.

Preparative procedure:

See 'Materials and Methods'.

Incubation conditions:

30mM L-histidine-HCl + 2mM Tris-ATP, pH 7.2, 37^o ± 0.1^oC.

Mg²⁺ (4mM), Na⁺ (100mM) and K⁺ (20mM) were included as indicated.

Na⁺ +K⁺-Mg²⁺-ATPase activity is the increment in enzyme activity obtained by the inclusion of Na⁺ and K⁺ with Mg²⁺.

Classification of affective status:

See text.

Drug therapy:

(A) N.D. = subjects not differentiated upon a drug therapy basis;

(B) NONE = those not receiving psychoactive drugs;

(C) CPZ = those receiving chlorpromazine.

(N) = normal subjects, not receiving psychoactive drugs.

P values shown are those derived by comparing data given for the groups designated, e.g. N+A), B+C).

ATPase ACTIVITY (nmoles P _i liberated/ml P.c.v./min)	DRUG THERAPY	VALUE	AFFECTIVE STATUS			
			NORMAL (N)	MANIC-DEPRESSION		ENDOGENOUS DEPRESSION
				MANIC PHASE	DEPRESSIVE PHASE	
Mg ²⁺ -ATPase	N.D. (A)	n $\bar{X} \pm 1 \times S.E.$ P	- - -	- - -	10 68.15 \pm 3.50 N+A) > 0.90	6 70.92 \pm 5.60 N+A) > 0.70
	NONE (B)	n $\bar{X} \pm 1 \times S.E.$ P	5 67.88 \pm 3.72 -	5 85.50 \pm 6.31 N+B) < 0.05	6 74.82 \pm 2.57 N+B) > 0.10	- - -
	CPZ (C)	n $\bar{X} \pm 1 \times S.E.$ P	- - -	- - -	4 58.12 \pm 4.42 N+C) > 0.10 B+C) < 0.01	- - -
Na ⁺ +K ⁺ -Mg ²⁺ -ATPase	N.D. (A)	n $\bar{X} \pm 1 \times S.E.$ P	- - -	- - -	10 32.23 \pm 1.73 N+A) > 0.05	6 34.87 \pm 5.57 N+A) > 0.20
	NONE (B)	n $\bar{X} \pm 1 \times S.E.$ P	5 27.28 \pm 1.31 -	5 40.82 \pm 4.24 N+B) < 0.02	6 34.93 \pm 2.12 N+B) < 0.02	- - -
	CPZ (C)	n $\bar{X} \pm 1 \times S.E.$ P	- - -	- - -	4 28.17 \pm 1.40 N+C) > 0.60 B+C) < 0.05	- - -

TABLE V-5. INFLUENCE OF AFFECTIVE STATUS AND PSYCHOACTIVE DRUG THERAPY UPON HUMAN ERYTHROCYTE Mg²⁺- AND Na⁺+K⁺-Mg²⁺-ATPase ACTIVITIES.

(i.e. when taken as group (A)). However, mean Mg^{2+} - and $Na^{+}+K^{+}-Mg^{2+}$ -ATPase activities were above normal in patients suffering either phase of manic-depression and receiving no psychoactive drugs (group (B)). The P values show that this elevation was statistically significant for all but the Mg^{2+} -ATPase activity of erythrocytes from depressive phase manic-depressives.

Administration of chlorpromazine to manic-depressive (depressed phase) subjects resulted in a statistically significant reduction of mean Mg^{2+} -ATPase ($P < 0.01$) and $Na^{+}+K^{+}-Mg^{2+}$ -ATPase ($P < 0.05$) activities with respect to those obtained for patients receiving no drugs. The extent of the chlorpromazine-mediated change was greater for Mg^{2+} -ATPase, giving a mean value below normal; $Na^{+}+K^{+}-Mg^{2+}$ -ATPase activity was reduced to normal. However, the P values show that neither enzyme activity was statistically different from that of normal humans.

The endogenous depressive subjects available for this study formed a heterogeneous group which could not be subdivided with regard to drug therapy. Thus, the data given in TABLE V-5 show that erythrocytes from such patients had mean Mg^{2+} - and $Na^{+}+K^{+}-Mg^{2+}$ -ATPase activities above normal, but that the sample of mentally disordered patients was more variable.

B. Acetylcholinesterase activity

Influence of adrenalectomy and post-operative fluid diet in rats

Acetylcholinesterase activity was assayed as given in Methods (d) and (e)(ii) (see above). The pooled results obtained from 4 control and 10 adrenalectomized rats are given in TABLE V-6. Animals sacrificed within 7 days of operation were maintained upon normal solid diet and distilled water fluid diet ad lib.; those retained for longer term (21 day) investigation were given 0.9% NaCl instead of distilled water. Control rats received distilled water. The results (see TABLE V-6) show that mean erythrocyte acetylcholinesterase activity increased gradually above control on days 3 and 7 post-adrenalectomy

FLUID DIET		DISTILLED WATER			0.9% NaCl
DAYS POST-ADX.		0 (control)	3	7	21
ACETYLCHOLINESTERASE ACTIVITY (μ moles ACh hydrolysed/ml pcv/30min)	n	4	4	3	3
	M	5.91	6.19	6.91	8.09
	S.E.	0.29	0.29	0.54	0.23
	P	-	-	> 0.10	< 0.01

TABLE V-6. INFLUENCE OF ADRENALECTOMY AND POST-OPERATIVE FLUID DIET UPON RAT ERYTHROCYTE ACETYLCHOLINESTERASE ACTIVITY.

Preparative procedure and incubation conditions:

Sae 'Materials and Methods'.

Post-operative fluid diet:

Distilled water or 0.9% NaCl ad lib., as indicated.

n = number of animals.

in rats receiving distilled water, but that application of Student's 't' test confirms that the day 7 value was not significantly different from control ($P = > 0.10$). The enzyme activity was therefore uninfluenced during the survival period of rats not receiving saline compensation. However, rats receiving 0.9% NaCl for 21 days post-operatively showed a 37% elevation of acetylcholinesterase activity above control, and this change was highly significant ($P = < 0.01$). These findings will be discussed in Chapter V-4 in conjunction with those of Vaccarezza & Willson (1965).

CHAPTER 4

Discussion of Experimental Results Obtained in Erythrocyte Studies

A. Influence of adrenalectomy and post-operative fluid diet upon rat erythrocytes

1. $[Na^+]_i$ and $[K^+]_i$.

The results reported in Chapter 2 do not entirely confirm those of Losert, et al. (1964). Although a slight reduction in $[K^+]_i$ occurred after adrenalectomy in both studies, Losert, et al. (1964) reported a small, though significant increase in $[Na^+]_i$ 3 days post-operatively. These authors' parallel studies of rat skeletal muscle are, moreover, at variance with those of Conway & Hingerty (1946, 1953) (see Section III), in that they report no changes in $[Na^+]_i$ or $[K^+]_i$ after adrenalectomy (Losert, et al., 1964).

In further considering the data of Losert, et al. (1964), it is noted that the plasma $[Na^+] / [K^+]$ ratio fell by 15% below normal in their animals. The magnitude of change is thus nearer that computed from the data of Jørgensen (1968) for 3 day adrenalectomized rats receiving saline compensation (20% reduction) than that derived for uncompensated animals (44% reduction).

Although this discrepancy may point to the use of different rat strains with differential susceptibility to the effects of adrenalectomy, it is more probable that the post-operative NaCl intake was sufficiently high to overcome the extreme hyponatraemia associated with total adrenalectomy in the report of Losert, et al. (1964). In the work presented in Chapter 2 (above), it is noteworthy that cells from saline compensated rats did show a transient, though not significantly supranormal, increase in mean $[Na^+]_i$ 2 days post-operatively (see TABLE V-1).

In the circumstances, it is not possible to conduct a strict comparison of the present results (see Chapter 2) with those of Losert, et al. (1964).

However, it is interesting that the post-operative fall in erythrocyte $[Na^+]_i$ (see Chapter 2) is directionally consistent with the change recorded for rat

skeletal muscle by Conway & Hingerty (1946, 1953) and for canine skeletal muscle by Flanagan, Davis & Overman (1950), although the latter investigators discovered no change for other dog tissues (erythrocytes were not considered). Flanagan, et al. (1950) were also unable to account for $[\text{Na}^+]_e$ loss in adrenalectomized dogs in terms of renal excretion alone: they were led to suggest that a fraction of $[\text{Na}^+]_e$ was transferred to a further body compartment, possibly bone. It is disappointing that they did not consider erythrocytes in this respect.

The reason for slight $[\text{Na}^+]_i$ regain by the tenth post-operative day in erythrocytes from rats surviving without 0.9% NaCl (see TABLE V-1) is not known. Although the animals were used because total adrenalectomy was known to have been performed successfully, it is conceivable that their prolonged survival and unexpectedly high erythrocyte $[\text{Na}^+]_i$ may be attributed to extra-adrenal steroid secretion.

2. Relationship of $[\text{Na}^+]_i$ to Na^+ flux

It is appropriate to consider whether the decreased $[\text{Na}^+]_i$ which follows adrenalectomy is related to altered Na^+ flux across erythrocyte membranes. Spach & Streeten (1964) found that the rate of $^{22}\text{Na}^+$ disappearance from plasma was elevated in blood from an adrenalectomized dog when incubated at 37°C in vitro; their result pointed to an enhanced net influx under these conditions. As they (Spach & Streeten, 1964) had incorporated glucose and not inordinately elevated $[\text{Na}^+]_e$ in their incubations, their results may be accounted for in terms of a change in the rate of active Na^+ efflux, and not only in that of passive Na^+ influx. Data presented above (see Chapter 2) suggest that, in the absence of an active transport component (at $4^\circ\pm 1^\circ\text{C}$), the loading characteristic of cells from normal and adrenalectomized rats does not differ. It is therefore relevant to look to the efflux characteristic for an explanation of the net influx (accumulation) of $[\text{Na}^+]_i$ observed by Spach & Streeten (1964). The $^{22}\text{Na}^+$ efflux rate indeed proved to be reduced in cells from adrenalectomized



rats not receiving 0.9% NaCl fluid diet (see Chapter 2).

Despite this, an explanation of Spach & Streeten's (1964) result in terms of adrenocorticosteroid influence upon Na^+ efflux must be accepted with reservation. Streeten & Moses (1968) later showed that hydrocortisone did not influence the phenomenon (see Chapter 1). In addition, there is evidence that Na^+ efflux rates do vary with species. Thus, the rate constant for efflux from human erythrocytes (see FIG. V-5) is close to that which may be derived from Garrahan & Glynn's (1967a) data for intact cells, and to that derived from their data for resealed ghosts containing ATP and Na^+ (Garrahan & Glynn, 1967c). This value is, however, smaller than that for cells from intact rats (see FIGS. V-6 and V-7), whilst intermediate value of -0.83hr^{-1} can be computed for rabbit erythrocytes from the data of Villamil & Kleeman (1969). The high $[\text{Na}^+]_i / [\text{Na}^+]_e$ ratio of 0.88 for normal canine erythrocytes (Bernstein, 1954) would suggest that Na^+ turnover may be rather slow in these cells. The possibility that the findings of Losert, et al. (1964) are supported by those of Spach & Streeten (1964) (see Chapter 1) may thus be regarded as tenuous.

It is clearly important to consider the direct effects of altered cation distribution upon Na^+ fluxes in erythrocytes from adrenalectomized animals. In vitro studies of rabbit erythrocytes (Villamil & Kleeman, 1969) and human erythrocytes (Sachs, 1970) have established that the rate constant for Na^+ efflux falls with reduction in $[\text{Na}^+]_e$ in the presence of a constant $[\text{K}^+]_e$. Sachs (1970) however, made a study of a $[\text{Na}^+]_e$ series, revealing that the rate constant declines negligibly within the range of plasma $[\text{Na}^+]$ reduction occurring in adrenalectomized rats maintained upon a low $[\text{Na}^+]$ diet (see, for instance, Conway & Hingerty, 1946). Moreover, the degree of hyperkalaemia occurring in such animals is neither associated with altered Na^+ efflux from intact human cells subjected to identical $[\text{K}^+]_e$ changes in vitro (Glynn, 1956), nor with an altered Na^+ influx rate (Garrahan & Glynn, 1967c). These facts

imply that adrenocorticosteroids are the agents responsible for the changes seen in erythrocytes from adrenalectomized animals, and not altered cation distributions. Nevertheless, it is evident that the prevention of extreme hyponatraemia by provision of 0.9% NaCl fluid diet is associated with an elevated $^{22}\text{Na}^+$ efflux rate, as discussed below.

Given that $^{22}\text{Na}^+$ efflux is influenced by adrenalectomy and, interestingly, by saline compensation, it is necessary to decide what component of the efflux mechanism is affected. The existence of Na^+ efflux components other than that ascribed to the ouabain-sensitive $\text{Na}^+\text{K}^+\text{Mg}^{2+}\text{-ATPase}$ of erythrocytes is a subject of current dispute (see Chapter 1), and it has never been proposed that they mediate fluxes of considerable magnitude in vivo. The ouabain-insensitive Na^+ efflux component (see Chapter 1) is small in rat erythrocytes, accounting for 10% of total efflux (see FIG. V-6) as compared with 33% in human cells (Garrahan & Glynn, 1967a) and 50% in rabbit cells (Villamil & Kleeman, 1969). In view of the considerable decline in $^{22}\text{Na}^+$ efflux rate after adrenalectomy in rats not receiving 0.9% NaCl, it seems appropriate to implicate the $\text{Na}^+\text{K}^+\text{Mg}^{2+}\text{-ATPase}$ in this change: a decline in efflux rate would most probably involve a decreased enzyme activity.

3. Relationship of ATPase activity and cation fluxes

Upon the basis of the $[\text{Na}^+]_i$ changes observed in rat cells after adrenalectomy, alterations in the $\text{Na}^+\text{K}^+\text{Mg}^{2+}\text{-ATPase}$ activity in erythrocytes would have been expected to follow the pattern set in skeletal muscle (see Section III) and kidney (see Section IV). The investigation of ATPase activities in erythrocytes revealed unexpected and interesting results. Instead of a loss of activity, there proved to be an initial marked activation of $\text{Mg}^{2+}\text{-}$ and $\text{Na}^+\text{K}^+\text{Mg}^{2+}\text{-ATPases}$, followed by a decline (see TABLE V-4). Although this result is difficult to interpret in the light of concomitant changes in $[\text{Na}^+]_i$ and $^{22}\text{Na}^+$ efflux, it is not necessary to consider that such activation reflects the state of affairs in intact cells: it may indeed reflect the degree of

accessibility of substrate or co-factors to the enzyme in membrane fragments in vitro. This concept again receives attention in connexion with tannic acid studies discussed below.

It is difficult to entertain the hypothesis that adrenalectomy results in altered accessibility of substrate or co-factors to erythrocyte membrane $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}$ in vitro without considering that a structural change must have occurred in the membrane fragments under investigation. Such a change may have occurred in vivo, or in vitro during the preparative sequence and as an outcome of primary ones in vivo. In this respect, it is noted from Chapter 1 that there is mounting evidence for adrenocorticosteroid/cell membrane interactions in several species, and, although little is understood about its nature (Ohtsuka & Koide, 1969), Dixon, Booth & Butler (1967) concluded that evidence favoured the existence of a hydrocortisone binding agent in human cells which assumes greater importance as plasma steroid levels fall. Since the plasma $t_{\frac{1}{2}}$ for steroid hormones is generally of the order of minutes, the accessibility of adrenocorticosteroids to erythrocytes would be expected to decline rapidly after adrenalectomy, and there would ensue a change in the amount associated with cells. The direction of this change would depend upon whether the view of Dixon, et al. (1967) is correct, and upon the period of time involved. It is possible that this change is reflected in the in vitro ATPase studies under consideration (see Chapter 3). However, it is evident that the activation of ATPases is a transient phenomenon (see TABLE V-4). Although this may be explicable in terms of the substitution of plasma cholesterol for steroid molecules dissociated from erythrocyte membranes (see Bruckdorfer, et al., 1969), alternative explanations for the enzyme activity changes need consideration. They are (i) that the altered plasma ACTH levels which follow removal of adrenocorticosteroidal feedback inhibition are responsible; (ii) that the removal of adrenocorticosteroids alters erythrocyte destruction and erythropoietic rates, thereby altering the age structure of the

population of cells under study; and (iii) that the decline in $[\text{Na}^+]_i$ which follows adrenalectomy may be involved.

The only detailed study of (i) is that of Brodish & Long (1956), who assayed the adrenal ascorbic acid disappearance in hypophysectomized recipient rats of parabiotic pairs at intervals of time after adrenalectomy of the donor animal. The response fell dramatically at first, but began to rise again 12hr post-operatively. A slightly supra-normal response was attained 3 and 7 days post-operatively, and a further rise occurred in ensuing weeks. It is thus concluded that plasma ACTH initially falls after adrenalectomy, and rises again during the first post-operative week. This pattern of events is to some extent reminiscent of that seen in erythrocyte ATPase activity studies reported in Chapter 3; but ACTH is not known to influence these enzymes.

Adrenocorticosteroidal regulation of erythropoiesis needs consideration because it is known that a number of erythrocyte enzyme activities is elevated in reticulocytosis, amongst them Mg^{2+} - and $\text{Na}^+\text{K}^+\text{Mg}^{2+}$ -ATPases in the rabbit (Yunis & Arimura, 1966) and cholinesterase in the rat (Pritchard, 1949; Bhatnagar, 1968). A doubling of ATPase activities was associated with the presence of a very high percentage of reticulocytes (10%-12%) in rabbit blood (Yunis & Arimura, 1966), and this was only obtained after repeated haemorrhage and separation by differential centrifugation. A doubling of rat erythrocyte cholinesterase also accompanies very considerable reticulocytosis (Pritchard, 1949). Since the reticulocyte count in rat blood alters insignificantly during the first 7 days of adrenalectomy, and rises to 1% above normal 21 days post-operatively (Gordon, Piliero & Landau, 1951), it follows that the considerable elevation of ATPase activities occurring in early adrenalectomy is not a concomitant of reticulocytosis. Moreover, the subsequent fall in ATPase activities would be inconsistent with such an event.

It is recalled that data presented in Sections III and IV show that the availability of adequate $[\text{Na}^+]_i$ is an important factor in the determination of

ATPase activities. It is likely that the reduction in $[\text{Na}^+]_i$ of erythrocytes which accompanies adrenalectomy in rats not receiving 0.9% NaCl would lead to similar changes in enzyme activity, and not to the activation apparent in in vitro membrane preparations. This point is further considered below.

4. Cholinesterase activity after adrenalectomy

The investigation-s of blood cell cholinesterase activity in adrenalectomized rats by Vaccarezza & Willson (1965) (see Chapter 1) are of interest in connexion with Pritchard's (1949) observation of its activity during reticulocytosis (see above), the observation that erythrocyte ATPase activities are affected markedly (see Chapter 3), and that erythrocyte cholinesterase is uninfluenced by Na^+ intake (Chan & Sanslone, 1969). Reinvestigation of this enzyme activity in erythrocytes from adrenalectomized rats (see Chapter 3) did not reveal the depression reported by Vaccarezza & Willson (1965). Instead, a gradual rise culminated in a 21 day mean value significantly greater than normal, and correlating with the elevated reticulocytosis reported by Gordon, et al. (1951). The reason for Vaccarezza & Willson's (1965) result remains obscure. Although they do not comment upon the post-operative maintenance of their experimental rats, the survival period indicates that salt compensation would have been employed. This measure would not have influenced cholinesterase activity (Chan & Sanslone, 1969), yet an initial fall is not apparent in non-compensated rats, and those maintained upon 0.9% NaCl until day 21 also failed to show a depression (see TABLE V-6). It seems unlikely that the changes reported by Vaccarezza & Willson (1965) are due to their use of the assay method of Biggs, Carey & Morrison (1958), despite the fact that the cellular components of blood are thereby un-differentiated. The slight reduction in haematocrit which occurs during the initial 14-21 days post-adrenalectomy (Gordon, et al., 1951) would have been compensated for by the use of this technique. ACTH is not known to influence this enzyme, and the slight elevation in blood cell cholinesterase activity which occurred after its administration by Vaccarezza

& Willson (1965) might be taken as indicating the presence of accessory adrenal tissue in their operated rats.

5. Tannic acid effects and the erythrocyte changes accompanying adrenalectomy

Experiments involving the treatment of intact and fragmented pig erythrocytes with tannic acid (see Chapter 2 and Radcliffe, Duncan & Bowler, 1971) may be regarded as illuminating the problem of apparent activation of Mg^{2+} - and $Na^+K^+-Mg^{2+}$ -ATPases by adrenocorticosteroid depletion. Although tannic acid inhibits both ATPases in rat brain microsomes (Radcliffe, et al., 1971) and also inhibits mammalian erythrocyte cholinesterase activity (Herz, 1968; Herz & Kaplan, 1968; Radcliffe, et al., 1971), treatment of intact or fragmented cells with low concentrations of the reagent results in non-specific activation of the ATPases (Radcliffe, et al., 1971). It is clear that the activation is not related to enhanced active transport of Na^+ , since $^{22}Na^+$ efflux from loaded erythrocytes was not affected by this agent in the experiments reported above (see Chapter 2).

This non-specific effect of tannic acid upon erythrocyte ATPases is indeed reminiscent of that initiated by adrenocorticosteroid depletion. However, as pointed out earlier in this discussion, it does not necessarily imply that ATPases are markedly activated in cells in vivo. An important distinction between the effects of tannic acid and adrenocorticosteroid depletion upon intact cells is that the latter treatment does affect $^{22}Na^+$ efflux (see Chapter 2). In the light of the evidence that active Na^+ efflux is mediated by $Na^+K^+-Mg^{2+}$ -ATPase (see Chapter 1), this implies an actual loss of $Na^+K^+-Mg^{2+}$ -ATPase activity, at least, in in vitro suspensions, and presumably in vivo.

It is suggested that the onset of reduced $^{22}Na^+$ loading in rat erythrocytes treated with tannic acid (see FIG. V-4) may not be regarded as reflecting a direct influence of this agent upon Na^+ permeability. The change is more probably a concomitant of the agent's action in reducing anion permeability (Edelberg, 1952), thereby influencing the magnitude of the potential difference

across the cell membrane (see Jay & Burton, 1969), and thus of cation permeability (LaCelle & Rothstein, 1966).

6. Relationship of erythrocyte survival to changes associated with adrenalectomy

The problem arises whether the declines in Na^+ efflux rate and $[\text{Na}^+]_i$ accompanying the loss of $[\text{Na}^+]_e$ in adrenalectomized rats influence the survival of intact cells. It is thought that the results of experiments testing their susceptibility to hypotonic haemolysis help to clarify this.

The finding that cells from non-saline compensated adrenalectomized rats are less susceptible to this treatment than cells from normal animals (compare FIG. V-11, (A) and (B)) is superficially in accord with the results of Megel & Gordon (1951), except that these authors used rats which had received 1% NaCl post-operatively, and could not distinguish the change in washed erythrocytes. There is no data to confirm whether altered adrenocorticosteroid/cholesterol exchanges at erythrocyte membranes mediate alterations in their susceptibility to hypotonic haemolysis after adrenalectomy, but it is possible that the lowered $[\text{Na}^+]_i$ would have been partly responsible for the decreased susceptibility observed in the experiments reported here (see Chapter 2).

In this regard, the fact is that, although erythrocytes from saline compensated adrenalectomized rats have normal $[\text{Na}^+]_i$ (see Chapter 1), these cells exhibit increased susceptibility to hypotonic haemolysis by comparison with those from normal rats (compare FIG. V-11, (A) and (C)). Although this result does refute that of Megel & Gordon (1951), it implies that, when $[\text{Na}^+]_i$ is normal, altered adrenocorticosteroid/membrane interaction may indeed influence susceptibility to hypotonic haemolysis. This does point to an influence of adrenocorticosteroids upon cell membrane organization, the effect of which may be masked by altered $[\text{Na}^+]_i$ in vivo and in vitro. Gordon, et al. (1951) concluded from their studies of serum bilirubin that erythrocyte destruction rate was not elevated in adrenalectomized rats, even though the animals were

saline compensated; however, $[\text{Na}^+]_i$ was unknown.

B. Influence of affective status upon human erythrocytes

1. $[\text{Na}^+]_i$ and $[\text{K}^+]_i$

In Section I of this thesis, the evidence was reviewed which suggests that distribution-s of body Na^+ and K^+ become abnormal in human subjects suffering depression and mania, and that they tend to return to normal upon recovery. This evidence stemmed mainly from the whole body isotope dilution studies of Coppen & Shaw(1963), Coppen, et al.(1966) and Shaw (1966). It was argued that, since their data suggest that the change in intracellular $[\text{Na}^+]_i$ must be very large, the alteration would be systemic, and therefore detectable in measurements of $[\text{Na}^+]_i$ of convenient cells (e.g. erythrocytes).

There are important reservations as regards the interpretation of these data, particularly because Coppen and his associates did not make direct observations of $[\text{Na}^+]_i$. They obtained values for residual body $[\text{Na}^+]_R$ ($[\text{Na}^+]_R$), which may be regarded as that fraction of total body $[\text{Na}^+]_T$ consisting of $[\text{Na}^+]_i$ plus the amount of exchangeable Na^+ which is associated with bone. Alterations in the magnitude of the latter fraction of $[\text{Na}^+]_R$ were not assessed in these studies, so that no account was taken of the buffering capacity of bone with reference to extracellular $[\text{Na}^+]_E$ changes. This matter is further discussed in Section VII of this thesis; it is appropriate, for the time being, to confine attention to the predicted and observed $[\text{Na}^+]_i$ changes associated with affective disorders.

The question has arisen whether the considerable increments in $[\text{Na}^+]_R$ which have been reported for depressive and manic patients (see, for instance, Coppen, et al., 1966) may be monitored as erythrocyte $[\text{Na}^+]_i$. If it is accepted that the total intracellular fluid volume in humans is 330 ml/kg body weight (Black, 1967), and that the total erythrocyte cell volume is 2.6% of body weight (Moore, Oleson, McMurrey, Parker, Ball & Boyden, 1963), erythrocyte cell volume may be re-expressed as 7.8% of total intracellular fluid volume. Thus, upon

the basis of a general elevation of intracellular $[Na^+]_i$ in mania and depression, it should be detectable in erythrocytes.

The data presented in Chapter 2 show that there was no significant difference in $[Na^+]_i$ for manic-depressive (manic or depressed phase) patients not receiving psychoactive drugs. It may therefore be concluded that either (i) erythrocytes are exempt from the general intracellular $[Na^+]_i$ changes which are taken to accompany mania and depression, or (ii) $[Na^+]_i$ changes do not accompany these affective states. These alternatives are further discussed below.

Coppen & Shaw (1963) presented evidence for a very slight and not significant increase in intracellular $[K^+]_i$ during depression, as assessed in the same subjects before and after recovery. This observation is interesting in connexion with the results given in Chapter 2 (TABLE V-2), which show that erythrocyte $[K^+]_i$ was significantly greater than normal in the drug-free manic-depressive patients under study. However, as pointed out by Shaw & Coppen (1966) themselves, their estimations of total intracellular $[K^+]_i$ in depressed and recovered subjects gave values below normal (as quoted from Moore, et al., 1963). Thus the direction of change in erythrocyte $[K^+]_i$ of depressives (see Chapter 2) is only partially consistent with the observations of Coppen & Shaw (1963). It is unfortunate that these authors were unable to obtain their own estimation of normal total intracellular $[K^+]_i$.

The results reported in Chapter 2 show that erythrocytes are not exempt from changes in electrolyte content, and to this extent are in accord with other reports of erythrocyte parameters in disease (see Chapter 1). Thus, if body electrolyte redistribution is a feature of affective status, these erythrocyte studies suggest that $[K^+]_i$ is markedly affected, and not $[Na^+]_i$, as suggested by the isotope dilution studies of Coppen and associates. It is interesting that the increase in erythrocyte $[K^+]_i$ proved to be greater in mania than in depression (see TABLE V-2), a pattern of events similar to that predicted for $[Na^+]_i$ from the $[Na^+]_R$ estimations of Coppen, et al. (1966). This point is

further discussed in Section VII in terms of the effects of altered $[K^+]_i$, and of the lack of evidence for altered erythrocyte $[Na^+]_i$ in the affective disorders under study.

2. Relationship of ATPase activities to cation content

Mean Mg^{2+} - and $Na^+ + K^+ - Mg^{2+}$ -ATPase activities were elevated to the greatest extent in manic-depressives suffering the manic phase. This change is therefore a concomitant of the more considerable $[K^+]_i$ increase in such patients, although mean $[Na^+]_i$ remains normal (see TABLE V-2). The normal ATPase activities of erythrocytes from endogenous depressives does correlate with the normal cation content of these cells, so that altered cation content and enzyme activity may indeed be considered to be related in manic-depression.

The features of erythrocytes from manic-depressives are in some degree similar to those of cells from hereditary spherocytotics. It is recalled from Chapter 1 that the high rate of Na^+ turnover by erythrocytes correlated with increased $Na^+ + K^+ - Mg^{2+}$ -ATPase and decreased Mg^{2+} -ATPase activities. Thus, if it is accepted that Mg^{2+} -ATPase is involved in the control of passive cation permeability, so that a fall in its activity results in a breakdown of a part of the mechanism which normally prevents Na^+ influx and K^+ efflux along their concentration gradients (Bowler & Duncan, 1967b), the state of affairs in these cells may be largely resolved. When both Mg^{2+} - and $Na^+ + K^+ - Mg^{2+}$ -ATPase activities are elevated, one would predict a net fall in $[Na^+]_i$ and net rise in $[K^+]_i$, caused by the combination of reduced passive permeability to these cations and their enhanced rate of active transport across the cell membrane. The evidence obtained in the present study suggests that this, at least in part, constitutes the situation in erythrocytes from manic-depressives.

3. Influence of haloperidol and chlorpromazine upon cation content and ATPase activities

These drugs are widely used in the treatment of affective disorders. An attempt

was therefore made to assess their effects upon the erythrocyte parameters measured in the present study, especially in view of their known effects upon these cells (see Chapter 1). The data indicate, however, that they are not entirely effective when administered to manic-depressives. The non-specific reduction of Mg^{2+} - and $Na^+K^+-Mg^{2+}$ -ATPase activities to normal in depressed phase subjects was remarkable in view of the fact that $[Na^+]_i$ and $[K^+]_i$ remained uninfluenced, although the effect upon ATPases is in accord with that recorded for brain microsomal preparations (see Section VI). In view of its effective reduction in $[Na^+]_i$ and $[K^+]_i$ of erythrocytes from manic phase patients, it is unfortunate that data remained unavailable for the influence of haloperidol upon ATPase activities.

C. Relationship of adrenocorticosteroidal determination of erythrocyte properties and those of erythrocytes from manic-depressive subjects

The results presented in this Section indicate that adrenocorticosteroids are regulators of erythrocyte properties which prove to be abnormal in manic-depressive disease. The observations are therefore interesting in view of proposals that altered adrenocorticosteroidal output, availability and target cell interaction are correlates of affective disorder (see Section I). Since further evidence concerning the relationship of these phenomena comes from work upon brain (see Section VI), an assessment of its extent is delayed until Section VII.

Main conclusions

1. Adrenalectomy results in reduction of erythrocyte $[Na^+]_i$ and $[K^+]_i$ in rats. The effect may be partially prevented by 0.9% NaCl supplementation.
2. Progressive fall in $[Na^+]_i$ is accompanied by a reduced capacity for Na^+ efflux by $^{22}Na^+$ -loaded erythrocytes from operated rats.
3. Although these factors would be consistent with lowered $Na^+K^+-Mg^{2+}$ -ATPase activity, assays of the activity in membrane fragments revealed an initial post-operative elevation. This is considered to have resulted from the altered

adrenocorticosteroid/cell membrane interactions which would follow adrenalectomy.

4. Normal $[\text{Na}^+]_i$ erythrocytes from 5-day adrenalectomized rats are more susceptible to hypotonic haemolysis than low $[\text{Na}^+]_i$ cells. This again points to the initial loss of a membrane stabilizing influence after adrenalectomy.

5. The subsequent return of ATPase activities towards normal may have resulted from the replacement of lost adrenocorticosteroid molecules by cholesterol in vivo. This may be regarded as membrane re-stabilization.

6. Work with tannic acid suggests that the apparent sequential elevation and return to normal of ATPase activities may not necessarily have occurred in vivo in adrenalectomized rats.

7. Alterations in ATPase activities are not accompanied by changes in acetylcholinesterase activity in intact cells after adrenalectomy. This finding contrasts that of Vaccarezza & Willson (1965).

8. Erythrocyte $[\text{Na}^+]_i$, $[\text{K}^+]_i$ and Mg^{2+} - and $\text{Na}^+\text{K}^+\text{Mg}^{2+}$ -ATPase activities are altered in manic depressives as distinct from endogenous and reactive depressives. However, the changes are not substantially consistent with those predicted from isotope dilution studies by other workers.

S E C T I O N VI

BRAIN STUDIES

CHAPTER 1Influence of Adrenocorticosteroids upon the Central Nervous SystemMonovalent cation distribution in the C.N.S.

The fundamental importance of monovalent cation distribution to the functioning of excitable cells is well known. In Section I it was pointed out that, should altered cation distribution be a systemic concomitant of affective disorders, altered behaviour may be causally related to the change affecting the nervous system.

The highly complex anatomical compartmentalization and cellular heterogeneity of the brain have generally impeded electrolyte distribution studies in ways similar to those previously outlined for skeletal muscle (see Section III). Not surprisingly, therefore, there are virtually no studies of this tissue in subjects suffering affective disorders. Shaw, Frizel, Camps & White (1969) analysed the Na^+ and K^+ content of the homogenates of brains taken from the cadavers of depressive suicides, but drew no definite conclusions with regard to their ante-mortem distributions.

Adrenocorticosteroidal interaction with the C.N.S.

It is not proposed to discuss problems relating to the accessibility of hormones to the brain, since this would involve a detailed consideration of the so-called blood-brain barrier. Discursive reviews of this phenomenon reveal that, whilst Dobbing (1961, 1968) has strongly questioned its existence, Davson (1972) remains confident of the concept's validity. Whichever view is accepted, there is growing evidence of steroid hormone interaction with C.N.S. regions. Donovan (1970) points out that the limbic structures of the mammalian brain currently assume ever greater prominence in studies of the neuroendocrine control of behaviour. They have been studied in connexion with olfaction and feeding behaviour, and (in conjunction with the hypothalamus) in connexion with the regulation of biological rhythms, motivation and emotion. Current evidence highlights the limbic hippocampal and amygdaloid structures respectively in exerting inhibitory and stimulatory effects upon

the hypothalamic release of corticotrophin releasing factor (C.R.F.) (Mangili, Motta & Martini, 1966; McEwen, Zigmoid & Gerlach, 1972), and in the feedback regulation of adrenocorticosteroid output (McEwen, et al., 1972). Recent work reveals that the nuclei of hippocampal, amygdaloid and cerebral cortical cells from adrenalectomized rats are especially capable of corticosterone binding (McEwen, Weiss & Schwartz, 1970). The means therefore exist whereby adrenocorticosteroids may influence the brain.

Influence of adrenocorticosteroids upon C.N.S. electrolyte distributions and excitability

The limited evidence which is available has been reviewed by Woodbury (1954, 1958) and Woodbury & Vernadakis (1966), and the essence of these reviews will be given here. Total adrenalectomy results in a lowered electroshock seizure threshold (E.S.T.) in rats, i.e. they become hyper-excitabile. This change is reputedly associated with brain cation redistributions; $[Na^+]_e$: $[Na^+]_i$ and $[K^+]_i$: $[K^+]_e$ are decreased as calculated upon the assumption that Cl^- space is a measure of extracellular volume. (This interpretation is accepted with the proviso that, since the Cl^- content of glial cells remains unknown, brain $[Cl^-]$ may not give a reliable estimate of extracellular volume (Van Harreveld, 1972). On the other hand, chronic deoxycorticosterone administration to intact rats results in an elevated E.S.T. (i.e. hypo-excitability), which is associated with increased cerebral cortical $[Na^+]_e$: $[Na^+]_i$ and $[K^+]_i$: $[K^+]_e$. As expected, this steroid prevents the E.S.T. change which follows adrenalectomy, but not all adrenocorticosteroids share this action. Chronic administration of hydrocortisone, cortisone or 11-deoxycorticosterone to intact rats actually lower E.S.T., whilst corticosterone does not influence it. Of great interest is the finding that combined adrenocorticosteroid doses yield intermediate E.S.T. values, and that the diurnal rhythm of corticosterone output in rats appears to be paralleled by E.S.T. changes (Woodbury & Vernadakis, 1966). E.S.T. may thus be regarded as a measure of the relative availabilities of these hormones to, and their interaction with, appropriate cerebral sites.

This conclusion is interesting in connexion with reports of affective changes in human subjects suffering hypo-adrenocorticism (Addison's disease) and hyper-adrenocorticism (Cushing's syndrome). Whilst such changes are held to be very frequent in Addison's disease, they infrequently accompany Cushing's syndrome (Bleuler & Stoll, 1962). When they do occur in the latter case, they are attributed to excess 'glucocorticoid' activity (Bleuler & Stoll, 1962). This suggests that altered adrenocorticosteroidal secretion or target cell sensitivity may be of aetiological significance in patients suffering affective disease (see Section I). This matter receives further consideration in Section VII.

Occurrence of Mg^{2+} - and $Na^+K^+-Mg^{2+}$ -ATPase in mammalian brain

There is abundant evidence for the presence of these enzymes in brain microsomal preparations. Järnefelt (1960, 1961) demonstrated their presence in rat brain, and for this species Somogyi (1964) showed that they possessed differential temperature liability. Bowler & Duncan (1968) subsequently extended such observations to reveal that the ATPases possess different sensitivities to thermal inactivation and different activation energies, i.e. that they behave as separate enzymes.

Influence of certain drugs and adrenocorticosteroids upon brain Mg^{2+} - and $Na^+K^+-Mg^{2+}$ -ATPase activities

Observations in this field are of interest in connexion with the pharmacology of erythrocyte membrane fragility (see Chapter V4). Ethanol inhibits rat brain $Na^+K^+-Mg^{2+}$ -ATPase at a concentration producing mild-severe intoxication in intact animals (Israel, Kalant & Laufer, 1965). Century & Horwitt (1956) noted that chlorpromazine slightly inhibited rat brain ATPase activity, but their assay conditions suggest that a Ca^{2+} -ATPase was under study. Subsequent workers (Järnefelt, 1962; Judah & Ahmed, 1964; Squires, 1965; Davis & Brody, 1966; Kraus & Šimáně, 1967; Akera & Brody, 1968; Robinson, Lowinger & Bettinger, 1968; Chowdhury, Rogers, Skinner, Spector & Watts, 1969) have demonstrated the inhibition of a true brain $Na^+K^+-Mg^{2+}$ -ATPase by this phenothiazine tranquillizer.

However, the role of Ca^{2+} may prove significant in chlorpromazine action: in accordance with findings in erythrocytes by Hauser & Dawson (1968) and Kwant & Seeman (1969), Robinson (1968) discovered a small, though significant fall in $^{45}\text{Ca}^{2+}$ retention by preloaded rat brain microsomes. This may have occurred through an effect upon a Ca^{2+} -ATPase (cf. Century & Horwitt, 1956), but it is not possible to say how $\text{Na}^{+}+\text{K}^{+}-\text{Mg}^{2+}$ -ATPase might be thus affected. Certainly, there is $\text{Ca}^{2+}-\text{Mg}^{2+}$ -ATPase activity in rat brain (Berl & Puszkin, 1970).

In the light of studies made upon other tissues (see Sections III - V), the question arises whether adrenocorticosteroids influence brain Mg^{2+} - and/or $\text{Na}^{+}+\text{K}^{+}-\text{Mg}^{2+}$ -ATPase activities. Whilst the present experiments were in progress, Gallagher & Glaser (1968) published data showing that these enzymes remain uninfluenced by adrenalectomy, even though the seizure threshold was lowered in their rats. However, their experiments were performed upon saline-maintained rats, and upon saline-maintained animals which subsequently had been given distilled water for 4 days. Since it has been shown that saline compensation modifies the altered monovalent cation stimulated ATPase activity of skeletal muscle (see Section III) and kidney (see Section IV) following adrenalectomy, it was felt that the effects of saline deprivation merited fuller investigation. Indeed, Takagi & Yamamoto (1969) have shown that rat adenohipophyseal $\text{Na}^{+}+\text{K}^{+}-\text{Mg}^{2+}$ -ATPase activity actually increases after adrenalectomy, and is depressed by corticosterone in vivo and in vitro. This is of interest because one feedback influence of the steroid upon its own output is considered to occur through its suppressing the effect of endogenous C.R.F. upon adenohipophyseal ACTH output (Yates, Brennan & Urquhart, 1969). The effect of adrenalectomy upon the brain Mg^{2+} - and $\text{Na}^{+}+\text{K}^{+}-\text{Mg}^{2+}$ -ATPase activities of rats is further reported in the following Chapter.

CHAPTER 2Effect of Adrenalectomy upon Rat Brain Mg^{2+} - and $Na^+K^+-Mg^{2+}$ -ATPase Activities

This Chapter reports the results of 3 series of experiments to investigate the influence of a distilled water fluid upon the brain Mg^{2+} - and $Na^+K^+-Mg^{2+}$ -ATPases of adrenalectomized rats.

MATERIALS AND METHODSMATERIALS

- a) Animals: ♂ CFHB albino rats, as described in Section II.
- b) Reagents: These are given in the text.

METHODS

- a) Bilateral adrenalectomy and maintenance of rats: The techniques are outlined in Section II. Operations were performed upon ether-anaesthetized rats. Control (intact) rats were ether-anaesthetized at the same time. Control (intact) and operated animals received normal solid diet and distilled water fluid diet ad lib..
- b) Sampling of brain tissue: Animals were sacrificed by cervical dislocation and whole brains removed, weighed and homogenized in 250mM sucrose +1mM $Na_2EDTA \cdot 2H_2O$ in 30mM L-histidine-HCl, pH 7.3 at $0^{\circ}-4^{\circ}C$.
- c) Preparation of microsomal fraction for ATPase assays: The preparative sequence is summarized in FIG.VI-1.
- d) Enzyme assays: Incubations of 0.5ml aliquots of freshly obtained microsomal resuspensions were made in duplicate for 10min at $37^{\circ} \pm 0.1^{\circ}C$ in the presence of 0.5ml Tris-ATP (2mM final concentration) and 1ml Mg^{2+} (4mM final concentration). Na^+ (100mM) and K^+ (20mM) were included with Mg^{2+} when required. The Cl^- salts of cations were used, and were made up in 30mM L-histidine-HCl, pH 7.3 at $37^{\circ} \pm 0.5^{\circ}C$.
Orthophosphate and protein determinations were made in duplicate as described in Section II.
Enzyme activities were expressed as nmoles P_i liberated/mg protein/min.

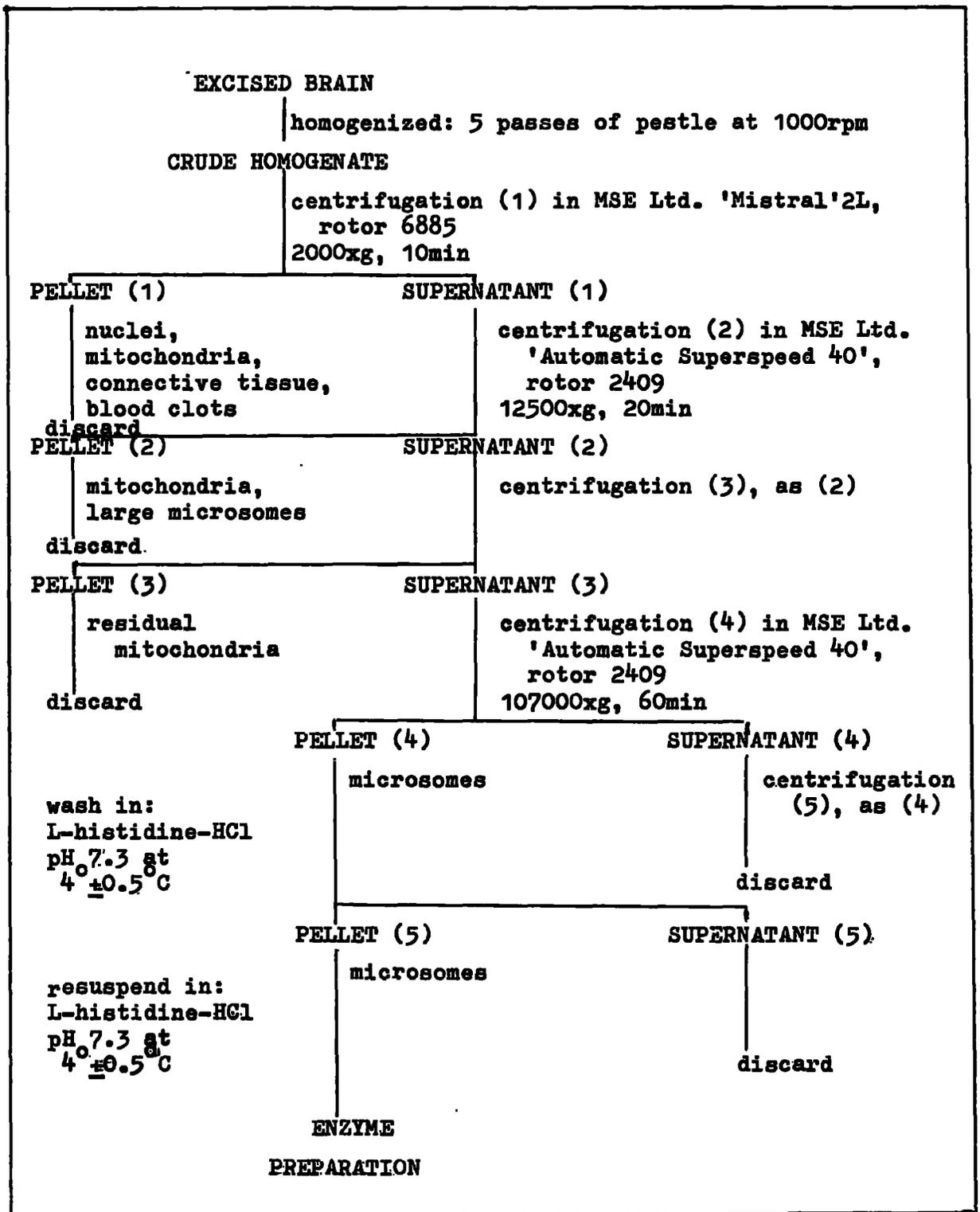


FIG. VI-1. PROCEDURE FOR THE EXTRACTION OF RAT BRAIN MICROSOMES.

All steps were performed at 0°-4°C.

RESULTS

The results of the 3 series of experiments are pooled and presented in TABLE VI-1. Animals were sacrificed 2, 4 and 7 days after the day upon which adrenalectomies were performed, and brain Mg^{2+} - and $Na^{+}+K^{+}-Mg^{2+}$ -ATPase activities assayed as described above. The mean values and their standard errors were calculated. It is clear from the data presented in TABLE VI-1 that adrenalectomy did not influence whole brain ATPase activities, even after the animals had been saline depleted for 7 days. The day 2 values reveal initial decreases in both Mg^{2+} - and $Na^{+}+K^{+}-Mg^{2+}$ -ATPase mean activities, but these did not prove to be statistically significant. Thereafter, the mean enzyme activities returned to very near control values.

The implications of these findings are discussed in the next Chapter.

FLUID DIET			DISTILLED WATER			
DAYS POST-ADX.			0 (control)	2	4	7
ATPase ACTIVITY (nmoles P _i /mg protein/min)	Mg ²⁺ -ATPase:	n	3	3	3	3
		M	279.7	261.9	284.2	281.3
		S.E.	11.7	13.1	15.4	20.5
ATPase ACTIVITY (nmoles P _i /mg protein/min)	Na ⁺ +K ⁺ - Mg ²⁺ -ATPase:	n	3	3	3	3
		M	346.0	295.8	316.3	335.4
		S.E.	27.9	19.3	11.3	13.9

TABLE VI-1. INFLUENCE OF ADRENALECTOMY UPON RAT BRAIN Mg²⁺- AND Na⁺+K⁺-Mg²⁺-ATPase ACTIVITIES.

Preparative procedure:

See 'Materials and Methods'.

Incubation conditions:

30mM L-histidine-HCl + 2mM Tris-ATP, pH 7.3, 37° ± 0.1° C.

Mg²⁺ (4mM), Na⁺ (100mM) and K⁺ (20mM) were added as indicated.

Na⁺+K⁺-Mg²⁺-ATPase activity is the increment in enzyme activity obtained by the inclusion of Na⁺ and K⁺ with Mg²⁺.

n = number of animals.

CHAPTER 3

Brain Mg^{2+} - and $Na^+K^+Mg^{2+}$ -ATPases as Targets for Adrenocorticosteroids

The experimental results shown in Chapter 2 support those of Gallagher & Glaser (1968), although these authors obtained a comparatively low microsomal $Na^+K^+Mg^{2+}$ -ATPase activity. These results remain unusual in the light of the effects of adrenalectomy and saline deprivation upon skeletal muscle, kidney and erythrocytes. They suggest that the brain ATPases are exempt from the primary and secondary effects of adrenocorticosteroid depletion, or that the preparative techniques employed rendered the effects undetectable. It is also possible that complete adrenocorticosteroid depletion removes a number of regulatory (steroidal) influences upon ATPase activities, and that there is no net change in their activities. However, the alterations in E.S.T. and in Na^+ and K^+ distribution which follow adrenalectomy (see Chapter 1) suggest that a fall in the active transmembrane fluxes of these cations does occur. This implies a decreased $Na^+K^+Mg^{2+}$ -ATPase activity, at least. In the experiments reported above (see Chapter 2), the fall in mean $Na^+K^+Mg^{2+}$ -ATPase activity which occurred on the second day after adrenalectomy was very small and not statistically significant. Mean Mg^{2+} -ATPase activity was also virtually unaffected.

The possibility remains that steroid hormone sensitivity is a property of very localized cerebral regions, in which case the effects upon Mg^{2+} - or $Na^+K^+Mg^{2+}$ -ATPase activity would be difficult to detect. This is because these enzymes are present throughout the brain, so that the whole brain preparation yields a very high activity. In these circumstances, it seems likely that, short of an attempt to isolate cells from such highly localized regions, a histochemical attempt to assay their ATPase activity might provide useful data. Nevertheless, the cerebral cortex, which represents the dominant fraction of whole brain homogenates, is reported to have steroid hormone binding properties (McEwen, et al., 1972; see Chapter 1).

It is concluded that Mg^{2+} - and $Na^{+}+K^{+}-Mg^{2+}$ -ATPase activities in whole brain homogenates from adrenalectomized rats resemble those obtained from intact rats, but that this does not preclude an influence of these hormones upon the enzymes in highly localized, steroid-sensitive brain regions.

S E C T I O N V I I

FINAL DISCUSSION OF THE SYSTEM UNDER STUDY

It must be immediately pointed out that, although a thorough consideration of the subject of this thesis requires the unification of a wide range of phenomenological knowledge, it has been necessary largely to disregard such aspects of adrenocorticosteroid action as the regulation of carbohydrate and fat metabolism, and of protein synthesis. Such is the nature of biological systems that these aspects are inextricably linked with electrolyte metabolism and membrane permeability control. In addition, no attempt has been made to assess the possible influence of adrenomedullary ablation upon the phenomena attributed to adrenocorticosteroid depletion. Again, certain hypotheses concerning the aetiology of affective disorders have remained unconsidered, in particular those implicating brain catecholamine distribution and turnover.

This Section seeks to consider the extent to which the observations reported in Sections III-VI may be viewed synoptically with regard to the working hypothesis proposed in Section I. The problems arise whether the effects of adrenocorticosteroid depletion upon the tissues under study have features in common, and whether it is possible to account for the discrepancy between those values obtained for erythrocyte cation content in manic and depressive subjects and those predicted from whole body determinations obtained by isotope dilution analysis.

Evidence obtained by many workers has shown that the $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+} - \text{ATPase}$ activity of kidney is essentially determined by the availability of Na^+ to the enzyme in situ, and that the passive permeability of the mucosal membrane of renal tubular epithelial cells is the factor under adrenocorticosteroidal control (see Section IV). The studies of monovalent cation stimulated $\text{Mg}^{2+} - \text{ATPases}$ in skeletal muscle microsomes reveal that maintenance of Na^+ availability indeed counteracts the loss of activity which follows adrenalectomy (see Section III), so that the situation in this tissue is superficially similar to that in kidney. However, one important distinction is that, whereas $\text{Mg}^{2+} - \text{ATPase}$ activity remains normal in kidney preparations, it is

markedly reduced in skeletal muscle ones. The loss of this ATPase activity component is not counteracted by post-operative saline administration, and, in preparations from rats not receiving saline, it proves to be actually inhibited by Na^+ in vitro.

This new observation suggests that Na^+ availability is not the only factor influencing ATPase activity, but that this is affected by adrenocorticosteroids in some other way. However, in view of the uncertainty with regard to the source of skeletal muscle microsomal ATPases, results obtained in erythrocytes are more worthy of consideration in this context (see Section V). The data obtained for these cells provide a case for a more direct steroidal influence upon membrane function, including that of Mg^{2+} - and $\text{Na}^+\text{K}^+\text{Mg}^{2+}$ -ATPases. Thus, despite their normal $[\text{Na}^+]_i$, erythrocytes from adrenalectomized rats are more susceptible to hypotonic haemolysis than those from intact rats. Furthermore, in vitro incubations of erythrocyte membrane fragments from adrenalectomized rats reveal considerable non-specific elevation of both Mg^{2+} -dependent ATPase activities above normal, an effect which would not have been predicted from the normal $^{22}\text{Na}^+$ -loading properties and reduced $^{22}\text{Na}^+$ efflux rate constant of intact erythrocytes from such animals. As in the instance of Na^+ -mediated inhibition of skeletal muscle Mg^{2+} -ATPase, these results suggest that adrenocorticosteroid depletion leads to changes in membrane properties which alter the pattern of membrane/cation interaction, at least in vitro. This most probably stems from altered membrane/hormone interaction.

It has already been pointed out that there is direct evidence for steroidal interactions with plasma membranes (Dixon, et al., 1967; Ohtsuka & Koide, 1969; see Section V), but this is a relatively new and difficult field of investigation. Of possible relevance to the abnormal cation/ATPase interactions characterizing skeletal muscle and erythrocyte preparations from adrenalectomized rats is the known influence of steroids upon water structure, particularly because these hormones have been found to act as potent ice structure-promoters

(Fukuta & Mason, 1963). Water constitutes about 30-50% of biological membrane mass, and is clearly important to membrane structure and function (Finean, 1957; Fernández-Morán, 1962; Hechter, 1965a,b). This is particularly because of the presence of membrane surface water layers which are highly structured and which remain 'unstirred' (i.e. uninfluenced by the rate at which bulk extracellular fluid is stirred in vitro). Kavanau (1964, 1965) has discussed this phenomenon in detail, and has drawn special attention to the relatively greater mobility of H^+ in ice than in liquid water. It is possible that local and interconnected alterations in membrane water structure could influence the functioning of single membrane enzymes or enzymatic circuits. Hechter (1965b) also believes that the degree of ordering of water molecules in the ice-like surface layer may influence the conformations of membrane proteins, and hence the properties of enzyme catalytic sites. Nevertheless, it is important to add the reservation that not all membrane-located enzymes need be directly influenced in this manner by adrenocorticosteroid depletion. Rat erythrocyte acetylcholinesterase is one example (see Section V), and brain Mg^{2+} - and $Na^+ + K^+ - Mg^{2+}$ -ATPases may prove to be others (see Section VI).

Thus, there is evidence that adrenocorticosteroid depletion does influence the active Na^+ transport and Mg^{2+} -dependent ATPase activity characteristics of a number of tissues. In certain cases, this effect may be directly attributed to altered membrane/steroid interaction.

As regards the involvement of electrolyte redistribution in mania and depression, it is necessary to further consider the discrepancy between the findings reported in Section V and those reported by Coppen & Shaw (1963), Coppen, et al. (1966), and Shaw & Coppen (1966), whose data have particularly suggested that Na^+ retention is a feature of these affective states (see Section I). As pointed out in Section V, the body Na^+ component which was estimated by the authors ($[Na^+]_R$) consists of total intracellular Na^+ plus

the exchangeable Na^+ associated with bone. A correct assessment of $[\text{Na}^+]_i$ is therefore dependent upon some knowledge of the latter fraction.

It is considered that a large proportion (approx. 30%) of body Na^+ is in bone (Manery & Bale, 1941), and that between 25% and 60% of this is rapidly exchangeable (Vaughan, 1970). A typical study (that of Edelman, James, Baden & Moore, 1954) showed that 45% of dog and human bone Na^+ exchanged with $^{24}\text{Na}^+$ within 4 hours. It appears that the size of the exchangeable bone Na^+ component may vary according to demand: thus, bone Na^+ is known to be mobilized in Na^+ -depleted rats (Bergstrom, 1952), and it is considered to act as a buffer source in metabolic acidosis (Lemann, Litzow & Lennon, 1966). It is therefore worthwhile considering other interpretations of the $[\text{Na}^+]_R$ change associated with affective disease.

Since approximately 8% of body weight in humans is attributed to dry, fat-free bone (Moore, et al., 1963), it follows that the approximate mean dry, fat-free bone weight in the depressive subjects studied by Coppen & Shaw (1963) was 5kg. This value is close to those listed by Moore, et al. (1963) for subjects in the appropriate age range. Edelman, et al. (1954) found that the mean $[\text{Na}^+]$ of human bone was 234mmoles/kg of dry, fat-free material, a value in close agreement with that of other authors. On this basis, the mean total bone Na^+ in the subjects under study would have been approximately 1200mmoles. Coppen & Shaw (1963) give data revealing an increment of 180mmoles in mean $[\text{Na}^+]_R$ in depressed as compared with recovered subjects. This value is 15% of that derived for mean total bone Na^+ in such subjects (see above), and it certainly lies within the limits of variability of the exchangeable bone Na^+ fraction (see above). Since proportionately small changes in bone Na^+ may therefore constitute apparently large changes in the remaining fraction of $[\text{Na}^+]_R$, it is conceivable that the altered $[\text{Na}^+]_R$ reported by Coppen & Shaw (1963) need not have entailed a change in $[\text{Na}^+]_i$, but rather in the exchangeability of bone Na^+ .

At present it appears that little is understood of the factors which govern the exchangeability or availability of bone Na^+ , but there is evidence for an adrenocorticosteroidal influence. Dosekun (1959) reported a loss of bone Na^+ in adrenalectomized rats not receiving saline compensation, and Stern, Cole, Bass & Overman (1951) a decrease in $^{24}\text{Na}^+$ uptake in bone from adrenalectomized dogs. Woodbury (1953) noted that the reduction of bone Na^+ in nephrectomized rats is prevented by hydrocortisone and ACTH administration. It is possible, therefore, that the degree of exchangeability of bone Na^+ is under adrenocorticosteroidal control, and notable that these hormones are among the factors which are influenced in affective disorders (see Section I). However, although it is known that altered adrenocorticosteroidal output is instrumental in the osteoporosis of Cushing's syndrome (Storey, 1963), there appears to be a paucity of data concerning bone Na^+ in adrenocortical disorders.

Despite the difficulties which arise in the interpretation of body Na^+ distribution data, there is circumstantial evidence to implicate monovalent cations in the aetiology of mania and depression. It stems from the treatment of the disorders by Li^+ administration (usually in the form of LiCO_3 ; see, for instance, Wharton & Fieve, 1966; Fieve, Platman & Plutchik, 1968), and it is relevant because Li^+ is known to influence the monovalent cation exchanges which characterize the action potential in excitable cells. Thus, although Li^+ and Na^+ may enter such cells with equal facility when they depolarize, Li^+ tends to accumulate intracellularly because its subsequent rate of extrusion is about 0.10 - 0.04x that of Na^+ , and the process appears to involve a Li^+ - Na^+ exchange mechanism (Keynes & Swan, 1969a,b). Thus, $[\text{K}^+]_e$ would increase as a result of a lowered rate of active Na^+ - K^+ exchange. This is borne out by the finding that the nerve cell respiratory increment associated with the increased ATP hydrolysis essential to active transport is abolished when $[\text{Na}^+]_e$ is replaced by $[\text{Li}^+]_e$ (Connelly, 1959;

Ritchie, 1966), an effect similar to that of externally applied ouabain (Baker & Connelly, 1966). Furthermore, transient Na^+ and K^+ diureses are associated with Li^+ administration to manic-depressive patients (Hullin, Swinscoe, McDonald & Dransfield, 1968; Baer, Platman & Fieve, 1970). It is therefore possible that Li^+ may act by reducing any increment in the cellular $[\text{K}^+]_i$, which may accompany mania and depression. In this context, if it were assumed that the 15-22% increase in $[\text{K}^+]_i$ of erythrocytes from manic and depressed subjects (see Section V) also occurs in other cells (including those of the C.N.S.) and in the absence of altered $[\text{K}^+]_e$ (Coppen & Shaw, 1963; Shaw & Coppen, 1966), one would predict a small increment in E_K (i.e. hyperpolarization) on the basis of the Nernst equation. The precise implications of this would depend upon the C.N.S. regions involved, a detailed consideration of which remains beyond the scope of this thesis (see Section VI).

Finally, it is of interest in connexion with the catecholamine hypothesis of affective disorders (see, for instance, Schildkraut & Kety, 1967) that $[\text{Na}^+]$ and $[\text{K}^+]$ influence the in vitro ^3H -noradrenaline uptake, storage and metabolism of rat sympathetic nerve endings and brain synaptosomes (Bogdanski & Brodie, 1966; Bogdanski, Tissari & Brodie, 1968; but see White & Keen, 1970), and that Li^+ administration has been shown to affect these phenomena in mouse brain synaptosomes (Kuriyama & Speken, 1970). High in vitro concentrations of oestradiol- 17β and progesterone have also been found to influence rat brain synaptosomal catecholamine uptake and efflux (Janowsky & Davis, 1970). It is in this context that three aspects of the disordered physiology of affective disorders may be considered in conjunction with each other and with regard to membrane phenomena. However, this more complex issue remains unconsidered for the purposes of this thesis.

From this consideration of the findings reported in Sections III - VI above,

it is clear that the working hypothesis proposed in Section I must be regarded as untenable. This is because the evidence for altered $[\text{Na}^+]_i$ in depression and mania remains equivocal. However, the outcome of investigations of body electrolyte distributions in the context of these affective states cannot be entirely abandoned, since the absence of definitive experimental evidence leaves open the possibility that bone Na^+ exchangeability is altered. This is relevant to the general perspective of body Na^+ balance in a way which may not, at present, be fully appreciated. In addition, it is possible that the altered erythrocyte $[\text{K}^+]_i$ of manic and depressed subjects may reflect a general intracellular change. There is also provision for the direct interaction of steroid hormones with plasma membranes in the regulation of their cation permeability, but we await a detailed investigation of this phenomenon in a wide variety of tissues in order to assess their hormone sensitivity with greater precision.

SUMMARY OF MAIN EXPERIMENTAL FINDINGS

1. Mg^{2+} -dependent ATP hydrolysis was evident in membrane fractions prepared from human and rat erythrocytes, and from rat skeletal muscle, kidney and brain. $Na^+ + K^+$ -stimulated, Mg^{2+} -dependent ATP hydrolysis occurred in all except rat skeletal muscle. (Sections III, IV, V and VI)
2. The application of a variety of extraction techniques in the preparation of rat skeletal muscle microsomal fractions failed to reveal a synergistically-stimulated $Na^+ + K^+ - Mg^{2+}$ -ATPase. Non-linked Na^+ - or K^+ -stimulation of Mg^{2+} -dependent ATPase activity was obtained, and some Na^+ - or K^+ -mediated ATP hydrolysis occurred in the absence of added Mg^{2+} . (Section III.)
3. Although there was electronmicroscopic and biochemical evidence for mitochondrial disruption during the course of preparation of rat skeletal muscle fractions, the evidence from incubations containing sodium azide suggested that microsomal Mg^{2+} -ATPase activity was not of mitochondrial origin. (Section III.)
4. Adrenalectomy resulted in non-specific reduction of Mg^{2+} -dependent and Na^+ - or K^+ -stimulated, Mg^{2+} -dependent ATP hydrolyses in rat skeletal muscle, specific reduction of $Na^+ + K^+ - Mg^{2+}$ -ATPase activity in rat kidney, and non-specific elevation of rat erythrocyte Mg^{2+} - and $Na^+ + K^+ - Mg^{2+}$ -ATPases; but it did not influence the Mg^{2+} -dependent ATPases of rat brain preparations. (Sections III, IV, V and VI.)
5. Mean $[Na^+]_i$ and $[K^+]_i$ were reduced in erythrocytes from adrenalectomized rats: mean $[Na^+]_i$ was significantly abnormal. (Section V.)
6. The magnitude of passive $^{22}Na^+$ influx into rat erythrocytes was uninfluenced by adrenalectomy, but reduced by $1 \times 10^{-5} M$ tannic acid in vitro. (Section V.)

7. The rate of active $^{22}\text{Na}^+$ efflux from $^{22}\text{Na}^+$ -loaded rat erythrocytes was reduced by adrenalectomy, but uninfluenced by $1 \times 10^{-5}\text{M}$ tannic acid in vitro. (Section V.)

8. Erythrocytes from adrenalectomized rats were less susceptible to hypotonic haemolysis than those from unoperated rats, provided that they contained low $[\text{Na}^+]_i$. (Section V.)

9. Dietary 0.9% NaCl administration to adrenalectomized rats counteracted the changes in ATPase activity associated with adrenalectomy in skeletal muscle and kidney preparations. It also counteracted those in erythrocyte $[\text{Na}^+]_i$, $[\text{K}^+]_i$, $^{22}\text{Na}^+$ efflux rate and susceptibility to hypotonic haemolysis, which were characteristic of cells from adrenalectomized rats. (Sections III, IV and V.)

10. Intact erythrocytes from adrenalectomized rats showed normal acetylcholinesterase activity during the period in which Mg^{2+} - and $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+}$ -ATPase activities were elevated. This finding contrasts that of Vaccarezza & Willson (1965). (Section V.)

11. Mean erythrocyte $[\text{Na}^+]_i$ remained normal in human subjects suffering affective disorders, but mean $[\text{K}^+]_i$ was elevated in manic-depression. These results are not substantially consistent with the abnormal monovalent cation distributions predicted from the whole-body isotope dilution studies of other workers. (Section V.)

12. Erythrocyte $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+}$ -ATPase activity was elevated in manic-depression; Mg^{2+} -ATPase activity was elevated in the manic phase alone. The greater elevation of erythrocyte $\text{Na}^+ + \text{K}^+ - \text{Mg}^{2+}$ -ATPase activity in the manic phase was accompanied by a greater $[\text{K}^+]_i$ increment. (Section V.)

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