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STUDIES ON THE CONTROL OF
CATION PERMEABILITY IN
SKELETAL MUSCLE OF THE
LABORATORY RAT

by

A. C. PARKIN, B.Sc.

Being a thesis submitted for examination for the
degree of Doctor of Philosophy at the University
of Durham, February, 1975.

Hatfield College
Durham



TO ANN AND MY PARENTS

The material contained in this thesis was the work of the author alone during the period 1968 - 1975, the practical study being undertaken during the period 1968 - 1971 whilst the author was maintained by a Science Research Council Studentship. None of this material has been published or submitted for a degree at any university.

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GLOSSARY

ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
CaATPase	calcium-activated ATPase
DOC	sodium deoxycholate
EDTA	ethylenediaminetetra acetic acid
MgATPase	magnesium-activated ATPase (not stimulated by monovalent cations)
NaKMgATPase	magnesium-activated ATPase synergistically stimulated by sodium and potassium
n.s.	not significant
p	probability
Pi	inorganic phosphate
r	correlation coefficient
S.E.M.	standard error of the mean
tris	tris(hydroxymethyl)aminomethane

ABSTRACT

The present study was undertaken in an attempt to examine the importance of the NaKMgATPase (the 'sodium pump' enzyme) in cation movements in rat skeletal muscle. Though the 'sodium pump' was first postulated in an attempt to explain some of the cation movements recorded in muscle tissue, at the commencement of this study clear evidence of the presence of the NaKMgATPase was still awaited.

Several approaches to the problem were made, using physiological and biochemical preparations. Isolated preparations of muscle, maintained in optimum conditions, were subjected to treatments with reagents known to affect the NaKMgATPase, and the changes in cation distribution were observed. Similar preparations were studied with respect to their oxygen consumption, to determine whether treatments known to cause changes in NaKMgATPase activity would in turn lead to alterations in oxidative metabolism as had been reported in a variety of other tissues, e.g. kidney, brain. Biochemical approaches consisted largely of attempts to isolate a fraction from skeletal muscle which demonstrated the properties of the NaKMgATPase that had been isolated from a wide range of tissues, and was especially active in those tissues in which a great deal of active cation movement was known to occur.

The studies of cation distribution were largely inconclusive, as though modified cation movements were clearly seen in various conditions known to inhibit NaKMgATPase activity, it was not possible to identify firmly the position of such an enzyme or indeed that it was the sole site of action.

Measurements of oxygen uptake failed to reveal any portion of oxidative metabolism which could be ascribed to metabolic activity linked to NaKMgATPase activity.

After a variety of isolation techniques had failed to produce a fraction of skeletal muscle containing a clearly-demonstrable NaKMgATPase, a procedure involving exposure of the muscle homogenate to 2 M NaI was successful in separating a membrane fraction exhibiting NaKMgATPase activity from rat diaphragm and hind-limb muscle. Whilst isolation of a 'pure' enzyme was not made, the study showed that the properties of the fraction isolated resembled those described for NaKMgATPases isolated from other mammalian tissues.

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CHAPTER ONE

INTRODUCTION

The ability of a cell to regulate its own internal environment is of prime importance if its functions are to be unimpaired. Whilst a passive barrier may serve to exclude large molecules, or prevent their loss, control of smaller molecules and ions cannot be so easily maintained. This problem is further complicated by the changing needs of the cell, so that cation levels, for example, may be constantly varying within the cell; thus, cells have evolved a wide variety of mechanisms to regulate their cation contents. The present study was undertaken in an attempt to elucidate the role played by one such mechanism, the 'sodium pump', in controlling cation movements in mammalian skeletal muscle. The enzyme responsible for the 'sodium pump', the NaKMgATPase, has been isolated from a wide variety of tissues, though evidence of its existence in muscle has been questioned. It is clear, however, that like other tissues muscle is capable of regulating its cation content, and that its plasma membrane, situated in the sarcolemma, plays an important part in this regulation.

The importance of the cell boundary in regulating cell activity has long been recognised; as early as 1855 Nageli introduced the term 'plasma membran' to describe a surface layer of cells which was self-healing and impermeable to dyes, and Pringsheim (1854) suggested that the osmotic responses of a cell were due to a limiting membrane.

With the introduction of radioactive tracers in the thirties, much attention was concentrated upon the penetration of membranes by labelled ions. Before the introduction of these useful tools



it had generally been supposed that the differences in the ionic content of the tissues and that of the surrounding extracellular fluid (E.C.F.) could be explained by variations in permeability of the cell membrane to these ions, indeed, it was believed that the membrane was impermeable to the sodium ion. The introduction of labelled-sodium showed that this was not the case, and new explanations were sought for the phenomena.

The first suggestion that the plasma membrane might contain a mechanism capable of actively pumping sodium ions out of the cell was probably made by Dean during discussions at the Cold Spring Harbor Symposium of Quantitative Biology after papers by Steinbach on potassium movements in frog muscle (Steinbach, 1940), and Davson on the cation permeability of erythrocytes (Davson, 1940). He suggested that phenomena observed by both workers could be explained by the existence of such a mechanism. Other workers returned to this theme, Ussing (1949) suggested that potassium entry might be linked with active sodium extrusion and Hodgkin (1951) suggested that the resting potential and ionic concentration differences may be maintained by a mechanism using a part of the cells metabolism to eject sodium against the electrochemical gradient. The sodium pump was first demonstrated by Lundberg and Oscarsson in nerve fibres in 1953, and the classical experiments of Hodgkin and Keynes in 1956 showed that the squid giant axon contained a coupled-pump, which caused potassium uptake and sodium extrusion and contributed little to the resting potential. In view of the major cation movements found in skeletal muscle it would seem likely that this tissue would contain such an active pump, indeed, Dean (1940) had postulated the sodium extrusion pump to explain the movement of the ion in

skeletal muscle. The extrusion of sodium ions and uptake of potassium by muscle against their electrochemical gradients was demonstrated by many workers (e.g. Conway and Hingerty, 1948; Steinbach, 1951, 1952). That part of this mechanism was due to the coupled-pump was indicated by the inhibition of these movements by the cardiac glycosides (Johnson, 1956; Edwards and Harris, 1957). This work was mainly carried out using the classical frog sartorius muscle preparation.

The amount of sodium extrusion which was caused by the pump was for a long time a point of contention, and the role of exchange diffusion was also widely disputed; Keynes and Swan (1959) suggested that this could be responsible for 50% of the sodium efflux whilst others (e.g. Ling and Ferguson, 1970) felt that there was no evidence for such a pump. It is becoming clear that in fact several mechanisms control sodium efflux, including the sodium pump (Whittam and Wheeler, 1970; Horowicz, Taylor and Waggoner, 1970). It is also clear that activity of each of these mechanisms is largely dependent on the preparation used, and more particularly on the treatment and conditions employed. The work of Sjodin and Beauge, and of Horowicz and his co-workers, have shown that the age of the preparation, the medium in which it is incubated, and particularly the internal sodium concentration of the tissue and the ions present in the surrounding medium will all have major effects on ion movements and will lead to conflicting results (e.g. Sjodin and Beaugé, 1968; Horowicz et al, 1970). This is hardly surprising considering the evidence that muscle may maintain a constant electrochemical gradient for sodium (Conway, Kernan and Zadunaisky, 1961; Fozzard and Kipnis, 1967; Williams, Withrow and

Woodbury, 1971b) and that sodium pumping may also be dependent on the transmembrane potential (Horowicz and Gerber, 1965; Fozzard and Kipnis, 1967). Since the majority of the experiments undertaken using muscle tissue have involved techniques using widely-varying sodium and potassium concentrations, often the substitution of other cations and/or the alteration of osmotic potential by the addition of other materials, e.g. sucrose, it seems clear that the anomalous results from such experiments are probably reflections of differing actions of several mechanisms, some of which may be relatively unimportant or even absent in physiological conditions.

In this light, one approach made in the present study was to select a mammalian skeletal muscle preparation which could be maintained 'in vitro' in a state as closely resembling that 'in vivo' as possible. Once this had been achieved, the modification of the conditions of the preparation in ways which were known to affect the functioning of the NaKMgATPase should give some indication of the importance of the role of this enzyme.

Two different approaches were used to measure the effect of NaKMgATPase modifiers on the 'in vitro' preparation. In the first instance, the change in the cation content of the muscle fibres were monitored, and thus the extent to which the NaKMgATPase played a part in maintaining the low intracellular sodium, and high intracellular potassium, of muscle tissue could be estimated. Secondly, the oxygen consumption of the 'in vitro' preparation was studied, since in some tissues, notably kidney and brain, it has been possible to demonstrate that a substantial portion of oxygen consumption was intimately linked with supplying energy to the

active sodium pump (e.g. Whittam, 1964; Willis, 1968), and that inhibition of this sodium pump would in turn lead to a measurable reduction in oxygen consumption.

In addition to 'in vitro' studies of physiological preparations, attempts were made to isolate a membrane fraction from skeletal muscle which exhibited the properties of the NaKMgATPase.

Since Skou first identified the enzyme responsible for the sodium-potassium active exchange mechanism (Skou, 1957) a great deal of work has been undertaken to establish the location and the mechanism of this pump. The same worker has written a detailed account of the properties and nature of the enzyme system (Skou, 1965) and more recent advances have been reviewed by Whittam and Wheeler (1970), Oxender (1972), Dahl and Hokin (1974) among others, whilst many authors have included reviews in monographs and compilations, e.g. Cole (1968), Jarnefelt (1968), Wallach (1972).

The enzyme has been isolated from a wide range of tissues; Bonting et al (1961) were able to detect its presence in twenty-nine out of thirty-six tissues from the cat. The general properties of the enzyme are that it hydrolyses ATP to ADP and Pi in the presence of magnesium, it is stimulated by the presence of sodium and potassium together but activity is inhibited by calcium or cardiac glycosides (Skou, 1965). Maximum activity is obtained when the sodium concentration is 100 mM or higher (Skou, 1957) and whilst the potassium concentration required for maximum activity varies with the sodium concentration, it is generally below 20 mM when the sodium concentration is 100 mM. The pH optimum for the enzyme

system is 7.5 - 7.6 (Bonting et al, 1961; Dunham and Glynn, 1961).

Skou (1965) pointed out that though he had first postulated an adenosine triphosphatase, i.e. a single enzyme, the growing evidence pointed to an enzyme system rather than an individual protein. The picture was further confused by the existence of MgATPase, an enzyme which utilized ATP and required magnesium ions, but was not synergistically stimulated by the presence of sodium and potassium. This concept of an enzyme system has been borne out by later work, much of which has been directed towards an analysis of the various stages of the NaKMgATPase action. Various phosphatases were demonstrated in microsomal preparations which would hydrolyse various substrates, e.g. p-nitrophenylphosphate (Ahmed and Judah, 1964; Albers and Koval, 1966), acetyl phosphate (Bader and Sen, 1966; Sachs, Rose and Hirschowitz, 1967; Robinson, 1970) and were suggested to be part functions of the NaKMgATPase complex. This has been strengthened by the work of Towle and Copenhaver (1970) and others who have produced highly-purified NaKMgATPases containing undiminished potassium-stimulated phosphatase activity. A sodium-activated ATP-ADP exchange activity (Fahn, Koval and Albers, 1966) is also believed to represent part of the complex (Whittam and Wheeler, 1970; Banerjee and Wong, 1972; Banerjee, Wong and Sen, 1972). The use of various reagents and drugs which will alter various part functions of the complex, e.g. ouabain- and nucleotide-binding capacities, has led to an increased understanding of the complex. Notable papers discussing this area are those of Atkinson, Gatenby and Lowe (1971); Siegel and Goodwin (1972) and Erdmann and Schoner (1973b) as well as the reviews previously mentioned. ATP is believed to act as an

activator as well as a phosphate donor (Post, Hegyvary and Kume, 1972; Siegel and Goodwin, 1972). There is also evidence that the NaKMgATPase can be reversed, i.e. that 'downhill' movements of sodium and potassium can lead to incorporation of Pi into ATP by the enzyme complex (for review see Whittam and Wheeler, 1970).

Though this enzyme complex had been the subject of intensive research for the past decade, remarkably little was known of its locality and functions in skeletal muscle by 1968 when this study was commenced. Work had naturally been concentrated on the frog leg muscles, though there had been studies made on a variety of mammalian tissues. Whilst studies on 'in vivo' and 'in vitro' preparations of whole muscle had demonstrated that cardiac glycosides would cause a marked increase in internal sodium level, and a fall in potassium, a convincing NaKMgATPase fulfilling the general properties described earlier had not been demonstrated in isolated fractions using a universally-repeatable technique.

Following the failure in the present study to produce any demonstrable NaKMgATPase activity using techniques reported in the literature (e.g. Samaha and Gergely, 1965, 1966; Rogus, Price and Zierler, 1969), preparations of membranes were made from rat skeletal muscle using a variety of methods found elsewhere to be effective in isolating NaKMgATPases from other tissues.

CHAPTER TWOSTUDIES OF THE SODIUM AND POTASSIUM CONTENTS OF
RAT DIAPHRAGM MUSCLES IN 'IN VITRO' CONDITIONSIntroduction

The use of 'in vitro' preparations has greatly helped in determining the effects of a variety of different agencies upon the characteristic properties of a tissue. Indeed, 'in vitro' studies have been essential in determining those functions of a tissue that are intrinsic and those that are controlled from an extrinsic site. One problem of 'in vitro' studies is to ensure that the tissue is functioning as it would 'in vivo', so that experimental findings are not merely artefacts.

It was necessary, therefore, before undertaking studies of the effects of various conditions upon the cation balance of the diaphragm muscle to establish a preparation which could maintain the 'in vivo' intracellular cation contents. To this end, preliminary investigations were undertaken to produce such a preparation following earlier work in the literature.

When this had been achieved, studies on the effect of ouabain, an inhibitor of the NaKMgATPase, ethacrynic acid, a diuretic agent, and rose bengal, a photooxidant, were undertaken in an attempt to elucidate the role of the NaKMgATPase in maintaining the cation balance in skeletal muscle.

The maintenance of mammalian muscle 'in vitro' that shows the properties of the 'in vivo' condition has long proved difficult. Much work has been undertaken in attempting to discover the various factors which are required for this 'in vitro' maintenance, and

whilst much has still to be done, there have emerged several important findings, which have led to improved 'in vitro' preparations. Though many of these findings have been inter-related, and complicated by differences in approach, conditions and preparations selected, there nevertheless have emerged the following priorities in selection and incubation of a preparation:-

- (1) A thin, intact muscle
- (2) A high oxygen tension
- (3) A saline containing bicarbonate ions
- (4) A saline containing serum proteins.

Selection of a thin preparation is essential if the high oxygen supply essential to mammalian muscle is to reach all parts of the preparation, as calculations of diffusion rates (e.g. Hill, 1928) will show. Suitable preparations in the rat are the diaphragm muscle and the extensor digitorum longus. If the diaphragm muscle is used, however, it has been common practice to trim the muscle away from the ribcage, vertebrae and xiphisternum, which leads to damage of many of the muscle fibres.

Zaimis (1962) has described how isolated muscles 'in vitro' show gross deterioration and altered function, and Barstad and Lilleheil (1968) have shown that resting potential declines near to the ends of damaged fibres, and that this decline spreads through the entire fibre when even only one end of the fibre is damaged. The same authors also demonstrated a marked shrinkage in the diameter of cut fibres, in the region of 30% reduction of the original diameter. Aebi (1953), Creese (1954) and many others have also shown that cut diaphragm preparations are incapable of maintaining their 'in vivo' sodium and potassium concentrations.

This decline in the preparation is so rapid that often within five minutes of isolation the muscle is no longer capable of conducting an action potential (Barstad and Lilleheil, 1968). Kipnis and Cori (1957) described an intact diaphragm preparation, modified from that of Bülbiring (1946), in which none of the muscle fibres were cut, and demonstrated that this closely resembled 'in vivo' muscle in many respects, including total tissue water content, intracellular and extracellular spaces and rate of pentose uptake, and Creese (1968) described a modified preparation of rat diaphragm which was capable of maintaining resting potential and sodium and potassium contents. A similar preparation was therefore selected for this study.

Calkins, Taylor and Hastings (1954) showed that hypoxia in the rat diaphragm caused a rapid fall in tissue potassium, and in the same year Creese found that potassium loss could only be avoided if a stream of O_2/CO_2 gas mixture was directed on to the preparation (Creese, 1954). Creese, Scholes and Whalen (1958) showed that hypoxia led to depolarisation of the central fibres of their diaphragm preparation, and that for adequate oxygenation bubbles of oxygen must continuously impinge directly upon the preparation from both sides. Creese has devised a simple chamber in which resting diaphragm muscle under tension can be incubated in conditions giving adequate oxygenation (Creese and Northover, 1961). Constantine and Kostyo (1961) have claimed, however, that their diaphragm preparation maintained 'in vivo' potassium content without this special oxygenation technique, gassing the medium with 95% O_2 : 5% CO_2 mixture proving satisfactory.

That muscle tissues require bicarbonate ions in the incubation medium has also been clearly demonstrated. Rona and Neukirch (1912) had demonstrated that full maintenance of the spontaneous contractions of rabbit intestine was only seen in bicarbonate-buffered medium, and that this phenomenon could not be explained by the pH of the saline. In 1950 Creese showed that the same held true for isolated rat diaphragm, and that this action was not due to carbon dioxide used in aeration but that the bicarbonate ion itself played some role. He suggested that the influence of the ion may be exerted via the metabolism, or by altering the intracellular pH of the muscle. That the CO₂-bicarbonate system is an important metabolic regulator is well known. It has been thought, however, that muscle cells were virtually indifferent to extracellular bicarbonate conditions, although freely permeable to carbon dioxide (Conway, 1957). Adler, Roy and Relman (1965a) have shown that in fact internal pH is sensitive to external bicarbonate as well as carbon dioxide, and have gone on to demonstrate that cellular pH is a complex function of carbon dioxide partial pressure and bicarbonate concentration (Adler, Roy and Relman, 1965b) though the influence of bicarbonate need not be as the direct result of the movement of bicarbonate ions across the membrane itself but may reflect movements of hydrogen or hydroxyl ions. Burnell (1968) and Roos (1971) have supported this work. Randle (1957) pointed out that in anaerobic conditions the increase in glucose uptake by rat diaphragm is marked in bicarbonate buffered saline, but not seen in phosphate-buffers. Bicarbonate has also been shown to regulate the passage of citrate through the

mitochondrial membrane (Simpson and Angielski, 1973). Caldwell (1968) has pointed out that the bicarbonate distribution does not fit the Donnan equilibrium, suggesting an active role for the ion. Hodson (1971) has produced evidence that the sodium pump of the corneal epithelium is dependent upon the presence of bicarbonate ions. In view of the role of bicarbonate ions recorded in several tissues (see Wareham et al, 1973) it may be that their presence is fundamental in the maintenance of normal membrane permeability.

In 1954 Carey and Conway noted that frog muscle incubated in serum had a low sodium content. Creese and Northover (1961) also found this to be true for their rat diaphragm preparation and were able to show that the level of fibre sodium was reduced when a crude globulin fraction of serum was present in the incubating medium. Without this serum extract there was a steady rise in fibre sodium during the course of the incubation, and Creese (1968) has demonstrated that the primary effect of the serum proteins is to reduce permeability of the membrane to sodium ions. He suggests that the protective action of the proteins is due to their ability to chelate heavy metals, present in trace amounts in the saline. Kernan has reported a slight hyperpolarisation of the resting potential in skeletal muscle fibres of the frog (Kernan, 1960) and the rat (Kernan, 1963) when kept in plasma, and Akaike (1971) produced evidence of an increased action potential in serum-incubated rat muscles.

Since these factors appeared to be extremely important for the maintenance of 'in vitro' preparations of mammalian muscle in optimum conditions, preliminary work consisted of attempts to

produce experimental conditions which included them all. The rat diaphragm appeared the obvious choice of preparation, and the long and thorough researches of Creese and his associates (see particularly Creese, 1968) greatly facilitated this work. In order to obtain the thin preparation required, diaphragms from weanling rats were used in incubations.

Materials

The rats used in these experiments were male albino rats, of the CFHB strain, either purchased from Carworth Europe or bred from stocks of this strain. Unless otherwise stated, they were used for experimental purposes when weighing between 40 and 65 g. They were fed ad libitum on Oxoid Breeding Diet pellets, and had unrestricted access to water.

Ouabain octahydrate (strophanthin-G) was obtained from Sigma Chemical Corporation; tetrabromtetrachlor-fluorescein (rose bengal) from G.T. Gurr, London; ethacrynic acid was kindly supplied by Merck, Sharpe and Dohme Ltd.; all other reagents were of 'Analar' grade and supplied by British Drug Houses Ltd. All water was glass-distilled. Serum was obtained from fresh pig's blood from a local slaughterhouse.

For radioactivity determinations inulin (carboxylic acid-¹⁴C) was obtained from the Radiochemical Centre, Amersham; 2,5-diphenyloxazole (PPO) from Sigma Chemical Corporation; naphthalene and 1,4-dioxan (scintillation grade) from B.D.H. Ltd.

Methods

1. Preparation of media.

The saline used was based on Krebs Bicarbonate Saline (Krebs and Henseleit, 1932) containing 118 mM NaCl, 5.9 mM KCl,

1.2 mM MgSO_4 , 25 mM NaHCO_3 , 1.25 mM NaH_2PO_4 , 1.25 mM CaCl_2 and 2 g.l^{-1} of glucose. The pH of the medium was adjusted to pH 7.4 at 37°C by the addition, where necessary, of a small amount of dilute hydrochloric acid.

Serum was prepared from fresh pigs blood which was stored at room temperature (c 20°C) for 24 hours to allow clotting to occur. The remaining liquid was then centrifuged for 15 minutes to give a clear serum and a pellet of blood cells which was discarded. It was found necessary to maintain the temperature of the serum at 20°C up to this point to prevent haemolysis of the erythrocytes with consequent contamination of the serum.

The clear serum was dialysed in visking tubing against two changes of distilled water and then three changes of the bicarbonate saline at 4°C for at least 24 hours. The sodium and potassium contents of the serum were measured by means of an S.P. 90 atomic absorption spectrophotometer (Pye Unicam Ltd.) to ensure that equilibration with the saline was complete. Further saline was then added as necessary to give a final solid content of 77 mg/cm^3 (salts and serum proteins), corresponding to that of the serum before dialysis. The serum was stored at -20°C in 50 cm^3 aliquots until required.

All media were stored without the addition of the glucose, and before use the media were brought to a temperature of 37°C , pre-equilibrated for at least 15 minutes with a mixture of 95% O_2 : 5% CO_2 , and the pH determined, after the addition of any special reagents and the glucose.

2. Preparation of diaphragm

The rat was killed by a blow on the head followed by cervical dislocation. The intact diaphragm was rapidly removed following the method of Kipnis and Cori (1957) and placed in oxygenated saline in a petri dish which was water-jacketed to maintain a temperature of 37°C. Excess tissue was trimmed away, and the diaphragm bisected as shown in Fig. 2:1 (cut No. 1). The vertebra, with the attached lumbar muscles, was then removed as shown in Fig. 2:1 (cut No. 2), these radial cuts ensured that as few as possible of the muscle fibres of the final hemidiaphragm preparations were damaged.

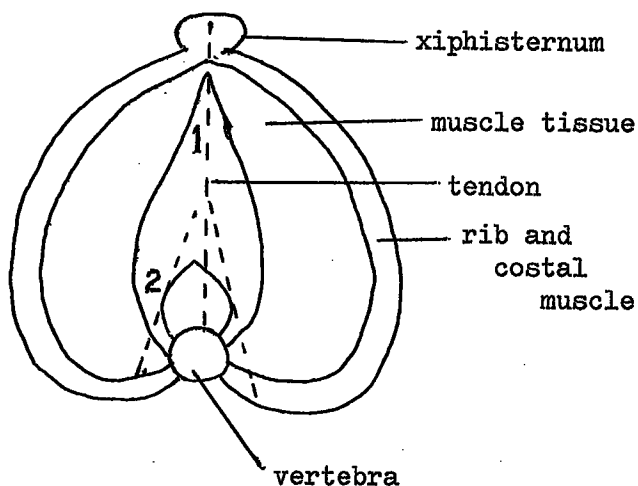


Fig. 2: 1 Diagram of intact diaphragm, ventral aspect.

The hemidiaphragm was kept in the oxygenated saline until transferred to an incubation chamber, this transfer occurring at the latest three minutes after the death of the animal.

3. Incubation of hemidiaphragm

In preliminary experiments the hemidiaphragms were incubated in simple flasks at 37°C containing 25 cm³ of bicarbonate saline, which might then be shaken. When these methods were found unsatisfactory, the incubation chambers shown in Fig. 2:2 were adopted.

These two chambers, of which only one is illustrated, were a modification of the type of chamber used by Creese and Northover (1961), each containing 25 cm³ of the appropriate incubation medium. The preparation was held by a hook, attached to the aeration tube, through the central tendon material below, and by a double hook through the costal muscle (but not the diaphragm fibres) above. To maintain tension in the diaphragm threads from the double hook led to a clip weighing approximately 8 g. A continuous stream of fine bubbles of a 95% O₂ : 5% CO₂ gas mixture passed over both sides of the suspended hemidiaphragm from the aeration tube, which consisted of glass tubing tipped by fine polythene tubing containing tightly-packed fibres of glass wool to act as a diffuser. Excessive frothing of the medium was reduced by means of a ring of silicone (Antifoam A, Hopkins and Williams Ltd.) applied to the inner surface of the chamber approximately 2 cm above the surface of the medium.

The two chambers were clipped into a box, light-tight apart from one aperture 3 cm in diameter, 6 cm from the chambers, through which light from a 100 watt bulb could fall on to the hemidiaphragms. This light gave an illumination of 5,000 lux at the muscle surface, measured by means of an EEL Lightmaster photometer, and was only used in experiments with rose bengal.

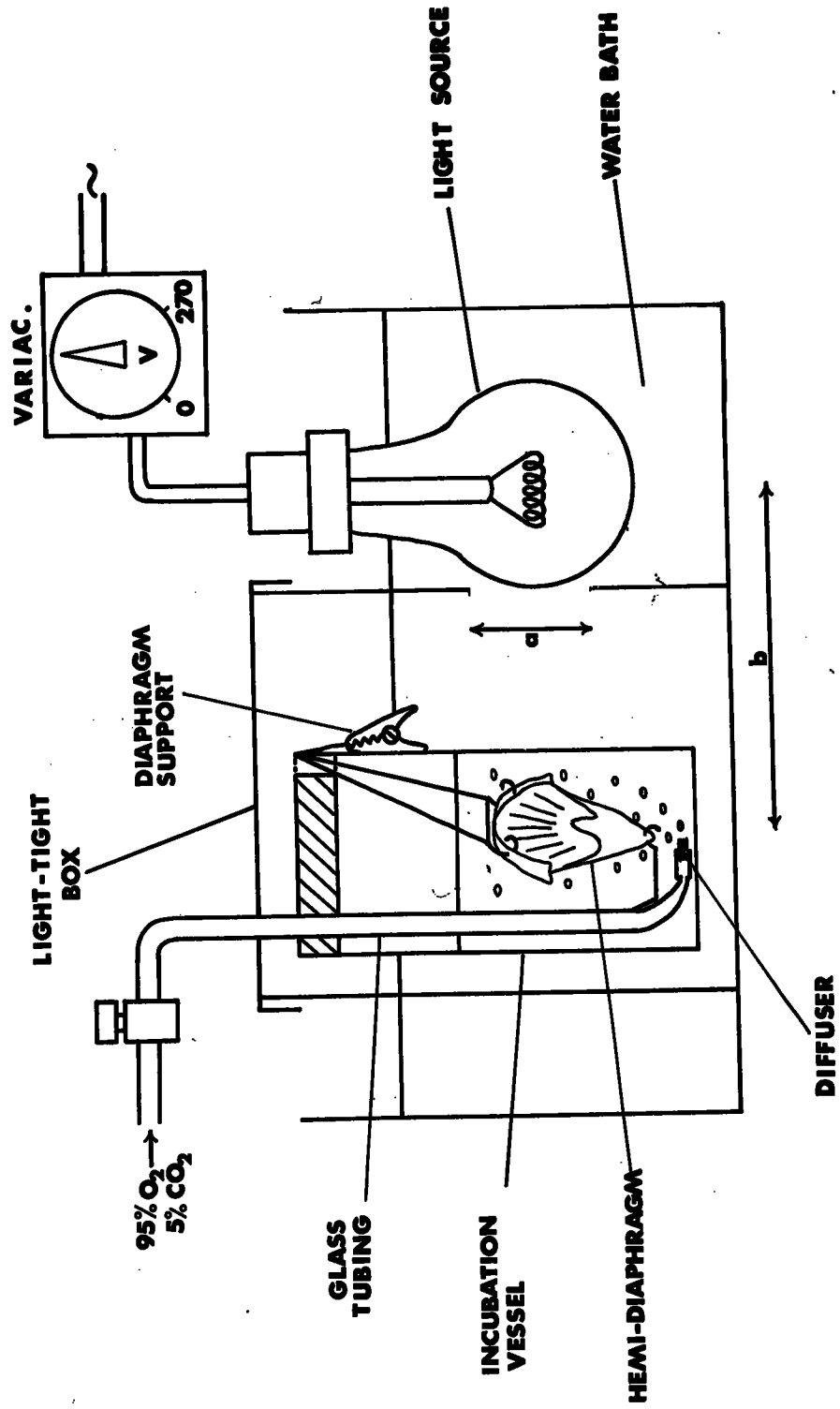
All incubations took place at 37°C (\pm 0.1°C) in a Shaking Reaction Incubator (Gallenkamp Ltd.) at a shaking rate of one complete stroke per second; the construction of the box was such that the water flowed freely past the incubation chambers whose internal temperature was maintained at 37°C \pm 0.2°C.

Fig. 2: E Incubation of intact hemidiaphragms

For greater clarity only one of the incubation chambers is shown. The water bath was maintained at $37^{\circ} \pm 0.2^{\circ}\text{C}$. The light source was only used in experiments using rose bengal, and the aperture (distance a) was sealed when light was to be excluded.

Distance a = 3 cms

Distance b = 6 cms



4. Analysis of muscle contents

The hemidiaphragm was withdrawn from the medium and tendon and rib quickly cut away. The muscle was rapidly blotted on both surfaces on Whatman No. 40 filter paper, divided into two, and each part placed in a dried, weighed boiling tube which was then reweighed to establish the wet weight of the muscle to an accuracy of 0.1 mg. The muscle was then dried to a constant weight at 70°C in a vacuum oven to determine the water content. The total sodium and potassium contents of the tissue were determined by a modification of the leaching technique of Kostyo and Schmidt (1963). Portions of diaphragm muscle were rapidly blotted on both sides on Whatman No. 40 filter paper and then placed in 10 cm³ aliquots of deionised water, obtained by passing glass-distilled water through an Elgastat Deionizer. Leaching of the cations from the muscle into the deionised water had reached equilibrium within twenty-four hours, the concentrations of cations being determined by means of an S.P. 90 Spectrophotometer (Pye-Unicam Ltd.). In many experiments the sodium and potassium contents were also determined by ashing a portion of muscle in a muffle-furnace at 350°C for twenty-four hours; the ash was then dissolved in deionised water and cation contents determined by spectrophotometry. The results obtained by the two techniques were indistinguishable. Control measurements were made to ensure that leaching of sodium and potassium from the walls of the glass tubes was insignificant (1% of the values measured in all cases).

Determinations of the sodium, potassium and water contents of the diaphragm muscle 'in vivo' were also made. Diaphragms of

freshly-killed rats were rinsed for thirty seconds in bicarbonate saline and then treated in a similar manner to the incubated preparations.

5. Estimation of extracellular space

The extracellular space of the muscles was estimated using inulin- ^{14}C obtained as a freeze-dried solid. The inulin was dissolved in the various incubation media to give an activity of approximately $1 \mu\text{Ci}/\text{cm}^3$, and diaphragm muscles were incubated therein in the usual manner. Preliminary experiments showed that inulin- ^{14}C entry into the muscle appeared to have reached an optimum within one hour, with further uptake apparently paralleling the gradual uptake of saline as the tissue deteriorated.

After incubation was completed the hemidiaphragm was rinsed for ten seconds in bicarbonate saline, blotted rapidly on both sides with Whatmans No. 40 filter paper and then dissected. Two pieces were removed from the central portion of each hemidiaphragm, each piece weighing approximately 10 mg, to avoid 'edge effects' (Harris and Burn, 1949), and a 0.2 cm^3 sample of the incubation medium was also taken. The muscle was placed in a pointed-bottom centrifuge tube, its wet weight determined, and then 0.2 cm^3 5N potassium hydroxide was added. After 24 hours the muscle had completely dissolved, and was ready for radioactivity measurements.

Counting was performed on a Beckman LS-200B Liquid Scintillation system, using a ^{14}C iso-set. The 0.2 cm^3 samples of incubation medium or dissolved muscle were placed in a scintillation tube containing 10 cm^3 of Beckman 'Cocktail D', composed of 5 g 2,5 diphenyloxazole, 100 g naphthalene in 1,4-dioxan giving a final volume of 1 litre. As the counting was performed at ambient temperature no antifreezing agent was required. Quenching was

measured, and found to be significant so quenching calibration curves were constructed using the combined external standard - channels ratio method. Counting efficiency was between 71% and 78% for muscle samples, and 75.5% and 76.5% for samples of medium; background counts were taken overnight and subtracted from experimental counts.

The extracellular space was calculated, based on the assumption that it was the only portion of muscle penetrated by the inulin. The concentrations of sodium and potassium in the extracellular space were assumed to be the same as those in the incubation medium, thus permitting calculation of the sodium and potassium contents of the muscle fibres.

Results

The estimations of the diaphragm cation contents 'in vivo' gave mean values of $49.6 \text{ m.moles.kg}^{-1}$ wet wt for sodium, and $86.3 \text{ m.moles.kg}^{-1}$ wet wt for potassium. Before an 'in vitro' preparation could be considered adequate, therefore, it must be capable of maintaining cation concentrations which were not significantly different from these values.

The results in Table 2:1 show that a simple preparation, in which the intact diaphragm was kept at 37°C in a well-oxygenated bicarbonate saline was by no means satisfactory. When the medium was stirred solely by the columns of $95\% \text{ O}_2 : 5\% \text{ CO}_2$ gas mixture bubbles there was a rapid increase in sodium, and fall in potassium content, to mean values of $70.7 \text{ m.moles.kg}^{-1}$ wet wt and $68.2 \text{ m.moles.kg}^{-1}$ wet wt respectively in one hour of incubation. When the stirring of the medium was increased by

placing the incubation chamber in a metabolic shaker the sodium uptake was reduced to a great degree, as was potassium loss, but the final cation contents of $61.5 \text{ m.moles kg}^{-1}$ wet wt for sodium and 79.7 for potassium were still significantly different from those of the 'in vivo' muscles. Further efforts were made to improve the incubation technique, culminating in the development of the apparatus shown in fig. 2:2.

As can be seen from Table 2:2, this incubation procedure was much more effective in maintaining the cation contents of the diaphragm. When the bisected diaphragm was incubated in bicarbonate saline for one hour at 37°C , the sodium content rose to a mean value of $57.4 \text{ m.moles kg}^{-1}$ wet wt, whilst potassium fell very slightly to a mean value of $82.9 \text{ m.moles kg}^{-1}$ wet wt. There was, however, a rise in water content from a mean value 'in vivo' of 727 g kg^{-1} wet wt to 750 g kg^{-1} wet wt. Since this increase might be either extracellular in nature, or an increase in fibre water, or both, and since an extracellular increase would naturally lead to higher apparent sodium content and lower potassium content of the muscles, as these were the conditions in the saline, it was obvious that studies of the volumes of the extracellular space, and the fibre water content, would have to accompany cation determinations.

Incubation in serum (Table 2:2 (c)) was much more effective in producing 'in vivo' concentrations in the bisected diaphragms, with water, sodium and potassium contents after one hour of incubation which were not significantly different from those measured in the freshly-dissected muscles. This indicated the suitability of the apparatus, which was therefore used throughout these investigations.

TABLE 2:1

Effects of inadequate oxygenation upon total sodium and potassium contents of incubated rat diaphragms

Muscle treatment	Total Na ⁺ content m.moles kg ⁻¹ wet wt	Total K ⁺ content m.moles kg ⁻¹ wet wt
A. ' <u>In vivo</u> '	49.6 ± 1.2 (12)	86.3 ± 1.3 (12)
B. Unshaken	70.7 ± 3.0 (8)	68.2 ± 1.8 (8)
C. Shaken	61.5 ± 1.8 (8)	79.7 ± 1.2 (8)
p A - B	.001	.001
A - C	.001	.01

Values expressed as means ± S.E.M. (no. of expts.)

Muscles 'A' taken from freshly-killed animals and rinsed.

Muscles 'B' and 'C' incubated for 1 hour at 37°C in Krebs

bicarbonate saline, pH 7.4, oxygenated by continuous

bubbling with 95% O₂ : 5% CO₂ (not impinging directly

upon muscles)

TABLE 2:2

Total sodium, potassium and water contents of hemidiaphragms
'in vivo', and when incubated in saline, serum, or saline
containing 10^{-3} M ouabain

Muscle treatment	Total H ₂ O content (g kg ⁻¹ wet wt)	Total Na ⁺ content (m.mole kg ⁻¹ wet wt)	Total K ⁺ content (m.mole kg ⁻¹ wet wt)
A. ' <u>In vivo</u> '	727 ± 2.6 (6)	49.6 ± 1.2 (12)	86.3 ± 1.3 (12)
B. Saline	750 ± 4.1 (8)	57.4 ± 1.2 (12)	82.9 ± 1.2 (12)
C. Serum	730 ± 4.2 (8)	47.9 ± 1.5 (11)	86.0 ± 1.2 (9)
D. Ouabain	763 ± 3.3 (7)	72.8 ± 1.6 (12)	67.3 ± 1.0 (12)
A - B	.01	.001	n.s.
A - C	n.s.	n.s.	n.s.
B - C	.01	.001	n.s.
B - D	.05	.001	.001

Values expressed as means ± S.E.M. (no. of expts.)

A - muscles taken from freshly-killed animals and rinsed.

Muscles B, C, D all incubated for 1 hour at 37°C (pH 7.4)

as shown in fig. 2:2, directly oxygenated by 95% O₂ : 5% CO₂
gas mixture in

B - Krebs bicarbonate saline

C - Krebs bicarbonate saline containing dialysed pig serum

D - Krebs bicarbonate saline containing 10^{-3} M ouabain

Estimation of the volume of the extracellular space, that is, that portion of the muscle not inside the fibres, was made using inulin-¹⁴C. Assuming that the ionic concentrations in the extracellular space were those of the bathing medium, this technique permitted calculation of the water and cation concentrations within the fibres; the results of such determinations are shown in Table 2:3 for saline- and serum-incubated hemidiaphragms. The inulin space can be seen to be significantly smaller in those muscles incubated in serum, and calculations indicate that there would probably be a significantly larger fibre water content when expressed as a proportion of the wet weight, a surprising finding. The presence of the serum proteins in the extracellular space are a complicating factor, not only may they influence the penetration of the inulin but they also contribute significantly to the increased total solid content recorded in the serum-incubated muscles, and hence directly alter the dry to wet weight ratio. When the extracellular values are allowed for, and the ratio of fibre solids to fibre water is determined, it is clear that there is no significant difference between saline- and serum-incubated muscles in this respect. Hence it seems improbable that there is any significant increase in uptake of water by fibres incubated in serum in comparison with those in saline.

The results obtained did appear to indicate a significantly lower uptake of sodium by fibres incubated in serum, and though variation from one diaphragm to another was relatively large, this reduced uptake was probably significant when expressed per

gram fibre water. There appeared to be no significant differences as far as potassium was concerned, there being apparently no significant loss of potassium in either condition.

These results clearly confirmed that to a great extent the problems of maintaining an 'in vitro' preparation had indeed been overcome, and that it closely resembled the preparation of Creese (e.g. 1968). The effect of various reagents upon the preparation were then tested.

As has been shown by many workers, the NaKMgATPase inhibitor ouabain has marked effects upon sodium, potassium and water contents of incubated tissues, and when 10^{-3} M ouabain was present gross changes in cation content were observable in the incubated diaphragm (Table 2:3). There was a marked increase in the water content, primarily due to uptake of water by the fibres, and a rise in sodium and fall in potassium contents. In a parallel series of experiments to those reported in Table 2:3 the effects of 10^{-3} M ouabain in serum-incubated muscles were determined, and values almost identical to those in Table 2:3 were observed; thus any possible improvement in sodium balance in serum appeared to have been abolished by the 10^{-3} M ouabain. The marked rise in fibre water content might be expected to lead to an increased fibre sodium content and a reduced fibre potassium content. It can be seen that there is a marked increase in fibre sodium even when expressed per gram fibre water, and similarly a marked fall in fibre potassium when similarly expressed, indicating that more than the simple entry of extracellular medium into the fibre, or some new or enlarged space, is involved, since this would lead to a rise in fibre sodium of the order of

TABLE 2: 3 Fibre water, sodium and potassium contents of hemidiaphragms incubated in saline, serum or saline plus 10^{-3} ouabain

Incubation conditions	A	B	C	P	
	Saline	Serum	Ouabain	A - B	A - C
Inulin space ($\text{cm}^3 \text{kg}^{-1}$ wet wt)	208 \pm 8.7(11)	172 \pm 6.4 (8)	186 \pm 6.0 (11)	.01	n.s.
Fibre water (g kg^{-1} wet wt)	543 \pm 9.1	570 \pm 7.4	578 \pm 6.4	.05	.01
Fibre solids (g kg^{-1} wet wt)	249 \pm 3.5	258 \pm 3.5	236 \pm 3.5	n.s.	.05
(g kg^{-1} fibre H ₂ O)	458 \pm 10.0	452 \pm 8.8	408 \pm 7.6	n.s.	.001
Fibre Na ⁺ (m.mole kg^{-1} wet wt)	27.2 \pm 1.7	23.0 \pm 1.6	45.8 \pm 1.8	n.s.	.001
(m.mole kg^{-1} fibre H ₂ O)	50.1 - 3.3	40.4 - 2.9	79.2 - 3.2	.05	.001
Fibre K ⁺ (m.mole kg^{-1} wet wt)	80.1 \pm 1.2	85.0 \pm 1.2	66.2 \pm 1.0	n.s.	.001
(m.mole kg^{-1} fibre H ₂ O)	148.4 \pm 3.3	149.1 \pm 2.8	114.5 \pm 2.2	n.s.	.001

Values expressed as means, \pm S.E.M. (no. of expts.)

All muscles incubated for 1 hour at 37°C, as shown in fig.

at pH 7.4 directly oxygenated by 95% O₂ : 5% CO₂ gas mixture, in

A - Krebs bicarbonate saline

B - Krebs bicarbonate saline containing dialysed pigs serum

C - Krebs bicarbonate saline containing 10^{-3} M ouabain

5 m.moles kg^{-1} wet wt. The rise in sodium content of the fibre over the incubatory period was 18.6 m.moles kg^{-1} wet wt.

Before the effects of the other reagents were monitored, there was a six months hiatus in these 'in vitro' experiments whilst work concentrated on the experiments described in subsequent chapters. Hence, when the effects of ethacrynic acid and rose bengal were to be determined, a new series of control experiments were also performed. As Table 2:4 shows, later analysis of the control results indicated that this new series had a significantly lower sodium content than the earlier series, total sodium being 51.3 m.moles kg^{-1} wet weight as against 57.4 m.moles kg^{-1} wet wt. ($p < 0.1$). There was, however, no significant differences in the potassium or water contents of these muscles. New determinations on non-incubated muscles to determine 'in vivo' levels showed sodium levels of 45.9 ± 1.5 (8) m.moles kg^{-1} wet weight, a marked reduction from 49.6 m.moles kg^{-1} previously recorded (Table 2:1), whilst potassium (84.8 ± 1.6 (8) m.moles kg^{-1}) and water (724 ± 3.3 g kg^{-1}) contents were not significantly different from those previously found. These new values were therefore adopted for the following experiments.

As might be expected, ethacrynic acid (10^{-4} M) caused a marked uptake of water by the incubated diaphragm, and a significant reduction in extracellular space indicated that this reflected a large increase in fibre water content, of the order of 20%. As was found with ouabain, this increase in water content was also accompanied by significant gains of sodium and loss of potassium which occurred steadily over the period of incubation (fig. 2:3). That these changes were also due to changes in the sodium and potassium contents of the fibres themselves is clearly

shown in Table 2:4, ethacrynic acid-exposed muscles having approximately 64% higher sodium, and 28% lower potassium in their fibres when expressed as m.moles kg^{-1} fibre water. Ethacrynic acid, therefore, also interfered with the fibres' ability to regulate cation balance, as well as tissue water. Calculations will show, however, that the increased entry of extracellular medium into an unmonitored space could lead to an increase in apparent fibre sodium content of up to 15 m.moles kg^{-1} wet weight. The increase in sodium content measured here was 19.3 m.moles kg^{-1} wet wt.

Rose bengal, the photooxidant, was the next reagent tested and because of its light-sensitive effects recorded elsewhere the incubations took place in the light-regulated box described earlier. Determinations were made in the presence or absence of light, and preliminary investigations showed that the light intensity used, 5,000 lux at the muscle surface, was not in itself detrimental to the cation or water regulation (Table 2:5B). If the incubations took place in the dark rose bengal, at a concentration of 10^{-4} M, also did not appear to affect the total sodium, potassium or water contents of the muscle (Table 2:5C). It was noticed that the preparation took up the reagent, and when the muscle was dissected free from the rib under a light source it gave vigorous twitches. Often these twitches would occur "spontaneously", that is, before the dissection had actually commenced but when the preparation had been illuminated. This appeared to suggest that this was a photooxidation effect upon the preparation. When incubation took place in the presence of both light and rose bengal (Table 2:5D,E) there was clearly a change in cation regulation, for one hours incubation with 10^{-4} M rose bengal cause a 54% increase in total

TABLE 2 : 4 Effect of (i) 10^{-4} M ethacrynic acid, (ii) 10^{-4} M rose bengal and 5,000 lux illumination, on water, sodium and potassium contents of incubated rat diaphragms

Incubation conditions	A	B	C	p	
10^{-4} M ethacrynic acid	-	+	-	A-B	B-C
10^{-4} M rose bengal + 5,000 lux	-	-	+		
Inulin space ($\text{cm}^3 \text{ kg}^{-1}$ wet wt)	$208 \pm 8.7(11)$	$138 \pm 1.6(8)$	$173 \pm 6.0(8)$.001	.01
Total H_2O (g kg^{-1} wet wt)	$756 \pm 3.7(8)$	$778 \pm 5.5(8)$	$756 \pm 1.8(8)$.01	n.s.
Fibre H_2O (g kg^{-1} wet wt)	549 ± 8.9	641 ± 7.0	583 ± 8.3	.001	.05
Fibre solid (g kg^{-1} wet wt)	243 ± 3.8	221 ± 6.3	243 ± 4.5	.01	n.s.
(g kg^{-1} fibre H_2O)	443 ± 10.0	345 ± 10.5	416 ± 9.7	.001	n.s.
Total Na^+ (m.mole kg^{-1} wet wt)	$51.3 \pm 1.3(8)$	$60.4 \pm 3.1(8)$	$79.0 \pm 2.5(8)$.05	.001
Fibre Na^+ (m.mole kg^{-1} wet wt)	21.1 ± 1.8	40.4 ± 3.2	54.0 ± 2.7	.001	.001
(m.mole kg^{-1} fibre H_2O)	38.4 ± 3.4	63.0 ± 4.9	92.6 ± 4.9	.01	.001
Total K^+ (m.mole kg^{-1} wet wt)	$81.4 \pm 1.4(8)$	$68.4 \pm 1.6(8)$	$61.2 \pm 1.3(8)$.001	.001
Fibre K^+ (m.mole kg^{-1} wet wt)	80.2 ± 1.4	67.6 ± 1.6	60.2 ± 1.2	.001	.001
(m.mole kg^{-1} fibre H_2O)	146.1 ± 3.5	106.0 ± 2.7	103.3 ± 2.7	.001	.001

Values expressed as means \pm S.E.M. (no. of expts.). All muscles incubated for 1 hour at 37°C , as shown in fig. 2:2, at pH 7.4, directly oxygenated by 95% O_2 : 5% CO_2 gas mixture, in

A - Krebs bicarbonate saline

B - Krebs bicarbonate saline containing 10^{-4} M ethacrynic acid

C - Krebs bicarbonate saline plus 10^{-4} M rose bengal, illumination at the muscle surface being 5,000 lux.

New 'in vivo' values for hemidiaphragms from freshly killed rats:-

Total water, $724 \pm 3.3 (8)$ g kg^{-1} wet wt; Total Na^+ , $45.9 \pm 1.5 (8)$ m.moles kg^{-1} wet wt; Total K^+ , $84.8 \pm 1.6 (8)$ m.moles kg^{-1} wet wt.

Fig. 2 : 3 Effect of 10^{-4} M ethacrynic acid
on sodium uptake and potassium loss
of incubated rat diaphragm

Values are means of six to eight determinations.

All incubations at 37°C and aerated by 95% O_2 : 5% CO_2
(pH 7.4). Graph shows sodium uptake in the absence ($\square\text{---}\square$)
and the presence (O---O) of 10^{-4} M ethacrynic acid, and
potassium loss in the absence (X---X) and the presence
($\nabla\text{---}\nabla$) of 10^{-4} M ethacrynic acid.

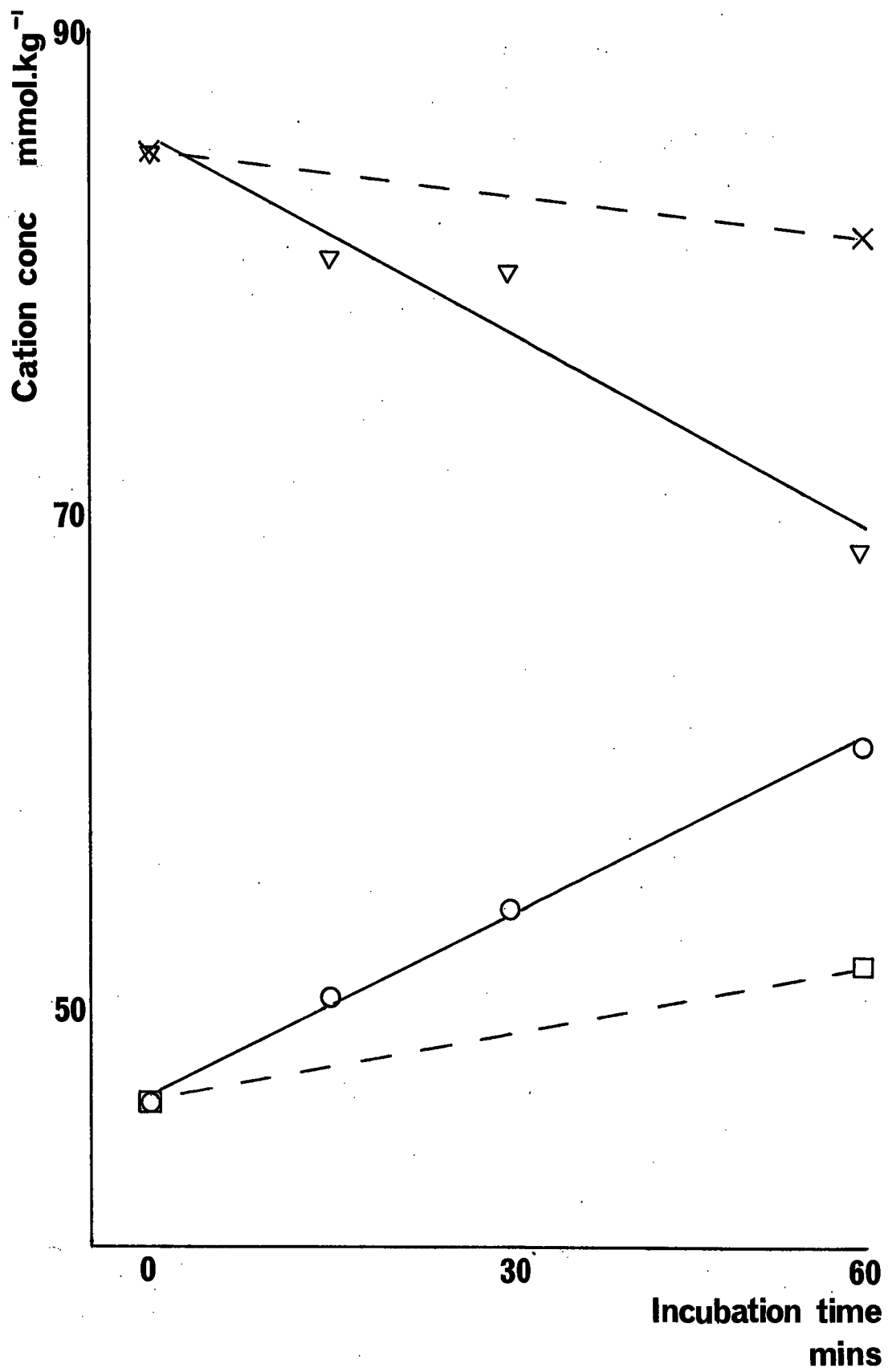


TABLE 2 : 5 Effect of rose bengal and/or illumination on total sodium, potassium and water contents of incubated rat diaphragms

	Incubation conditions			Total Na ⁺	Total K ⁺	Total H ₂ O
	Light 5000 lux	Rose bengal 10 ⁻⁵ M 10 ⁻⁴ M		content (m.moles kg ⁻¹ wet wt)	content (m.moles kg ⁻¹ wet wt)	content ^f (g kg ⁻¹ wet wt)
A	-	-	-	51.3 ± 1.3(8)	81.4 ± 1.4(8)	756 ± 3.7 (8)
B	+	-	-	52.3 ± 1.5(6)	82.9 ± 1.8(6)	750 ± 2.8 (6)
C	-	-	+	53.8 ± 2.7(4)	82.5 ± 1.0(4)	755 ± 2.0 (4)
D	+	+	-	58.7 ± 1.3(7)	77.9 ± 2.3(7)	748 ± 3.0 (7)
E	+	-	+	79.0 ± 2.5(8)	61.2 ± 1.3(8)	756 ± 1.8 (8)
p	A - B, A - C			n.s.	n.s.	n.s.
	A - D, B - D			.01	n.s.	n.s.
	A - E, B - E			.001	.001	n.s.

Values expressed as means ± S.E.M. (no. of expts.).

All incubations for 1 hour at 37°C in Krebs bicarbonate saline (pH 7.4), directly oxygenated by a 95% O₂ : 5% CO₂ gas mixture.

Values A and E also in Table 2:5.

sodium content, and a 25% fall in potassium content, whilst there was no significant change in total water content.

Table 2:4 shows that a significant decrease in inulin space indicated a rise of 6% in fibre water content, but an apparent fall of 6% in the fibre solid to water ratio is not significant by the t-test, owing to the large standard errors obtained by calculation. It seems probable, however, that there was some water uptake by muscle fibres incubated in rose bengal. There were slightly significant gains in fibre sodium, and falls in fibre potassium, when the muscles were incubated in these conditions (Table 2:4). Fig. 2:4 shows that the time course of these changes was different from those seen with ethacrynic acid, with initially high rates of cation movement which declined over the hour of incubation and sodium uptake being more markedly affected than potassium loss. Again, calculation shows that entry of medium into an enlarged intracellular space unmonitored by inulin would only have led to a maximum increase of 5 m.moles kg^{-1} in fibre sodium, compared to the some 33 m.moles kg^{-1} increase observed.

It was clear that, in the presence of light, rose bengal also seriously affected the diaphragm's ability to regulate its cation content, and probably its water content.

Discussion

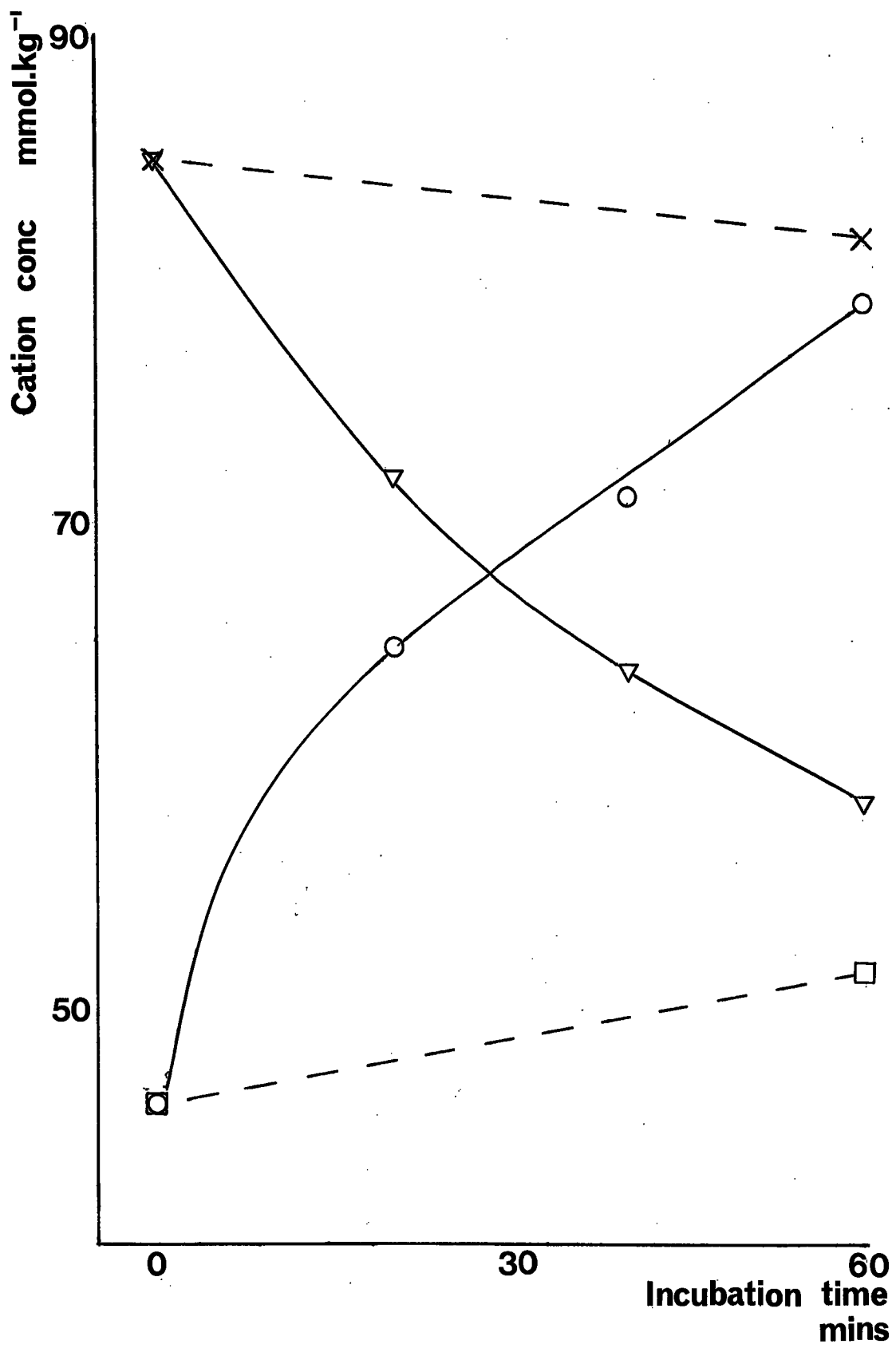
As is common in work on 'in vitro' preparations there are large discrepancies in the literature as to those conditions which are essential for the maintenance of 'in vivo' conditions. Consequently, before a study was undertaken, it proved necessary to undertake preliminary investigations into the conditions under

Fig. 2 : 4. Effect of 10^{-4} M rose bengal on sodium uptake and potassium loss of incubated rat diaphragm muscle

Values are means of between four and eight determinations.

All incubations at 37°C and aerated by 95% O_2 : 5% CO_2 gas mixture (pH 7.4).

Graph shows sodium uptake in the absence ($\square-\square$) and the presence ($\circ-\circ$) of 10^{-4} M rose bengal, and potassium loss in the absence ($\times-\times$) and the presence ($\nabla-\nabla$) of 10^{-4} M rose bengal.



which the muscle should be maintained in order to sustain its sodium and potassium contents.

In the light of Kostyo and Schmidt's (1963) findings that the 'intact' diaphragm (the Bübring preparation) could preserve 'in vivo' sodium and potassium concentrations, experiments using a similar preparation and technique were first attempted, but proved fruitless, probably due to inadequate oxygenation (see below). The preparation itself, though, being composed of a narrow sheet of fibres which are mostly undamaged in dissection, appeared suitable for the study.

Various accounts in the literature pointed towards Krebs's bicarbonate saline as a suitable medium for incubation. The importance of the presence of the bicarbonate ion, if tissue potassium was to be sustained, was demonstrated by Creese (1950) on a stimulated rat diaphragm preparation. He showed that in the absence of bicarbonate the muscles showed a continuous decline in tension, which could be reversed by re-substitution of a bicarbonate-buffered medium. The effects of the bicarbonate ion have been discussed previously (Introduction).

The values determined for the 'in vivo' sodium, potassium and water contents of the rat diaphragm varied somewhat from those of Creese and his associates, though a survey of the literature reveals that quite a range of values have been recorded previously, e.g.

	Total sodium m.moles kg ⁻¹ wet wt	Total potassium m.moles kg ⁻¹ wet wt	Total water g kg ⁻¹ wet wt
Creese (1954)	42.0	86.2	770
Krnjevic & Miledi (1958)	47.7	94.3	759
Creese & Northover (1961)	32.9	97.1	758
Kleinzeller & Knotkova (1964)	28.6	102.2	756
Creese (1968)	42.2	93.6	777
Manninen (1970)	31.6	98.9	765

(where necessary the values have been calculated from the data supplied)

The 'in vivo' sodium values found here are higher, and the potassium and water values lower, than has generally been found. The reason for this disparity in cation content is unclear but the consistency of the results, and the use of two different analytical techniques giving identical values, would appear to imply their reliability. Furthermore, any fault in technique would be most likely to cause both sodium and potassium values to show an apparent shift in the same direction, i.e. both higher or both lower than the true value, since the same sample was used to determine the concentrations of both ions. Determinations on larger animals than used in the present study, weighing between 130 g and 150 g, but of the same stock, and kept in identical conditions, showed mean values of 40 m.moles kg⁻¹ wet wt for sodium content, and 92 m.moles kg⁻¹ wet wt for potassium content. It may be, therefore, that the values recorded in the present study differed because of the immaturity of the animals used. Several authors have recorded high sodium and low potassium concentrations in muscles of immature animals (Dickerson and Widdowson, 1960;

Vernadakis and Woodbury, 1964) which, along with other evidence, has led to the concept of 'chemical maturity' and Creese (1968) reported higher sodium contents than found previously in heavier rats of the same stock (Creese and Northover, 1961).

Determinations of 'in vivo' sodium and potassium values made some time later in the course of these experiments (see Table 2:4) may also indicate the presence of periodic variations in these values, possibly due to seasonal changes, or to unknown factors in the rearing of the animals.

When preliminary experiments were performed to test the ability of the muscle to sustain these 'in vivo' values, it was immediately apparent that adequate oxygenation of the tissue was of the utmost importance. Calkins, Taylor and Hastings (1954) have demonstrated that inadequate oxygenation leads to a fall in tissue potassium levels, and in the muscles tested here even incubation under saline that is being constantly oxygenated by a 95% O₂ : 5% CO₂ gas mixture and shaken in a metabolic reaction bath, was not sufficient (Table 2:1). As had been found by Creese (1954), direct oxygenation of the tissue was essential if values approaching those of fresh muscles were to be achieved. Furthermore, diaphragm muscles from larger rats failed to preserve sodium and potassium levels even under these conditions, probably because of anoxia in the central regions removed from the direct bubbling (Creese, Scholes and Whalen, 1958).

The effectiveness of serum in helping maintain 'in vivo' sodium, potassium and water contents, as demonstrated by Creese and Northover (1961), was also found here (Table 2:2). Insulin will reduce the sodium content of muscle (Flückiger and Verzár,

1954; Creese and Northover, 1961) and raise the potassium content (Leupin and Verzár, 1950; Zierler, 1957) and the resting potential (Zierler, 1957). It is possible that insulin present in the serum exerted this effect to some degree here, but this is unlikely in view of the demonstration that serum in which the insulin has been inactivated by cysteine will still exert these effects (Creese, 1968). Akaike (1971) observed that the action of serum on cation exchange was temperature-dependent; at lower temperatures the serum was less effective in maintaining low internal sodium concentrations. He suggested that the effect of the serum may be mediated via the sodium pump or through some metabolic process.

Determinations of intracellular levels of ions required an accurate measurement of the volume of the extracellular space, and the assumption that in this space ionic concentrations closely resemble those in the external medium. The volume of the extracellular space of tissues has traditionally been studied by incubation in the presence of chemicals whose molecular structure is such that they are able to penetrate freely the extracellular volume of the tissue but are unable to penetrate the cells. The most commonly used chemicals have been polysaccharides of high molecular weight, e.g. inulin. Varying values have been reported in the literature for the inulin space of rat diaphragm muscle, probably due to variations in diaphragm size and incubation techniques, but the values reported here (Table 2:3) of $208 \text{ cm}^3 \text{ kg}^{-1}$ wet weight in saline and $172 \text{ cm}^3 \text{ kg}^{-1}$ wet weight in serum agreed closely with those of Creese and Northover (1961) ($200 \text{ cm}^3 \text{ kg}^{-1}$ wet wt and $163 \text{ cm}^3 \text{ kg}^{-1}$ wet wt respectively), who

used a similar preparation and incubation procedure. A further complication is the variation in space revealed by use of different molecules, since those giving high values for this space may well be penetrating the fibre to some degree, whilst those giving low values might not be occupying the entire space. Kipnis and Cori (1957) observed that raffinose, thiosulphate and inulin measurements produced different volumes for the extracellular space, and these authors also noted that three factors markedly influenced the estimated space, (a) the degree of trauma to which the diaphragm was exposed during its preparation; (b) the availability of glucose; (c) the temperature to which the diaphragm was exposed. It has been suggested that inulin may be hindered by steric barriers present in the extracellular space (Ogston and Phelps, 1960; Goodford and Leach, 1966) and Creese (1968) demonstrated that the use of mannitol or labelled-sodium gave larger estimates than inulin. Therefore, whilst it is difficult to state with certainty the absolute extracellular volume, it seems probable that the values measured here are accurate in relation to one another, but possibly an underestimate of that portion of the fibre occupied by cations at concentrations resembling those in the surrounding medium. This would in turn mean that the values of intracellular sodium would be overestimates, whilst those of intracellular potassium would be underestimates.

The ameliorative influence of serum proteins was not as clearly demonstrated when the intracellular values of sodium concentration were calculated. The results would appear

to suggest that serum-incubated hemidiaphragms did accumulate less intracellular sodium, though the statistical significance of this is questionable. It seems likely that the indirect methods of computation used, with the resulting accumulation of standard errors, obscured this difference and that a more direct method using labelled-isotopes of sodium and potassium would have been more successful in clearly demonstrating the reduced uptake seen by Creese (1968).

The effect of ouabain upon the preparation agrees with the work of many authors suggesting the presence of an ouabain-sensitive active sodium extrusion by skeletal muscle. Johnson (1956) and Edwards and Harris (1957) showed such a pump in frog muscle, and Kleinzeller and Knotkova (1964) and Akaike and Kowa (1970) showed that sodium extrusion in sodium-enriched muscle was limited by ouabain. The sensitivity of this mechanism to the action of glycosides varies from species to species and the relative insensitivity of the rat is probably due to some differences in the structure of the NaKMgATPase (Allen and Schwartz, 1969). For this reason the high concentration of 1 mM ouabain was used here. Kostyo and Schmidt (1963) found that their rat diaphragm preparation showed a more marked potassium loss in 10^{-3} M ouabain than in 10^{-5} M ouabain, though Akaike and Kowa (1970) claimed that the maximal effect of ouabain upon sodium and potassium balance occurred at 10^{-5} M ouabain in their preparation. However it is clear that the ability of the rat diaphragm to maintain 'in vivo' sodium and potassium levels is markedly affected by ouabain when otherwise it is in optimal conditions, thus it seems certain that a

NaKMGATPase is to be found in the tissue, and that this enzyme plays an important part in cation regulation.

The action of the diuretic ethacrynic acid upon the preparation resembled that of the glycoside, in that incubation in its presence led to increased sodium and water contents and a loss of potassium by the diaphragm. Interpretation of this finding is made more difficult, however, by the fact that ethacrynic acid may here be affecting at least three factors which influence the results. As was shown earlier, much of the sodium uptake could be accounted for by the enlargement of an 'extracellular space' which is not penetrated by inulin but in which cation conditions resemble those of the surrounding medium. The diuretic may also act upon a portion of cation exchange which is not dependent upon the NaKMGATPase sodium pump, either active or passive cation movements, as well as a possible action upon the ouabain-sensitive pump. There is, in fact, evidence of all three of these possible actions. Birks and Davey (1969) have demonstrated that the sarcoplasmic reticulum will swell under conditions of osmotic stress, and Keynes and Steinhardt (1968) have suggested that this could form an important compartment for sodium which would help to explain anomalies of labelled sodium washout. Several authors have shown that ethacrynic acid will block a ouabain-insensitive component of active sodium efflux from muscle (e.g. Bittar, 1966; Erlij and Leblanc, 1971) whilst others have shown that ethacrynic acid will inhibit the NaKMGATPase sodium pump (e.g. Duggan and Noll, 1965; Charnock, Potter and McKee, 1970). The action of ethacrynic acid upon this preparation will be considered more fully, after subsequent

subsequent experiments, in Chapter six.

The dye rose bengal caused an even more marked effect upon cation regulation by the diaphragm when illuminated, though water uptake was not as great. Sodium entry, and potassium loss, occurred rapidly and to a greater extent than was observed with ouabain. implying, if the ouabain concentration was high enough to cause maximum inhibition (vide infra), that more than inhibition of an NaKMgATPase was involved. Physiologists using the frog gastrocnemius preparation had observed that the addition of dyes to the saline often led to contracture of the muscle when illuminated. Lippay (1929) showed that the effective wavelength of illumination was the absorption wavelength of the dye, and in 1930 he demonstrated that oxygen was required for the photosensitivity effect. Lillie, Hinrichs and Kosman (1935) observed that incubation of muscles in salines containing only sodium salts would also lead to spontaneous contracture, but that this was much more marked in the presence of photo-sensitizing dyes. These authors suggested that the dyes were unlikely to penetrate the cells which, with the speed of the effect, suggested that the surface membrane was the site of action. This was supported by the effects of photooxidant dyes observed on other preparations (for review see Blum, 1941). Allison, Magnus and Young (1966) have studied the localisation of some of the photodynamic damage by various reagents. They studied the effects of photosensitizers upon various cultured cells; red blood cells and protozoans, and were able to identify two types of action. Some photooxidants penetrated the cells and caused damage to the lysosomes, leading to general cell damage, whilst others

did not penetrate the cells and had an action at the cell membrane only, leading to increased permeability. They could find no evidence of uptake of rose bengal, and suggested that the sole site of action was the cell membrane. Thus it seems clear that the increase in cation flux observed here in the presence of light and rose bengal represents photodynamic damage of some components of the fibre membrane. Further consideration of the site of action will follow the experiments upon isolated membranes in subsequent chapters.

CHAPTER THREE

OXYGEN CONSUMPTION OF THE RAT DIAPHRAGM

Introduction

If the sodium pump is to be maintained it requires a constant supply of energy from the cells metabolism. A popular approach used in the study of active sodium extrusion has been to examine the effect of this mechanism upon the metabolism of the cell, either by monitoring oxygen consumption or by evaluating the rates of turnover of the substrates involved in the many metabolic pathways. The interdependence of active transport and metabolism have been discussed by Whittam (1964).

The parameters of the isolated rat diaphragm (see Chapter Two) are such that the preparation lends itself to the study of oxygen consumption 'in vitro', and it has long been used for this purpose. Consequently, a study of the oxygen consumption of the rat diaphragm was undertaken, using animals similar to those used in the previous chapter, in an effort to evaluate the relationship between sodium transport and oxygen consumption in this tissue.

Materials and Methods

The rats used were in all respects identical to those used in Chapter Two. All reagents as previously described.

1. Diaphragm preparation. The intact diaphragm was first removed as described in the previous chapter. Subsequent treatment varied according to the experimental procedure adopted.

In the case of the diaphragms subjected to oxygen

consumption measurements in the Warburg apparatus, the treatment was as follows. The diaphragm muscle was trimmed from the ring of rib-cage, and as much as possible of the central tendon and any adhering fat were removed. The muscle was then cut into four portions, each of which was transferred from the saline dissection-bath to a Warburg flask. After incubation was completed the muscle was removed from the flask, blotted rapidly on both sides and then the wet weight was measured to an accuracy of 0.1 mg.

Oxygen consumption measurements using the oxygen electrode required two different techniques. In some cases, where the tissue was used immediately after its removal from the animal, the preparative procedure resembled that described above. Those muscles which were not used immediately were preincubated in the incubation chambers described in the previous chapter, in a variety of media, as intact hemidiaphragms. After preincubation the surplus tissue was trimmed away rapidly and transferred to the Oxygen Monitor incubation chambers. The wet weight of the tissue was later determined as described above.

2. Manometric techniques. Oxygen consumption was determined using classical manometric techniques with Warburg constant volume manometers (Umbreit *et al.*, 1964). Twenty cm^3 flasks, each containing 3 cm^3 of Krebs phosphate saline (NaCl 131 mM, KCl 5.9 mM, MgSO_4 1.2 mM, Na_2HPO_4 1 mM, NaH_2PO_4 0.25 mM, CaCl_2 1.25 mM, and glucose 2 g l^{-1} , pH 7.4) were used, where appropriate the CO_2 evolved was absorbed by 0.2 cm^3 of 10% KOH solution on a Whatman No. 40 filter paper fan in the centre well of the flask.

After the addition of the hemidiaphragm the flask was flushed with 100% oxygen, and equilibrated at 37°C for ten minutes

at a shaking rate of two strokes per second, before oxygen consumption was measured over one hour.

3. Polarographic techniques.

Oxygen consumption was also measured by means of a polarographic electrode (Yellow Springs Instrument Co. Inc., Ohio, Model 53, Biological Oxygen Monitor). This is a design based on the Clark oxygen electrode (Clark, 1956); the principle of the oxygen electrode has been described by Davies and Brink (1942).

In this model the electrodes are situated at the tips of two probes which also act as seals on the incubation chambers. The electrodes were covered by molar KCl solution enclosed by a thin teflon membrane. Before each experiment this membrane was examined with a 10 X eyepiece for signs of physical damage, and the efficiency of the probe was checked by altering the polarising voltage applied; where the signal alteration was greater than 3% two minutes after changing the polarising voltage from 0.8 V to 0.65 V the membrane was discarded.

The signal was monitored on a Servoscribe pen recorder (Goerz Electro) which was calibrated in the manner recommended in the Y.S.I. Handbook, using oxygen solubility data from Umbreit et al (1964). The apparatus included a constant temperature bath containing four sample chambers and temperature stability within these chambers was $\pm 0.02^{\circ}\text{C}$, equilibration being complete in less than three minutes, at 37°C (see fig. 3:1).

Oxygenation of the incubation chamber was achieved via fine (0.05 inch diameter) teflon tubing which was inserted down the access slot into the chamber, where this contained 5 cm^3

Krebs phosphate saline 100% oxygen was bubbled through the saline from a gas-filled bladder, in the case of 5 cm³ Krebs bicarbonate saline a 95% oxygen : 5% carbon dioxide mixture was used.

Flushing of an overflow groove with the gas ensured that diffusion of gas down the access slot caused a negligible loss of oxygen from the sample chamber during the course of an experiment.

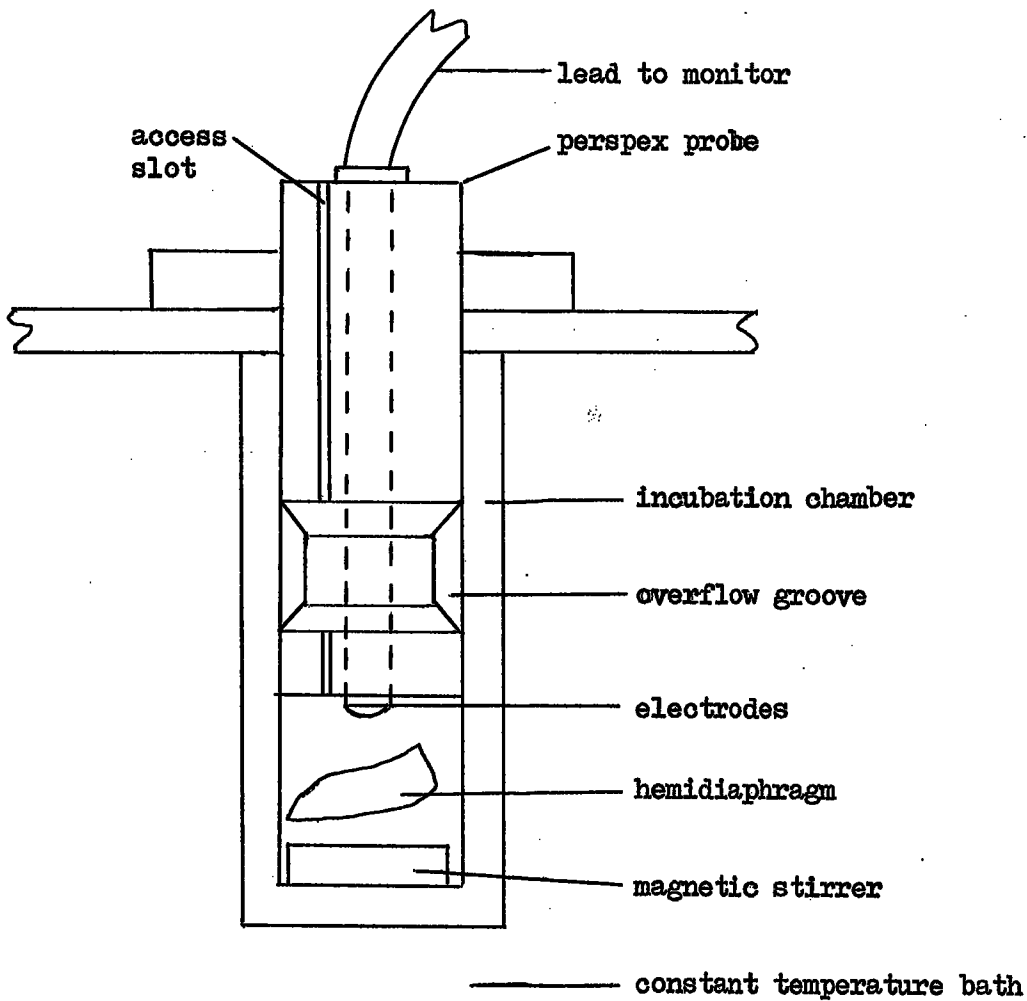


Fig. 3: 1 One incubation chamber and probe of Oxygen Monitor

This access slot enabled tests to be performed on the effect of potassium cyanide, added in 0.1 cm^3 saline via the teflon tubing, to give a final cyanide concentration of 2 mM. After the modified oxygen consumption rate was measured the hemidiaphragm was removed, rinsed rapidly in two changes of oxygenated saline at 37°C , and oxygen consumption was again measured.

In other experiments the effects of preincubation and/or incubation in various modified media were studied. All preincubations were performed in the apparatus described in the previous chapter, and details of the modified media will be given along with the appropriate results in the next section.

Results

The first assessment of oxygen consumption was made by manometric techniques, and the results are shown in Table 3:1. The monitoring of the effect of ouabain by addition from the side-arm of the flask proved impractical, as a slight fall in activity was noted in both control and test flasks. This was almost certainly due to the change in volume of saline in which the diaphragm was floating, leading to different physical movements of the suspended diaphragm. The effect of ouabain was therefore monitored by comparing oxygen consumption of different portions of diaphragm incubated in the presence or absence of 10^{-3} M ouabain.

As Table 3:1 shows, there was no significant difference between the oxygen consumption of diaphragm muscles in the two conditions. There were large variations in the results, however, which were not related to the presence of the glycoside nor to the weight of tissue incubated and there was a distinct fall in oxygen consumption during the course of the experiment, probably

indicating deterioration of the preparation.

It was felt, therefore, that the Warburg apparatus was not very suitable for monitoring oxygen consumption of the diaphragm tissue because of the length of time required. The apparent deterioration was not unexpected, bearing in mind the conditions shown to be essential for the prolonged maintenance of 'in vivo' cation concentrations in the previous chapter.

All further measurements of oxygen consumption were made using the polarographic techniques, which enabled readings to be taken within minutes of the removal of the tissue from the animal.

The first experiments performed using the oxygen monitor revealed that the oxygen consumption by the rat hemidiaphragm was not linear (see fig. 3:2), possibly due to progressively inadequate oxygenation, deterioration of the tissue, modification of respiratory rate with variation in oxygen availability, or any combination of these factors. That this fall in rate was partly due to the oxygenation of the medium can be seen from fig. 3:2, when an initial oxygen consumption of $1.545 \text{ cm}^3 \text{O}_2 \text{ h}^{-1} \text{ g}^{-1}$ wet wt declined to $1.029 \text{ cm}^3 \text{O}_2 \text{ h}^{-1} \text{ g}^{-1}$ wet wt after about ten minutes but was then restored to $1.202 \text{ cm}^3 \text{O}_2 \text{ h}^{-1} \text{ g}^{-1}$ wet wt upon transfer to another incubation chamber containing fresh medium at an oxygen tension similar to the original. It was not found possible to maintain a steady, high rate of oxygen consumption even when the incubation chamber was repeatedly flushed with oxygen to maintain a constant oxygen tension; therefore the decline in rate must also represent in part a deterioration of the tissue itself, or some metabolic change.

TABLE 3 : 1

Oxygen consumption of rat diaphragm as measured
by Warburg manometry

	Time interval	Ouabain 10 ⁻³ M	Oxygen consumption cm ³ O ₂ h ⁻¹ g ⁻¹ wet wt
A	60'	-	1.462 ± 0.120 (7)
	60'	+	1.432 ± 0.280 (7)
B	0 - 20'	-	1.613 ± 0.178 (7)
	20 - 40'	-	1.457 ± 0.119 (7)
	40 - 60'	-	1.344 ± 0.086 (7)

Values expressed as means ± S.E.M. (no. of expts).

All incubations for one hour in 3 cm³ Krebs phosphate
saline (pH 7.4) at 37°C, in 20 cm³ Warburg flasks
flushed with 100% O₂.

Fig. 3:2.

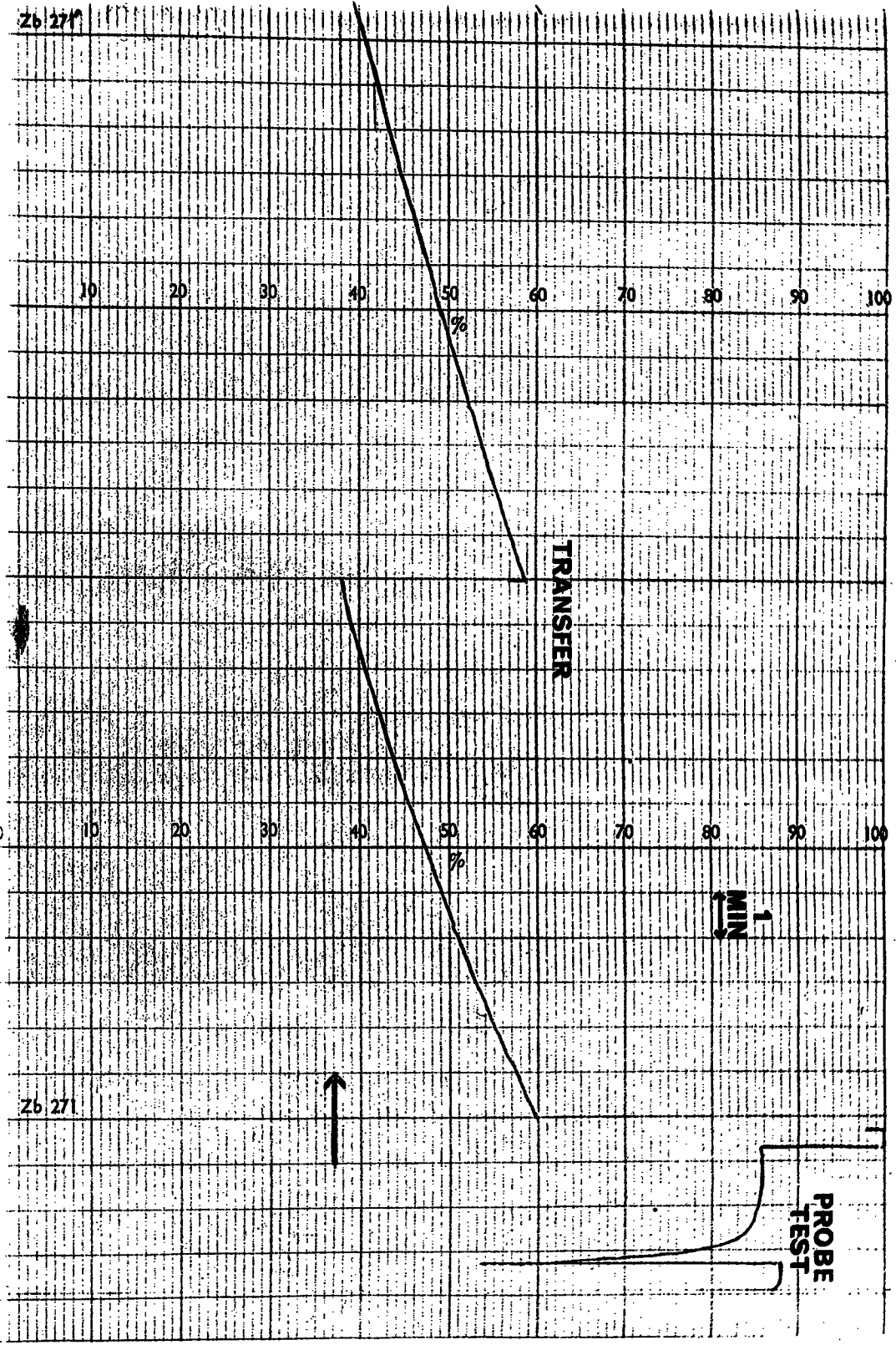
Record of preliminary investigation of rat diaphragm muscle
oxygen consumption by polarographic techniques

After probe test, hemidiaphragm (wet wt 76.2 mg)
incubated for 12 min in 5 cm³ Krebs phosphate saline
at 37°C, before transfer to a second, freshly oxygenated
chamber.

All incubations at 37°C using Y.S.I.

Oxygen Monitor.

Z6.271



10 20 30 40 50 60 70 80 90 100

%

TRANSFER

10 20 30 40 50 60 70 80 90 100

%

1 MIN

Z6.271

PROBE TEST

For this reason it was decided to measure oxygen consumption over the first five minutes, which appeared virtually linear, at an oxygen tension of between 8 and 9 $\text{cm}^3\text{O}_2 \text{ l}^{-1}$ phosphate saline (the 'initial rate') and then measure the consumption again in a second incubation chamber in similar conditions but in the presence or absence of 10^{-3} M ouabain. The four incubation chambers of the apparatus, with two probes, meant that transfers could be achieved rapidly into pre-equilibrated media. These experiments (Table 3:2) again failed to show any significant effect of ouabain upon the respiratory rate. Oxygen consumption declined markedly between the primary and secondary determinations in both control and ouabain-incubated muscles indicating that this technique also was not satisfactory. The initial values for oxygen consumption corresponded closely with those obtained by Warburg manometry.

To test whether the presence of carbon dioxide, and the bicarbonate ion, had any effect upon the oxygen consumption of the diaphragm muscle a third experimental procedure was adopted. The two hemidiaphragms from a single rat were each placed in an incubation chamber, one containing Krebs phosphate saline (pH 7.4) equilibrated at 37°C under 100% oxygen, and the other containing Krebs bicarbonate saline (pH 7.4) equilibrated at the same temperature under a mixture of 95% O_2 : 5% CO_2 . The oxygen consumption of each was then monitored, with the oxygen tension of the medium remaining within the range of 7 - 9 $\text{cm}^3\text{O}_2 \text{ l}^{-1}$ medium through intermittent reoxygenation via the access slot, and the results of these experiments are shown in Table 3:3.

TABLE 3:2

Effect of ouabain upon oxygen consumption of rat diaphragm

Primary incubation		Secondary incubation		P
10^{-3} ouabain	O_2 consumption	10^{-3} ouabain	O_2 consumption	
-	1.496 ± 0.019 (6)	-	1.302 ± 0.043 (6)	.01
-	1.522 ± 0.026 (6)	+	1.317 ± 0.031 (6)	.001
p	n.s.		n.s.	

Values for oxygen consumption expressed as mean $cm^3 O_2 h^{-1} g$ wet wt,
 \pm S.E.M. (no. of expts.) Incubations in Krebs bicarbonate
saline (pH 7.4) at 37° , flushed with 95% O_2 : 5% CO_2 .
Hemidiaphragms incubated in one chamber for 5 minutes
(primary incubation) and then transformed to fresh medium
in second chamber (secondary incubation).

TABLE 3 : 3

Oxygen consumption of rat diaphragm in bicarbonate- and phosphate-buffered media

Incubation buffer	Oxygen consumption		p
	Initial	After 15 min	
Bicarbonate	1.502 \pm 0.049 (6)	1.302 \pm 0.035 (6)	.01
Phosphate	1.495 \pm 0.023 (6)	1.302 \pm 0.071 (6)	.05
P	n.s.	n.s.	

Values for oxygen consumption expressed as mean

$\text{cm}^3 \text{O}_2 \text{ h}^{-1} \text{ g}^{-1} \text{ wet wt} \pm \text{S.E.M. (no. of expts)}$.

Initial oxygen consumption determined over first four minutes of incubation. Oxygen tension maintained at 7 - 9 $\text{cm}^3 \text{ l}^{-1}$ medium throughout.

Oxygen consumption over the first few minutes closely resembled that recorded in the previous experiment, and there was obviously no difference in this respect between the phosphate-buffered medium and that containing the bicarbonate. A marked decline in oxygen consumption was again observed, despite maintenance of oxygen tension, so that mean oxygen consumption rates of $1.302 \text{ cm}^3 \text{ O}_2 \text{ h}^{-1} \text{ g}^{-1}$ wet wt were recorded in both phosphate and bicarbonate media after 10 minutes of incubation; thus the bicarbonate and carbon dioxide were not effective in maintaining a steady rate of oxygen consumption.

Other workers have reported stimulation of oxygen consumption in tissues which have previously been submitted to a 'leaching' procedure during which the absence of potassium and glucose from the incubation medium has led to a marked accumulation of sodium ions. These are then removed, with an accompanying increase in oxygen consumption, when the tissue is returned to media containing potassium and glucose. Some tests were therefore performed to see whether this might be true for the present preparation. 'Intact' hemidiaphragms, prepared as described earlier, were preincubated for one hour in the apparatus described in the previous chapter. One hemidiaphragm from each rat was preincubated in full Krebs bicarbonate saline whilst the other was preincubated in bicarbonate saline from which the KCl (5.9 mM) and glucose (2 g l^{-1}) had been omitted. After one hour of preincubation the hemidiaphragms were removed, the tendon and rib rapidly trimmed away, and oxygen consumption measurements taken over five minutes by means of the polarographic techniques already described. As Table 3:4 shows, oxygen consumption in

TABLE 3 : 4

Effect of preincubation on oxygen consumption of rat diaphragm

	Preincubation conditions		Oxygen consumption $\text{cm}^3 \text{O}_2 \text{ h}^{-1} \text{ g}^{-1} \text{ wet wt}$
	KCl	Glucose	
A	No preincubation		$1.484 \pm .034$ (7)
B	+	+	$1.428 \pm .061$ (7)
C	-	-	$1.415 \pm .070$ (7)

Values expressed as means \pm S.E.M. (no. of expts).

All incubations at 37°C in Krebs bicarbonate saline

(pH 7.4) oxygenated by 95% O_2 : 5% CO_2 .

Oxygen consumption determined over first four minutes of incubation.

Preincubation:-

A - no preincubation

B - Krebs bicarbonate saline (containing 5.9 mM KCl,
0.2% glucose)

C - Krebs bicarbonate saline omitting KCl and glucose.

All preincubations for 1 hour in apparatus described in Chapter Two.

the 'leached' preparation exactly matched that recorded in the muscle maintained under optimal conditions, though both are a little lower than the value obtained for muscles in this medium without preincubation. This relatively slight deterioration in oxygen consumption may indicate that it is the actual cutting of the preparation which leads to the marked decline in activity noted above, whilst this leaching procedure, known to be effective in raising the sodium content of the muscle from previous experiments, has been ineffective in measurably raising oxygen consumption.

The effect of the introduction of potassium cyanide on the oxygen consumption of the preparation was then tested, and the results are shown in Table 3:5, with a typical record of such an experiment being given in fig. 3:3. The potassium cyanide was added in 0.1 cm^3 of the bicarbonate saline via the access slot, to give a final cyanide concentration of 2 mM. The first result was a marked, but short-lived, elevation of oxygen consumption which then rapidly declined so that after about one minute there remained a gradually decreasing consumption some one-quarter of the original. If the muscle was left in the cyanide medium this remaining rate was abolished, so that some fifteen minutes after the introduction of the cyanide no oxygen uptake was recorded. Generally, however, after the preparation had been exposed to the cyanide for five minutes it was removed, rinsed in two changes of fresh saline and the oxygen consumption remeasured. Since a similar transfer procedure had already been shown to lead to a reduced oxygen consumption, controls were also performed, using the other hemidiaphragm from each animal, which were treated

TABLE 3 : 5

Effect of KCN on oxygen consumption of rat hemidiaphragms

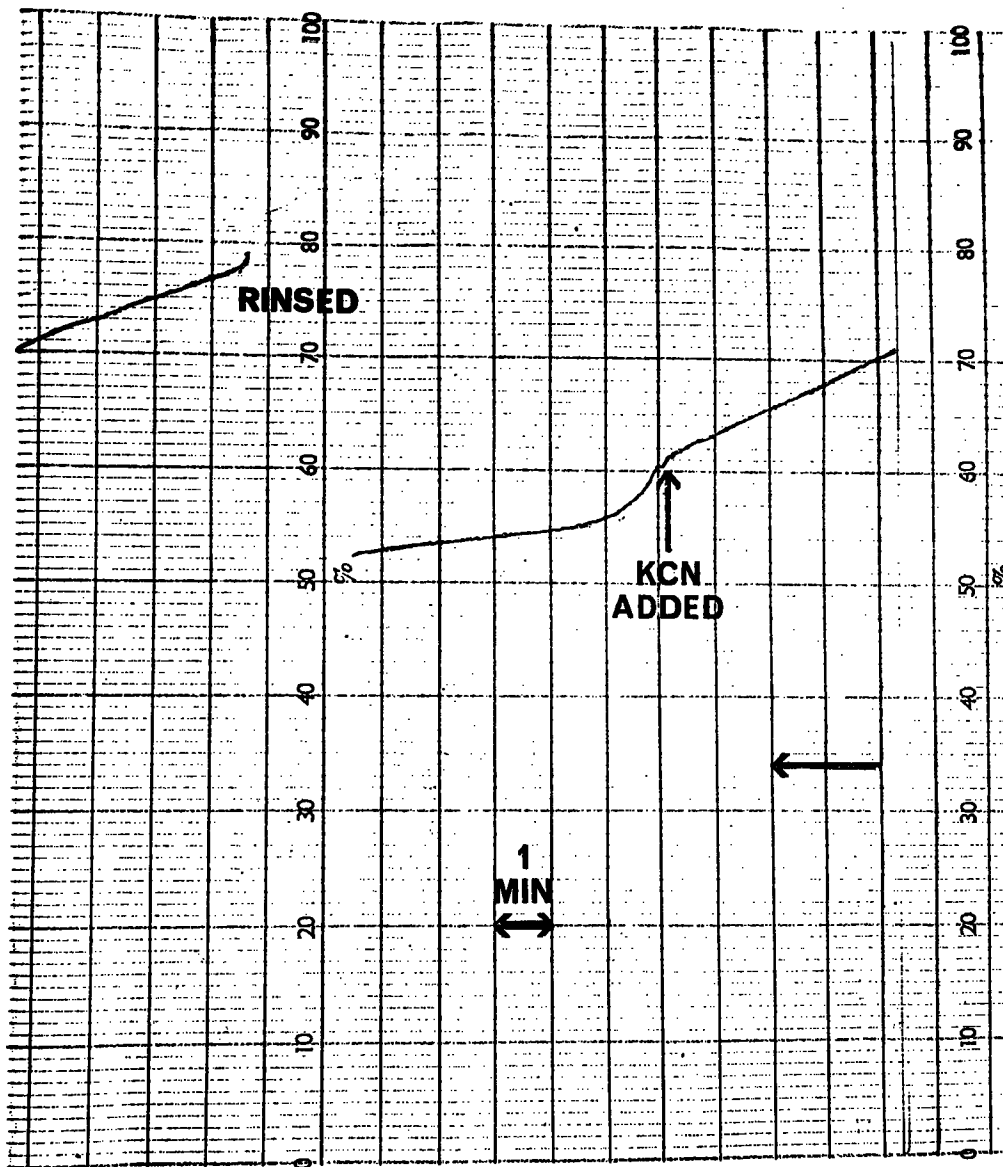
Incubation condition	Oxygen uptake ($\text{cm}^3 \text{O}_2 \text{ h}^{-1} \text{ g}^{-1} \text{ wet wt}$)		p
	A (rinsed only)	B (KCN incubated)	
Initial rate	1.475 ± 0.052	1.440 ± 0.043	n.s.
After KCN addition	-	0.334 ± 0.029 (23)	-
In fresh saline	1.295 ± 0.035 (88)	1.071 ± 0.041 (74)	.01

Values expressed as means \pm S.E.M. (nos. in parenthesis are values expressed as % initial rate). No. of expts in each case was 6. All incubations using polarographic techniques, in Krebs bicarbonate saline (pH 7.4) at 37°C, oxygenated by 95% O₂ : 5% CO₂ gas mixture. KCN added in solution to give final conc. of 2 mM.

Both KCN-treated diaphragms (B) and untreated (A) rinsed rapidly in two changes of warm bicarbonate saline before final determination in fresh saline.

Fig. 3:3. Polarographic record of oxygen consumption of hemidiaphragm submitted to KCN treatment

Hemidiaphragm (wet wt 45.1 mg) incubated for 4 mins in 5 cm³ Krebs bicarbonate saline (pH 7.4) before addition of 0.1 cm³ saline containing KCN to give final conc of 2 mM KCN. After further 5 mins the hemidiaphragm was removed, rinsed rapidly in two changes of bicarbonate saline, then oxygen consumption determined in second incubation chamber containing 5 cm³ bicarbonate saline. All incubations at 37°C using Y.S.I. Oxygen Monitor.



identically in all respects except for the exposure to cyanide. These control muscles gave values for oxygen consumption, $1.475 \text{ cm}^3 \text{O}_2 \text{ h}^{-1} \text{ g}^{-1}$ wet wt initially and $1.295 \text{ cm}^3 \text{O}_2 \text{ h}^{-1} \text{ g}^{-1}$ wet wt after the rinsing procedures, which closely resembled those recorded earlier in similar experiments (Table 3:2 A). In the case of the muscles exposed to cyanide there was a marked restoration of oxygen consumption, though this was still a little lower than was recorded in the control experiments. This restored oxygen consumption was then retained, and then gradually declined in a manner indistinguishable from that observed in the control muscles.

Following these experiments a slight modification of procedure, involving continuous oxygenation of the chambers throughout the preparation of the diaphragm and temperature equilibration enabled the measurements of oxygen consumption of the diaphragms to be made at far higher oxygen tensions than had previously been used, at values ranging from $10 \text{ cm}^3 \text{ l}^{-1}$ to $16 \text{ cm}^3 \text{ l}^{-1}$ and occasionally even higher. It was immediately apparent that elevating the oxygen tension also increased the oxygen consumption of the tissue, and in fig. 3:4 are shown the rates of oxygen uptake in the first five minutes of twenty-two hemidiaphragms incubated at various oxygen tensions. There is a marked linear positive correlation between oxygen consumption and the oxygen tension in the medium ($r = + 0.971$) through the entire range monitored. It was clear therefore that the hemidiaphragm, although respiring at rates resembling those recorded by Warburg manometry, was not respiring maximally in the previous determinations, nor could the maximal respiratory

rate be reached with this apparatus. It was also noticed that the rate of oxygen consumption often appeared to decline more rapidly when oxygen tension was high. As fig. 3:5 shows, in those preparations where oxygen tension was maintained for some fifteen minutes there appears to be a negative correlation ($r = - 0.610$) between the oxygen consumption of the preparation after fifteen minutes, expressed as a percentage of the original rate, and the oxygen tension, which might imply the presence of such an oxygen-consuming process which deteriorates rapidly after excision of the muscle.

It might have been that an oxygen-requiring, active sodium extrusion formed part of this rapid deterioration, and hence had not been revealed in earlier experiments. To check this possibility a series of experiments were performed in which the two halves of each diaphragm were introduced into two incubation chambers at high oxygen tensions, one containing 10^{-3} M ouabain and one without the glycoside, and the high initial rates of oxygen consumption were compared, as were the rates after some fifteen minutes of incubation. Fig. 3:6 shows a typical recording from this series of experiments, with the gathered results being given in Table 3:6. As can be seen, there was no significant difference between the rates in the presence or absence of the glycoside, either initially or after the fifteen minutes incubatory period, hence none of the higher oxygen consumption measured at these high values of oxygen tension could be ascribed to an active sodium pump powered by a NaK₂MgATPase.

Fig. 3 : 4. The effect of oxygen tension upon the
rate of oxygen consumption of rat
diaphragm muscle

Oxygen consumption of hemidiaphragms determined
by polarographic techniques in Krebs bicarbonate saline
(pH 7.4) at 37°C. Measurements based on the first five
minutes of recorded oxygen uptake.

Regression line drawn using method of least
squares ($r = 0.971$; $p = < .001$)

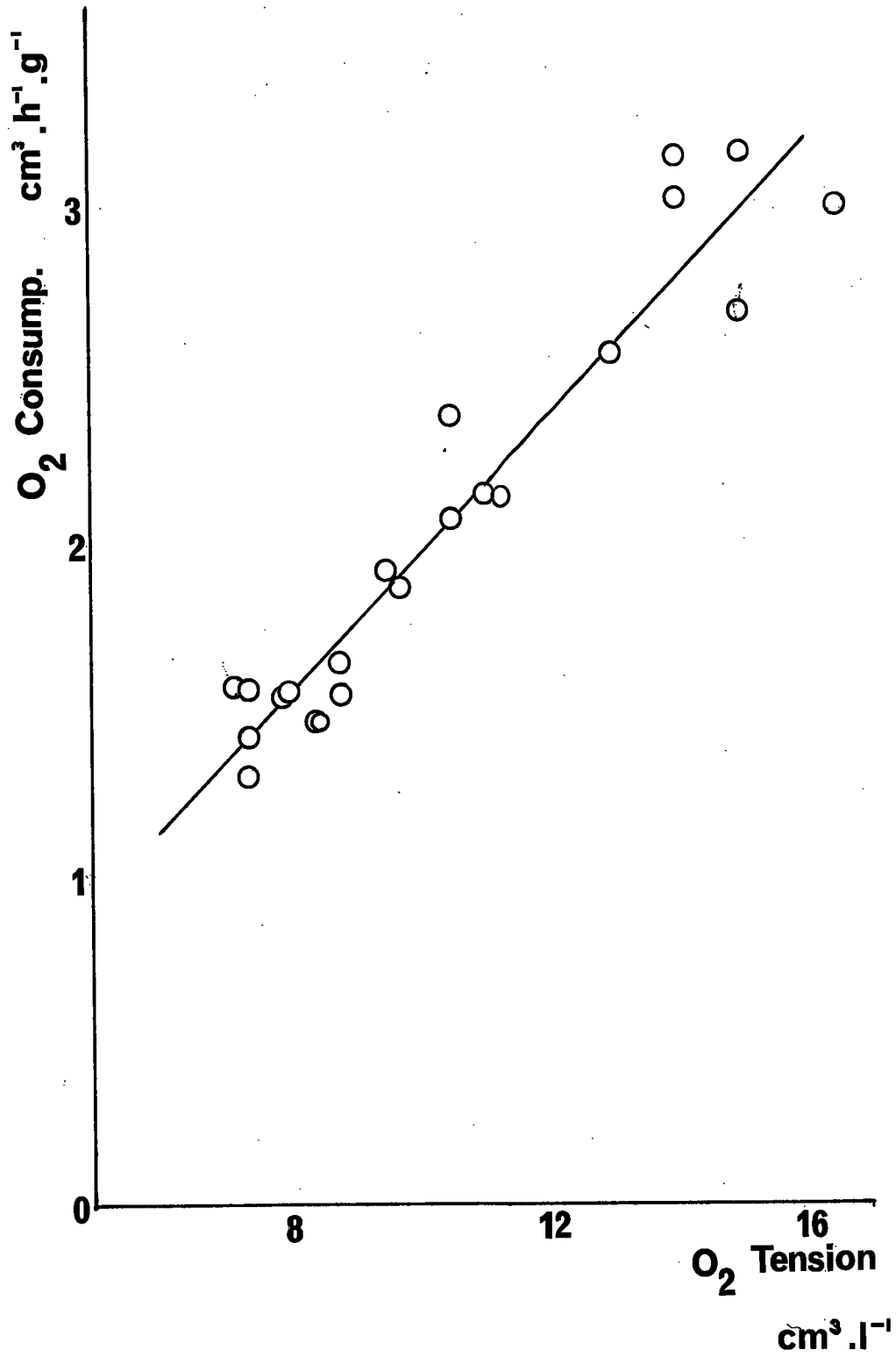
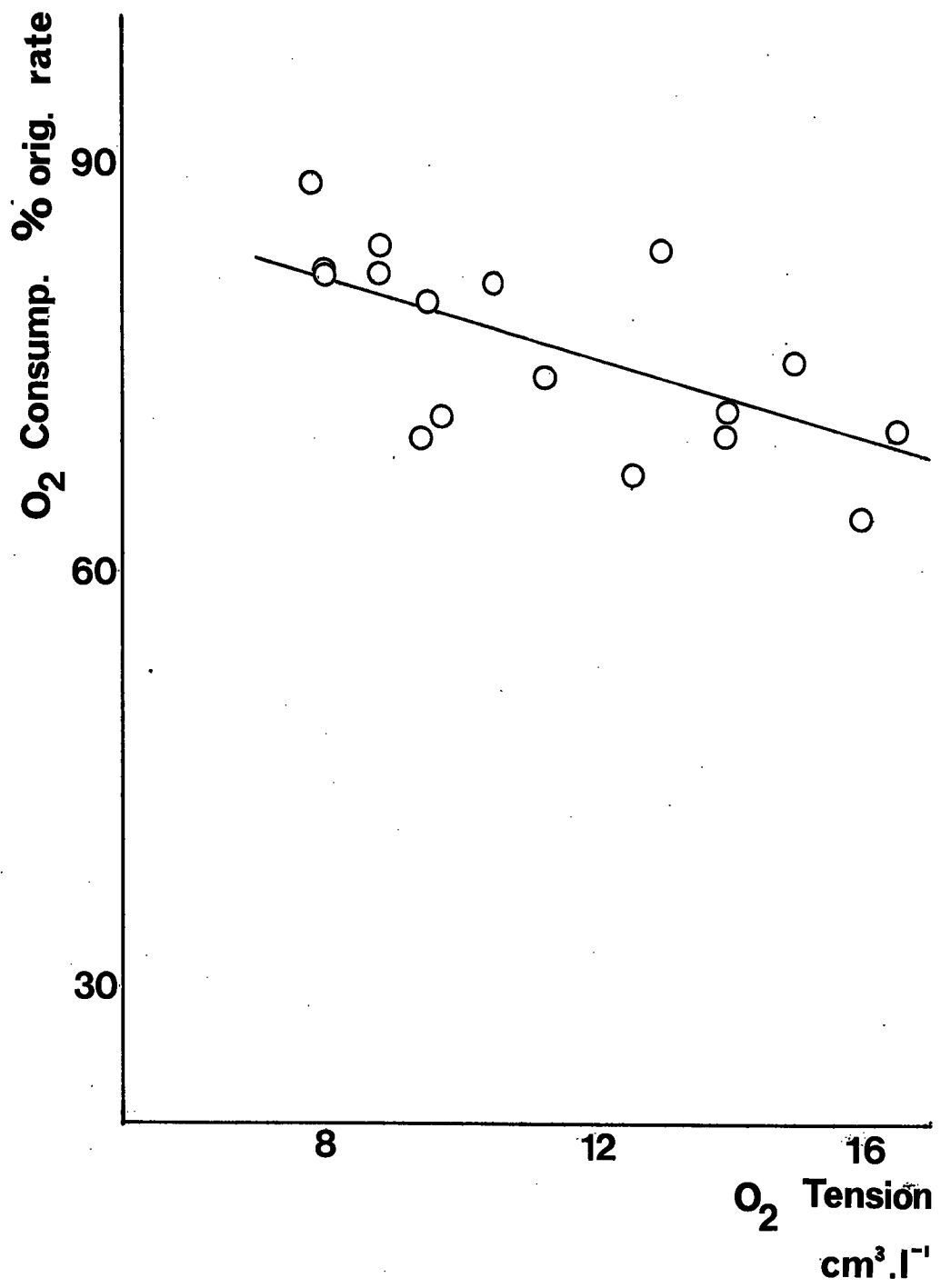


Fig. 3 : 5. Effect of oxygen tension upon maintenance
of oxygen consumption of rat diaphragm

Oxygen consumption of hemidiaphragms determined by polarographic techniques in Krebs bicarbonate saline (pH 7.4) at 37°C. Measurements taken after 15 minutes incubation and expressed as a percentage of the original rate of oxygen consumption.

Regression line drawn using method of least squares ($r = 0.610$; $p = < .01$)



63.

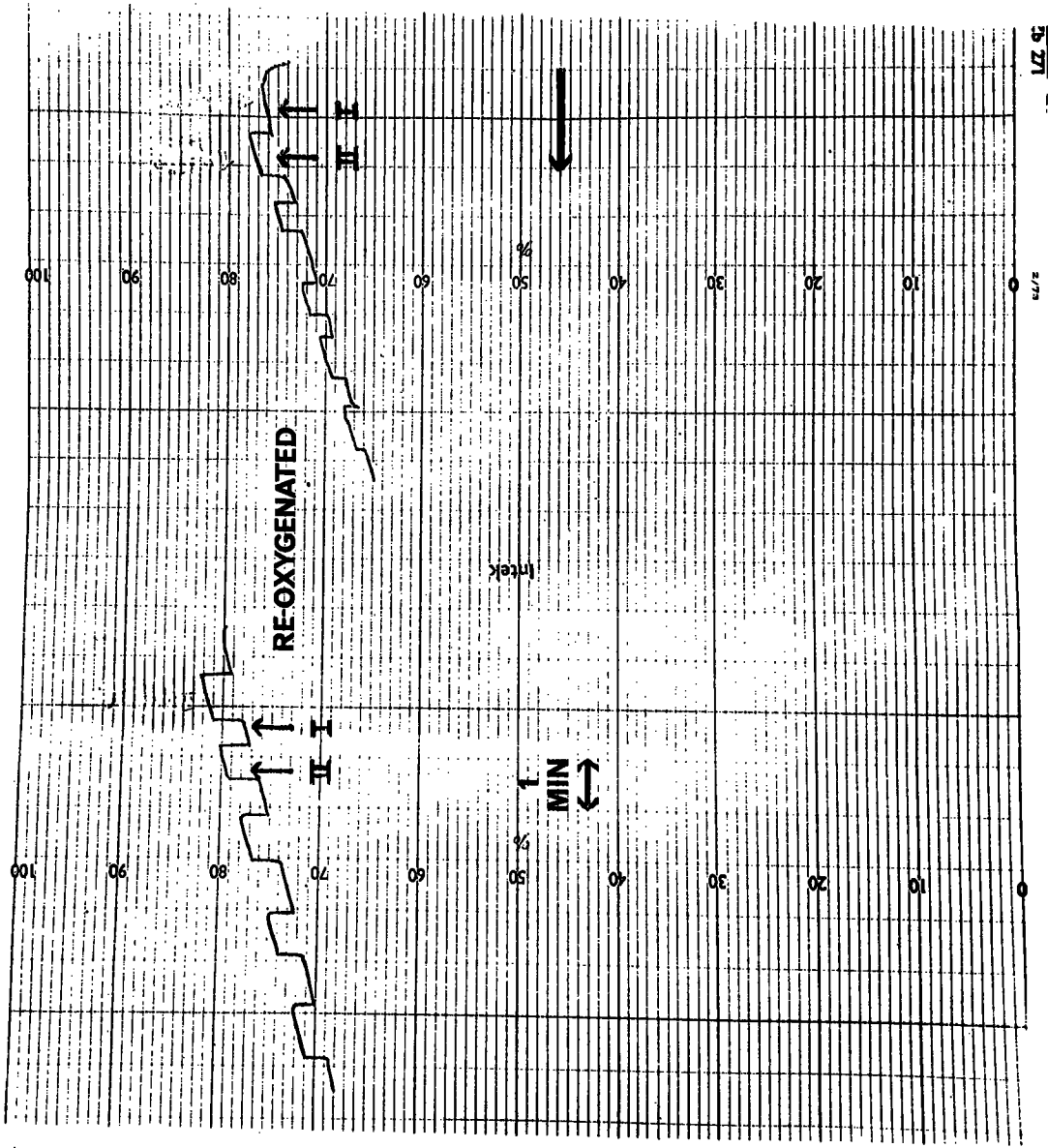
Fig. 3 : 6. Polarographic record showing effect of
 10^{-3} M ouabain on oxygen consumption
of a rat diaphragm

I - first hemidiaphragm (wet wt 57.2 mg) incubated
in 5 cm³ Krebs bicarbonate saline (pH 7.4)
containing 10^{-3} M ouabain

II - second hemidiaphragm (wet wt 59.8 mg) incubated
in 5 cm³ Krebs bicarbonate saline (pH 7.4)
as control

After 8 minutes both incubation chambers
subjected to reoxygenation via access slot.

All incubations at 37°C using Y.S.I. Oxygen Monitor.



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56/2

TABLE 3 : 6.

Effect of ouabain on oxygen consumption of rat diaphragm
at high oxygen tensions

	10^{-3} M. ouabain	Oxygen consumption		p
		$\text{cm}^3 \text{O}_2 \text{ h}^{-1} \text{ g}^{-1} \text{ wet wt}$		
		0 - 5 mins	10 - 15 mins	
A	-	$2.044 \pm 0.066 (12)$	$1.671 \pm 0.052 (12)$	n.s.
B	+	$1.937 \pm 0.074 (12)$	$1.592 \pm 0.069 (12)$	n.s.
p		n.s.	n.s.	

All values expressed as means \pm S.E.M. (no. of expts).

All incubations in Krebs bicarbonate saline (pH 7.4) at

37°C, oxygenated by 95% O_2 : 5% CO_2 gas mixture.

Oxygen tensions maintained at $> 13 \text{ cm}^3 \text{ O}_2 \text{ l}^{-1}$.

Discussion

While Warburg manometry has been a standard procedure used in determining oxygen consumption of various tissues, it did not appear to lend itself to the present preparation. The shape and size of the preparation are such that as it is agitated the hemidiaphragm tends to fold over upon itself, which will cause interference with oxygen diffusion into the tissue, and in certain cases anomalous results obtained here could possibly be ascribed to this phenomenon. In addition, it was observed that changing the amount of saline in which the hemidiaphragm was suspended also altered the apparent rate of oxygen consumption, though whether this was due to changes in the physical movements of the diaphragm or to an alteration in diffusion into the medium is unknown. The values measured for the oxygen consumption of diaphragm muscle using this technique corresponded well with those found in the literature, e.g. Nissan et al (1966) 1.19 - 1.50, Czaczkes et al (1969) 1.15, Rowlands (1969a) 1.46 (all values adjusted to $\text{cm}^3 \text{O}_2 \text{h}^{-1} \text{g}^{-1}$ wet wt). Kypson, Triner and Nahas (1968) reported values as low as $0.84 \text{ cm}^3 \text{O}_2 \text{h}^{-1} \text{g}^{-1}$ wet wt, but these workers used air as the gas phase which may well have limited oxygen consumption.

From the work described in the previous chapters it is clear that after one hour the cut hemidiaphragm preparation will be markedly changed from the 'in vivo' condition. In the light of Creese's experiments on oxygenation it seems certain that the centre of the diaphragm will have become hypoxic, and depolarisation will also have occurred. Tests performed on the

incubated diaphragms confirmed that there had also been a marked uptake of sodium and loss of potassium as described in Chapter 2. Not only ionic and electrical changes occur, but also changes in metabolism. Rowlands (1969a) showed that manometrically-incubated diaphragms had a markedly lower lactate production than that in perfused preparations, and Rookledge (1971) has demonstrated that, after incubation, the ATP and hexose phosphate contents of diaphragms are markedly reduced, whilst AMP and glucose contents are markedly higher. This would seem to imply impaired metabolic activity. In view of the length of incubation of the cut preparation, and the major impairment of normal metabolic and ion-regulatory functions, it seemed unlikely that the Warburg incubation technique would give a very useful picture of processes 'in vivo', hence attention turned to the use of the oxygen electrode technique.

Oxygen consumption values in normal medium corresponded closely with those measured in the Warburg apparatus, but there was a marked reduction in the variation of the results from one muscle to another, supporting the hypothesis that some of this variation was due to physical factors in the Warburg apparatus. Whilst the decline in the rate of oxygen consumption linked to oxygen availability was marked in the oxygen electrode chamber, the speed at which a determination of oxygen consumption could be made meant that conditions in the diaphragm were much closer to those pertaining 'in vivo'. It is possible that one factor playing a part in this decline in consumption was metabolic changes which occurred during the death of the animal. Hollanders (1968) and Rowlands (1969b) have both attributed

high lactate production and low glycogen levels in their preparations of perfused diaphragm in part to adrenaline release caused by handling and killing the animal, which would probably be accompanied by elevated oxygen consumption. This may explain differences in the proportional decline in rates which were not related to oxygen tension, but it is unlikely that it represented a major factor in the decline since hemidiaphragms which had been preincubated for one hour before introduction into the oxygen apparatus also exhibited the falling rate of oxygen consumption.

A further advantage of the polarographic technique is that it greatly facilitates the use of bicarbonate buffers. In view of the evidence that bicarbonate is essential if normal metabolic processes are to continue unaltered (see Chapter 2) it might have been expected that oxygen consumption in bicarbonate-buffered medium differed from that seen in phosphate-buffered, bicarbonate-free saline. This was not the case here, nor did bicarbonate medium reduce in any way the decline in oxygen consumption, as Table 3:3 shows. As Randle (1956) showed that the ability of bicarbonate to stimulate glucose uptake was most marked in anaerobic conditions rather than aerobic, and Roos (1971) and Adler, Roy and Relman (1965a, b) have demonstrated the importance of the carbon-dioxide-bicarbonate balance in maintaining intracellular pH and ion distribution, the present results would appear to suggest that the primary action of bicarbonate does not affect oxygen consumption itself, but the metabolic pathway must be intimately related. Dyson, Anderson and Nordlie (1969) showed that physiological levels of bicarbonate

influenced the activity of G-6-P phosphohydrolase in rat liver microsomes, and suggested that the action of bicarbonate caused conservation of G-6-P and led to increased glycogen accumulation.

In the earlier experiments (Table 3:2) it was not possible to demonstrate that the action of ouabain affected oxygen consumption. This might imply that active sodium transport has its energy requirements met in part or in total by ATP generated in glycolysis and so is responsible for a very small proportion of oxidative metabolism of resting muscle; this agreed with the findings of Kypson, Triner and Nahas (1968). To increase the activity of the sodium pump it has been common practice to preincubate the tissue in medium from which potassium and glucose have been omitted, and then to monitor oxygen consumption in normal medium as the pump adjusts the ionic balance. It was found here that this was not successful in revealing oxygen consumption by an activated pump. Czaczkes et al (1969) have demonstrated a portion of oxygen uptake which was inhibited in the absence of potassium, after a 90 minute leaching procedure, and showed that oxygen uptake could be still further depressed by use of high sodium levels and added ouabain. It is not clear why such a portion was not demonstrably activated here by the leaching procedure, though the trauma to which the preparation of Czaczkes et al (1969) was exposed was undoubtedly greater than that used here, and would probably have led to a more marked accumulation of sodium and loss of potassium. Nissan et al (1966) were also able to demonstrate that elevated sodium levels in the bathing medium would increase oxygen consumption in the rat diaphragm, an effect removed by ouabain hence attributable to

the sodium pump. It would seem therefore that a direct effect of the sodium pump upon oxygen consumption is best demonstrated by muscles in a 'stress' condition.

As was expected, cyanide proved a powerful but reversible inhibitor of oxygen consumption by the preparation. Caldwell (1968) has pointed out, however, that the action of such inhibitors preventing sodium extrusion by preventing an adequate ATP supply will only occur if internal ATP concentrations are low. Keynes and Maisel (1954) were unable to demonstrate any effect of cyanide upon sodium extrusion using frog muscle, and Akaike and Kowa (1970) have found this to be true for rat diaphragm also. Sodium-loaded muscles have been found to show reduced extrusion in the presence of cyanide (Carey, Conway and Kernan, 1959) but this effect can be abolished if there is an adequate supply of potassium in the medium (Conway, Kernan and Zadunaisky, 1961). Since low internal ATP levels are likely to cause muscles to go into rigor (Bendall, 1951) and sodium-loading also causes marked changes in muscle (see previous chapter) cyanide does not appear to be a suitable tool in the investigations reported here.

The discovery that the oxygen consumption of the preparation was influenced by the oxygen content of the medium was surprising considering the evidence to the contrary in other tissues (see Creese, Scholes and Whalen, 1958). Hill (1948) demonstrated that oxygen consumption in frog muscle was independent of oxygen tension until very low levels were reached. Consideration of the work of Creese and his associates, and the evidence presented in the previous chapter, which established that continuous oxygenation of the medium was not

enough to support full activity of the diaphragm, even at rest, but that a fine stream of bubbles must envelope the preparation, makes this linkage of oxygen consumption and oxygen tension much more acceptable. Values of oxygen consumption found here, up to $3.165 \text{ cm}^3 \text{ O}_2 \text{ h}^{-1} \text{ g}^{-1}$ wet wt, are higher than have generally been reported in the literature, and it is clear that adequate oxygenation to meet this consumption cannot be achieved by simple diffusion from an oxygenated medium. Rookledge (1971) has observed that a perfused diaphragm preparation under ideal conditions exhibited evidence of metabolic changes indicating hypoxia even though a high oxygen level remained in the effluent perfusate. It would seem that a process requiring a very high oxygen supply deteriorates rapidly after excision of the diaphragm. Fig. 3:5 shows that at high oxygen levels there is a proportionately larger fall in oxygen consumption, so that after ten to fifteen minutes of incubation oxygen consumption by the diaphragm is similar at a wide range of oxygen concentrations. It is unlikely that this oxygen consumption would be monitored by the Warburg apparatus due to the time required to flush the flasks and equilibrate them following introduction of the diaphragm.

The hypothesis that this rapidly-declining process might represent a portion of oxygen consumption concerned with active sodium transport was an attractive one, but was not supported by the evidence in Table 3:6; whilst oxygen consumption by the hemidiaphragm in the normal saline was occasionally slightly higher than that in the presence of 10^{-3} M ouabain, this was not statistically significant. Since the effect of the glycoside upon the metabolic processes is believed to be secondary to its

action upon active transport, it is possible that the active process had ceased before ouabain could exert its effect upon the oxygen consumption, but this seems unlikely in view of the demonstration by Czaczkes et al (1969) that ouabain could still depress oxygen consumption after a 90 minute leaching procedure.

The difficulty in demonstrating a marked reduction in oxygen consumption could well be indicative that sodium transport only involves a small proportion of the total oxidative metabolism. Clausen (1966) demonstrated that though ouabain did not reduce glucose uptake, it produced a marked change in glucose catabolism, with inhibited lactate production, and an increased glycogen pool; this was also observed by Kypson, Triner and Nahas (1968). Since ouabain caused a 30% reduction in glucose catabolism, and other workers had measured a 30% reduction in oxygen consumption by other tissues, e.g. kidney (Whittam and Willis, 1963), in the presence of ouabain Clausen suggested that active cation transport in the diaphragm requires about this proportion of oxidative energy production. From their results Czaczkes et al suggest that in their preparation the oxygen consumption of the muscle linked to the pump is approximately 20% of the total, though the proportion will vary with the conditions pertaining. Creese (1968), however, has calculated the work required if active secretion of sodium is utilized to remove all the sodium entering the resting muscle fibre. Using oxygen consumption values of $7.07 \text{ cm}^3 \text{ O}_2 \text{ h}^{-1} \text{ g}^{-1}$ wet wt (Creese, Scholes and Whalen, 1958) he demonstrated that less than 2% of the total metabolism could be used in secretory work. This was an upper limit, since either higher oxygen consumption or movements of sodium which did not require the

expenditure of energy would reduce the proportion of energy required.

It would seem, therefore, that mammalian muscle has a high metabolic requirement, but that unlike brain and kidney, which can be shown to have ouabain-sensitive oxygen consumption, much of this energy requirement is not involved with NaK Mg ATPase activity.

CHAPTER FOUR

ATTEMPTS TO ISOLATE A NaKMgATPase FROM

RAT DIAPHRAGM MUSCLE

Introduction

Since Skou (1957) identified the NaKMgATPase as an important cation-transport enzyme it has been isolated and identified in a wide range of tissues (see Skou, 1965). Its presence in skeletal muscle was strongly implied by the sensitivity of the cation balance of this tissue to the cardiac glycoside ouabain, but attempts to isolate a skeletal muscle fraction which exhibited a clear, highly-active NaKMgATPase were complicated by the presence of a large number of ATPases in the tissue.

In 1962 Skou, using a rabbit, and Bonting *et al.*, using frog, reported low NaKMgATPase activities in skeletal muscle preparations. Other workers, however, were unable to demonstrate an ATPase activity which was clearly the NaKMgATPase, as their preparations were not ouabain-sensitive, or were not synergistically stimulated by sodium and potassium (e.g. Engel and Tice, 1966; Duggan, 1965). A ouabain-sensitive ATPase was demonstrated in cardiac tissue (e.g. Schwartz, 1962; Lee and Yu, 1963).

Using bile salt, deoxycholate, Samaha and Gergely isolated by centrifugation a fraction of human skeletal muscle exhibiting the properties of the NaKMgATPase (Samaha and Gergely, 1965, 1966). Again, others were unable to demonstrate

such an enzyme using similar techniques (Radcliffe, 1968).

In view of the uncertainty surrounding the presence of this enzyme in skeletal muscle an investigation was undertaken using the bile salt deoxycholate and the separation techniques of Ahmed and Judah (1964). Shortly after the commencement of this study Rogus, Price and Zierler (1969) published evidence of a NaKMgATPase in a fraction of rat skeletal muscle using these techniques, and their methods were adopted for the present work.

Materials

Male albino rats, weighing between 200 and 300 g, of the CFHB strain, were used, fed and watered ad libitum as previously described.

L-Histidine, tris(hydroxymethyl)aminomethane (Tris), 7-7-deoxycholic acid (as sodium salt), phosphatidylserine (bovine brain extract type III - reputedly 80% phosphatidylserine), adenosine triphosphate (Sigma grade), bovine serum albumin (Folch fraction V), L α lecithin (prepared from egg-yolk), ouabain (strophanthin G), ethylenediaminetetra-acetic acid (EDTA), and Fiske and Subba Row reducing agent were obtained from the Sigma Chemical Corporation. All other chemicals used were 'Analar' grade, obtained from British Drug Houses Ltd.

Methods

(a) Extractions. A variety of extraction techniques, based on those of Ahmed and Judah (1964), and Rogus, Price and Zierler (1969) were used. A general description of the techniques will be given here, and variations will be described alongside the appropriate results in a later section.

The rat was killed by a blow on the head followed by cervical dislocation. Approximately 1 g of hind-limb muscle, or the whole diaphragm (weight 0.6 - 1 g), was rapidly excised and placed in a petri dish on a bed of ice. Tendon and connective tissue, plus any adipose tissue, were cut away and the remaining muscle fibres were minced using a pair of fine scissors. This minced muscle was then transferred to a homogenisation tube containing 10 cm³ of extraction medium, containing 0.25 M mannitol, 30 mM histidine, 5 mM EDTA, and usually 0.1% deoxycholate, the final pH being 6.8. An homogenate was then prepared using a Potter-Elvehjem homogeniser with a teflon pestle (clearance 0.1 mm - 0.15 mm); this generally took three minutes, during which time the tube was surrounded by ice. The homogenate was then centrifuged at 600 x g for ten minutes, at 4°C, in a 32 x 10 cm³ angle-head of a Mistral 2L centrifuge (Measuring and Scientific Equipment Ltd.) to remove a 'nuclear' fraction, plus any unhomogenised fibres. The supernatant was decanted and submitted to two centrifugations at 10,000 x g for twenty minutes each, at 4°C, in the 16 x 15 cm³ angle-head of a High Speed 18 centrifuge (M.S.E. Ltd.) to remove the 'mitochondrial' fraction. The remaining supernatant, corresponding to Ahmed and Judah's S1 fraction, was carefully pipetted off and was then either submitted immediately to higher speed centrifugations or was frozen and stored at -20°C for various time periods as part of the 'ageing' process.

Treatment of this S1 fraction varied to give differing preparations. Generally the procedure of Ahmed and Judah was followed, the S1 fraction being submitted to a further

centrifugation of 100,000 x g for thirty-five minutes at 4°C in an 8 x 10 cm³ angle-head of a Superspeed 40 centrifuge (M.S.E. Ltd.) to give a pellet corresponding to the R1 fraction of Ahmed and Judah. This pellet was resuspended in a medium containing 0.25 M mannitol, 1 mM EDTA and 30 mM Tris-HCl (pH 7.4) at 0°C using gentle homogenisation with a hand-held pestle. The suspension was then centrifuged at 20,000 x g for fifty minutes in the 8 x 10 cm³ angle-head of the Superspeed 40 centrifuge to give a pellet corresponding to the R2 fraction of Ahmed and Judah, which was resuspended again in the same medium. This preparation was then used for the enzyme assay, either immediately or after storage at -20°C.

In some cases this 'R2' fraction was further submitted to a lipoprotein extraction technique resembling that of Ahmed and Judah. To the 'R2' pellet in 1 cm³ of the resuspension medium, a solution containing 2.1 M sucrose, 5 mM EDTA and 20 mM Tris-HCl (pH 7.4) was added drop by drop, with continuous stirring, until the density of the medium was 1.22, and homogenised gently to give a uniform preparation. Centrifugation for three hours at 100,000 x g in the Superspeed 40 followed, and after this treatment the surface layer was carefully removed by means of a spatula, this fraction being the lipoprotein fraction which was resuspended in the mannitol-tris-EDTA medium described above.

(b) Incubations. Incubations took place in boiling-tubes in a water-bath maintained at 37°C. Each tube contained 1 cm³ of incubation medium containing 60 mM Tris-HCl (pH 7.4), 0.2 mM EDTA,

plus two-fold concentrations of $MgCl_2$, NaCl and KCl as appropriate, to which was added 0.5 cm^3 of a four-fold concentration of Tris-ATP (pH 7.0), prepared by passing the sodium salt through a Dowex exchange resin and neutralizing with Tris solution. These tubes were equilibrated at $37^\circ C$ for 10 minutes before the incubation was started by the addition of 0.5 cm^3 of the membrane preparation, except in the case of the control tubes when the enzyme was not added until after the addition of trichloroacetic acid (see below). In some experiments preincubation with various reagents preceded the incubation, and details of this, along with details of variations in incubation procedure and the precise composition of the incubation media will be given with the results of each experiment. Incubation was terminated by the addition of cold trichloroacetic acid to give a final concentration of 6% w/v.

(c) Assay procedures. The amount of inorganic phosphate (Pi) released during the incubation was assayed by the technique of Fiske and Subba Row (1925). The incubated preparations were centrifuged at $2,000 \times g$ for fifteen minutes to remove the suspended protein, and then 1 cm^3 aliquots were added to an equal volume of the ammonium molybdate-sulphuric acid mixture. After ten minutes 0.25 cm^3 of Fiske and Subba Row reducing agent was added, and fifteen minutes later the absorption at a wavelength of $660\ \mu m$ was read in matched glass cuvettes on a Hilger-Watts Spectrophotometer. Zero readings were made using aliquots from the control tubes which were incubated without addition of the enzymes. Control tubes also monitored any possible interference caused by additions to the incubation medium. The values obtained were calibrated against determinations made on

a standard phosphorous solution.

Protein content was determined by the method of Lowry et al (1951) using bovine serum albumin (Folch fraction V) as standard.

Results

Initially, a series of experiments following the method of Rogus, Price and Zierler (1969) were performed in an attempt to produce an enzyme preparation exhibiting the characteristic properties of a NaKMgATPase. A freshly prepared 'R2' fraction was incubated in the presence of 3 mM MgCl, 100 mM NaCl or 10 mM KCl alone or in combination. Results of two typical experiments from each of two preparations are shown in Table 4:1.

Comparison of duplicate experiments performed on each preparation showed that there was good replication between individual experiments under the standard conditions. Comparison of activity levels found in the two preparations revealed the wide range of specific activity found in these extracts. When activity was expressed as a percentage with reference to the activity observed in the presence of Mg⁺⁺, as the sole cation, replication between both experiments and preparations was far closer. In the presence of Na⁺ alone, K⁺ alone, or both Na⁺ and K⁺ ATPase activity was never found. Typically, the ATPase activity found in the presence of Mg⁺⁺ alone was the highest, with the addition of the monovalent cations inhibiting this activity some 20-40% in the case of Na⁺, 5- 15% with K⁺, and some 20-40% in the presence of both Na⁺ and K⁺. In a long series of such experiments similar results were obtained, and stimulation of ATPase activity in the presence of Mg⁺⁺ by the addition of either Na⁺ or K⁺, or both together, was never observed.

TABLE 4 : 1 ATPase activity of the 'R2' fraction
of rat skeletal muscle in the presence of various cations

Preparation	Expt.	Incubation cations			
		Mg ⁺⁺	Mg ⁺⁺ Na ⁺	Mg ⁺⁺ K ⁺	Mg ⁺⁺ Na ⁺ K ⁺
22.11	(a)	41.96 (100)	30.47 (73)	38.43 (92)	31.40 (75)
	(b)	42.65 (100)	31.17 (73)	38.43 (90)	31.89 (75)
28.11	(a)	83.95 (100)	58.70 (70)	82.82 (99)	65.11 (77)
	(b)	82.95 (100)	58.23 (70)	78.20 (95)	64.25 (77)

Activities expressed as m. moles Pi h⁻¹ g⁻¹ protein.

Figures in parentheses are activity expressed as % activity in presence of Mg alone. All incubations at 37°C in 30 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 2 mM ATP plus 3 mM MgCl₂, 100 mM NaCl, 10 mM KCl as appropriate.

Determinations of activity with either 100 mM NaCl or 10 mM KCl, or 100 mM NaCl, 10 mM KCl, but in the absence of MgCl₂ revealed a total absence of ATPase activity in all experiments.

The addition of 10^{-3} M ouabain to incubations of an enzyme preparation should inhibit any ATPase activity due to the action of the NaKMgATPase. In a series of experiments in which this inhibitor was added it failed to produce any significant effect on the ATPase activity recorded (Table 4 : 2).

Various modifications of the basic method of preparation were then tested in an attempt to reveal the presence of NaKMgATPase.

Varying the length of time the preparation 'R2' was stored at -20°C failed to reveal an increase in such activity. ATPase activity declined over a period of storage (fig. 4:1 (a)) and in fact activity when measured in the presence of Na^{+} and/or K^{+} had declined more rapidly than that measured in the presence of Mg^{++} alone (fig. 4:1 (b)) so that a $\text{Na}^{+} + \text{K}^{+}$ inhibition of 23% was increased to 50% after storage for nine days.

Varying the sodium deoxycholate treatments of the preparation was then tried: the reagent was added at a strength of 0.1% to the extraction medium, or 0.05% in the case of the incubation medium. The 'R2' fraction was then extracted as described previously, with the exception that one half of the muscle was extracted in medium containing the DOC, whilst the other half was extracted in medium omitting this reagent. Incubations were also performed in the presence or absence of DOC, and typical results can be seen in Table 4:3. The treatments failed to produce any marked effect on ATPase activity, though the presence of DOC in the incubation medium did appear to lower overall activity without markedly altering the effect of Na^{+} and K^{+} . Further exploratory experiments with concentrations of 0.05 - 0.2% DOC in the extraction medium, and of 0.025 - 0.1%

TABLE 4 : 2

The effect of ouabain on the ATPase activity of the R2 fraction
of rat skeletal muscle

Incubation conditions	Mg ⁺⁺	Mg ⁺⁺ Na ⁺ K ⁺	Mg ⁺⁺ Na ⁺ K ⁺ + ouabain
Preparation			
12.1	85.3 (100)	49.6 (58)	48.0 (56)
12.2	59.4 (100)	47.9 (81)	48.6 (82)
12.3	46.4 (100)	39.2 (85)	36.5 (80)
12.4	51.8 (100)	37.6 (73)	39.6 (76)

Activity expressed as m. moles Pi h⁻¹ g⁻¹ protein.

Figures in parentheses represent activity as a % activity
in presence of Mg alone. All incubations in 30 mM Tris-
HCl (pH 7.4), 0.1 mM EDTA, 2 mM ATP at 37°C, plus
3 mM MgCl₂, 100 mM NaCl, 10 mM KCl and 10⁻³ M ouabain
as appropriate.

Fig. 4 : 1. Effect of ageing on ATPase activity of
R2 fraction of rat skeletal muscle

(a) Effect on ATPase activity in presence of Mg^{++} alone.

(b) Effect on ATPase activity in presence of Mg^{++} plus
monovalent cations (expressed as a % of activity
with Mg^{++} alone)

All preparations stored at $-20^{\circ}C$ in neutralised, deionised
water. All incubations at $37^{\circ}C$ in presence of 30 mM Tris-
HCl (pH 7.4), 0.1 mM EDTA, 2 mM ATP, plus

Δ 3 mM $MgCl_2$

\circ 3 mM $MgCl_2$, 100 mM NaCl

∇ 3 mM $MgCl_2$, 10 mM KCl

\square 3 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl

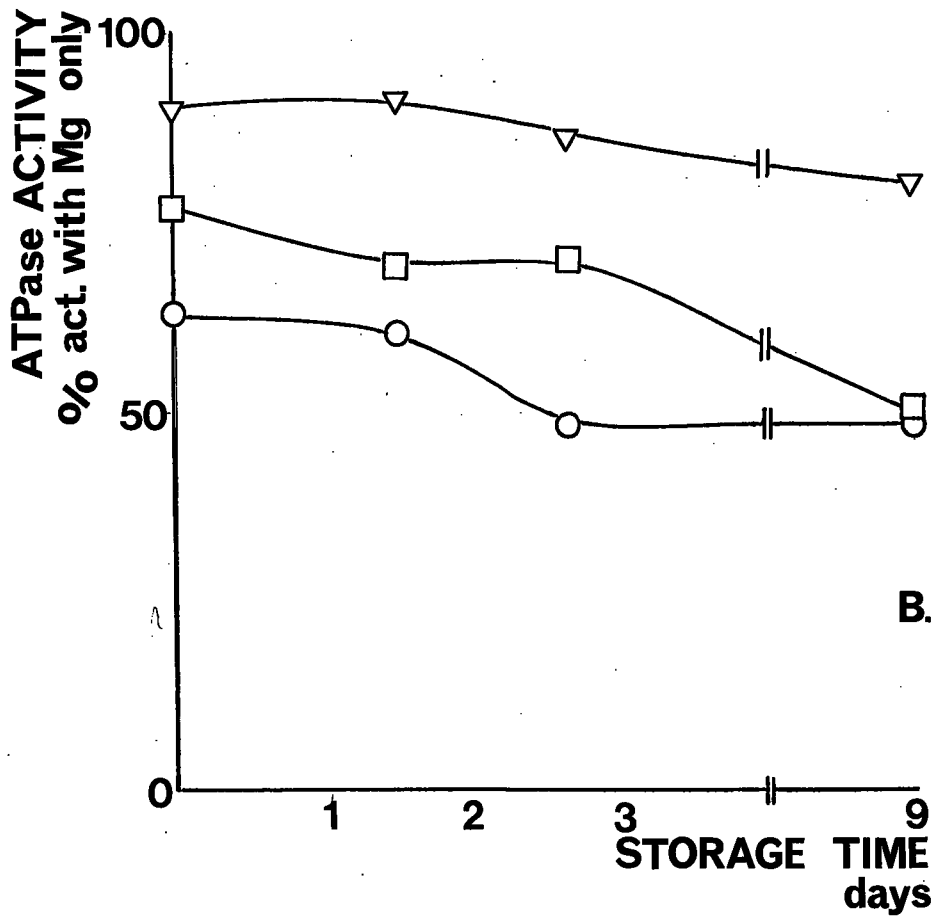
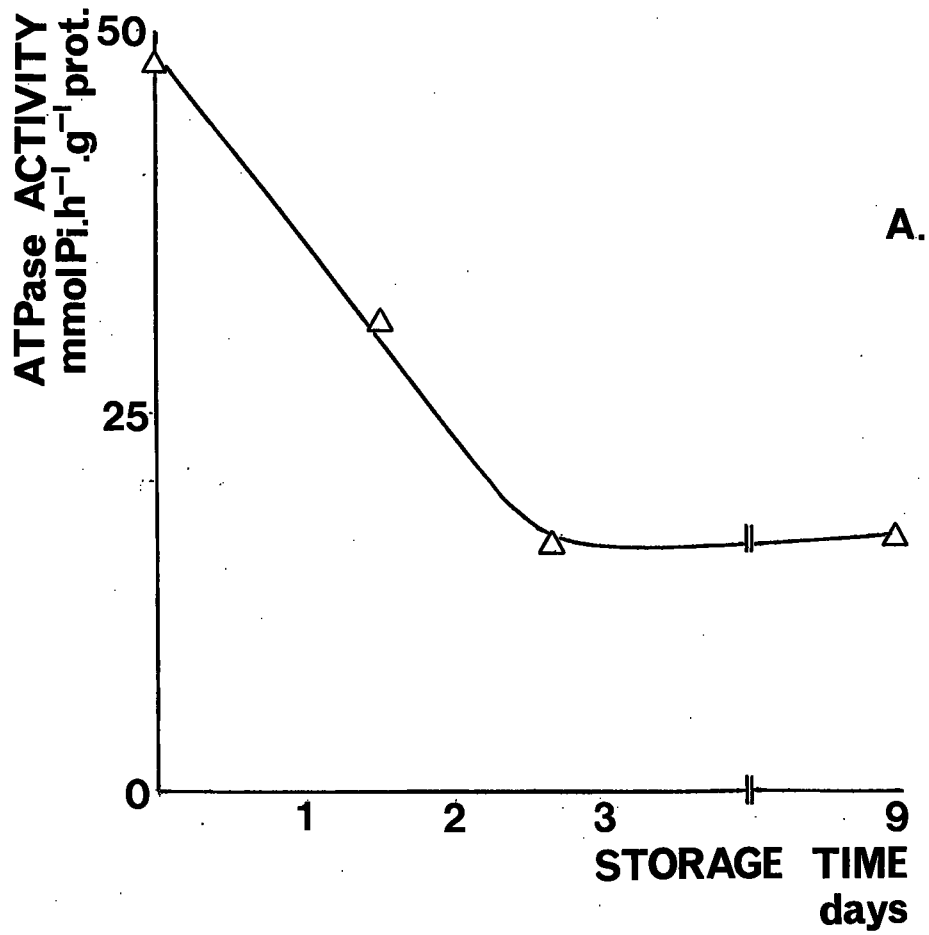


TABLE 4 : 3.

Effect of sodium deoxycholate treatments on ATPase activity
of R2 fraction of rat skeletal muscle

Deoxycholate		Incubation cations			
Extract- ion	Incub- ation	Mg	MgNa	MgK	MgNaK
-	-	61.1 (100)	42.0 (69)	55.3 (91)	34.5 (56)
-	+	56.3 (100)	41.2 (73)	50.7 (90)	22.6 (40)
+	-	65.2 (100)	44.8 (69)	65.7 (101)	29.7 (46)
+	+	57.4 (100)	40.6 (71)	51.6 (90)	24.1 (42)

Activity expressed as m. moles Pi $h^{-1} g^{-1}$ protein.

Figures in parentheses represent activity as a % activity
in presence of Mg only.

Preparations extracted in presence/absence of 0.1% sodium
deoxycholate. Incubations were performed at 37°C in
30 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 2 mM ATP, plus 3 mM
MgCl₂, 100 mM NaCl, 10 mM KCl and 0.05% sodium
deoxycholate as appropriate.

DOC in the incubation medium, produced similar results and in no case was stimulation of ATPase activity in the presence of Na^+ and K^+ recorded.

Since the calcium ion is known to have a marked effect on many ATPases the preparation was incubated in various conditions which included this ion (Table 4:4). The 'R2' fraction used was found as usual to contain a MgATPase which was inhibited some 40% with the addition of 100 mM NaCl and 10 mM KCl. There was, however, also present a Ca ATPase, with an activity level of about one-quarter of that of the MgATPase. This CaATPase was stimulated by some 20% in the presence of the monovalent cations. In the presence of both calcium and magnesium the two ATPase activities appeared to be additive.

Incubations with tannic acid in concentrations up to 5×10^{-5} M failed to reveal any significant difference in percentage inhibition of activity in the presence or absence of the monovalent cations (fig. 4:2). Activity was reduced by 20% in a tannic acid concentration of 5×10^{-6} M and some 60% in the 5×10^{-5} M concentration, with no observable effect of sodium or potassium on this inhibition.

Since these experiments had failed to reveal a NaKMgATPase present in the 'R2' fraction, but had shown high MgATPase activity, an attempt was made to separate such an enzyme by further purification of the preparation. A lipoprotein fraction was prepared by means of a sucrose density gradient (see Methods). Table 4:5 shows the ATPase activities measured in five such preparations in the presence of magnesium, sodium, potassium and

TABLE 4 : 4.

Effect of calcium upon ATPase activity of R2 Fraction
of rat skeletal muscle

Incubation Mg ⁺⁺	cations		ATPase activity m.moles Pi h ⁻¹ g ⁻¹ protein
	Ca ⁺⁺	Na ⁺ K ⁺	
-	-	+	0
+	-	-	44.62
+	-	+	27.25
-	+	-	11.86
-	+	+	14.35
+	+	-	57.72
+	+	+	58.97

All incubations at 37°C in presence of 30 mM Tris-HCl
(pH 7.4), 2 mM ATP, plus 3 mM CaCl₂, 3 mM MgCl₂,
100 mM NaCl, 10 mM KCl as appropriate.

Fig. 4 : 2.

Effect of tannic acid on ATPase activity of R2 fraction
of rat skeletal muscle

All incubations at 37°C in presence of 30 mM Tris-HCl
(pH 7.4), 0.1 mM EDTA, 2 mM ATP, plus

○ 3 mM MgCl₂

▽ 3 mM MgCl₂, 100 mM NaCl, 10 mM KCl.

Activity expressed as a % of activity recorded in
control incubations in the absence of tannic acid.

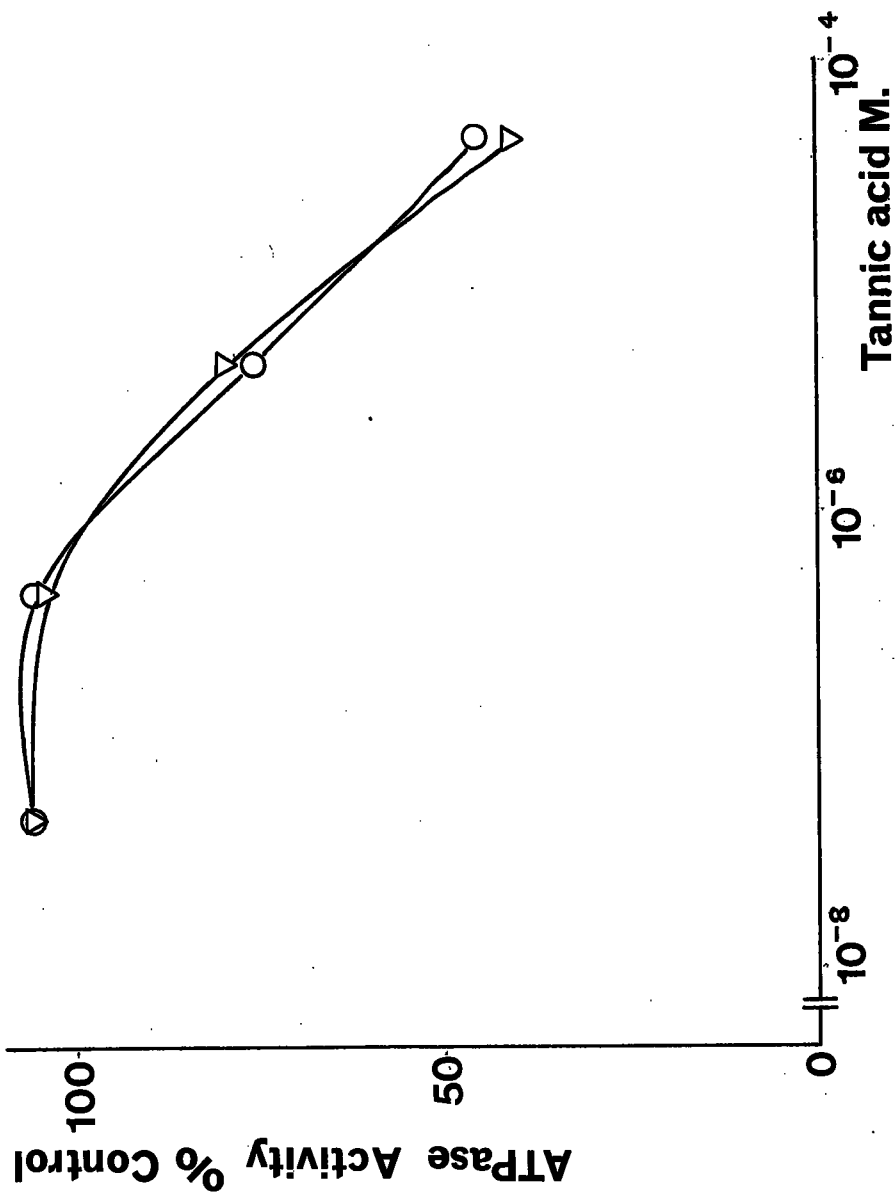


TABLE 4 : 5

ATPase activity of lipoprotein fractions of rat skeletal muscle

Incubation conditions	Mg	MgNa	MgK	MgNaK	MgNaK ouabain
Expt. 1	24.1 (100)	-	-	35.0 (145)	31.7 (132)
2	19.9 (100)	18.1 (91)	16.5 (83)	20.8 (105)	20.4 (103)
3	26.8 (100)	29.4(110)	-	31.3 (117)	31.5 (118)
4	32.0 (100)	18.0 (56)	24.4(76)	23.2 (73)	18.8 (59)
5	25.9 (100)	20.2 (80)	22.7 (85)	18.8 (73)	19.8 (76)

Values expressed as m. moles Pi h⁻¹ g⁻¹ protein.

Figures in parentheses are % activity in presence of Mg only. Incubations were performed at 37°C in the presence of 30 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 2 mM ATP, plus 3 mM MgCl₂, 100 mM NaCl, 10 mM KCl and 10⁻³ M ouabain as appropriate.

ouabain. In the first preparation the monovalent cations did stimulate activity by some 45%, and this was partly sensitive to ouabain (29%). In further preparations however only slight stimulation by the monovalent cations, not ouabain-sensitive, was recorded. In some cases the ATPase activity of this lipoprotein fraction closely resembled that of the R2 fraction (e.g. expts. 4 and 5, Table 4:5.).

Discussion

Deoxycholate treatment followed by differential centrifugation did not prove successful in producing a fraction of rat skeletal muscle exhibiting the properties of the NaKMgATPase. The 'R2' fraction, as observed by Rogus, Price and Zierler (1969), contained substantial MgATPase activity, which was inhibited by the further addition of sodium or potassium alone. Contrary to the findings of these authors, however, this MgATPase activity was not further stimulated by the addition of these two monovalent cations together, but was in fact inhibited by such an addition. Furthermore 10^{-3} M ouabain, shown by these authors to completely inhibit the monovalent cation stimulated portion of MgATPase activity, failed to cause any significant alteration in such activity here. Thus it seems that the present preparation, despite the use of techniques apparently identical in all respects to those of Rogus, Price and Zierler, does not contain a demonstrable NaKMgATPase. This was also found by another worker in the laboratory (Radcliffe, 1972). It seemed clear, therefore, that as with the discrepancies reported earlier in the

literature in this area a highly-active consistent NaK₂MgATPase was not demonstrable by this technique. It seemed likely, however, that minor modifications might be successful in revealing such activity.

Ahmed and Judah (1964) had observed that storage of their preparations below 5°C led to a fall in total ATPase activity, but that the decline in activity of the sodium and potassium stimulated portion was less marked, so that there was a proportional increase in this activity. Samaha and Gergely (1965) and Rogus, Price and Zierler (1969) also recorded these changes in ATPase activity. In the present preparation the decline in total ATPase activity was clearly seen with storage, but in fact activity in the presence of sodium and potassium fell even more markedly. This would appear to indicate a proportional increase in MgATPases which were inhibited by the monovalent cations, such as the sodium-inhibited ATPase often seen in heart muscle (e.g. Tashima et al, 1966). The presence of MgATPases which are inhibited by monovalent cations would obviously hinder attempts to demonstrate clear NaK₂MgATPase activity.

Skou (1962) demonstrated that deoxycholate could produce an increase in the NaK₂MgATPase portion of a brain preparation, and the use of DOC and detergents to solubilize components of cell membranes (see Penefsky and Tzagoloff, 1971) has proved useful for preparations from a wide variety of tissues. Samaha and Gergely (1965) and Rogus, Price and Zierler (1969) found that deoxycholate treatment of skeletal muscle would reveal a NaK₂MgATPase but this was clearly not seen in the present study. It is probable that DOC acts by differentially removing protein

and lipid portions of the membrane, at low concentrations removing inactive portions, but at higher ones inactivating the NaKMgATPase itself, though this can be reversed by reconstitution (Philipott, 1968). The extraction concentrations used here covered the range which has been found successful elsewhere. Incubation with DOC has been shown to inhibit NaKMgATPase (Skou, 1962) but the inhibition of activity recorded here is slight and could be attributable to a wide variety of mechanisms. After the completion of this study Jorgensen and Skou (1971) showed that preincubation of rabbit kidney medulla microsomes with concentrations of DOC similar to those used in the present study could produce marked activation of NaKMgATPase after the DOC concentration was reduced. These authors have suggested that DOC activates by exposing latent sites of NaKATPase activity in the preparation, possibly by increasing permeability of the microsomal vesicles to the substrates and activators. It has been demonstrated that DOC does not act by conversion of MgATPase to NaKMgATPase (Ellory and Smith, 1969), hence the demonstration of DOC-exposed NaKMgATPases in skeletal muscle by other authors is not likely to be due to such an action. The reason for it not being found here is unclear, but as several authors have reported inconsistent results with these techniques (e.g. McNamara, Sulakhe and Dhalla, 1971) it seems possible that preparative stages such as homogenisation may well be critical in producing a fraction containing this enzyme without contamination from other fractions. Homogenisation is believed to lead to the formation of small vesicles of outer membrane by a liquid shearing action (Novikoff, 1960). The complexity

of muscle tissue, and the difficulty found in homogenisation, may well lead to contamination of membrane vesicles from several sources. This aspect has been reviewed recently (Wallach and Lin, 1973).

A variety of calcium-activated ATPases have been reported in microsomal extractions from muscle, including one believed to bring about relaxation of muscle by calcium uptake (e.g. Hasselbach and Makinose, 1961; review Sandow, 1970). The CaATPase found in the present preparation was not examined in detail, and may well be a mixture of CaATPases, but with reservations consideration of its response to cations may be informative. As it was stimulated by high calcium levels alone, it was unlikely to be the 'relaxing factor' CaATPase, since this latter requires the presence of added magnesium (Rubin and Katz, 1967; MacLennan, 1970) and is stimulated by much lower calcium levels than used here (Lee, Tanaka and Yu, 1965; MacLennan, 1970). Nor was the present fraction likely to be the CaATPase recorded in mammalian sarcolemma, since it was additive to the MgATPase and was further stimulated by monovalent cations, unlike the sarcolemmal CaATPase (Peter, 1970; McNamara, Sulakhe and Dhalla, 1971). Lee and Yu (1963) found calcium exerted a diphasic effect upon the CaATPase activity of a microsomal preparation from guinea pig heart, with optima at approximately 10^{-6} M and 10^{-3} M calcium concentrations. The lower optimum probably represented the 'relaxing factor' CaATPase whilst the higher, which was stimulated by the addition of 100 mM sodium, closely resembles the CaATPase recorded here. It is possible that the present preparation may have also contained the relaxing factor CaATPase,

and indeed a combination of several ATPases, but this was not further examined.

Using a rat brain microsomal preparation Radcliffe, Duncan and Bowler (1971) were able to demonstrate a quantitatively-varying effect of tannic acid upon the NaKMgATPase and the MgATPase. Concentrations greater than 10^{-7} M tannic acid led to a fall in the activity of the preparation, with MgATPase declining steadily up to 10^{-4} M, where some 70% of the activity remained, whilst the extra activity in the presence of sodium and potassium was not affected up to 10^{-5} M tannic acid, above which it rapidly declined to zero at approximately 5×10^{-5} M tannic acid. In the present preparation activity declined in a similar fashion in the presence or absence of sodium and potassium when exposed to tannic acid. This, together with the failure of ouabain to affect the preparation, would appear to confirm the absence of an active NaKMgATPase activity in this preparation.

Since Ahmed and Judah (1964) demonstrated that ATPase could be floated on sucrose solutions, their suggestion that this may imply the presence of lipoprotein has been verified by others, e.g. Uesugi et al (1969). Some workers with muscle tissue have been able to demonstrate a clear NaKMgATPase activity, after removal of inactive protein, and have used sucrose density gradients to purify their preparations (e.g. Boegman et al, 1970; Kidwai et al, 1973). The failure here to demonstrate such a purification, with the inconsistent results observed, may probably be attributed to a certain extent to the relative crudity of the technique employed, but it also seems probable that adequate separation of inactive protein had not occurred before the preparation was placed on the sucrose solution.

CHAPTER FIVE

SODIUM IODIDE-TREATED PREPARATIONS OF RAT DIAPHRAGM MUSCLE

Section A Preliminary investigations

Introduction

In the light of the difficulty encountered in revealing an NaKMgATPase in rat diaphragm by use of DOC, an alternative method was sought to isolate the enzyme.

In 1963 Nakao et al described the separation of MgATPase from erythrocytes by use of differing concentrations of NaI. Nakao et al (1965) went on to show that exposure of various tissue homogenates to NaI could help in the isolation of NaKMgATPases from various tissues in the rabbit, including cardiac muscle, and Tashima et al (1966) showed that the preparation of NaKMgATPase obtained from cardiac tissue by NaI treatment was in several respects superior to the one derived by the use of deoxycholate.

In view of the success of these authors in isolating a NaKMgATPase from cardiac muscle by use of the NaI, similar experiments were undertaken using the rat diaphragm muscle to see whether skeletal muscle would also respond to such a treatment.

Materials

The rats were identical to those described in the previous chapter, and maintained in a similar fashion. Sodium iodide was obtained from B.D.H. Ltd. ('Analar') and lubrol W flakes were kindly supplied by I.C.I. Dyestuffs Division. All other chemicals were as previously described (Chapter Four).

Methods.

1. Extractions. The extraction methods used were based on those of Ahmed and Judah (1964), accompanied by treatments with sodium iodide developed from those described by Nakao et al (1965).

In preliminary experiments a microsomal fraction corresponding to Ahmed and Judah's 'R1' fraction, prepared as previously described, was exposed to 2 M sodium iodide. The microsomes were suspended in 10 cm³ of neutralized, deionised water to which was added 10 cm³ of a double-strength sodium-iodide solution. This solution was prepared by mixing equal volumes of a solution containing 8 M sodium iodide with one containing 10 mM MgCl₂, 6 mM disodium ATP, and 20 mM EDTA (as the tris salt), the pH being adjusted to 8.0 with Tris-HCl buffer. The microsomes were exposed to this mixture for thirty minutes, after which time 30 cm³ of distilled water was added to give a final sodium iodide concentration of 0.8 M. All these operations were performed in an ice-bath.

The microsomes were centrifuged at 20,000 x g for thirty minutes, and the pellet obtained was resuspended in a washing medium containing 5 mM NaCl, 1 mM EDTA (pH 7.4). Recentrifugation at 20,000 x g followed, and this washing procedure was repeated twice more. The final pellet obtained was suspended in 10 cm³ of neutralized, deionised water and an assay of ATPase activity was performed as described in the previous chapter.

In later experiments the two procedures were reversed, exposure of the whole muscle homogenate to sodium iodide being followed by extraction of the microsomal fractions. The rat

diaphragm was removed, mixed and homogenized in 10 cm³ of a medium containing 0.25 M mannitol, 30 mM histidine, 5 mM EDTA and 0.1% DOC (pH 6.8). To this homogenate was added 10 cm³ of the double-strength sodium iodide medium described above, and the mixture was gently rehomogenised to give a uniform suspension. After a period of exposure to the sodium iodide (usually thirty minutes) in an ice-bath, the homogenate was diluted with distilled water to give a final sodium iodide concentration of 0.8 M. Two centrifugations at 10,000 x g for twenty minutes each followed to remove the 'mitochondrial' fraction, then a 'heavy microsome' fraction was obtained by centrifugation for thirty minutes at 50,000 x g. A 'light microsome' fraction was separated by submitting the supernatant of the 50,000 x g centrifugation to a further centrifugation of 100,000 x g for one hour. These two microsome fractions were then washed three times as described above and the final pellets again suspended in 10 cm³ of neutralized, deionized water.

2. Incubations. Incubation techniques were similar to those described in the previous chapter. Aliquots of 2 mM ATP (0.5 cm³) were added to 1 cm³ of medium containing 60 mM imidazole-HCl buffer (pH 7.4) 0.2 mM EDTA, and twofold concentrations of MgCl₂, NaCl and KCl (and any other additions) as appropriate. Incubations were started by addition of 0.5 cm³ of enzyme preparation to tubes after pre-equilibration to 37°C for ten minutes, and continued for one hour.

3. Assay procedures. The amount of inorganic phosphate released during the incubation was measured by a procedure developed from that of Atkinson and Gatenby (private

communication)(see Atkinson, 1970). Incubations were terminated by the addition to each tube of 4 cm³ of a mixture containing 1% lubrol W and 1% ammonium chloride in 1.8 N sulphuric acid at room temperature. The tubes were then left at room temperature for ten minutes before transfer to an ice-bath, during which time development of a yellow colouration indicated the presence of inorganic phosphate. Absorption by these solutions at 390 nm was measured on a Hilger-Watts spectrophotometer, and calibration measurements performed using standard phosphorus solutions. Activity calculations using this technique exactly matched those made by the procedure of Fiske and Subba Row previously described.

Protein concentrations were again determined by the method of Lowry et al (1951).

Results

The results of the preliminary experiments in which the whole microsome fraction (equivalent to Ahmed and Judah's 'R1' fraction) was exposed to the sodium iodide treatment can be seen in Table 5:1. In a series of such experiments increased activity could be seen when either Na⁺ or K⁺ was present in addition to Mg⁺⁺, but the synergistic stimulation typical of NaKMgATPase activity was not seen. Furthermore it was found difficult to harvest a pellet after exposure to the sodium iodide, due to the number of centrifuge tubes in which the preparation was dispersed prior to the final centrifugations.

In all further experiments other techniques were tried, submitting the whole homogenate to the sodium iodide procedure. The ATPase activities of typical microsomal fractions obtained after such treatments can be seen in Table 5:2.

TABLE 5 : 1

Effect of NaI treatment of rat skeletal muscle microsomes
(R1 fraction) on ATPase activity

Incubation cations			ATPase activity
Mg ⁺⁺	Na ⁺	K ⁺	m.moles Pi h ⁻¹ g ⁻¹ protein
-	+	+	0
+	-	-	39.4 (100)
+	+	-	57.1 (145)
+	-	+	53.0 (134)
+	+	+	51.5 (131)

R1 fraction exposed to 2 M NaI for 30 min at 0°C.

Incubations performed at 37°C in 30 mM Tris-HCl
(pH 7.4), 0.1 mM EDTA, 2 mM ATP, plus 3 mM MgCl₂,
100 mM NaCl, and 10 mM KCl as appropriate.

Figures in parentheses are activity expressed as
% activity in presence of Mg⁺⁺ only.

TABLE 5 : 2.

ATPase activity of microsomal fractions from NaI-treated
homogenates of rat diaphragm muscle

Incubation additions				ATPase activity	
Mg ⁺⁺	Na ⁺	K ⁺	ouabain	10,-50,000 x g	50,100,000 x g
+	-	-	-	51.0 (100)	14.4 (100)
+	+	-	-	47.6 (93)	11.8 (82)
+	-	+	-	47.3 (93)	11.8 (82)
+	+	+	-	54.1 (106)	21.7 (151)
+	+	+	+	49.5 (97)	13.6 (95)

Whole diaphragm homogenate exposed to 2 mM NaI at 0°C for
60 mins, then heavy microsomes (10,000 - 50,000 x g) fraction
and light microsomes (50,000 - 100,000 x g fraction) obtained.

Values expressed as m. moles Pi h⁻¹ g⁻¹ protein.

Figures in parentheses are % activity in presence of Mg⁺⁺ only.

Incubations performed at 37°C for 60 mins in 30 mM Tris-HCl
(pH 7.4), 0.1 mM EDTA, 2 mM ATP plus 3 mM MgCl₂, 100 mM NaCl,
10 mM KCl and 10⁻³ M ouabain as appropriate.

In the heavy microsome fraction (10,000 -50,000 x g fraction) addition of either 100 mM Na⁺ or 10 mM K⁺ resulted in slightly reduced levels of ATPase activity compared with that in the presence of Mg⁺⁺ alone, whilst in the presence of both monovalent cations the level of activity was slightly enhanced. In the light microsome (50,000 - 100,000 x g fraction), however, though there was still slight inhibition of ATPase activity by either monovalent cation alone, when both Na⁺ and K⁺ were present there was a marked stimulation, of the order of 50%, of this activity. This stimulation could be completely negated by the presence of 10⁻³ M ouabain in the incubation; thus it appeared that the preparations contained an enzyme which exhibited the properties of a NaKMgATPase.

Some further investigations were made in an attempt to increase the NaKMgATPase activity relative to the MgATPase activity. Prolonging storage of the homogenised preparation prior to the addition of sodium iodide failed to produce any significant increase in the activity rate. Storage for 24 hours at -20°C reduced overall ATPase activity by some 40% in the light-microsomal fraction though the stimulation by Na⁺ and K⁺ was unaffected. Prolongation of exposure of the homogenate to sodium iodide did increase the activity ratio, but at the expense of total ATPase activity (Table 5:3). With an exposure time of thirty minutes to 2 mM sodium iodide ATPase activity in the presence of Na⁺, K⁺ and Mg⁺⁺ was over twice that in the presence of Mg⁺⁺ alone, but after 24 hours storage of the preparation in 2 M sodium iodide the ratio had increased to about six to one. There had been a marked reduction in

TABLE 5 : 3

Effect of prolonged exposure to 2 mM NaI on ATPase activity
of rat diaphragm muscle

Exposure to NaI (hours)	Incubation Mg	cations MgNaK	Activity ratio $\frac{\text{MgNaK}}{\text{Mg}}$
0.5	13.35	28.72	2.15
1.0	13.40	26.90	2.01
24	0.51	3.04	5.96
40	0.27	2.02	7.48
48	0.23	1.75	7.61

Whole diaphragm homogenate exposed to 2 M NaI, then light
microsome fraction (50,000 - 100,000 x g) obtained for
activity determinations. Activity expressed as
m.moles Pi h⁻¹ g⁻¹ protein. Incubations performed at
37°C for 60 mins in 30 mM Tris-HCl (pH 7.4), 0.1 mM EDTA,
2 mM ATP, plus 3 mM MgCl₂, 100 mM NaCl and 10 mM KCl as
appropriate.

activity however, with activity in the presence of Mg^{++} being only some 4%, and with Mg^{++} , Na^+ and K^+ some 9% of the original values. Further extension of exposure time increased this ratio still further whilst again decreasing overall ATPase activity.

Attempts to store the final preparation of light-microsomes at $-20^{\circ}C$ proved unsuccessful, so it was found that the ATPase activity of the preparation in the presence of Mg^{++} , Na^+ and K^+ fell to only 10% of the values measured in the fresh preparation within 24 hours.

Section B Studies on the NaKMgATPase in the light microsomal fraction of NaI-treated rat diaphragm muscle

Introduction

It seemed likely that the results obtained in the previous section could be explained best by the presence of a MgATPase and a NaKMgATPase in the light microsomal preparation.

A standard preparation procedure was now adopted, and some studies of the properties of the preparation were undertaken in an attempt to determine whether a NaKMgATPase was indeed present, and to try to characterize this enzyme to some degree.

Materials and Methods

The rats used were described in the previous chapter, and all reagents have been described earlier.

1. Extractions. The rat diaphragm was excised, homogenized, exposed to 2 M sodium iodide for thirty minutes and then a 'light microsome' fraction extracted as described in the previous section. The final pellet obtained was resuspended in up to 20 cm^3 of neutralized, deionised water.



2. Pre-incubations. In a series of experiments to determine the effect of rose bengal upon the preparations, the entire 10 cm^3 of preparation was added to 10 cm^3 of 60 mM imidazole buffer (pH 7.4) containing 3.33×10^{-6} M rose bengal in a foil-wrapped, stoppered tube. Subsequent operations were performed in a dark-room under illumination by light reflected from a single, dim safety-light. 0.5 cm^3 aliquots of preparation were added to 1 cm^3 aliquots of incubation medium in a glass cuvette and placed in a light-tight box containing a tungsten-filament bulb, which could be moved to alter the intensity of light falling on the cuvettes. Light intensities of 500, 1,000, 2,000 and 3,000 lux on the cuvettes were calibrated by means of an E.E.L. Lightmaster photometer.

After pre-incubations, performed at room temperature, the contents of the cuvettes were transferred to foil-wrapped, stoppered incubator tubes on ice prior to incubation.

3. Incubations. Incubations were performed as in the previous section. In those incubations involving rose bengal incubation was started by the addition of 0.5 cm^3 aliquots of ATP; elsewhere incubation was started by the addition of the enzyme preparation.

4. Assay procedures. As described in section 5A.
N.B. In this section the ATPase activity in the presence of Mg^{++} as the sole cation will be referred to as 'MgATPase' activity; activity in the presence of Mg^{++} , Na^+ and K^+ will be referred to as 'total ATPase' activity, whilst 'NaMgATPase' activity will be calculated from 'total ATPase' minus 'MgATPase' activities.

Results

Although the preparative technique had been standardized there remained a wide variation in the overall ATPase activity of the preparation when expressed as millimoles of inorganic phosphate released per gram protein per hour. Table 5:4 shows the activities measured in fourteen different preparations. Whilst the mean values in the Table reflect the type of activation by Na^+ and K^+ generally found in these preparations, the total ATPase activities ranged from 50% to 200% greater than the MgATPase activity figure. This made direct comparison of results from two preparations extremely difficult, hence indirect comparisons were generally used when attempting to qualify the effects of various conditions upon the ATPase activities (see below). Typically, though, the NaKMGATPase (total ATPase minus MgATPase) activity was greater than the MgATPase activity, giving an activity ratio of between 1:1 and 1.5:1.

Because of the relatively large number of incubation tubes used in each experiment, hence the necessity for increased dilution of the preparation, incubation periods of one hour were generally used. To establish that the ATPase activities were in fact constant over that period of time, a series of incubations of different time-periods were tried (see fig. 5:1) which revealed that the rates of ATP breakdown were indeed linear over the one hour of incubation for both the MgATPase and the NaKMGATPase.

In further characterizations it proved desirable to combine the results from several preparations, often with

TABLE 5 : 4.

ATPase activity of 'light microsomes' from NaI-treated
homogenates of rat diaphragm

ATPase	Activity m.moles Pi h ⁻¹ g ⁻¹ protein
MgATPase	10.42 ± 0.77 (14)
Total ATPase	21.98 ± 1.75 (14)
NaKMgATPase	11.56 ± 1.19 (14)
Activity ratio $\frac{\text{NaKMgATPase}}{\text{MgATPase}}$	1.125 ± 0.09 (14)

Values expressed as means ± S.E.M., based on determinations
from fourteen different preparations.

Incubations performed in 30 mM imidazole (pH 7.4),
0.1 mM EDTA, 2 mM ATP plus 3 mM MgCl₂, 100 mM NaCl,
and 5 mM KCl as appropriate.

All incubations for 1 hour at 37°C.

Fig. 5 : 1

MgATPase and NaKMGATPase activity of rat diaphragm
muscle light microsomes

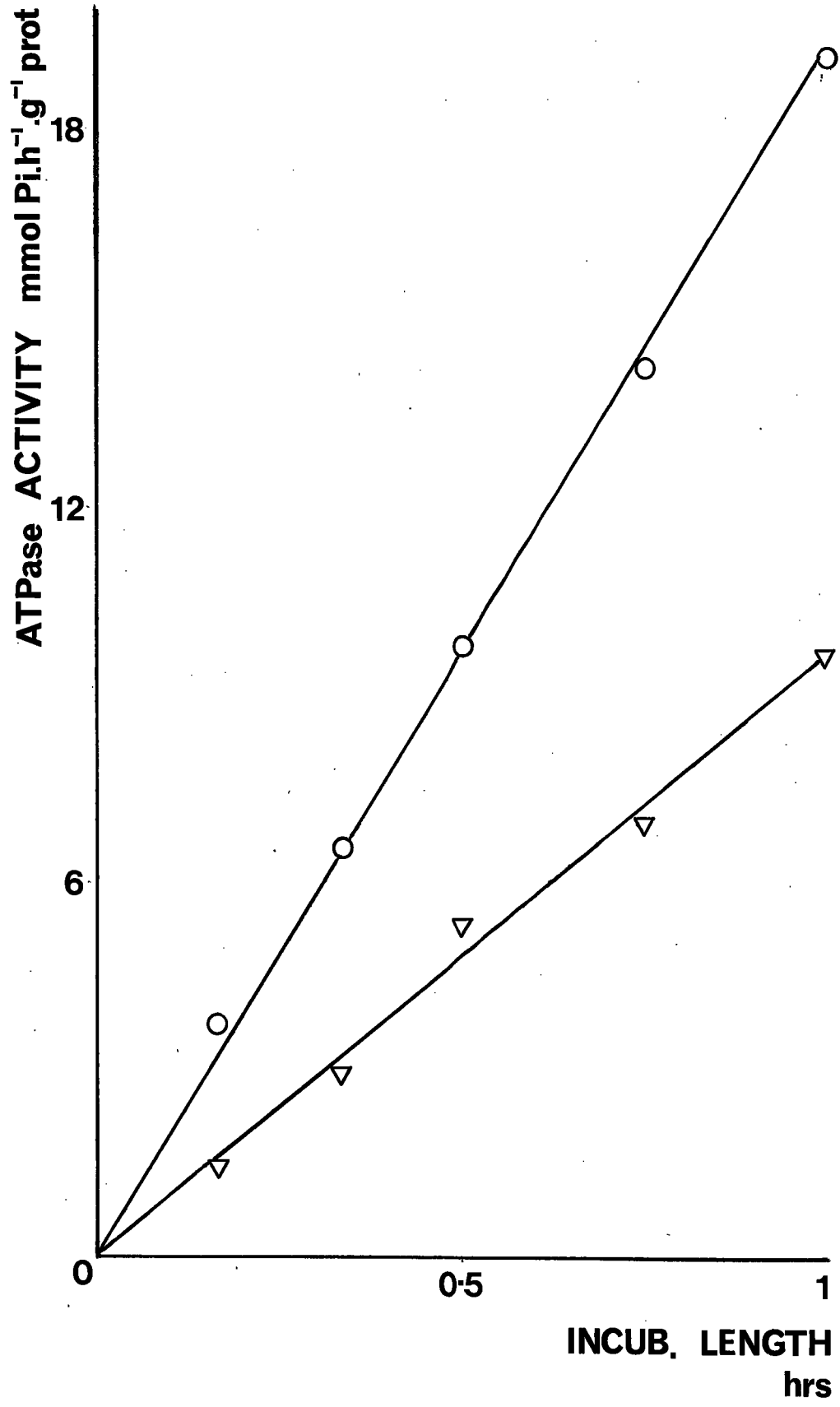
Each point is a mean value of four determinations.

Preparation of light microsomes from NaI-treated
homogenate of rat diaphragm muscle.

Incubations at 37°C in presence of 30 mM imidazole
(pH 7.4), 0.1 mM EDTA, 2 mM ATP, plus

▽ 3 mM MgCl₂

○ 3 mM MgCl₂, 100 mM NaCl, 5 mM KCl.



varying activity levels and ratios. Consequently the results given are either expressed as a percentage of the maximum activity observed or as a percentage of the activities recorded in the absence of a reagent.

Previous experiments had demonstrated that the ATPase activity of the preparation was not stimulated by the presence of Na^+ or K^+ alone, but by the presence of both cations, and tests were performed to discover the optimum concentrations of these cations to give the greatest stimulation of NaKMgATPase activity. As Table 5:5 shows, the highest stimulation of activity was found when the Na^+ concentration was 100 mM and the K^+ concentration was 5 mM, although greatly stimulated activity could be seen in a wide range of Na^+ and K^+ concentrations. These concentrations - 100 mM Na^+ and 5 mM K^+ - were adopted as the standard concentrations used in those incubation tubes where total ATPase activity was being measured for all the determinations in this section.

The effect of adding ouabain, ranging in concentration from 10^{-9} to 10^{-3} M, to the incubation medium was then measured, and the results are shown in fig. 5:2. Concentrations of below 3.3×10^{-8} M failed to inhibit activity of either the MgATPase or the NaKMgATPase. Higher concentrations produced an increasing inhibition of NaKMgATPase until at a concentration of 3.3×10^{-4} M it was abolished completely. 50% inactivation was produced by a concentration of 5×10^{-7} M. Although in some instances there appeared to be slight, though insignificant, falls in MgATPase activity, this enzyme was probably basically unaffected by the presence of the ouabain.

TABLE 5 : 5

Effect of Na^+ and K^+ on NaKMgATPase activity
of rat diaphragm muscle

		Relative NaKMgATPase activity (as % optimum)						
Na^+ conc. (mm)	K^+ conc. (mm)	25	50	75	90	100	110	120
1.25		-	-	-	59	64	47	49
2.5		-	-	-	90	86	83	63
5.0		76	87	91	90	<u>100</u>	96	95
10.0		56	68	84	89	92	90	84
25.0		37	47	65	-	72	-	-
50.0		35	44	54	-	62	-	-

NaKMgATPase activity expressed as a percentage of maximum activity observed.

Incubations for 1 hr at 37°C , in 30 mM imidazole (pH 7.4), 0.1 mM EDTA, 2 mM ATP plus 3 mM MgCl_2 . NaCl and KCl were added to give appropriate concentrations.

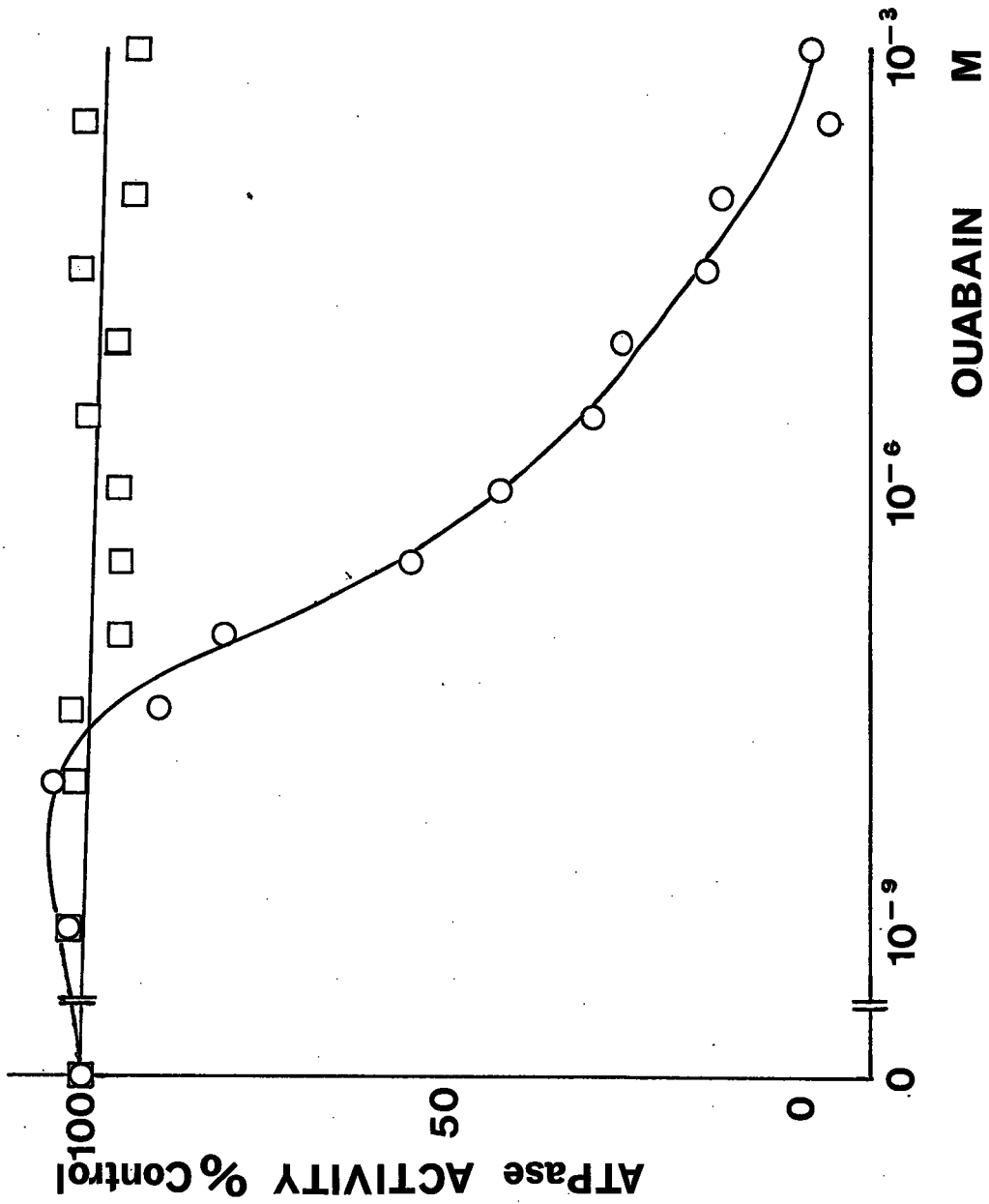
Fig. 5 : 2. Effect of ouabain on MgATPase and NaKMgATPase activity of rat diaphragm light microsomes

Each point is a mean value of four determinations.

Incubations were performed for 1 hour at 37°C, in the presence of 30 mM imidazole (pH 7.4), 0.1 mM EDTA, 2 mM ATP, plus

□ 3 mM MgCl₂

○ 3 mM MgCl₂, 100 M NaCl, 5 mM KCl.



The effect of varying the concentrations of Mg^{++} and ATP in the incubation upon the MgATPase and NaKMgATPase activity can be seen in fig. 5:3. Both enzymes showed activity variations that were dependent upon not only the concentration of these two substances but also on the relationship between them. In the case of the MgATPase, whilst the highest activity was recorded at concentrations of 4 mM Mg^{++} , 6 mM ATP, activity was almost equally as high in 6 mM Mg^{++} , 6 mM ATP. The NaKMgATPase had a measured optimum of 4 mM Mg^{++} , 4 mM ATP, and increasing ATP concentration to 6 mM caused a reduction in activity to some 80 - 90% of the maximum. The standard concentrations of 3 mM Mg^{++} , 2 mM ATP used in the other determinations performed in this section would appear therefore to be close to the optimum for the NaKMgATPase.

The optimum pH for the NaKMgATPase also approximated to that used throughout the incubations. As fig. 5:4 reveals, the NaKMgATPase had a measured optimum of pH 7.5, with a sharp decline in activity in acid conditions until at pH 5 there was no measurable activity by this enzyme: there was also a sharp decline in activity of this enzyme as alkalinity increased. In the case of the MgATPase increased pH appeared to lead to increased activity, with optimum activity recorded at pH 9, the highest tested. Furthermore the decline in activity with increased acidity was relatively slight, and appreciable activity was still present at pH 5. Control measurements showed that acid hydrolysis of ATP, noticeable at pH 5.5, increased markedly below this value, making accurate determinations of enzyme hydrolysis difficult.

Fig. 5 : 3. Effect of Mg^{++} and ATP concentrations on MgATPase and NaKMgATPase of rat diaphragm muscles

MgATPase and NaKMgATPase values expressed as a percentage of the maximum observed activity, each point being the mean of four determinations.

All incubations in the presence of 30 mM imidazole (pH 7.4), 0.1 mM EDTA (Tris salt), plus

△ 1 mM $MgCl_2$

○ 4 mM $MgCl_2$

▽ 2 mM $MgCl_2$

□ 6 mM $MgCl_2$

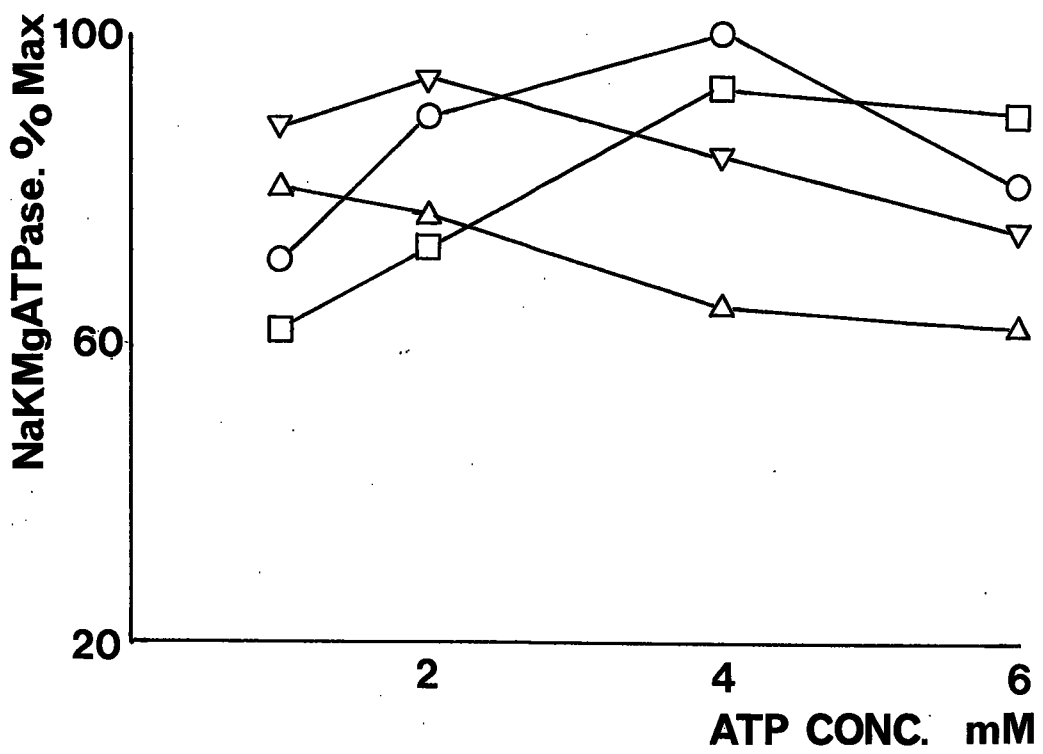
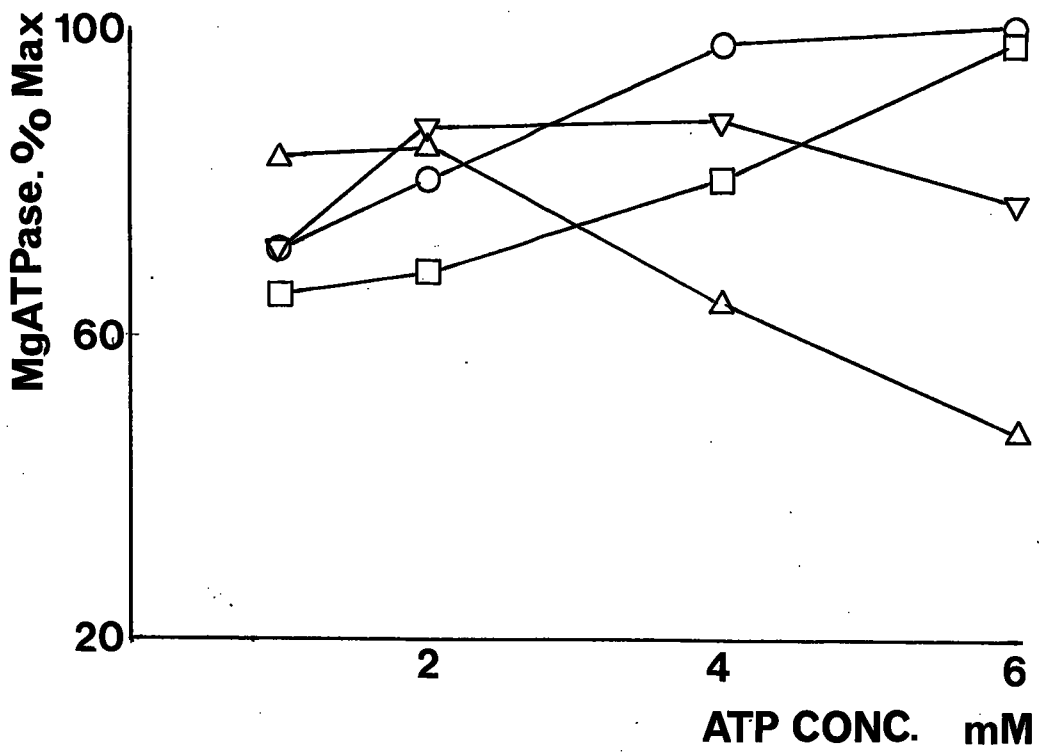


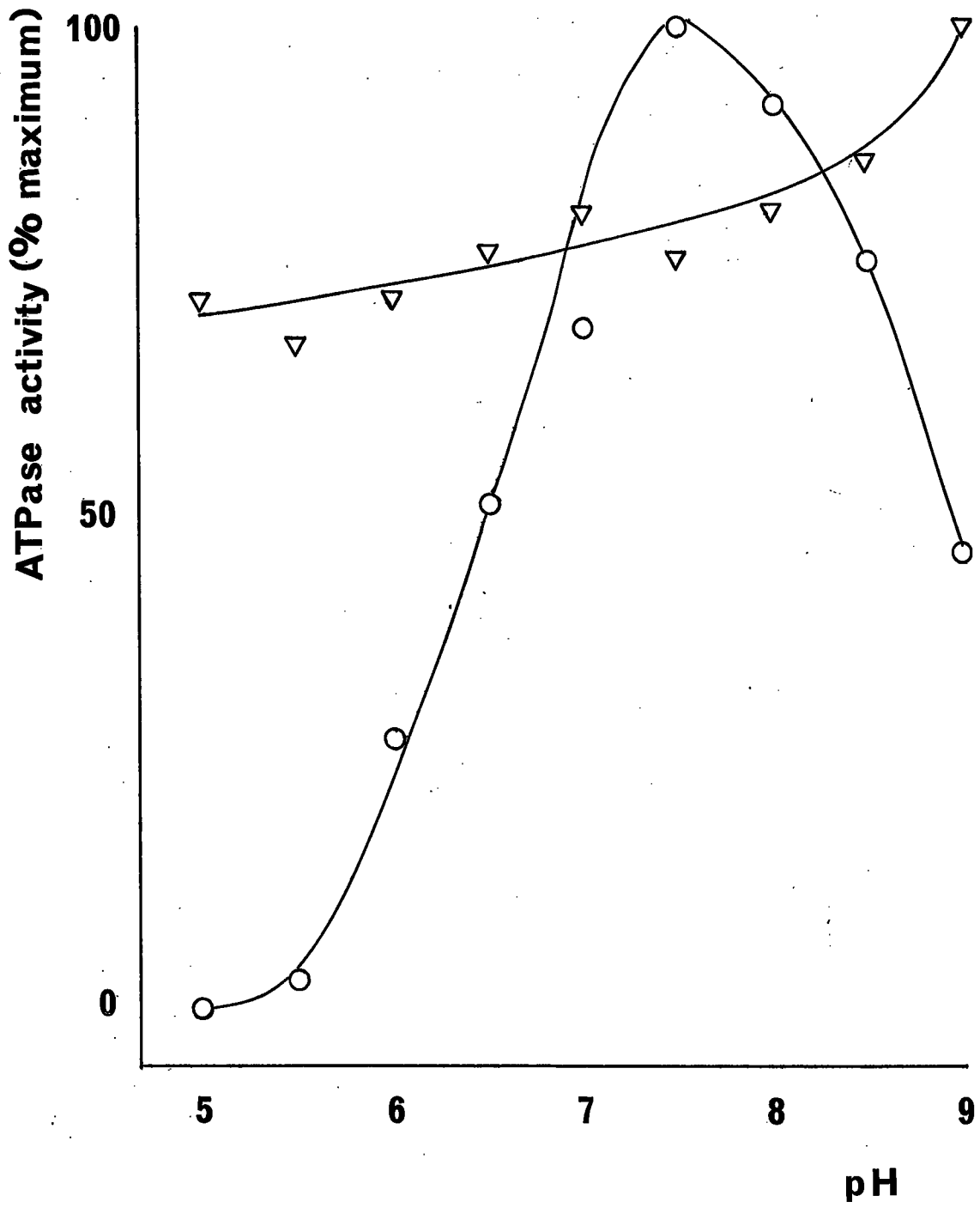
Fig. 5 : 4. Effect of pH on MgATPase and NaMgATPase activities of rat diaphragm muscle

MgATPase and NaMgATPase values expressed as a percentage of maximum observed activity, each point being the mean of four determinations.

All incubations in the presence of 30 mM imidazole (pH 7.4), 0.1 mM EDTA (Tris salt), 2 mM ATP, plus

▽ 3 mM MgCl₂

○ 3 mM MgCl₂, 100 mM NaCl, 5 mM KCl.



Preliminary investigations of the effect of ethacrynic acid on the two ATPases revealed that marked inhibition of NaKMgATPase was only achieved by high concentrations of the acid. (Fig. 5:5) At concentrations of 10^{-3} M and 10^{-9} M ethacrynic acid there appeared to be slight stimulation of the NaKMgATPase activity but this was almost certainly due to an excessively low MgATPase baseline recorded at these concentrations. Higher concentrations of the acid caused a gradually increasing inhibition of the NaKMgATPase activity until at 10^{-4} M it was some 68% of that recorded in the absence of ethacrynic acid. At 10^{-3} M ethacrynic acid there was a much greater inhibition of the NaKMgATPase to about 6% of its unmodified level. Despite fluctuations in the results, ethacrynic acid appeared to have no effect on the MgATPase.

The effects of rose bengal and illumination upon the preparation were measured by preincubations in varying illumination intensities, and then incubation as usual for one hour in complete darkness. Preliminary experiments showed that 3.33×10^{-6} rose bengal did not affect either MgATPase activity or NaKMgATPase activity in the absence of illumination, nor did exposure of the preparation to the levels of illumination, in the absence of rose bengal, which were used in the subsequent tests. The combined effects of rose bengal and illumination at various intensities can be seen in fig. 5:6. At each intensity tested it became apparent whilst 3.3×10^{-6} M rose bengal did inhibit the MgATPase activity, this inactivation never exceeded some 30% of the control activity, though the length of exposure required to

Fig. 5 : 5 Effect of ethacrynic acid on MgATPase
and NaKMgATPase activities of rat
diaphragm muscle

MgATPase (Δ — Δ) and NaKMgATPase (\bigcirc — \bigcirc) activities
expressed as a percentage of the activity observed in the
absence of ethacrynic acid.

Incubation medium contained 30mM imidazole (pH 7.4),
0.1mM EDTA (tris salt), 2mM ATP, plus :-

Δ 3mM $MgCl_2$

\bigcirc 3mM $MgCl_2$, 100mM NaCl, 5mM KCl

All incubations at 37°C for 1 hour.

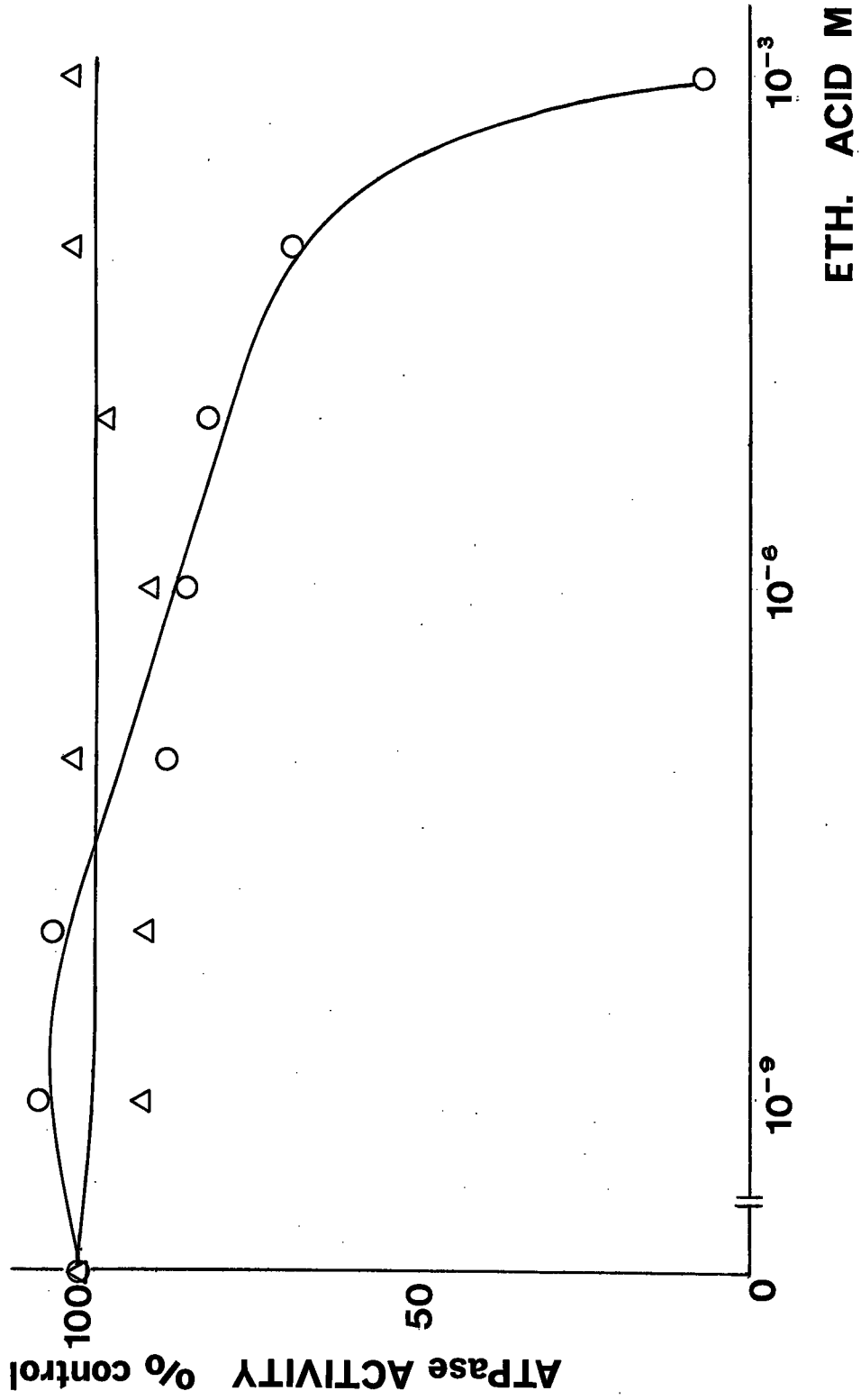


Fig. 5 : 6. Effect of rose bengal and illumination on MgATPase and NaKMgATPase activity of rat diaphragm muscle

- A - at 500 lux illumination
- B - at 1,000 lux illumination
- C - at 2,000 lux illumination
- D - at 3,000 lux illumination

MgATPase (□—□) and NaKMgATPase (○—○)

activities expressed as % residual activity after exposure to varying light intensities in the presence of 3.33×10^{-6} M rose bengal.

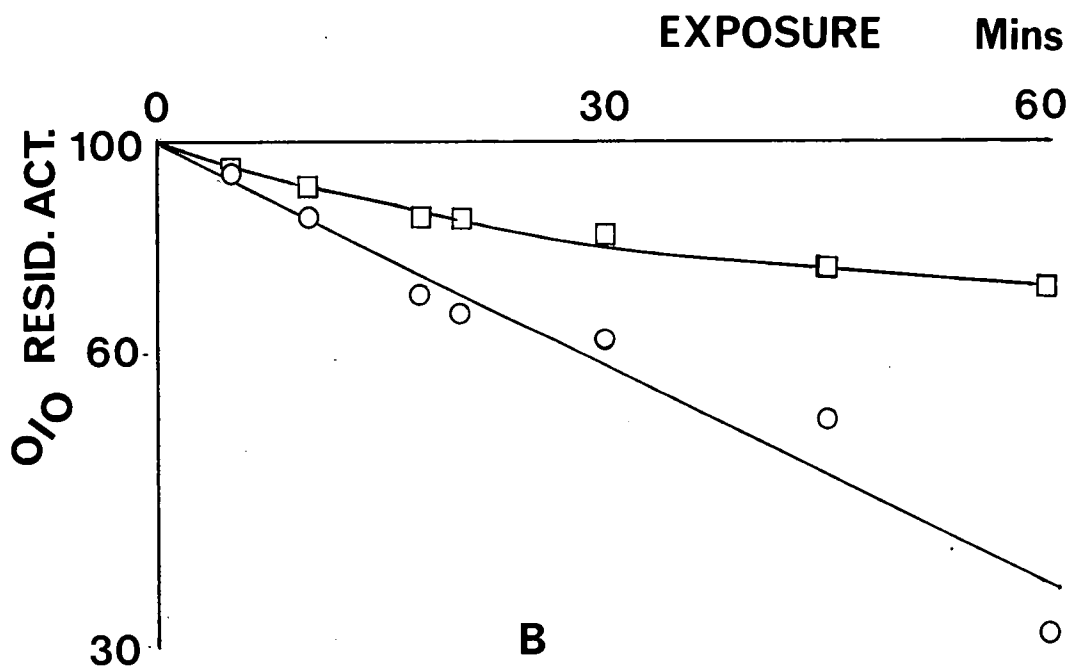
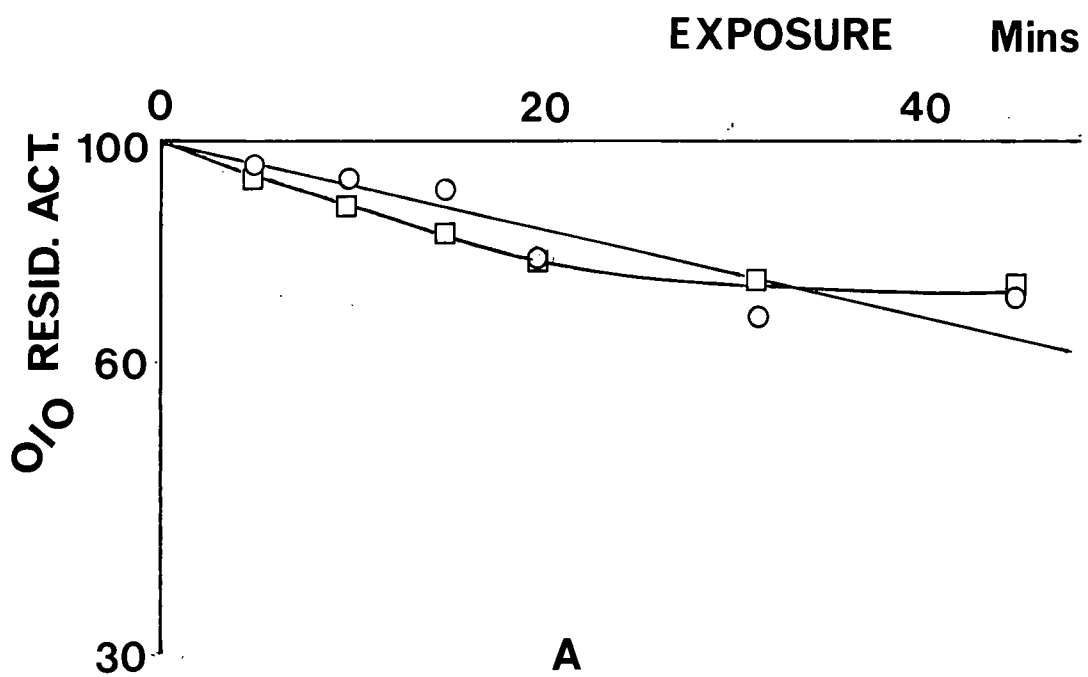
Exposure and incubation in 30 mM imidazole (pH 7.4), 0.1 mM EDTA, plus

□ 3 mM MgCl_2

○ 3 mM MgCl_2 , 100 mM NaCl, 5 mM KCl.

Exposure at room temperature (c 21°C).

Incubation at 37°C for one hour, started by addition of 2 mM ATP.



reach this level of inactivation did generally decrease as the intensity of illumination increased. In the case of NaKMgATPase activity, however, when plotted as percentage residual activity on a logarithmic scale against time of exposure there was an obviously linear relationship, with the rate of fall of NaKMgATPase activity being greatest at the highest light intensity. When the data is expressed as a double log. plot of the reciprocal of the intensity of illumination and the time for 30% inactivation of the NaKMgATPase a linear relationship is revealed (fig. 5:7), the importance of which will be discussed below.

Section C. Electron-microscopy of isolated fractions of rat diaphragm muscle

Introduction

It was clear that NaI treatment of rat diaphragm muscle homogenate prior to centrifugation led to the production of a light microsomal fraction which exhibited the properties of a NaKMgATPase, whilst a similar fraction isolated from homogenates which had not been exposed to the NaI did not reveal such an enzyme.

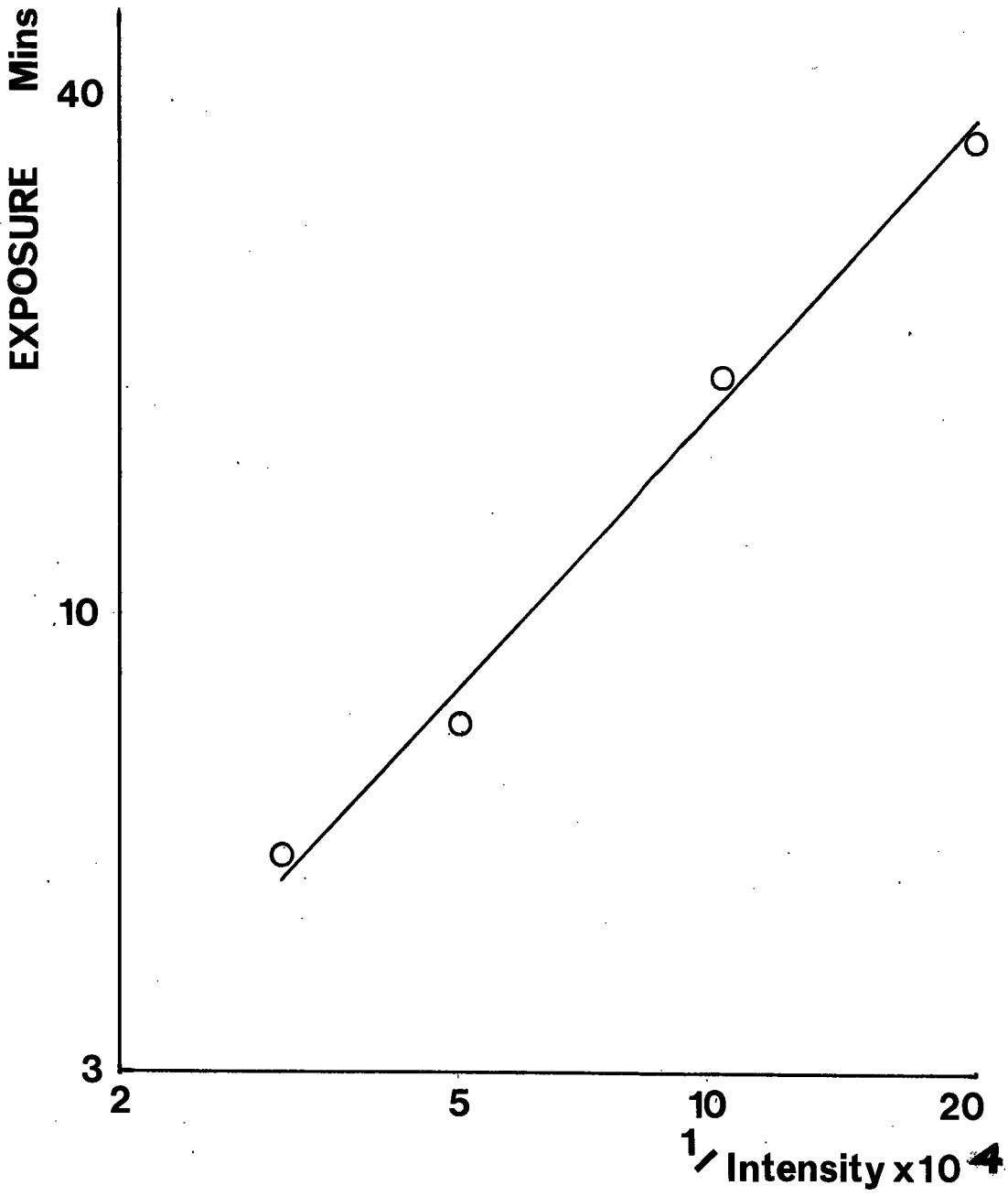
Since the NaKMgATPase is known to be membrane-bound, it seemed likely that the NaI treatment must in some way be altering the membranes in the light microsomal fraction, either by alteration of the membrane structure itself or by altering the type of membrane isolated in this fraction. An electron-microscopical study was undertaken to examine the fractions

Fig. 5 : 7. Double log. plot : reciprocal of intensity
of illumination against time, for 30% inactivation of
NaKMgATPase activity of rat diaphragm muscle

Values derived from expts. shown in fig. 5:6.

Rose bengal concentration 3.33×10^{-6} M,

light intensities 500, 1,000, 2,000 and 3,000 lux.



isolated from NaI-treated and non-NaI-treated preparations to see whether there were obvious physical differences in the membranes isolated.

Materials

The rats and reagents used were as described in the previous sections.

Epon 812, dodecenylsuccinic anhydride hardener 964 (DDSA), methyl nadic anhydride (MNA), 2,4,6-tri(dimethylaminomethyl)phenol (DMP 30) and sodium cacodylate were obtained from TAAB Laboratories. Osmium tetroxide was from Johnson Mathey Ltd. All these chemicals were specifically prepared for use in electron microscopy. Propylene oxide (1,2, epoxypropane) and alcohol were obtained from British Drug Houses Ltd. and were reagent grade.

Methods

A rat diaphragm was removed, divided into two, and then each half was minced and homogenised as previously described. One half was exposed to sodium iodide as usual, whilst the other remained untreated, and 10,000 x g, 50,000 x g and 100,000 x g fractions were prepared.

The pellets were each resuspended in 5 cm³ of fixative containing 1% osmium tetroxide and 0.1 M sodium cacodylate adjusted to pH 7.2 by HCl, and recentrifuged for one hour to ensure formation of a hard, discrete pellet for subsequent treatment, as described by Glauert (1965).

After further fixation for one hour, the pellets were removed whole and washed in 0.1 M sodium cacodylate buffer for thirty minutes. Dehydration through a series of alcohols

followed - fifteen minutes in 50% ethanol, overnight in 70% ethanol, fifteen minutes in 95%, and thirty minutes each in two changes of absolute alcohol.

The pellets were broken into pieces approximately 2 mm square and embedded in epon following the method of Luft (1961). They were first soaked in two changes of propylene oxide for fifteen minutes each, and then overnight in a mixture of 50% propylene oxide, 50% epon. The epon was made up as follows:-

Mixture A	Epon 812 (62 cm ³)	Mixed in equal proportions
	DDSA (100 cm ³)	
Mixture B	Epon 812 (100 cm ³)	2% DMP 30 added to final mixture
	MNA (89 cm ³)	

The specimens were left overnight in epon solution and incubated at 60°C for forty-eight hours. Thin sections of about 75 nm were cut on a Reichert OmU2 ultratome, and examined on an A.E.T. 8012 electron microscope.

Results

The mitochondrial fractions (10,000 x g) of both preparations were a heterogenous collection of mitochondria and general debris, and there was no obvious difference between the two (see Plates 5:1 and 5:2). Whilst many of the mitochondria appeared to be highly intact, there were some which had been broken, and the upper portion of the mitochondrial pellet of both NaI-treated and non-NaI-treated preparations contained some large fragments of double-membrane which were probably broken portions from mitochondria.

The heavy microsomal fractions (10,-50,000 x g) were vesicular, and careful examination failed to reveal portions

which were obviously mitochondrial in origin. Comparison of the NaI-treated and non-NaI-treated preparations showed that in the former (plate 5:3) the vesicles were generally larger than those in the latter (plate 5:4) and were much less regular in appearance and size. The occurrence of 'double vesicles', i.e. vesicles which had formed within another vesicle, was also more frequent in the NaI-treated preparation, and some had inclusions which may have indicated a mitochondrial origin, though, as mentioned above, the clear double-membrane seen in the mitochondrial fraction (plate 5:1) was not seen. Both pellets were virtually homogenous, though some non-vesicular debris was found at the base of each, which probably indicated contamination from the previous fraction. There was a general decrease in average vesicular size from the base to the top of the pellets.

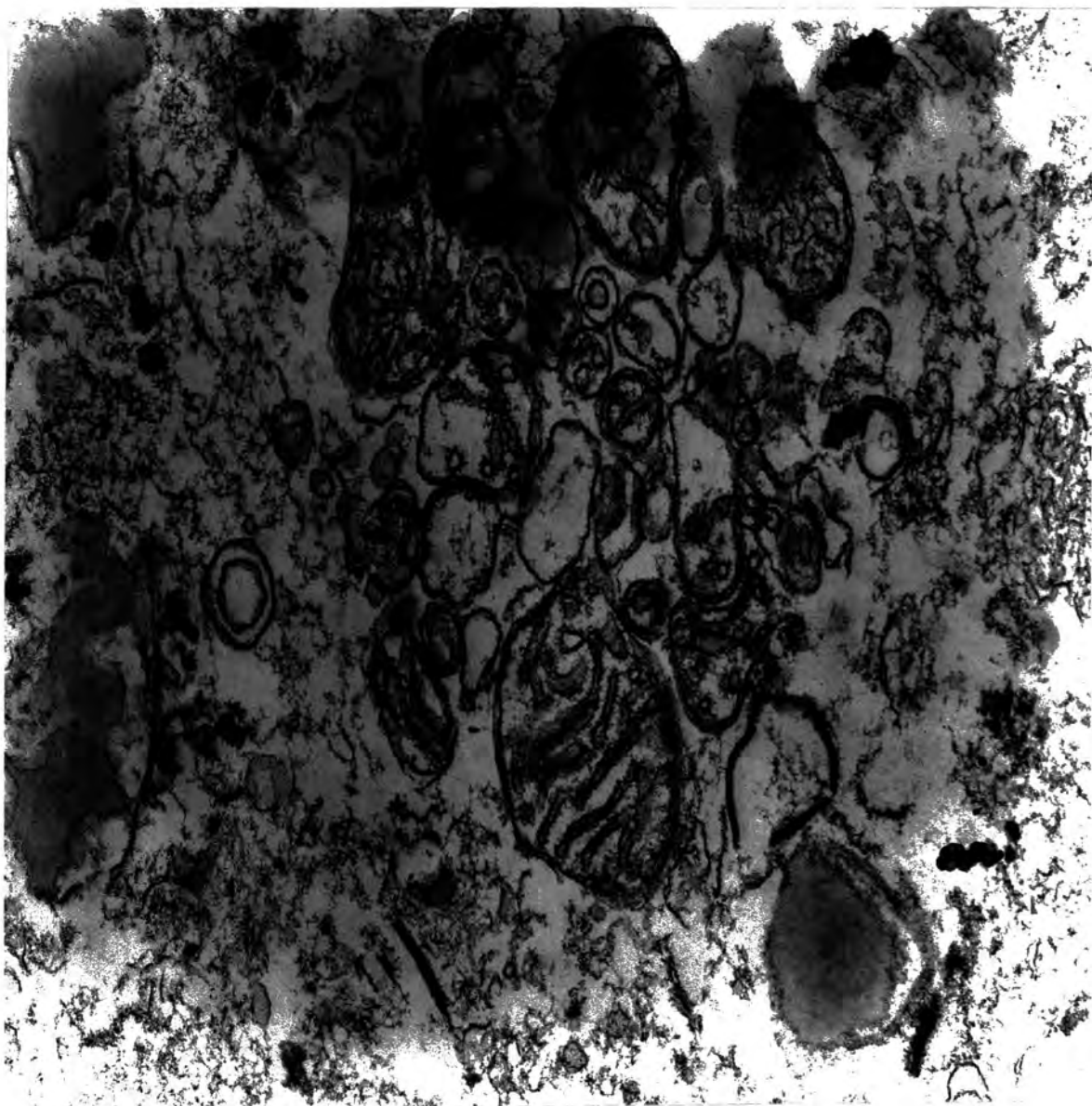
The light microsomal fractions (50,-100,000 x g) were clearly dissimilar in the NaI-treated and non-NaI-treated preparations. This fraction in the NaI-treated membranes was composed largely of vesicles which resembled those of the heavy microsome fraction, though of a smaller size (plate 5:5). There also occurred at the upper surface, and to some extent among the vesicles, a certain amount of material which appeared slightly filamentous but generally granular, without clear structure, and definitely not membranous, probably being protein material which had been precipitated along with the microsomes. In the non-NaI-treated fraction there also occurred vesicles, but these were much smaller than those seen in the NaI-treated preparation and were relatively regular in size (plate 5:6). There also occurred amongst these vesicles membrane fragments

which appeared to have formed into fine tubules, incomplete in some cases, a formation which was not seen in the NaI-treated fraction. The non-membranous material deposited along with the light microsomes was indistinguishable from that seen in the NaI-treated fraction. Light microsomal fractions of both preparations failed to show any clear indication of mitochondrial contamination.

Discussion

It was clear from the preliminary investigations (Section A) that whilst NaI treatment of the microsomes led to monovalent cation-stimulated ATPase activity, either cation alone was equally effective and the synergistic stimulation of ATPase activity by Na^+ and K^+ which characterises the NaKMgATPase was not seen. Rogus, Price and Zierler (1969) found that treatment of their microsomal pellet with 2 M NaI apparently solubilised protein rich in MgATPase, since the proportion of NaKMgATPase to MgATPase was increased with this treatment whilst the protein content declined. Tashima et al (1966) also found that a cardiac microsomal preparation yielded a NaKMgATPase after treatment with 2 m NaI which resembled that obtained by treatment of the whole homogenate. Since there was no demonstrable NaKMgATPase present in the microsomal fraction before treatment with NaI, it is possible that the microsomes have differed in origin or activity from those prepared by the above authors, hence no NaKMgATPase was present. An alternative possibility is that the treatment with 2 M NaI was not effective in the present preparation in separating the MgATPase-rich protein which therefore remained to obscure

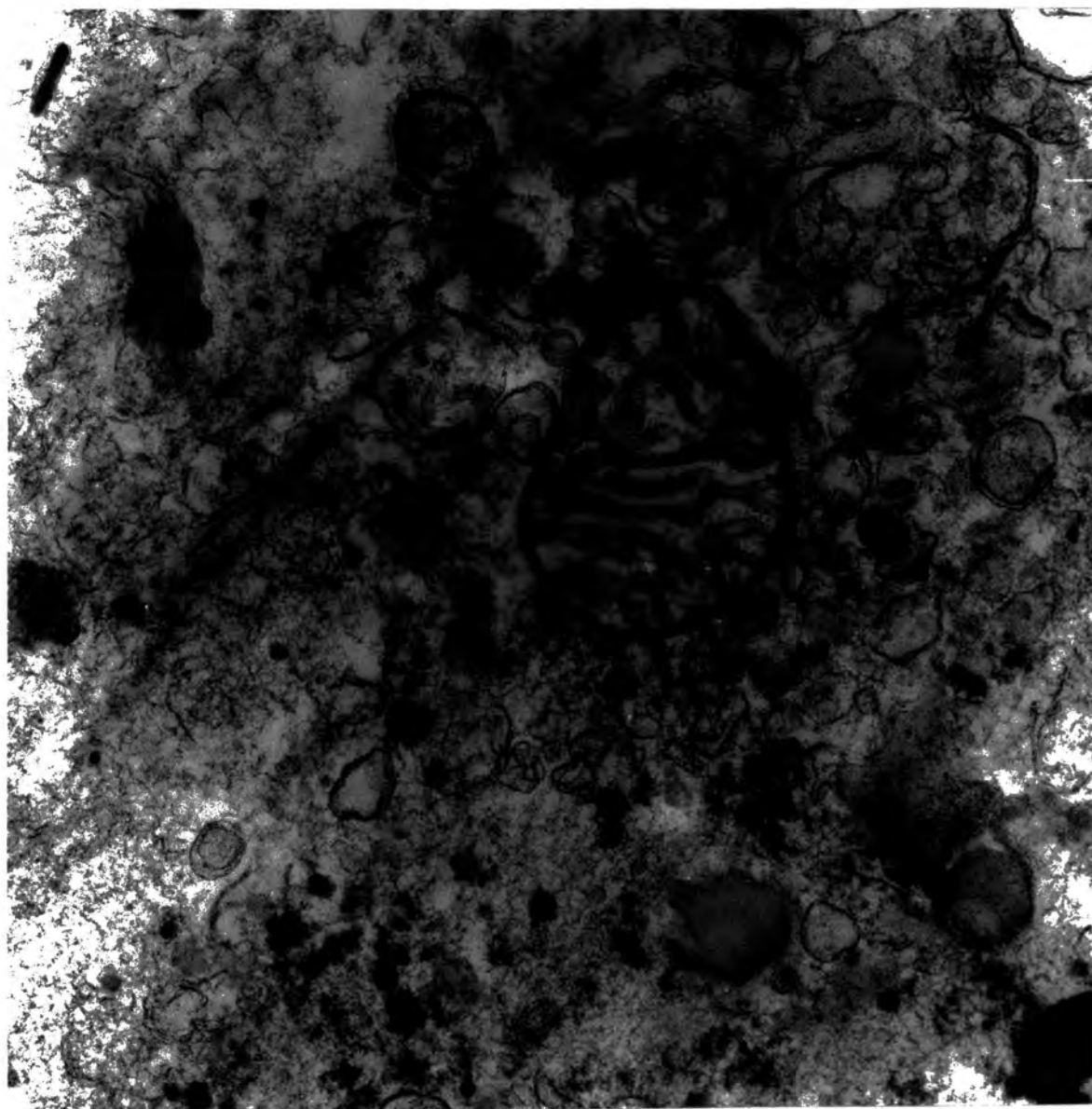
PLATE 5:1 Electronmicrograph of 'mitochondrial' fraction
from NaI-treated preparation of rat diaphragm
muscle



x 25,000

10,000 x g fraction from muscle exposed to NaI, prepared
as described in Methods

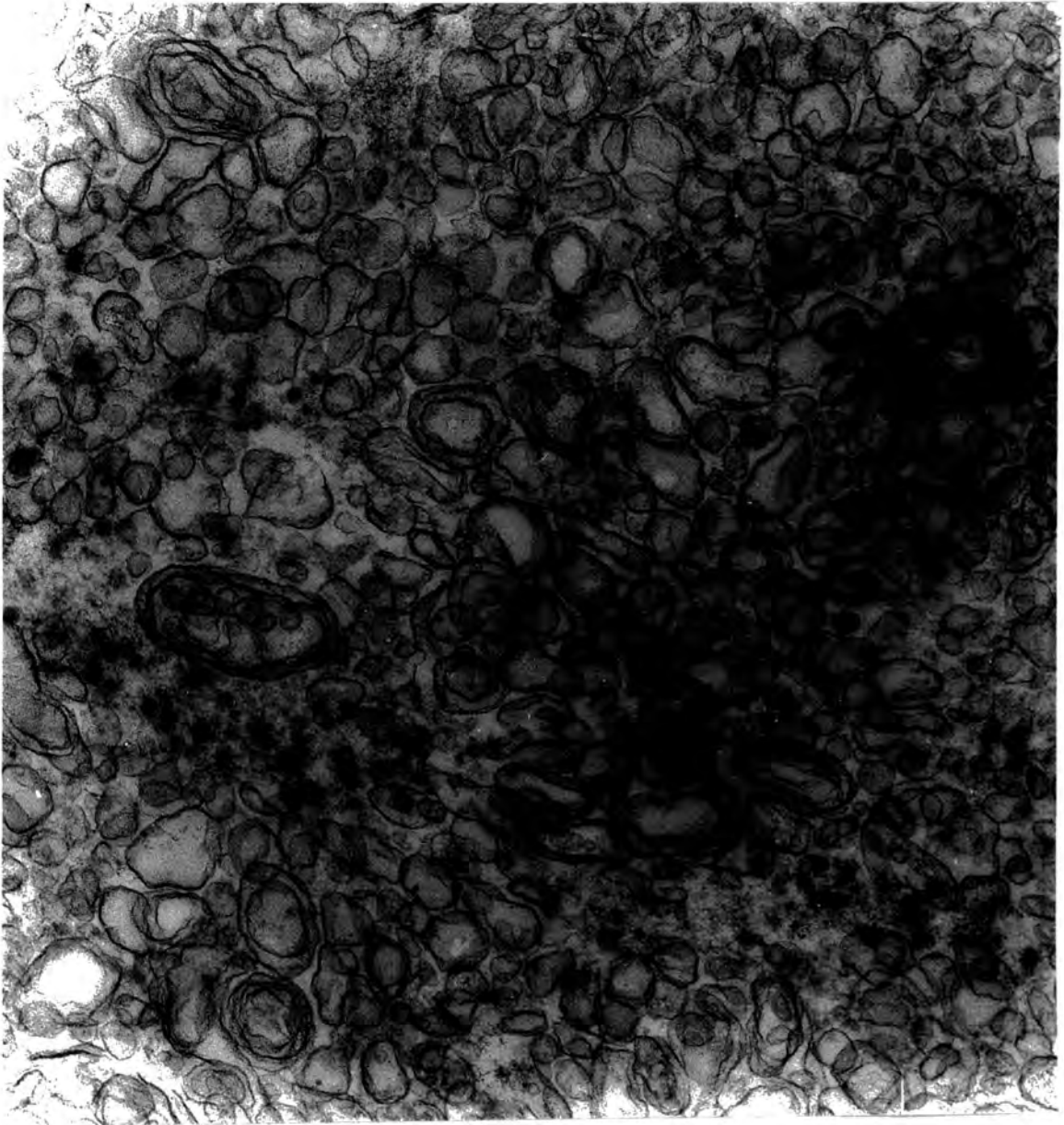
PLATE 5:2 Electronmicrograph of 'mitochondrial' fraction
from non-NaI-treated preparation of rat diaphragm
muscle



x 25,000

10,000 x g fraction from muscle prepared without
NaI exposure, otherwise treated as described in Methods.

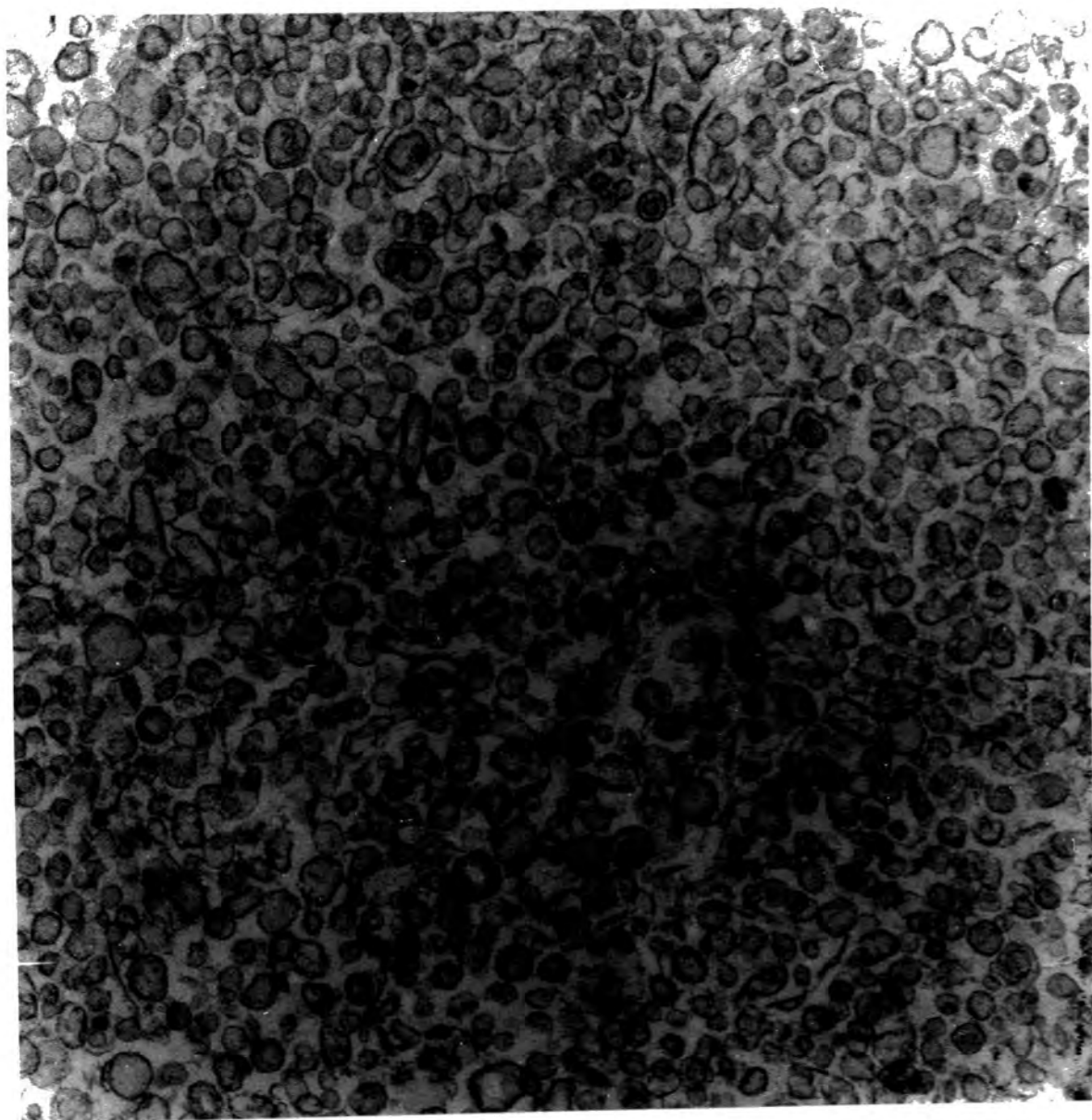
PLATE 5:3 Electronmicrograph of 'heavy microsomal' fraction
from NaI-treated preparation of rat diaphragm muscle



x 25,000

10,000 - 50,000 x g fraction from muscle exposed to NaI,
prepared as described in Methods.

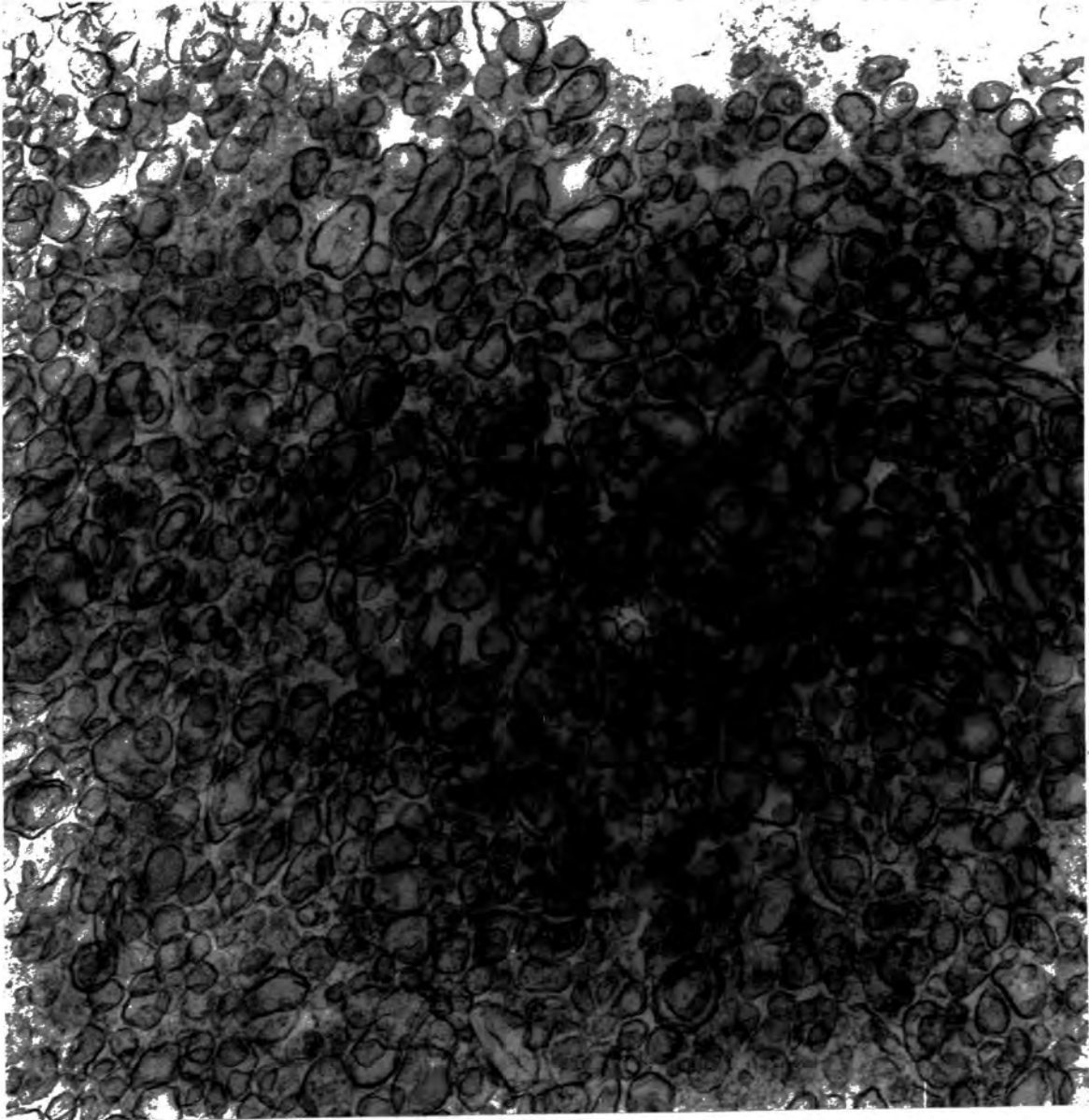
PLATE 5:4 Electronmicrograph of 'heavy microsomal' fraction
from non-NaI-treated preparation of rat diaphragm
muscle



x 25,000

10,000 - 50,000 x g fraction from muscle prepared without
NaI exposure, otherwise treated as described in Methods.

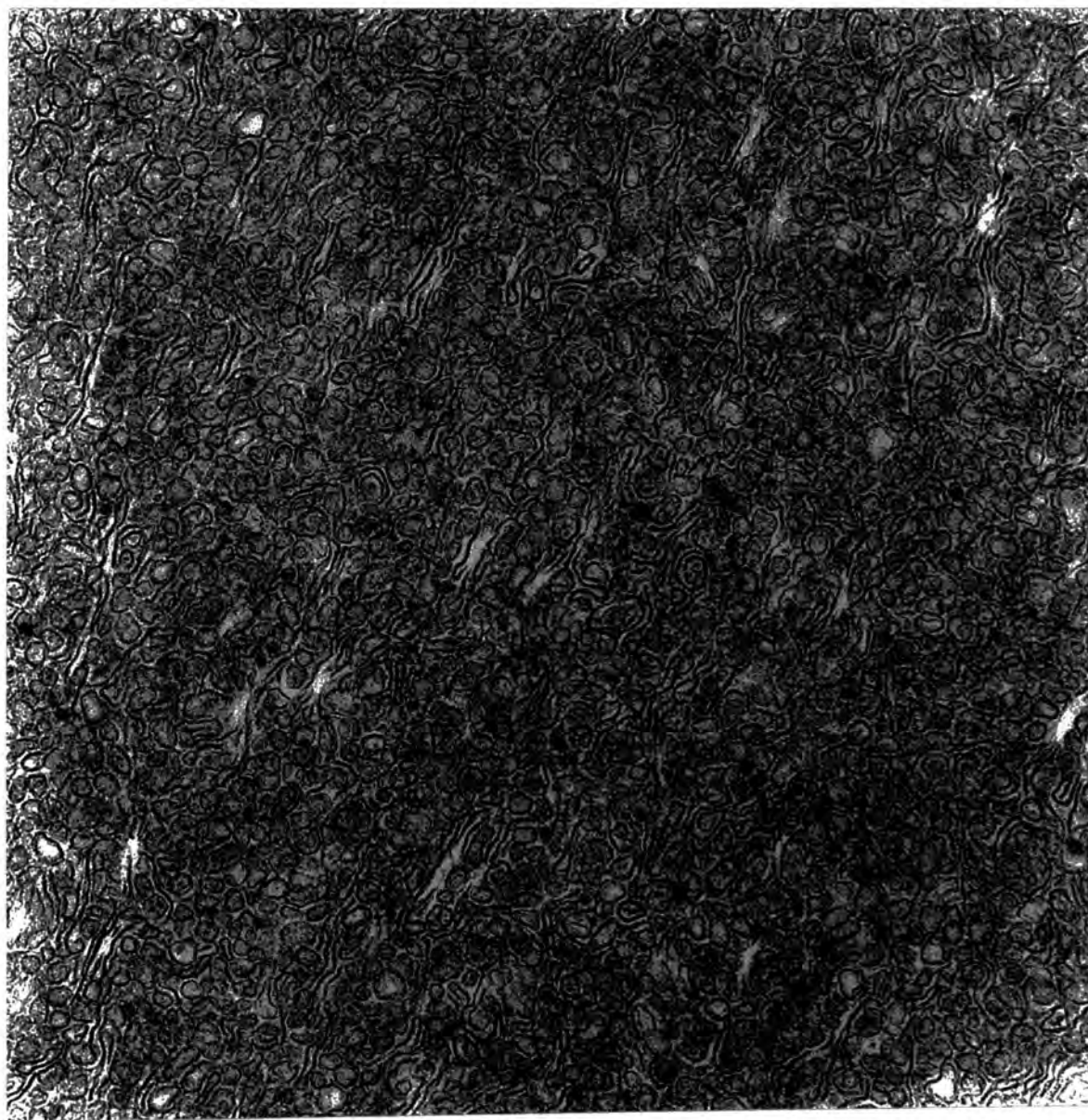
PLATE 5:5 Electronmicrograph of 'light microsomal' fraction
from NaI-treated preparation of rat diaphragm muscle



x 25,000

50,000 - 100,000 x g fraction from muscle exposed to
NaI, prepared as described in Methods.

PLATE 5:6 Electronmicrograph of 'light microsomal' fraction
from non-NaI-treated preparation of rat diaphragm
muscle



x 25,000

50,000 - 100,000 x g fraction from muscle prepared without
NaI exposure, otherwise treated as described in Methods.

NaKMgATPase activity. Whilst the latter explanation is rendered less likely by the successful isolation of NaKMgATPase by the same technique from whole homogenates, it is supported by the presence of detectable monovalent-cation stimulated activity following NaI treatment of the microsomes. It is noticeable, however, that the ouabain-sensitive NaKMgATPase derived from the whole homogenate was not stimulated by the presence of either Na^+ or K^+ alone, hence even in this respect the activity recorded in NaI-treated microsomes more closely resembles the single-monovalent cation-stimulated activity recorded elsewhere (e.g. Duggan, 1965; Engel and Tice, 1966) than the NaKMgATPase.

The Na^+ and K^+ -stimulated activity from the NaI-treated homogenates in the preliminary investigations much more closely resembled the classical NaKMgATPase, consequently it was this preparation which was selected for more detailed study (section B).

The specific activities of the ATPases recorded were similar to those measured in a similar preparation from cardiac muscle by Tashima et al (1966); MgATPase of 10.1 and NaKMgATPase of 9.1 compared with values of 10.4 and 11.6 m.moles $\text{Pi h}^{-1} \text{g}^{-1}$ protein respectively found in the present preparation. Both the specific activity of the NaKMgATPase and the NaKMgATPase : MgATPase activity ratio of this preparation are markedly higher than those seen in the DOC preparation of Rogus, Price and Zierler (1969), which had an MgATPase of 6.5 and an NaKMgATPase of 4.4 m.moles $\text{Pi h}^{-1} \text{g}^{-1}$ protein. Whilst this study was in progress other workers reported the successful isolation of a NaKMgATPase fraction from skeletal muscle by exposure to high salt concentrations. Boegman et al (1970)

were able to separate MgATPase and NaKMgATPase activities in frog skeletal muscle by means of high salt concentrations followed by density gradient centrifugation, and Ash and Schwartz (1970) used 0.6 M KCl treatment to obtain NaKMgATPase activity in a fraction of rat skeletal muscle. The NaKMgATPase activity recorded by these authors, $9.0 \text{ m.moles Pi h}^{-1} \text{ g}^{-1}$ protein is slightly lower than that found with the present preparation, and the high MgATPase recorded by these authors ($19.7 \text{ m.moles Pi h}^{-1} \text{ g}^{-1}$ protein) meant that the activity ratio of their preparation was relatively low.

It has been common practice to store NaKMgATPase preparations at -20°C , when it has generally been found that activity has remained steady for quite a time. Indeed, even where a decline in ATPase activity has been recorded it has often been noted that the MgATPase is more sensitive than the NaKMgATPase; thus prolonged storage could lead to improved activity ratios (e.g. Rogus, Price and Zierler, 1969). The present preparation, however, showed a marked decline in activity when stored at 5°C , or -20°C , for periods as short as twenty-four hours. This was also found to be the case for the similar preparation of Ash and Schwartz (1970) who noted that though the preparation was stable for several hours at 0°C , overnight storage led to reduced activity levels.

Activation by monovalent cations (Table 5:5) resembled that recorded for many other preparations (review, Skou, 1965), with optima of c. 100 mM NaCl and 5 mM KCl. The preparation of Rogus, Price and Zierler (1969), whilst similar in overall pattern of response to monovalent cations, had a measurable optimum of approximately 50 mM NaCl, 10 mM KCl, and the decline

in activity away from this optimum was less marked than that seen in the present preparation. Samaha and Gergely (1965) reported an optimum of cation-stimulated activity at values of some 120 mM NaCl, 20 mM KCl for NaKMgATPase from human striated muscle.

That the present preparation contained a true NaKMgATPase was strongly reinforced by the response to ouabain (fig. 5:2). The curve resembled that of Samaha and Gergely (1965) and was virtually identical with that recorded by Rogus, Price and Zierler (1969), who showed inhibition of NaKMgATPase by ouabain concentrations greater than 10^{-8} M, with 50% inhibition occurring between 10^{-7} and 10^{-6} M and 100% inhibition at about 10^{-3} M. These authors also recorded, as was found here, slight activation of NaKMgATPase activity by ouabain concentrations lower than 10^{-8} M. Whilst the level of activation found here was very low, and could be ascribed to experimental error, activation by low concentrations of ouabain, below 10^{-3} M, has been reported in the literature (Lee and Yu, 1963; review Lee and Klaus, 1971).

The response of the enzyme to varying concentrations of Mg^{++} and ATP also resembled the characteristic response to an NaKMgATPase. The finding that activity was generally greater when the Mg^{++} and ATP concentrations were equal is in agreement with other preparations (e.g. Bourgoignie et al, 1969).

Charnock and Potter (1969) have shown that the incorporation of P^{32} from γ -ATP 32 into the NaKMgATPase proceeded most quickly when the Mg^{++} :ATP ratio was 1:1, and it has been suggested that a Mg^{++} -ATP complex may be the substrate for the reaction (Epstein and Whittam, 1966).

The effect of pH upon the preparation also resembled that

of the classic NaKMgATPase, with a pH optimum of approximately 7.5 (review, Skou, 1965). Tashima et al (1966) observed a similar pattern of NaKMgATPase activity with an optimum activity of c pH 7.5, with a marked fall at pH 5.5 and pH 9, and of MgATPase activity which increased up to pH 9, for their cardiac NaKMgATPase preparation, and basically similar patterns were recorded for skeletal muscle preparations by Samaha and Gergely (1966) and Rogus, Price and Zierler (1969), though the latter measured a pH optimum of 7.0.

The response, therefore, of the present preparation to variations in monovalent cations, ouabain, Mg^{++} , ATP and pH was in accordance with the presence in the preparation of the MgATPase and NaKMgATPase combination now isolated from a wide variety of tissues. Whilst others (Samaha and Gergely, 1965; Rogus, Price and Zierler, 1969) have isolated such a preparation using DOC, their techniques have not easily been duplicated, and in view of the present findings, and those of Boegman et al (1970), it would seem that the use of high salt concentrations is a more reliable method than DOC to reveal the NaKMgATPase. Others have used the technique described here and have isolated a NaKMgATPase, where other techniques have failed to do so, from chicken skeletal muscle (Wareham, 1971) and the hindgut of insects (Peacock, ~~Bowler~~ and Anstee, 1972). In the light of the findings of Peter (1970) that an isolated preparation of sarcolemma from skeletal muscle contains NaKMgATPase, it may be that the role of the NaI is to solubilize away other portions of the muscle homogenate which will contain high levels of ATPase activity which mask the presence of the NaKMgATPase.

Since Duggan and Noll (1965) demonstrated that the

NaKMgATPase from guinea pig renal cortex was inhibited by the diuretic ethacrynic acid, many other workers have been able to demonstrate this inhibitory effect on NaKMgATPase preparations. In view of its efficacy as a diuretic, work has largely centred on preparations from renal material (e.g. Banerjee et al, 1970; Charnock et al, 1970) but it has also been shown to inhibit NaKMgATPases from other tissues, including turtle bladder epithelium (Bourgoignie et al, 1969), insect hindgut (Peacock et al, 1972) and canine cardiac muscle (Song and Scheuer, 1968), as well as reducing active sodium efflux from a wide variety of tissues. Whilst only preliminary investigations were made here, the pattern of inhibition of NaKMgATPase by ethacrynic acid matched that recorded elsewhere. Values of ethacrynic acid above 10^{-7} M appeared to slightly inhibit activity, marked inhibition was seen at 10^{-4} M, and 10^{-3} M produced 94% inhibition. The requirement for high levels of ethacrynic acid ($> 10^{-4}$ M) before marked inhibition of NaKMgATPase is seen has been generally reported (e.g. Bourgoignie et al, 1969; Charnock et al, 1970), though Song and Scheuer (1968) reported that a concentration of 1.66×10^{-5} M ethacrynic acid caused an inhibition of 37% in their cardiac preparation. Pre-incubation of the enzyme with the diuretic has been reported to increase inhibition (Duggan and Noll, 1965; Banerjee, Khanna and Sen, 1970); whilst there is still controversy as to whether the inhibition of NaKMgATPase is responsible for the diuretic action of ethacrynic acid (review, Suki et al, 1973) it is clear that in the present preparation, as in many others, high concentrations of the diuretic will cause such inhibition.

Both the MgATPase and the NaKMgATPase were also inhibited

by photooxidation, using rose bengal, in a similar fashion to that described for these activities in erythrocyte ghosts (Duncan and Bowler, 1969). The enzymes in the present preparation were not as sensitive as those in the erythrocyte, the NaKMgATPase being more sensitive than the MgATPase which only appeared to decline by some 30%. As was found by Duncan and Bowler (1969) the photodynamic inhibition of NaKMgATPase appeared to follow the Schwartzchild equation: $I \times t^p = K$ (where I = light intensity, t = time for 30% inactivation, K and p are constants) which was developed to describe photodynamic effects on photographic plates (Schwartzchild, 1899). The value of p found in the present preparation was 1.3, which is rather nearer unity, and the Bunsen-Roscoe reciprocity law, than the 1.5 found for the erythrocyte ghosts (Duncan and Bowler, 1969). This value of 1.3 matches that found by Blun and Hyman (1939) for photodynamic haemolysis of erythrocytes. The Schwartzchild equation has also been said to describe the photodynamic effect of rose bengal on whole skeletal muscle (Lillie et al, 1935).

Thus the response of the monovalent-cation stimulated MgATPase activity to both ethacrynic acid and rose bengal is similar to the responses of NaKMgATPases recorded from other preparations, giving further support to the hypothesis that the activity recorded here represents the existence of such an NaKMgATPase.

The examination of the fractions by means of the electron microscope revealed that both NaI-treated and non-NaI-treated light microsome fractions were composed mainly of vesicles of smooth membrane. Whilst many of the mitochondria seen in the mitochondrial preparation appeared to be relatively intact,

there was certainly some damage to mitochondria which may have led to contamination by vesicles of mitochondrial membrane in the light and heavy microsome fractions. The origin of the vesicle fractions is difficult to determine since it is not possible to distinguish between the plasma membrane, sarcoplasmic reticulum, smooth endoplasmic reticulum, outer mitochondrial membranes and nuclear membranes with negative staining. The non-NaI-treated light microsome fraction resembles in appearance the 'sarcotubular' fraction of Muscatello et al (1962), which was composed of similar vesicles and contained numbers of 'tubules'. In view of the complexity of the muscle tissue, and the difficulty of homogenising it, it seems likely that the vesicles originate from several sources. The different sizes of vesicle seen in the non-NaI-treated and NaI-treated preparations may well in part be attributable to the greater density of the NaI mixture even after dilution, and this may also explain the absence of the fine tubules in the NaI-treated light microsome fraction. In view of the solubilisation effect of the NaI, however, it may be that treatment with this salt has led to changes in the structure or type of membrane isolated. In view of Peter's discovery of NaKMgATPase activity in the sarcolemmal tubule emptied of contents (Peter, 1970), the general finding that the NaKMgATPase is located in the plasma membrane (review, Whittam and Wheeler, 1970), and the lack of demonstrable NaKMgATPase activity in the non-NaI-treated preparation, it is tempting to speculate that the action of NaI may lead to solubilization of other fractions of homogenate so that the NaI light microsome fraction contains at least a large proportion of plasma membrane whose NaKMgATPase is not marked by other ATPases in other membrane fragments.

CHAPTER SIXTHE NaKMgATPase and RAT DIAPHRAGM MUSCLE

The successful isolation of a fraction of rat diaphragm muscle which exhibited the characteristic properties of the NaKMgATPase confirmed the hypothesis that this enzyme was present in mammalian skeletal muscle, playing a role in cation regulation. It seems probable, however, that it is the complexity of muscle tissue, with its several 'compartments' and variety of transport ATPases, that makes estimations of the extent and importance of the role of the 'sodium pump' extremely difficult.

Assuming that the action of ouabain in the 'in vitro' experiments in Chapter Two is solely that of an NaKMgATPase inhibitor, it is clear that normal functioning of the 'sodium pump' is essential if the low intracellular sodium content is to be maintained. As mentioned in Chapter One, muscle is believed to maintain a constant electrochemical gradient for sodium, and the uptake of sodium in the presence of ouabain seen in Chapter Two would indicate that the NaKMgATPase must normally have exercised a regulatory function, operating according to the levels of sodium on either side of the muscle membrane to help maintain this gradient. This is in accordance with the role of the 'sodium pump' in other tissues.

The discovery that it was not possible to demonstrate clearly the activity of this pump by monitoring oxygen consumption was at first surprising considering the widespread success of this technique when used on other tissues. As has been discussed in Chapter Three, there are several possible reasons for this, but the fact that the pump is 'powered' by ATP, which is found in muscle 'in vivo', and hence presumably 'in vitro' in the conditions

used here, and the relatively low proportion of the 'energy budget' of respiring muscle needed for sodium pumping would seem to be likely contributors.

This issue is further complicated by the fact that the tissue used in these studies, the rat diaphragm, though ideal in many respects for 'in vitro' studies, cannot be considered as a uniform tissue but rather an 'organ' composed of a variety of muscle-types with varying glycolytic, oxidative and ATPase enzyme activities. Furthermore, there may well have been maturational changes occurring between the ages of the rats used in these 'in vitro' studies and those used in the later work on the isolated fractions, changes which might have involved both oxidative metabolism and ATPase activity.

It has long been realised that 'skeletal muscle' may be in fact a mixture of tissue types. Difference in colour, 'red' and 'white', were described by Kühne as long ago as 1865, and Ranvier detected variations in contraction speeds in 1873. The heterogeneity of mammalian skeletal muscle was described by Grützner in 1884, and since this time a great deal of work has been concentrated upon characterizing the various types of muscle fibres (for general reviews see Peachey (1968), Sandow (1970), Close (1972), Fuchs (1974)).

Various morphological and cytochemical factors have been used in the characterization processes, notably fibre diameter, glycolytic and oxidative enzyme activities, ATPase activities, in addition to physiological characteristics such as contraction speed and other constants, and electrical phenomena, e.g. membrane conductance, resistance, etc. Histochemically, fibres have been

divided into two types based on reciprocal differences in glycolytic and oxidative enzyme activities (Dubowitz and Pearse, 1960) and differences in myosin ATPase activities (Engel, 1962), though later work has tended to point to three main fibre types (e.g. Padykula and Gauthier, 1967). Whilst the work is hindered by dubious techniques, such as ATPase histochemistry which is markedly pH sensitive in addition to other problems, and variations between one muscle and another in the same animal, in different individuals and in different species, Close (1972) has postulated that these three fibre types can be distinguished:-

(a) Fast-twitch white fibres, having relatively short isometric twitch contraction times, high myosin ATPase activity, well-developed glycolytic enzyme systems, a low mitochondrial content and oxidative activity, and which fatigue rapidly.

(b) Fast-twitch red fibres, having relatively short isometric twitch contraction times, high myosin ATPase activity, moderately-developed glycolytic enzyme systems, a high mitochondrial content and oxidative activity, and which are more resistant to fatigue than type (a).

(c) Slow-twitch intermediate fibres, with a slow speed of contraction, low myosin ATPase activity, poorly-developed glycolytic enzyme system, a high mitochondrial and oxidative enzyme activity, and which show little or no susceptibility to fatigue.

Gauthier (1969) determined that the rat diaphragm was composed of 20% of white fibres, 20% of intermediate fibres and 60% of red fibres. The fibres appeared to be randomly distributed throughout the diaphragm (Zolovick, Norman and Fedde, 1970), and

these authors were unable to demonstrate any connection between membrane constants and diameter in these fibres, though membrane resistance and type of innervation did appear to be related.

That innervation plays an important part in muscle fibre function has been known since 1958, when Eccles showed that denervation of slow muscles caused them to change to an action similar to that of fast fibres, and that crossinnervation led to the relevant changes in contraction speeds. The influence of nerve on muscle has recently been reviewed by Guth (1968) and Close (1972).

Zolovick, Norman and Fedde (1970) found that the small diameter fibres (20 - 30 μm) in rat diaphragm muscle showed the high oxidative enzyme activity characteristic of 'red' muscle, and though speed of contraction in frog muscle may be related to its myosin ATPase activity (Engel and Irwin, 1967) and certain membrane constants (Adrian and Peachey, 1965), they were unable to find any such relationships in the mature rat diaphragm.

It is therefore difficult to classify the 'type' of muscle found in the diaphragm preparation used in the present study. Furthermore, it has been shown that in the early post-natal period the contractions of all muscle fibres in the rat are relatively slow (Close, 1964) and that at the age of the rats used in the experiments demanding thin preparations, i.e. less than three weeks old, 'fast' and 'slow' muscles were not fully differentiated (Guth, 1968) : this is not due to innervational changes only but to some change in the muscle itself (Ridge, 1967). Much of the maturation of myofibres in the hind-limb muscles of the rat occur postnatally (Bergman, 1962) but is virtually complete at birth in the inter-costal muscles (Kelly and Zacks, 1969), and there appears

to be a cephalo-caudal gradient of maturation. Close (1964) suggested that these changes were possibly linked to changes in the biochemistry of the excitation-contraction coupling of the muscle, and Boëthuis (1969) has demonstrated a cephalo-caudal gradient for the development of the muscle membrane potential, the neck muscles reaching the high potentials recorded in the adult well before the hind-limb muscles. Many of the changes which occur appear to be related to the contraction mechanism, e.g. the myosin MgATPase of fast muscle increases in activity over the first five post-natal weeks (de Villafranca, 1954), and Trayer and Perry (1966) have suggested that these myosin ATPase changes may reflect changes of isoenzymes or combinations of isoenzymes as required for the muscle activity. Other maturation changes appear to act directly at the membrane level, and may well be extremely important in the present study. Fudel-Osipova and Martynenko (1964) showed that the resting potential of gracilis membrane 'in vivo' was low at birth and did not reach a maximum until after more than 15 days, and Hazelwood and Nichols (1967) showed that the resting potential in rat gastrochemins had not reached the mature level after 27 days. These authors also found that the internal potassium concentration did not change significantly from birth, hence the change in resting potential was not attributable to this. Vernadakis and Woodbury (1964) also found that intracellular potassium and water did not change significantly but that there was a marked fall in intracellular sodium post-natally. Luff and Goldspink (1970) believe however that there is a high intracellular water content which diminishes post-natally, and

which is also complicated by changes in the volume of the extracellular space. A major maturational membrane change was demonstrated by Diamond and Miledi (1962) when they showed that in neonatal rats the entire muscle membrane was sensitive to acetylcholine, but that this sensitivity declines to the adult stage, where only the neuromuscular junctions are sensitive, a few weeks afterwards. This process can be reversed by denervation, when sensitivity will gradually spread from the neuromuscular junctions until the entire muscle is again receptive.

The type of innervation is also known to affect the ATPase activity of muscle. CaATPase, myosin ATPase and myofibrillar ATPase activities are all higher in 'fast' muscle than 'slow', but will decline to the lower level after cross-innervation (Buller, Mommaerts and Seraydarian, 1969). The hypothesis that neural influences may determine dynamic properties of muscle via effects on the ATPases is strongly supported by the work of Barany and Close (1971). Dockry, Kernan and Tangney (1966) have demonstrated that the nerve exerts a positive trophic effect on sodium extrusion and potassium uptake in rat muscle, and Akaike (1971) has shown that acetylcholine will accelerate this exchange.

In the light of these findings it is possible that lack of maturity and of neuronal stimuli may mean that the rat diaphragms involved in the hemidiaphragm incubations were different in certain respects from those used in the NaKMgATPase extraction experiments. Certainly the neonatal changes in resting potential, in other ATPase activities, and

in ionic content may well suggest that those membrane structures controlling permeability, possibly including the NaKMgATPase, had not yet attained the adult state. Choice of the diaphragm, rather than limb muscles, has lessened this probability though the ion contents recorded in Chapter Two would seem to suggest that the mature state had not yet been acquired.

The simple 'in vitro' studies used in Chapters Two and Three cannot, taken in isolation, tell much about the mechanism of the 'sodium pump'. The use of radioactively-labelled ions, along with detailed histochemical and anatomical studies of the tissue, might assist in shedding more light on this issue, but as yet these approaches have not produced a clear-cut picture (vide infra), for example, analysis of ^{24}Na loss from muscle cannot be accurate until it is known what portion the ion can enter freely. Nor is it possible to interpret the role of the NaKMgATPase until its location, or locations, in the muscle membranes have been identified with certainty.

Interpretation of these 'in vitro' studies is greatly assisted, however, if the results are considered along with the evidence obtained from the isolated NaKMgATPase preparations in Chapter Five, and in the light of the most recent advances in the field of muscle morphology and histochemistry. Furthermore, during the course of the present study, and during the time it has been in preparation, other workers have successfully isolated NaKMgATPase fractions from skeletal muscle, and a study of their preparations will also help in the interpretation of this work. Consideration of the roles of ouabain, ethacrynic acid and rose bengal on the present preparations will therefore be made following an examination of these recent developments.

In 1970, Peter, using the isolation techniques of Rosenthal et al (1965), was able to obtain a preparation of sarcolemma from rat skeletal muscle which had been emptied of contractile protein and was not visibly contaminated by other subcellular components of muscle fibre. This preparation had a MgATPase activity of some 10 m.moles Pi h⁻¹ g⁻¹ protein, with a NaKMgATPase of some 10 - 72% (mean value 39%) of the MgATPase. There was also a CaATPase in the preparation which was not additive to the MgATPase.

Most authors, however, have utilised techniques which incorporated exposure to high salt concentrations before an extraction procedure. Ash and Schwartz (1970) exposed rat skeletal muscle homogenates to 0.6 M KCl before centrifugation. They obtained a 10,000 x g fraction with a MgATPase activity of 19.7 m.moles Pi h⁻¹ g⁻¹ protein, and a NaKMgATPase of 9.0 m.moles Pi h⁻¹ g⁻¹ protein which was stable for some hours at 0°C but declined overnight. Boegman et al (1970) used a sophisticated technique involving exposure of frog muscle homogenate to 0.4 M LiBr and 0.6 M KCl before sucrose density gradient extraction procedures which largely separated Na⁺ and K⁺-sensitive activity from the MgATPase, with a high, widely-varying activity which could be inhibited up to 94% by 0.25 mM ouabain. Sulakhe et al (1971) used a similar technique of exposure of hamster skeletal muscle homogenate to 0.5 M LiBr and 0.6 M KCl, but without sucrose density gradient centrifugation, to give an MgATPase of around 40 m.moles Pi h⁻¹ g⁻¹ protein, with an NaMgATPase of some 18 m. moles Pi h⁻¹ g⁻¹ protein. These authors also recorded elevated NaKMgATPase activities of 40 - 55 m.moles Pi h⁻¹ g⁻¹

protein in preparations from genetically-dystrophic hamsters. Bray (1973) exposed muscle fibre fragments, obtained by straining minced muscle through terylene mesh, to 1 M NaI for 2 hours, before centrifugation. He detected NaKMgATPase activity in all fractions but found the greatest activity in the microsomal fraction (8,000 - 100,000 x g). The high concentrations of salts, such as LiBr, KCl and NaI, which are effective in solubilising proteins, may well also disrupt the membrane-bound proteins such as the NaKMgATPase (see Penefsky and Tzagoloff, 1971). Kidwai et al (1973), however, have developed a preparative technique which avoids the use of these salts. They removed the unwanted contractile protein elements by a filtration device before submitting the membranes to sucrose density-gradient centrifugation. The interface of the medium and the sucrose gradient yielded a membrane fraction with an MgATPase activity of some 60 m. moles Pi h⁻¹ g⁻¹ protein with ouabain-sensitive NaKMgATPase of some 15 m. moles Pi h⁻¹ g⁻¹ protein.

It seems clear, therefore, that skeletal muscle does contain an NaKMgATPase which is membrane-bound and ouabain-sensitive, but that it is necessary to remove - either by filtration or solubilisation - a large proportion of the muscle before this activity can be clearly demonstrated. The location of the membranes upon which this enzyme is found is, however, by no means certain.

The preparation of Peter (1970) indicates clearly that at least some, if not all, of the NaKMgATPase is situated in the sarcolemma. The sarcolemma is known to consist of four layers (Mauro and Adams, 1961): (a) an outer layer of fine filaments, randomly orientated, up to 10 nm in thickness; (b) a layer of collagen filaments approximately 30 nm thick; (c) an amorphous

layer resembling basement membrane matrix, 30 - 50 nm thick; (d) the plasma membrane. In view of the finding that plasma membrane in other tissues are rich in NaKMgATPase it would appear likely that this layer contains this enzyme in muscle also. This does not rule out the possibility that other membranes may also contain NaKMgATPase.

Many of the workers producing NaKMgATPase preparations have mentioned homogenisation as a critical factor in producing successful results (e.g. Kidwai et al, 1973) and it has been pointed out (Wallach and Lin, 1973) that homogenisation can lead to vesicles from a wide variety of sources within the cell. The difficulty of morphological identification of smooth membranes has also been pointed out (Boegman et al, 1970; Wallach and Lin, 1973). Whilst certain enzymes are said to be biochemical markers of particular membranes, their presence or absence cannot be used to show that all of a fraction originates from a particular locality within the cell, merely that a portion of a fraction is from that locality, or occasionally that it is probable that a particular organelle is absent from the fraction. Nevertheless, several of those isolating membrane fractions exhibiting NaKMgATPase activity have tentatively identified them as being plasma membrane in origin. Boegman et al (1970) treated his membrane fragments with saponin, since Dourmashkin et al (1962) had shown that external cell membranes so treated formed a clearly-visible hexagonal lattice structure. This was also clearly visible in their preparation of NaKMgATPase, and was definitely not shown in mitochondrial preparations, but a fraction tentatively identified as sarcoplasmic reticulum also showed

this lattice structure in parts, thus positive identification of plasma membrane as the sole site was not made. Sulakhe et al (1971) identified their preparation as sarcolemmal in origin since preparations of nuclear, mitochondrial and myofibrillar fractions obtained by conventional techniques did not contain such activity, microsomal fragments may be contaminated with sarcolemma, and the fact that the majority of workers believe that 'transport' ATPase is a constituent of the cell membrane (Whittam and Wheeler, 1970). Bray (1973) has also suggested that his preparation is probably sarcolemmal in origin, in view of the large numbers of workers who have demonstrated the absence of Na^+ , K^+ -stimulated, ouabain-sensitive activity in preparations from myofilaments, mitochondria and sarcoplasmic reticulum. Kidwai et al (1973) have claimed that the sucrose density gradient employed in their preparation will separate plasma membrane from sarcoplasmic reticulum, as the former, being less dense, will stay upon the surface of the gradient, whilst the sarcoplasmic reticulum, though morphologically similar, will enter the gradient to give a distinct lower band. They found NaKMgATPase activity to be associated with the 'plasma membrane' fraction; other workers have found that NaKMgATPase activity is associated with 'light' membranes which formed the upper fraction on density gradients (e.g. Ahmed and Judah, 1964; Uesugi et al, 1969) because of their lipoprotein nature. Kidwai et al (1973) have however pointed out that the complex linkage of sarcolemma, T-tubule system and sarcoplasmic reticulum could mean that connected portions of plasma membrane and sarcoplasmic reticulum could occur in each preparation fraction. The proximity of

the two membranes does not imply similarity, however, since it has been shown that the T-tubule system arises from invaginations of the sarcolemma, whilst the sarcoplasmic reticulum is formed by tubular evaginations of the endoplasmic reticulum (Ezerman and Ishikawa, 1967). This problem of cross-contamination is not restricted to the plasma membrane preparations from muscle; Korn (1969) has pointed out that even preparations of plasma membrane of the much-studied liver cells are often contaminated, with more than 25% of preparations containing more than 5% of membranes originating from the endoplasmic reticulum.

Recently Schapira et al (1974) reported a careful study of the membrane fractions obtained by treatment of skeletal muscle with high salt concentrations followed by sucrose density gradient centrifugation. They also found the length and strength of homogenisation to be critical if mitochondrial damage was not to occur, and using a variety of techniques, including marker enzymes, electron-microscopy and cholesterol: phospholipid ratio, they reported that the salt treatments eliminated most of the sarcoplasmic reticulum, so that the light fraction that they obtained by density gradient centrifugation was relatively rich in NaKMgATPase activity, and appeared to be composed of plasma membrane vesicles. These authors again reported absence of NaKMgATPase activity in mitochondrial and sarcoplasmic reticulum fractions.

Whilst the preparative techniques in the present work and those reported above vary in several ways, an overall look permits some speculation. Schapira et al (1974) have

not published their electron-micrographs, but the NaKMgATPase-containing preparations of Kidwai et al (1973), Bray (1973) and to some degree Boegman et al (1970) resemble the 'light microsome' fraction found here to contain the NaKMgATPase. The preparations of Kidwai et al (1973) are particularly interesting, since these authors have included electron-micrographs of their 'sarcolemmal reticulum' fraction, and comparison with the present work shows that the appearance of this fraction closely resembles the 'light microsome' fraction of the non-NaI-treated preparation, whilst the 'plasma membrane' preparation is similar to the NaI 'light microsomes'. Since these authors did not use salt treatments but filtration and sucrose density gradient centrifugation, this would appear to lend support to the hypothesis that the strong salt solutions play a major role in separating sarcolemmal reticulum from plasma membrane, whilst differential centrifugation following salt treatment leads to purification of the fraction by separating the lighter plasma membrane fractions from remaining contaminants, as proposed by Schapira et al (1974). Fricke and Klaus (1974) have also produced an active NaKMgATPase fraction from cardiac muscle very similar in appearance to that shown in the electron micrograph in Chapter Five (fig. 5: 13c).

It is difficult to see at the present time how there can be conclusive proof that the NaKMgATPase resides solely in the plasma membrane of muscle tissue, but the direct and indirect evidence available at this time would point to this being the case.

Consideration of these various preparations, with the effects of the NaKMgATPase inhibitors ouabain, ethacrynic acid

and rose bengal upon the isolated preparation here, can give some indication as to the role these drugs may play in bringing about the increased intracellular sodium concentrations and decreased intracellular potassium concentrations recorded in Chapter Two.

Ouabain is well known as a specific inhibitor of NaKMGATPases (Skou, 1965; Whittam and Wheeler, 1970) and at 10^{-3} M the NaKMGATPase from rat diaphragm muscle was completely inhibited. Ouabain inhibition is generally enhanced by sodium, and decreased by potassium, and Matsui and Schwartz suggested that ouabain bound to the phosphorylated intermediate (Matsui and Schwartz, 1968); this was supported by the findings of Albers, Koval and Siegel (1968) and Sen, Tobin and Post (1969). Some conflicting evidence as to the performance of ouabain in the presence of various ligands has made an exact definition of its effects difficult (see e.g. Erdmann and Schoner, 1973a). Tobin et al (1974) have added weight to the evidence in favour of an effect upon the phospho-enzyme, so that K^{+} -induced dephosphorylation is inhibited.

Extension of the finding that 10^{-3} M ouabain is sufficient to cause 100% inhibition of the NaKMGATPase in an extracted form to an 'in vitro' preparation of whole muscle, as used in Chapter Two, is made difficult by the unknown variables of, firstly, the proportion of NaKMGATPase binding sites in proportion to the concentration of ouabain, and secondly, the amount of non-specific ouabain binding, i.e. to sites other than NaKMGATPases, that will occur in the gross tissue preparation. However, the apparent specificity of the drug action upon this enzyme would

support the hypothesis that the sodium gains and potassium losses reported in Chapter Two can be attributed to ouabain-inhibition of the NaKMgATPase.

The effects of ethacrynic acid upon the whole preparation are not so easily explained. Whilst the unknown variables of binding ratios still cloud the issue, it seems unlikely that a concentration of ethacrynic acid shown to be far less effective an inhibitor of NaKMgATPase than 10^{-3} M ouabain could cause effects in the whole tissue so different from those of the cardiac glycoside merely by a single action upon the NaKMgATPase. The marked sodium accumulation, though lower than that with the glycoside, and the vast increase in intracellular water, greater than that with the glycoside, would apparently indicate that other processes concerned with permeability are involved.

It is clear that ethacrynic acid is a NaKMgATPase inhibitor but there is still controversy as to whether the diuretic effects of this drug 'in vivo' can be attributed to this action (for review, see Suki et al, 1973). Duggan and Noll (1972) and Nechay and Contreras (1972) have shown that enzyme inhibition follows 'in vivo' administration of ethacrynic acid in the dog, but Landon and Fitzpatrick (1972) were unable to find inhibition of NaKMgATPase activity after high levels of ethacrynic acid in rabbits led to natriuresis. Furthermore, concentrations of ethacrynic acid far higher than those required to produce diuretic effects 'in vivo' have been needed to cause inhibition of NaKMgATPase, though it has been shown that renal tissue can accumulate ethacrynic acid (Charnock and

Almeida, 1972). Hence it is possible that the sodium and water uptake recorded in Chapter Two may not be attributable to a direct effect upon the NaKMgATPase.

There is evidence that ethacrynic acid may exert an effect upon cellular metabolism, with mitochondrial inhibition (Landon and Forte, 1971) and reduction of lactate formation (Klahr et al, 1971). Whilst this could lead to indirect effects on the NaKMgATPase by altering ATP availability, there is once again controversy as to whether ethacrynic acid exerts a direct effect upon metabolism (see Suki et al, 1973). It has also been suggested that ethacrynic acid may increase the passive permeability of cell membranes to bring about its effects, though as yet evidence for such a mechanism has not been produced (Suki et al, 1973). However, some workers have produced results which appear to indicate that ethacrynic acid may exert its effects by inhibition of a sodium pump other than the NaKMgATPase. The existence of such a pump was proposed by Hoffman (1966) to explain the ouabain-insensitive uphill movements of Na^+ from erythrocytes, movements also seen in frog muscle (e.g. Sjodin and Beaugé, 1968). Inhibition of $\text{Na}^+:\text{Na}^+$ exchange in red blood cells by ethacrynic acid in the presence of ouabain has been demonstrated by Lubowitz and Whittam (1969) and in frog muscle by Erlj and Leblance (1971), and Whittam and Wheeler (1970) believe that such movements represent a passive carrier-mechanism rather than a true pump. On the other hand, Whitembury and Proverbio (1970) demonstrated that ethacrynic acid appeared to inhibit Na^+ extrusion accompanied by Cl^- , rather than $\text{Na}^+:\text{K}^+$ exchange or $\text{Na}^+:\text{Na}^+$ exchange, and postulated a sodium pump

which was balanced by movements of Cl^- and water, which would obviously play a major role in volume regulation, a tempting hypothesis in view of the diuretic role of ethacrynic acid. Whilst not conclusive, the dose-response effects on ion movements in leached and rewarmed kidney slices in the presence of ouabain or ethacrynic acid (Proverbio et al., 1970) strongly supports the suggestion that ethacrynic acid exerts its physiological effect via Na^+ and Cl^- movements rather than upon the characteristic $\text{Na}^+:\text{K}^+$ exchange of the NaKMgATPase or the $\text{Na}^+:\text{Na}^+$ exchange mentioned above.

The entry of water into the rat diaphragm, so much greater than that seen in the presence of ouabain, would appear to support this separate action of ethacrynic acid, but the results must be viewed with care since it is not clear as to what portion of the complex muscle tissue is taking up the water. The reduction in inulin-space, along with water uptake, would appear to point to entry of sodium and water into the fibre, but outside the true 'intracellular' space in which low levels of sodium are maintained. Whilst the present study will not permit such a distinction to be made, there is evidence in the literature that such an enlargement of a space may play at least some part in the action of ethacrynic acid seen here.

Water movements in muscle are extremely complex, and changes in the tonicity of the bathing medium lead to volume changes which are not predictable by the Van't Hoff equations (Rothstein, 1968). Determination of 'extracellular' space using different molecules gave different results, with small molecules apparently entering a space not reached by larger ones, and the

degree of entry varying with the toxicity of the bathing medium (Tasker et al, 1959). Dydynska and Wilkie (1963) and Blinks (1965) found that exposure of muscle to varying bathing media showed discrepancies between changes in the calculated volume of fibre water, from weight and sucrose space determinations, and the cell volume as calculated by optical studies. These discrepancies could be explained by a portion of the muscle, within the fibre 'optically', being freely permeable to sucrose and hence acting 'extracellularly'. Movements of labelled ions from muscles give rise to similar discrepancies. Levi and Ussing (1948) and Carey and Conway (1954) reported a muscle compartment, other than the extracellular space, which could rapidly exchange Na^+ , with a high Na^+ and low K^+ content. This could also be explained by the existence of an 'intracellular' compartment freely accessible to 'extracellular' ions (Simon et al, 1957). Hodgkin and Horowicz (1960) postulated that the T-tubule system could be this compartment, but Harris (1963) calculated that such a compartment should comprise about 12% of the volume, and suggested that the sarcoplasmic reticulum (shown by Peachey, 1965, to be some 13% of muscle volume at normal osmotic pressure) might form such a compartment. In his studies on the 'in vitro' rat diaphragm preparation, Creese (1968) has assumed that muscle is composed of merely two compartments, the intracellular, sodium-regulated compartment, and the extracellular compartment with ionic conditions resembling those of the surrounding medium. This treatment was also adopted for the present study.

Recently, however, Rogus and Zierler (1973) have suggested that two intracellular compartments exist, the sarcoplasm, containing less than 4% of the intracellular Na^+ , a slowly-exchanging component, and the sarcoplasmic reticulum, a rapidly-exchanging component containing over 96% of the intracellular Na^+ . If this is the case, then quantitative evaluation of the incubation experiments in Chapter Two, including the ouabain and rose bengal experiments as well as those involving ethacrynic acid, is rendered extremely difficult, since it cannot be established whether the changes in 'intracellular' cation and water contents represent alterations in volume of a non-regulated sarcoplasmic reticulum or actual uptake by a regulated sarcoplasm, or a combination of both. Furthermore the various drugs used may well be exerting different effects upon these two components.

There is strong evidence from elsewhere that the sarcoplasmic reticulum may well form such a separate component. Keynes and Steinhardt (1968) found that such a model fitted better the washout of ^{24}Na observed in frog muscle. The difficulty of explaining access of medium to the sarcoplasmic reticulum has apparently been resolved by the demonstration of bridge-structures between the T-tubules and the sarcoplasmic reticulum at the triad sacs (Birks and Davey, 1969; Franzini-Armstrong, 1970). Birks and Davey (1969) also demonstrated that the sarcoplasmic reticulum swelled with increasing osmotic pressure, though Freygang et al (1964) showed in their preparation that T-tubule swelling occurred but not swelling of the

sarcoplasmic reticulum. Birks and Davey (1972) showed that these differences could be explained by the different fixation methods used on the preparations, and that the sucrose treatment and osmium fixation used by Freygang et al (1964) led to T-tubule swelling, whereas their fixation technique using aldehyde appeared to be less damaging. Free access to the sarcoplasmic reticulum by extracellular medium has been further supported by the finding that horseradish peroxidase, an extracellular marker, will enter this muscle component (Rubio and Sperelakis, 1972; Kulczycky and Mainwood, 1972).

This, in turn, has significance when considering the site of NaKMgATPase action. As has been pointed out by Birks and Davey (1969), free access of extracellular medium to the sarcoplasmic reticulum would suggest that a similar potential difference should exist across the sarcoplasmic reticulum membrane as exists across the sarcolemma, and that depolarisation of the sarcoplasmic reticulum should spread through the muscle. This has been supported by the observations of Constantin and Podolsky (1966) that local depolarisation of the triad sacs appears to lead to muscle contraction. It would then appear likely that there should exist in the walls of the sarcoplasmic reticulum a sodium pump to help maintain this potential difference, in all probability the NaKMgATPase. Rogus and Zierler (1973) have put forward such a hypothesis, pointing out that the sole effect of 10^{-5} M ouabain is to increase the sodium content of the sarcoplasm, not the reticulum. They also cite their earlier finding that NaKMgATPase was associated

with microsomes of probable sarcoplasmic reticulum origin (Rogus, Price and Zierler, 1969), and Constantin and Podolsky's (1967) demonstration that muscles from which the sarcolemma has been removed are still sensitive to cardiac glycosides.

There is also evidence of NaKMgATPase activity in the sarcoplasmic reticulum from histochemical studies. In experiments not reported here, using histochemical techniques based on those of McClurkin (1964) for demonstration of NaKMgATPase, it was found impossible to demonstrate such activity in the rat diaphragm muscles used in the present study. These procedures involved fixation processes which have been shown to cause inhibition of NaKMgATPase (Moses et al, 1966) but even modifications and omissions of such processes were found to be unsuccessful here in permitting demonstration of a NaKMgATPase. Other serious objections to these techniques have been raised, however, Rosenthal et al (1966) demonstrated that lead, present in the incubation medium used (Wachstein and Meisel, 1957) to deposit at the site of ATP hydrolysis, was itself capable of causing ATP hydrolysis, and Moses et al (1966) showed that the localisation of lead deposits could be radically altered by altering the concentrations of ATP and lead nitrate used. Tormey (1966) studied the technique carefully, and concluded that it could not possibly be used to localise NaKMgATPase, and there is no doubt that much evidence from the literature supports his claim. Recently the technique has been improved by the introduction of the medium of Schulze and Wollenberger (1969), composed of

35 mM sodium potassium tartrate, 2.5 mM lead acetate, 30 mM Tiron, 2.5 mM ATP, 65 mM NaCl, 0.2 M sucrose and 0.1 M tris-maleate buffer (pH 7.2). The low concentration of lead, along with the sodium potassium tartrate, tris-maleate and Tiron which will complex the lead, is believed to greatly reduce inhibition of NaKMgATPase and the spontaneous hydrolysis of ATP. Forbes and Sperelakis (1972) have studied NaKMgATPase histochemistry in rat skeletal muscle, combining techniques of short fixation time, the avoidance of inhibitory buffers and of the freezing and re-thawing of the preparation, with the Schulze and Wollenberger incubation medium. Their preparations showed clear, ouabain-sensitive lead deposition in the sarcoplasmic reticulum, particularly in the terminal cisternae, along with some deposition at the sarcolemma and in the T-tubules. Whilst many of the criticisms levelled at histochemical techniques, such as the presence of artefacts and possible movement of the products should be borne in mind, nevertheless this would appear to lend support to the hypothesis that the sarcoplasmic reticulum contains NaKMgATPase in addition to the plasma membrane.

This runs contrary to the hypothesis put forward earlier in the chapter based on the evidence from membrane preparations of rat muscle. As has been stated previously, however, the positive identification of the membranes obtained in such preparations is extremely difficult, and while most workers have postulated that their preparations were of plasma membrane origin, the presence of sarcoplasmic reticulum has not been

ruled out. Indeed, Rogus and Zierler (1973) have suggested that sarcoplasmic reticulum vesicles in all probability do contain the NaKMgATPase that they have recorded. It is difficult to explain, however, why in the present study, and in those of many workers, it has not been possible to demonstrate NaKMgATPase activity in preparations which would clearly contain a large proportion of sarcoplasmic reticulum, nor why optimum NaKMgATPase activity has been said to depend on minimum homogenisation, which would tend to increase the proportion of plasma membrane and decrease the proportion of sarcoplasmic reticulum, found in the final preparations.

It is clear that a much fuller understanding of the techniques of histochemistry, salt extraction, centrifugation etc. is required before the answer to this problem can be resolved with certainty.

That whole muscle preparations were sensitive to photo-oxidation, with a site of action which was probably at the surface membrane, was well known (e.g. Lillie, Hinrichs and Kosman, 1935). Yu et al (1967) demonstrated that in an isolated preparation of sarcotubular fragments both MgATPase and Ca^{+} -sequestering activity were inhibited by photooxidative agents such as rose bengal. In the present preparation it has also been demonstrated that the NaKMgATPase is sensitive to rose bengal, with this enzyme being more sensitive than the MgATPase, as was found in red blood cells (Duncan and Bowler, 1969). It would seem unlikely, however, that simple inactivation of the active cation transport by the NaKMgATPase complex was solely responsible for the changes observed in the whole muscle

preparation in Chapter Two. The rapid Na^+ uptake and K^+ loss observed, greater than was observed with 10^{-3} M ouabain, would imply other sites of action, and Duncan and Bowler (1969) have demonstrated that rose bengal must affect other sites controlling permeability, suggesting the MgATPase as a possible contender. It seems likely, however, in view of the variety of ion-transport mechanisms which are now emerging (Whittam and Wheeler, 1970) that photooxidative damage may well be affecting several such enzymes present within the cell membrane. The mechanisms of photooxidative effects are not yet fully understood. Akera and Brody (1968) suggested that the synergistic effect of ultraviolet illumination and chlorpromazine upon inhibition of rat brain NaKMgATPase was due to the formation of a free radical of chlorpromazine which was unstable but pharmacologically active. Duncan and Bowler (1969), however, suggested that the photooxidative effect of rose bengal may be related to the conformational changes of enzymes such as the NaKMgATPase. Sun (1972) has also produced evidence that photooxidation may cause configurational changes. He has suggested that oxidative membrane damage is probably due to changes in phospholipid poly-unsaturated fatty acids important in membrane configurations. He showed that lipxygenase and hydrogen peroxide produced synergistic inhibition of NaKMgATPase, and a conformational change, with a major effect probably at the K^+ site, was suggested by kinetic studies.

The importance of phospholipid integrity for normal NaKMgATPase activity is well known. Schatzmann (1962) and

Swanson, Bradford and McIlwain (1969) showed that treatment with phospholipase C would reduce NaKMgATPase activity, and Ohnishi and Kawamura (1964) and Emmelot and Bos (1968) that phospholipase A would also have this effect. Controversy over the reactivation of NaKMgATPases by phospholipids (see Whittam and Wheeler, 1970) has been resolved by the recent findings of Stahl (1973), Chipperfield and Whittam (1973) and Specht and Robinson (1973). These authors demonstrated that at least in part the effect of phospholipids could be simulated by EGTA, that this was due to calcium sequestration, and that calcium contamination of phospholipid samples may explain variability of results. Taniguchi and Tonomura (1971) and Stahl (1973) have clearly demonstrated that phospholipids play an important function in the activity of the NaKMgATPase other than mere calcium binding, and Specht and Robinson (1973) have suggested that the excellence of phosphatidylserine in activating NaKMgATPase preparations may be due to its efficacy in both chelation and in restoration of the lipid environment. It has been suggested that the presence of the charged membrane phospholipids is essential for correct conformation of some membrane proteins (Wallach, 1972). The increasing evidence for conformational changes of proteins in membranes, and the possible sites of photodynamic action have also been reviewed by this author (Wallach, 1972).

The discovery that chelators can play an important part in maintenance of membrane protein function may have relevance to the role of serum in helping preserve 'in vivo' ionic conditions in muscles incubated 'in vitro'. Gabel et al (1970) have demonstrated that serum is effective in preventing the

mild fatigue normally seen in incubated muscle due to weakening of the intensity of the active state, and deterioration of the preparation in more marked fatigue was reduced, though it was not effective in major stress. The addition of serum to saline-incubated muscles led to improved performance, and the serum need not be species-specific. These authors found that the effective factor in the serum was heat-stable, extractable in chloroform:ethanol, and chromatographed like a phospholipid. They suggested that the action of the factor was probably sited at the muscle membrane, and that it affected calcium mobility. In view of the increasing evidence of the importance of calcium in controlling active and passive movements of monovalent cations at the cell membrane (Whittam and Wheeler, 1970) it may well be that factors in the serum are important in controlling calcium levels at the membrane sites.

Thus, as has been found elsewhere, the NaKMgATPase enzyme of muscle requires a specific conformation on the membrane dependent upon phospholipids, and is sensitive to photooxidation and the presence of calcium. Various models have been proposed to explain the structure and function of these enzymes, a notable example being the tetrameric model of Stein (Stein et al., 1973) which appears to fit the available data on the sodium pump enzyme.

The present study has shown that in skeletal muscle the NaKMgATPase plays an important role in the regulation of cation balance. If this enzyme is inhibited when the muscle is 'in vitro' there follows a marked uptake of sodium and loss of potassium, though the precise portion of the tissue affected

requires further examination. The tissue does not lend itself to studies of the metabolic requirements of the pump via the measurement of oxygen consumption due to the practical problems in making a suitable preparation, though studies of changes in biochemical content may be possible. Production of an isolated membrane fraction bearing NaKMgATPase activity has permitted some analysis of the characteristics of this enzyme in mammalian skeletal muscle, which have shown it to resemble closely the NaKMgATPases isolated from other tissues. The response of the isolated preparation to the diuretic ethacrynic acid, and the photooxidant rose bengal, would seem to indicate that though the NaKMgATPase is inhibited by these reagents, that it is not likely to be the sole site of action in the whole muscle, as evidenced by changes in cation and water contents in the 'in vitro' studies.

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