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METABOLIC REQUIREMENTS FOR COUPLED Na^+-K^+ EXCHANGE AND SPONTANEOUS CONTRACTIONS IN THE RAT MYOMETRIUM

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Metabolic Requirements for Coupled Na^+-K^+ Exchange and Spontaneous Contractions in the Rat Myometrium," submitted by Dr. Patangi Srinivasa Kumar Rangachari in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The metabolic requirements for contractions and Na^+ pumping in the rat myometrium were studied by following the recovery of ion contents and contractions by Na^+-rich tissues. Under aerobic conditions, omission of D-glucose from the medium did not hamper the recovery of either process. Both anoxic conditions and dinitrophenol (DNP) reduced but did not abolish the recovery of either ion movements or contractility, provided that suitable glycolytic substrates (D-glucose or D-mannose) were present. 2 deoxy-D-glucose (2DG) proved an effective inhibitor only under anoxic conditions. Iodoacetic acid (IAA) inhibited both processes; the inhibition being dose-dependent. Inhibition of ion movements produced by 0.2 - 0.5 mM IAA was partially overcome by 25 mM pyruvate, whereas contractility failed to recover. β-hydroxybutyrate proved an effective substitute for pyruvate. The recovery seen was abolished by 1 mM ouabain and anoxia. The differential effects of pyruvate on the recovery of ions and contractility was not due to a limitation of internal K^+; there were no significant changes in the contents of exchangeable Ca^{2+} either. There was no clear correlation between the contents of ATP and the presence or absence of contractility. That the inability of IAA-treated tissues to contract may be related to a direct effect on contractility was suggested by studies with iodoacetamide (IAAmide); the precise

mechanics remains unclear. Thus these studies showed that energy production from endogenous stores or from aerobic and anaerobic glycolysis could support ion movements and contractions. Energy produced solely by oxidative mechanisms could support Na^+ pumping but not contractions.

Under a number of different metabolic conditions, there was an apparent dissociation between net Na^+ and K^+ movements. These discrepancies were explicable on the basis of tissue swelling and gain of isotonic fluid. Changes in extracellular space were not clearly correlated with gains in weight. Tissues treated with IAAmide gained water and Na^+ even with normal contents of ATP, suggesting the involvement of -SH groups. Na^+-rich tissues rewarmed in K^+-free Krebs lost weight and also exhibited a transient contracture; both these processes were abolished by papaverine. Furthermore, there was a significant correlation between the inability of a tissue to contract and the swelling observed under diverse metabolic conditions. It was suggested that this correlation was causal.

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And it came to pass, as was oft before and will be in years to come, after many drowsy days, sleepless nights, many trials, fond hope, despair, with a little help from my friends, some living, some dead, this task was partly done. To the many gentle rats who stretched their necks as they gazed at "that undiscovered country", a deep debt I owe, which words ill convey. Many thanks, I do have for David Murray Paton (may he pardon this license) who, suffering much in stony silence, steered safely this unsure ship twixt the Scylla of smug satisfaction and the Charybdis of utter despair. Much did I learn from Daniel - of ions, bound and free, fluxes, great and small, vesicles, open (and shut?), that lay beneath the membrane in rows; soap bubbles? He gave me wings to soar - but not too close to the sun. And when in a rash fierce blaze of riot, I ventured into the murky depths of diverse nucleotide pools - and sank, J.C. fished me out from down under. He trod waters such as those with ease. Squirting loving spoonfuls of labelled sucrose into solutions complex, spending two Sundays, soiling his hands to boot, Fikry aided much. And Julie Munson, may her tribe increase, saved me from disgrace with fortune and men's eyes, by doing things I'd already done, but in her own sweet way - the results, needless to say, were much the same. Thus did Truth triumph. And Bob and Lorraine, Fred and Alice did in many ways, too diverse to spell, help this work see the light of day. From the Medical Research Council, bounty did I

receive, far more than my deserts; from the fifth floor, comfort and solace, through long, bitter, wintry nights; from this City of Dreadful Night, lost amidst the prairies, that slumbers in frigid sunlight, much incentive did I get - to labour hard to leave soon so that I, like Stout Cortez, could stare with eagle-eyes at the Pacific.

TABLE OF CONTENTS

TABLE OF CONTENTS - continued.

TABLE OF CONTENTS - continued. Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

This work is dedicated to my parents for affection beyond the call of duty and to my sister, Prabha, who tinted my world with a fine touch of whimsy.

"To find solutions to problems of grave importance requires mere intelligence; to ask questions of profound irrelevance and answer them needs talent."

P. K. Rangachari in The Exuberant Pursuit of the Trivial: Studies in Academic Life. (unpublished as yet)

I. INTRODUCTION

GENERAL INTRODUCTION

The Greek philosopher, Heraclitus, observed that all things existed in a state of flux or that there was nothing so constant as change. The word "metabolism" derived from the Greek - to change (metabolos) epitomises the dynamic nature of living cells. The energy produced by metabolic reactions in "unresting cells" (a term coined by the physiologist Gerard) is expended in a variety of ways - for the maintenance of cellular integrity, growth, replication and the performance of specialized functions. Since muscle cells are specialized for the purposes of contraction, it is reasonable to expect that a certain proportion of the energy thus produced would be utilized in the efficient operation of the contractile process. The contraction of any muscle is the end result of several processes; the linkage of electrical to mechanical events being termed excitation - contraction coupling. Under normal circumstances there does appear to exist a certain relationship between the ionic composition of the cell and its mechanical and electrical activities. This relationship, however, may not be a simple or a direct one. Metabolic energy is required both for maintaining the ionic composition of the cell (the basis of the electrical excitation) and for the efficient functioning of the contractile mechanisms. The present study is concerned with the metabolic requirements for two processes in the rat myometrium smooth muscle viz. the transport of ions and contractions.

Daniel and Robinson (1960) showed that anaerobic conditions and most inhibitors of oxidation such as cyanide had relatively little effect on Na^+ extrusion and K^+ reaccumulation by cat and rabbit uterine segments that had been made Na^+-rich. On the other hand, glycolytic inhibitors such as Iodoacetic acid (IAA) and fluoride inhibited active transport of ions. They suggested that aerobic glycolysis provided the major share of energy for active transport of ions. Although Na^+ loss and K^+ gain appeared to occur in a 1:1 ratio, it was observed that dinitrophenol (DNP) had a greater effect on inhibition of K^+ accumulation rather than on Na^+ extrusion. Using the estrogen-dominant rabbit myometrium, Kao (1967) obtained results that he interpreted as showing that net Na^+ extrusion depended mainly on exogenously supplied glucose whereas net K^+ gain derived energy largely from endogenous substrates. He also suggested that when the supply of energy was exhausted, K^+ accumulation was dissociated from Na^+ extrusion.

Recently Taylor, Paton and Daniel (1969) have produced evidence for an electrogenic sodium pump in the rat uterus. The operation of this pump could be best demonstrated in sodium-rich tissues. The addition of potassium "switched on" the pump, led to an extrusion of sodium, the creation of a potential difference and the consequent accumulation of potassium. Given these conditions, it did not seem likely that potassium accumulation could occur in the absence of sodium extrusion, as suggested by Kao (1967). The other characteristics of this pump, viz., temperature dependence, ouabain

sensitivity, cation requirements suggested the involvement of a Na^+-K^+ ATP-ase and thus of metabolic energy.

In isolated rat uterine horns, metabolic inhibitors like IAA and DNP produced complex changes in unidirectional Na^+ and K^+ fluxes (Daniel and Robinson, 1971). Some of these effects were apparently related to depletion of cellular ATP; although it was highly likely that a number were non-specific. These compounds had effects on Na^+ influx and K^+ efflux that were greater than those produced by ouabain.

Metabolic inhibitors have marked effects on uterine contractility. Csapo and Gergely (1950) showed that in the IAA-treated uterus, the capacity to contract fell off rapidly and they suggested that this was due to depletion of high energy phosphates although no actual measurements were made. Marshall and Miller (1964) showed that IAA (2×10^{-4} M) irreversibly abolished all electrical activity in the pregnant rat uterus without affecting the level of the resting membrane potential, unless the tissues had been in contact with the drug for about 3 hours. The inhibited tissues failed to exhibit either spontaneous or stimulated contractions. On the other hand, the effects produced by DNP (10^{-3} M) were reversible and consisted mainly of a depolarization of the cell membrane with abolition of contractility. The authors pointed out that the effects produced by DNP, being rapid, could be unrelated to metabolism and may in fact represent some non-specific effects on the membrane. The contractility of immature rat uterine horns was irreversibly abolished

by IAA but reversibly inhibited by DNP (Paton 1968).

In the studies discussed above, the metabolic pathways providing energy for sodium pumping and for contractility were not clearly defined; the metabolic inhibitors being used primarily to produce depletion of high-energy phosphates. No attempts were made to by-pass the metabolic inhibition produced by suitable substrates and thus it was uncertain whether the effects noted were related to metabolism. The present study sought to determine the metabolic requirements for coupled Na^+-K^+ exchange in the rat myometrium and compare these with the energy requirements for spontaneous contractions. That these may be different had been suggested by some preliminary observations (Daniel, unpublished). Prasad and Macleod (1969) had argued that such a distinction based on a source of energy supply existed in cardiac muscle, with energy derived from glycolysis being used preferentially for membrane events rather than for contractility. On the contrary, Bueding et al., (1967) believed that at least in the guinea-pig taenia coli, ATP produced by oxidative processes was localized and rendered unavailable for contractile events. Webb (1966) cautioned against the general assumption that ATP generated intracellularly was used indiscriminately for all processes and suggested that intracellular compartmentalization was a real possibility. It was thus not inconceivable that the metabolic pathways providing energy for ion transport and contractions could be different. Furthermore, this study sought to examine carefully the net movements of Na^+ and K^+ in order to determine whether these

could be dissociated by the use of metabolic inhibitors under appropriate conditions. Such a scrutiny, it was believed, would also provide some information regarding the specificity of the metabolic inhibitors used.

To summarize, the questions posed at the outset of this study were:

a) Can energy obtained by either glycolytic or oxidative processes be utilized for active transport of ions and spontaneous contractions?

b) Can the coupled movements of Na^+ and K^+ be themselves dissociated by suitable metabolic alterations?

II. LITERATURE REVIEW

METABOLISM AND ION TRANSPORT

Living cells possess mechanisms that regulate the ionic composition of the intracellular fluid and the role of the cell membrane in this regard has been studied since the latter years of the 19th century. Intracellular fluid contains K^+ as the principal cation but contains much lower concentrations of Na^+ and Cl^-. Ostwald (1890) pointed out that when solutions of different ionic composition were separated by precipitation membranes, a potential difference would be generated. Bernstein (1902) applying the physico-chemical concepts developed by Ostwald to living cells, sought to explain the genesis of the "injury" potential by postulating a selective permeability of the membrane to K^+, the membrane potential being given by the Nernst equation. This simple view had to be modified when it became apparent that the cell membrane was permeable to Cl^- ions as well; Boyle and Conway (1941) showed that the skeletal muscle fibre membrane appeared to obey a Donnan equilibrium, being impermeant to Na^+. Further revisions became necessary when tracer studies showed that the cell membrane was permeable to Na^+ as well. (See Katz 1966, Dowben 1969 for historical review). There thus evolved the concept that the distribution of ions across the cell membrane involved a combination of passive and active forces and the term active transport was coined by Wilbrandt (1937). It was later defined as the movement of an ion against an electrochemical gradient (Ussing 1949). The energy for such an active transport can be obtained either by utilizing the free

energy present in an electrochemical gradient of another species or by a direct coupling to a vectorial metabolic process (Stein 1967).

The ability of tissues to utilize diverse substrates provides information regarding the metabolic pathways supporting specific processes. Combined with the use of metabolic inhibitors, such studies have provided information regarding the pathways energizing ion transport in a variety of tissues (see reviews by Webb, 1966; Conway, 1957; Shanes, 1958; Koefoed-Johnsen and Ussing, 1960). Thus, for instance, Van Bruggen and Zerahn (1960) showed that the frog skin was able to utilize exogenous supplied lactate and acetate for transporting Na^+. Similar studies had been reported earlier by Huf (1935) and Francis and Gatty (1938). This suggested that the Krebs cycle was operative in this tissue. Later work by Skjelvale *et al.*, (1960)showed that supplying intermediates from the Krebs cycle increased the O_2 consumption of the isolated epithelium of the frog skin, providing further evidence for the above contention.

Efforts have been directed not only at determining the metabolic pathways supporting active transport in different systems but also at identifying the actual energy donors and these have led to the evolution of two divergent concepts - the Redox-Pump hypothesis and the High-Energy Phosphate hypothesis (see Charnock and Opit (1968) for review).

As early as 1928, Lund sought to explain the genesis of bioelectric potentials by postulating the operation of a spatially

separated redox chain. At one end of the chain hydrogen atoms obtained from metabolic substrates were stripped of their electrons, leaving behind hydrogen ions. These electrons were transferred down the chain to the final acceptor - molecular oxygen (see Schoffeniels, 1967). Friedenwald and Stiehler (1938) suggested that the hydrogen ions left behind could either be secreted or exchanged for other cations. This view was subsequently developed and elaborated by Lundegardh (1939, 1954), Davies and Ogston (1950) and principally by Conway (1957). But there are several serious problems with this concept.

The Redox pump hypothesis sets a quantitative limitation on the number of cation equivalents transported. The reduction of each molecule of oxygen leaves behind four hydrogen ions that can be exchanged - and this is at maximal efficiency. Studies by Zerahn (1956) showed that the net oxygen consumption in the frog and toad skin using this model could only account for about 20% of the Na^+ transported. Studies by Leaf et al., (1959) on the toad bladder also showed that the amount of cations transported was in excess of that predicted. In several tissues, there is an appreciable transport of Na^+ under anaerobic conditions and the efficiency of energy utilization under aerobic and anaerobic conditions may not be markedly different (Schoffeniels, 1967). Dinitrophenol would be expected to stimulate ion transport since it stimulates mitochondrial redox reactions. In several systems - e.g., the toad bladder (Davis

et al., 1964) and Ehrlich's ascites cells (Hempling, 1966), DNP in fact inhibits active Na^+ transport.

Recently Dydynska and Harris (1966) found a close correlation between ion transport and stores of creatine phosphate and ATP. They showed that the number of energy-rich phosphates utilized for the transport of Na^+ under anaerobic conditions was constant for the same muscle, irrespective of the ultimate source (be it ATP, creatine phosphate or from glycolytic phosphorylation). Creatine phosphate was usually the main component depleted in the absence of an inhibitor like dinitrofluorobenzene (DNFB). This drug prevented the rephosphorylation of ADP to ATP by creatine phosphate and in its presence, the contents of ATP decreased. After inhibition with iodoacetate (IAA), transport seemed to be entirely at the expense of the two preformed phosphate esters. These findings not only raise problems for the Redox-pump hypothesis but provide considerable support for the alternative view - emphasising the central role played by ATP.

The concept that ATP may be the direct source of energy for ion transport is based on its ubiquitious nature and its synthesis from both respiring and glycolytic systems. Early studies by Fuhrman (1952), Schoffeniels (1955a) and Hodgkin and Keynes (1955 a,b) using dinitrophenol, produced indirect evidence for the involvement of ATP. The persistence of cation transport even under anaerobic conditions in a variety of tissues, e.g., duck red cells (Tosteson and Robertson 1956), frog skin (Leaf and Renshaw 1957 a,b) etc.,

suggested the existence of an energy donor common to both aerobic and anaerobic pathways - presumably ATP. However, the most convincing evidence for the direct involvement of high energy phosphates is derived from studies on the squid axon and erythrocyte ghosts.

In early studies on cephalopod axons, Hodgkin and Keynes (1955) showed that sodium efflux could be markedly inhibited by metabolic inhibitors (like DNP, CN or azide). Shanes and Berman (1955) showed that anoxia reduced Na^+ efflux but IAA had little effect. Caldwell (1960) attempted to correlate sodium transport in squid axons with contents of high energy phosphate esters. He showed that CN treatment led to a rapid but reversible disappearance in the tissue contents of arginine phosphate (which corresponds functionally to creatine phosphate in vertebrates) and ATP, and that the time course of these changes correlated well with those induced in Na^+ efflux by similar treatment. The sensitivity of the squid axon to DNP was pH dependent; the axon was more sensitive in alkaline solutions and more DNP was taken up at pH 8.0 than at pH 6.5. Yet even with this metabolic inhibitor, changes in ATP and arginine phosphate content ran parallel with changes in Na^+ efflux. This was strong circumstantial evidence in favour of high energy phosphates being involved in Na^+ transport. Further studies were carried out to see if injected ATP or arginine phosphate could restore normal ion transport in poisoned axons (Caldwell *et al.*, 1960 a,b). Seven compounds were tested: arginine phosphate, ATP, ADP, phosphoenol-pyruvate, GTP, ITP and creatine phosphate. All except ITP (slight effect)

and creatine phosphate (no effect) produced considerable increases in Na^+ efflux. Arginine phosphate and phosphoenol-pyruvate not only increased the Na^+ efflux but also restored to this process the requirement for external K^+ that is normally observed. ATP on the other hand, did not do so. It was suggested that the arginine phosphate and phosphoenol-pyruvate could provide sufficient ATP at the membrane site through interaction with arginine phosphokinase and pyruvate phosphokinase respectively. Injected ATP, on the other hand, might be dissipated throughout the axoplasm. Baker and Shaw (1965), using a squid axon preparation where the axoplasm had been extruded and replaced with artificial salt solutions, obtained data that suggested that approximately three Na^+ ions were extruded for each molecule of ATP hydrolysed.

Studies carried out on erythrocyte ghosts have produced further evidence for the involvement of ATP. Gardos (1954) introduced ATP into erythrocyte ghosts and showed that these preparations accumulated K^+. Hoffmann (1960) showed that ATP (but not other high energy phosphates) could be utilized by erythrocyte ghosts to extrude Na^+. Whittam (1962) showed that ATP was hydrolyzed only when it was present on the inside of the membrane preparation suggesting that the orientation of the enzyme system concerned may be responsible for vectorial transport.

Garrahan and Glynn (1967) loaded erythrocytes with K^+ and incubated them in a K^+-free Ringers solution containing Na^+. The absence of external K^+ prevented the operation of the Na^+ pump and

the ions moved down their concentration gradients. This, in essence, reversed the sodium pump and under such conditions there was in fact a net synthesis of ATP.

The existence of a membrane-bound enzyme system capable of hydrolyzing ATP was demonstrated in crab nerves by Skou (1957). This ATP-ase, which was stimulated by the combined presence of Na^+ and K^+ has been subsequently demonstrated in a wide variety of tissues (e.g., red cells, brain, nerve, kidney, muscle, liver, etc.; see Schoffeniels (1967) for list). There appears to be a close analogy between the properties of this enzyme system and those of the active transport system for Na^+ (the sodium pump). These similarities have been amply elaborated in a number of recent reviews (Charnock and Opit 1968) and only a few crucial points will be listed. These are: (1) the requirement of metabolic energy in the form of ATP for both systems; (2) the requirement for the combined presence of Na^+ and K^+ ions; (3) the vectorial properties of the Na^+ transport system can be "duplicated" in certain preparations, e.g., erythrocyte ghosts, where Na^+ and K^+ need to be on opposite surfaces of the membrane for ATP hydrolysis; and, (4) the inhibition of both the sodium pump and the transport ATP-ase by low concentrations of ouabain.

The circumstantial evidence seems overwhelmingly in favour of ATP being the direct source of energy but several pieces of evidence suggest the participation of other phosphorylated intermediates in energising ion transport.

The formation of ATP by oxidative phosphorylation presumably proceeds via the formation of several intermediates in accord with the scheme shown below:

$$\text{Respiration} \rightleftharpoons \sim 1 \rightleftharpoons \sim 2 \rightleftharpoons \sim P \rightleftharpoons ATP$$

In fact, Slater (1953) suggested that these intermediates may be utilized directly by energy requiring processes without the involvement of ATP. Two metabolic inhibitors, oligomycin and DNP have been used to explore the role of these intermediates in energising ion transport. Oligomycin presumably inhibits the reaction in the region of the second intermediate, allowing the formation of the first, but inhibiting the formation of ATP (van Rossum, 1964). DNP, on the other hand, probably promotes the hydrolysis of the first intermediate. (Borst and Slater 1961) It is believed that under anaerobic conditions, glycolytically generated ATP is used to form the intermediates by a reversal of the above sequence. (van Rossum, 1964)

Bricker and Klahr (1966) studied anaerobic Na^+ transport in the isolated turtle bladder. In this tissue, anaerobic transport is closely coupled to anaerobic glycolysis and DNP should have had no effect. But the drug markedly inhibited anaerobic Na^+ transport without inhibiting anaerobic glycolysis. In fact, glycolysis was stimulated as estimated by glycogen utilization or lactate production. The authors suggested that the DNP-sensitive intermediate was directly coupled to cation transport. A similar situation may hold in the toad bladder (Davis et al., 1969) and liver slices (van Rossum,

1964). To quote Davis (1969): "All the evidence in favour of ATP as the direct energy donor for translocation of sodium, and other ions as well, would be equally compatible with the hypothesis that high-energy phosphorylated intermediates are the donors of energy for ion transport".

Attempts have been made to obtain quantitative information on the relation between ATP hydrolysis and sodium transport. The ratio of the number of sodium ions transported to the number of energy-rich phosphate bonds utilized ($Na^+ : \sim P$ ratio) was approximately three in red blood cells (Glynn 1962, Sen and Post, 1964). In the frog skin and toad bladder, a similar value was obtained from measurement of the extra oxygen consumption (Zerahn, 1956; Leaf, Page and Anderson, 1959). Caldwell et al., (1960) correlated the number of Na^+ ions ejected out of the squid axon to the energy-rich phosphates injected and obtained a value of approximately 0.7. Later, Baker and Shaw (1965) showed that over 70 - 80% of the endogenous energy-rich phosphate of the squid axon was utilized in the axoplasm. If injected energy-rich phosphates underwent a similar fate, only 20 - 30% would be utilized for increasing Na efflux and the $Na^+ : \sim P$ ratio would be between 2.3 - 3.5. Garrahan and Glynn (1967) calculated that in red blood cells, ATP hydrolysis under intracellular conditions would leave 13017 cals. of free energy available and of these 9311 calories would be utilized in ion transport.

The concept of ATP as an energy donor has been criticized by several workers (Hill and Morales, 1951, Morales et al., 1955).

More recently, Banks and Vernon (1970) have marshalled more arguments against the high-energy concept. They contend that such a concept leads to undue emphasis on free energy changes, particularly those associated with the hydrolysis of ATP. The basic premise underlying such a viewpoint is that the free energy of hydrolysis of ATP directly transfers its energy to biosynthetic reactions or other functional events. Banks and Vernon (1970) argue that such thermodynamic considerations, although applicable to closed systems may be misleading in open systems like living cells. Calculations of free energy changes assume that equilibrium conditions exist. However, in living systems, such conditions do not hold and the content of ATP exists in a steady-state due to a balance between production and utilization. Furthermore, the hydrolysis of ATP does not seem to occur in biochemical reactions and thus energy-linked reactions may be meaningless. On the other hand, ATP could well form a chemical link between various reactions and this, in fact, may be its importance in living cells. Thus, the authors do not deny the relevance of ATP to biochemical reactions, but merely wish to eliminate the obsession with thermodynamic parameters on the part of biochemists studying such reactions.

If metabolism can regulate ion transport, it is reasonable to expect that ion transport can in turn influence metabolism. Quastel (1961) pointed out that both respiration and glycolysis may be partly controlled by the rate of ion transport. Whittam and

Willis (1963) measured O_2 consumption and the K^+ content (an index of ion transport) in kidney cortex slices from adult rabbits. They showed that various concentrations of ouabain caused parallel reductions in O_2 consumption and tissue K^+ contents. When the slices were incubated in Na^+-free solutions (choline being used as a substitute), oxygen consumption and tissue K^+ contents were reduced to constant levels. Increasing the external Na^+ in graded amounts led to an increase in O_2 consumption and in tissue K^+. They suggest that O_2 consumption may consist of two fractions - a constant basal rate and a variable rate that is a function of ion transport. They propose a model wherein ADP generated by the action of transport ATP-ase stimulates oxidative phosphorylation. Regulation of at least a part of respiration by ion transport has been demonstrated in several other tissues, e.g., frog skin (Ussing, 1959), sartorius muscle (Conway and Mullaney, 1961), toad bladder (Leaf et al., 1959) and mammalian brain slices (Whittam 1961, 1962). In kidney cortex (Whittam and Willis, 1963), the fraction of respiration controlled by and therefore presumably supplying energy for active transport amounts to 35 - 45% of the total at 25^o and 38^o.

Similar results have been obtained in glycolytic systems like the erythrocyte. Whittam and Ager (1965) showed that lactate production by human erythrocytes was stimulated by internal Na^+ and external K^+ and this effect was nullified by ouabain. The presence of K^+ was essential for the effects of ouabain, suggesting the

involvement of membrane ATP-ase. There seemed to exist a basal level of energy production that was unaffected by ouabain and a portion controlled by ion transport that varied from 20 - 75% of the basal energy level. In fresh cells, about 20% of the normal energy production was regulated by active cation transport whereas in stored cells with higher Na^+ contents, the comparable value was approximately 43%. They pointed out that control could be exercised through ADP and there were thus two possible loci for its action - the phosphofructokinase step or the phosphoglycerate reaction. Parker and Hoffman (1967) showed that the activity of phosphoglyceratekinase was modified by ion transport in human red blood cells. Since ADP was both a product of the ouabain-sensitive Na^+-K^+ ATP-ase and a substrate for phosphoglyceratekinase, it appeared to be a possible intermediary, although the effects of ouabain could not be correlated with levels of ADP. They therefore postulated a compartmentalization of ADP that controlled the phosphoglyceratekinase step. This study showed that the rate of lactate production was controlled (at least in part) by the Na^+ pump. These studies thus emphasized the close relationship between metabolism and active ion transport.

However, the ionic environment of cells is not determined solely by active transport systems and the influence of diverse manipulations on the passive permeability properties of the cell membrane has been studied (Lepke and Passow, 1968). A "leaky" membrane would allow more external Na^+ to enter the cell with a

consequent influence on the activity of the Na^+-pump. It is uncertain whether the determinants of passive permeability are physico-chemical factors like fixed positive charges, lipid composition, etc., or whether metabolic energy is involved. Metabolic inhibitors (e.g., IAA, fluoride and lead) along with Ca^{2+} cause a selective increase in passive K^+ movements and this permeability change can be aggravated by metabolites like adenosine. Gardos (1966) showed that bisulphite ions caused a hydrolysis of 2,3 diphosphoglycerate in the presence of Ca^{2+} ions and there was an increased loss of K^+. He suggested that metabolic interference with 2,3 diphosphoglycerate caused changes in the passive permeability to K^+ ions. Parker (1969) argued that the effects of bisulphite were unrelated to its effects on the metabolism of 2,3 diphosphoglycerate. These studies show that while using metabolic inhibitors, interpretations should not ignore the effects on passive permeability properties of the cell membrane.

The involvement of metabolic energy in the transport of ions, other than Na^+ and K^+ has not been extensively investigated. However, several studies have pointed out the relationship between metabolism and Ca^{2+} transport. Hasselbach, Makinose and Fiehn (1970) point out that perhaps all cells may possess transport or binding mechanisms for the elimination of Ca^{2+} ions from the cytoplasm. This is of particular importance in muscle cells, where depolarization is associated with a rapid increase in the concentration of Ca^{2+} ions in the myoplasm (Sandow, 1970). In fact, isolated sarcoplasmic membranes (which form vesicles) provide an elegant model of a calcium

accumulating system. Weber, Herz and Reiss (1966) showed that these vesicles took up Ca^{2+} from solutions containing ATP and Mg^{2+}. When a calcium precipitating agent like oxalate was present, large amounts of calcium oxalate were stored in these vesicles and this, in the absence of an active mechanism for transport of phosphate or oxalate, provides tentative evidence for an active calcium transport system (Hasselbach and Makinose, 1961, 1963). Their membrane preparation could use all nucleoside triphosphates and the energy requirements for the pump reached a maximum value of 5,000 cal/mole. A calcium pump has been demonstrated in human red cells by Schatzmann (1966), Schatzmann and Vicenzi (1969). Using the technique of reversible haemolysis, Schatzmann introduced Ca^{2+}, Mg^{2+} and ATP into red cells. Calcium extrusion occurred and was dependent on the ATP hydrolyzed. The ATP-ase activity was stimulated by internal but not external Ca^{2+} and was insensitive to Na^{+}, K^{+}, ouabain and oligomycin, thus rendering it independent of the Na^{+} pump. Van Breeman et al., (1967) have suggested an active Ca^{2+} exclusion mechanism in uterine smooth muscle since metabolic inhibition caused a net Ca^{2+} uptake, an increase in Ca^{2+} influx with no effect on efflux.

In the squid axon, on the other hand, a calcium extrusion mechanism exists that is dependent on the electrochemical gradient for Na^{+} ions. (Baker et al., 1969, Blaustein et al., 1969) The evidence suggesting that such a Na^{+}-Ca^{2+} link exists has been summarised by Baker (1971). These are "(a) Ca^{2+} extrusion occurs in axons fully poisoned with CN, (b) both in the presence and absence

of CN, Ca^{2+} extrusion is dependent on the presence of external Na^{+} ions and (c) reversal of the Na^{+} gradient by replacing external Na^{+} by Li^{+}, choline or sugar results in an increased Ca^{2+} influx coupled to Na^{+} efflux". Some studies suggest that a similar situation may exist in vascular smooth muscle. Thus Briggs and Malvin (1961) noted that in the rabbit aorta, Ca^{2+} influx increased with low Na^{+} and Keatinge (1968) showed that one fraction of the Na^{+} efflux in arterial muscle required extracellular Ca^{2+}.

Ca^{2+} is known to affect several metabolic functions, e.g., activation of phosphorylase a in tissues (Krebs, Graves and Fischer 1959, Williamson et al., 1967 a,b), stimulation of respiration and increased electron transporting mitochondria (Chance 1965) etc. Thus the alterations in Ca^{2+} fluxes with activity can be expected to have diverse effects on metabolism. That Ca^{2+} enters the nerves with electrical stimulation was shown by Hodgkin and Keynes (1957) and Baker, Hodgkin and Ridgeway (1970). Recently Landowne and Ritchie (1971 a,b) monitored the fluorescence changes of non-myelinated nerves following electrical stimulation. In such a preparation, a decrease in fluorescence represented presumably a reduction in the level of NADH, an increase in the level of NAD and by inference, a stimulation of mitochondrial oxidative phosphorylation. Conversely, an increase in fluorescence indicated a stimulation of glycolysis leading to an increased production of NADH. This study showed that Ca^{2+} entry induced both an increase and a reduction in fluorescence.

They argued that the decrease in fluorescence implied an increased rate of mitochondrial oxidative phosphorylation and the increase in fluorescence was caused by an activation of phosphorylase a or phosphofructokinase. They do not, however, have any measurements of metabolites to support their argument. However, this study does suggest that newer techniques in the future may provide elegant tools for unravelling the complex interactions between transport of ions and metabolic functions in the intact cell.

METABOLISM AND SMOOTH MUSCLE CONTRACTILITY

Most varieties of smooth muscle exhibit spontaneous contractions, but the basic cause for this automatic myogenic activity is uncertain. Bozler (1948) suggested that fluctuations in cellular metabolism led to alterations in membrane potential and these were in turn accompanied by mechanical changes. His suggestion, though not conclusively tested, has served to generate considerable interest in this field. However, even now "far less is known about mammalian smooth muscles..... than about skeletal or cardiac muscles. Many factors are responsible for this difference. Some of them are due to the vagaries of smooth muscle preparations....." Kao (1967).

Such vagaries include amongst others the problems of tissue variability that makes it difficult to extrapolate results obtained from one tissue to another, even in the same species. These are best exemplified by information obtained on vascular smooth muscle (Reviewed by Somlyo and Somlyo 1968). Thus the oxygen consumption of different aortic segments is reported to be unequal (Christie and Bahl, 1957); the extent of aerobic glycolysis may be subject to seasonal variations (Lundholm and Mohme-Lundholm, 1960); the relative proportions of glycolysis and oxidative metabolism may vary in different vascular tissues even in the same species.

It is not unlikely, however, that the basic mechanics of contraction would be similar in all types of muscle. In essence, this

consists of two interacting elements -- the contractile proteins and energy. The series of biochemical reactions linking metabolism and contractions in skeletal muscle have been worked out as follows: (reviewed by Needham 1960):

1. ATP is regarded as the most probable source of immediate energy.

2. The rapid replenishment of ATP occurs by the Lohmann reaction

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow[\text{transferase}]{\text{creatine phosphoryl}} \text{creatine} + \text{ATP}$$

3. Carbohydrate breakdown leads to the slower synthesis of ATP and the Lohmann reaction acting in reverse leads to replenishment of creatine phosphate.

Studies on different smooth muscles from different sources have emphasized the importance of carbohydrates as a source of energy. Thus carbohydrates appear to provide a major source of energy in vascular smooth muscle. However, in substrate-depleted, anaerobic vascular strips, fatty acids and Krebs cycle intermediates provided energy for contractions (Furchgott, 1966). Glycolysis, the citric acid cycle and the pentose pathway for the metabolism of carbohydrates have also been demonstrated in this tissue (Pantesco et al., 1962; Sbarra et al., 1960). The relative proportions of carbohydrates metabolized by oxidation and glycolysis varies in arterial and venous musculature. Preformed glycogen may be preferentially used (quoted by Somlyo and Somlyo, 1968) and the content of endogenous glycogen

in bovine mesenteric arteries is apparently sufficient to maintain activity in response to drugs for varying periods - for 8 hours under aerobic and 7 hours under anaerobic conditions. (Lundholm et al., 1960, 1962).

The ATP content of vascular smooth muscle is of nearly the same magnitude as in skeletal muscle. Lundholm et al., (1966) showed that iodoacetic acid (IAA) relaxed vascular smooth muscle under anaerobic conditions and this was accompanied by a reduction in the content of ATP and creatine phosphate. Later studies by the same group (Beviz et al., 1968), showed that in a glucose-free solution, the K^+ contractures of isolated bovine mesenteric arteries under anaerobic conditions was accompanied by a significant decrease in the content of ATP and creatine phosphate and by an increased lactate content. Resynthesis of high energy phosphates occurred within three minutes after the addition of K^+. Vascular smooth muscle contains little creatine phosphate and its contribution to regeneration of ATP is less than in skeletal muscle (Beviz et al., 1965). It is therefore easier to demonstrate a reduction in ATP with contraction without interference from the Lohmann reaction. Apparently much of the ATP required by vascular smooth muscle is synthesized during contraction. (Daemers-Lambert 1964, quoted by Somlyo and Somlyo 1968). Oxidative phosphorylation as determined by P/O ratios, is fairly efficient in vascular smooth muscle (Wollemann and Kocsar, 1964; Ritz and Kirk, 1967) and the more significant contribution of glycolysis to energy production in the

intact muscle may be due to a relative paucity of mitochondria. (suggested by Somlyo and Somlyo, 1968).

Bülbring and her colleagues (Bülbring and Lullmann 1957; Born and Bülbring 1955; Bülbring 1955) have studied the mechanical and electrical activities of the guinea-pig taenia coli using metabolic inhibitors (Axelsson and Bülbring 1961). Bülbring (1955, 1957) showed that the tension developed was inversely related to the membrane potential but directly related to the frequency of spikes and their duration. Later studies showed that this close coupling could be dissociated by several means, e.g., by interfering with the supply of metabolic energy. Using 2,4-dinitrophenol (DNP) to disrupt oxidative metabolism, Bülbring and Lullmann (1957) showed that in the initial stages, the frequency of spikes increased and the duration of spikes was prolonged. However, the tension which should have increased declined. At a later stage, DNP reduced the frequency of spike discharge, and ultimately membrane activity ceased. Later Axelsson, Bueding and Bülbring (1959) showed that exposure to a glucose-free solution produced similar results. Electrical activity persisted for several hours in the absence of glucose, but the tension declined rapidly. Thus there did seem to exist a differential sensitivity to metabolic inhibition (Axelsson and Bulbring, 1961). Born (1956) attempted to link these functional events to the levels of high energy phosphates. He showed a correlation between the content of creatine phosphate (CP) and the stimulated tension in this

tissue. On removing external glucose, a fall in the content of CP occurred concomitant with the decline in tension, but the levels of ATP were not significantly altered even after 180 minutes. Thus at a time when the expected dissociation between electrical and mechanical events would have occurred, the levels of ATP remained within the normal range. Bülbring and Kuriyama (1963) suggest that under these conditions bound calcium ions may be lost from the membrane leading to the dissociation. Later work by Timms (1964) showed that a brief exposure to a glucose-free medium reduced the level of glucose to a very low level but significant amounts of glycogen still persisted. Therefore, the dissociation between electrical and mechanical activity would have occurred at a time when glycogen stores would have been adequate for generating high energy phosphates (Axelsson, Hogberg and Timms, 1965). Timms (1964) suggested that under such conditions, the breakdown of glycogen may be slower and thus be insufficient to maintain the link to the contractile process. Thus these studies focused on the importance of carbohydrates as provenders of energy in smooth muscle.

The provision of energy in the myometrium is similar to that in other types of muscle. The high energy phosphates (ATP and CP) which are utilized during contraction are replenished largely by the metabolism of carbohydrates, although the uterus is capable of metabolizing fatty acids as well (Gergely 1964). This tissue is capable of storing considerable amounts of glycogen which act as a reserve. The enzymes concerned with glycolysis, the citric acid

cycle and the pentose pathway have been demonstrated (Needham and Shoenberg, 1967). The reactions involved in glycolysis are similar to those in skeletal muscle, but the whole process is slower - the stages responsible for the lower rate being the aldolase step and the oxidoreduction between triosephosphate and pyruvate (Hollmann, 1949). This tissue exhibits a Pasteur effect, i.e., anaerobic sugar utilization is greater than aerobic. Although the control steps have not been clarified, these may be similar to those in other tissues (Passonneau and Lowry 1962).

In the case of the uterus, the inherent tissue variability that plagues investigators on smooth muscle is further aggravated by the complex effects of hormonal steroids - estrogens and progesterone. These hormones affect the excitability and contractility and alter several biochemical parameters. Furthermore, where biochemical aspects are being studied, it is important to distinguish between the myometrium and mixed samples containing both the myometrium and the endometrium. Thus for example, the glycogen content of the rat and the mouse myometrium is reported to be higher than that of the endometrium (Leathem, 1959) but the endometrium of the non-pregnant rhesus monkey contains twice as much glycogen as the myometrium (Van Dyke and Chen 1936). Thus in studies on the effects of diverse procedures on the glycogen content of the uterus, this distinction between muscle and mixed samples assumes great importance. Then again age, as well as pregnancy, profoundly alters uterine metabolism and function. This surfeit of complexities makes evaluation

of results more difficult.

A feature of considerable importance is the marked effect of the estrogenic hormones on metabolism. The oxygen consumption of uteri from castrated rats that had received estrogen was higher as compared to the controls (Khayyal and Scott, 1931). Waalas et al., (1952) showed that oxygen consumption, glucose uptake and lactate production increased when estradiol was injected into spayed rats. In a series of papers, Singhal and his colleagues (1966, 1967a,b, 1969) have shown that estrogenic hormones, estradiol 17 β-diethyl-stilbesterol etc., increased the activities of a number of key glycolytic enzymes (viz., hexokinase, phosphofructokinase, and phosphohexoisomerase). Since the hormone-stimulated increases in the activities of these enzymes were blocked by agents like actinomycin D, cycloheximide and ethionine, it was inferred that the increase in enzyme activities was due to de-novo protein synthesis. Progesterone seemed to antagonize the effects of estrogen, though the amounts required were considerable.

Estrogenization has pronounced effects on the glycogen content of the uterus. This, as has been mentioned earlier, forms an important carbohydrate reserve in the myometrium. The increase in glycogen content during estrous or induced by estrogenization has been amply demonstrated (Boettiger, 1946; Kostyo, 1957; Telfer and Hisaw, 1957). Estradiol induces the synthesis of the enzyme glycogen synthetase (Singhal et al., 1969). Furthermore, the glycogen content

of the uterus increases markedly with pregnancy (Connolly et al., 1962); this may provide substrate for carbohydrate breakdown and replenishment of ATP at term. Studying the rate of glycogenolysis in the isolated estrogen-primed rat uterus, Schane and Leonard (1965), showed that the decrease in glycogen content had two phases. In the first 15 minutes, there occurred a rapid decrease in glycogen which was not affected by the presence of external substrates. The second slower phase was, however, markedly affected by the presence of substrates like glucose. In their presence, the content remained virtually at a standstill; however, in the absence of external substrates, the glycogen content declined slowly with time. Under anaerobic conditions, the glycogen content decreased very rapidly; half the glycogen content being metabolized within 10 minutes. Earlier West and Cervoni (1955) had shown that a certain critical amount of glycogen may be necessary for contractility. In the rat, maintenance of contractility became uncertain if the glycogen content was reduced to 2 μmoles/g wet weight (approximately). Waalas et al., (1952) found that glycogen breakdown was increased in uteri obtained from castrated animals treated with estradiol. Thus both glycogen deposition and breakdown may be promoted by estrogenization.

Estrogen administration increases the ATP and creatine phosphate content of whole uteri (Volfin et al., 1957). However, Waalas and Waalas (1950 a,b) found no significant increase in the ATP content of myometrial samples after estrogenization. Pregnancy, however, led to an increase in the ATP content. Part of the

differences may lie in the techniques employed, since Menkes and Csapo (1952) showed that removal of the endometrium led to a great reduction in the ATP content. In rabbit uteri, the above workers obtained an average value of 1.5 μmoles of energy-rich phosphate per gram of tissue. Later studies produced more conflicting data. Thus Volfin et al., (1957) showed that estradiol injection caused an increase in the high-energy phosphate and the content of ATP was doubled in ovariectomized rats that were estrogenized. Cretius (1957 a,b) showed that in the human myometrium, creatine phosphate, ATP and ADP were approximately twice as high at the end of pregnancy as compared to the non-pregnant uterus. In general, however, pregnancy and estrogenization bring about an increase in the stores of those substances involved in the energy transfer process - i.e., glycogen, high energy phosphates and key glycolytic enzymes.

Estrogenization also appears to affect the contractile elements themselves. Csapo (1948, 1950 a,b) obtained actomyosin from uterine extracts and showed that although the content was low in ovariectomized animals it increased with estrous. The ability to develop tension seemed to parallel the increase in actomyosin content. (Csapo and Corner, 1953). Studies by Needham and Williams (1963 a) showed that although the actomyosin content of uterine muscle was low as compared to skeletal muscle, the ratio of actin to myosin remained 1:4. The actomyosin obtained possessed ATP-ase activity. The enzymic activity showed changes similar to the actomyosin content following ovariectomy and at estrous; however, the increase

in ATP-ase activity preceded the increase in actomyosin content. Uterine actomyosin ATP-ase activity differed from that of skeletal muscle in several respects (Needham and Cawkwell, 1956). It showed little or no activation by Ca^{2+} ions at low ionic strength and negligible activation by Mg^{2+} at either low or high ionic strength. Since the energy for skeletal muscle contraction appears to be associated with a Mg^{2+} activated ATP-ase, this lack of stimulation by Mg^{2+} seemed surprising. However, studies by Hasselbach and Ledermair (1958) and Briggs (1963) showed that the tension induced by ATP in glycerinated fibres required external Mg^{2+}. Needham and Williams (1959) suggested that the enzymic activity of actomyosin may have been altered by the extraction procedures and have attempted to assess the ATP-ase activity of native myofilaments. They claimed that the actomyosin ATP-ase activity of such filaments was stimulated by Mg^{2+} as well as by Ca^{2+}. Goodall (1968) pointed out that estrogenization leads to the synthesis of several proteolytic enzymes and these may rapidly degrade actomyosin during extraction.

Given these diverse biochemical effects of estrogenization, it is not surprising that striking functional changes occur in the myometrium after the administration of estrogen. In the castrated rat, the uterus is electrically and mechanically quiescent (Marshall, 1962; Csapo, 1962). The membrane potential recorded is fairly low, approximately 35 mv. On estrogenization, the membrane potential is raised to a critical level (average 57 mv.) and this is accompanied by the onset of spiking activity and rhythmic contractions. From a

practical point of view, estrogenization increases the size of the uteri and the tissues can be handled with greater ease. Furthermore, the endometrium can be more easily stripped off if the uterus is obtained from estrogenized animals. It was shown by Csapo and Corner (1953) that the maximal isometric tension developed by rabbit uterine strips was related to the degree of estrogenization and this could be further correlated with the increased actomyosin contents seen in the latter instance. Also the capacity of the uterus to maintain contractility under anaerobic conditions was related to the extent of estrogenization. The ability of the uterus to contract is thus influenced by estrogenization which alters not only the excitability but also the provision of energy and the contractile proteins.

The effects of metabolic inhibitors on uterine contractility, the transport of sodium in this tissue, their relation to metabolism and the specific studies that led to the present project have been discussed earlier.

III. METHODS AND MATERIALS

METHODS AND MATERIALS

TISSUE PREPARATION

Female rats (Wistar strain), weighing 150 - 170 grams, were used in all experiments after being pretreated with 50 µg estradiol-17B, injected subcutaneously, three times a day for 2 - 3 days. After the animals had been killed by a blow on the head, their uterine horns were removed and dissected free of surrounding tissue. The endometrium was then removed and the remaining paired myometrial pieces from each animal cut to equal sizes. Na^+-rich tissues were obtained by incubating the tissues thus obtained in glucose-free, K^+-free Krebs overnight for 18 - 20 hours at 4^oC. The tissues usually weighed between 40 - 80 mg and each pair was incubated separately in 250 mls of the K^+-free solution. Any variations in the above procedure are mentioned in the text.

RECORDING OF CONTRACTIONS

After being rendered Na^+-rich, as described above, tissues were mounted in individual organ baths (25 ml capacity) and contractions recorded isometrically with force-displacement transducers (Grass Model FT03C) connected either to a Beckman dynograph or a Grass polygraph. The temperature of the organ bath was maintained

at 37°C by circulating warm water, through an outer jacket with a constant temperature circulator. The pieces of myometrium were usually allowed to equilibrate in glucose-free, K^+-free Krebs for at least 20 minutes before any manipulations were carried out. A resting tension of 0.5 - 1.0 g was applied to all tissues. The dynograph was calibrated with the use of weights and the sensitivity suitably adjusted to obtain decent tracings. The chart speed was set at 2.5 or 5 mm/min. In general, only spontaneous contractions were monitored. However, when these were feeble or non-existent, responses were elicited to 2×10^{-5} gm of acetylcholine, a supra-maximal concentration for this tissue. At the end of the experiment, the tissues were removed for estimation of ion contents.

DETERMINATION OF ION CONTENTS OF TISSUES

Tissues were rapidly removed at the end of the experiment and spread out on Whatman filter paper (Grade 1) and gently dabbed with tissue paper to remove excess fluid. The tissues were rapidly transferred to pre-weighed test-tubes and the wet weights obtained. The tissues were then dried in an oven at 105°C for 36 hours and the total tissue water obtained by calculating the difference in wet and dry weights. To each sample, 0.1 mls of 35% hydrogen peroxide and nitric acid solution were added and samples dried on a sand bath kept at 200°C. Further lots of nitric acid and hydrogen peroxide were

added till a whitish residue was obtained. In each instance, blank test-tubes were processed concurrently to correct for any Na^+ that may have been leached out of the glass by the acid. The residues obtained were dissolved in de-ionized distilled water and the volumes made up to 25 ml. The ion contents (Na^+ and K^+) were determined by flame photometry using an EEL flame photometer. Standard solutions of NaCl and KCl were used to construct a standard curve for each experiment. Values obtained from the blanks were subtracted from the sample readings and the ionic contents read off from the standard curve. The ionic contents were expressed in terms of final dry weight of the tissue. An Olivetti Underwood Programma 101 Desk computer was used to eliminate tedium.

MEASUREMENT OF EXTRACELLULAR SPACE

The extracellular space under several different conditions was measured using (^{14}C)-inulin or (^{14}C)-sucrose as extracellular markers. Although the protocol followed varied slightly between experiments, all tissues were exposed to the tracer dissolved in 10 ml of the appropriate solution. The incubation in the tracer solution was carried out in stoppered Ehrlenmeyer flasks to prevent evaporation. After the incubation, the tissues were quickly removed, blotted on filter paper, rinsed rapidly to remove superficial radioactivity and then blotted once more. The tissues were then

weighed and digested in a scintillation vial using NCS solubiliser. When the tissues were dissolved, 10 mls of Bray's phosphor (Bray, 1960) was added and the (^{14}C) content measured. Duplicate aliquots of the media used were also treated with 10 ml of Bray's phosphor and counted. The (^{14}C) contents were measured in a Picker Nuclear Liquid Scintillation Counter (Liquimat Model 110). All the vials were counted for 10 minutes and each was corrected for tissue size, background and quenching.

To correct for quenching of the samples, the channels-ratio method was used. Quenched (^{14}C) standards were counted on two channels, one of which covered the entire spectrum whereas the other covered approximately one-third of the total spectrum. Since the samples contained a known amount of radioactivity, the counting efficiency could be correlated with the channels-ratio observed and a calibration curve constructed. The experimental samples were counted using the same channels and after subtraction of background activity, the channels-ratio calculated and the corresponding efficiences read from the graph.

Where 2,4-dinitrophenol was used, a further correction was applied because colour quenching was pronounced. The percentage error had previously been estimated to be 16% (Osman, personal communication) and had been obtained by counting samples containing the same amount of radioactivity before and after addition of known amounts of 2,4-dinitrophenol.

The (^{14}C)-inulin and (^{14}C)-sucrose spaces were expressed in terms of ml/100 gm wet weight and calculated from the ratio

$$\frac{\text{dpm/100 gm wet wt. of tissue}}{\text{dpm/ml incubation medium}}$$

MEASUREMENT OF EXCHANGEABLE CALCIUM

Exchangeable tissue calcium was estimated with the use of tracer amounts of ^{45}Ca added to Krebs solution containing 1.5 mM Ca^{2+} (van Breeman, 1965). Tissues were exposed to the labelled solution for 120 minutes. At the end of that period, the tissues were quickly removed, blotted on filter paper, rinsed rapidly to remove superficial radioactivity and then blotted once more. The tissues were then weighed and digested in scintillation vials using NCS solubiliser (as described above). After digestion, tissues were dissolved in Bray's phosphor and counted in a Picker Nuclear Liquid Scintillation Counter (Liquimat 110). 1 ml aliquots of the appropriate media were dissolved in Bray's phosphor and counted. Appropriate corrections for background activity were applied.

Uterine concentration of Ca^{2+} was calculated as follows:

Specific activity of $^{45}Ca^{2+}$ (cpm/mmole) =

$$\frac{\text{medium } ^{45}Ca^{2+} \text{ content (cpm/litre)}}{\text{medium } Ca^{2+} \text{ content (mmole/litre)}}$$

Tissue content of Ca^{2+} (mmoles/kg wet wt) =

$$\frac{\text{tissue content of } ^{45}Ca^{2+} \text{ (cpm)}}{\text{specific activity of } ^{45}Ca^{2+} \text{ (cpm/mmole)}} \times \frac{10^6}{\text{wt of tissue (mg)}}$$

ESTIMATION OF ATP

References: (Kahlben and Koch, 1967; Lin and Cohen, 1968; Wirth, Daniel and Carrol, 1970).

Reagents: 0.1 M Arsenate buffer pH 7.4

2.0 mM Glycine buffer pH 9.5 - 10.0

Buffered Firefly Extract (Sigma)

Methods:

a) Preparation of Standards

A stock solution of ATP in 2 mM glycine buffer was prepared (1 mg/ml) and frozen in small quantities. From this stock solution, ATP standards were prepared afresh each time an assay was carried out. The range of standards prepared was determined by the anticipated contents of ATP in the tissues; usually the standards contained 0.25 - 1.0 µg/ml. All working standards were prepared in glycine buffer about an hour before assays were performed and stored in the cold till use.

b) Preparation of Enzyme Solution

Vials containing a crude Luciferin-luciferase preparation were used. The vials, each containing soluble extract from 50 mg of dried lanterns, were stored at $0^{o}C$ and were reconstituted in arsenate buffer about 60 minutes before use. The vials were broken, the contents emptied into a mortar and ground firmly with a pestle. The

powdered extract was dissolved in 300 - 400 ml of cold buffer solution. $MgSO_4$ was added to the enzyme solution to make up a final concentration of 0.02 M. The enzyme extract was pipetted into scintillation vials (5 ml) and stored in ice till just before use.

Extraction of ATP from Tissues

Tissues were incubated in oxygenated normal Krebs solution for 30 - 40 minutes and their "fresh" weights recorded at the end of that period. These tissues were then made Na^+-rich before the experiments were done. 4 - 6 mls of glycine buffer were heated to boiling in graduated test-tubes in a water-bath. At the end of the experiment, tissues were removed and quickly plunged into the hot glycine buffer and heating continued for 10 minutes. The tubes were then removed, buffer added to appropriate volume and the tubes stored in ice till the assays were performed.

Recoveries by this method of extraction were estimated in two ways:

a) A known amount of ATP (from the stock solution) was added to hot glycine buffer and boiled for 10 minutes. The extract was then assayed.

b) Two halves of the same uterus were boiled separately in glycine buffer and a known amount of ATP added to one tube. The difference in ATP contents was an estimate of that "recovered".

By both methods, the recoveries were virtually complete (98%).

Counting

A liquid scintillation counter (Unilux Nuclear-Chicago) was used for the assay. The instrument was set up for integral measurements on one channel that was kept wide open. All operations were carried out in the manual mode. Before the start of the assay, a few vials were counted for background activity which was usually negligible. The standards prepared were counted to obtain a standard curve. Since the enzyme activity (and thus the actual counts) varied considerably, such a curve had to be constructed afresh, each time an assay was done. 0.5 ml of each standard was added to 5 ml of the enzyme, mixed and the counting started exactly 30 seconds later. Three consecutive counts (0.1 minutes each) were taken of each standard. The extracts were then counted, using the same procedure. The extracts were counted in duplicate or triplicate and the standards recounted in between as a check. The entire counting period usually took less than 120 minutes and no significant decline in enzyme activity was noticed. Since the machine used had no cooling unit, all the vials were kept on ice during the experiment. Erratic results were obtained if the enzyme solution was not kept cool. A standard curve was obtained by plotting the average counts obtained against the concentration of standard on log-log paper. The amount of ATP in the extract was read off from the graph and its concentration in the

tissue calculated as follows:

Tissue ATP content (μmoles/gm) =

$$\frac{\text{Amount of ATP in aliquot (μg)}}{\text{Mol. wt of ATP}} \times \frac{\text{Tissue extract vol. (ml)}}{\text{aliquot vol. (ml)}} \times \frac{1000}{\text{wt of tissue (mg)}}$$

SOLUTIONS AND DRUGS

All the solutions were made using double-distilled water. The Krebs solution used had the following ionic composition (mM): NaCl 116; $NaHCO_3$ 22; NaH_2PO_4 1.2; KCl 4.6; $CaCl_2$ 1.5; $MgSO_4$ 1.2. Substrates were weighed out separately and added in concentrations of 10 - 20 mM into the final solution. K^+-free Krebs had the ionic composition of the solution shown above with the sole omission of K^+. Pyruvate Krebs was made by dissolving 25 mM sodium pyruvate in a modified Krebs solution containing 91 mM NaCl. All organic substrates and metabolic inhibitors were weighed out daily as required and added directly to the final solution. Wherever any substances were omitted from the solution, isosmolarity was maintained by sucrose. All solutions were equilibrated with either 95% O_2/5% CO_2 or 95% N_2/5% CO_2. The pH of all solutions was between 7.3 - 7.5. The pH of pyruvate Krebs was 7.8 but was reduced to 7.4 after equilibration with 95% O_2/5% CO_2.

The chemicals and drugs used in the present study and their sources are given below:

The substrates used were sucrose, D-glucose, D-galactose, D-mannose, sodium succinate (all obtained from Fisher Scientific Co.), sodium pyruvate (Sigma Chemical Co.) and β-(OH)butyrate (Mann).

The metabolic inhibitors used were 2,4-dinitrophenol (Fisher Scientific Co.), iodoacetic acid (Eastman Organic Chemicals), N-ethylmaleimide and α-iodoacetamide (Calbiochem) and 2-deoxy-D-glucose (from Sigma Chemical Co., Aldrich Chemical Co. and Mann).

The other drugs and miscellaneous chemicals used were acetylcholine chloride (BDH), histamine dihydrochloride (Sigma), ouabain (Nutritional Biochemicals Corp.), sodium arsenate and glycine (from Fisher Scientific Co.) and Luciferase-luciferin Enzyme extract (Sigma Chemical Co.).

The radiochemicals used were carboxyl (^{14}C)-inulin, 1.45 - 3.65 mc/g, mol. wt. 5000 - 5500 from New England Nuclear Corp. and U (^{14}C)-sucrose, 10.4 mc/mmole from Amersham/Searle Corp., $Ca^{45}Cl_2$, (Amersham/Searle Corp.).

STATISTICAL ANALYSIS

The variability of samples is expressed as mean ± standard error. The significances of differences between paired samples were determined using Student's t-test for paired data. Where multiple comparisons were performed, Scheffe's test was used (see Edwards, 1968). The 0.05 level of significance was chosen.

IV. RESULTS

RESULTS

That tissues soaked in low K^+-Ringer accumulated Na^+ in exchange for internal K^+ was demonstrated by Fenn and Cobb (1934). Steinbach (1940) showed that when such tissues were incubated in a solution with a higher K^+ content, the process was reversible. The extrusion of Na^+ by Na^+-rich rat uterine horns was studied by Daniel (1963).

In the present study, Na^+-rich segments were used for the following reasons:

1. The changes in total Na^+ content and K^+ content can be taken as reasonable indices for active cation transport, since the extrusion of Na^+ is "far beyond the small amount that could be attributed to diffusion from interspaces to equilibrate with the external fluid". (Carey, Conway & Kernan, 1959).

2. In the absence of external K^+, the Na^+ pump is inhibited. The addition of sufficient K^+ would be expected to activate the Na^+ pump at a maximal rate in Na^+-rich tissues and the effect of metabolic alteration would therefore be more pronounced under such conditions.

3. Earlier investigations (Taylor, Paton and Daniel, 1969) have demonstrated that Na^+-rich uterine pieces from pregnant rats were quiescent, with a low membrane potential, 15 mV inside negative. The addition of 4.6 mM K^+ at 37° led to an initial rapid hyperpolarization (to about 70 mV) which subsequently decreased to 46 mV and

spontaneous electrical and mechanical activity started. Thus the recovery of spontaneous contractions by Na^+-rich segments would provide a convenient end-point.

Before using Na^+-rich segments to study the effects of metabolic alterations, it became necessary to delineate some of the characteristics of this model system. The two aspects investigated were:

1. the alterations in ionic composition of Na^+-rich myometrial segments incubated in a K^+- containing medium.
2. the contractile properties of Na^+-rich myometrial pieces.

I. CHANGES IN IONIC COMPOSITION

When myometrial pieces were incubated in an oxygenated Krebs-Ringer medium for 30 - 45 minutes after dissection, they had the electrolyte composition shown in Table 1 (mean values, water 823 g/kg; Na^+ 533 mmoles/kg; K^+ 267 mmoles/kg). When such tissues were incubated for 17 - 20 hours at 4^o in a glucose-free, K-free Krebs-Ringer solution, containing 140 mM Na, they lost K^+ and gained Na^+. Tissues removed from the cold had a Na^+ content of 852 mmoles/kg and a K^+ content of 5 mmoles/kg. On rewarming in a K^+ free solution (glucose-free), there was an extrusion of water and Na^+ that occurred rapidly (15 minutes). Further rewarming for 2 hours produced little alteration. When Na-rich tissues were allowed to recover in a medium containing 4.6 mM K^+ (i.e., normal Krebs-Ringer), they extruded Na^+ and reaccumulated K^+.

TABLE 1

Ion contents of fresh and Na^+-rich tissues. All tissues were incubated in solutions equilibrated with 95% O_2/5% CO_2.

Group	Condition	TISSUE CONTENTS OF H_2O (g/Kg wet wt)	Na^+ (mmoles/Kg dry wt)	K^+ (mmoles/Kg dry wt)
I	Fresh [a]	823.2 ± 2.5	533.5 ± 15.5	266.5 ± 15.7
II	Na^+-rich tissues [b] (from the cold)	852.1 ± 5.8*	876.9 ± 34.5*	5.1 ± 2.4*
III	Na^+-rich tissues [c] (rewarmed K-free 15 min.)	837.6 ± 5.0*†	766.0 ± 23.5*†	6.6 ± 2.8*
IV	Na^+-rich tissues [d] (rewarmed K-free 120 min.)	831.9 ± 5.9*†	800.5 ± 44.7*†	8.7 ± 1.9*
V	Na^+-rich tissues (in Normal Krebs 120 min.)	836.9 ± 8.7*	602.6 ± 48.3*	252.1 ± 6.2

[a] Tissues incubated in Krebs solution for 45 minutes after dissection.

[b] Tissues made Na^+-rich as described in Methods and removed for ion analysis, straight from the cold.

[c] Na^+-rich tissues incubated in glucose-free, K^+-free Krebs for 15 minutes at 37°C.

[d] Na^+-rich tissues rewarmed in the same medium (as c) for 120 minutes.

* Differences found significant ($p < 0.05$) by Scheffe's test, using fresh tissues as the control.

† The Na^+ and H_2O contents in Groups III and IV were significantly different from those in Group II ($p < 0.05$, Scheffe's test).

The number of tissues in each experiment was 4 - 6.

The time-course of this recovery process was studied by incubating Na^+-rich tissues in normal Krebs-Ringer solution (140 mM Na^+, 4.6 mM K^+ and 20 mM D-glucose) and removing samples for ion analysis after varying periods. As the graph (Figure 1) shows, the tissues, which initially had a high Na^+ and a low K^+ content, rapidly extruded Na^+ and reaccumulated K^+. A steady-state was approached within an hour. The ionic contents are expressed in terms of final dry weight, since expression in terms of wet weight may be complicated by changes in water content. There appears to be little or no change in the H_2O content. As shown in the table, shifts in water took place fairly rapidly and in all these experiments, tissues were rewarmed in K^+ free solution for 15 - 20 minutes before the addition of K^+.

II. CONTRACTILE PROPERTIES OF Na^+-RICH TISSUES

Although the earlier studies by Taylor et al (1969) had shown that the pregnant rat uterus made Na^+-rich was quiescent and exhibited contractions only after the addition of K^+, no systematic investigation of the contractile properties of Na^+-rich tissues had been carried out.

Experiments were therefore designed to answer the following questions:

1. Can a Na^+-rich tissue contract in response to acetylcholine (Ach) used as a stimulant?

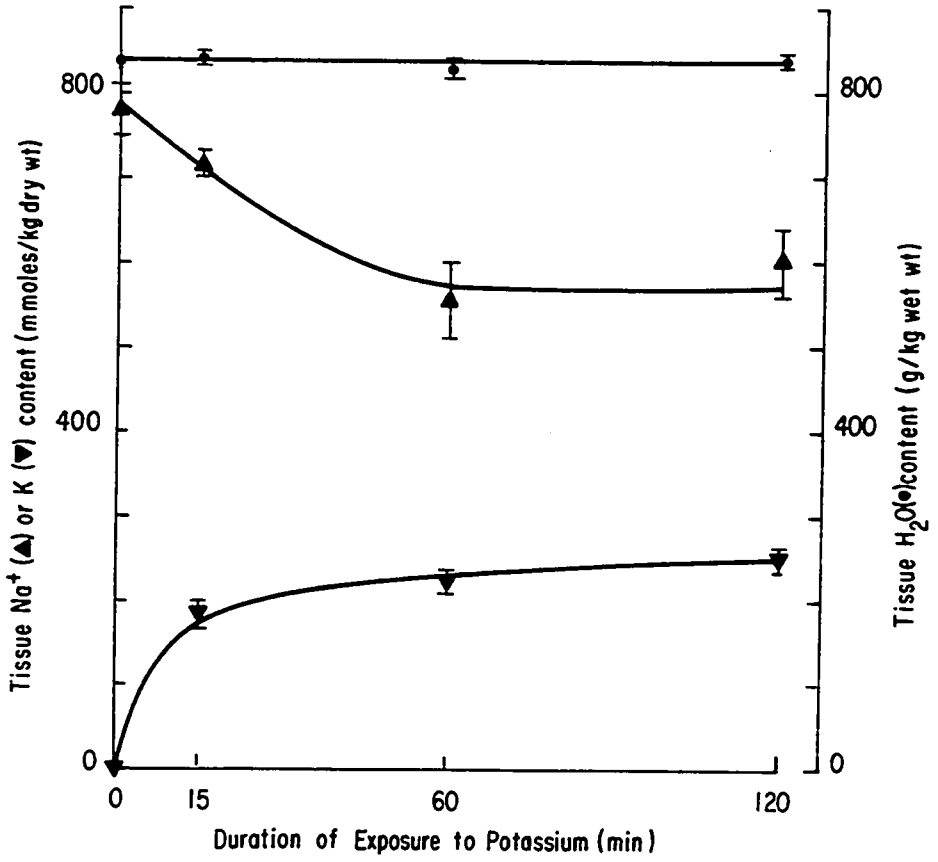

Figure 1. The graph shows the extrusion of Na^+ (▲-▲) and reaccumulation of K^+ (▼-▼) by Na^+-rich tissues exposed to normal Krebs solution (4.6 mM K^+, 140 mM Na^+, 20 mM D-glucose) and equilibrated with 95% O_2/5% CO_2. The changes in H_2O content (●-●) are also shown. Each point is the mean ± standard error for 4 - 6 tissues. All tissues were rewarmed in glucose-free, K^+-free Krebs for 20 minutes before the addition of K^+.

2. Does the presence of an exogenous substrate (eg., D-glucose) have any effect on contractility?

3. What is the effect of adding K^+?

Na^+-rich tissues were suspended in a substrate-free K^+-free medium at 37^o. In general, such tissues were rewarmed in the above medium for 15 - 20 minutes before tension was recorded. However, when tissues removed from the cold were suspended immediately in warm K^+-free Krebs, a contracture of varying amplitude and duration was seen.

The response of such tissues to a supramaximal dose of Ach (2×10^{-5} g/ml) was feeble. But for each individual piece, this response was reproducible for several hours under aerobic conditions. The addition of 20 mM D-glucose alone did not improve the contractile response. On the addition of K^+, the tissue underwent a prompt relaxation. After a variable period (usually 15 - 20 minutes), spontaneous contractions resumed. The response to stimulation was also markedly improved. This effect of K^+ occurred even in the absence of exogenous glucose (Figure 2, 3).

Thus, the preliminary investigations of this model system showed that:

1. Na^+-rich tissues recovered spontaneous contractions only after K^+ was added. By contrast, the addition of glucose in the absence of K^+ failed to restore spontaneous contractions.

2. the recovery of ions attained equilibrium within 2 hours.

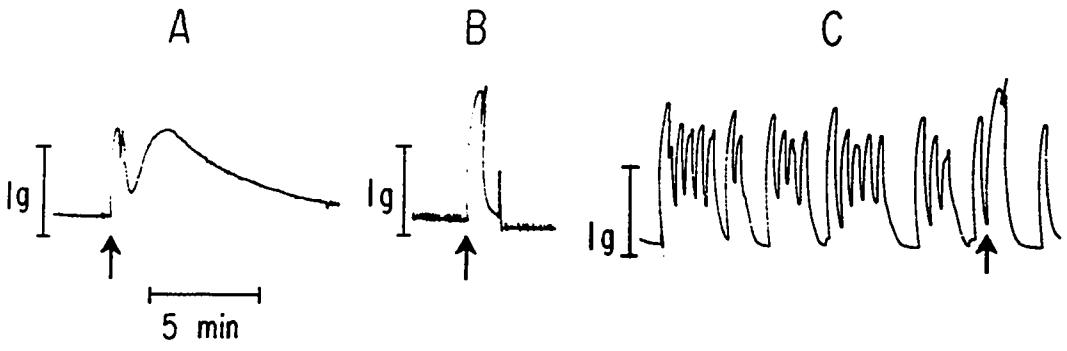

Figure 2. Contractile properties of Na^+-rich myometrium.

A. Na^+-rich tissue in glucose-free, K^+-free Krebs at 37°C; response to 2.5 x 10^{-5} g/ml acetylcholine (Ach) at ↑.

B. Response to same dose of Ach, 10 minutes after exposure to normal Krebs solution (4.6 mM K^+, 20 mM D-glucose).

C. 60 minutes later.

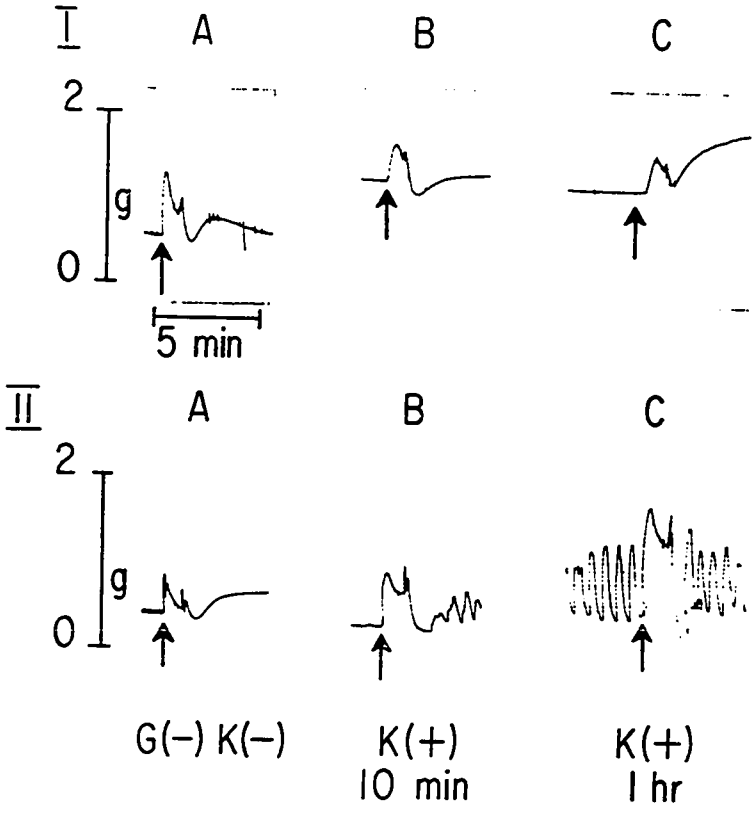

Figure 3. Contractile properties of Na^+-rich myometrium.

I. Effect of adding glucose, in the absence of K^+. Ach stimulation at ↑. (A) Tissues in glucose-free, K^+-free Krebs solution. (B) 10 minutes after addition of 20 mM D-glucose, no K^+. (C) 60 minutes later.

II. Effect of adding K^+ in the absence of glucose. Induced contractions with Ach (↑). (A) Tissues in glucose-free, K^+-free Krebs solution. (B) 10 minutes after addition of normal Krebs solution. (C) 60 minutes later.

Therefore, in subsequent experiments, the recovery of spontaneous contractions was monitored and tissues were removed for ion analysis at the end of 2 hours incubation in a medium containing K^+. Where spontaneous contractions were feeble or absent, stimulation with acetylcholine was tried to exclude effects on excitability alone.

EFFECT OF METABOLIC ALTERATIONS ON THE RECOVERY OF Na^+-RICH TISSUES

III. EFFECTS OF OXIDATIVE CONDITIONS

The effects of oxidative and hypoxic conditions on the recovery of spontaneous contractions and ion movements by Na^+-rich tissues were investigated. Wherever possible paired horns were used. Although the length of myometrial segments used in different experiments varied, each member of a pair was cut to equal size.

Paired horns incubated in a medium containing 4.6 mM K^+ and 20 mM D-glucose extruded Na^+, regained K^+ and recovered spontaneous contractions; no significant differences in ion contents or contractions were noted between tissues. Omission of D-glucose alone did not significantly reduce the recovery of either ion movements (Table 3) or spontaneous contractions (Figure 4). This suggested that the oxidative metabolism of endogenous substrates provided adequate energy for both processes.

TABLE 2

Recovery of ion contents and contractility of paired Na^+-rich rat myometrial segments. Tissues were exposed to Krebs solution containing 4.6 mM K^+ for 120 minutes.

Uterine Horn	TISSUE CONTENT OF			SPONTANEOUS CONTRACTILITY	
	H_2O	Na^+	K^+		Max. tension
	(g/Kg wet wt)	(mmoles/Kg dry wt)	(mmoles/Kg dry wt)	No. contracting	development (g)
Left	824.2 ± 11.3 [a]	610.4 ± 91.4	163.5 ± 13.8	6	2.9 ± 0.4
Right	823.6 ± 15.6	648.5 ± 92.3	182.0 ± 17.5	6	3.0 ± 0.5

[a] mean ± standard error of the mean of 6 observations in all cases.

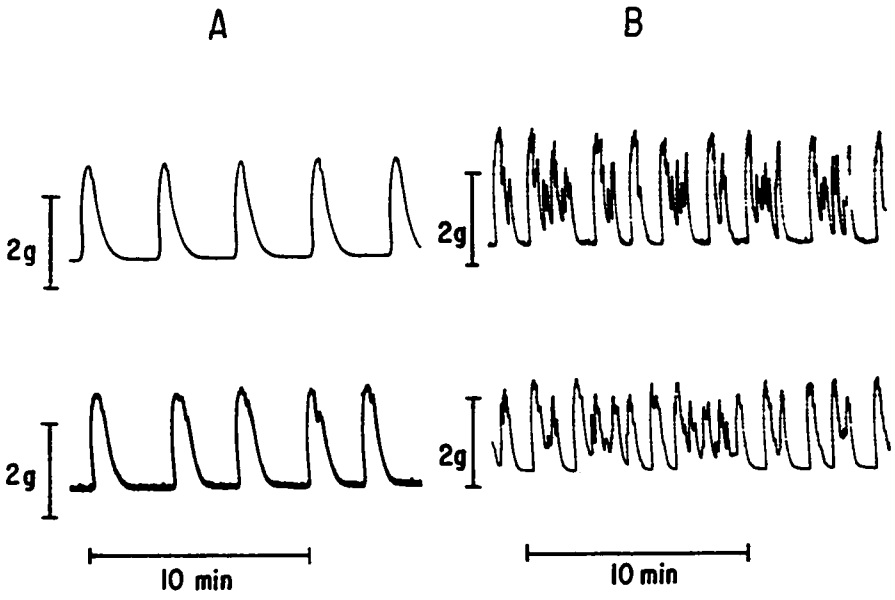

Figure 4. Spontaneous contractions of Na^+-rich tissues incubated in normal Krebs solution, equilibrated with 95% O_2/5% CO_2. Paired tissues in both experiments.

A. Paired horns incubated in normal Krebs containing 20 mM D-glucose.

B. Upper tracing: tissue incubated in Krebs (containing 20 mM D-glucose). Lower tracing: tissue in glucose-free Krebs, (containing 20 mM sucrose).

IV. RECOVERY UNDER HYPOXIC CONDITIONS

The recovery of contractions and ion movements by Na^+-rich tissues under hypoxic conditions was studied by incubating such tissues in a medium gassed with N_2 (95%) and CO_2 (5%). The paired controls were incubated in an oxygenated medium.

The results tabulated (Table 3) show that the Na^+ content of hypoxic tissues (704 mmoles/kg) was significantly higher than that of the control tissues (573 mmoles/kg). On the other hand, the K^+ content of hypoxic tissues (188 mmoles/kg) was significantly lower than that of the controls (243 mmoles/kg). That the hypoxic tissues had extruded Na^+ and regained K^+ can be seen when these contents are compared to those in an Na^+-rich tissue (see Table 1). The hypoxic tissues had also gained water. Under hypoxic conditions, the recovery of spontaneous contractions was also affected; not only was the amplitude of individual contractions lower, but the pattern was also irregular (see Figure 5).

More stringent conditions were applied -- i.e., tissues were incubated in a glucose-free medium under hypoxic conditions. As can be seen from Table 3, and Figure 5, both the recovery of ions and contractions was drastically impaired. Thus the hypoxic tissues had increased water and Na^+ contents and contained significantly less K^+ than the paired control. The spontaneous contractions exhibited were feeble and irregular.

TABLE 3

Effect of oxygen and D-glucose on recovery of ion contents and of contractility. Na^+-rich tissues were exposed to Krebs solution for 120 minutes under the conditions indicated.

Treatment			Tissue Content of			Spontaneous Contractility[a]
	20 mM D-glucose[b]	Oxygen[c]	H_2O (g/Kg wet wt)	Na^+ (mmoles/Kg dry wt)	K^+ (mmoles/Kg dry wt)	Maximal tension development (g)
Expt. I	+	+	849.1 ± 6.4[d]	640.1 ± 35.2	214.1 ± 12.1	3.3 ± 0.2
	-	+	843.1 ± 6.7	629.9 ± 34.0	196.7 ± 16.7	2.9 ± 0.2
Expt. II	+	+	841.9 ± 4.5	572.5 ± 19.8	242.7 ± 31.4	3.9 ± 0.9
	+	-	853.8 ± 5.6*	704.1 ± 48.1*	187.7 ± 25.4*	2.8 ± 0.9*
Expt. III	+	+	844.2 ± 4.4	668.5 ± 30.1	251.6 ± 4.1*	3.8 ± 0.4
	-	-	871.4 ± 11.5*	1037.6 ± 137.8*	84.6 ± 12.6*	0.9 ± 0.2*
Extp. IV	+	-	844.7 ± 4.5	647.0 ± 28.7	215.7 ± 12.5	2.1 ± 0.2
	-	-	855.8 ± 5.1*	794.9 ± 33.8*	95.1 ± 4.3*	0.4 ± 0.1*
Expt. V	-	-	849.6 ± 6.5	871.3 ± 44.6	77.1 ± 13.1	Not recorded
	-	- †	858.4 ± 5.1	959.8 ± 24.8*	33.2 ± 1.4*	Not recorded

[a] All tissues contracted spontaneously.
[b] Solutions contained either 20 mM D-glucose (+) or 20 mM sucrose (-).
[c] Solutions equilibrated with either 95% O_2/5% CO_2 (+) or 95% N_2/5% CO_2 (-).
[d] Mean ± standard error of the mean for 6 observations in all cases.
* $p < 0.05$ using paired 't' test.
† 1 mM ouabain used for 150 minutes - initial 30 minutes in glucose-free K^+-free Krebs solution.

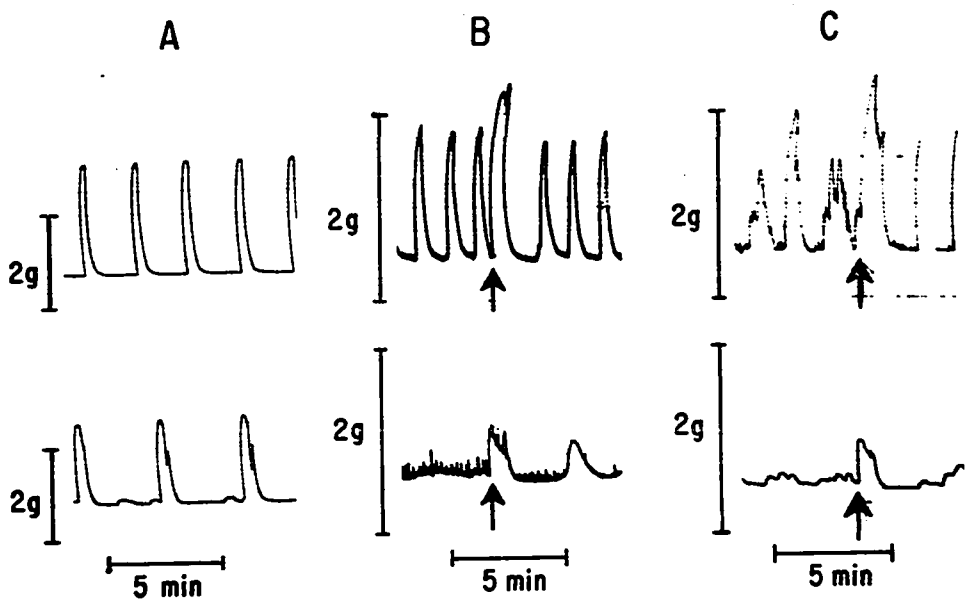

Figure 5. Recovery of spontaneous and drug-induced contractions of Na^+-rich tissues in Krebs solution (4.6 mM K^+), with (+) or without (-) 20 mM D-glucose. Solutions were equilibrated with 95% O_2/5% CO_2 (O_2) or 95% N_2/5% CO_2 (N_2). Paired horns in all experiments. Response to Ach (↑).

A. Upper tracing: Krebs (+) glucose, (O_2)
Lower tracing: Krebs (+) glucose, (N_2)

B. Upper tracing: Krebs (+) glucose, (O_2)
Lower tracing: Krebs (-) glucose, (N_2)

C. Upper tracing: Krebs (+) glucose, (N_2)
Lower tracing: Krebs (-) glucose, (N_2)

The above results suggested that although residual glycogen could support ion transport and contractions, recovery would be improved if D-glucose were supplied. When paired tissues were incubated under hypoxic conditions, it was noticed that the presence of 20 mM D-glucose brought about a distinct improvement. As the Table shows, tissues incubated in the absence of D-glucose had higher H_2O and Na^+ contents and lower K^+ contents. The spontaneous contractions exhibited by such tissues were feeble and their response to acetylcholine (2×10^{-5} g/ml) was less (0.6 ± 0.1 gms tension) than that observed with the control tissues (3.1 ± 0.1 gms tension).

Since the accumulation of K^+ and the extrusion of Na^+ seemed relatively poor, ouabain (1 mM) was used to determine whether this residual K^+ accumulation was occurring through the action of a coupled Na^+-K^+ pump. It was anticipated that hypoxic tissues treated with ouabain would contain less K^+ and more Na^+ than those not treated. A concentration of 1 mM ouabain was used, since this high a dose is necessary for inhibiting sodium transport in rat tissues (Daniel, 1963a, 1964). The tissues were pre-incubated with ouabain in glucose-free K^+ free solution for 30 minutes and then incubated with the drug in a glucose-free medium containing K^+ and gassed with N_2. As can be seen, the ouabain-treated tissues have higher Na^+ contents and lower K^+ contents than the paired controls.

V. STUDIES WITH 2,4 DINITROPHENOL (DNP) (see next page)

The experiments with hypoxic tissues suggested that anaerobic glycolysis could provide energy for both ion movements and contractions. If this were so, the disruption of oxidative metabolism with an uncoupler of oxidative phosphorylation should have little effect, provided suitable glycolytic substrates were provided. The experiments with DNP were designed to check this point and thus to obtain corroborative evidence for the role of glycolysis in this tissue.

Na^+-rich tissues were exposed to two different concentrations of the inhibitor (0.1 mM and 1.0 mM). In the initial 30 minutes of incubation, the tissues were exposed to the drug dissolved in K^+-free Krebs containing 20 mM D-glucose. After this pre-incubation period was over, the tissues were incubated in normal Krebs-Ringer (4.6 mM K^+ and 20 mM D-glucose), containing the same concentration of the drug for 120 minutes. Thus the total period of exposure to the drug was 150 minutes. The paired controls were incubated in identical solutions, DNP alone being omitted.

The ion contents of DNP-treated tissues are shown in Table 4. With either concentration of the inhibitor (i.e., 0.1 or 1.0 mM DNP) the treated tissues contained more water and Na^+ and less K^+ than the corresponding controls. Although the recovery of ion movements was reduced, it was not eliminated because marked recovery of K^+ contents occurred in the treated tissues. There did not appear to be any significant differences between the two concentrations of the inhibitor. Thus the differences in Na^+ contents between the treated and control tissues were 188 mmoles/kg with 0.1 mM DNP and

TABLE 4

Effect of 2,4-dinitrophenol (DNP) on recovery of ion contents and of contractility. Tissues were exposed to K^+-free solutions with (+) or without (-) DNP for 30 minutes; 4.6 mM K^+ was then added for an additional 120 minutes. All solutions contained 20 mM D-glucose.

DNP (mM)	Tissue Contents of			Contractility Maximum tension developed	
	H_2O (g/Kg wet wt)	Na^+ (mmoles/Kg dry wt)	K^+ (mmoles/Kg dry wt)	spontaneous	induced
Expt. I					
-	841.8 ± 13.0[b]	680.3 ± 100.4	316.9 ± 27.9	1.4 ± 0.1	1.9 ± 0.8
0.1 (+)	865.3 ± 9.3*	868.9 ± 84.3*	249.3 ± 17.0*	0.3 ± 0.1*	0.9 ± 0.2*
Expt. II					
-	835.1 ± 5.9	604.6 ± 40.8	230.1 ± 21.3	1.5 ± 0.2	2.3 ± 0.2
1.0 (+)	857.6 ± 4.1*	783.5 ± 31.0*	189.8 ± 20.1*	0.1 ± 0.04*	0.8 ± 0.1*

[b] Mean ± standard error of the mean of 9 observations in all cases.

* $p < 0.05$ using paired 't' test, compared to control without DNP.

179 mmoles/kg with 1.0 mM DNP. The corresponding values for K^+ were 67 mmoles/kg and 40 mmoles/kg respectively.

Both doses of DNP appeared to have marked effects on spontaneous contractions (Table 4, Figure 6). With 0.1 mM DNP, only 5 pieces exhibited spontaneous contractions and even these were feeble. All the pieces, however, responded to a supramaximal dose of Acetylcholine (2×10^{-5} g/ml). This suggests that treatment with DNP may have affected excitability rather than the ability of the tissue to contract. Approximate calculations showed that the spontaneous contractions in the control tissue were 70% of that observed after stimulation. However, with 0.1 mM DNP, the spontaneous contractions were only 12% of that seen with drug stimulation. Similar results were obtained with 1 mM DNP.

To study the ability of different sugars to support ion pumping and contractions, the design described below was adopted. Na^+-rich tissues underwent a 150 minutes exposure to 0.1 mM DNP; in the initial 30 minutes, the drug being dissolved in K^+ free Krebs. 20 mM of the test sugar was present in the incubation medium during the entire experiment. The sugars used were sucrose, D-fructose, D-galactose, D-mannose and D-glucose. The results (Table 5, Figure 6) show that whereas sucrose, D-fructose and D-galactose were ineffective, D-mannose substituted for D-glucose in supporting both ion transport and contractions. Thus the K^+ contents of tissues incubated in D-glucose and D-mannose were significantly higher than those of tissues incubated in the other substrates. Furthermore, the tissues that failed to recover contractions also had higher H_2O

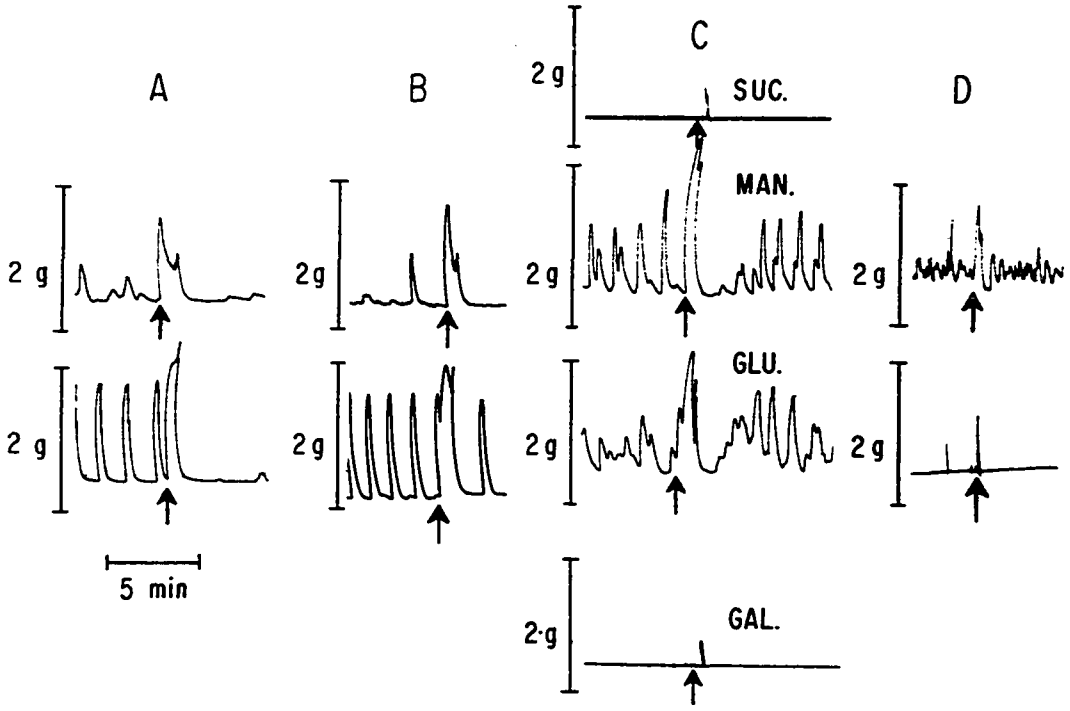

Figure 6. Recovery of spontaneous and drug-induced contractions of Na^+-rich tissues treated with 2,4-dinitrophenol (DNP). All solutions were equilibrated with 95% O_2/5% CO_2; responses to ACh (↑). Paired tissues used except in C.

A. Upper tracing: Tissue in 1.0 mM DNP for 150 minutes; 30 minutes in glucose-free, K^+-free Krebs solution and then in normal Krebs, 4.6 mM K^+ and 20 mM D-glucose.
Lower tracing: Similar incubation; DNP, omitted.

B. As in (A); except 0.1 mM DNP used. Upper tracing: treated tissue. Lower tracing: control.

C. Recovery of Na^+-rich tissues treated with 0.1 mM DNP, 150 minutes as in (B), except that various sugars substituted for D-glucose (Glu). These were sucrose (SUC), D-mannose (MAN) and D-galactose (GAL).

D. Upper tracing: tissue in 1.0 mM DNP, as in (A).
Lower tracing: Paired horn; treated with 0.5 mM IAA for 30 minutes and then incubated in 25 mM sodium pyruvate (for explanation, see later).

TABLE 5

Effect of glycolytic substrates on the recovery of ion contents and contractility in the presence of 2,4-dinitrophenol. All tissues were incubated in K^+-free Krebs solution containing the indicated substrate and 0.1 mM DNP for 30 minutes. 4.6 mM K^+ was then added for an additional 120 minutes.

Substrate (20 mM)	No. of tissues	Tissue Contents of			Max. tension development	
		H_2O (g/Kg wet wt)	Na^+ (mmoles/Kg dry wt)	K^+ (mmoles/Kg dry wt)	Spontaneous	Induced
D-glucose	5	835.5 ± 10.6	697.2 ± 76.6	169.1 ± 13.2	0.6 ± 0.2	1.3 ± 0.2
D-mannose	5	845.6 ± 9.2	730.9 ± 60.1	168.7 ± 15.9	0.6 ± 0.2	1.5 ± 0.3
D-galactose	4	863.4 ± 11.2*	947.1 ± 81.6*	60.5 ± 8.4*	Nil	Nil
D-fructose	4	869.7 ± 6.8*	1011.5 ± 51.7*	51.7 ± 7.5*	Nil	Nil
Sucrose	8	847.4 ± 6.1	842.6 ± 41.2*	54.5 ± 3.9*	Nil	Nil

* $p < 0.05$, compared to values obtained in presence of D-glucose (using Scheffe's test).

contents, with the sole exception of tissues incubated in sucrose. The lower H_2O contents in the latter case could be explained by the osmotic effect of sucrose, since it is relatively impermeant.

Thus these experiments with DNP show that:

1. In the presence of D-glucose, disruption of oxidative metabolism by DNP reduces but does not abolish ion pumping as judged by K^+ accumulation.

2. DNP reduces the recovery of spontaneous contractions, but the response to stimulation is affected to a lesser degree.

3. D-mannose alone of the sugars used, proves an adequate substitute for D-glucose under these conditions.

VI. EFFECTS OF 2 DEOXY-D-GLUCOSE (2DG)

The results obtained with DNP had shown that Na^+-rich tissues recovered ions and contractions when supplied with glycolytic substrates. To study the utilization of oxidizable substrates by the myometrium, it was necessary to inhibit glycolysis. Experiments were performed with 2 deoxy-D-glucose which had been shown to inhibit glycolysis in a variety of tissues (Webb 1966 for references).

Na^+-rich tissues were incubated with 20 mM 2 deoxy-D-glucose for a total period of 180 minutes; in the first 60 minutes, the inhibitor was present in K^+ free Krebs and in the subsequent period, it was dissolved in normal Krebs-Ringer solution; D-glucose was omitted throughout the duration of the experiment. The control tissues were incubated in glucose-free, K^+ free Krebs for 60 minutes

and in glucose-free Krebs for 120 minutes, sucrose being used as a substrate for glucose. The results (Table 6) show that tissues incubated in 2 deoxy-D-glucose extruded Na^+ and accumulated K^+ to a significant extent and exhibited spontaneous contractions as well. In fact, there was no significant difference in either ion contents or contractions from control tissues. Thus under oxidative conditions, 2 deoxy-D-glucose, even in high concentrations, did not inhibit the supply of energy for ion movements and contractions. In two experiments, tissues were incubated in 50 mM 2 deoxy-D-glucose. No significant inhibition occurred and therefore no attempt was made to use higher concentrations.

The experiments were repeated under hypoxic conditions. In the first set of experiments, Na^+-rich tissues were treated with 20 mM 2 deoxy-D-glucose for a total period of 180 minutes, in the complete absence of exogenous glucose (as described earlier). The control tissues were incubated in a medium containing 20 mM D-glucose. Under such conditions, the recovery of ions and spontaneous contractions by inhibited tissues was significantly lower. In the second set of experiments, the tissues treated with 2 deoxy-D-glucose were compared with tissues incubated in glucose-free Krebs. Under these conditions also, inhibited tissues contained significantly higher amounts of Na^+ and water and lower amounts of K^+. This suggested that 2 deoxy-D-glucose could inhibit the breakdown of residual glycogen under hypoxic conditions. However, this inhibitory effect of 2 deoxy-D-glucose was relatively weak, since addition of 5 mM

TABLE 6

Effect of 2 deoxy-D-glucose (2DG) on the recovery of ions and contractions under oxidative and hypoxic conditions. Tissues were equilibrated with either 95% O_2/5% CO_2 or 95% N_2/5% CO_2.

2 Deoxy-D-glucose[a] (20 mM)		D-glucose (mM)	O_2/N_2	Tissue Contents of H_2O (g/Kg wet wt)	Na^+ (mmoles/Kg dry wt)	K^+ (mmoles/Kg dry wt)	Spontaneous Contractility Maximum tension development (g)
Expt. I	+	-	O_2	838.0 ± 3.9[b]	639.3 ± 32.6	188.6 ± 24.7	1.2 ± 0.5
	-	+(20)	O_2	830.7 ± 7.5	668.9 ± 23.7	215.0 ± 14.4	2.0 ± 0.4
Expt. II	+	-	N_2	860.1 ± 10.3*	1066.7 ± 27.5*	53.9 ± 5.3*	0*
	-	+(20)	N_2	843.7 ± 6.6	781.9 ± 23.1	123.5 ± 0.7	1.0 ± 0.2
Expt. III	+	-	N_2	864.6 ± 8.4	999.5 ± 68.6*	52.7 ± 3.1*	0*
	-	-	N_2	853.8 ± 7.1	892.2 ± 56.8	73.6 ± 5.3	0.3 ± 0.2
Expt. IV	+	+(5)	N_2	851.7 ± 5.8	790.9 ± 29.6	81.4 ± 7.3	0.4 ± 0.1
	-	+(5)	N_2	852.2 ± 1.7	818.6 ± 13.0	100.0 ± 8.8	0.6 ± 0.1

[a] Tissues were exposed to 2DG for a total period of 180 minutes. (for 60 minutes in K^+-free, 120 minutes in K^+ containing Krebs solution).

[b] Mean ± standard error of at least 4 observations.

* $p < 0.05$ using paired 't' test.

D-glucose was able to reverse the inhibition (Table 6, Experiment IV). Since D-glucose was added along with the inhibitor, this suggested that this reversal was perhaps related to a competition at the carrier site.

Since 2 deoxy-D-glucose proved ineffective under aerobic conditions, it was not possible to use it to study the utilization of oxidizable substrates by the myometrium. Therefore, studies were carried out with another inhibitor of glycolysis-iodoacetic acid (IAA).

VII. STUDIES WITH IODOACETIC ACID (IAA)

Iodoacetic acid (IAA) inhibits the glycolytic enzyme, 3 phospho-glyceraldehyde dehydrogenase, but is neither specific nor even very selective. If, however, the dose and the duration of exposure are chosen appropriately, the selectivity of the drug for the above enzyme can be enhanced (Webb, 1966) (p. 95-96).

Na^+-rich tissues were exposed to IAA dissolved in glucose-free K^+ free Krebs for 30 minutes. The drug was then washed out and tissues incubated in Krebs solution containing the appropriate substrate. IAA (0.5 mM or 1.0 mM) prevented the recovery of either ion movements (Table 7) or contractions (Figure 7) when 20 mM D-glucose was supplied as the exogenous substrate. Treated tissues had significantly higher contents of water than controls. Furthermore, compared to Na^+-rich tissues rewarmed for 120 minutes in K^+ free Krebs, IAA-treated tissues had significantly higher contents of Na^+. Thus

TABLE 7

Effect of iodoacetic acid on recovery of ion contents and of contractility. Tissues were exposed to K^+-free Krebs solution with or without the inhibitor for 30 minutes. All tissues were then incubated for a further 120 minutes in Krebs solution free of inhibitor.

Iodoacetic acid (mM)		Tissue Contents of			Spontaneous Contractility
		H_2O (g/Kg wet wt)	Na^+ (mmoles/Kg dry wt)	K^+ (mmoles/Kg dry wt)	Maximum tension developed (g)
Expt. I	-	817.9 ± 7.1[a]	476.8 ± 48.1	169.7 ± 13.1	3.4 ± 0.5
	0.5	877.5 ± 6.1*	954.0 ± 55.2*	33.6 ± 7.8*	Nil[b]
Expt. II	-	814.3 ± 7.9	473.0 ± 38.0	178.4 ± 24.2	2.3 ± 0.4
	1.0	861.8 ± 9.1*	904.3 ± 33.3*	16.6 ± 2.2*	Nil[b]

[a] Mean ± standard error of the mean of 4 observations in all cases.

[b] There were no responses to drug-stimulation either.

* $p < 0.05$ using paired 't' test.

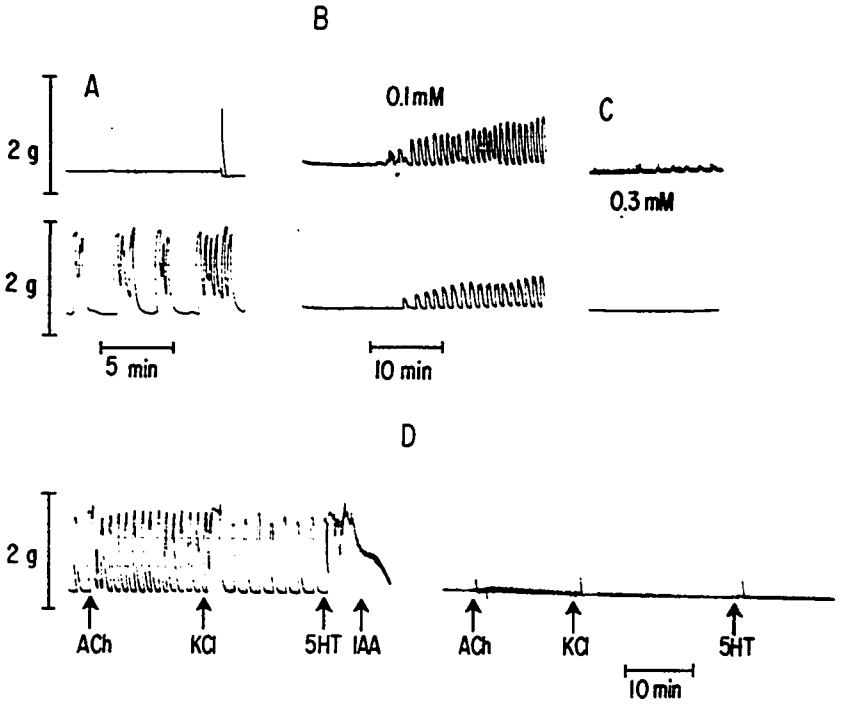

Figure 7. Effect of Iodoacetic acid (IAA) on the recovery of contractions by Na^+-rich tissues. All solutions were equilibrated with 95% O_2/5% CO_2.

A. Upper tracing: 0.5 mM IAA, in glucose-free, K^+-free Krebs solution for 30 minutes; then normal Krebs solution (20 mM D-glucose). Lower tracing: Tissue in normal Krebs solution, no IAA.

B. Upper tracing: 0.1 mM IAA, 30 minutes; then incubation in pyruvate Krebs (25 mM sodium pyruvate, 4.6 mM K^+). Lower tracing: 0.1 mM IAA, 30 minutes; then in normal Krebs (20 mM D-glucose).

C. Upper tracing: 0.3 mM IAA, 30 minutes; then in pyruvate. Lower tracing: 0.3 mM IAA, 30 minutes; then in glucose Krebs.

D. Responses of fresh myometrium to 2.5×10^{-5} g/ml Ach, 50 mM K^+, 2×10^{-5} g/ml 5HT; followed by a 30-minute exposure to 0.5 mM IAA. The tissue was subsequently allowed to recover in pyruvate Krebs and responses to the same agonists obtained.

inhibited tissues failed to accumulate K^+ and gained Na^+ and H_2O.

In order to determine whether the above effects of IAA were related to glycolytic inhibition, the ability of 25 mM sodium pyruvate to reverse the effects of IAA (0.1 - 1.0 mM) was studied. Since the substrate was used as the monosodium salt, an equivalent amount of Na Cl was removed from the incubation medium. After a 30 minute exposure to 0.1 mM IAA, contractions and ion movements recovered in the presence of either D-glucose or pyruvate, (Table 8) suggesting that glycolytic inhibition was incomplete. However, greater K^+ accumulation occurred in the presence of pyruvate and the average tension developed was significantly higher. With increasing concentrations of the inhibitor, contractility failed to occur in the presence of either substrate. D-glucose proved ineffective in restoring ion gradients whereas pyruvate partially restored ion movements. However, with increasing concentrations of the inhibitors, the accumulation of K^+ seen with pyruvate was gradually reduced. The water and Na^+ gain seen with increasing concentrations of IAA did not appear to be significantly affected by pyruvate.

In the above experiments, tissues had been removed after they had been exposed to K^+ and substrate for 120 minutes, since it had been observed that recovery of ions was complete by that period in untreated tissues. However, a slower recovery may have occurred in poisoned tissues. To check this point, the time course of recovery of IAA-treated tissues in pyruvate was studied. Tissues were exposed

TABLE 8

Effect of pyruvate on recovery of ion contents and of contractility of iodoacetate-treated tissues. All tissues were exposed to K^+-free Krebs solution containing iodoacetic acid for 30 minutes. Tissues were then incubated for an additional 120 minutes in Krebs solution containing the substrate indicated but free of inhibitor.

Iodoacetate (mM)	Substrate (25 mM)	Tissue Content of			Spontaneous Contractility
		H_2O (g/Kg wet wt)	Na^+ (mmoles/Kg dry wt)	K^+ (mmoles/Kg dry wt)	Maximal tension development (g)
Expt. I	D-glucose	886.9 ± 6.8	991.9 ± 59.0	211.2 ± 64.0	0.6 ± 0.1
0.1	pyruvate	865.6 ± 10.7[a]	844.5 ± 56.5	244.4 ± 51.5*	1.6 ± 0.2
Expt. II	D-glucose	858.1 ± 7.6	926.6 ± 66.8	89.3 ± 31.9	nil[b]
0.2	pyruvate	843.5 ± 8.4*	725.1 ± 66.0*	203.4 ± 30.0*	
Expt. III	D-glucose	880.5 ± 7.5	1074.6 ± 74.1	69.2 ± 4.8	nil[b]
0.3	pyruvate	868.7 ± 10.0*	898.3 ± 64.8*	177.3 ± 9.3*	
Expt. IV	D-glucose	872.7 ± 2.9	1008.3 ± 57.9	48.6 ± 3.5	nil[b]
0.5	pyruvate	853.5 ± 4.7*	803.8 ± 46.8*	166.5 ± 43.4*	
Expt. V	D-glucose	903.5 ± 10.3	1402.6 ± 18.4	58.7 ± 16.1	nil[b]
1.0	pyruvate	897.8 ± 10.8	1330.2 ± 18.8	115.4 ± 14.0*	

[a] Mean ± standard error of the mean of 4 - 7 observations
[b] No spontaneous or induced contractions occurred.
* $p < 0.05$ using paired 't' test.

to 0.5 mM IAA for 30 minutes, the inhibitors washed out and the tissues allowed to recover in pyruvate Krebs for varying periods of time, prior to removal for ion analysis.

The results obtained are shown in the graph (Figure 8). The contents of Na^+ and K^+ are expressed as mmoles/kg dry weight and the water contents as g./kg. As can be seen, the tissues rapidly accumulated K^+, the process attaining a steady-state within 120 minutes. There did not, however, appear to be any extrusion of Na^+ and the mean Na^+ contents appeared to increase with time; there was, however, considerable variability. The water content of the tissues increased gradually during the incubation period. The results obtained here were in sharp contrast to those observed earlier with untreated tissues, where K^+ gain was accompanied by loss of Na^+. Thus under these conditions there appeared to be a dissociation between K^+ accumulation and Na^+ extrusion.

Further experiments were carried out to clarify this point and specifically to answer the following questions:

1. Is the K^+ accumulation noticed sensitive to inhibition by ouabain? That is, is there a coupled movement of Na^+ and K^+ occurring under these conditions?

2. Is the K^+ gain noticed dependent on the oxidative metabolism of pyruvate?

(i) Effect of Ouabain on the Accumulation of K^+

To determine the effects of ouabain on K^+ accumulation,

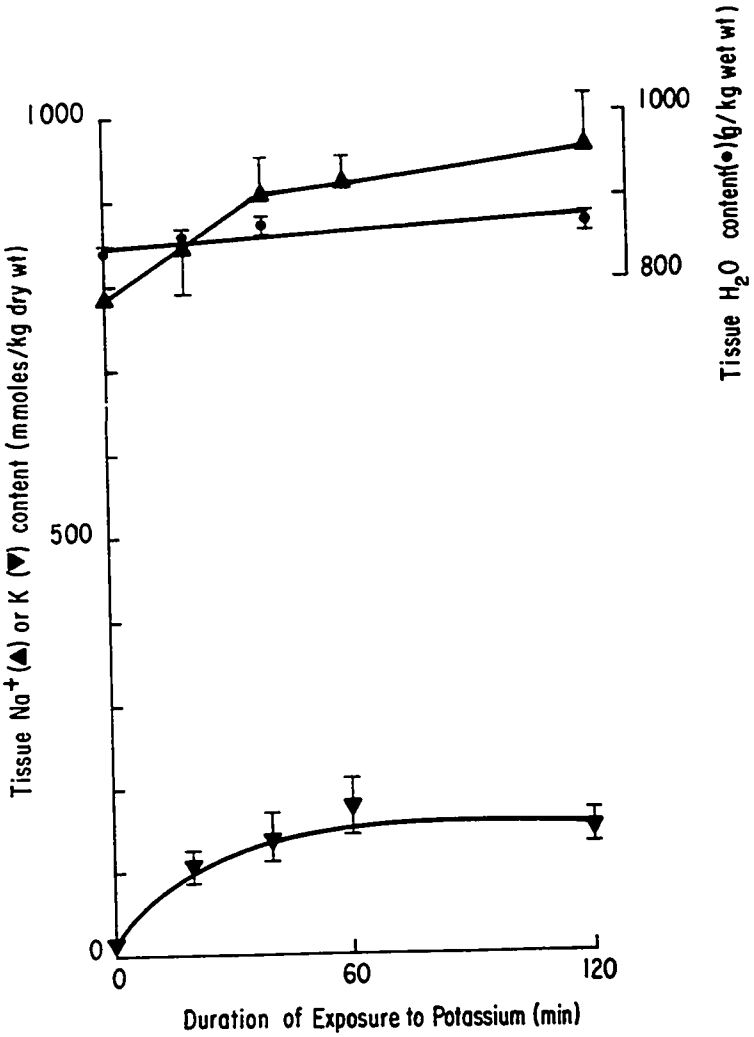

Figure 8. Recovery of IAA-treated tissues in pyruvate Krebs.

Tissues were treated with 0.5 mM IAA in glucose-free, K^+-free Krebs for 30 minutes and then exposed to Krebs (4.6 mM K^+, 25 mM sodium pyruvate) for 120 minutes. The changes in Na^+ (▲-▲), K^+ (▼-▼) and H_2O (●-●) contents are shown. Each point is the mean ± standard error of 4 - 6 tissues.

test horns were exposed to 0.5 mM IAA and 1.0 mM ouabain dissolved in glucose-free, K^+-free Krebs. At the end of 30 minutes, the tissues were washed in K^+-free Krebs and then allowed to recover in pyruvate Krebs, containing 1.0 mM ouabain, for 120 minutes. Thus the total period of exposure to ouabain was 150 minutes. Control tissues were treated identically except for the omission of ouabain.

The results show that ouabain-treated tissues had significantly higher contents of Na^+ and lower contents of K^+. (See Table 9) Both the controls and treated horns had increased contents of water (850 g/kg) compared to untreated Na-rich tissues recovering in normal Krebs (820 g/kg). The net changes in Na^+ and K^+ appeared to be in an approximate 1:1 ratio suggesting the operation of a coupled movement of these two monovalent cations. The increased water content (presumably reflecting swelling of the tissue) did not appear to be affected by ouabain.

(ii) Effect of Anoxia on K^+ Accumulation

If the oxidative metabolism of pyruvate was providing the energy for the accumulation of K^+, anoxia should reduce or abolish this process. Tissues were exposed to 0.5 mM IAA for 30 minutes and allowed to recover in pyruvate Krebs, as described. The solutions bathing the test horns were continuously gassed with 95% N_2 and 5% CO_2 whereas those added to control horns were aerated with 95% O_2 and 5% CO_2. The results (Table 9) showed that tissues incubated under

TABLE 9

Effect of 1 mM ouabain and hypoxia on the K^+ gain seen in IAA-treated tissues incubated in 25 mM pyruvate. Ouabain was used for a total period of 150 minutes. Contractions were not recorded. All tissues equilibrated with 95% O_2/5% CO_2 except where stated.

Treatment (0.5 mM IAA, 25 mM Pyruvate)	Tissue Contents of H_2O (g/Kg wet wt)	Na^+ (mmoles/Kg dry wt)	K^+ (mmoles/Kg dry wt)
Expt. I			
+ ouabain	851.5 ± 3.0	884.8 ± 16.0	52.7 ± 1.4
- ouabain	852.6 ± 8.6	796.6 ± 29.6*	155.8 ± 11.3*
Expt. II			
+ N_2 [a]	860.3 ± 7.3	994.2 ± 79.1	40.8 ± 2.5
- N_2	851.9 ± 4.6	782.9 ± 35.3*	195.9 ± 24.4*

* $p < 0.05$ using paired 't' test.

[a] Tissues equilibrated with 95% N_2/5% CO_2

The number of tissues in each experiment was: Expt. I - 4, Expt. II - 5.

anaerobic conditions had significantly higher contents of Na^+ and lower contents of K^+. This further suggested that the K^+ gain was accompanied by a Na^+ extrusion which, however, was obscured by other changes. Both sets of tissues had increased contents of water.

(iii) Effect of Oxidizable Substrates on IAA-Treated Tissues

The ability of other oxidizable substrates to replace pyruvate in providing energy for ion movements was investigated. Tissues were treated with IAA as before and then exposed to the appropriate substrate. 25 mM β-hydroxybutyrate and 25 mM acetate were used as the monosodium salt whereas 12.5 mM succinate was used as the disodium salt. The results are shown in Table 10. Although K^+ accumulation occurred in tissues treated with β-hydroxybutyrate, both acetate and succinate appeared to be much less effective. Pyruvate was the most effective of the oxidizable substrates used. Contractility (either spontaneous or stimulated) failed to occur.

These results showed that:

1. Oxidizable substrates like pyruvate and β-hydroxybutyrate could partially resuscitate IAA-treated tissues.

2. The K^+ accumulation that occurred was ouabain-sensitive and was accompanied by Na^+ extrusion.

3. Changes in Na^+ content were not observed, apparently because Na^+ extrusion in exchange for K^+ was balanced by Na^+ gain accompanying tissue swelling.

4. Contractility (spontaneous or stimulated) failed to

TABLE 10

Effect of oxidizable substrates on action of iodoacetic acid on the recovery of ion contents and of contractility. All tissues were exposed to K^+-free Krebs solution containing 0.5 mM iodoacetic acid for 30 minutes. They were then transferred to Krebs solution containing the indicated substrate for an additional 120 minutes. No tissues contracted either spontaneously or when drugs were added.

Substrate[a]	No. of tissues	Tissue Contents of		
		H_2O (g/Kg wet wt)	Na^+ (mmoles/Kg dry wt)	K^+ (mmoles/Kg dry wt)
D-glucose	4	862.6 ± 7.4	955.6 ± 49.5	34.7 ± 5.8
Pyruvate	4	856.9 ± 4.9	831.6 ± 43.0*	205.3 ± 42.6*
β-hydroxybutyrate	5	861.6 ± 2.6	901.0 ± 33.2	128.1 ± 23.1*
Succinate	5	848.5 ± 3.8	874.5 ± 27.3	93.6 ± 4.6
Acetate	3	859.1 ± 2.2	906.1 ± 5.0	60.7 ± 12.1

[a] Present as 25 mM, except for succinate which was 12.5 mM.

* $p < 0.05$ compared to value in presence of D-glucose, using Scheffe's test.

occur in IAA-treated tissues, even when suitable substrates were supplied and ion movements recovered partially.

VIII. DIFERENTIAL EFFECTS OF IAA ON CONTRACTILITY: POSSIBLE EXPLANATIONS

The failure of IAA-treated tissues to recover contractions even when supplied with suitable substrates suggested several possible explanations. In the experiments to be described, attempts were made to eliminate several of these, by seeking answers to the following questions:

1. Does insufficient internal K^+ limit the recovery of contractions?

2. Does treatment with IAA affect specifically the receptor to acetylcholine (used here as a stimulant)?

3. Is the effect on contractility related to the ability of IAA to affect -SH groups in general -- i.e., is this effect unrelated specifically to glycolytic inhibition?

4. Is the effect related to changes in Ca^{2+} content of tissues?

5. Does insufficient ATP prevent the recovery of contractions?

6. Is there a preferential utilization of oxidizable substrates by membrane processes?

A. Use of K^+-Loaded Segments

The first question posed was, whether insufficient internal

K^+ limited the recovery of contractions in spite of an adequate supply of energy since muscle function may be adversely affected by inadequate K^+ (Kernan 1965, Ussing 1960). If this were so, tissues loaded with K^+ and then treated with IAA should exhibit contractions when incubated in pyruvate Krebs.

K^+ loaded tissues were obtained by incubating myometrial pieces in a solution containing 116 mM Na^+ and 27 mM K^+ for 18 - 20 hours at 4°. The corresponding pairs were made Na^+-rich in the usual fashion. Both sets of tissues were incubated in 0.5 mM IAA for 30 minutes. After the drug had been washed out, the tissues were allowed to recover in pyruvate Krebs. The Na^+-rich tissues were incubated in a solution containing 4.6 mM K^+ whereas that bathing the test tissues contained 27 mM K^+.

The results show that after IAA treatment Na^+-rich tissues had a very low K^+ content (8 mmoles/kg) (see Table 11) whereas the test tissues had far higher K^+ contents (250 mmoles/kg). After the 120 minute incubation period in pyruvate, both sets had fairly high contents of K^+. However, neither tissue exhibited spontaneous or stimulated contractions. Also shown for comparison, are the ion contents of untreated Na^+-rich tissues recovering in normal Krebs solution. These have lower K^+ contents (214 mmoles/kg) than the K^+-rich tissues and yet exhibited regular, rhythmic spontaneous contractions. Thus the K^+ content did not appear to be the limiting factor.

TABLE 11

Effects of IAA on K^+-loaded tissues. Na^+-rich and K^+-rich tissues were incubated with 0.5 mM IAA for 30 minutes and then with 25 mM pyruvate for 120 minutes. For further details, see text.

Condition	IAA (0.5 mM)	Substrate	Tissue Contents of K^+ (mmoles/Kg dry wt) In IAA 30 min.	Substrate 120 min.	Spontaneous Contractility Maximal tention development (g)
Expt. I					
Na^+-rich	+	Pyruvate	8.4 ± 0.9	195.4 ± 21.3	Nil[a]
K^+-rich	+	Pyruvate	248.2 ± 8.2*	348.3 ± 29.0*	Nil
Expt. II					
Na^+-rich	-	Glucose	-	214.1 ± 12.1	3.3 ± 0.2

[a] No response occurred to drug stimulation with acetylcholine (1×10^{-4} M).
* $p < 0.05$ using paired 't' test.

B. Use of Other Agonists

In all the experiments reported above, stimulated contractions had been elicited with acetylcholine. It was possible that IAA may have affected the receptor site for acetylcholine and therefore the responses to several other agonists were studied. But, IAA-treated tissues failed to respond to supramaximal concentrations of serotonin (1×10^{-5} g/ml) or KCl (50 mM) (Figure 7).

C. Use of Other Sulphydryl Reagents

(i) Studies with N-Ethyl Maleimide

The effects observed with IAA on contractility could be related to the effects of the drug on sulphydryl groups in general. Experiments were carried out with other -SH reagents to investigate this possibility.

N-ethyl maleimide is reported to have a high selectivity for sulphydryl groups and being uncharged penetrates reasonably well into cells. However, the metabolic effects of NEM are not well defined (Webb 1966).

The experiments with NEM were carried out in a manner similar to that adopted with IAA. Na^+-rich tissues were exposed to the drug, dissolved in a glucose-free, K^+-free solution for 30 minutes. After this preincubation period, the drug was washed out and the tissues allowed to recover in normal Krebs-Ringer solution, containing

TABLE 12

Effect of N-ethylmaleimide (NEM) on the recovery of ion contents and of contractility. Tissues were exposed to K^+-free solutions with (+) or without (-) inhibitor for 30 minutes and then incubated in 4.6 mM K^+ solution containing either 20 mM D-glucose or 25 mM pyruvate.

N-ethyl maleimide (mM)	Substrate	Tissue Contents of [a]			Contractility
		H_2O (g/Kg wet wt)	Na^+ (mmoles/Kg dry wt)	K^+ (mmoles/Kg dry wt)	Maximal tension development (g)
Expt. I (1.0)					
+	Glucose	860.9 ± 8.1*	982.9 ± 81.2*	49.4 ± 14.8*	Nil*
-	Glucose	832,2 ± 3.7	582.5 ± 16.0	298.6 ± 12.2	2.4 ± 0.6
Expt. II(0.2)					
+	Pyruvate	846.3 ± 8.8	765.4 ± 70.0	101.7 ± 38.4	0.15 ± 0.09
+	Glucose	853.6 ± 7.6	789.8 ± 67.7	92.2 ± 16.9	0.2 ± 0.14
Expt.III(0.3)					
+	Pyruvate	853.0 ± 8.8	857.1 ± 63.9	58.1 ± 6.3	Nil
+	Glucose	865.0 ± 8.3	935.1 ± 55.8	64.0 ± 7.4	Nil
Expt. IV(0.5)					
+	Pyruvate	858.2 ± 9.8	967.0 ± 89.7	30.1 ± 4.7	Nil
+	Glucose	858.7 ± 8.4	988.9 ± 68.0	33.3 ± 3.1	Nil

[a] Mean ± standard error of the mean for 4 - 6 observations.
* $p < 0.05$ using paired 't' test.

either glucose or pyruvate. In the first set of experiments, the effect of 1 mM NEM on the recovery of ions and contractions was compared to that of an untreated control. The results are shown in Table 12. When tissues were exposed to 1 mM NEM for a brief period, they failed to extrude Na^+ or reaccumulate K^+. The water content of treated tissues was also higher. Neither spontaneous nor stimulated contractions occurred.

This effect of NEM could have been due to an inhibition of glycolysis since NEM has been shown to depress glycolysis in several tissues. (See Webb 1966 for references). Therefore, the ability of sodium pyruvate to resuscitate the poisoned tissues was studied. In the experiments to be described, the effects of lower doses of NEM were studied and the relative abilities of pyruvate and glucose to act as suitable substrates compared.

When tissues were exposed to 0.2 mM NEM, they gained K^+ to a limited extent (100 mmoles/kg) on subsequent incubation in either substrate and no significant differences were noticed in Na^+ and water contents. Spontaneous contractions were negligible and the response to drug stimulation feeble. With increasing doses of NEM, the K^+ accumulation was inhibited and the Na^+ and H_2O contents increased; contractions failed to occur. No differential effects were noticed with either pyruvate or glucose.

Thus no selectivity could be attributed to NEM, used under these conditions. These findings suggest that there may be an inhibition of a number of cellular processes that is dose-dependent.

These may include contractility, glycolysis, respiration, ion transport, etc. In view of these results, the effects observed on contractility with IAA, may well be related to an effect on -SH groups. The evidence, however, is largely circumstantial.

(ii) Studies with Iodoacetamide

Iodoacetamide (abbreviated as IAAmide) is similar to iodoacetic acid in many respects. However, it penetrates cells with greater ease, reacts more readily with -SH groups and appears to be less effective as an inhibitor of glycolysis. (Webb 1966) If this be true, an inhibition of contractility may occur without a concommitant inhibition of glycolysis. The relative abilities of pyruvate and glucose to act as substrates after treatment with IAAmide was taken as an index of glycolytic inhibition. Thus if no significant differences were observed between glucose and pyruvate as substrates, it would be argued that there was little or no inhibition of glycolysis.

Na^+-rich tissues were treated with IAAmide for 30 minutes and subsequently allowed to recover in either glucose or pyruvate. As in previous experiments with IAA, the period of contact with 4.6 mM K^+ was limited to 120 minutes. Three different concentrations of IAAmide were used (0.1, 0.3 and 1.0 mM). The results are shown in Table 13. At all three concentrations of the inhibitor, there was no significant difference between the ion contents of tissue treated with either glucose or pyruvate.

TABLE 13

Effect of iodoacetamide on the recovery of ions and contractility. Tissues were exposed to K^+-free Krebs solution with (+) or without (-) iodoacetamide for 30 minutes. All tissues were then incubated for a further 120 minutes in Krebs solution containing either 25 mM pyruvate or 20 mM glucose without the inhibitor. The solutions were equilibrated with 95% O_2/5% CO_2 except in Expt. VII where 95% N_2/5% CO_2 was used.

Iodoacetamide (mM)		Substrate	Tissue Contents of			Induced Contractility
			H_2O (g/Kg wet wt)	Na^+ (mmoles/Kg dry wt)	K^+ (mmoles/Kg dry wt)	Maximum tension development (g)
Expt. I 0.1	(+)	Glucose	856.8 ± 4.0[a]	772.1 ± 59.3	219.9 ± 27.1	< 0.1
	(+)	Pyruvate	861.5 ± 8.1	798.7 ± 85.7	242.6 ± 29.6	< 0.1
Expt. II 0.3	(+)	Glucose	858.2 ± 4.8	810.9 ± 37.6	200.3 ± 19.5	nil
	(+)	Pyruvate	856.9 ± 8.5	854.3 ± 34.3	186.5 ± 11.9	nil
Expt. III 1.0	(+)	Glucose	871.1 ± 3.2	1046.2 ± 52.0	64.3 ± 11.7	nil
	(+)	Pyruvate	859.3 ± 8.6	949.7 ± 90.8	53.2 ± 9.9	nil
Expt. IV 0.1	(+)	Glucose	852.6 ± 3.6*	755.7 ± 20.4*	216.7 ± 19.2	< 0.1*
	(-)	Glucose	834.2 ± 3.0	629.3 ± 33.9	230.6 ± 14.2	3.4 ± 0.3
Expt. V 0.2	(+)	Glucose	854.3 ± 4.8*	767.3 ± 33.3*	182.9 ± 18.7	nil*
	(-)	Glucose	825.3 ± 5.3	622.2 ± 21.7	204.9 ± 19.3	2.9 ± 0.2
Expt. VI 0.3	(+)	Glucose	854.8 ± 7.6*	732.2 ± 54.7*	161.7 ± 28.5	nil*
	(-)	Glucose	814.9 ± 8.9	507.5 ± 29.1	224.8 ± 25.6	2.8 ± 0.2
Expt. VII 0.3	(+)	Glucose	865.4 ± 3.6	872.8 ± 14.9	112.9 ± 14.4	nil*
	(-)	Glucose	863.4 ± 2.5	825.9 ± 26.5	146.9 ± 15.2	2.0 ± 0.3

[a] Mean ± standard error of 4 - 6 observations

* $p < 0.05$ using paired 't' test.

With 0.1 and 0.3 mM of the drug, the tissues gained K^+ to an appreciable extent (approximately 200 mmoles/kg). This accumulation of K^+ was considerably reduced when a higher concentration (1 mM) of inhibitor was used. The tissues treated thus also had increased contents of Na^+ and H_2O. Even with the lowest concentration, contractions (both spontaneous and stimulated) were drastically impaired. Here again no distinction could be drawn between either substrate. In view of the earlier statement, these results seem to indicate that with the doses of IAAmide used, no significant inhibition of aerobic glycolysis occurs.

It was also found that the K^+ content of tissues treated with 0.1 mM IAAmide was comparable to untreated controls, when glucose was provided as the substrate. Contractility, however, was drastically reduced (only 2 out of 6 pieces responded to any stimulation with a maximal tension of less than 0.1 gm). Tracings from one such experiment are shown (Figure 9). With a higher dose of IAAmide (0.3 mM), K^+ accumulation occurred both under aerobic and hypoxic conditions, although no contractions could be elicited. The K^+ content of tissues treated with 0.3 mM IAAmide was comparable to untreated controls under both conditions. Therefore, with the dose of IAAmide used, no significant inhibition of anaerobic glycolysis occurred. This suggests that IAAmide, in contrast to IAA, inhibits contractility in concentrations which do not significantly inhibit glycolysis or oxidative phosphorylation. Therefore, the effects on contractility observed with IAAmide and possibly IAA may not be related to metabolic

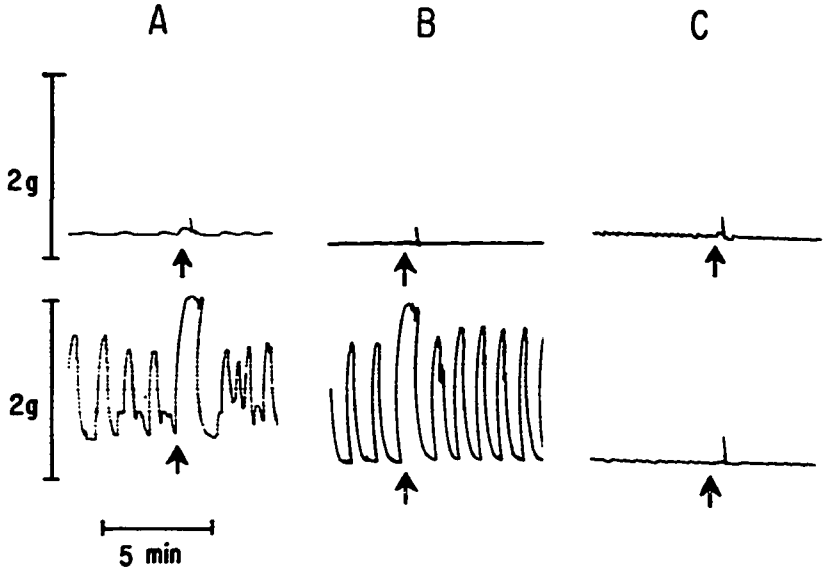

Figure 9. Effects of Iodoacetamide (IAAmide) on the recovery of spontaneous and drug-induced contractions of Na^+-rich tissues. All solutions equilibrated with 95% O_2/5% CO_2; responses to Ach at ↑. Paired tissues were used in all experiments.

A. Upper tracing: 0.1 mM IAAmide in glucose-free, K^+-free Krebs for 30 minutes; then in inhibitor-free Krebs (4.6 mM K^+, 20 mM D-glucose) for 120 minutes. Lower tracing: Tissue in normal Krebs solution.

B. As in (A), except 0.3 mM IAAmide used (upper tracing).

C. Both tissues treated with 0.2 mM IAAmide, for 30 minutes and then incubated in Krebs solution containing either 20 mM D-glucose (upper tracing) or 25 mM sodium pyruvate (lower tracing).

inhibition. However, ATP measurements were carried out to test the above suggestion. (see later)

D. Changes in Calcium Content of IAA-Treated Tissues

The failure of IAA-treated tissues to exhibit spontaneous or drug-induced contractions could be due to alterations in tissue Ca^{++} content. Van Breeman (1965) had shown that incubation of tissues in Krebs solution containing labelled Ca^{45} for 120 minutes was sufficient to label the exchangeable Ca^{++} and the procedure described by him was followed. The exchangeable Ca^{++} contents were measured under three different experimental conditions:

a) Na^{+}-rich tissues recovering in oxygenated Krebs, containing 20 mM D-glucose were used as controls.

b) Na^{+}-rich tissues were treated with 0.1 mM IAAmide for 30 minutes and then incubated in Krebs containing 20 mM D-glucose.

c) Na^{+}-rich tissues were treated with 0.5 mM IAA for 30 minutes and then incubated in pyruvate Krebs.

Although K^{+} accumulation occurred under all three conditions, only the control tissues recovered spontaneous drug-induced contractions. But as the results (Table 14) show there was no significant difference in the tissue contents of exchangeable Ca^{45} between any groups. The values for Ca^{++} contents reported here are higher than those obtained by Van Breeman, et al. (1966) for fresh, undissected uterine horns. However, Van Breeman (1965) and later Hodgson (1971) demonstrated that dissection of tissues raised the contents of exchangeable Ca^{++}. Furthermore, the tissues used here had been made

TABLE 14

Changes in contents of exchangeable calcium of myometrial pieces. All tissues were incubated in solutions equilibrated with 95% O_2/ 5% CO_2. The duration of exposure to K^+ and to Ca^{45} was 120 minutes in all cases.

Treatment	No. of tissues	Ca Content (mmoles/Kg wet wt)
Control[a]	5	2.46 ± 0.04
IAA + pyruvate[b]	4	2.65 ± 0.05
IAAmide + Glucose[c]	5	2.63 ± 0.15

[a] Na^+-rich tissues incubated in normal Krebs (20 mM D-glucose).

[b] Tissues treated with 0.5 mM IAA for 30 minutes and then incubated in 25 mM pyruvate.

[c] Tissues treated with 0.1 mM IAAmide for 30 minutes and then in incubated in 20 mM D-glucose.

Na^+-rich and this too may have contributed to the higher values noted.

Thus the failure of tissues treated with IAA and IAAmide to contract was not associated with marked changes in tissue contents of exchangeable Ca^{++}. Earlier studies by Van Breeman, et al. (1966) and Batra and Daniel (1970) had shown that more prolonged treatment of uteri with IAA led to an increased uptake of labelled Ca^{45}; however, this was not associated with an increase in resting tension.

E. Difference in Energy Requirements

The sodium pump might be able to function at a lower level of ATP than the contractile mechanism. If so, when the level of ATP is low, membrane phenomena may recover in preference to contractions. It could be argued that the amount of ATP generated by the oxidation of pyruvate in IAA-treated tissues may not be adequate for the recovery of contractions. Thus, the inability of IAA-treated tissues to contract, even in the presence of suitable substrates, could be related to a reduction in their ATP content. Therefore, experiments were carried out to investigate this possibility. The specific questions asked were:

1. Is there any correlation between total content of ATP and the occurrence of pumping activity and contractions in the myometrium?

2. Under conditions where sodium pumping alone occurs, do the tissues have a lower content of ATP than where both processes occur?

ATP measurements were carried out under a limited number of conditions, which are listed below:

a) "Fresh" tissues: These were tissues that had been incubated in normal oxygenated Krebs solution for at least 30 - 40 minutes after the endometrium had been removed. These tissues have fairly high contents of K^+ and exhibit regular, rhythmic spontaneous contractions. In all these experiments, K^+ contents rather than Na^+ contents were taken as an index of pumping activity, since swelling and permeability changes appeared to introduce more complexities where Na^+ contents were concerned (to be discussed later).

b) Na^+-rich tissues: These tissues have low K^+ contents, do not exhibit spontaneous contractions but contract feebly in response to acetylcholine.

c) Na^+-rich tissues that have recovered in oxygenated normal Krebs solution. These tissues have fairly high contents of K^+ and exhibit regular spontaneous contractions.

d) Tissues treated with 0.5 mM IAA and incubated with sodium pyruvate. Such tissues have appreciable amounts of K^+ but neither spontaneous nor stimulated contractions occur.

e) Tissues treated with 0.5 mM IAA and incubated in glucose Krebs, which do not recover either ion gradients or contractility.

f) Tissues treated with 1.0 mM DNP and incubated in glucose, which have K^+ contents approximately equivalent to these seen in (d) but also exhibit feeble spontaneous contractions and respond well to drug stimulation. The results of paired experiments with conditions

(d) and (f) showed that there was no significant difference in the K^+ contents under these conditions. The mean K^+ contents in the two cases were 195 mmoles/kg dry weight and 170 mmoles/kg dry weight respectively. (Also see Figure 6D).

g) Tissues treated with 0.1 mM IAAmide for 30 minutes and then incubated in glucose for 24 hours. The K^+ content of such tissues is not significantly different from untreated controls, but these tissues contain more water and Na^+ and their ability to contract is severely affected.

The procedures followed under each of these conditions has already been described. At the end of the incubation period, tissues were extracted with hot glycine buffer and the ATP measured as described (Methods). Ion contents and contractions were not measured in these experiments, but as the conditions were analogous, it was assumed that these would not be markedly different from the values obtained earlier. Samples were occasionally removed for ion analysis and no discrepancies were found with results obtained earlier.

Initial experiments showed that there was no significant difference between paired myometrial pieces from the same animal, whether these were fresh, Na^+-rich or were Na^+-rich tissues that were allowed to recover (see Table 15). Fresh tissues contained significantly more ATP than those made Na^+-rich. Incubation of Na^+-rich tissues under optimal conditions did not alter the ATP contents significantly. IAA-treated tissues incubated in pyruvate contained

significantly lower amounts of ATP than untreated controls. However, IAA-treated tissues incubated in glucose had no measurable amounts of ATP. Treatment with DNP (1 mM) reduced ATP levels and the amounts present were similar to those noted in IAA-treated tissues, incubated in pyruvate. Tissues treated with IAAmide did not differ significantly from untreated controls in their ATP contents. A comparison of these ATP contents, the K^+ contents and the occurrence of contractions is shown in the diagram (Figure 10).

It is fairly obvious from the diagram that the absence of spontaneous contractions in Na^+-rich tissues is more likely to be due to a failure to utilize rather than to produce ATP. The recovery of K^+ by Na^+-rich tissues incubated under aerobic conditions was substantial even though the ATP contents were usually lower than with fresh tissues. Considerable K^+ gain occurred in tissues treated with DNP or with IAA and incubated in pyruvate, even though the ATP contents were considerably lower than normal. Although the tissues treated thus had equivalent amounts of ATP and accumulated equivalent amounts of K^+, the DNP treated tissues alone exhibited contractions. It is thus difficult to suggest that the lowered ATP contents of an IAA-treated tissue is the sole cause for the inability of such a tissue to contract. This does not, however, exclude the possibility that the localization of ATP in a particular cellular compartment where it is produced may be a deciding factor.

Tissues treated with IAAmide and incubated in glucose had fairly high levels of ATP (not significantly different from corres-

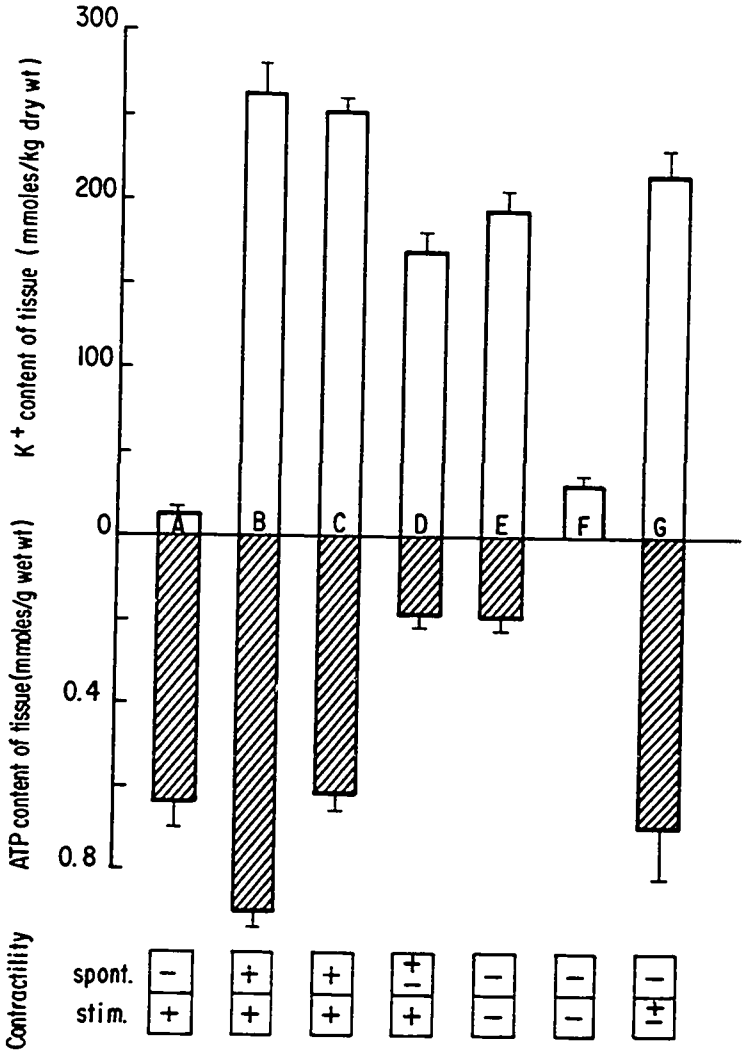

Figure 10. The K^+ (□) and ATP (▨) contents are shown for tissues that had undergone the treatments listed below. In each instance, the presence (+) or absence (−) of spontaneous and drug-induced contractions is also shown. All the results and experimental details have been described previously. (A) Na^+-rich tissue in glucose-free, K^+-free Krebs, 120 minutes. (B) "Fresh" tissues. (C) Na^+-rich tissue, in oxygenated normal Krebs. (D) Na^+-rich tissue treated with 1.0 mM DNP for 150 minutes (30 minutes in glucose-free, K^+-free Krebs; 120 minutes in normal Krebs). (E) Na^+-rich tissue, treated with 0.5 mM IAA, 30 minutes; then incubated in 25 mM pyruvate. (F) Na^+-rich tissue, treated with 0.5 mM IAA, 30 minutes; then incubated in Krebs (20 mM D-glucose). (G) Na^+-rich tissue; 0.1 mM IAAmide, 30 minutes; then incubated in Krebs (20 mM D-glucose).

ponding untreated controls). Although such tissues accumulated significant amounts of K^+, contractions were drastically reduced; only two out of six pieces responded to a supramaximal dose of acetylcholine with a tension of less than 0.2 g. As discussed earlier, it was unlikely that significant inhibition of glycolysis had occurred under these conditions. Therefore, the ATP produced would in all likelihood have been obtained from glycolysis as well as oxidation. Previous results showed that ATP produced thus could support contractions. Thus this finding supports the contention that the effects of IAAmide (and perhaps IAA too) on contractility may be related to a direct effect on the contractile mechanism.

SUMMARY

1. Under the conditions used, there did not appear to be any correlation between the total content of ATP and the occurrence of pumping activity and contractility.

2. Under conditions where Na^+ pumping (K^+ accumulation) alone occurred, the tissues did not have a lower content of ATP than where both processes occurred.

3. It was still possible that the localization of ATP in a particular cellular compartment may prove an important factor.

F. Studies with Substrate-Depleted Tissues

The experiments with IAA had shown that oxidizable substrates, like pyruvate, could provide energy for ion transport. But

as contractions failed to recover under such conditions, it was suggested that ATP obtained through oxidative pathways was compartmentalized and was thus unavailable to the contractile system. The alternative glycolytic inhibitor tried, 2 deoxy-D-glucose failed to inhibit glycolysis sufficiently under aerobic conditions and this prevented a rapid test of the above hypothesis. Attempts were therefore made to study the utilization of oxidizable substrates by substrate-depleted tissues. In the experiments to be discussed below, diverse manouevres were tried but in all cases the general principle remained the same; this was to obtain substrate-depleted tissues, render them sodium-rich and then follow their recovery in the presence or absence of substrates.

1. Bueding and Bülbring (1967) were able to deplete the guinea-pig taenia coli of endogenous glycogen by incubation in a substrate-free medium with successive anaerobic (45 minutes) and aerobic (60 minutes) incubation. Although the glycogen stores in the estrogenized uterus are likely to be extensive (see Introduction), it was felt that with suitable modifications of the above techniques, substrate-depleted tissues could be obtained.

Preliminary experiments were carried out to determine whether incubation under anoxic conditions would reduce the recoveries of ion movements and/or contractions. One horn from each pair was incubated for 120 minutes in glucose-free Krebs solution under hypoxic conditions (see Methods). The corresponding pair was

incubated in normal Krebs solution under aerobic conditions for the same duration. After Na^+-enrichment in the absence of glucose, the tissues were incubated in glucose-free Krebs and O_2. It was found that the recovery of ion movements and contractions by depleted tissues was significantly reduced as compared to the paired control. In six experiments, depleted tissues had higher contents of water (854.5 $\pm$ 7.0 g/kg) and Na^+ (914.1 $\pm$ 45.7 mmoles/kg dry wt) but lower contents of K^+ (74.0 $\pm$ 10.3 mmoles/kg dry wt) than the controls (water 809.7 $\pm$ 16.5 g/kg, Na^+ 579.5 $\pm$ 47.3 mmoles/kg dry wt and K^+ 230.1 $\pm$ 17.0 mmoles/kg dry wt). A tracing from one such experiment is shown to emphasize the marked effects of "depletion" on contractility. (Figure 11)

To study the effects of substrates, the above design (i.e., incubation under anoxic conditions for 120 minutes PRIOR to Na^+-enrichment) was adopted. Both horns of a pair were depleted and after Na^+-enrichment, one horn was allowed to recover in glucose-free Krebs and O_2 as a control whereas the other was incubated with D-glucose. In five experiments with 20 mM D-glucose there was no significant difference from the paired control incubated in a substrate-free medium. In two cases, both tissues recovered ions and contractions whereas they failed to do so in the others (see Figure 11).

In the above experiments, the period of pre-incubation had been fixed at 120 minutes and since contractions had not been monitored, it was likely that these variable results could be explained on the

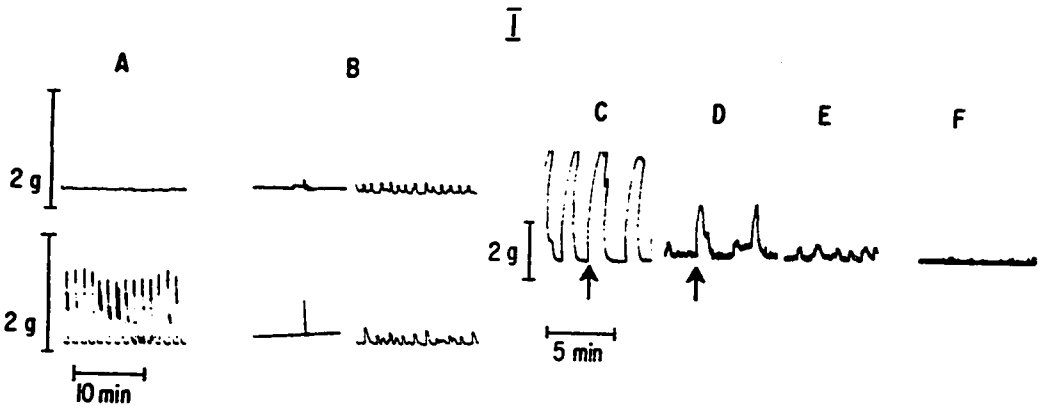

Figure 11. Studies on Substrate-Depleted Tissues.

I. (A) Upper tracing: Recovery of "depleted" tissue in glucose-free Krebs, (O_2). (see text) Lower tracing: Recovery of control tissue (not depleted) in glucose-free Krebs, (O_2). (Paired tissues used)

I. (B) Recovery of "depleted" tissues, two separate experiments. In each case, upper tracing: Tissue in normal Krebs, (O_2). Lower tracing: Tissue in glucose-free Krebs (O_2).

I. (C, D, E) Fresh tissues incubated in glucose-free Krebs, equilibrated with 95% N_2/5% CO_2 for 1, 8 and 9 hours. (to show "depletion").

I. (F) Depleted tissue from E, made Na^+-rich and then incubated in normal Krebs solution, (O_2).

basis of variable endogenous stores. If this were true, then a 120 minute incubation may have been inadequate for some tissues and been far too excessive for others. In subsequent experiments, tissues were mounted in organ baths and contractions monitored till drug stimulation failed to elicit a response. The tissues were then made Na^+-rich and their recoveries followed. It was found that tissues incubated in a substrate-free medium under hypoxic conditions continued to contract for long periods (4 to 9 hours). The Na^+-rich tissues thus obtained failed to recover, even when incubated in glucose + O_2. Similar studies have already been reported (Daniel and Robinson 1971b) where substrate-depleted Na^+-rich tissues failed to recover ion gradients. The probable reasons for this will be discussed later.

2. In earlier experiments with DNP, it was found that incubation of tissues in the absence of a suitable glycolytic substrate, prevented recovery of ion movements and contractions. This could be attributed to the increased glycolytic flux resulting from the uncoupling of oxidative phosphorylation and failure of endogenous stores to compensate for the absence of exogenous substrates. This appeared to provide a more rapid technique for obtaining substrate-depleted tissues and therefore several experiments were carried out with DNP.

a) Na^+-rich tissues were incubated with 1.0 mM DNP dissolved in glucose-free, K^+-free Krebs for 30 minutes. The drug was then washed out and substrates supplied. One horn was incubated in glucose-free Krebs and the other in Krebs containing 25 mM of

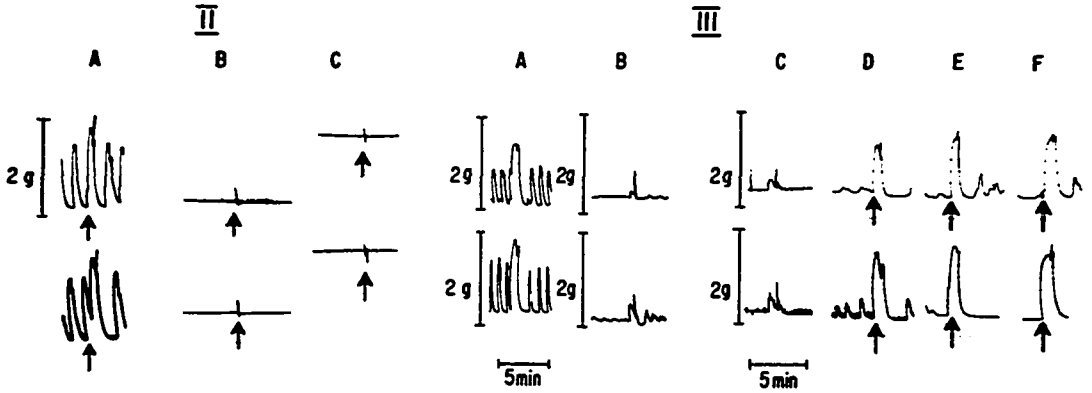

Figure 12. Studies on Substrate-Depleted Tissues.

II. All experiments were done with paired tissues. (A) Na^+-rich tissue in 1.0 mM DNP, glucose-free, K^+-free, 30 minutes; then DNP washed out. Upper trace: Recovery in 25 mM β-hydroxybutyrate, (O_2), 120 minutes. Lower trace: Recovery in glucose-free Krebs, 120 minutes. (B, C) Na^+-rich tissues in 1.0 mM DNP, glucose-free, K^+-free Krebs solution, 90 minutes; then tissues incubated in Krebs solution (± substrates). (B) Upper trace: Recovery in 25 mM β-hydroxybutyrate, (O_2). Lower trace: Recovery in glucose-free Krebs (O_2). (C) Upper trace: Recovery in 25 mM pyruvate, (O_2). Lower trace: Recovery in glucose-free Krebs (O_2).

III. Paired tissues used in all experiments. (A) Both tissues depleted <u>prior</u> to Na^+ enrichment (60 minutes in G-free Krebs (N_2), 60 minutes in Krebs containing 20 mM 2-deoxy-D-glucose (2DG), (N_2)). Upper trace: Recovery in pyruvate Krebs. Lower trace: Recovery in glucose-free Krebs. (B) Both tissues depleted <u>prior</u> to Na^+ enrichment. Upper trace: Recovery in Krebs containing 25 mM pyruvate, 20 mM 2DG. Lower trace: Recovery in Krebs, containing 20 mM 2DG, 20 mM sucrose (i.e., glucose-free). (C, D, E, F) Tracings from the same experiments. All solutions equilibrated with 95% O_2/5% CO_2. (C) Na^+-rich tissue in glucose-free, K^+-free Krebs solution containing 20 mM, 2DG, 60 minutes. (D) Upper trace: Tissue allowed to recover in Krebs solution (4.6 mM K^+, 2.5 mM pyruvate, 20 mM 2DG), 20 minutes. Lower trace: Tissue incubated in glucose-free Krebs solution (4.6 mM K^+, 20 mM 2DG), 20 minutes. (E) 60 minutes later. (F) 60 minutes after pyruvate concentration increased to 10 mM.

β-hydroxybutyrate as a substrate. In three experiments, contractions recovered in both pairs and no significant differences were observed. Substrate depletion therefore appeared to be inadequate (see Figure 12).

b) In subsequent experiments, the exposure to DNP was increased to 90 minutes before substrates were supplied. It was found that no recoveries occurred either in the presence of oxidizable substrates (pyruvate, glucose, β-hydroxybutyrate) or in their absence.

3. Although 2 deoxy-D-glucose (20 mM) failed to inhibit aerobic glycolysis sufficiently, it was argued that a combination with substrate-depletion may render it more effective and thus provide a model for studying the utilization of oxidizable substrates. Three different experimental procedures were tried.

a) Tissues were incubated in glucose-free Krebs and N_2 at 37^{o} for 60 minutes and then in a medium containing 20 mM 2 deoxy-D-glucose for another 60 minutes. The depleted tissues were made Na^{+}-rich and their subsequent recoveries followed. It was found that all tissues recovered spontaneous contractions when incubated in glucose-free Krebs alone. Addition of pyruvate (25 mM) did not lead to any improvement (see Figure 12).

b) The above experimental procedure for obtaining substrate-depleted Na^{+}-rich tissues was followed. However, the tissues were incubated for 30 minutes in a K^{+}-free medium containing 2 deoxy-D-glucose (20 mM) before the addition of K^{+} and substrates. The

inhibitor was present in the medium during the entire duration of the experiment (150 minutes). Hardly any recovery of contractility occurred, either in pyruvate or in glucose-free Krebs.

c) The last variation played on the 2 deoxy-D-glucose theme was to use Na^+-rich tissues that had not undergone any prior anoxic incubation. These tissues were exposed to K^+-free Krebs containing 20 mM 2 deoxy-D-glucose for 60 minutes and then incubated in Krebs containing 4.6 mM K^+ and 20 mM of the inhibitor for another 60 minutes. Such tissues recovered spontaneous contractions and responded to acetylcholine. Addition of pyruvate (2.5 mM or 10 mM did not produce any dramatic improvement (see Figure 12).

The experiments described suggest that attempts to obtain substrate-depleted tissues are fraught with difficulties, for the drastic metabolic alterations required to do so produced perhaps, some irreparable damage to the cells. Thus these experiments do not exclude the possibility that oxidizable substrates are preferentially used for membrane phenomena and that this could explain the failure of IAA-treated tissues to recover contractions when incubated in pyruvate. However, the experiments described with IAAmide provided stronger evidence for the view that a non-specific effect on the contractile system was a more likely explanation.

IX. STUDIES ON TISSUE SWELLING

In the experiments reported earlier, it was found that

IAA-treated tissues incubated in pyruvate appeared to gain K^+ without a concomitant loss of Na^+. But, this K^+ accumulation was shown to be ouabain-sensitive and therefore occurring presumably through the operation of a coupled Na^+-K^+ pump. This raised the possibility that there could exist an alternative mode of Na^+ extrusion. It was noticed, however, that under a number of different metabolic conditions (see Tables), such a discrepancy between K^+ gain and Na^+ extrusion occurred, along with changes in the water content of the tissues. This observation prompted the following questions:

a) Was this discrepancy due to (or at least be explained by) a gain of isotonic fluid?

b) Did metabolic inhibition produce changes in tissue weight?

The results previously presented have been rearranged in Table 16 to facilitate calculations. (Only a few sample conditions have been chosen). The mean H_2O contents of the tissues have been expressed as g/g solids using the relation:

$$\text{Tissue } H_2O \text{ content (g/g tissue solids)} = \frac{\text{Tissue } H_2O \text{ content (g/Kg wet wt)}}{\text{Tissue solids content (g/kg wet wt)}}$$

where,

$$\underset{\text{(8 kg wet wt)}}{\text{Tissue solids content}} = 1000 - \underset{\text{(g/kg wet wt)}}{\text{Tissue } H_2O \text{ content}}$$

The differences in the Na^+ and K^+ contents of the treated

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$$H_2O \text{ (gms/gm solids)} = \frac{H_2O \text{ (gms)}}{\text{gm } H_2O + \text{gm solid}_{\text{(final)}}} \times \frac{\text{gm } H_2O + \text{gm solid (final)}}{\text{gm solid}}$$

$$= H_2O \text{ (g/g tissue)} \times \frac{\text{wet wt.}}{\text{dry wt.}}$$

$$= \frac{H_2O \text{ (g/kg)}}{1000} \times \frac{1000}{1000\text{-wet wt.}}$$

The differences in the Na^+ and K^+ contents of the treated

TABLE 16

To demonstrate that the fluid gained by tissues under different metabolic conditions had the composition of isotonic fluid. All the values listed have been recalculated from data presented earlier.

Condition[a]	H_2O g/g solid	ΔH_2O	Δ Na (mmoles/Kg dry wt)	Δ K (mmoles/Kg dry wt)	$\frac{\Delta Na - \Delta K}{\Delta H_2O}$
Glucose (-) N_2	6.78	1.36	370	167	149
Glucose (+) N_2	5.42				
1.0 mM DNP	6.01	0.95	179	41	145
Glucose (+) O_2	5.06				
1.0 mM IAA + Glucose	6.24	1.85	432	162	146
Glucose (+) O_2	4.39				
0.5 mM IAA + Pyruvate (N_2)	6.16	0.41	211	155	136
0.5 mM IAA + Pyruvate (O_2)	5.75				
0.1 mM IAAmide + Glucose	5.78	0.75	126.4	14	149
Glucose (+) O_2	5.03				

[a] All the conditions have been described earlier in Tables 3, 4, 7, and 13 respectively.

and control tissues have been calculated from the mean contents, ignoring standard errors of the mean, and expressed as $\Delta\ Na^+$ and $\Delta\ K^+$. The difference between $\Delta\ Na^+$ and $\Delta\ K^+$ has been calculated and this value $\Delta\ Na^+ - \Delta\ K^+$ gives the "extra" Na^+ that must be accounted for by a gain in H_2O, assuming that a 1:1 exchange occurs between Na^+ and K^+. Division of the "extra" Na^+ by the change in H_2O content, gives the approximate Na^+ composition of the fluid gained. As the calculations show, in all the conditions tabulated, the fluid gained has approximately 140 mM/litre of Na^+, which is the composition of the isotonic fluid in the bathing medium.

To answer the second question, a number of different experiments were performed. Tissues were incubated in oxygenated normal Krebs for 30 - 40 minutes after dissection and the weights (called fresh weights) noted. These tissues were then made Na^+-rich by incubation in a glucose-free, K^+-free medium. On the following day, the tissues were removed from the cold, reweighed and returned to the cold solution. This procedure took less than 30 seconds and the weight was recorded as the Na^+-rich weight. The tissues were then rewarmed in glucose-free, K^+-free Krebs for 20 minutes (at 37°C). This procedure duplicated that followed in the contractility experiments where tissues were equilibrated for 20 minutes before the addition of metabolic inhibitors. Weights were taken at the end of this equilibration period. In all cases, fresh weights were used as reference weights, but changes in weight referred to the conditions

compared.

The results (Figure 13) show that tissues usually gained weight after Na^+-enrichment. On being rewarmed, in a glucose-free, K^+-free medium for 15 minutes, all tissues lost weight. This change in weight was, however, variable (range 10 - 15%). Since the incubation medium was devoid of K^+, the loss in weight was apparently unrelated to the operation of a coupled Na^+-K^+ exchange. When these tissues were allowed to remain in glucose-free, K^+-free Krebs, they gradually regained weight. Incubation in normal Krebs (containing D-glucose and K^+) resulted in similar changes.

After incubation in 0.5 mM IAA, for 30 minutes, tissues gained weight (4 - 5%). Further gains in weight occurred when these tissues were incubated in substrates, either D-glucose or pyruvate. Since the weights gained in both sets were approximately equivalent, (12 - 13%) it appeared that pyruvate did not abolish the weight gain, despite its ability to restore K^+ accumulation. However, these tissues had not been paired at the start and the variability was considerable. Therefore, paired horns were incubated in 0.5 mM IAA for 30 minutes and then transferred to media containing glucose or pyruvate. The results of such experiments showed that the weight gains in tissues incubated in D-glucose ($11.0 \pm 2.5\%$) was significantly greater than the weight gained in tissues incubated in pyruvate ($8.0 \pm 3.4\%$). Thus 25 mM pyruvate reduced but did not abolish the gains in weight observed on metabolic inhibition.

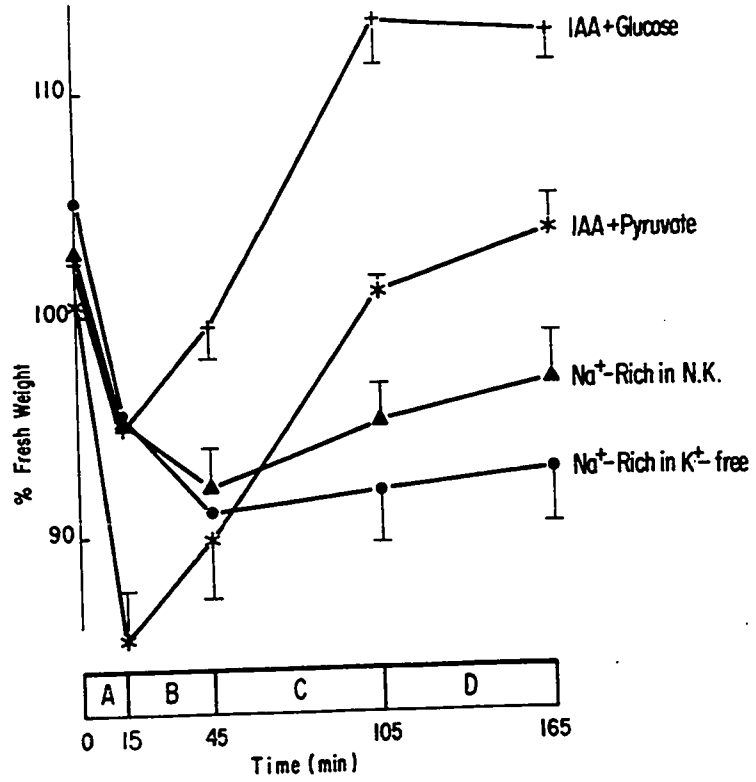

Figure 13. Shows the weight changes in Na^+-rich tissues on rewarming. Tissues removed from the cold were weighed and then rewarmed for 15 minutes in glucose-free, K^+-free Krebs (A).

B. Tissues were incubated for a further 30 minutes in the same medium with or without 0.5 mM IAA.

C, D. Tissues treated with the inhibitor, were incubated for 120 minutes in Krebs solution (4.6 mM K^+) containing 20 mM D-glucose or 25 mM sodium pyruvate.

C, D. Tissues, not treated with IAA, were incubated for the same duration in normal Krebs solution (4.6 mM K^+, 20 mM D-glucose) or in glucose-free, K^+-free Krebs.

All weights were expressed as a percentage of fresh weights recorded prior to Na^+ enrichment.

Earlier experiments had shown that IAA-treated tissues incubated in glucose had ATP contents that were immeasurable whereas those incubated in pyruvate had low (0.2 μmoles/gm) but significant amounts of ATP. It seemed reasonable to argue that the greater swelling observed in inhibited tissues incubated with glucose could be due solely to the absence of ATP. Tissues which had virtually no ATP also failed to recover K^+ contents. Tissues treated with 1.0 mM DNP in the complete absence of glucose did not recover K^+ and had no measurable amounts of ATP. If there existed a correlation between the ATP contents and the swelling of tissues, then those tissues treated with IAA and incubated in glucose should exhibit gains in weight comparable to tissues treated with DNP in a glucose-free medium. However, the results of paired experiments did not support the above contention (Table 17). Thus IAA-treated tissues had K^+ and ATP contents that were comparable to DNP-treated tissues, but their contents of water and Na^+ were greater and so were the weight gains observed. Thus the gain of isotonic fluid seen in tissues treated with IAA is perhaps <u>not solely</u> related to lack of ATP. In subsequent experiments, it was found that tissues treated with 0.1 mM IAAmide and incubated in glucose gained weight ($7.4 \pm 2.0\%$). The ATP contents of such tissues (as measured in similar experiments) were fairly high (0.7 μmoles/gm) and compatible with the conclusion arrived at above.

The results described raised the following question: Was the gain of isotonic fluid due to a swelling of cells or did it imply

TABLE 17

Comaprison of ionic contents, weight gains and ATP contents in paired tissues treated with either DNP or IAA. All solutions were equilibrated with 95% O_2/5% CO_2.

Treatment	Tissue Contents of[c]			Final weight (%age fresh wt)	ATP[d] (µmoles/Gm wet wt)
	H_2O (g/Kg wet wt)	Na^+ (mmoles/Kg dry wt)	K^+ (mmoles/Kg dry wt)		
1.0 mM DNP, glucose-free[a]	839.0 ± 3.7	760.6 ± 26.8	29.0 ± 1.9	106.7 ± 1.5	Nil
0.5 mM IAA; glucose[b]	862.7 ± 2.3*	873.8 ± 21.3*	35.2 ± 2.0	113.2 ± 1.2*	Nil

[a] Total period of exposure to DNP was 150 minutes; 90 minutes in K^+-free. D-glucose was omitted throughout.
[b] Exposure to IAA limited to 30 minutes.
[c] Results are means ± standard errors of 7 observations.
[d] ATP measured in separate tissues under identical conditions.
* $p < 0.05$, using paired 't' test.

a gain of extracellular fluid? The extracellular space measured by suitable markers should diminish in the former instance and increase in the latter and these changes should be related to the weight gains observed. Therefore, ^{14}C-sucrose and ^{14}C-inulin spaces were measured under different conditions. The interpretation of measurement of spaces in smooth muscle occupied by various extracellular markers is problematic (Goodford 1968) and there is, in fact, no ideal marker. However, for comparative purposes, sucrose and inulin being of widely different molecular sizes are reasonable markers to choose and give a measure of two different spaces or of the upper and lower limits of the true extracellular space.

The experimental procedure followed was similar to that described already (see preceeding pages) and the results are presented in Table 18. The inulin and sucrose spaces of a fresh tissue were taken as the control values. The inulin space of a Na^+-rich tissue incubated either in a glucose-free, K^+-free medium or in normal Krebs containing glucose and K^+ was not significantly different from that in a fresh tissue. Furthermore, treatment with 0.1 mM IAAmide did not produce any significant change although these tissues had gained weight. Treatment with 0.5 mM IAA on the other hand, led to an increase in the inulin space; tissues incubated in glucose but not those incubated in pyruvate had significantly larger spaces than fresh tissues; both types of tissue gained weight. A similar result was obtained when inhibited values were compared to those of tissues recovering in Krebs Ringer solution.

TABLE 18

^{14}C Sucrose and ^{14}C Inulin spaces in rat myometrium. All media were equilibrated with 95% O_2/5% CO_2.

Expt. No.	Condition	Weight of Tissue (as % fresh wt)	^{14}C Sucrose Space (ml/100 Gm)	^{14}C Inulin Space (ml/100 Gm)
I	Fresh tissues[a]	100	52.5 ± 3.0	43.5 ± 1.7
II	Na^+-rich (rewarmed in K^+-free 120 minutes)[b]	93.7 ± 2.6	61.2 ± 0.7*	47.1 ± 0.6
III	Na^+-rich (recovered)[c]	97.2 ± 1.6	57.2 ± 1.8	45.9 ± 0.3
IV	0.1 mM IAAmide + Glucose[d]	107.4 ± 2.0	61.5 ± 0.6*	48.1 ± 2.2
V	0.5 mM IAA + Glucose	118.7 ± 2.2	75.4 ± 1.1*†	58.6 ± 1.5*†
VI	0.5 mM IAA + Pyruvate	109.9 ± 2.6	73.7 ± 1.9*†	51.3 ± 0.6
VII	1.0 mM DNP, Glucose-free[e]	104.8 ± 1.9	68.9 ± 2.9*†	52.4 ± 3.6

a Tissues incubated in Krebs solution containing tracer, for 45 minutes after dissection.
b Tissues rendered Na^+-rich and incubated in glucose-free, K^+-free Krebs containing tracer for 120 minutes.
c Na^+-rich tissues incubated in Normal Krebs (4.6 mM K^+, 20 mM D-glucose) containing tracer for 120 minutes.
d In Expts. IV, V and VI, tissues were incubated in K^+-free Krebs containing the inhibitor for 30 minutes and then in Normal Krebs containing the tracer and the appropriate substrate for 120 minutes.
e Tissues incubated in DNP for 120 minutes in K^+-free Krebs and K^+, then added for 120 minutes. Tracer present for 120 minutes. D-glucose omitted.
* $p < 0.05$ using Scheffe's test for multiple comparisons; all values compared to Group I.
† $p < 0.05$ using Scheffe's test; values compared to Group 3.
The number of tissues in each experiment was 4.

The sucrose space in a fresh tissue was higher than the inulin space, suggesting either that sucrose penetrated cells or was gaining access to a portion of the extracellular space denied to inulin. The sucrose space in Na^+-rich tissues recovering in normal Krebs was not significantly different from that in fresh tissues. If, however, these tissues were rewarmed in glucose-free, K^+-free Krebs, they did have a larger sucrose space than that in a fresh tissue despite having lost weight. That Na^+-rich tissues consistently had larger sucrose spaces than fresh tissues was observed by Daniel and Robinson (1970) and they attributed this to a real difference in the size of the extracellular space. In the experiments reported here, the larger sucrose space was found in tissues that had lost rather than gained weight. Tissues treated with 0.1 mM IAAmide had larger sucrose spaces than fresh tissues and these tissues also gained weight. Metabolic inhibition with either IAA or DNP led to an increase in the sucrose space, compared either to fresh or recovered tissues. There was no significant difference in the spaces in IAA-treated tissues incubated in either glucose or pyruvate.

The results suggest that metabolic inhibition leads to alterations in the extracellular space. It is also likely that permeability to these markers (particularly sucrose) may be affected as well. This finding is consistent with earlier results (Daniel and Robinson 1971). Electron microscopy was carried out on tissues treated in a similar fashion.[†] It was found that treatment with metabolic inhibitors caused cellular damage, especially to the plasma membrane and mitochondria; it was possible that cells thus damaged

would behave as part of the extracellular space by allowing marker molecules to permeate. Sucrose, a smaller molecule, would penetrate normal cells with greater ease than inulin and the difference could be exaggerated after metabolic inhibition. In fact, the sucrose spaces in general were larger than the inulin spaces and the difference appeared greater after metabolic inhibition.

Thus metabolic inhibition led to a gain of isotonic fluid which was, perhaps, extracellular in location. However, tissues rewarmed in K^+-free Krebs lost weight and it thus seemed likely that some active process was also involved in extruding water. It had also been noted that in a number of instances, tissues which showed little or no contractions had increased contents of water and this suggested that there could be a correlation between these two parameters. In Table 19, the results reported earlier, have been abstracted to emphasize the point. In each case, the control condition is listed below the experimental condition. It is evident that in all the cases listed, there is a correlation between reduced contractions and increased contents of water, but whether such a correlation is causal, is not clear.

It was described earlier, that a Na^+-rich tissue removed from the cold and suspended in K^+-free Krebs exhibited a transient contracture. Under similar conditions (see Figure 14), such tissues lost weight on rewarming. It was possible that the contracture could mechanically squeeze out water and thus cause the loss in weight observed. If this be so, abolishing the contracture should reduce

TABLE 19

Correlation between changes in water content and contractions. All tissues were equilibrated with 95% O_2/5% CO_2, unless specified.

Expt.	Condition[a]	Changes in H_2O Content (g/Kg)	Changes in Contractility		Ref. Tables
			Spontaneous	Stimulated	
I	Glucose(-) N_2 Glucose (+)	↑	↓	Not Recorded	3
II	Glucose (-) N_2 Glucose (+) N_2	↑	↓	Not Recorded	3
III	1.0 mM DNP + Glucose Glucose (+)	↑	↓	↓	4
IV	0.1 mM DNP + Glucose Glucose (+)	↑	↓	↓	4
V[b]	2DG (+) N_2 Glucose (+)	↑	↓	Not Recorded	6
VI	0.5 mM IAA + Glucose Glucose (+)	↑	↓	↓	7
VII	1.0 mM IAA + Glucose Glucose (+)	↑	↓	↓	7
VIII	1.0 mM NEM + Glucose Glucose (+)	↑	↓	↓	12
IX	0.3 mM IAAmide + Glucose Glucose (+)	↑	↓	↓	13

[a] All experimental conditions and other details have been described earlier.
[b] In Expts. V - IX, contractility was reduced to zero in treated tissues.

or abolish the loss in weight that occurs. It was found that when Na^+-rich tissues were treated with papaverine (1×10^{-4} M), they did not show any contracture on being rewarmed in K^+-free Krebs; tissues undergoing a contracture, relaxed promptly when treated with the same concentration of papaverine. Furthermore, the loss in weight that occurs in rewarming was abolished by the drug (1×10^{-4} M)(Table 20). This suggests that the loss in weight could be related to the operation of a contractile process, and as a corollary, a gain in weight could be linked to the inefficient functioning of a contractile mechanism. The evidence is, however, largely circumstantial and will be discussed later.

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* The E. M. experiments referred to were carried out in collaboration with Robert Garfield and will be reported by him in greater detail in his dissertation.

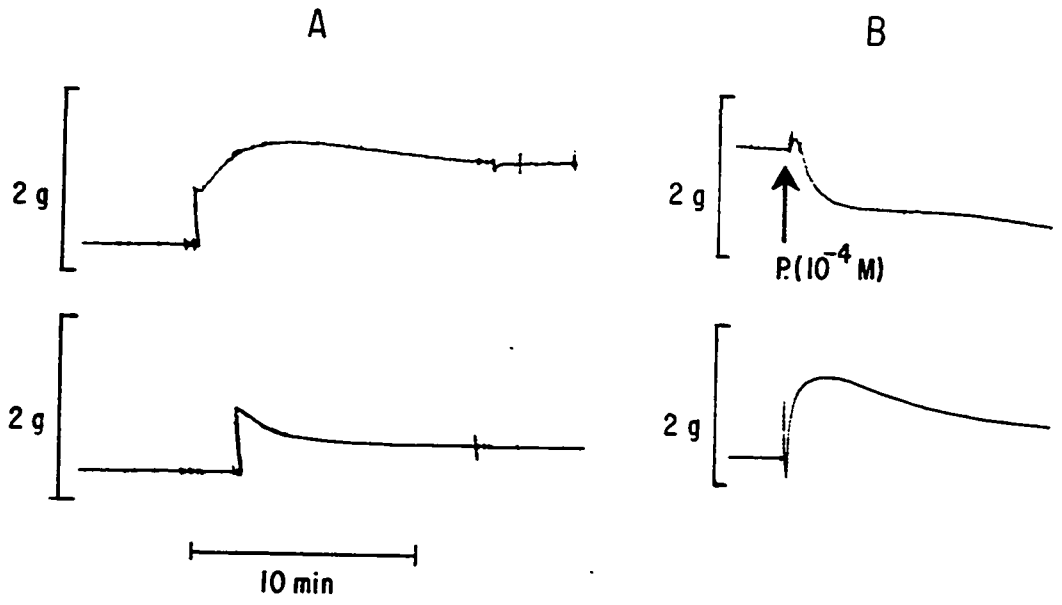

Figure 14. Effect of papaverine on the contracture observed when Na^+-rich tissues are rewarmed in K^+-free Krebs, 37°C. Paired tissues used.

A. Upper trace: Na^+-rich tissue, removed from the cold, rewarmed in glucose-free, K^+-free Krebs, 37°C, equilibrated with 95% O_2/5% CO_2. Lower trace: Paired tissue, treated identically except that 10^{-4} M papaverine was present in the solution.

B. Upper trace: Papaverine (10^{-4} M) added to a Na^+-rich tissue undergoing a contracture. Lower trace: paired control, to show contracture.

TABLE 20

Weight changes in Na^+-rich tissues treated with 10^{-4} M papaverine. Na^+-rich tissues were incubated in glucose-free, K^+-free Krebs at 37°C under conditions similar to those described earlier (see Figure 14). The weights after varying periods of incubation are expressed as a percentage of the Na^+-rich weight before rewarming.

Papaverine 10^{-4} M	Weights (% Na^+-rich wt)		
	15 min.	30 min.	60 min.
+	100.7 ± 21.	99.9 ± 0.9	100.0 ± 0.7
-	96.2 ± 2.3	95.9 ± 2.6	93.9 ± 2.0

V. DISCUSSION

DISCUSSION

PROPERTIES OF THE MODEL SYSTEM

The prime purpose of the present project was to define the pathways providing energy for sodium pumping and spontaneous contractions in the rat myometrium. To this end, the recovery of ions and spontaneous contractions by Na^+-rich tissues was studied. The observation that such tissues extruded Na^+ and regained K^+ when rewarmed in a K^+-containing medium is in accord with earlier reports on cat and rabbit uterine segments (Daniel and Robinson, 1960), pregnant rat myometrium (Kao and Nishiyama, 1969). Daniel and Robinson (1960) showed that the ionic contents of rabbit uterine segments that had recovered in oxygenated Krebs-Ringer solution was similar to those in "fresh" tissues that had been exposed for several hours to Krebs-Ringer solution.

It was shown by Kao and Nishiyama (1969) that the time course of the net loss of Na^+ from the estrogen-dominant myometrium could be described in terms of first-order kinetics. The net Na^+ loss and K^+ gain were in fair correspondence suggesting a stochiometric relationship. They also observed that shifts in water between intra and extracellular compartments occurred within the initial 30 minutes of rewarming in normal Krebs (5.9 mEq/litre of K^+ present). In the experiments described here, a rapid extrusion

of water occurred on rewarming but since this was observed after incubation in a K^+-free solution, it is uncertain whether these corresponded to the shifts in water suggested by Kao and Nishiyama (1969).

Na^+-rich tissues rewarmed in glucose-free, K^+-free Krebs did not exhibit spontaneous contractions and their response to stimulation was feeble. The addition of external K^+ led to a rapid but transient relaxation which was particularly noticeable if the initial tone was high. The tissues exhibited spontaneous contractions, a variable duration after the initial relaxation. Barr, Headings and Bohr (1962) studied the recovery of contractions by arterial strips that were rewarmed after cold storage. They showed that replacing the cold storage by a warm solution caused an initial rapid contraction of "unpredictable amplitude". Such a contraction was only occasionally observed in the present experiments, as in most instances the tissues were rewarmed for 15 - 20 minutes before tension was recorded. However, when tissues removed straight from the cold were suspended in warm K^+-free Krebs, an initial contracture of varying duration and amplitude was noticed. This tension change was prevented by exposure to papaverine (1×10^{-4} M). Barr and his associates also observed that the addition of K^+ led to a protracted relaxation which was dependent on external K^+. They attributed this to the reaccumulation of K^+ and suggested that the rate of relaxation may be related to the rate of reaccumulation. From earlier studies

by Taylor, Paton and Daniel (1969, 1970), it appears likely that the transient relaxation noticed here could be attributed to hyperpolarization resulting from the operation of an electrogenic Na^+ pump. The reduction in the ability of Na^+-rich tissues to contract could be due to the low level of the resting membrane potential. The operation of the electrogenic pump by altering the membrane potential may ultimately render the tissue more excitable and thus lead to the onset of spontaneous contractions.

There are, however, other possible explanations. Thus, internal K^+ may be required for the efficient functioning of the contractile apparatus (Kernan 1965, Ussing 1960). Furthermore, it was noticed that the addition of glucose alone failed to improve contractility and it could be argued that the absence of K^+ may have caused an inhibition of metabolism. Several glycolytic enzymes (principally pyruvate kinase) are critically dependent on K^+ for their activity (Sols, 1968; Suelter, 1970; Ussing, 1960 and Kernan, 1965) and it was found (in experiments not reported here) that supplying pyruvate did bring about a slight but significant improvement in the maximal response to acetylcholine. This suggests that some glycolytic inhibition could be responsible for the inadequate response to stimulation.

EVIDENCE FOR GLYCOLYSIS

The enzymes responsible for glycolysis and oxidative

phosphorylation have been demonstrated in uterine tissues (Needham and Shoenberg, 1967); the present study showed that both glycolytic and oxidative mechanisms were operative in this tissue. Thus, evidence was obtained that glycolysis could provide energy for coupled Na^+-K^+ exchange and spontaneous contractions. Both processes recovered when tissues were incubated under hypoxic conditions or with DNP, provided that suitable glycolytic substrates were supplied. Furthermore, the glycolytic inhibitor IAA prevented recovery of both processes.

Of the various glycolytic substrates supplied, D-mannose alone proved an effective substitute. This is not surprising, since it is a good substrate for hexokinase and as such it is utilized in a variety of tissues; the hexophosphate thus formed undergoes isomerization to fructose-6-phosphate before entering the main glycolytic pathway (Sols, 1968). It has been shown that uterine tissue metabolizes D-mannose as readily as D-glucose and that both proved equi-effective in inhibiting the transport and phosphorylation of 2-deoxy-D-glucose (Smith and Stultz, 1971). D-mannose supported contractions in the rabbit aorta (Coe, Detar and Bohr, 1968), and sodium pumping in the rabbit detrusor muscle (Munson and Paton, 1971). D-fructose, D-galactose and sucrose proved ineffective substrates both in the present study and in those carried out on the rabbit detrusor muscle (Paton, 1968; Munson and Paton, 1971). However, Coe, Detar and Bohr (1968) showed that D-galactose partially supported

contractility in rabbit aortic strips, although Shibata and Briggs (1967) did not observe this effect.

The results obtained with IAA further underscored the importance of glycolysis in this regard. Tissues treated thus, failed to recover ions and contractions when glucose was supplied as the substrate. This also indicated the inadequacy of endogenous oxidizable substrates under those conditions. Beatty, et al., (1969) found that the pregnant rhesus myometrium had a low respiratory quotient (0.7) and only a small fraction of the CO_2 produced (5%) was derived from (^{14}C)-glucose. This, they argued, suggested that the major energy-source for resting myometrium was lipid rather than carbohydrate. There could be a species difference or IAA may interfere with the utilization of endogenous oxidizable substrates. However, mitochondrial function was not seriously compromised with doses of IAA in the 0.1 to 0.5 mM range, since exogenously supplied oxidizable substrates like pyruvate, could bypass the metabolic block induced.

Since pyruvate, β-hydroxybutyrate and succinate partially restored ion movements in IAA-treated tissues, it is clear that the operation of the Krebs cycle can provide energy for at least coupled Na^+-K^+ exchange in this tissue. This was further confirmed by the adverse effects of anoxia and ouabain on the recovery induced by pyruvate. The inability of IAA-treated tissues to recover contractions suggested several explanations and attempts were made to

eliminate these in turn.

DIFFERENTIAL EFFECTS OF IAA ON CONTRACTILITY: POSSIBLE EXPLANATIONS

The three major possibilities considered were:

(a) A quantit ative difference in the ATP requirements for ion transport and contractility.

(b) A preferential utilization of oxidative energy for membrane ion transport because of some compartmentalization of the resultant ATP.

(c) A direct effect of IAA on the contractile mechanism.

There could exist a quantitiitative difference in the ATP requirements for these processes. Thus the Na^+ pump might be able to function at a lower level of ATP than the contractile mechanism. If this be true, then when the level of ATP was low, membrane phenomena may have recovered in preference to contractility. As discussed earlier (see Introduction), studies on the guinea-pig taenia coli had raised the possibility that there could exist a differential sensitivity of electrical and mechanical events to metabolic inhibition. But Born (1956) could not link ATP levels to these events. In the experiments described here, attempts were made to correlate ATP contents of tissues to the occurrence of pumping activity and spontaneous contractions. There did not appear to be any definite correlation between the ATP content of tissues

and the presence or absence of spontaneous contractions. Thus tissues treated with IAAmide had near normal contents of ATP but did not exhibit spontaneous or stimulated contractions. DNP-treated tissues with far lower contents of ATP exhibited some spontaneous contractions and responded well to drug stimulation.

The total content of ATP under steady-state conditions is the net result of synthesis and utilization and it has been assumed that changes (particularly a reduction) in ATP reflect altered (i.e., decreased) synthesis. It is possible that in the presence of DNP or anoxia a higher rate of utilization occurred, and this allowed a recovery of both ion transport and contractions and led to the low ATP contents observed. Stephens (1971) showed that in the trachealis smooth muscle, hypoxia actually led to a reduction in the rate of energy utilization as measured by a reduction in the b constant derived from force-velocity curves. Though hazardous to extrapolate such findings to the uterus, it is clear that to explain how such differences in energy utilization occurred would require answers in terms of differences in availability (compartmentalization) of ATP or in a differential inhibition of ATP utilization, resulting from an additional action of IAA.

It is not inconceivable that the ATP obtained through different pathways could be localized in specific compartments. As mentioned earlier, Webb (1966) cautioned against the general assumption that the ATP generated intracellularly was used indiscriminately

for all intracellular processes. Wu and Racker (1957, 1959) found that the ATP formed during oxidative phosphorylation in mitochondria was less readily available for the phosphorylation of glucose than that formed by glycolysis. Harary, Seraydarian and Gerschenson (1967) found that small changes in ATP affected the spontaneous contractions of cultured heart cells and reflected on the possibility that this may actually represent a large change in a localized compartment. Bueding, et al., (1967) showed that β-hydroxybutyrate could prove an adequate substrate in guinea-pig taenia coli preparations that had been depleted of glycogen. The tension development associated with β-hydroxybutyrate was, however, slower than with D-glucose, although the ATP and CP contents were equivalent. The addition of D-glucose doubled the tension produced. They felt that these findings could be explained on the basis of a compartmentalized store of ATP since D-glucose would provide ATP by both glycolysis as well as by oxidative mechanisms whereas β-hydroxybutyrate would only generate mitochondrial ATP. In contrast, Prasad and Macleod (1969) suggested that in the guinea-pig papillary muscle, ATP produced by oxidative mechanisms was available for both membrane phenomena and contractility whereas that originating from glycolysis alone was used preferentially for membrane phenomena.

Thus, it was argued that in this study, the failure of contractility to recover when IAA-treated tissues were incubated in oxidizable substrates pointed to a localization of mitochondrial ATP

that allowed membrane processes to recover preferentially. Two methods were used to test the above suggestion: (a) the provision of oxidizable substrates to tissues depleted of endogenous substrates and (b) the use of the inhibitor 2-deoxy-D-glucose.

STUDIES ON SUBSTRATE-DEPLETED TISSUES

Bueding and Bülbring (1967) depleted the guinea-pig taenia coli of endogenous substrates with successive periods of anaerobic (45 minutes) and aerobic (60 minutes) incubation. On supplying oxidizable substrates, the tissue regenerated high energy phosphates. Attempts were made to obtain substrate-depleted tissues by several methods. But the results obtained were rather erratic and it is likely that these could be due to variable stores of endogenous substrates. Of those available in the myometrium, glycogen is in all likelihood the major source. Estrogenization is known to increase the stores of glycogen (Boettiger 1946, Kostyo 1957) but whether these prove adequate is uncertain. The metabolic changes that occur in prolonged hypoxia can only be conjectured, since no measurements of either glycogen or adenine nucleotides were carried out. However, Daniel and Robinson (1971) found that after prolonged incubation in a glucose-free medium, rat uterine pieces had low contents of ATP (0.04 μmoles/gm) and ADP (0.06 μmoles/g). Subsequent incubation in an oxygenated medium containing substrates did not lead to any improvement. Substrate-depleted tissues thus obtained failed to

recover ion gradients.

In cardiac muscle, Deuticke and Gerlach (1966) found that the nucleotide degradation of ATP to AMP continued further to adenosine, inosine, hypoxanthine and xanthine which rapidly diffused out and were irretrievably lost. Similar findings were reported by Benson, et al., (1961) who found increased contents of deaminated nucleosides and bases in the effluent leaving isolated perfused dog hearts that were rendered anoxic. Imai, et al., (1964) showed that in ischaemic skeletal muscle, contents of IMP, inosine and hypoxanthine increased. Kroeger and Stephens (1971) found that in the trachealis smooth muscle, hypoxia caused a greater decrease in ATP levels than an increase in ADP and AMP. This, they inferred, could be due to conversion of AMP to hypoxanthine and inosine monophosphate. Furthermore, Daniel and Robinson (1971, b) found inosine and hypoxanthine in the medium surrounding tissues incubated with IAA. It is thus possible that under conditions of prolonged metabolic stress, irreversible nucleotide degradation accompanied by leakage of purine bases occurs. Although the extent of such leakage is uncertain, it could explain the erratic recoveries obtained in the experiments on substrate-depleted tissues reported in this study.

INEFFECTIVENESS OF 2-DEOXY-D-GLUCOSE

2-deoxy-D-glucose has been shown to be an effective

inhibitor of glycolysis in a variety of tissues - diaphragm (Nakada and Wick, 1956), adipose tissue (Webb, 1966), lymph node cells (Helmreich and Eisen, 1959), non-myelinated nerves (den Hertog and Ritchie, 1969), and cerebral cortex slices (Tower, 1958). This drug has several possible loci of action. Thus it may competitively inhibit hexokinases, may interfere with active transport of hexoses, inhibit phosphoglucose isomerases and by depleting ATP, reduce transport and phosphorylation of hexoses. It is generally believed (Webb, 1966) that 2-deoxy-D-glucose is phosphorylated and the derivative thus obtained undergoes no further metabolism. This, in fact, may be the actual inhibitor. Smith and Gorski (1968) showed that uterine tissue metabolized (^{14}C)-2-deoxy-D-glucose by a system that exhibited saturation kinetics, a high temperature coefficient and was competitively inhibited by D-glucose. Later Smith and Stultz (1971) showed that D-mannose could also inhibit the transport and phosphorylation of 2-deoxy-D-glucose. However, the precise mode of action of the drug in this tissue is still uncertain. In the experiments described here, 2-deoxy-D-glucose did not prove to be an effective inhibitor under aerobic conditions, even when D-glucose was omitted from the medium. It is unlikely that this was due to insufficient permeation of the inhibitor which was used in a fairly high concentration of 20 mM. By comparison, the concentration required to inhibit glucose metabolism in non-myelinated nerves was of the order of 0.5 - 5.0 mM (den Hertog and Ritchie, 1969). Furthermore, the animals used in the present study

had been treated with large doses of estrogen and Smith and Gorski (1968) showed that estrogenization accelerated transport and/or phosphorylation of 2-deoxy-D-glucose.

Incubation of Na^+-rich tissues in glucose-free Krebs under hypoxic conditions reduced recoveries of ions and contractions. However, in the presence of 20 mM 2-deoxy-D-glucose, this reduction was accentuated. The inhibition thus induced was overcome with small amounts of D-glucose added simultaneously. This suggests that there could be a competition for transport and/or phosphorylation. The ineffectiveness of 2-deoxy-D-glucose under oxidative conditions may be related to the existence of endogenous substrates in this tissue. If the major locus of action of 2-deoxy-D-glucose in this tissue were the hexokinase step, breakdown of glycogen to glucose-1-phosphate would bypass the inhibition. This, under aerobic conditions, would support contractions and ion movements adequately. The absence of endogenous substrates in myelinated nerves may explain the greater effectiveness of 2-deoxy-D-glucose in that tissue (den Hertog and Ritchie, 1969). In ascites carcinoma cells, Christensen, et al., (1961) found that 2-deoxy-D-glucose simultaneously inhibited glycolysis and increased the oxidation of glucose. Webb (1966) suggested that this may be due to the stimulation of the pentose pathway. The activity of the pentose shunt in uterine tissue is still uncertain. Thus Beatty, et al., (1969) calculated that only about 0.2% of the glucose uptake was metabolized by the pentose

pathway in the pregnant rhesus myometrium. On the contrary, Smith and Stultz (1971), Barker and Warren (1966), Webb (1966) and Smith (1967), argued that the high CO_2 production noted with C-1 labelled sugars denoted an active pentose shunt in uterine tissue. Whatever be the precise explanation for the inability of 2-deoxy-D-glucose to reduce recovery of ion gradients and contractions, it was clear that attempts to use the drug to determine the utilization of oxidizable substrates were unsuccessful. Thus it was not possible to exclude conclusively the suggestion that there existed a compartmentalization of mitochondrial ATP; however, the results obtained with other -SH inhibitors rendered that possibility less likely.

STUDIES WITH -SH INHIBITORS

A direct effect of IAA on the contractile apparatus, unrelated to metabolic inhibition, was another possibility considered to explain the differential effects of the inhibitor on contractility. The problems associated with interpreting data obtained with metabolic inhibitors will be discussed later; it is sufficient to comment here that IAA is notorious for a number of non-specific effects on a variety of systems. This is related to its ability to alkylate a number of ligands, principally -SH groups. The importance of -SH groups in the functioning of diverse proteins and enzymes has been well documented. Recently, Rothstein (1970) has critically discussed

some of the problems involved in the use of -SH reagents. He points out that the -SH groups of proteins vary considerably in their reactivity and this depends on a number of factors - the protein concerned, the agent used, and the conditions of the test. Thus the pH, for instance, may prove crucial, since it determines the ionization state of the -SH proton and some reagents react with RSH and others with RS groups. Then again, some of the -SH groups involved may be crucial for function whereas others may be relatively insignificant. Thus on studies with lyophilized membranes of human erythrocytes, Green (1967) found that IAAmide which bound to over 30% of the -SH groups produced no effects on RH antigen activity whereas NEM that bound to 20% produced far greater effects.

It is difficult to evaluate the effects of -SH reagents on myosin ATP-ase activity. The -SH inhibitors, p-chlormercuribenzoate and iodoisobenzoate strongly inhibited myosin ATP-ase activity. Heavy metals completely inhibited contraction and splitting of ATP in model systems. IAA on the other hand, did not inhibit contraction of actomyosin threads whereas IAAmide was only effective in large concentration (Hasselbach and Weber, 1955). Sulphydryl groups on the myosin molecule are involved both in its ATP-ase activity as well as in its combination with actin. Barany and Barany (1959) showed that treatment of actomyosin with high concentration of IAAmide abolished the enzymatic activity but not the actin-combining activity of myosin. This suggested that these sulphydryl groups may be functionally distinct. This view was subsequently elaborated by

Stracher (1965). It is also likely that the myosin ATP-ase activity may involve different functioning -SH groups. Thus two -SH sites have been postulated in the functioning of myosin ATP-ase. Of these, one is "activated" by N-ethylmaleimide while the other is inactivated. (see review by Dreizen and Gershman, 1970). Wachsberger and Kaldor (1971) showed that p-chloromercuribenzoate inhibited the Ca^{++} moderated ATP-ase activity of uterine myosin A although the drug has been reported to activate the skeletal enzyme. They found other differences in the ATP-ase activities of uterine and skeletal myosin A which suggested that the configuration of the active sites of these enzymes were not identical. It is thus difficult to predict the effects of -SH reagents on contractility.

The results obtained with N-ethylmaleimide were equivocal. This drug reacts rapidly with thiols forming addition products. As an -SH reagent, it is believed to possess certain advantages - a high selectivity for -SH groups and good penetration into cells due to its lack of charge. It is reported to react only with certain accessible -SH groups on enzymes raising hopes of a more selective action (Webb, 1966). In the experiments performed here, N-ethylmaleimide appeared to behave as a non-specific inhibitor. At each concentration used, there were no significant differences in the ion contents or contractility of tissues incubated in glucose or pyruvate. With the lowest concentration, (0.2 mM), K^{+} gain occurred with both glucose and pyruvate and there was a feeble response to drug stimulation. With higher concentrations, tissues failed to exhibit either

spontaneous or stimulated contractions; the K^+ gain that occurred was reduced but not abolished. Although this may be taken as an indication of the greater susceptibility of the contractile process to -SH inhibition, the results were not sufficiently conclusive. It has thus been found in this study that the inhibitory effects of NEM may be critically dependent on the concentration used.

The results obtained with IAAmide were more informative. This drug reacts more readily than IAA with -SH groups (1.9 times as fast at pH 7.1) whereas IAA reacts more rapidly with $-NH_2$ groups (Webb, 1966). Thus it was anticipated that if -SH groups were involved, IAAmide would produce more rapid and potent inhibition. In the experiments with IAAmide, it was found that even with very low concentrations (0.1 mM) used for a brief period (30 minutes), recovery of contractions was drastically reduced. However, K^+ gain, taken as a measure of Na^+-K^+ exchange, was considerable. No differential effects were noted with glucose and pyruvate as substrates under aerobic conditions. This suggested that glycolytic inhibition was negligible and in fact the ATP contents of IAAmide-treated tissues was comparable to those of untreated controls. It could be argued that the results could be explained by an inhibition of glycolysis and a channelling of glucose through the pentose pathway. However, even under hypoxic conditions, significant K^+ gain occurred, suggesting adequate functioning of anaerobic glycolysis. This

provided fairly strong evidence that the results obtained with IAAmide (and by analogy, IAA) was not related to inhibition of metabolism. This does not, however, specify the precise mechanism involved. Thus the effects of -SH reagents on contractility may be related to an inefficient formation of actomyosin or an inadequate functioning of the contractile proteins. As described earlier, loading the tissues with K^+ did not lead to recovery of contractions either and the effects of IAA and IAAmide were not associated with any changes in tissue contents of exchangeable Ca^{2+}. Further studies are clearly necessary to delineate the mechanics of the inhibition described.

TISSUE SWELLING AND THE SECOND PUMP

The experiments with IAAmide also suggested that the swelling of tissues observed under a number of different conditions could have a non-metabolic component. As has been mentioned earlier, this study was primarily concerned with metabolism in relation to coupled Na^+-K^+ exchanges and sought to obtain evidence that these could be dissociated. It was noticed that there often appeared to be a discrepancy between K^+ gain and Na^+ extrusion. This was particularly conspicuous in the experiments with IAA. Thus tissues treated with that drug and incubated in pyruvate accumulated significant amounts of K^+ with little change in Na^+ content. A study of the time-course of K^+ accumulation showed clearly that this was not accompanied by Na^+ extrusion as would have occurred under normal

circumstances. However, this K^+ gain was inhibited by ouabain and anoxia. Although this suggested the operation of a coupled Na^+-K^+ exchange dependent on metabolism, the extrusion of Na^+ was masked by other changes leading to a gain of Na^+. Calculations showed that the Na^+ gain could be explained by a gain of isotonic fluid. This may explain the discrepancy between the observations made here and those reported by Kao (1967) on the rabbit myometrium. He found that net Na^+ extrusion of tissues recovering in the absence of glucose was markedly hampered whereas K^+ accumulation continued. This suggested to him that Na^+ extrusion depended mainly on exogenous glucose whereas net K^+ accumulation could derive energy from endogenous substrates. Thus swelling of tissues with alterations in Na^+ content may have been responsible (at least partly) for the "dissociation" observed between Na^+ and K^+ movements. The crucial question raised at that juncture was related to the mechanics of the Na^+ gain - whether it was due to an increase in passive permeability or due to inhibition of some process that was actively extruding Na^+ and water as occurs for instance in kidney cells (Whittembury and Fishman, 1969).

In recent years there has been mounting evidence for the existence of diverse modes of Na^+ extrusion not coupled to K^+ accumulation. Hoffman (1966) suggested that part of the Na^+ movements that occurred after ouabain treatment might represent the activity of a second pump. In red blood cells, he described the existence of three pumps. Of these pumps, IA and IB were sensitive

to ouabain, although only the former required external K^+. The third pump (Pump II) required external Na^+, showed no requirements for external K^+ and was not inhibited by ouabain. It was, however, inhibited by ethacrynic acid. Whittembury and his associates (Whittembury and Fishman, 1969) have described two pumps in the kidney. One of these exchanged Na^+ for K^+ and was inhibited by ouabain, and the other extruded Na^+ from the cell accompanied by Cl^- and water movements followed to maintain osmolarity; this second pump was sensitive to inhibition by ethacrynic acid and thus seemed analogous to Hoffman's Pump IIA in erythrocytes. Whittam and Wheeler (1970) are skeptical of results based on the use of ethacrynic acid since the compound also decreases ATP-ase activity and the Na and K movements associated with Na pumping. MacKnight (1969) also regards the effects of ethacrynic acid as relatively non-specific. So do Daniel et al., (1971) who showed that ethacrynic acid had marked metabolic effects on the rat uterus - it inhibited oxidative phosphorylation partly and glycolysis completely and thus depleted the tissue of ATP and ADP.

The above studies on kidney cells were based on an initial finding by Kleinzeller and Knotkova (1964) who, however, reached different conclusions. They showed that cells from the renal cortex of rabbits swollen after incubation at 0^oC in a K^+-free medium, extruded Na^+ and water when rewarmed in the same medium. This extrusion occurred even in the presence of ouabain and therefore

could not be explained on the basis of Na^+-K^+ exchange. These workers suggested that some contractile system existed which squeezed or filtered a Na^+ and Cl^- solution out of the swollen cell. They were unable to obtain evidence for a similar process in liver cells. Most of the above workers regard the NaCl pump as being responsible for regulation of cellular volume.

Daniel, et al., (1970) argued on the basis of flux data from estrogenized rat uterine horns that an ATP-dependent Na^+ pump could exist which controlled cellular volume, but was unaffected by ouabain. They found that even after inhibition of transport ATP-ase by ouabain, a certain component of Na^+ efflux persisted and this was reduced by ATP depletion. Ethacrynic acid did not appear to inhibit this second pump. Daniel and Robinson (1971 a,b) showed that Na^+-rich tissue rewarmed in K^+-free solutions lost weight and this was not prevented by ouabain. However, if IAA or DNP were added before the rewarming was started, no loss of weight occurred and in fact the tissues gained weight. In the experiments performed as part of this study, their findings were confirmed. The weight changes resulting from Na^+ enrichment were variable. However, tissues when rewarmed in a K^+ free medium lost considerable weight within 15 - 20 minutes. On further incubation in a K^+-containing medium, the tissues gradually gained weight and yet did not attain their fresh weights even at the end of a 120-minute incubation. However, in the presence of DNP or IAA, the tissues gained weight rapidly and at the end of a 120-minute incubation, their increase in weight compared to fresh weights amounted

to approximately 7% for DNP-treated tissues and 13% for IAA-treated tissues. Incubation with high concentration of pyruvate reduced but did not abolish the weight gain. This suggested that this effect of IAA was independent of its metabolic effect or alternatively, the ATP produced was either insufficient or compartmentalized and thus unavailable for extruding Na^+ and water. It was also found that DNP-treated tissues incubated in glucose-free Krebs gained less weight than corresponding pairs treated with IAA and subsequently incubated in glucose. The ATP contents of such tissues (in parallel experiments) were virtually nil (unmeasurable). This again suggests that IAA may have an effect unrelated to ATP reduction. The results with IAAmide produced more evidence for the above contention. Thus IAAmide-treated tissues had normal contents of ATP, gained K^+ but failed to extrude Na^+ and water. Thus the Na^+ and water gain noticed appeared to be independent of metabolism. It has already been argued that glycolytic inhibition was either inadequate or non-existent under those conditions, and as such it is difficult to suggest a compartmentalized store of ATP that was unavailable for Na^+ and water extrusion.

Perhaps the most interesting aspect of these experiments with IAAmide was the close correlation between water gain and virtual absence of contractility in inhibited tissues. Such a correlation was also found under a variety of metabolic conditions (Table 19). As mentioned earlier, Kleinzeller and Knotkova (1964) suggested the existence of a contractile mechanism responsible for squeezing

water out of cells. It is likely that a similar mechanism may be operative in this tissue.

Daniel and Robinson (1971, c) have proposed a model to explain the complex movements of Na^+ in the rat uterus. Their model is based on certain ultrastructural features of smooth muscle cells, namely, the presence of pinocytotic vesicles in the plasma membrane. Goodford, et al., (1968) had argued that these vesicles, being derived from plasma membrane, would have fixed negative charges and thus serve as cation-binding sites. If the vesicular membrane were postulated to be selectively permeable to Na^+, a very high intravesicular concentration of Na^+ (approximately 900 mM) would be attained and the opening of the vesicles onto the cell surface might be responsible for Na^+ extrusion. The Daniel model postulates a dynamic existence for the vesicles, with ATP being required both for their formation as well as for maintaining their selectivity towards Na^+. Thus in a metabolically active cell, the vesicles form, soak up Na^+ from the cytoplasm and extrude the Na^+ by reverse pinocytosis. The formation of the vesicles is ATP dependent but insensitive to ouabain. On the other hand, the filling of the vesicles occurs through the agency of an ouabain-sensitive transport ATP-ase. With a reduction in ATP, the vesicles would lose their ability to accumulate and bind Na^+ and with more progressive depletion of ATP, the formation would be hampered as well. This would, in a sense, "unbind" Na^+ and the concentration of this ion in the cytoplasm would increase, alter the osmotic pressure and thus induce swelling. The vesicles thus become

the seat for volume control. Electron microscopic studies showed that in the myometrium metabolic inhibition led to swelling of the vesicles and their ultimate disappearance. Reduction of ATP to zero led to extensive cellular damage and disappearance of vesicles (Garfield, et al., unpublished). Under similar conditions, sucrose and inulin spaces increased, suggesting a drastic alteration in permeability to these markers. All these ultrastructural changes occurred in tissues that had higher contents of water. The results presented here can provide an alternative mechanism for volume regulation. It is not difficult to imagine the existence of a contractile protein in the vicinity of the vesicles, which could squeeze out the contents effectively (as suggested by Kleinzeller and Knotkova, 1964). In recent years, considerable interest has been aroused in the role played by microfilaments in a number of cellular processes - from morphogenesis to ciliary motility or function of contractile vacuoles (Wessells, et al., 1971). Thrombesthenin, a contractile protein that is present as filaments 80 - 100° A in diameter, is believed to participate in clot retraction (Bettex-Golland and Luscher, 1965; Zucker-Franklin, 1969). In the cortex of amphibian eggs, Ca^{++}-activated contractions have been observed (Gingell, 1970). Studies on red cell membranes have shown the existence of several proteins. Spectrin, described by Marchesi and Steers (1969) polymerized to form filaments in the presence of divalent cations. Rosenthal, Kregenow and Moses (1971) isolated a group of fibrillar proteins associated with ouabain-

insensitive ATP-ase activity from human erythrocyte membranes.

Recently, Palek, Curby and Lionetti (1971 a,b) have studied the effects of Ca^{++} on human red cell ghosts. Using the technique of reversal haemolysis, they introduced Ca^{++} into red cell ghosts. This introduction resulted in a marked decrease in cell volume and an alteration in osmotic behavior. There were two phases observed in the decrease in volume. The early or isosmotic phase, occurred when Ca^{++} was added to the hypotonic medium before restoration of isotonicity. This phase was rapid and not affected by ouabain. The second phase produced a greater decrease in volume and occurred when NaCl was added to raise the tonicity. These workers argued that the early phase was due to a contraction of the erythrocyte membrane and the second phase was an increased response to changes in osmolarity. Later work showed that Ca^{++} and ATP added externally did not cause a volume change nor was ATP hydrolyzed. However, introduction of Ca^{++} and ATP internally, resulted in marked shrinkage of the ghosts and hydrolysis of ATP occurred as well. Thus they present a persuasive case for a link between contraction of the membrane and the Ca^{++}-activated ATP-ase.

An interesting model linking -SH groups, membrane contraction and vesicular function has been proposed by Kosower and his colleagues based on their studies on transmitter release at frog neuromuscular synapses (Werman et al., 1971, Kosower and Werman 1971). They showed that the thiol-oxidizing agent, diamide, provoked

an increased release of Ach. They suggested that oxidation of reduced glutathione occurred and this, in turn, converted membrane dithiols into disulphides. The formation of disulphides led to contraction of certain regions of the membrane. If the vesicles were attached to the membrane, the strain induced would lead to transmitter release. They also suggested that reduction in the distance between the thiol groups on the membrane and those on the vesicles could occur if disulphides were formed. This in turn would produce a strain on the vesicles. The authors postulated that the formation of calcium dithiolate under normal conditions may cause release of transmitter.

How these studies relate to the problem under review is uncertain. They do, however, suggest that contractile proteins in membranes may have a wide distribution and exhibit diverse functional adaptations. To evoke the existence of a contractile protein as a regulator of cell volume in the uterus, is thus not unreasonable. However, further work is clearly necessary for refuting the above suggestion. As mentioned earlier, Na^+-rich tissues undergo a contracture on rewarming and under similar conditions exhibit a considerable reduction in weight. This could result either from a mechanical squeezing of fluid from the extracellular spaces or from an expulsion of fluid from the pinocytotic vesicles. Papaverine not only prevented the contracture on rewarming but also abolished the loss in weight. If the hypothetical contractile protein possessed properties similar to actomyosin, it can be expected to be stimulated

or inhibited by similar treatments. Thus the papaverine experiment does not clearly distinguish between the two possibilities.

It must be emphasized at this stage, that one of the major assumptions underlying this work is that Na^+-K^+ exchanges take place across a membrane that separates two electrolyte solutions and that the membrane does in essence control this exchange. This viewpoint has been developed over several decades and is based on evidence obtained from a variety of systems (see Introduction). That this may be an oversimplification has been suggested by several studies and alternative non-membrane models have been proposed (Ling 1962, Troshin 1966). Jones (1970) has presented the case for viewing ion movements in smooth muscle on the basis of one such model - the Association-Induction hypothesis. In essence, the non-membrane models view the processes of ionic transfer as occurring in a continuum; and the proponents for this view argue that proteins, ions and water exist in close association within the living cell. Thus an ion that enters the cytoplasm does not freely diffuse, but undergoes interactions with a number of relatively immobile tissue elements such as proteins. The charges on macromolecules afford sites for interaction with cations and the degree of interaction being associated with "associational energies" of such moieties. Such an association forms the basis of selective accumulation of ions. Furthermore, the distribution of charges on the macromolecules and thus the accumulation of ions can be affected by other molecules such as ATP. This more complex model derives support from recent studies on the physico-chemical state

of Na^+, K^+ and water in cells. (Cope 1967, 1970; Ling, 1962). Ling and Cope (1969) showed that the greater portion of Na^+ in skeletal muscle fibres after Na^+-enrichment existed in a "complexed" state. This, they suggest implied that K^+ was similarly bound and Na^+ adsorption occurred on sites "vacated" by K^+ ions lost from the fibres. Recently, Armstrong and Lee (1971) measured intrafibre K^+ and Na^+ concentrations as well as the activities of these ions with cation-selective electrodes. They argued that the increase in Na^+ activity noticed was far less than could be expected on the basis of an increased concentration of "free" Na^+. Conversely, the decrease in K^+ activity was much less than the decrease in K^+ concentration. Thus they implied that the K^+ lost must have existed in a sequestered state. Na^+ and K^+ may also be complexed in barnacle muscles (Hinke and McLaughlin, 1967). Evidence for the occurrence of "ordered" water has also been obtained by N.M.R. studies (Hazelwood, Nichols and Chamberlain, 1969). Similar conditions may exist in the myometrium, although no actual evidence is available (Jones, 1970). Ling (1962) suggested on the basis of the association-induction hypothesis, that there would be a quantitative relationship between ATP and K^+ contents of cells, with each mole of ATP critically determining the selective accumulation of about 34 moles of K^+ ion. As shown in (Figure 10), such a close relationship did not seem to exist. The ATP/K^+ ratios were calculated for the various conditions tested (using wet weight values). These ratios were as follows:

Fresh tissues, - 50; Na^+-rich tissues, - 2; recovered Na^+-rich tissues, 65; IAA-treated tissues incubated in pyruvate, 141; DNP-treated tissues, 132; and IAAmide-treated tissues, 46. Where the ATP content was very low (i.e., in IAA or DNP-treated tissues), far more K^+ accumulation occurred. This implies that either a small fraction of total cellular ATP is sufficient for K^+ accumulation or that the rate/efficiency of utilization of ATP is increased under such conditions. Whatever be the precise explanation, it is difficult to bear out Ling's predictions under the conditions studied.

PROBLEMS IN THE USE OF METABOLIC INHIBITORS

Any study such as this, where the results have been based largely on the use of metabolic inhibitors faces certain general problems of interpretation. Although most of these have been touched upon in the course of this discussion, at this terminal stage, a more comprehensive critique is essential. The difficulties stem from several sources (Webb, 1966). These are:

(a) Lack of specificity of metabolic inhibitors.

(b) Variations in concentration of the inhibitor at different sites in the cell.

(c) Problems associated with correlating observations on cell-free systems with intact cells.

(d) The existence of complex regulatory pathways.

The vast literature on metabolic inhibitors makes it abundantly clear that these agents are neither specific nor even very selective. Thus IAA is commonly listed as an inhibitor of glycolysis since it inhibits the enzyme 3-phosphoglyceraldehyde dehydrogenase. Webb (1965, 1966) has compiled an impressive list of other enzymes affected - 21 involved in glycolysis, 26 in electron transport, 14 in the tricarboxylic acid cycle, 26 in proteolysis, 21 in the pentose shunt and related pathways, 31 in lipid metabolism, 62 in amino acid metabolism and 53 in miscellaneous functions. This lack of selectivity stems from the fact that most -SH reagents also react with a number of other ligands, e.g., carboxyl, amino, imidazole, phosphoryl, etc. Thus selectivity is the exception rather than the rule. However, selectivity may be improved by careful choice of the drug and incubation conditions. Webb (1966) points out that it may be possible to produce a specific inhibition of 3-phosphoglyceraldehyde dehydrogenase by using concentrations in the range 0.05 - 0.2 mM, although the Embden-Meyerhof pathway itself may not be completely inhibited in all cases. The use of specific concentrations to produce certain effects rests on the unwarranted assumption that the concentration of the inhibitor in the bathing medium is the same as its concentration at the site of action.

Webb (1966) discusses the problems involved, in relation to the penetration of IAA. Being a weak acid (pKa 3.12) the drug would be largely ionized at physiological pH. If permeability

barriers to anions exist, then the effects of IAA should exhibit a marked pH dependence. Although this occurs in plant cells, the results are not so definitive in mammalian tissues. Many mammalian tissues are inhibited by IAA at physiological pH, provided large enough concentrations are used. This may indicate barriers to permeation. Furthermore, undissociated IAA entering the cell will dissociate into iodoacetate and H^+ and this in turn would have effects on intracellular pH. Any reduction in intracellular pH may retard the reaction between iodoacetate ion and -SH groups and the change in pH may affect metabolic processes directly. Rothstein (1970) has discussed in greater detail the problems of permeability in relation to -SH reagents. These drugs may react with diverse ligands present on the membrane, in the cytoplasm and on organelles. The accessibility of ligands and the existence of diffusion channels plays an important role in determining the reactivity of different -SH reagents. The binding and distribution of organic mercurials in intact cells varies markedly from their binding properties on purified membranes. The -SH reagent p-chlormercuriphenylsulphonic acid (PCMBS) diffuses into the red blood cell through two anion channels and one of these can be specifically blocked by amino-reactive agents (Rothstein, 1970). Information regarding the existence of such mechanisms in other systems is sadly lacking. Recently, Piccoli and Lajtha (1971) have shown that slices of mouse brain accumulated 2,4-dinitrophenol against a concentration gradient and this transport process was related to that of tyrosine and

phenylalanine. Thus concentrations of metabolic inhibitors within cells may be vastly different from that in the incubation medium and that at the locus of action can only be conjectured.

The complexities discussed above emphasize the problems in attempting to extrapolate data obtained in cell-free systems and purified enzymes onto intact cells or tissues. Further complications arise because enzymes within cells may exist in different conformational states and may be protected by a variety of substances. It is known that 3-phosphoglyceraldehyde dehydrogenase isolated from muscles exists predominantly (80 - 90%) in the oxidized state and is relatively resistant to IAA inhibition. However, incubation with cysteine or glutathione "activates" it (Webb, 1966). Since the conformational state of the enzyme within the cell is unknown, inhibition by IAA may or may not occur. Since the cell contains organelles and other intracellular structures, it is possible that enzymes may be sequestered and thus be inaccessible to metabolic inhibitors. Heart hexokinase, for instance, appears to exist in equilibrium between a form combined to intracellular structures and a freely soluble form (Hernandez and Crane, 1966). Such information is not available for the majority of cellular enzymes. Then again, a large number of enzymes are protected from inhibition by their substrates and other substances in the cell. Thus alcohol dehydrogenases are protected by their substrates and NAD, creatine kinase is protected by Mg^{++} (Webb, 1966) and 3-phosphoglyceraldehyde dehydrogenase is

protected by 3-phosphoglyceraldehyde (Webb, 1966; Krimsky and Racker, 1954). The rapidity with which 3-phosphoglyceraldehyde is formed and the concentration it attains may therefore influence the inhibition of the enzyme by drugs. Thus the steady-state level of 3-phosphoglyceraldehyde may determine the degree of inhibition by IAA (Holzer and Holzer, 1953).

The above findings suggest that complex regulatory mechanisms could alter the response to metabolic inhibition. That regulatory mechanisms do exist have been amply demonstrated and the theoretical basis for such control systems have been developed (Chance, 1965; Garfinkel, 1965). Of special importance to the present study are the mechanisms controlling the rate of glycolysis. These have been studied in a variety of tissues (brain, ascites cells, heart, etc.). Glycolytic control mechanisms are an example of multisite control reactions and possible control steps include phosphorylase, hexokinase, phosphofructokinase, 3-phosphoglyceraldehyde dehydrogenase and pyruvate kinase (Williamson, 1965). The steps catalyzed by the above enzymes are characterized by being widely displaced from equilibrium (Hess, 1963); this displacement from equilibrium renders them suitable for precise control. Phosphofructokinase which catalyzes the relatively irreversible phosphorylation of fructose-6-phosphate to fructose-1, 6-diphosphate is inhibited by ATP (Mansour and Mansour, 1963; Lowry and Passonneau, 1964). As the level of intracellular ATP increases, it exerts a feedback control

on glucose entering the glycolytic pathway. This enzyme is also inhibited by citrate. This dual inhibition by ATP and citrate may be the explanation of the Pasteur effect-viz that the glycolytic flux increases when respiration is prevented by anaerobiosis. The reverse phenomenon, the inhibition of respiration by addition of glucose, is termed the Crabtree effect. The inhibitory effects of 2-deoxy-D-glucose in Ehrlich ascites cells has been attributed to the existence of the Crabtree effect in such tissues (Webb, 1966).

Given such complexities, it becomes difficult to interpret the results obtained with metabolic inhibitors. Several attempts have been made to develop procedures that help localize the sites of action of metabolic inhibitors. Chance and Williams (1956) developed the "cross-over" theorem which was initially used to determine the locus of inhibition in the electron transport chain. The concept is based on the assumption that when an inhibitor is introduced into an active series of redox components of the respiratory chain in the steady-state, those components on the electronegative side of the block become more reduced while those on the electropositive side become more oxidized. Later, Williamson (1967) used the cross-over theorem to study the action of IAAmide in the perfused rat heart. He showed that after 10 minutes of perfusion with IAAmide in the presence of glucose and insulin, hexose monophosphates increased slightly, fructose-1,6-di-phosphate and triose phosphates showed a two-fold increase whereas 3-phosphoglycerate and lactate decreased.

On continued perfusion, these changes were intensified (fructose-1,6-di-phosphate increased 133-fold, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate increased 40-fold and 28.5-fold whereas glycerophosphate and 3-phosphoglycerate decreased). Thus a cross-over occurred just at the 3-phosphoglyceraldehyde dehydrogenase step. The locus of action of fluoroacetate was determined in the same system by using a cross-over plot (Williamson, 1967). Thus measurement of metabolites affords a sensitive method for locating the site of action of an inhibitor.

In the present study, no metabolites were measured and the assumption was made that the metabolic inhibitors used acted at the same sites as suggested in other tissues. However, attempts were made to bypass the metabolic block induced by suitable substrates and this affords another means for locating the site of action of an inhibitor (Webb, 1966). Thus in the presence of DNP, the anticipated sequence of events would be an uncoupling of oxidative phosphorylation, reduction in ATP levels and alteration in the ATP/ADP ratio, with consequent release of inhibition of phosphofructokinase (Lowry and Passonneau, 1964). The increased glycolytic flux would provide ATP if sufficient (suitable) glycolytic substrates were available. This expectation was borne out since tissues failed to recover in the absence of exogenous D-glucose or D-mannose. In the case of IAA, the ability of pyruvate to restore ion movements at least partially, provided some information regarding the locus of action of IAA. In

other systems, the use of pyruvate has yielded variable results. Thus isolated kidney tubules accumulated phenol red by a mechanism sensitive to IAA. However, pyruvate overcame this inhibition only partially (Beck and Chambers, 1935). In erythrocytes, the reversal of IAA inhibition was partial (Wilbrandt, 1940) or negligible (Maizels, 1951). Similar results were obtained in frog muscle (Van der Kloot, 1958). A crucial problem in such studies is the permeability of the cells to pyruvate. In the present study, fairly high concentrations of pyruvate (25 mM) were used to overcome such problems. Furthermore, if compartmentalization of ATP occurs, that produced by the operation of the Krebs cycle may not be able to replace that from glycolysis. It is thus clear that if pyruvate is not able to resuscitate IAA-treated tissues, it is not wise to conclude that IAA is not inhibiting glycolysis alone. Since pyruvate (as well as other oxidizable substrates) partially restored ion movements in the present study, it is clear that oxidative processes were not drastically affected.

To summarize then, metabolic inhibitors, though not very selective, can yield useful information provided the problems inherent in their use are clearly borne in mind. When these drugs are used in conjunction with suitable substrates to circumvent the metabolic block produced, useful information can be obtained regarding the pathways involved in providing energy for specific processes. If measurement of intermediates and metabolites are included in such a study, it becomes more meaningful.

The present study has rested on several assumptions that need to be critically assessed and has also suggested several possible areas for future work. An attempt will be made to comment briefly on some areas that may bear further investigation.

a) It is clear that more information is required concerning the distribution and mode of action of metabolic inhibitors in this tissue, since it has been assumed that their loci of action corresponds to those reported for other tissues. Of particular relevance would be a study of the binding and distribution of -SH reagents, since it is possible that these drugs may affect certain processes selectively. Recently Kirsten and Kuperman (1970 a,b) have investigated the effects of different -SH reagents (PCMB, PCMBS and NEM) on frog skeletal muscle and have correlated differences in the effects observed to differences in permeability and cellular sites of action. Solberg and Forte (1971) showed that -SH reagents modify H^+ and Cl^- transport by gastric mucosa and that these may be differentially affected by PCMBS. The subcellular distribution of IAA and IAAmide in the myometrium would provide useful information regarding the differential effects of IAAmide on contractility.

That 2-deoxy-D-glucose is transported and phosphorylated in the uterus has been demonstrated by Smith and Gorski (1968), but its precise locus of action in this tissue is uncertain. Measurement of glycolytic intermediates and the application of the cross-over theorem should provide the answer.

b) This study has been largely qualitative or at best semi-quantitative in character. Although it has been demonstrated that both glycolytic and oxidative pathways are operative in this tissue, the quantitative contributions of these processes under various conditions has not been evaluated. As mentioned earlier, the contribution of the pentose pathway in the myometrium is still disputed. The questions that need to be answered are:

i) What is the proportion of carbohydrate metabolized by glycolytic and oxidative processes under basal conditions?

ii) Does the proportional contribution of diverse pathways alter with activity? Thus it has been demonstrated that in polymorphonuclear leukocytes, phagocytosis is associated with an increase in glucose and O_2 consumption and the proportion of glucose metabolized by the pentose pathway is sharply increased. Platelet phagocytosis, on the other hand, is associated with an increase in aerobic and anaerobic glycolysis with an increase in CO_2 production due to oxidation via the citric acid cycle (Sbarra and Karnovsky, 1959; Karnovsky, 1962; Kuramota et al., 1970). Thus measurement of basal activity in Na^+-rich tissues (where pumping is absent) can be compared to tissues that have been incubated in Krebs for varying periods. This may also provide information regarding the effects of ion transport on metabolism similar to those demonstrated in several other tissues (see Whittam and Wheeler, 1970). It has been suggested that the absence of spontaneous contractions and the reduction in

induced contractions seen after Na^+ enrichment may be related to a reduced activity of pyruvate kinase. This point can be tested by measurement of glycolytic intermediates which could also help to elucidate the control steps for glycolysis in this tissues.

c) The pathways for adenine nucleotide degradation and the turnover of adenine nucleotide pools in relation to activity needs further investigation.

d) Perhaps the most interesting area for future research would be to explore the relation between metabolism and water movements in the myometrium. The crucial questions that need to be answered are:

i) Is the reduction in weight observed when Na^+-rich tissues are rewarmed, due to an extrusion of intracellular water or is it due to a squeezing out of extracellular fluid?

ii) What is the precise relationship between contractility and swelling?

It has been proposed that a contractile protein is involved in the extrusion of water from the pinocytotic vesicles. The location of this protein and its functional characteristics need investigation. Thus this protein may be present either around the vesicles or in the membrane adjacent to the vesicles. The hypothetical protein may be related to actomyosin or may be a Ca^{++}-dependent ATP-ase. On the other hand, microfilaments may be involved in the extrusion process. These possibilities need further investigation. Selective pharmacological agents may prove helpful. Thus drugs like cytochalasin and vinblastine

are known to affect microfilaments (Wessels, et al., 1971). Polyamines like spermidine affect Mg^{2+}-activated ATP-ase activities of myofibrils, myosin B and actomyosin but do not affect Ca^{2+}-activated ATP-ases. The use of these and similar agents combined with E.M. studies may provide the answers.

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AFTERWORD

1. It was argued that the physiological significance of the present study was obscure since sodium-enrichment imposed an artificial condition that would have little bearing on the situation in vivo. This argument can be countered in two ways:

a) The removal of any tissue from an animal necessarily introduces an element of artifice into any study - a problem that has not deterred physiologists to any great extent.

b) Furthermore, on incubation in normal Krebs, sodium-rich tissues recover ions as well as contractions - i.e. they recover functionally. Since it was this recovery process that was studied, it can be argued that the use of Na^+ rich tissues served as a reasonable model for the situation in vivo. As such this procedure was analogous to studies that impose artificial stresses to evaluate physiological function.

2. No attempt was made to list the problems concerning ion analysis by flame photometry since these are well-documented. (See below).

a) Dean, J. A. and Rains, T. C. (1969). Flame Emission and Atomic Absorption Spectrometry. Vol. 1. Dekker, New York, N.Y.

b) Evans Electroselenium Ltd., (1967). Atomic Absorption Analytical Methods. Vol. 1. Halstead, Essex, England.

3. Those abbreviations that have been repeatedly used are listed below:

IAA for iodoacetic acid

IAAmide for iodoacetamide

DNP for dinitrophenol

NEM for N-ethylmaleimide

ATP for adenosine triphosphate.