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THE NATURE OF SODIUM PUMPING IN PREGNANT RAT UTERUS

by

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ABSTRACT

When incubated in potassium-free Krebs solution at $4^{\circ}C$ for 18 hours, pregnant rat uteri became "Na-rich" i.e. gained sodium (Na) and lost potassium (K), exhibited no spontaneous contractility and had a membrane potential (V_m) of -15 mV. Changes in membrane potential and contractility of Na-rich tissues were followed at different temperatures in the presence of media of varying ionic composition. The addition of K (4.6 mM) at $37^{\circ}C$ to Na-rich tissues, initially bathed in K-free Krebs solution, resulted in a rapid hyperpolarisation, V_m exceeding the calculated potassium equilibrium potential (V_K); Na was extruded and K reaccumulated, V_m gradually decreasing towards the value found in fresh tissues (-50 mV) and spontaneous electrical and mechanical activity then commenced. The addition of higher concentrations of K (46 mM and 120 mM) to Na-rich tissues also produced hyperpolarisation, V_m exceeding V_K. These findings indicate that electrogenic Na-pumping was occurring during the recovery of Na-rich tissues. The initial rapid hyperpolarisation was absolutely dependent upon the presence of external K; however, rubidium (Rb) and to a lesser extent cesium (Cs), could be substituted for K; lithium (Li) could not substitute for K. The hyperpolarisation was temperature dependent and inhibited reversibly by ouabain; it was essentially independent of chloride and of external Na but was abolished when internal Na was depleted or replaced by Li. These properties of the hyperpolarising

response are similar to those of (Na + K) activated ATPases in many other tissues and suggest that the response may be associated with the action of a similar ATPase in this tissue. In contrast, fresh tissues depolarised rapidly in high K-containing solutions (46 mM or 120 mM), showed no significant change in membrane potential in K-free solutions and showed a small initial depolarisation in response to ouabain (10^{-3}M). An electrophysiological study of the effects of an alpha (α) adrenergic agonist (noradrenaline in the presence of propranolol) and a beta (β) adrenergic agonist (isoproterenol) on the Na-pump provided no evidence that α or β adrenergic agonists could modify Na-pumping in tissues recovering from the Na-rich state. In addition evidence was obtained that K permeability was in some way related to the activity of the Na-pump. Na-rich tissues incubated in Cs-containing solutions (4.6 mM) showed action potentials with large overshoots and a prolonged repolarisation phase. Furthermore, the permeability to Cs appeared to be smaller than that of K.

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I INTRODUCTION

I INTRODUCTION

Ia Ionic basis of the resting membrane potential in smooth muscle.

Attempts to evaluate the magnitude of the potential difference existing across smooth muscle cell membranes were made as long ago as 1935 (Bacq 1935) with the aid of external electrodes. Membrane potential measurements have also been made more recently using extracellular wick electrodes, the sucrose gap method, or intracellular electrodes (see Burnstock et al 1963 for references). Intracellular microelectrodes were first used to determine the membrane potential in rabbit's sphincter pupillae by Bülbring and Hooton in 1954; and in the same year by Woodbury and McIntyre in pregnant myometrium.

Many smooth muscles are spontaneously active and show fluctuations in the resting potential during activity. Thus quantitative descriptions of resting membrane potential in smooth muscles will depend upon the particular phase of activity during measurement. The maximum polarisation developed across the membrane during quiescent periods has been taken by many workers to be a reliable measure of the resting membrane potential in spontaneously active tissues (Burnstock et al 1963). Estimations of the absolute values of the resting membrane potential using either the sucrose gap technique or intracellular microelectrodes is also limited by several technical problems. The latter technique in particular is often subject to difficulties involving (i) the small size of the smooth

muscle cells (ii) injury during impalement and damage during spontaneous activity (Holman 1968, Kuriyama 1968) (iii) tip and liquid junction potentials (Adrian 1956; Agin and Holtzman 1966).

Estimates of the resting membrane potential range from 50 to 90 mV (inside negative) in smooth muscles using various techniques in different smooth muscles (Burnstock et al 1963).

Attempts have been made for many smooth muscles to relate the magnitude of the resting membrane potential to the concentration differences of Na, K and Cl across the cell membrane and the permeability of these ions (Goodford 1968; Kao 1967; Burnstock et al 1963). Early formulations of the relationship between the ionic distribution and resting potential for skeletal muscle led to the following equation which considered the cell membrane permeable only to K and Cl at a time when the tissue was in a steady state with respect to the ionic gradients across the cell membrane (Boyle and Conway 1941).

$$V_K = \frac{RT}{F} \ln \frac{K_o}{K_i} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

Where V_K = the equilibrium potential for K, R is the gas constant, T the absolute temperature and F Faraday's Constant.

K_o = Extracellular K ion activity *

K_i = Intracellular K ion activity *

* Often it is assumed that the intracellular activity coefficient of an ion is the same as that in free solution and concentrations are used in this equation not activities.

This formula does not consider changes in the distribution of Na ions due to leakage or the active transport process that maintains a constant intracellular Na concentration.

In a non-steady state certain assumptions have to be made concerning the properties of the cell membrane and the way in which ions can cross it. The constant field theory of Goldman (1943) assumes that (1) all ions cross the membrane by diffusion, under the influence of electrical and concentration gradients in a manner essentially the same as that in free solution; (2) the electric field is constant throughout the membrane; (3) the concentrations of ions at the edges of the membrane are directly proportional to those in the solutions on either side of the membrane; (4) the membrane is homogenous. If there is no ionic current flowing across the cell membrane the net movement of Na, K and Cl ions must cancel out. Under these conditions the value of the membrane potential is given by:-

$$V = \frac{RT}{F} \ln \frac{P_K K_o + P_{Na} Na_o + P_{Cl} Cl_i}{P_K K_i + P_{Na} Na_i + P_{Cl} Cl_o} \quad .. \quad .. \quad .. \quad (2)$$

Where P_K, P_{Na}, P_{Cl} are the permeability coefficients for these ions

V = resting membrane potential

Na_o, K_o, Cl_o are the extracellular concentrations of Na, K and Cl.

Na_i, K_i, Cl_i are the intracellular concentrations of Na, K and Cl.

The remainder of the symbols as in equation (1).

In Cl-free solutions the intracellular concentration of Cl should fall rapidly and the value of $P_{Na}/ P_K = b$ can be determined

from the following equation:-

$$V = \frac{RT}{F} \ln \frac{K_o + b\, Na_o}{K_i + b\, Na_i} \quad \cdots \quad \cdots \quad \cdots \quad \cdots \quad \cdots \quad (3)$$

Where $b = P_{Na}/P_K$

The remainder of the symbols as in equation (2).

In frog sartorius muscle fibres the ratio b was found to be 0.01 by Hodgkin and Horowicz (1959). Assuming a value of b of 0.01 Hodgkin (1958) had earlier demonstrated in squid axons reasonable agreement between the calculated and observed value of the resting membrane potential. Few attempts have been made to estimate P_{Na}/P_K for smooth muscles. Kao and Nishiyama (1964) estimated b to be about 0.1 for rat myometrium and a similar value has been reported by Casteels and Kuriyama (1965) in the same tissue. For taenia coli Bennett (1966) has reported a value of 0.05 for P_{Na}/P_K and concluded that for values of $K_o > 25$ mM the distribution of K and the resting membrane potential were in agreement with the constant field equation; Casteels and Kuriyama (1966) did not substantiate this finding. For intestinal smooth muscle a value of b = 0.06 has been proposed by Goodford (1968) to account for the difference between V_K and the observed range of V_m of 40-70 mV.

Assuming that V_K defines the resting membrane potential, equation (1) predicts an inverse relationship between the membrane

potential and the external K concentration. (Strictly, ionic activities should be used not concentrations). As in nerve and skeletal muscle, the deviation of the slope of this relationship from the theoretical 58 mV per tenfold change in K_o, observed in smooth muscle probably indicates some degree of permeability to other ions. In general for smooth muscles the relationship is linear for higher external K concentrations but deviates at lower external K concentrations. A slope of 26 mV per tenfold change of K concentration has been reported by Burnstock and Straub (1958) in guinea-pig taenia coli; Holman (1958) using the sucrose gap technique and Kuriyama (1963) using microelectrodes reported a slope of 30-38 mV per tenfold change of K concentration in the presence of chloride in the bathing medium. Kuriyama (1963) also demonstrated that in taenia coli the slope of the line relating the membrane potential to the K-equilibrium potential was dependent upon the anion used. In the presence of foreign anions and excess K the maximum change of membrane potential per tenfold change in K_o was 49 mV in sulphate, 47 mV in ethanesulphonate, but only 15 mV in nitrate containing Krebs solution.

In attempting to relate the distribution of ions across the cell membrane to the observed membrane potential several problems occur. The first of these concerns the calculated values for the intracellular concentration of Na and K which are in turn used for an estimate of the equilibrium or resting potentials. Intracellular concentrations can be calculated from whole tissue electrolyte analysis, after subtracting contributions of bound ions and partitioning into

intra and extracellular content by means of extracellular space

measurements.   Estimates of the extracellular space made by

determining the volume of distribution of a marker substance (e.g.

inulin) which either does not enter cells or does so at a rate much

slower than that at which it penetrates extracellular fluid depend

upon certain assumptions.   First, it is assumed that the concentration

of marker substance in the extracellular space is the same as in the

incubating medium.   That the marker substance occupies the whole

volume of the solution located extracellularly and that this substance

is not bound by any constituent of the extracellular fluid or

chemically modified by the extracellular fluid.   Analytical methods

for extracellular space determination also depend upon the size of the

molecular species and upon the electrical charge on the substance used

as a marker.   In general large extracellular markers measure a small

extracellular compartment and vice versa (Goodford 1968).   Radioactive

tracers including ^{14}C-inulin, ^{35}S-ethanesulphonate, ^{14}C-sucrose,

^{131}I-serum albumin, have also been employed by many workers and

provide similar values to analytical methods for the extracellular

fluid (see Goodford 1968, Schoffeniels 1967, for references).

Electron microscopy has been used by some workers for

extracellular space measurements.   The technique involves cutting

and weighing of the actual micrograph and necessitates the selection

of a large random sample of micrographs in order to be sure that all

parts of the tissue are adequately represented.   During fixation

physical changes in the tissue structure are likely to occur and may

thus influence the extracellular space estimate. Some typical values for the extracellular space of various smooth muscles and the methods employed are summarised in Table I.

Calculation of intracellular concentrations of ions can be achieved by the use of the following formula after the determination of the extracellular space (Daniel and Robinson 1960).

$$X_t = X_{bd} + X_e V_e + X_i V_i \quad \cdots \quad \cdots \quad \cdots \quad \cdots \quad \cdots \quad \cdots \quad \cdots \quad (4)$$

Where X_t = tissue content of ion (m equiv/kg)

X_{bd} = content of ion chemically bound (m equiv/kg)

X_e = extracellular concentration of ion (m equiv/l)

X_i = intracellular concentration of ion (m equiv/l)

V_e = volume of extracellular fluid (l/kg tissue)

V_i = volume of intracellular fluid (l/kg tissue)

Account must be taken of ion binding during this calculation as there is increasing evidence that quantities of cellular electrolytes are bound in skeletal muscle (Wilde 1962; McLaughlin and Hinke 1966) kidney and brain (Cope 1967) and smooth muscle (Daniel and Daniel 1957)

Equation (4) involves the determination of volume of the intracellular fluid which can be obtained from the difference between the water content of the tissue and the extracellular space provided a correction is made assuming the density of intracellular fluid to be the same as that in the bathing medium (Goodford 1968). Calculation of membrane potentials using equation (1) assumes a passive distribution of chloride ion. Assuming an accurate estimate of extracellular space has been made, calculated values of intracellular

TABLE I

Extracellular space values and methods used for various smooth muscles

Tissue	Method	Space ml/Kg	Reference
Cat ileum	Inulin	101 ± 1.7	Barr (1959)
Cat small intestine	Electron Microscopy	125	Prosser et al (1960)
Rat Uterus Pregnant	Inulin	365-376	Casteels and Kuriyama (1965)
	Ethanesulphonate	557-570	
Rabbit Uterus Estrogen	Inulin	390	Kao and Siegman (1963)
Guinea-pig ileum longitudinal muscle	Inulin	380 ± 7	
	Sucrose	379 ± 10	Weiss (1966)
	Mannitol	382 ± 8	
Toad Stomach Muscle	Albumin ^{131}I	286	Burnstock et al (1963)

chloride content in guinea-pig taenia coli (Casteels 1965; Goodford 1964; Goodford 1968) and in rabbit myometrium (Kao and Nishiyama 1964) are not compatible with passive distribution of chloride. Casteels (1965) has postulated an active chloride pump in taenia coli to account for the relatively high intracellular chloride content. At the present time there is insufficient data concerning chloride movements in smooth muscle to be able to predict the contribution of this ion to the resting membrane potential.

From the above brief discussion of extracellular space measurements and calculated intracellular ion content and their use

in determining the membrane potential are fraught with many problems. According to Goodford (1968) four regional terms may be applied to the distribution of electrolytes in smooth muscle: these are (1) extracellular (2) membrane (3) intracellular (4) sequestered.

. Ib Uterine Membrane Potentials.

Considerable data is currently available concerning the values of resting membrane potential of uteri from a variety of preparations (Kao 1967; Kuriyama 1961a). Resting membrane potentials between 40-60 mV (inside negative) have been reported for mouse (Kuriyama 1961b) rat (Marshall 1959; Casteels and Kuriyama 1965; Kuriyama and Csapo 1961; Marshall 1962; Marshall and Miller 1964; Csapo and Kuriyama 1963) and rabbit (Goto and Csapo 1959; Kao and Nishiyama 1964; Kuriyama and Csapo 1961). Membrane potential changes occur during pregnancy and under differing hormonal regimes. For example, Casteels and Kuriyama (1965) demonstrated a gradual increase in membrane potential from 42-61 mV at the fifteenth to sixteenth day of a twenty-two day pregnancy cycle in the rat. During the last week of pregnancy there was a decline, a value of 55 mV being recorded just before parturition. Post-partum the potential fell to a value found in non-pregnant tissue. Several workers have concluded that these changes are probably due to permeability changes during pregnancy and not due to changes in intracellular concentrations of Na,

K or Cl (Casteels and Kuriyama 1965; Kao 1961; Kao and Siegman 1963).
Other differences in recorded values of membrane potential are to be
found according to the area of penetration of myometrial cells.
Penetrations from areas overlying placenta gave higher values than
interplacental areas (Thiersch et al 1959; Goto and Csapo 1959).

Both estrogen (Marshall 1959; Marshall 1963; Kuriyama 1961;
Csapo and Kuriyama 1963; Casteels and Kuriyama 1965; Jung 1964)
and progesterone (Marshall 1959; Goto and Csapo 1959) pretreatment
have been reported to increase the resting membrane potential in
myometrium. Kao (1967) has pointed out that during estrogenic
stimulation the increase in size of the myometrial cells may facilitate
microelectrode penetration resulting in a higher average value of
resting potential. Kao (1967) also dismisses the elevation of membrane
potential due to progesterone administration as being an artifact of
technique and concludes that there is no difference between the resting
potentials of the estrogen-dominated and progesterone-dominated
non-pregnant rabbit myometrium.

Electrolyte determinations in several different myometrial
preparations have demonstrated considerable variability (Kao and
Siegman 1963; Daniel 1958; Horvath 1954; Casteels and Kuriyama
1965). Apart from species differences there are differences
associated with hormonal dominance of the tissue and probably more
important with conditions of handling before analysis (Goodford 1968).
Kao (1967) has obtained a value of 100mEq/kg (wet weight) for total K
content and 75mEq/kg for Na. These values were obtained without
preincubation of the tissue and represent values some 20-30mEq/kg

greater for K and 10-30mEq/kg less for Na than after preincubation for
1 hour. The calculated K-equilibrium potential using an extracellular
space of 312 ml/kg as determined with inulin (Kao 1967) is 90 mV
(inside negative). Although an extreme case this example again
demonstrates the discrepancy between the measured membrane potential
(approximately 50 mV) and the K-equilibrium potential in myometrium.
Estimations of extracellular space are by no means consistent and
depend upon many factors including the marker employed (see Discussion).
Values in the rat may range from 557 ml/kg (ethanesulphonate) to
376 ml/kg (inulin) in the 19-20 day pregnant rat (Casteels and Kuriyama
1965). Calculations of intracellular concentrations will further be
biased according to the amount of bound ions per kilogram of wet tissue.
The amount of bound ions have been estimated for estrogen-dominated
rabbit myometrium to be as follows:-

Na   29;   K   14;   Cl   1;   mEq/kg wet weight (Kao 1961).

Using several corrections in order to reduce intracellular
chloride, Kao and Nishiyama (1964) reported a discrepancy between
the chloride equilibrium potential and the resting potential in
rabbit myometrium which these authors concluded was evidence for
active transport of chloride. At the present time the problem of
active chloride transport in rat myometrium has not been investigated.

The slope of the line relating the resting potential to .
the external K concentration has been shown by many workers to be
less than the theoretical 58 mV in myometrium (Marshall 1962; Goto
and Csapo 1959; Csapo and Kuriyama 1963; Casteels and Kuriyama
1965). Assuming that an increase in Na permeability is the cause

of this discrepancy, values have been obtained for the ratio b (P_{Na}/P_K)

in equation (2) of 0.11 for estrogen-dominated rabbit myometrium and

0.17 for progesterone-dominated tissue (Kao and Nishiyama 1964). For

rat myometrium Casteels and Kuriyama (1965) estimated the value to be

0.1. Kuriyama (1968) has concluded that the membrane potential in

smooth muscle is probably determined by the K and chloride equilibrium

potential and to some degree by the Na equilibrium potential.

Ic The Sodium Pump: Electrogenic or Neutral ?

In recent years the stoichiometric relationship of Na and

K movement has received considerable attention. Although it is well

documented that living cells can actively extrude Na and accumulate

K under various conditions, the ratio of Na extruded to K gained

has not been established for many tissues.

Some of the conditions pertinent to the operation of either

an electrogenic or neutral Na pump have been summarized by Straub

(1967) and are shown in Table II. An electrogenic Na pump may be

defined in terms of its ability to separate charge across the membrane

by the movement of a positive charge (Na) out of the cell not

compensated for by the inward movement of a cation or the outward

movement of an anion. On the other hand an electrically neutral

pump would involve the tight coupling of Na extrusion to K uptake in

a 1:1 manner, not involving the separation of charge across the

membrane.

Evidence for the existence of electrogenic Na pumps has

been obtained for several tissues (Izquierdo and Izquierdo 1967).

In frog sartorius muscle Kernan (1962) found that during net extrusion

of Na from Na-rich muscles V_m exceeded V_K by about 12 mV. Frumento (1965)

TABLE II

Properties of Neutral or Electrogenic Na-Pumps.

PUMP CHARACTERISTICS

ELECTRICAL:	NEUTRAL	ELECTROGENIC	
CHEMICAL:	FULLY COUPLED	PARTIALLY COUPLED	UNCOUPLED

(A) EQUILIBRIUM CONDITIONS

$$\frac{M^o_{Na}}{M^i_K} = q = \qquad 1 \qquad\qquad >1 \qquad\qquad \infty$$

$$V_m = \frac{-RT}{zF} \ln x \; \frac{P_K K_i + P_{Na} Na_i}{P_K K_o + P_{Na} K_o} \qquad \frac{qP_K K_i + P_{Na} Na_i}{qP_K K_o + P_{Na} K_o} \qquad \frac{K_i}{K_o}$$

(B) NET Na EFFLUX

$$\frac{V_K}{V_m} \qquad\qquad >1 \qquad\qquad \lessgtr 1 \qquad\qquad <1$$

M^o_{Na} and M^i_K are active fluxes.

also reported a rapid increase in the membrane potential in frog skeletal muscle upon rewarming a Na-rich tissue which could not be accounted for in terms of the measured tissue K content. Similar studies by Keynes and Rybova (1963) and later Cross et al (1965) confirmed these findings and demonstrated that when Na-extrusion was blocked with ouabain V_m and V_K became almost identical. Mullins and Noda (1963) suggested that if there was a linkage between the Na and K movements the coupling ratio could not be higher than one K ion pumped in for each three Na pumped out; thus implying an electrogenic mechanism.

In view of the fact that Adrian (1964) had shown that the permeability of muscle fibres was much less to Rb than to K, Adrian and Slayman (1966) measured the membrane potential during recovery of Na-rich muscles in the presence of Rb. The recorded membrane potentials were 10-20 mV more negative than during extrusion of Na in the presence of K, but this increase in membrane potential could not account for all the inward Rb movement leading the authors to conclude that about 90% of the inward movement of Rb was coupled to the efflux of Na. An increased resistance to K movement may be produced by the addition of local anesthetic or antihistamines (Shanes 1950). Using procaine, cocaine, amytal and mepyramine Harris and Ochs (1966) demonstrated an increased membrane potential during Na-extrusion, a finding to be predicted if the inward movement of K was passive in response to the potential generated by Na-extrusion.

For red blood cells (Whittam 1964) the evidence points in favour of coupled movements of Na and K across the cell membrane although agreement has not always been reached on the ratio of Na to

K moved by the pump.   (Harris 1954;   Post and Jolly 1957;   Tosteson and Hoffman 1960;   Garrahan and Glynn 1967).   There is evidence for a system transporting two K ions into the cell for every three Na extruded (Post et al 1967).   Hodgkin and Keynes (1955) working with squid axons concluded that Na efflux and K influx were coupled but that the linkage was not rigid;   further experiments by these authors (Hodgkin and Keynes 1956) showed that a hyperpolarising response could be elicited by the microinjection of Na ions into squid giant axons.   More recently Kerkut and Thomas (1965) and Chiarandini and Stefani (1957) have applied a similar technique to snail neurones and observed a large hyperpolarisation in response to injected Na ions.   Thomas (1969) has also shown that the injection of similar quantities of lithium (Li) or K did not cause hyperpolarisation and that the response was blocked by removal of external K or the application of ouabain.   In conclusion, Thomas (1969) states that the reason for the electrogenic effect of the pump is that the extrusion of every three Na ions is directly coupled with the uptake of only two K ions and that the stoichiometry of the pump in snail neurones is the same as in red blood cells. Changes in membrane potential following a change in pO_2 in the bathing medium surrounding snail neurones have been interpreted by Kerkut and York (1969) to reflect the activity of the electrogenic pump stimulated by the intracellular injection of Na ions.   Similarly, changes in oxygen consumption have been reported by Rang and Ritchie (1968c) to reflect electrogenic pump activity in mammalian non-myelinated nerve fibres in the presence of external K.   The post-tetanic hyperpolarisation observed in mammalian non-myelinated nerves has been attributed to the

activity of an electrogenic Na-pump by Rang and Ritchie (1968a). The

pump activity was inhibited by ouabain and Li and activated by K,

thallium, Rb, cesium (Cs) and ammonium.   These authors also showed

(Rang and Ritchie 1968a)that under certain conditions the linkage

ratio for Na and K could vary, a finding not confirmed by Thomas (1969)

in snail neurones.

Hyperpolarisation of cardiac muscle to a maximum of

267.7 mV has been reported to be due to the activity of an

electrogenic Na-pump in cat ventricle by Tamai and Kagiyama (1968).

The magnitude of the hyperpolarisation, was claimed by these authors,

to be dependent upon the duration of hypothermia before rewarming.

The existence of an electrogenic pump in this tissue had been shown

earlier by Page and Storm (1965) when they reported a discrepancy

between the calculated K equilibrium potential and the observed

membrane potential during rewarming of muscles made Na-rich by 2

hours exposure to Ringer's solution at 2-3°C.

Changes in membrane potential produced by increasing the

temperature have been noted in lobster axons (Senft 1967).   The

potential changes recorded were about twice that predicted if the

axon behaved as a K-ion electrode.   Metabolic inhibition reduced

the temperature dependence to that expected for a K-ion electrode

and led Senft to conclude that the potential changes with temperature

were due to the activity of an electrogenic Na-pump.   In Aplysia

neurones a 50% increase in the membrane potential upon increasing the

temperature from 3° to 25° was attributed to electrogenic Na-pump

activity which was inhibited by ouabain, Li and required external K

(Carpenter and Alving 1968). Cell bodies and synaptic processes in leech central nervous sysrem have been shown by Nicholls and Bayler (1968) to hyperpolarise due to electrogenic Na-pump activity after stimulation; similar observations have been made in crutacean stretch receptors (Nakajima and Takahashi 1966).

For many tissues there is now evidence that Cs as well as Rb is able to substitute for K in promoting Na efflux from resting and Na-enriched cells (Hoffman 1966; Sjodin and Beaugé 1967a, b; Beaugé and Sjodin 1968; Sjodin and Beaugé 1968). In frog skeletal muscle Beauge and Sjodin (1968) showed that the amount of Cs entering the fibres was dependent upon the intracellular Na concentration and that the inward movement of Cs was to a large extent chemically coupled to the outward movement of Na. The hyperpolarisation observed by these workers during recovery of Na-rich muscles in Cs-containing media does suggest some imbalance between the movements of Na and Cs and an electrogenic component may therefore exist during the early recovery phase of Na-extrusion.

On the basis of the above studies it is possible to summarise some of the conditions associated with, and the properties of, electrogenic pumps from a variety of tissues. It is clear from the many reports mentioned above that electrogenic pump activity has been observed most readily during net Na-extrusion. In fresh tissues, attempts to observe electrogenic pump activity are not usually as successful because the rate of Na movement is not as large as in Na-rich tissues recovering from the Na-rich state.

In summary:-

1. An electrogenic pump potential is most readily demonstrated when the cell is not in a steady state:-

e.g.
(i) After intracellular injection of Na

(ii) Following a period of tetanic activity

(iii) Increases in temperature following hypothermia

(iv) Rewarming of Na-loaded cells. (Loaded in normal or K-free medium).

(v) Following the application of certain metabolic inhibitors (Horowicz and Gerber 1965a, b).

2. Electrogenic pump activity demonstrates an absolute requirement for one of the following cations in the bathing medium during Na extrusion:-

K, Rb, Cs, Li, Thallium, Ammonium.

3. The intracellular potentials recorded during extrusion of Na exceed the calculated value of the K-equilibrium potential.

4. The differences between V_m and V_K are generally temperature sensitive and abolished by ouabain.

5. Drugs known to increase membrane resistance to K movement give rise to an increased membrane potential during net Na extrusion.

6.   The presence of Li ions intracellularly quickly reduces the membrane potential generated by the pump (Keynes and Swan 1959; Ritchie and Straub 1957).

7.   The observed hyperpolarisation is independent of chloride in the bathing medium.

8.   Changes in pO_2 or oxygen consumption follow closely changes in Na-induced hyperpolarisation and may reflect activity of an electrogenic pump.

Id The Effect of catecholamines on the electrophysiology of smooth muscle.

Many catecholamines activate both alpha (α) and beta (β) receptors (Ahlquist 1966; Ahlquist and Levy 1959) in a variety of tissues. In most tissues studied both α and β receptors are present and although non-specific properties may be ascribed to most blocking agents a selective action on one receptor can be sought by the use of a selective blocking agent for the other receptor (Furchgott 1964). In many instances such differentiation of receptors has not been attempted and the experimental results must be attributed to stimulation of both types of receptor.

In general stimulation of   receptors in smooth muscles causes a depolarisation and action potentials when concomitant actions on β receptors are inhibited. In the presence of α blocking agents   receptor activation is generally accompanied by relaxation and hyperpolarisation (Schatzmann 1968). Intestinal smooth muscles provides an important exception to these generalisations

for relaxation and hyperpolarisation have been reported after stimulation of either α or β adrenergic amines (see Daniel et al 1969c for references; Daniel 1968; Burnstock and Holman 1966). Increased permeability to K occurs in intestinal smooth muscle in response to α adrenergic stimulation (Bülbring et al 1966; Jenkinson and Morton 1965; Jenkinson and Morton 1967a,b,c; Setekleiv 1966). Bülbring and Tomita (1967) observed a decreased resistance in response to α stimulation which was potentiated by high K. Replacement of chloride with a non-penetrating anion (ethanesulphonate) reduced the lowering of the membrane resistance, suggesting an involvement of chloride in this response. The reduction in barium induced plateau-type action potentials in taenia-coli has been attributed by Bülbring and Tomita (1968) to an increase in K permeability. Prolonged exposure to calcium-free medium abolishes this response which suggests that calcium may be necessary for this action of epinephrine.

Inhibition, produced by β adrenergic amines on intestinal smooth muscle, has not been conclusively attributed to changes in electrolyte contents or fluxes (see Daniel et al 1969c for references). Recent studies by Bülbring and Tomita (1967) have suggested that inhibition by β stimulation may involve increased calcium binding. Calcium flux studies by Schatzmann (1964) showed that epinephrine did not alter ^{45}Ca uptake in taenia-coli and Nagasawa (1965) found the rate of ^{45}Ca efflux was greatly increased by epinephrine. The activation of an electrogenic Na-pump by epinephrine has been postulated by Bülbring (1962) to account for the hyperpolarisation in

intestinal smooth muscle. As this effect was observed in K-free solutions it seems unlikely that epinephrine is causing hyperpolarisation through activity of an electrogenic pump which, if similar to other electrogenic pumps,would show an absolute requirement for external K, Rb, Cs, etc. (see Section Ic). It is possible that Na pump activity was maintained in K-free solutions by a small leak of intracellular K, and that the action of epinephrine was to cause further stimulation of the Na pump at a time when Na pumping was not maximal. Epinephrine has also been reported by Bülbring (1953) to have a biphasic action on oxygen consumption. During induced relaxation the oxygen consumption was lowered to about 20% of control and subsequently increased by about 50%-100% of control. In view of the demonstrated increase in oxygen consumption during electrogenic Na pumping (Kerkut and York 1969) the time course of the hyperpolarisation observed becomes important. Hyperpolarisation occurring after an initial delay of 10 minutes may well result from electrogenic Na pumping although it is difficult to reconcile the early decrease in oxygen consumption with hyperpolarisation occurring immediately upon the addition of epinephrine as shown by Bülbring and Kuriyama (1963).

Catecholamine induced inhibition of membrane potential changes associated with contractions have also been demonstrated on various intestinal muscles of the dog by Daniel and Irwin (1968).

α receptor stimulation of uteri from several species usually produces depolarisation, action potentials and contraction after β receptor blockade (Bülbring et al 1966; Diamond and Brody 1966; Marshall 1967; Paton 1968; Tothill 1967). Inhibition of

spontaneous contractions and subsequent hyperpolarisation has been reported by Marshall (1967, 1968) to be the response to β adrenergic stimulation. The induced hyperpolarisation was not abolished by Li substituted Na-free Krebs solution, a finding which prompted Marshall to reject increased Na pumping as a cause of the hyperpolarisation. This argument is based on the assumption that Li cannot substitute for Na in maintaining the Na pump.

Subsequent experiments reported in this thesis and elsewhere (Daniel et al 1969c)will provide more information concerning catecholamine actions in pregnant rat myometrium.

β adrenergic stimulation, of single unit (Somlyo and Somlyo 1968) vascular smooth muscle, has been reported to produce depolarisation of rat portal vein (Johansson et al 1967) and hyperpolarisation in rabbit portal vein (Holman et al 1968). Somlyo and Somlyo (1968) have suggested that the different results may reflect different coupling ratios of Na and K movements due to different concentrations of K in the bathing medium, or may be a result of species differences. α receptor stimulation of spike-generating vascular smooth muscle has also been reported to cause depolarisation in a variety of vascular smooth muscle preparations (see Somlyo and Somlyo 1968 for references).

A recent review (Daniel et al 1969c) of catecholamine actions on ion fluxes, electrophysiology and contractility, which includes a portion of the work included in this thesis, concluded the following:-

1. α receptor stimulation causes increased K fluxes,

probably by increasing membrane permeability, in a variety of, but not all, tissues. Increased K influx may occur as the result of hyperpolarisation in some tissues.

2. β receptor stimulation produces inconsistent changes in K fluxes. Present available evidence does not permit any general conclusions to be made concerning the effects on Na efflux or influx. β activated hyperpolarisation may occur as a result of increased Na efflux or decreased Na influx (reduction in Na permeability).

le <u>The objectives of the Thesis</u>.

There have been numerous studies on the properties of the membrane potential of smooth muscles. Many of these studies have investigated the properties of the membrane potential of fresh tissues during drug application or changes in the ionic environment bathing the tissue. An exceedingly useful tool, that of Na-enrichment, has been successfully applied to a variety of tissues in order to obtain information of certain cell parameters e.g. membrane potential, permeability, during the re-establishment of equilibrium conditions within the cell. Na-rich tissues are prepared by an overnight immersion at $4^\circ C$ in cold K-free Krebs bicarbonate medium. During this treatment the tissue may lose 50-60 mEq of K and gain a corresponding quantity of Na and show an increase in water content. Upon rewarming such tissues to $37^\circ C$ in the presence of K for an hour about half the gained Na will be extruded and a similar quantity of K gained and a loss of water will be observed. Daniel and Robinson (1960a, b, 1963a, b, 1969a, b, c) have applied this technique to the

movements of Na and K in uterine smooth muscle; much information has been obtained about ion movements in smooth muscle although complimentary electrical studies have not been carried out.

As discussed earlier (Section Ic) electrogenic pump activity may be most readily defined during conditions of net Na-extrusion. In view of the fact that Na-extrusion can be stimulated upon rewarming ·Na-rich rat myometrium in the presence of K it was of interest to ask whether the Na-pump in this tissue was electrogenic or neutral in operation during recovery from the Na-rich state. Potentiation of hyperpolarisation associated with electrogenic pumping of Na has been reported in response to modifications of the recovery medium. It would be interesting to know if modifications in recovery media could increase or decrease the magnitude of the Na-pump generated hyperpolarisation in smooth muscle.

In view of the postulated action of certain catecholamines on electrogenic Na-pumping, it would be of interest to observe the effects of catecholamines on the membrane potential during conditions of either maximal or minimal Na-pumping. Continuous recordings from the same cell before and after the application of adrenergic amines may provide more positive proof of membrane potential changes. Specifically, this thesis will attempt to answer the following questions:-

1. What are the changes in membrane potential accompanying Na-extrusion in Na-rich rat uteri ?

2. What factors control the membrane potential of
(a) Na-rich tissues during recovery from the Na-rich state and

(b) fresh tissues?

3. If electrogenic Na-pumping occurs during the recovery of Na-rich tissues, what are the properties of this pump ?

4. If electrogenic Na-pumping occurs during the recovery of Na-rich tissues can α or β adrenergic agonists influence the activity of the pump ?

II   METHODS

II   METHODS

IIa   Tissue preparation.

Non-pregnant Wistar rats, weighing 100-150 g, were injected subcutaneously for 5 days with diethylstiboestrol, 50 ug per day, and killed by a blow on the head.   The uterine horns were then removed, dissected free from surrounding tissues and opened along their mesenteric borders.

Pregnant rats (Wistar strain) were killed as near term as possible (about 20 days pregnant) and, after removal of foetuses and placenta, a segment of the uterus (70 mm x 30 mm) was mounted serosal surface uppermost in an organ bath as shown in Fig.1.   Where non-pregnant uteri were used the tissue was mounted in a similar manner.   Samples of post-partum uteri were never used.

Uteri from either pregnant or non-pregnant rats were incubated in K-free Krebs solution (see Table III - Solutions and Drugs, section IIg) for 18-24 hours at $4^{o}C$ in the absence of oxygen, to render them "Sodium-Rich" (Na-Rich).   During recovery from cold storage all tissues were gassed with a mixture of 95% O_2: 5% CO_2 and maintained at $37^{o}C$ unless otherwise stated in the text.

IIb   Recording of mechanical and electrical activity.

Fresh or Na-rich tissues were mounted on a neoprene block by means of two pins at one end and two silk ties at the other end attached to a small plastic lever (see Fig.1).   Tension changes were monitored via a fulcrum from the horizontally mounted piece of tissue
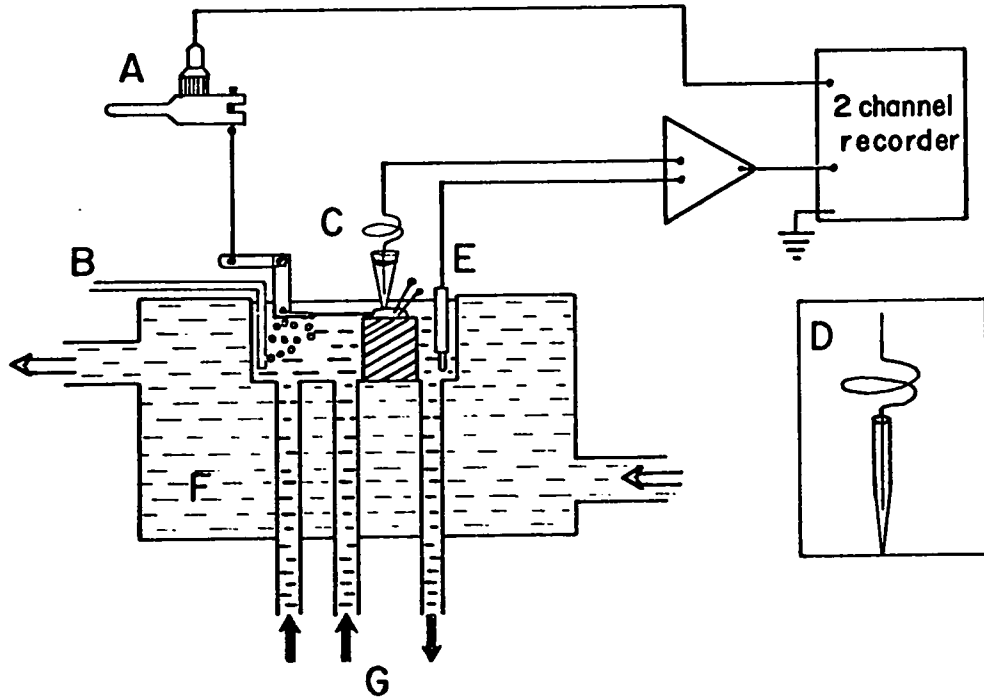
Figure 1. Schematic diagram of apparatus for recording electrical and mechanical activity of rat uterus. A, Grass force displacement transducer; B, Inlet for gassing medium with 95% O_2 and 5% CO_2; C, microelectrode suspension; D, enlargement of microelectrode suspension showing glass microelectrode suspended on a platinum wire coil; E, Ag:AgCl Reference electrode; F,constant temperature water jacket surrounding organ bath; G, Inlet for introducing solutions to the organ bath, The tissue was mounted on a neoprene block (shown cross-hatched) in the organ bath: Mechanical activity was recorded by means of silk ties and a plastic lever connected to a Grass Force displacement transducer (A) and thence to a 2 channel Grass polygraph; electrical activity was recorded from a glass microelectrode (C) on a 2 channel Grass polygraph.

by means of a Grass force displacement transducer (Model FT.03C)
connected to a Grass Polygraph (Model 5A). A resting tension of
0.5-1 gm. was applied to all tissues.

Membrane potentials were measured using glass capillary
microelectrodes filled with 3M KCl and of 50-80 MΩ resistance.
The microelectrodes were prepared from glass tubing (Corning; 7740
tubing,1.18 mM O.D.x 0.44 mm I.D.) and drawn into fine pipettes with
a commercial microelectrode puller (Industrial Science Associates).
After filling with 50:50 v/v water:methanol mixture under reduced
pressure the electrodes were left to stand overnight in 3M KCl to fill
by diffusion. Microelectrodes prepared in this way were used on the
day after preparation. In most instances microelectrodes stored for
longer than one day broke very easily upon attempting penetration of
uterine cells.

The microelectrode suspension consisted of 0.002" diameter
platinum wire formed into a helix of 1 cm diameter and approximately
$1\frac{1}{2}$ revolutions. Such a suspension had flexibility and would often
allow a microelectrode tip to remain within a cell during contractions
of the whole tissue. Membrane potentials were relayed from the
microelectrode to a high input impedance probe head connected to a
negative capacitance electrometer (Model A-35, Medistor Instrument
Co.) and displayed on a Grass Polygraph (Model 5A). A Ag:AgCl
reference electrode completed the circuit.

Tip potentials were measured by breaking the electrode after
a series of penetrations; data from electrodes whose tip potential
was greater than 5 mV was rejected. In some experiments thorium

chloride (1 μM) was added to the organ bath to abolish tip potentials (Agin and Holtzman 1966). No correction was made for differences in junction potentials between microelectrode and intracellular fluid. Any small changes in zero baseline of potential that occurred when the bathing medium was changed were zeroed out before penetrations were made.

Recorded membrane potentials were accepted if they fulfilled the following criteria: (a) were produced by a sharp negative deflection upon penetration (b) remained stable for at least 4 secs. and (c) returned to the original baseline upon withdrawal from the cell. Membrane potential measurements in the body of the text are expressed without a negative sign, for example 50 mV refers to a membrane potential of -50 mV (inside negative). Hyperpolarisation is referred to as an increase in potential; depolarisation is referred to as a decrease in potential. In all membrane potential recordings illustrated the top of the vertical calibration bar represents zero potential. In certain instances membrane potential records are shown for one experiment; such data were not included in this thesis unless similar changes were observed in at least 3 more identical experiments.

IIc Ion content determination.

50-100 mg samples of whole uterine tissue were used for ion content analysis and were maintained under similar tension to the tissues used for membrane potential measurements. Samples were removed from the organ bath, blotted carefully, weighed, dried in an oven at 105°C for 48 hours and reweighed. The samples were then digested in 0.2 ml concentrated Nitric acid and 0.1 ml hydrogen peroxide at 200°C and dried to a white powder. The residue was

dissolved in 25 ml distilled water and the ion content determined using

an Eel Flame Photometer (Evans Electroselenium) by comparison of the

emission of the unknown sample to that of a standard curve constructed

using appropriate proportions of known amounts of Na and K.   A new

standard curve was prepared for each set of samples analysed.   Ion

content was expressed as mmoles/kg wet weight of tissue.   In the

latter stages of this work ion content and water content were calculated

by the use of an Olivetti Underwood Programma 101 Desk computer.

Intracellular ion content was calculated using the method of Daniel

and Robinson (1960) (see Section Ia).

IId Determination of extracellular space with ^{14}C-inulin.

Extracellular space measurements were made on pregnant rat

uterus prepared in two ways.   Tissues were either taken fresh from

the animal and incubated in normal Krebs for 30 min. at $37^{\circ}C$, or

Na-rich tissues were used that had been incubated at $37^{\circ}C$ in normal

Krebs for 40 min.   Tissues pretreated as described above were

subsequently treated identically for determination of the extracellular

space.   Two samples of tissue, weighing between 50 mg-100 mg, were

incubated on stainless steel hooks in centrifuge tubes in 5 ml normal

Krebs medium containing ^{14}C-inulin (0.05 μC/ml:33.6 μg/ml).   The media

was gassed with a mixture of 95% O_2: 5% CO_2 during the uptake of

tracer inulin.   Samples were incubated in each of 6 centrifuge tubes

for 10, 20, 30, 60, 120, 240 min. respectively; after which the

tissues were rapidly removed, rinsed for one to two seconds in

isotope-free Krebs, and blotted gently.   The tissues were then

weighed and each tissue was then digested in a scintillation vial

using NCS solubuliser (Amersham-Searle), 1 ml of solubuliser per 100 mg

of tissue, at $37^{\circ}C$ for 18 hours   To each vial 15 ml Bray's phosphor

(Bray 1960) was then added and total ^{14}C-counted in a Picker-Nuclear

'Liquimat' scintillation spectrometer (Model 650-513) that had a

practical counting efficiency for ^{14}C of over 90%.   Duplicate 1.0 ml

aliquots of the media were counted for ^{14}C (as described for the

tissues above).   In all cases, vials were counted for at least 10 min.

each and corrected for quenching, using the channels ratio method, and

for tissue size and background.

   ^{14}C-inulin uptake was expressed as ml per 100 gm tissue using

the following equation:-

$$dpm/100 \text{ gm tissue} \div dpm/ml \text{ incubation medium}$$

IIe **Uptake of Cesium (^{137}Cs) into rat uterus.**

   Small samples (50-100 mg) of Na-rich pregnant uterine tissue

were suspended by means of stainless steel hooks in a K-free Krebs

solution for 30 min. before transfer to tracer uptake medium.   The

uptake media consisted of K-free Cs-substituted Krebs to which was

added either 4.6 or 46 mM CsCl containing $^{137}CsCl$ (5×10^{-5} mM).

All uptake media were maintained at $37^{\circ}C$ and gassed with 95% O_2: 5% CO_2.

At the end of an uptake period the tissue samples were removed from

the tracer solution, dipped rapidly as described above in non-radioactive

uptake media and carefully blotted dry.   After weighing the samples

were counted in a Well Type Scintillation Detector (Picker X-Ray

Engineering Ltd., Canada) in conjunction with an all transistorised

Spectroscalar III using a 240 Kev window across a 661 Kev peak.

Each sample was counted for at least 10 min. or 200,000 counts,

whichever was completed first.   Background samples were estimated

using at least 5, 10 ml samples of non-radioactive uptake media, and

samples of tissue that had not been exposed to ^{137}Cs (tissue blanks)

were counted with every experiment.   Cs uptake was expressed as:-

$$\frac{cpm/kg\ tissue}{cpm/mmoles\ media} = mmoles/kg\ wet\ weight$$

During Cs uptake the concomitant loss of Na was followed by Na

content analysis of the tissue samples after counting.

IIf Efflux of ^{137}Cs or ^{42}K from rat uterus.

All efflux experiments were performed on Na-rich tissues

that had been incubated for 90 min. in solutions containing either

^{137}Cs or ^{42}K according to the experimental design.   Single pieces

of Na-rich uterus weighing 50 mg-100 mg were suspended in 10 ml

uptake media by means of hollow stainless steel tubes which also

served to gas the tissues with a mixture of 95% O_2: 5% CO_2.   The

uptake media used for loading the tissue with ^{137}Cs consisted of

either, 5 ml 4.6 mM Cs-Krebs or 5 ml 46 mM Cs-Krebs and ^{137}CsCl

(about 4 µc/ml and 10^{-3} mM). ^{42}K was obtained as K_2CO_3 and the

powder neutralised with 0.1 N HCl and diluted further with deionised

water.   The total K of Krebs solution was adjusted to allow for the

addition of ^{42}K to the incubation media.   After 90 min. incubation

in uptake media the tissues were rinsed in isotonic choline chloride

and placed in a series of efflux tubes containing 10 ml tracer free

media.   The samples were then transferred along a series of tubes

after the following consecutive time periods:-

  2,5,10,15,25,35,50,60,90,120,150,165,180,210,240,300,360 min.

At the end of the efflux period the tissues were removed,

blotted gently and counted as described below.   A 2 ml sample from

each efflux tube containing ^{137}Cs was transferred to a counting vial

and the radioactivity present determined in a Picker Well Counter as

described above for ^{137}Cs uptake experiments (see section IIe).

The efflux of ^{42}K, from tissues loaded with isotope in an

analogous manner to that described for ^{137}Cs, was counted in a Picker

Well type Scintillation detector.   The spectroscaler III was set with

a lower level of 1.35 MeV with a 0.300 MeV window to reach an upper

level of 1.65 MeV.   The decay factor of 10 min.= 0.00932 was

incorporated into the computer programme (discussed below) to calculate

the radioactivity in the efflux tubes and the tissue at zero time.

Efflux data for ^{42}K and ^{137}Cs was expressed as efflux coefficients by

use of the following formula:-

The rate coefficient for the efflux of ^{137}Cs or ^{42}K was

defined as $\dfrac{\Delta C}{\Delta t.Cm}$ and has the units min^{-1}.   Where, ΔC is the amount

of radioactivity lost during the time period Δt, and Cm is the mean

of the ^{137}Cs or ^{42}K content of the tissue at times t and t + Δt.

Efflux data was analysed according to the method of Daniel

and Robinson (1969a) with the aid of an APL 360 computer programme

designed in conjunction with Dr. D.A. Cook (Cook and Taylor 1969).

The computer provided the following print out:-

(a) a graph of the logarithm (log) of the counts per minute

(c/m) remaining in the tissue, against time;

(b) an estimate of the bound or slowly releasable fraction, according to the method of Dick and Lea (1964). Briefly, this method consists of extrapolation of the arithmatic plot of rate of efflux against the corresponding c/m, to zero rate of efflux;

(c) the values of the efflux coefficients calculated according to the formula given above;

(d) a log-log plot of the rate of efflux (c/m^2), against the corresponding c/m of the efflux data.

A log-log plot of the rate of efflux against the corresponding c/m of the efflux data from 120-360 min. showed a slope of approximately one (see insert of Fig.34) indicating efflux from a single compartment (Keynes and Swan 1959; Persoff 1960). The effect of drugs or modified solutions on the efflux of ^{137}Cs or ^{42}K was observed 120-210 min. after the beginning of efflux.

IIg Solutions and Drugs.

Table III shows the composition in mM/Litre of Normal Krebs solution and K-free Krebs solution.

Krebs solutions containing varying concentrations of K were prepared by substitution of KCl for NaCl. For example, 9.2 mM K Krebs solution was prepared by the replacement of 4.6 mM NaCl in Normal Krebs solution with 4.6 mM KCl.

Solutions containing Rb or Cs were prepared by the substitution of CsCl or RbCl for KCl. In solutions containing varying amounts of KCl, RbCl or CsCl the concentration of $NaHCO_3$, NaH_2PO_4, $CaCl_2$, $MgSO_4$ and glucose was the same as shown in Table III.

TABLE III

Composition of Normal and K-free Krebs Solution

	Normal Krebs Solution mM/l	K-free Krebs Solution mM/l
NaCl	115.5	120.1
NaHCO$_3$	21.9	21.9
NaH$_2$PO$_4$	1.2	1.2
KCl	4.6	-
CaCl$_2$	2.5	2.5
MgSO$_4$	1.2	1.2
Glucose	49.2	49.2

Na-free solutions were prepared by the complete replacement of Na (138.5 mM) with sucrose (250.8 mM) or Li chloride (138.5 mM) and the addition of KHCO$_3$ (5.7 mM) for maintenance of a pH of 7.4. "Low-Na" solutions were prepared by replacing 31.6 mM sucrose in Na-free (sucrose) Krebs with 23 mM Na (present as 21.9 mM NaHCO$_3$ and 1.2 mM NaH$_2$PO$_4$), the K concentration of this solution being 4.6 mM.

Solutions containing low quantities of chloride (less than 10 mM as KCl and CaCl$_2$) were prepared by the replacement of the total Na content of normal Krebs with Sodium Methyl Sulphate (NaCH$_3$SO$_4$). A pH of 7.4 was maintained in this solution by the addition of TRIS (0.7 mM) buffer. A K-free NaCH$_3$SO$_4$ solution was prepared similarly with the omission of K.

All solutions above had initially a pH of 7.3-7.5, and were gassed with a mixture of 95% O_2: 5% CO_2 at 37°C.

Stock solutions of all drugs were prepared fresh before an experiment where catecholamines were used. Concentrations of drugs are expressed in terms of the free base.

Chromatographically pure inulin-carboxyl-^{14}C with a specific activity of 1.49 mC/g (M.W. 5000-5500) was obtained from New England Nuclear Corp.

^{137}CsCl (31.8 mC/mg) radiometic purity 99%, was obtained from New England Nuclear Corp.

^{42}K$_2$CO$_3$ was obtained from Atomic Energy of Canada.

The following drugs and chemicals were used and obtained from the sources indicated:-

Ouabain	Nutritional Biochemicals Corp;
d-l Isoproterenol HCl	Sigma Chemical Co;
l-Noradrenaline HCl	Sigma Chemical Co;
l-epinephrine bitartrate	Calbiochem;
Propranolol	Ayerst, McKenna & Harrison;
Phenoxybenamine HCl	Smith Kline and French;
Thorium Chloride, Rubidium Chloride, Cesium Chloride	Fisher Scientific Co.

IIh Statistical Methods.

The variability of samples is expressed as mean $\pm$ standard error. Significant differences between samples was established using Student's t test.

III RESULTS

III RESULTS

IIIA (a) <u>Electrical and mechanical activity of non-pregnant rat uterus.</u>

In view of the considerable data (see Daniel and Robinson 1969a,b,c for references) available concerning tracer ion movements in non-pregnant rat uterus preliminary experiments were made in order to determine the feasibility of obtaining data on electrical activity from single cells of rat myometrium under similar conditions.

Considerable difficulty was encountered when attempts were made to penetrate non-pregnant rat uterus with microelectrodes. Several factors appeared to be responsible for the difficulty in obtaining penetrations which met with the established criteria (see Methods). Although some penetrations were achieved the majority of these did not remain stable within a cell long enough to meet the established criteria despite variations in microelectrode size, and various forms of microelectrode suspensions. Experiments were also attempted using non-pregnant tissue after Na-enrichment. Although spontaneous contractions resumed after about 40 min. exposure to a K-containing Krebs solution changes in membrane potential were difficult to measure due to difficulty in obtaining stable penetrations.

Haffeman and Miller (1967) reported a method for enzymic softening of connective tissue in visceral ganglion cells of <u>Aplysia vaccaria</u> in order to aid penetration of these cells with microelectrodes. Briefly, this method consisted of immersing the

ganglia in a solution of collagenase and elastase for about 30 min. The electrical activity of the ganglia was apparently unaltered by enzymic treatment. This method was also attempted on non-pregnant rat uterus in an attempt to facilitate cell penetration. Incubation of fresh spontaneously active uteri in a solution, containing collagenase and elastase, for 30 min. abolished spontaneous mechanical activity and the membrane potential had decreased to an undetectable level. From these results it was concluded that treatment with these enzymes destroyed the cells and that this method could not be used to facilitate penetration of microelectrodes into non-pregnant rat uterus.

Experiments involving non-pregnant myometrium were terminated shortly after it was found that penetrations could be made quite easily into pregnant tissue using similar microelectrodes and the same form of microelectrode suspension that had failed to give results with non-pregnant tissue. All experiments reported therefore in this thesis were carried out on pregnant rat uterus.

IIIB (a) Ion content and electrical and mechanical activity of pregnant rat uterus.

One of the problems associated with the use of uterine tissue from pregnant rats was the difficulty of separation of endometrium and myometrium. Several different techniques were tried in order to achieve a good separation of the two types of tissue such that viable, undamaged myometrial strip could be used for electrophysiological experiments and ion content analysis. However, after numerous attempts at separation a satisfactory myometrial preparation was not obtained.

In view of this difficulty whole uterine samples were used for this study and mounted serosal surface uppermost to record electrical events occurring in the myometrium.

Typical spontaneous mechanical and electrical activity of 4 different pregnant rat uteri is shown in Fig.2. Rhythmical contractions recorded from the whole tissue are shown accompanied by volleys of action potentials recorded from a single cell. The mean membrane potential recorded from 162 penetrations in 12 such tissues was 50.5 ± 0.34 mV (mean $\pm$ SE). In Fig. 2B, action potentials are shown at a faster sweep speed. The irregularity of spontaneous spikes, sometimes recorded in normal tissues, is also shown in Fig. 2D.

Table IV shows the ion content of pregnant uteri a) immediately upon removal from the animal; b) after 40 min. incubation in normal Krebs solution and c) after overnight immersion in K-free Krebs solution at 4°C. Over a period of about 18 hours in K-free Krebs solution at 4°C fresh tissues lose K and gain Na.

IIIB (b) <u>Recovery of Na-rich pregnant uterus.</u>

After approximately 18 hours in K-free Krebs solution in the cold (4°C) and after re-warming to 37°C in K-free Krebs solution, Na-rich tissues had a low membrane potential 15.4 ± 0.6 mV (91 penetrations in 15 tissues) and were quiescent. Upon the addition of K to the bathing medium at 37°C a large rapid increase in the membrane potential to about 70 mV (22 penetrations in 8 tissues) was observed. The membrane potential recorded during this early recovery period exceeded
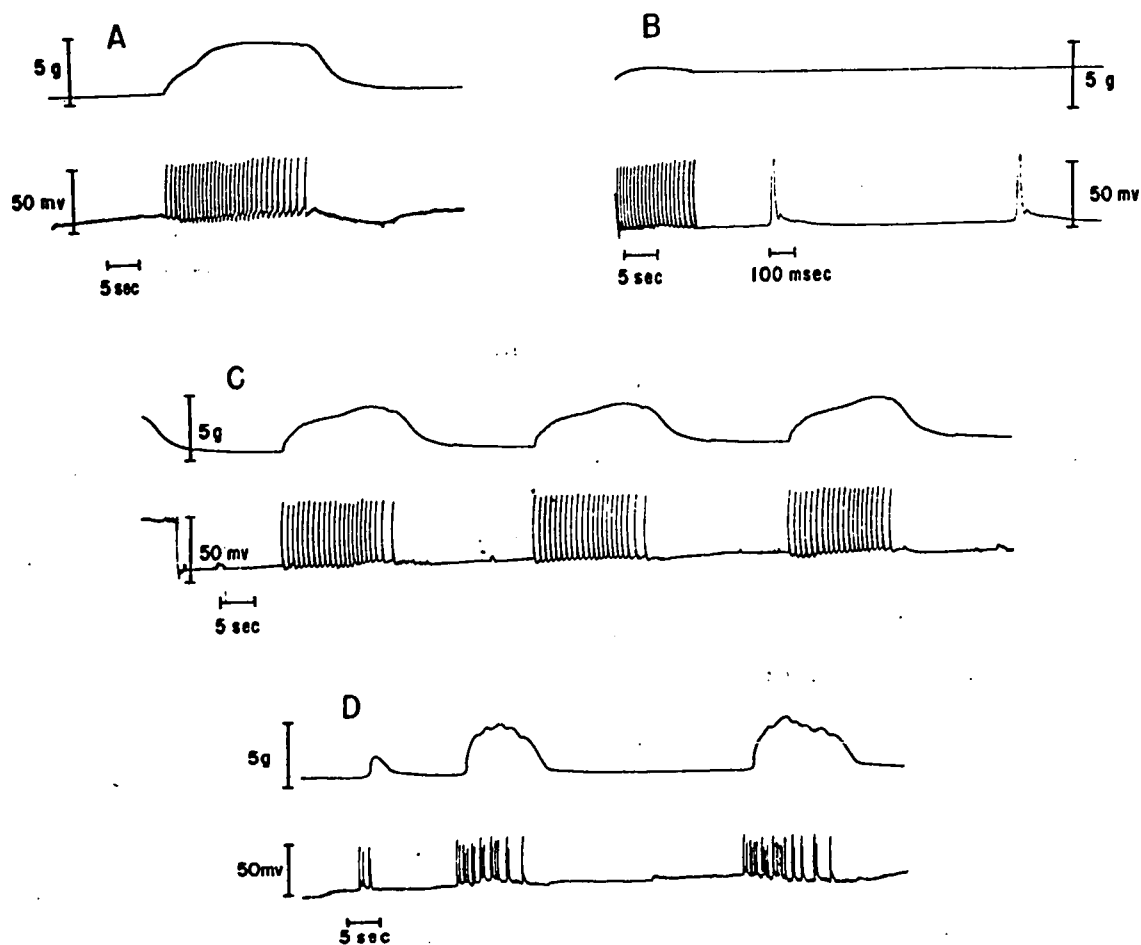
Figure 2. Spontaneous electrical and mechanical activity of pregnant rat uterus. The upper recordings show changes in isometric tension, the lower recordings changes in membrane potential. A,B,C,D,typical spontaneous contractions accompanied by action potentials; B shows action potentials at a faster sweep speed; A,B,C,D, represent activity from 4 different tissues.

TABLE IV

	Treatment	Na_t	K_t	H_2O_t
A	Fresh	79 ± 1.8 (29)	80.8 ± 1.6 (35)	814 ± 2.3 (47)
B	40 min. Normal Krebs solution	90.9 ± 2.6 (26)	69.0 ± 1.4 (20)	838 ± 1.9 (47)
C	Overnight in K-free Krebs solution "Na-rich"	138.9 ± 3.9 (28)	13.0 ± 1.3 (28)	830 ± 3.9 (28)

Values are shown in mEq/kg wet weight of tissue, Water in gH_2O/kg tissue (mean $\pm$ SE).

Numbers in parenthesis indicate number of tissues.

the normal resting potential recorded in fresh tissues by about 20 mV. As shown in Fig. 3, hyperpolarisation beyond the control resting membrane potential of about 50 mV (fresh tissue) was obtained within 2 min. after the introduction of K (4.6 mM) to the organ bath. After about 30-40 min. in normal Krebs solution the membrane potential had gradually declined to a resting value of about 46 mV (81 penetrations in 12 tissues) and spontaneous bursts of action potentials were recorded accompanying each contraction. Tissues allowed to recover in normal Krebs solution produced action potentials resembling those found in fresh tissue (c.f. Fig.2 and Fig.3). The changes in ion content during recovery of Na-rich tissues are shown in Table V along with calculated equilibrium and observed values of membrane potential. Samples were analysed for Na and K after Na-enrichment, 2 min. after changing the bathing medium from K-free to normal Krebs solution (4.6 mM K) and at the first spontaneous contraction (usually 30-40 min. after changing to normal media). Intracellular values for K and Na were calculated according to Daniel and Robinson (1962) as discussed on page 8 using an extracellular space of 370 ml/kg determined using ^{14}C-inulin (see Methods IIc and Results IIIB (f).

The small changes in tissue Na and K observed 2 min. after adding 4.6 mM K to the organ bath are not significant. From Table V it can be seen that the measured membrane potential exceeds the K-equilibrium potential by about 15 mV during the early recovery in 4.6 mM K-Krebs solution at 37°C. The absolute values of V_K in these tissues may not be meaningful since K may be bound inside cells and
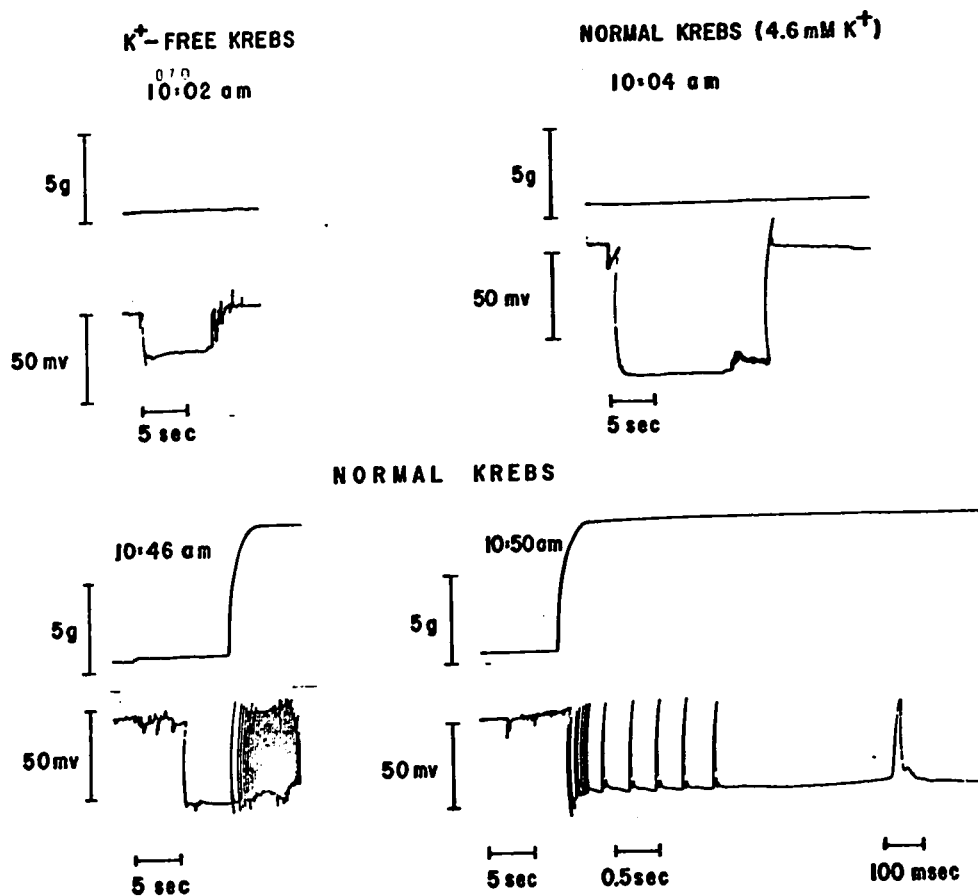
Figure 3. The recovery of the membrane potential and contractility of Na-rich rat uterus in normal Krebs solution. Upper left trace (10:02 a.m.) shows the membrane potential recorded in a Na-rich tissue; 10:04 a.m. (upper right trace) the membrane potential is shown as a large negative deflection upon penetration of the cell 2 min. after the addition of normal Krebs solution to a Na-rich tissue. Withdrawal from the cell is shown as a return to the original base line; 10:46 a.m. (lower left trace)the return of the membrane potential to about 50 mV and the onset of spontaneous contractile activity. 10:50 a.m. (lower right trace) cell penetration and action potentials at a faster sweep speed.

TABLE V

Total Ion Content, Calculated Intracellular Ion Content, and Calculated Equilibrium Potential for Rat Uterus when Na-Rich; when Hyperpolarised; and at First Spontaneous Contraction.

Medium	Na-Rich K-free Krebs	At Hyperpolarisation K-containing Krebs (2 minutes)	1st Contraction K-containing Krebs (40 minutes)
Na_t	130.9 ± 1.7 (23)	128.4 ± 1.5 (24)	113.1 ± 2.1 (24)
K_t	16.5 ± 1.3 (23)	17.8 ± 0.7 (24)	32.2 ± 1.6 (24)
Na_i (inulin)	169.3	165.3	131.9
K_i (inulin)	35.7	34.5	65.0
H_2O_t	836.8 ± 4.0 (23)	843.3 ± 2.6 (24)	845.6 ± 5.7 (24)
V_m	-15.4 ± 0.6 (91)	-69.4 ± 1.7 (22)	-46.2 ± 0.5 (81)
V_K (inulin)	*	-54.7	-70.2
V_{Na}	-7.6	-4.7	$+1.2$

* Bathing fluid contains no added K although a low concentration may be present near the cell membrane; V_K cannot be calculated but must be very large.

Na_t, K_t: Total ion content of tissue (mEq/kg wet weight).

Na_i, K_i: Calculated intracellular ion content using ^{14}C-inulin space (mEq/l).

H_2O_t: Water content of tissue (g H_2O/kg tissue).

V_m: Observed mean membrane potential (mV). (See text).

V_K; V_{Na}: Calculated equilibrium potentials (mV).

Numbers in parenthesis refer to number of tissues except in the case of V_m where the numbers in parenthesis indicate numbers of penetrations.

since the external concentrations of K near the cell membrane may not be identical with that in the bath. However, at a time when V_K must have been decreasing from a very high value to a relatively low one, after the addition of K to the medium, V_m was increasing by 54 mV. It is unlikely that the increase in V_m can be attributed to an increase in V_K.

In summary the results of this section show that:-

(1) After overnight incubation in K-free Krebs solution, fresh tissues gain Na and lose K.

(2) The low membrane potential recorded in Na-rich tissues was rapidly increased beyond the resting membrane potential 2 min. after adding normal Krebs solution to these tissues.

(3) After 30-40 min. in normal Krebs solution the membrane potential of Na-rich tissues approached the value recorded in fresh tissues and spontaneous contractions accompanied by bursts of action potentials were recorded.

(4) The hyperpolarisation observed during the early recovery of Na-rich tissues was greater than the calculated K-equilibrium potential.

IIIB (c) The effect of temperature on recovery of the membrane potential.

No significant increase in membrane potential was observed in 2 experiments in which the temperature of the K-free bathing medium surrounding Na-rich tissues was increased from 4°C to 37°C. Fig.4 shows the membrane potentials recorded in two Na-rich tissues for
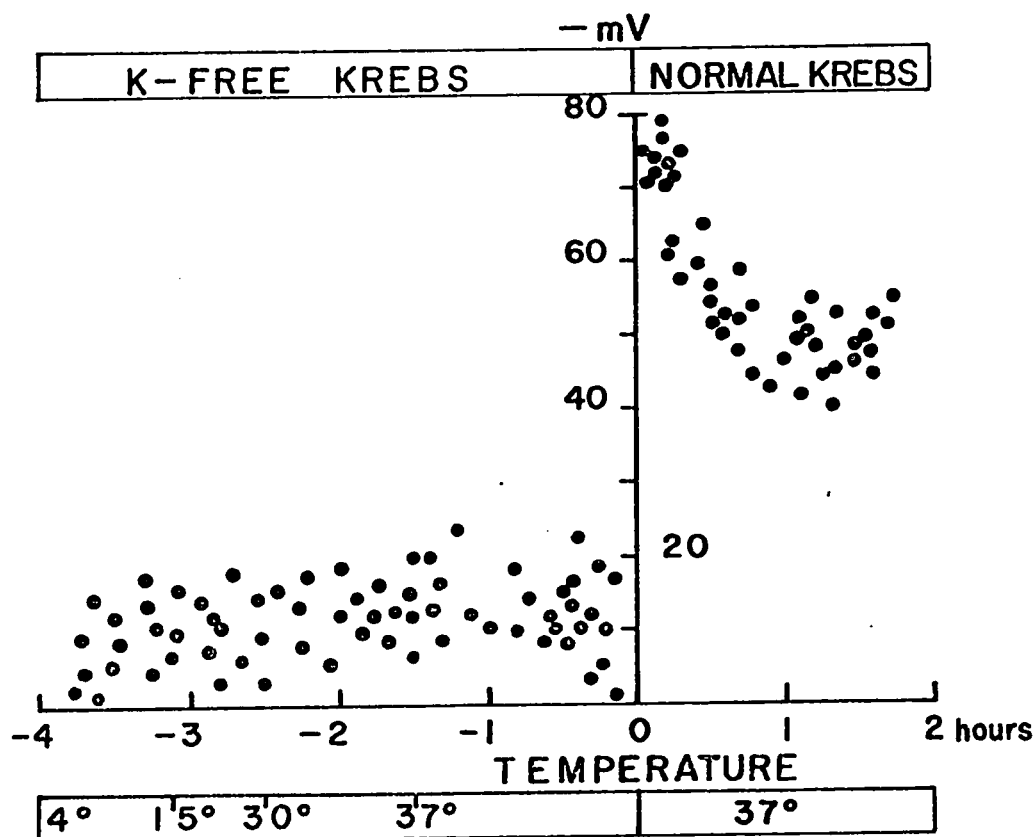
Figure 4. The membrane potential of Na-rich rat uterus at different temperatures in K-free Krebs solution and at 37°C in the presence of normal Krebs solution. Two Na-rich tissues were incubated in K-free Krebs solution and the membrane potential recorded as the temperature of the bathing medium was increased from 4°C to 37°C over a 4-hour period (shown to the left of the ordinate). At zero time normal Krebs solution was added to the Na-rich tissues at 37°C and the membrane potential is shown to the right of the ordinate. Each point represents 1 penetration. Ordinate, membrane potential (-mV).

4 hours in K-free Krebs solution whilst the temperature was slowly increasing from 4°C to 37°C. However, the addition of K to the bathing medium at 37°C produced the same rapid increase in membrane potential as described in Section IIIB (b). Fig.4 also demonstrates the time course of the subsequent decline in membrane potential.

If Na-rich tissues were allowed to recover at 25°C, in the presence of 4.6 mM K, a slower rise and fall in the membrane potential was observed (Fig.5). Hyperpolarisation developed slowly over a period of 45-70 min; the membrane potential remaining at this value for about 30 min. Although not shown, spontaneous contractions were recorded as the membrane potential approached 50 mV (about 90 min. after changing the bathing medium to normal Krebs solution).

Experiments were carried out in which normal Krebs solution at 4°C was added to an Na-rich tissue bathed in K-free medium at 4°C. Neither recovery of the membrane potential nor spontaneous contractile activity had resumed up to 5 hours after the addition of normal Krebs solution.

In summary these results indicate that:-

(a) The recovery of the membrane potential is dependent on the presence of K in the bathing medium and is temperature dependent.

(b) At 25°C in the presence of K (4.6 mM), the membrane potential increased slowly to a maximum value of 77 mV (Range 60 mV to 70 mV, recorded 45-70 min. after the introduction of K). Spontaneous mechanical activity commenced at a membrane potential of about 50 mV, approximately 90 min. after the addition of K.
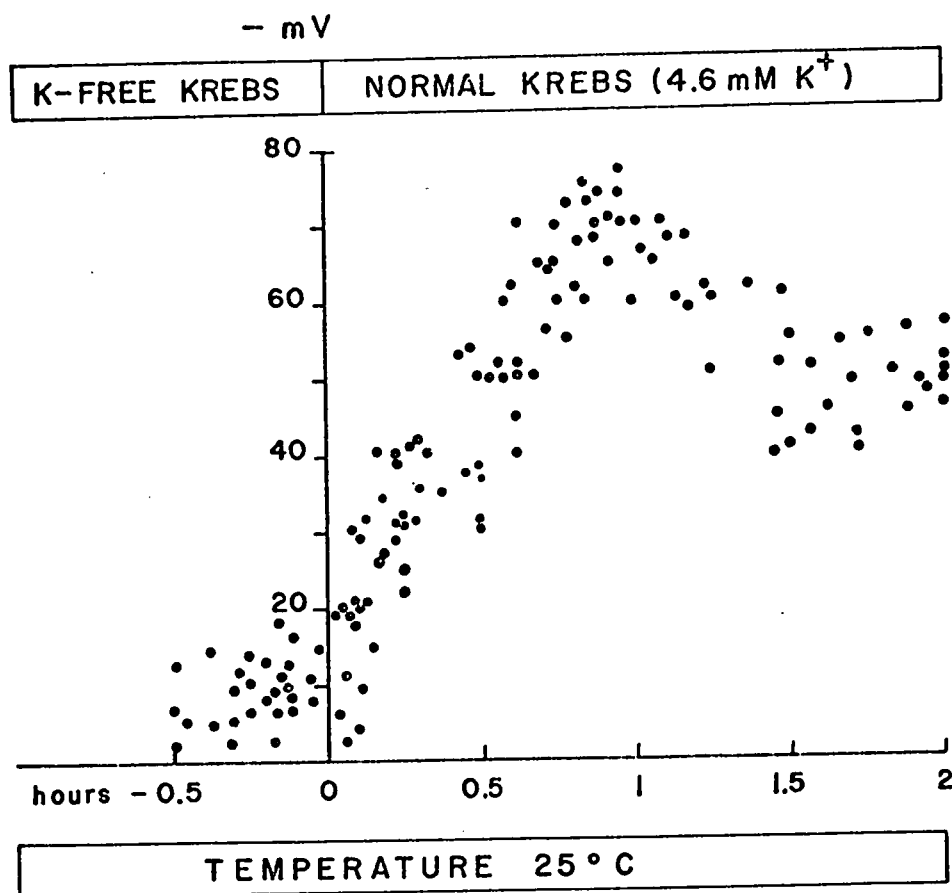
Figure 5. The recovery of the membrane potential of Na-rich rat uterus in the absence and presence of K (4.6 mM) at 25°C. A Na-rich tissue was incubated in K-free Krebs solution at 25°C and the membrane potential recorded (shown to the left of the ordinate). Zero time represents the addition of normal Krebs solution to the tissue and the increase in membrane potential is shown to the right of the ordinate. Ordinate, membrane potential (-mV). Each point represents 1 penetration. Data plotted from 3 experiments.

(c) At 4^OC, in the presence of K, the membrane potential was not significantly different from that found in a Na-rich tissue and spontaneous contractions did not occur.

IIIB (d) The effect of Ouabain on the recovery of the membrane potential.

In view of the known lack of sensitivity of tissues from rat to ouabain, 10^{-3}M ouabain was used throughout these experiments as this concentration has been shown by Daniel (1963a,1964) to be necessary for the induction of downhill ion movements or prevention of uphill ion movements in rat uterus.

Preliminary electrophysiological experiments were carried out to determine the effect of ouabain on the fresh pregnant rat uterus.   The addition of ouabain (10^{-3}M) to a fresh spontaneously active tissue produced a small but significant decrease in membrane potential within 8 min., followed by a large decrease in membrane potential after 60 min., as shown below.

Fresh Tissue	5-10 min. after Ouabain 10^{-3}M mV	50-60 min. after Ouabain 10^{-3}M mV
48.3 $\pm$ 0.5 (26)	44.1 $\pm$ 0.8 (23)	33.2 $\pm$ 0.6 (22)

Data from 4 different tissues, mean $\pm$ SE.

Numbers in parenthesis refer to number of penetrations.

A sustained contraction, lasting about twice the duration of a spontaneous contraction was observed in response to the addition of ouabain.

After 60 min. exposure of fresh spontaneously active tissues to 10^{-3}M ouabain the membrane potential had fallen to 33 mV, whereas fresh tissues incubated in the absence of ouabain did not show any significant fall in membrane potential.   In the presence of ouabain spontaneous contractions were present, superimposed upon a sustained increase in tension, the spontaneous contraction correlated with irregular spike activity.   Occasionally "double spikes" occurred during a contraction and the frequency of firing of action potentials was always reduced from that prior to the addition of ouabain (Fig.6C).

Fig.7 shows the effect of ouabain (10^{-3}M) on the recovery of the membrane potential of Na-rich pregnant uterus.   Ouabain (10^{-3}M) was added to the organ bath 10 min. before changing the bathing medium from K-free Krebs to normal Krebs solution.   The presence of ouabain inhibited recovery of the membrane potential.   Upon washing out the ouabain with normal Krebs solution, a slow increase in membrane potential was observed indicating the reversible nature of the ouabain inhibition of recovery of the membrane potential.   15-20 min. after washing out ouabain with normal Krebs solution the membrane potential in 3 experiments was 54.1 $\pm$ 1.0 mV (12 penetrations).   Approximately 30 min. later spontaneous contractile activity commenced.

The results of the experiments involving ouabain show that:-

(1) Fresh tissues contracted in response to 10^{-3}M ouabain and a small decrease in membrane potential was observed.   After 60 min. exposure to ouabain, a significant decrease in membrane potential, accompanied by a sustained increase in tension was demonstrated.
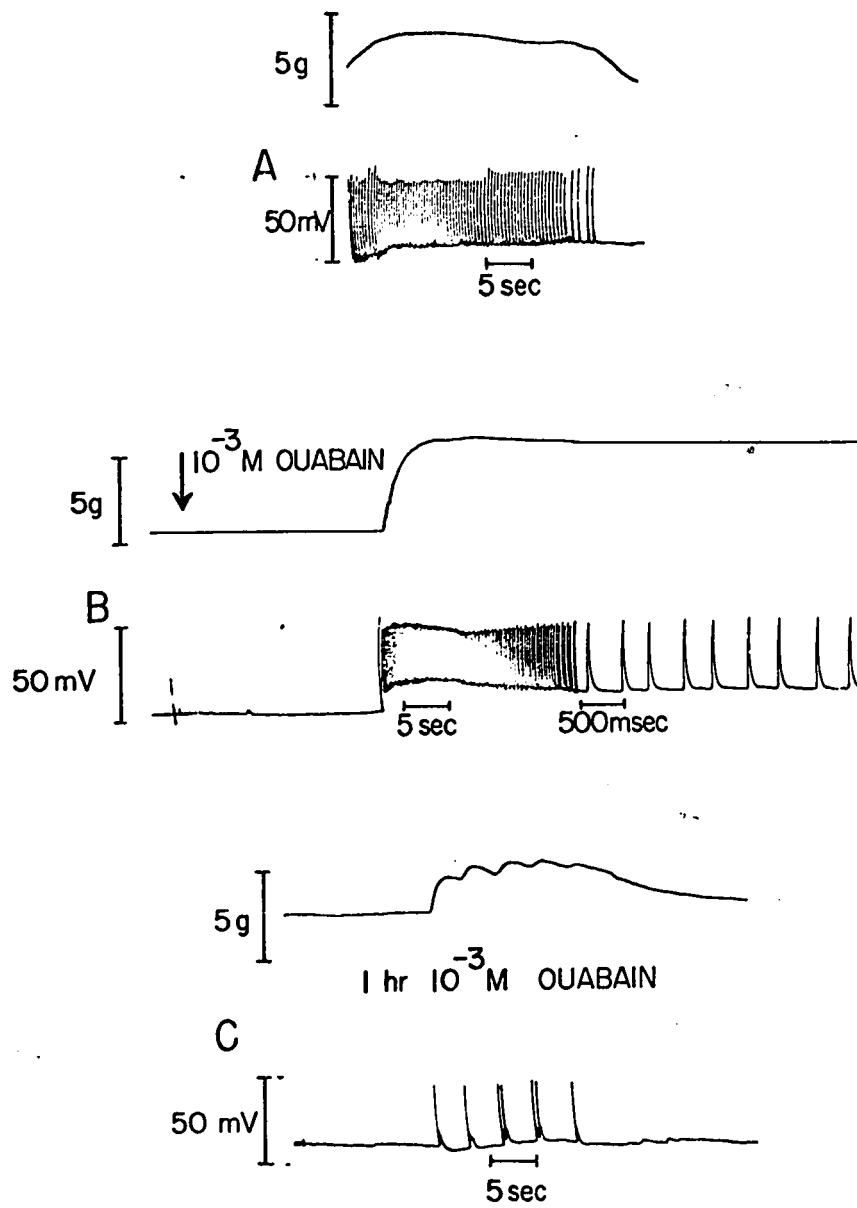
Figure 6. Effect of Ouabain (10^{-3}M) on the mechanical and electrical activity of fresh rat uterus. A, control spontaneous contraction in normal Krebs solution; B, the response to the addition of 10^{-3}M ouabain to a fresh tissue; C, mechanical and electrical activity of fresh tissue after 1 hour in normal Krebs solution containing 10^{-3}M ouabain.
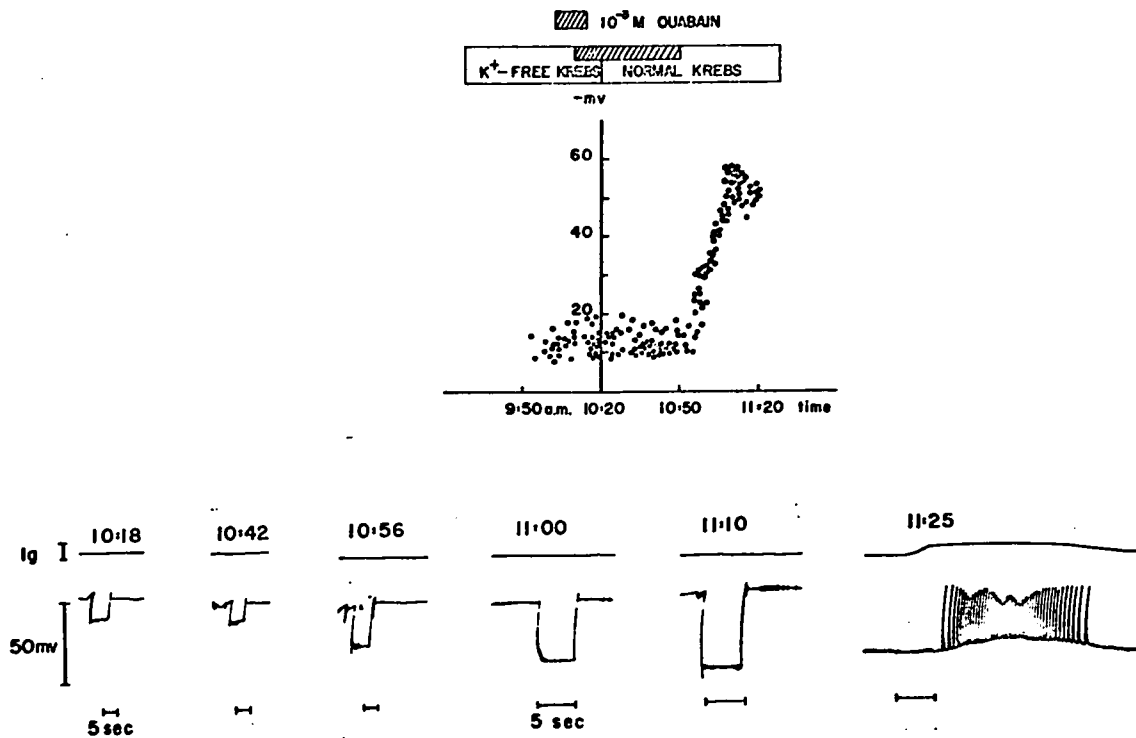
Figure 7. Effect of Ouabain (10^{-3}M) on the recovery of the membrane potential and mechanical activity of Na-rich rat uterus. The graph in the upper half of the Figure shows the membrane potential of a Na-rich tissue in K-free Krebs solution, and in K-free Krebs solution containing ouabain (10^{-3}M) for 10 min. to the left of the ordinate. At 10:20 a.m. the bathing medium was changed to normal Krebs solution containing ouabain 10^{-3}M for 30 min. Upon removal of ouabain the membrane potential gradually increased as shown to the right of the ordinate. Each point represents 1 penetration. The recordings below show microelectrode cell penetrations and membrane potentials corresponding to the times shown on the upper graph.

(2) 10^{-3}M ouabain prevented recovery of the membrane potential in Na-rich tissues.

(3) Inhibition of recovery of the membrane potential produced by ouabain could be reversed upon washing out ouabain from the bathing medium. Only a small hyperpolarisation over the resting potential in fresh tissues (54.1 as opposed to 50 mV) was recorded before spontaneous contractions occurred.

IIIB (e) <u>Modification of the recovery medium.</u>

(i) Potassium

As discussed in section IIIB (b), lack of K in the recovery medium prevented the hyperpolarisation and subsequent recovery of the membrane potential and contractility of Na-rich tissues.

A reduction in the K concentration (below 4.6 mM) in the recovery medium of Na-rich tissues delayed the restoration of the membrane potential and the onset of spontaneous contractions; with 0.58 mM K spontaneous contractile activity resumed after 360 min; with 1.15 mM K mechanical activity resumed after 180-200 min. As shown in Table Vi higher concentrations of K produced more rapid recovery of spontaneous contractions.

Na-rich tissues, incubated for 2 hours, in media containing less than 2.3 mM K did not attain membrane potentials greater than 60 mV in 4 separate tissues. Spontaneous contractions developed after about 6 hours in 0.5 mM K-Krebs, and after about 3 hours in 1.1 mM K-Krebs. Membrane potential changes were not monitored for

TABLE VI

Effects of K on the Recovery of Spontaneous Contractions in Na-Rich

Tissues.

K concentration (mM)	Time Until First Contraction after addition of K (range in min.)	
0.5	360-390	(4)
1.1	180-200	(4)
2.3	70-100	(3)
4.6	25-40	(22)
9.2	20-35	(4)
18.4	18-25	(4)
46	8-15	(7)
120	5-10	(3)

Number of tissues shown in parenthesis.

longer than 2 hours in these low K solutions.   Changes in membrane

potential of Na-rich tissues could be induced by altering the K

concentration of the bathing medium during recovery from the Na-rich

state.   For example, Na-rich tissues allowed to recover in 1.1 mM

K-Krebs had a membrane potential of 40-50 mV (4 tissues) after

60 min. in this medium.   The addition of 18.4 mM K to the bath at

this time increased the membrane potential to about 70 mV, as shown

in Fig.8B where the microelectrode remained within a single cell

whilst the bathing medium was changed.   Conversely, removal of K
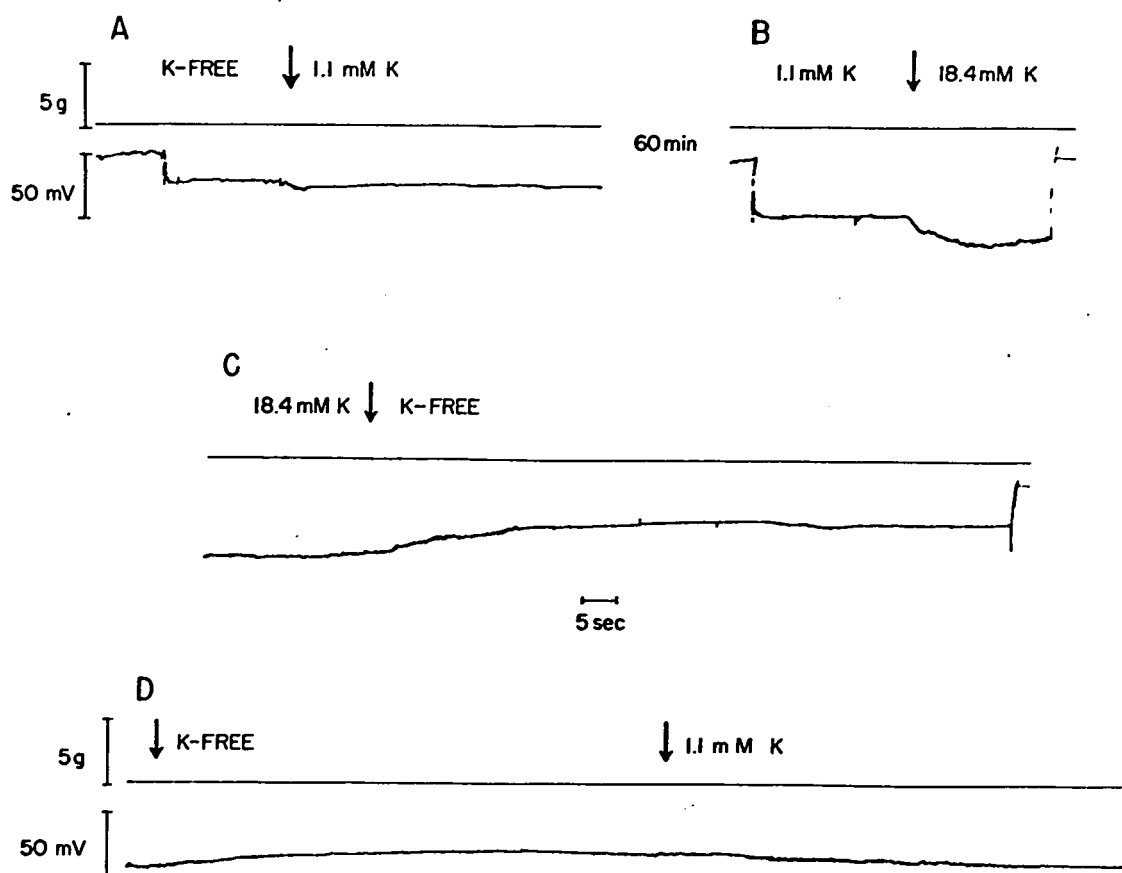
Figure 8. The effect of 1.1 mM K-Krebs solution and 18.4 mM K-Krebs
solution on the recovery of the membrane potential of Na-rich
rat uterus. A, the effect of the addition of 1.1 mM K-Krebs
solution on the membrane potential of a Na-rich tissue bathed
in K-free Krebs solution; B, the effect of 18.4 mM K-Krebs
solution on the membrane potential of a Na-rich tissue incubated
for 60 min. in 1.1 mM K-Krebs solution; C, recording taken
3 min. after B showing the effect of K-free Krebs solution on
the membrane potential; D, the effect of K-free Krebs solution
on the membrane potential of a Na-rich tissue after 70 min. in
1.1 mM K-Krebs solution and the effect of reintroducing 1.1 mM
K-Krebs solution on the membrane potential.

decreased the membrane potential to the value found before the addition of 18.4 mM K (Fig.8C). Fig.8A shows one of 3 experiments in which a small increase in membrane potential was produced by changing the bathing medium from K-free to one containing 1.1 mM K. The addition of 1.1 mM K-Krebs solution to a Na-rich tissue did not produce an immediate hyperpolarisation as was observed in response to higher concentrations of K. Fig.8D shows the effect of K-free Krebs solution on the membrane potential of a Na-rich tissue after 70 min. in 1.1 mM K Krebs solution. The decrease in membrane potential of about 8 mV (mean of 3 similar experiments) could be reversed by the addition of 1.1 mM K-Krebs solution.

Double the normal concentration of K (9.2 mM) in the recovery media also caused an initial hyperpolarisation. The mean membrane potential in 4 tissues was 14.3 ± 0.6 (16 penetrations) before the addition of 9.2 mM K-Krebs solution and 76.2 ± 0.8 (19 penetrations made within 3 min. of addition of K). The hyperpolarisation produced by the addition of 9.2 mM K-Krebs solution is shown in Fig.9.

Recordings from the same cell shown in Fig. 9 also demonstrate the fall in membrane potential (about 41 mV) produced when K-free Krebs solution replaced 9.2 mM K-Krebs solution during the early hyperpolarisation. The average decrease in membrane potential produced by removal of K in 3 similar experiments was 39 mV. In two out of four experiments an irregular oscillation of the membrane potential occurred before the onset of recorded spontaneous contractility; this may represent electrical activity in neighbouring cells propagated electrotonically to the cell from which the record was taken.
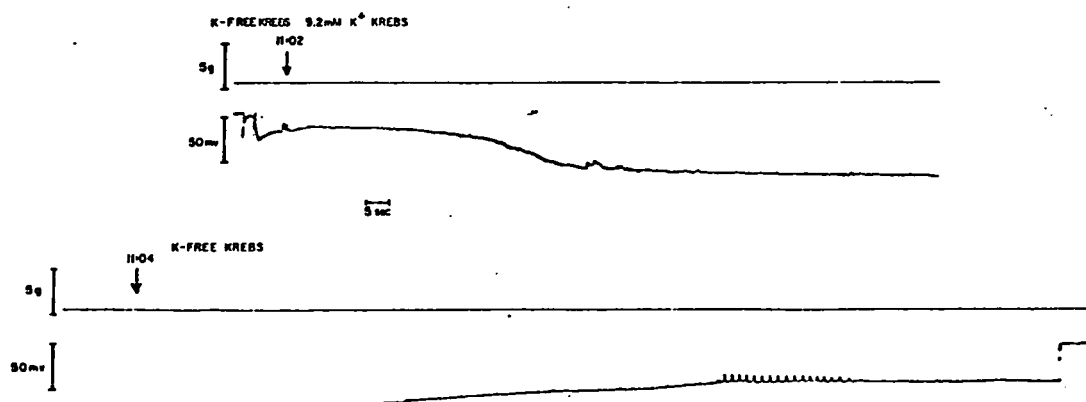
Figure 9. The effect of 9.2 mM K-Krebs solution on the recovery of the membrane potential of Na-rich rat uterus. Upper trace shows the effect of the addition of 9.2 mM K-Krebs solution to a Na-rich tissue (11:02 a.m.) whilst the microelectrode remained within the same cell. Lower trace shows the effect of removal of K 2 min. after adding K (11:04 a.m.) and the decrease in membrane potential accompanied by electrical activity. See text.

Hyperpolarisation induced by addition of 18.4 mM K to a
Na-rich tissue is shown in one of four similar tissues in Fig.10.
Note the rapid increase of the membrane potential under these
circumstances.   Removal of K, 1 min. after the induction of
hyperpolarisation gave rise to a reduction in membrane potential and
the generation of action potentials (Fig.10B).   An increase in tension
accompanied by contractions was observed in 18.4 mM K for up to 2 hours
as shown in Fig.10C,D.

Increasing the concentration of K in the bathing medium
surrounding Na-rich tissues to 46 mM produced marked hyperpolarisation
(77.4 ± 0.4 mV - 56 penetrations in 7 tissues) within 2 min. after
changing from K-free medium.   Fig.11 shows some records taken from a
typical experiment with 46 mM K in the recovery medium.   46 mM K
Krebs solution was introduced at 10:40 a.m., 1 min. before record B
was taken.   Spontaneous contractions began 11 min. later and were
followed by a sustained contraction and depolarisation with constant
firing as shown on the left of Fig.11D.   The introduction of K-free
Krebs solution to such a preparation produced an increase in membrane
potential from 24.2 ± 0.5 to 49.5 ± 1.1 in 16 penetrations made before
and after the introduction of K-free Krebs solution in 2 tissues.
This response was the reverse of that observed when K-free Krebs
solution was introduced shortly after the induction of hyperpolarisation
with several lower concentrations of K, at which time a depolarisation
was the observed response (see Figs.9, 10).

A series of experiments were carried out to determine the
ion content under similar conditions to those in which the recordings
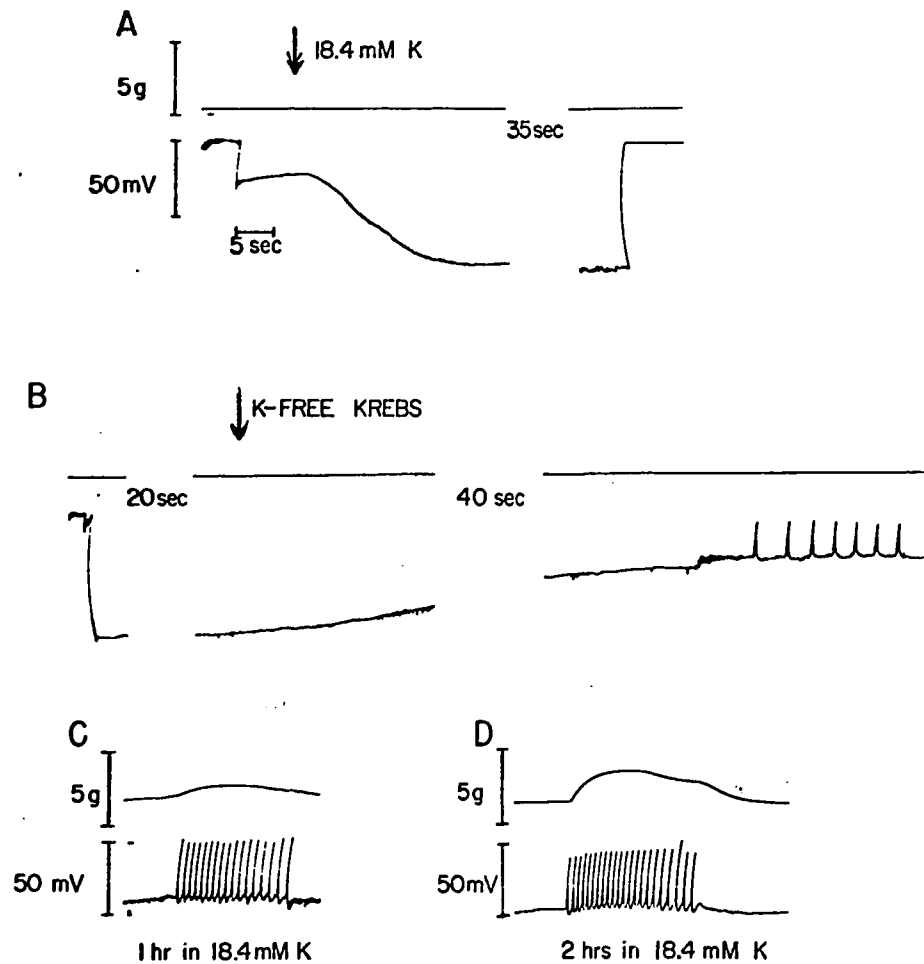
Figure 10. The effect of 18.4 mM K-Krebs solution on the recovery of the membrane potential and mechanical activity of Na-rich rat uterus. A, the addition of 18.4 mM K-Krebs solution to a Na-rich tissue, after 35 seconds the microelectrode was removed from the cell; B, the effects of K-free Krebs solution on the initial hyperpolarisation caused by the addition of 18.4 mM K-Krebs solution to a Na-rich tissue. The decrease in membrane potential in K-free Krebs solution was accompanied by action potentials; C and D show the spontaneous electrical and mechanical activity of a Na-rich tissue after, respectively, 1 and 2 hours in 18.4 mM K-Krebs solution.
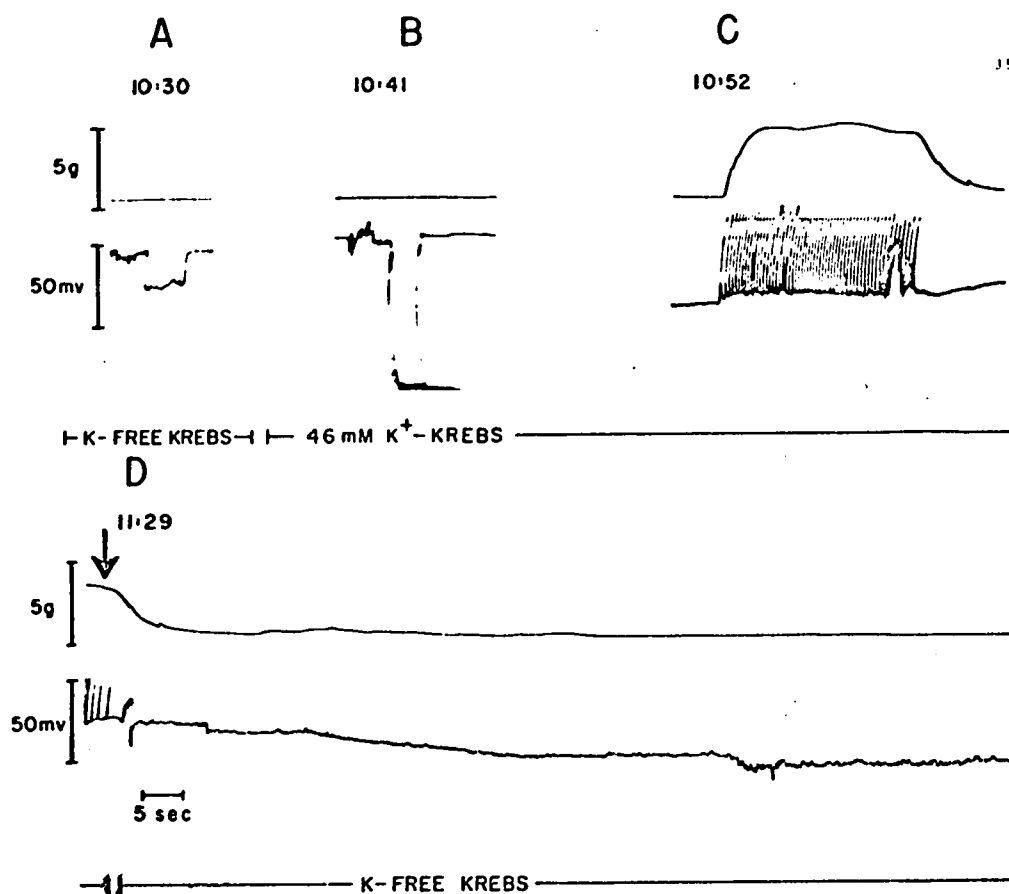
Figure 11. The effect of 46 mM K-Krebs solution on the electrical and mechanical activity of Na-rich rat uterus. A, membrane potential of Na-rich tissue in K-free Krebs solution; B, membrane potential recorded 1 min. after changing bathing medium to 46 mM K-Krebs solution; C, spontaneous contraction 12 min. after introduction of 46 mM K-Krebs solution to Na-rich tissue; D, the change in membrane potential produced by the addition of K-free Krebs solution to a Na-rich tissue exposed to 46 mM K-Krebs solution for 49 min.

shown in Fig.11 were made. Samples of Na-rich tissue were analysed for Na and K content (a) immediately before changing the bathing medium from a K-free Krebs solution to one containing 46 mM K (b) 2 min. after the introduction of 46 mM Krebs solution whilst hyperpolarisation of the membrane potential was occurring (c) at the first contraction (see Table VII.

The K-equilibrium potential calculated during the early hyperpolarisation (2 min. after the addition of 46 mM K-Krebs solution) was +9.1 mV. A large discrepancy between the observed membrane potential of -77 mV and the calculated K-equilibrium potential was most readily observed when 46 mM K-Krebs solution, instead of 4.6 mM K-Krebs solution, was used to induce hyperpolarisation. Furthermore, at the time of the first contraction the calculated K-equilibrium potential still remained less than the observed membrane potential in 4 tissues examined.

Hyperpolarisation of the membrane potential was also the observed response when very high concentrations of K were used in the recovery medium after Na-enrichment. Fig.12 shows one of three experiments in which the bathing medium was changed from K-free to a Krebs solution containing 120 mM K. In two similarly treated tissues the membrane potential increased to 79 $\pm$ 0.9 (16 penetrations made within 2 min. of the addition of 120 mM K-Krebs solution). 5 min. later the membrane potential had fallen to about 40 mV and 1 min. later a small contraction was recorded in response to an irregular action potential showing a considerable plateau phase (Fig.12B). Displacement of the electrode from the cell may have been responsible

TABLE VII

Recovery of ion content and membrane potential in 46 mM K-Krebs.

	Na-Rich	At Hyperpolarisation	1st Contraction
	K-free Krebs	46 mM K-Krebs (2 min.)	46 mM K-Krebs (12 min.)
Na_t	138 ± 3.9 (35)	120 ± 2.0 (39)	97 ± 1.5 (39)
K_t	13 ± 1.3 (35)	32 ± 1.1 (39)	56 ± 1.4 (39)
H_2O_t	829 ± 3.9 (38)	831 ± 3.4 (39)	824 ± 3.5 (39)
V_m	-16.4 ± 0.8 (42)	-77.0 ± 0.9 (31)	-47.9 ± 0.9 (30)
K_i	28.7	32.7	86.9
V_K (inulin)	*	+9.1	-16.9
V_{Na} (inulin)	-7.3	-12.8	-11.1

* Bathing fluid contains no K although a low concentration may be present near the cell membrane; V_K cannot be calculated but must be very large.

Na_t, K_t :   Total ion content of tissue mEq/kg.

K_i :   Calculated intracellular ion content using inulin space of 370 ml/kg (mEq/1).

H_2O_t :   Water content of tissue g H_2O/kg tissue.

V_m :   Observed mean membrane potential (mV).

V_K : V_{Na} Calculated equilibrium potentials (mV).

Numbers in parenthesis indicate numbers of tissues used except in the case of V_m where numbers in parenthesis indicate numbers of penetrations.
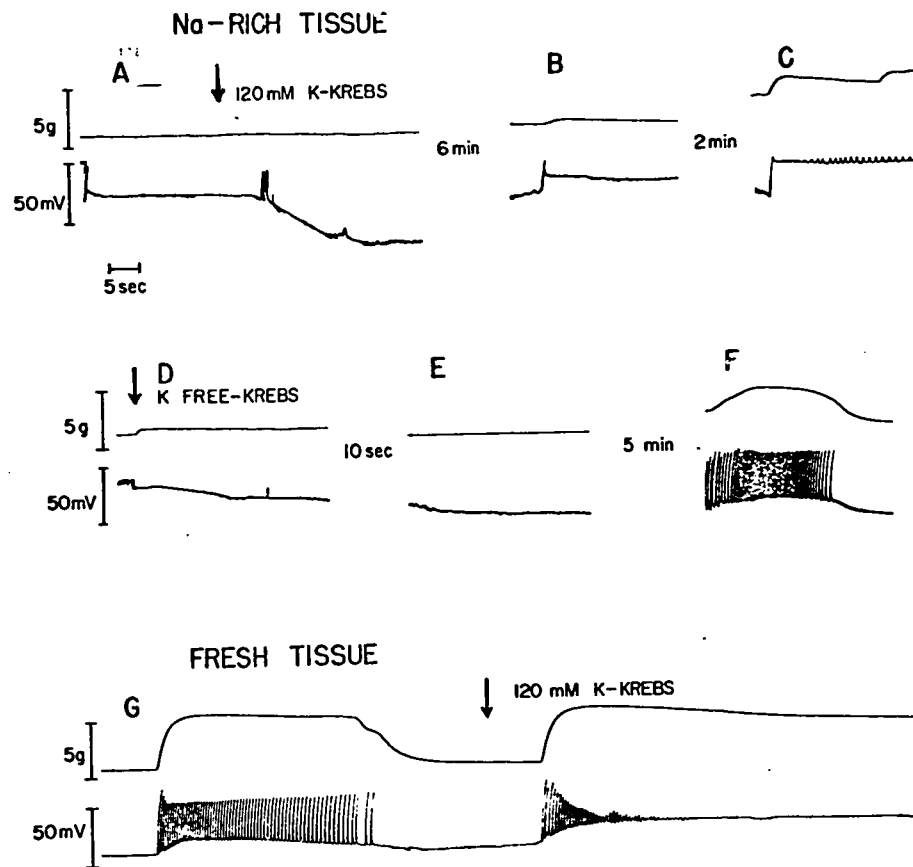
Figure 12. The effect of 120 mM K-Krebs solution on the electrical and mechanical activity of Na-rich and fresh rat uterus.
A, marked increase in membrane potential observed in response to the addition of 120 mM K-Krebs solution to a Na-rich tissue;   B, first spontaneous contraction in 120 mM K-Krebs solution and irregular action potential;   C, record taken 2 min. after trace B in the same tissue in 120 mM K-Krebs solution;   D,E, the increase in membrane potential produced by the removal of K from the same tissue as records A, B, and C, after 12 min. exposure to 120 mM K-Krebs solution;   F, spontaneous activity recorded in the same tissue 5 min. after introduction of K-free Krebs solution. G, the effect of 120 mM K-Krebs solution on a fresh tissue initially bathed in normal Krebs solution.

for the large plateau, although in some instances repolarisation preceeded the next action potential. The long plateau was typical of three tissues allowed to recover in very high K concentrations. Fig.12C shows one such record taken 2 min. after the first spike shown in trace B. At this time the plateau phase of the action potential showed superimposed spikes. The effects of removal of K from a tissue depolarised within 12 min. in 120 mM K as described above is seen in records from another tissue shown in Fig.12D. The increase in membrane potential from 18 mV to 45 mV upon removing K can be seen, followed 5 min. later by bursts of action potentials and spontaneous contractions (Fig.12F).

The addition of 120 mM K-Krebs solution to a fresh tissue produced a sustained contracture and a decrease in membrane potential to about 20 mV. Fig.12G shows a control contraction on the left and the effect of 120 mM K recorded from the same cell on the right. Although not shown, removal of K after 5 min. exposure of fresh tissues to 120 mM K produced a slow increase in membrane potential followed by spontaneous electrical and mechanical activity similar to that observed after removal of K from tissues initially Na-rich which have recovered and become depolarised in high K (see Fig.11).

Changing the bathing medium of a fresh tissue from normal Krebs solution to a K-free Krebs solution, did not produce any detectable change in membrane potential. In tissues where spontaneous contractions were few the frequency of contractions could sometimes be increased by the omission of K from the bathing medium. In such a quiescent tissue the contraction induced by K-free Krebs solution, is
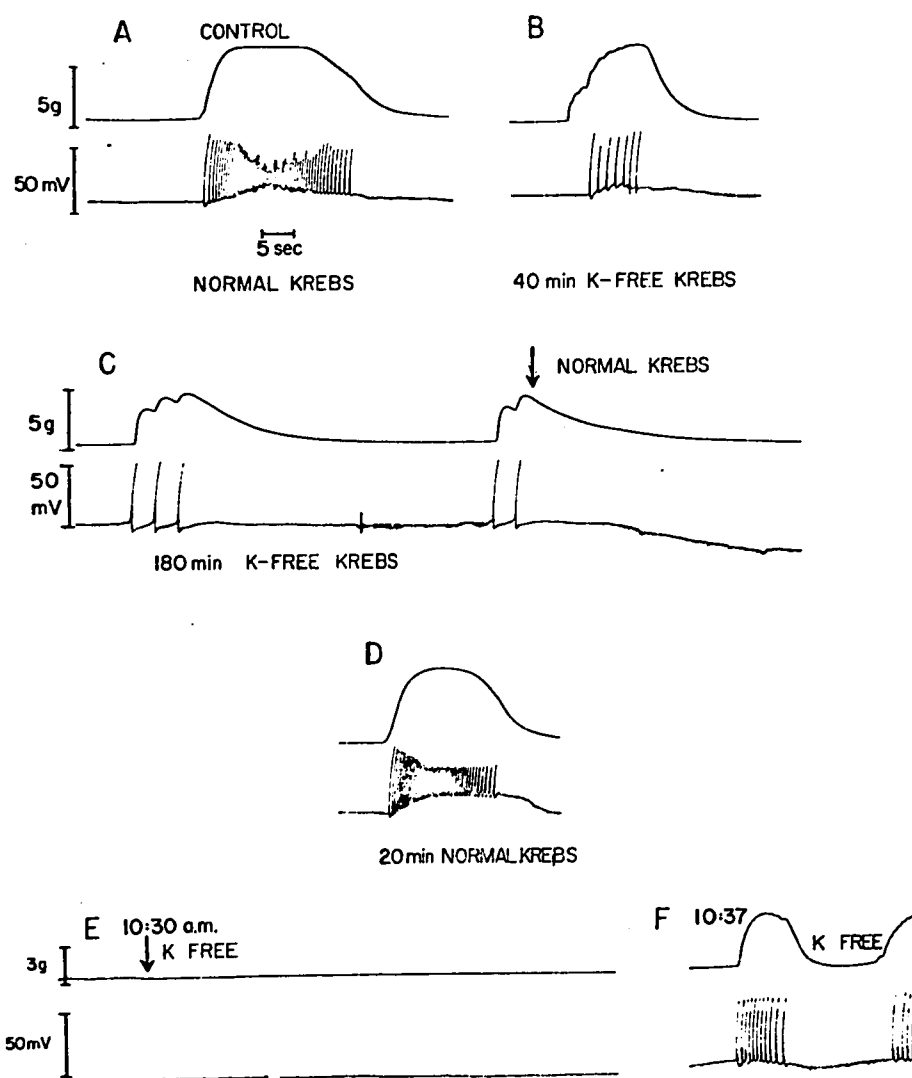
Figure 13. The effect of K-free Krebs solution on the mechanical and
electrical activity of spontaneously active, and quiescent,
fresh tissues.   A, control spontaneous contraction recorded
in normal Krebs solution;   B, 40 min. after changing to
K-free Krebs solution in the same tissue;   C, 180 min. after
changing to K-free Krebs solution and the increase in membrane
potential caused by the addition of normal Krebs after 180 min.
in K-free Krebs solution;   D, spontaneous activity in same
tissue as C, after 20 min. in normal Krebs solution;   E, the
effect of K-free Krebs solution on the membrane potential of
a fresh quiescent rat uterus and F, the induction of spontaneous
activity 7 min. later in K-free Krebs solution in the same
tissue as E.

shown in Fig.13 (E & F). Fig.13 also shows the effect of prolonged exposure to K-free Krebs solution in a fresh tissue. 40 min. after removal of K from the bathing medium the frequency of firing of action potentials was reduced and each spike appeared to be correlated with a small superimposed peak of contractile activity (Fig.14B). After 3 hours in K-free Krebs solution the membrane potential (49.3 ± 0.7, 27 penetrations in 3 tissues) was not significantly different from the control value (50.2 ± 0.3, 39 penetrations in 3 tissues) and a correlation between spike activity and contraction could be clearly observed (Fig.13C). Furthermore, upon the addition of normal Krebs solution (containing 4.6 mM K) to two of these tissues at this time, a marked increase in membrane potential to about 71 mV occurred, as shown in Fig.13C. The increase in membrane potential was accompanied by relaxation of the tissue. During the next 15 min. the membrane potential gradually declined from about 71 mV to 50 mV and spontaneous activity resumed 20 min. after the introduction of K to the organ bath (Fig.13D).

If ouabain 10^{-3}M was included in the K-free bathing medium surrounding a fresh tissue a different electrical and mechanical pattern was observed. Fig.14B shows the increase in tension and repetitive irregular firing induced by K-free Krebs solution containing ouabain. After 60 min. incubation in ouabain containing K-free Krebs solution the membrane potential decreased to a mean value of 43 ± 0.8 mV (20 penetrations in each of 3 different tissues). The irregular pattern of contractile activity was characteristic in this tissue of prolonged exposure to either K-free solutions (3 tissues) or ouabain (10^{-3}M)
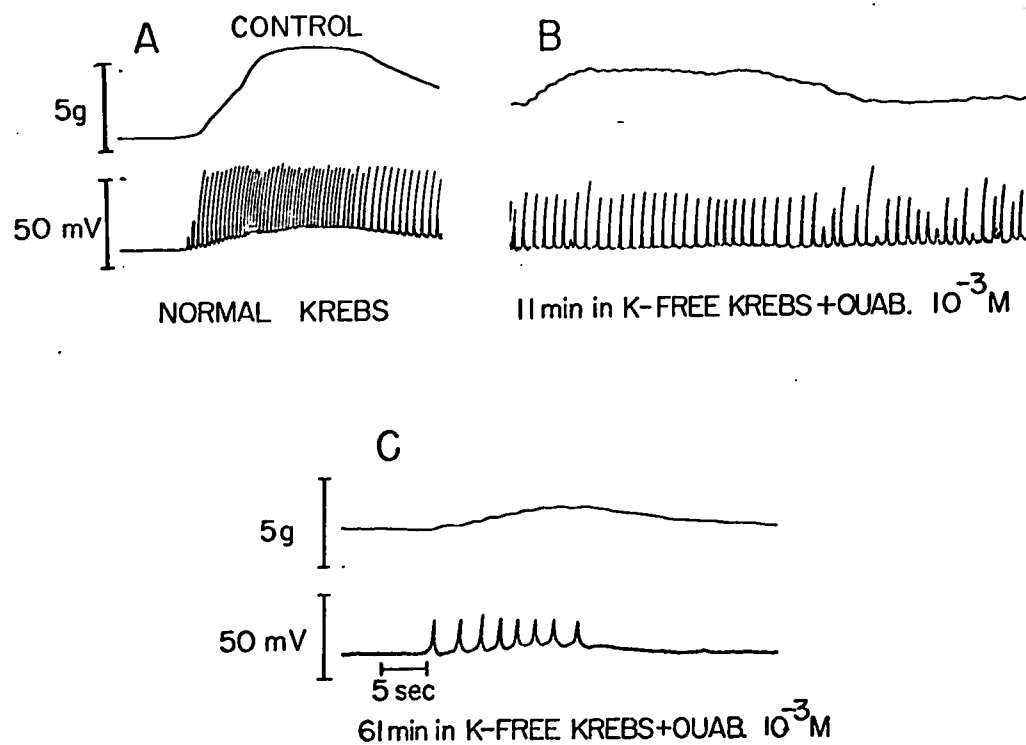
Figure 14. The effect of K-free Krebs solution containing ouabain $(10^{-3}M)$ on the mechanical and electrical activity of fresh pregnant rat uterus. A, control spontaneous contraction and action potentials recorded in normal Krebs solution; B, electrical and mechanical response of the same tissue as A after 11 min. in K-free Krebs solution + ouabain $10^{-3}M$; C, electrical and mechanical response of the same tissue after 61 min. in K-free Krebs solution + ouabain $10^{-3}M$.

containing solutions (3 tissues).

This section of results may be summarised as follows:-

(1)   Changes in K concentration from normal (4.6 mM) in the recovery medium of Na-rich tissues may decrease (low K concentrations) or increase (high K concentrations) the rate of recovery of the membrane potential and spontaneous contractile activity.   The hyperpolarisation induced by the presence of K in the recovery medium, was reversible upon the removal of this ion during the early time period of the recovery of the membrane potential.

(2)   The hyperpolarisation (77 mV) observed when 46 mM K was added to a Na-rich tissue was not explicable in terms of the K-equilibrium potential (+ 9 mV).   Very high concentrations of K (120 mM) also caused an initial hyperpolarisation followed by rapid depolarisation.

(3)   Removal of K, from Na-rich tissues depolarised with high concentrations of K, (46 mM or 120 mM) caused an increase in membrane potential.

(4)   Fresh tissues as opposed to Na-rich tissues, did not hyperpolarise when placed in either 46 mM or 120 mM K-containing Krebs solution.   Such concentrations of K depolarised fresh tissues.

(5)   After prolonged exposure of fresh tissues to K-free solutions, a hyperpolarising response could be elicited by the addition of normal Krebs solution containing 4.6 mM K.

(6)   K-free Krebs solutions did not depolarise fresh tissues but induced spontaneous activity in some quiescent preparations.

K-free Krebs solutions containing ouabain produced a decrease in membrane potential after 60 min. exposure to this medium.

IIIB (e) Modification of the recovery medium.

(ii) Substitution of Rubidium for Potassium

Replacement of K with 4.6 mM Rb in the bathing medium of a fresh tissue in two tissues produced no observable changes in the size of the contraction and the resting membrane potential. The membrane potential in fresh tissues before the addition of 4.6 mM Rb-Krebs solution was 49.3 $\pm$ 0.6 (27 penetrations in 2 tissues) and 2 hours after the replacement of normal Krebs solution with Rb 4.6 mM Rb-Krebs solution (containing no K) the membrane potential was 47.8 $\pm$ 0.5 (23 penetrations in two tissues).

Na-rich tissues allowed to recover in solutions containing Rb in place of K showed similar recovery profiles to tissues allowed to recover in solutions containing corresponding amounts of K.

Fig.15 illustrates one of 5 experiments in which a Na-rich tissue was placed in a recovery solution containing 4.6 mM Rb. The graph (inset) shows the changes in membrane potential from 3 of these experiments. The hyperpolarisation developed after 2 min. in the presence of Rb was similar to that developed in the presence of 4.6 mM K. Furthermore, spontaneous activity occurred about 40 min. after changing to an Rb-containing solution (Fig.15) and was monitored for up to 3 hours.

18.4 mM Rb-Krebs solutions applied to a Na-rich tissue produced a marked hyperpolarisation as shown in continuous recordings
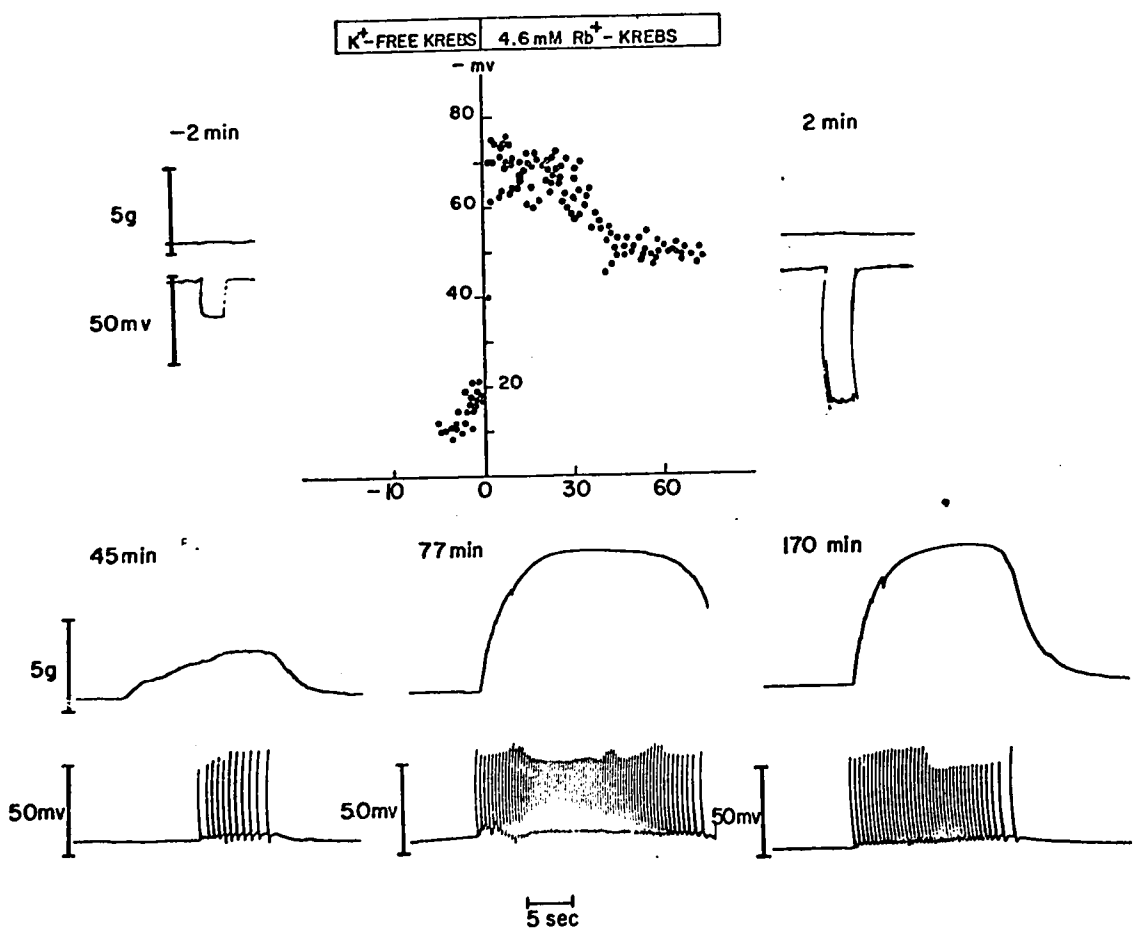
Figure 15. The effect of 4.6 mM Rb-Krebs solution on the recovery of mechanical and electrical activity of Na-rich rat uterus. The graph (inset) shows the changes in membrane potential produced by the addition of 4.6 mM Rb-Krebs solution (at zero time) to a Na-rich tissue. Data plotted from 3 experiments. Each point represents 1 penetration. Records surrounding the graph show the membrane potential and mechanical activity from a typical experiment. The recordings to the left and right of the graph show the membrane potential of a Na-rich tissue 2 min. before (left) and 2 min. after (right) the addition of 4.6 mM Rb-Krebs solution. The records below the graph show the electrical and mechanical activity 45, 77 and 170 min. after the addition of 4.6 mM Rb-Krebs solution to a Na-rich tissue.

made in a single cell whilst changing the bathing medium in Fig.16A. Experiments involving hyperpolarisation induced with 18.4 mM K had shown that removal of K, shortly after the rapid increase in membrane potential caused by this ion, could produce a decrease in membrane potential, and the generation of spikes. Similarly, the removal of Rb, shortly after the hyperpolarising response to this ion, caused a depolarisation. The fall in membrane potential which occurred in the absence of Rb produced spikes of low frequency and a small increase in tension. Fig.16B shows the spikes produced by removal of Rb 3 min. after the initial hyperpolarising response to this cation. After 5 min. in Rb-free solution Fig.16C was obtained. The slow frequency spikes and small contractions were terminated by the reintroduction of 18.4 mM Rb to the bathing medium, which caused a rapid increase in membrane potential. 20 min. exposure to Rb solution produced spontaneous mechanical and electrical activity as shown in Fig.16D,E. The repetitive action potentials accompanying the contraction in this solution invariably showed an unusual depolarisation during the maximum tension of the contraction. Records D and E were taken from different cells; E being recorded 3 min. after D. The sustained depolarisation during contraction showed small superimposed spikes and lasted for about 75% of the contraction. For purposes of comparison record 16F shows the electrical and mechanical activity recorded from another segment of the same Na-rich uterus 30 min. after transfer to a solution containing 18.4 mM K. Note continued firing of action potentials in spite of a reduction in membrane potential during the contraction.

A   ↓18.4mM Rb KREBS   B Rb-FREE KREBS   C   ↓18.4 mM Rb KREBS

5g

50mV

D   E

F 32 min 18.4mM K KREBS

5sec
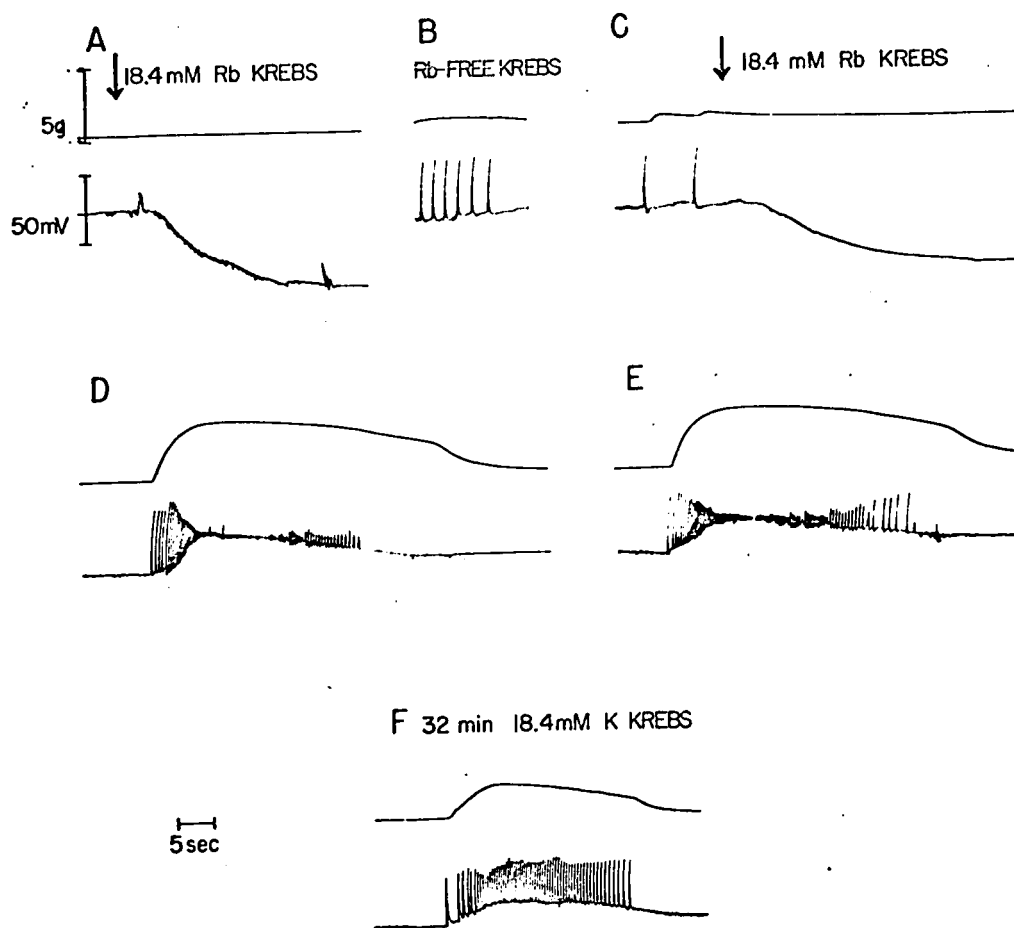
Figure 16. The effect of 18.4 mM Rb-Krebs solution on the recovery of the mechanical and electrical activity of Na-rich uterus. A, the effect of 18.4 mM Rb-Krebs solution on the membrane potential of a Na-rich tissue; B, spontaneous action potentials recorded 3 min. after removal of Rb from a Na-rich tissue previously hyperpolarised by 3 min. exposure to 18.4 mM Rb-Krebs solution; C, the effect of reintroducing 18.4 mM Rb-Krebs solution on the membrane potential of the same tissue shown in B. Record C taken 5 min. after record B; D and E, two spontaneous contractions recorded 20 min. after the addition of 18.4 mM Rb-Krebs solution to a Na-rich tissue. For comparison, F, shows the spontaneous activity of another segment of the same Na-rich tissue, shown in D and E, after 32 min. incubation in 18.4 mM K-Krebs solution.

The hyperpolarisation developed in response to 18.4 mM Rb was not statistically different than the hyperpolarisation developed in response to 18.4 mM K in tissue samples taken from the same animal. The mean membrane potential from 19 penetrations in two Na-rich tissues in response to 18.4 mM Rb was found to be 77.8 ± 1.23 mV (SE of mean) in 19 penetrations made within 8 min. of introducing the Rb-containing solution. Similar measurements from two tissues exposed to 18.4 mM K-Krebs solution involving 17 penetrations made in the same time period showed a membrane potential of 76.6 ± 0.8 mV.

In summary, the experiments with Rb substituted media reported above show that:-

(1) The incubation of fresh tissues in media containing Rb (4.6 mM) in place of K showed no significant change in membrane potential after 2 hours. Furthermore, spontaneous contractions accompanied by action potentials were present after this time.

(2) Na-rich tissues hyperpolarise in response to varying concentrations of Rb and recover spontaneous contractile activity more rapidly the higher the Rb concentration in the recovery medium.

(3) Membrane potential changes, similar to those found with K, may be induced by the removal or addition of Rb to the bathing medium in Na-rich tissues. An increase in membrane potential was caused by the addition of Rb and a decrease by removal of Rb from Na-rich tissues during the early recovery phase.

(4) Exposure of Na-rich tissues to 18.4 mM Rb for about 30 min. generally gave rise to a prolonged depolarisation accompanying

each contraction; superimposed spikes or oscillations of membrane
potential were often visible during the depolarisation phase of the
contraction.

The effects of concentrations of Rb greater than 18.4 mM
on the recovery of the membrane potential of Na-rich tissue were
not investigated.

IIB (e) Modification of the recovery medium.

(iii)Substitution of Cesium for Potassium

Further experiments were carried out in which Krebs solutions
were prepared containing differing concentrations of CsCl substituted
for KCl. The effects of these modified solutions on the recovery of
the membrane potential of Na-rich rat uteri are described below.

As the effects of Cs on rat uterine muscles are unknown
preliminary experiments were carried out to determine any effect
this ion may have on fresh tissues. The membrane potential of 2
fresh tissues incubated for 90 min. in normal Krebs solution was
49.5 ± 0.6 (19 penetrations made between 70-90 min.) After
replacement of 4.6 mM K with 4.6 mM Cs the membrane potential in
two different tissues was 49.2 ± 0.5 (20 penetrations made 70-90 min.
in Cs-Krebs solution). After 90 min. in 4.6 mM Cs-Krebs solution
no abnormalities could be detected in the shape of the action
potentials (within the limits of the pen recording system) and
spontaneous contractions. The activity of fresh tissues in 4.6 mM
Cs-containing solutions was not investigated for longer than 90 min.

Having established that fresh tissues maintained activity in

Cs-containing solutions a series of experiments were performed using

Cs as a substitute for K in order to determine whether or not Cs would

promote recovery of the membrane potential in Na-rich tissues.

TABLE VIII

Effects of Cesium on the recovery of spontaneous contractions in Na-rich

tissues.

Cesium Conc. (mM)	Time until 1st Contraction After addition of Cesium (min,range)
1.15	not contracted after 180 min. (2)
2.3	not contracted after 70 min. (2)
4.6	70-90 (7)
9.2	25-35 (7)
18.4	20-30 (5)
46.0	15-20 (6)

Value in parenthesis indicates number of tissues.

After 3 hours incubation of Na-rich tissues in a medium

containing 1.15 mM Cs the resting membrane potentials was 15.3 ± 0.3 mV

(39 penetrations in two tissues). The corresponding membrane potential

of these two tissues before the addition of Cs was 14.6 ± 0.5 mV

(23 penetrations in 2 tissues). Spontaneous contractions had not

resumed after 3 hours in 1.15 mM Cs-Krebs solution. A small, but

significant, increase in membrane potential from 15.05 ± 0.7

(20 penetrations) to 19.7 ± 1.1 (14 penetrations) was observed in 2

Na-rich tissues after one hour in 2.3 mM Cs-Krebs.   Spontaneous

contractions had not resumed after 70 min. in this media.

When the Cs concentration was increased to 4.6 mM (the

same concentration as K in normal Krebs solution) changes in membrane

potential were observed in Na-rich tissues as demonstrated in Fig.17.

The graph (inset) shows the gradual increase in membrane potential

produced by exposure to 4.6 mM Cs.   This slow increase in membrane

potential without hyperpolarisation before the onset of spontaneous

contraction is in contrast to the rapid increase and then slow

decline of membrane potential produced by similar concentrations of

either Rb or K (see Figs.4, 15). After 2 hours incubation in 4.6 mM

Cs-Krebs solution the membrane potential in 4 Na-rich tissues was

48.9 ± 0.3 (42 penetrations).   Fig.17 shows the slow increase in

membrane potential from one of 7 such tissues allowed to recover in

media containing 4.6 mM Cs.   Note the presence of "plateau-type"

action potentials with large overshoots (22 mV) and the continued

activity after 180 min. in Cs-Krebs solution.   In some instances

the duration of the plateau was greater than 20 sec.   After prolonged

exposure to Cs-Krebs solution (about 2 hours) changing the bathing

medium to normal Krebs solution (i.e. containing no Cs and 4.6 mM K)

produced a change in spike activity although no change in membrane

potential after 20 min. (see last frame of Fig.17).

If the Cs concentration in the recovery medium was doubled

(9.2 mM) the recovery of contractility and electrical activity in
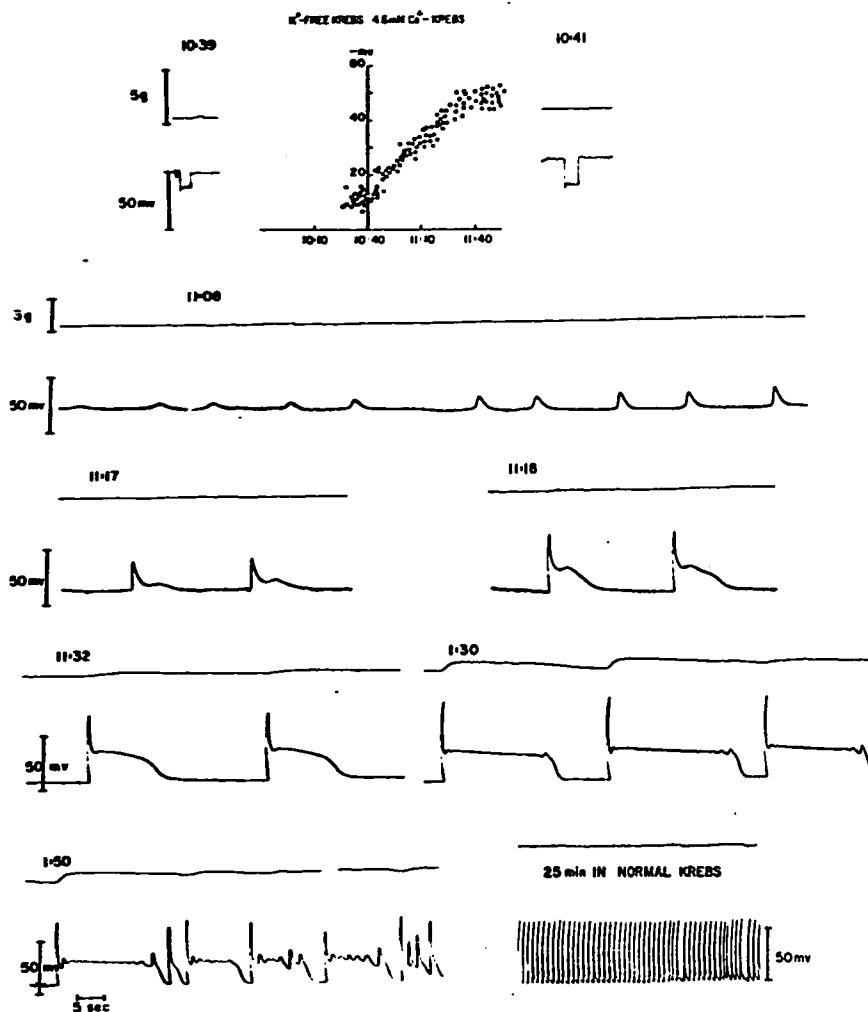
Figure 17. The effect of 4.6 mM Cs-Krebs solution on the recovery of the mechanical and electrical activity of Na-rich rat uterus. The graph (inset) shows the recovery of the membrane potential before and after the addition of 4.6 mM Cs-Krebs solution to a Na-rich tissue. 4.6 mM Cs Krebs solution was added to the tissue at 10:40 a.m. and the surrounding records show the characteristics of the development of spontaneous "plateau-type" action potentials, with large overshoots. After 190 min. incubation in 4.6 mM Cs-Krebs solution the bathing medium was changed to normal Krebs solution. The last frame (bottom right) shows the change in spike activity after 25 min. incubation in normal Krebs solution.

Na-rich tissues was as shown in Fig.18. The increase in membrane potential produced by 9.2 mM Cs is shown graphically to correspond in time with the microelectrode penetrations shown surrounding the graph. In 7 tissues the initial spontaneous contractile response was accompanied by a single action potential; subsequent contractions were much larger and were accompanied by action potentials often resembling the plateau-type as previously shown in Fig.17. After 2 hours in 9.2 mM Cs solution the sustained increase in tension was accompanied by continuous firing of action potentials showing a large overshoot and delayed repolarisation, and the occasional plateau-type action potential was also observed as shown in Fig.18.

Even after 2 hours exposure of a Na-rich tissue to 9.2 mM Cs-Krebs solution a rapid reduction in membrane potential was achieved by washing the tissue with a Cs-free Krebs solution. The depolarisation produced by this procedure is shown in a record from the same cell in Fig.19A. The depolarisation induced by Cs-free Krebs solution could be reversed and repolarisation of the membrane achieved once again by the addition of Cs-containing Krebs solution (Fig.19 middle frame). The lower portion of Fig.19 (Fig.19B) demonstrates the effect of 10^{-3}M ouabain on the membrane potential after repolarisation of the membrane induced by 9.2 mM Cs from one of 4 similar experiments. In this record taken from the same cell throughout, the increase in tension caused by ouabain and subsequent depolarisation and decrease in tension are demonstrated.

After 20-30 min. in 18.4 mM Cs-Krebs solution Na-rich tissues

Figure 18. The effect of 9.2 mM Cs-Krebs solution on the recovery of the mechanical and electrical activity of Na-rich rat uterus. The graph (inset) shows the effect of 9.2 mM Cs-Krebs solution on the recovery of the membrane potential of Na-rich uterus. The horizontal line to the left of the ordinate represents the mean membrane potential ($\pm$ SE) and extends over the period during which penetrations were made in a Na-rich tissue in K-free Krebs solution. Each point to the right of the ordinate represents 1 penetration after the addition of 9.2 mM Cs-Krebs solution to a Na-rich tissue. The recordings surrounding the graph show the changes in membrane potential and mechanical activity before (9:47 a.m.) and at various times after the addition of 9.2 mM Cs-Krebs solution. The record taken at 10:29 a.m. shows the first spontaneous contraction and action potential with a large overshoot. At 10:47a.m. the spontaneous contraction was accompanied by a "plateau-type" action potential. The lower recording (11:50 a.m.) was taken 2 hours after the addition of 9.2 mM Cs-Krebs solution to a Na-rich tissue.

A
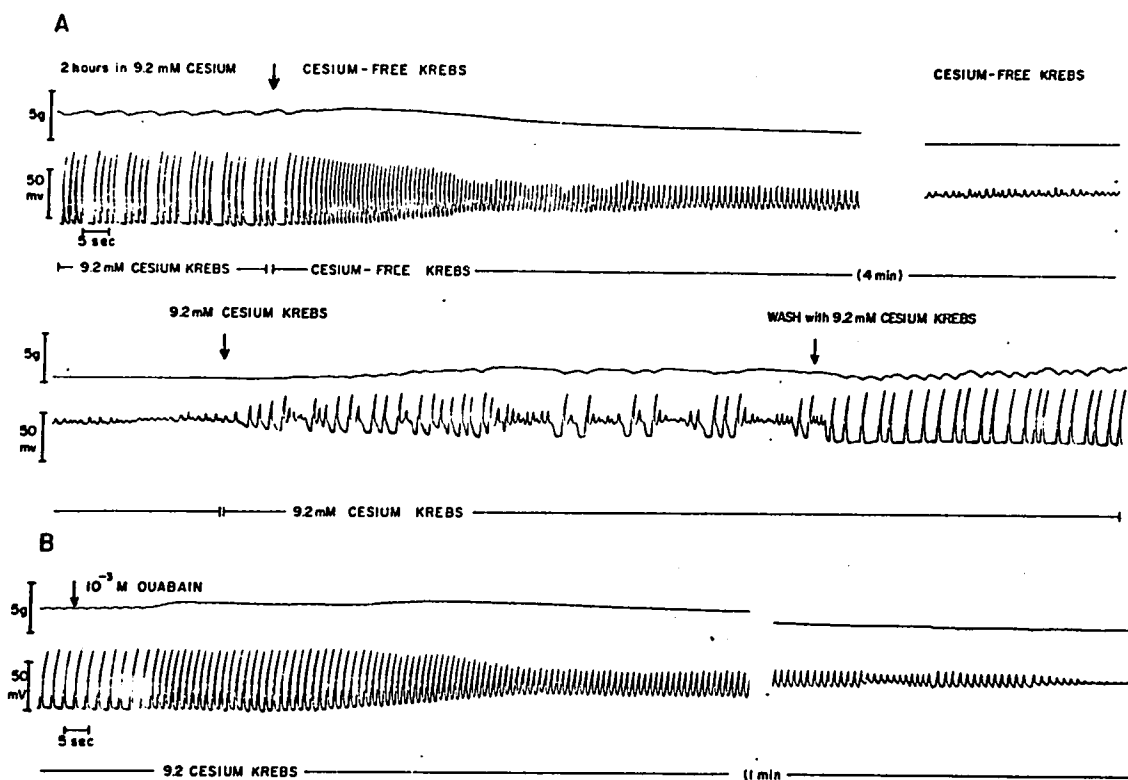
Figure 19. The effect of removal and addition of Cs on the mechanical and electrical activity of Na-rich uterus after 2 hours in 9.2 mM Cs-Krebs solution. A, the decrease in membrane potential after the removal of Cs from a Na-rich tissue incubated in 9.2 mM Cs-Krebs solution for 2 hours. The middle membrane potential recording was taken from the same cell as A and shows the effect of reintroducing 9.2 mM Cs-Krebs solution to the tissue; B, the effect of ouabain $(10^{-3}M)$ on the membrane potential of another Na-rich tissue incubated for 2 hours in 9.2 mM Cs-Krebs solution.

showed spontaneous bursts of action potentials accompanied by contractions. The increase in membrane potential, after transfer of Na-rich tissues to 18.4 mM Cs-Krebs solution, was slow in onset and after 15 min. in this medium the membrane potential had attained 61.3 $\pm$ 0.7 mV (average of 10 penetrations) in 2 out of 5 tissues examined. The large membrane potentials noted above occurred after 15 min. in Cs-Krebs solution and were not recorded as an immediate hyperpolarisation as observed upon the addition of similar concentrations of Rb or K to Na-rich tissues. Fig.20 demonstrates the type of activity recorded after 46 min. in 18.4 mM Cs-Krebs solution. After 1 hour in this medium, the removal of Cs from the bathing medium produced a fall in membrane potential accompanied by a slight relaxation. Whilst the microelectrode remained within the same cell, 18.4 mM Cs was reintroduced to the organ bath as shown in Fig.20 (lower frame); an increase in membrane potential was observed. A fall and then an increase in membrane potential was observed repeatedly in 3 different tissues by respectively the removal and then addition of Cs to recovered Na-rich tissues.

A rapid increase in membrane potential (78.3 $\pm$ 0.5 mV, 44 penetrations) was observed within 2 min. of the introduction of 46 mM Cs-Krebs solution to a Na-rich tissue. The subsequent decline in membrane potential is shown in Fig.21 for 4 tissues. Spontaneous contractility occurred 15-20 min. after introducing the Cs-containing solution (Fig.21A). Plateau-type action potentials were not observed in 4 experiments where 46 mM Cs was added to Na-rich tissues. Records B to E of Fig.21 were taken from a different cell than A, whilst the microelectrode
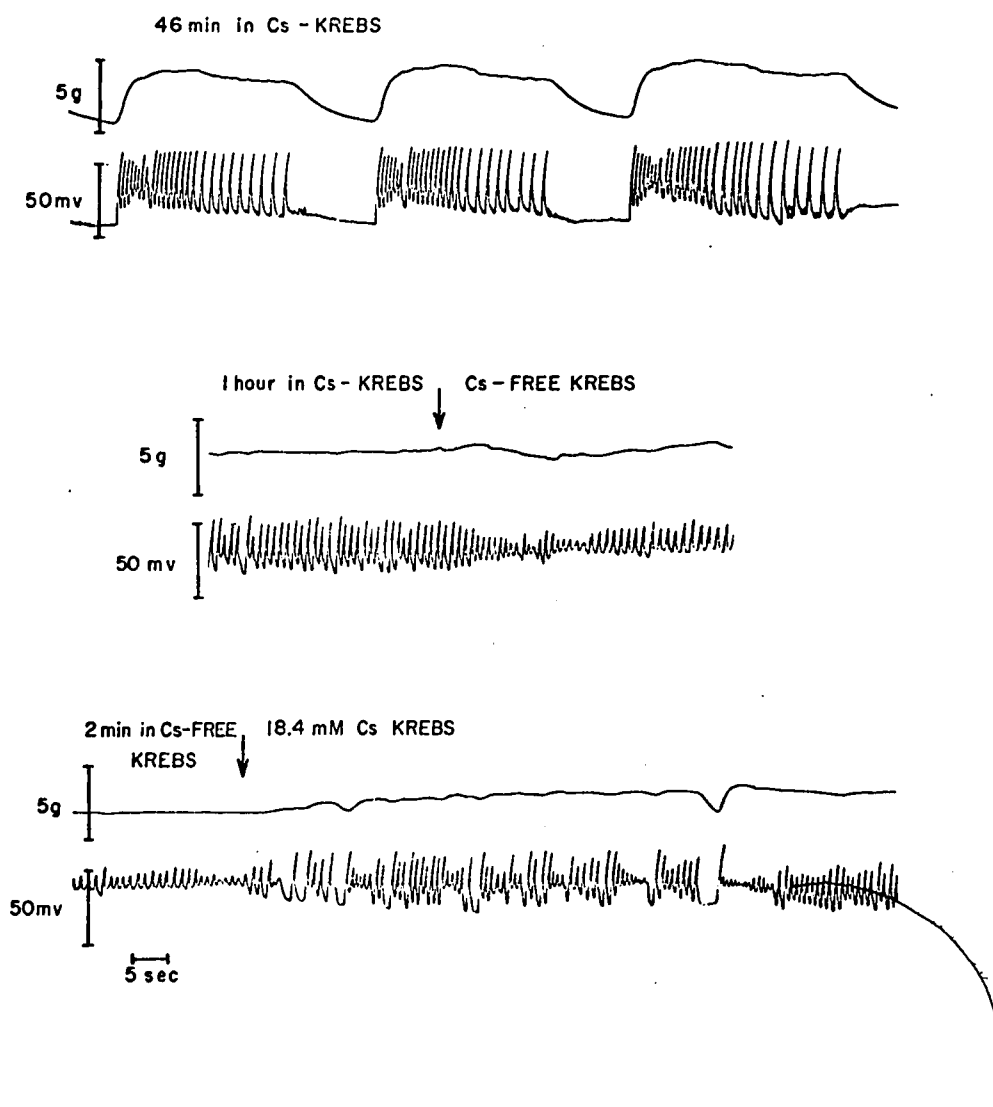
Figure 20. The effect of removal and addition of Cs on the mechanical activity and membrane potential of a Na-rich tissue after prolonged exposure to 18.4 mM Cs-Krebs solution. The upper recording shows the electrical and mechanical activity of a Na-rich tissue after 46 min. exposure to 18.4 mM Cs-Krebs solution. The effect of Cs-free Krebs solution on the membrane potential is shown in the middle recording after 1 hour incubating of a Na-rich tissue to 18.4 mM Cs-Krebs solution. After 2 min. in Cs-free Krebs solution, the membrane potential change in response to reintroducing 18.4 mM Cs-Krebs solution to the same tissue is shown in the lower recording.
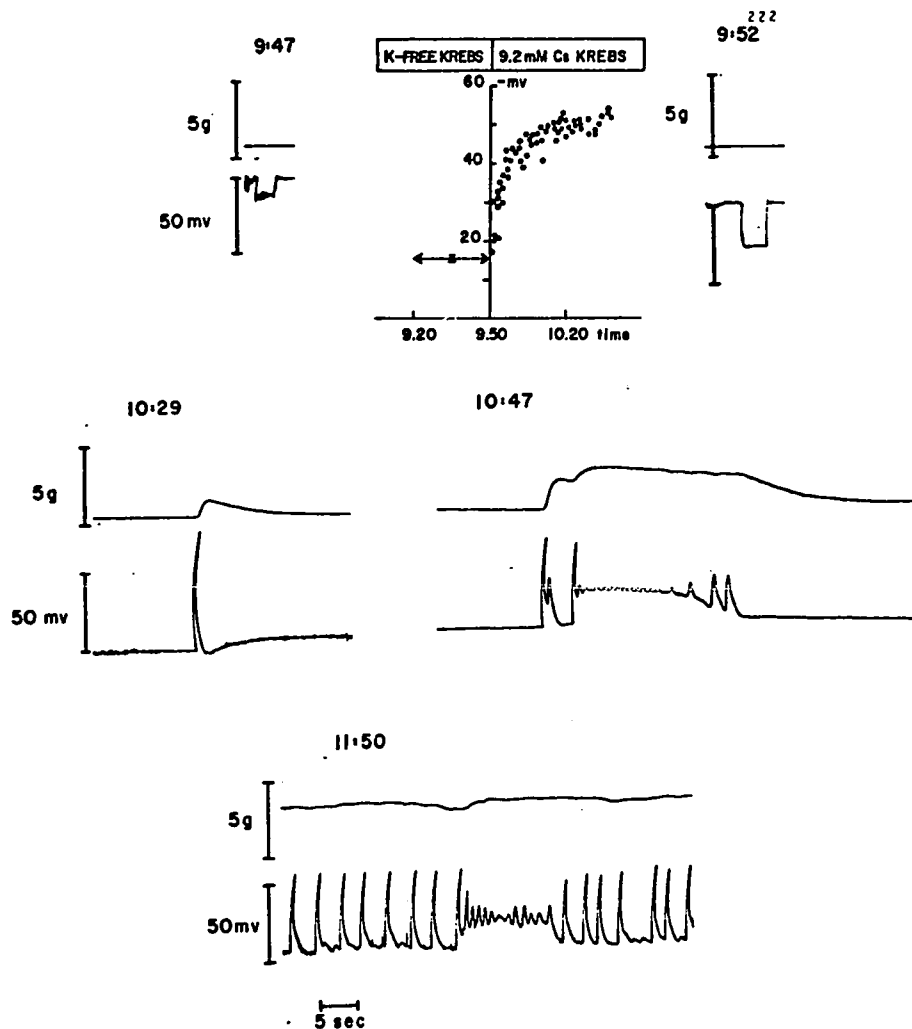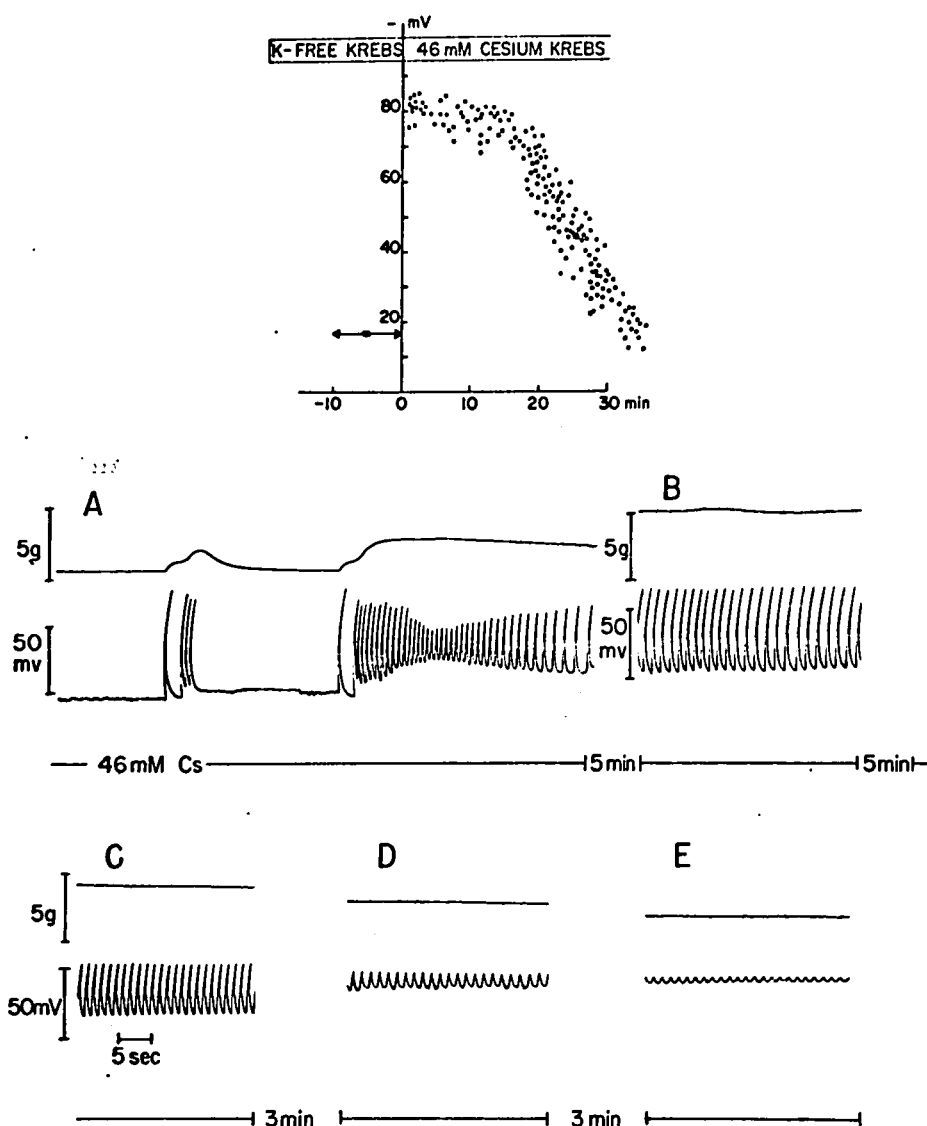
Figure 21. The effect of 46 mM Cs-Krebs solution on the recovery of the mechanical and electrical activity of Na-rich rat uterus. The graph shows the rapid increase in membrane potential when 46 mM Cs-Krebs solution was added to a Na-rich tissue. To the left of the ordinate the horizontal line represents the mean ($\pm$ SE) membrane potential and the length of line the time during which penetrations were made in the Na-rich tissue. Each point to the right of the ordinate represents 1 penetration in 46 mM Cs-Krebs solution. (Data from 4 tissues); A, shows the first spontaneous mechanical and electrical activity of a Na-rich uterus in the presence of 46 mM Cs-Krebs solution. Records B to E, show the decrease in membrane potential over the next 16 min. in the presence of 46 mM Cs-Krebs solution.

remained in the cell for 16 min. 25-30 min. after the introduction of 46 mM Cs the membrane potential had decreased to 20.6 $\pm$ 0.5 mV (39 penetrations in 4 tissues). After 30 min. exposure to 46 mM Cs-Krebs solution, removal of Cs did not produce a significant increase in the membrane potential of 4 tissues. The membrane potential before the removal of 46 mM Cs was 20.6 $\pm$ 0.5 mV (39 penetrations in 4 tissues) and 19.8 $\pm$ 0.7 mV (24 penetrations in 4 tissues) in Cs-free Krebs solution.

After 30 min. incubation of fresh tissues in 46 mM Cs-Krebs solution the membrane potential had decreased to 41.6 $\pm$ 1.0 mV (19 penetrations in 2 tissues). In contrast the membrane potential of fresh tissues exposed to 46 mM K-Krebs solution had decreased to 23 mV (mean 16 penetrations in one tissue) after 5 min. exposure to this medium.

Two experiments were carried out to investigate the effect of ouabain on the membrane potential changes induced by removal of K from a Na-rich tissue depolarised with 46 mM K-Krebs solution. After 40 min. in 46 mM K-Krebs solution the membrane potential of two tissues was 24.2 $\pm$ 0.5 (11 penetrations). The removal of 46 mM K from the bathing medium caused the membrane potential to increase to 49.5 $\pm$ 1.1 (16 penetrations in 2 tissues). A similar type of experiment was carried out in which ouabain 10^{-3}M was added for 10 min. to two tissues depolarised in 46 mM before K-free Krebs solution was added to these tissues. The addition of K-free Krebs solution containing ouabain increased the membrane potential from about 24 mV to only 32.5 $\pm$ 1.9 (16 penetrations in 2 tissues). In

this instance ouabain had reduced the increase in membrane potential, produced by the addition of K-free Krebs solution to a Na-rich tissue depolarised in 46 mM K-Krebs solution, by about 17 mV.

In summary, the results of substituting Cs for K show that:-

(1) Fresh tissues incubated in 4.6 mM Cs-Krebs solution for 2 hours did not show any appreciable decline in membrane potential or contractile activity.

(2) Concentrations of Cs below 4.6 mM were unable to restore contractility or the membrane potential of Na-rich tissues with the experimental time periods discussed above.

(3) Na-rich tissues placed in a medium containing 4.6 mM Cs showed a slow increase in membrane potential and abnormal spike activity, including plateau-type action potentials and spontaneous contractions. The membrane potential did not increase above the value found in fresh tissues, incubated in normal Krebs solution, either before or after the onset of action potentials and spontaneous contractions.

(4) Higher concentrations of Cs 18.4 mM produced a hyperpolarising response in 2 out of 5 tissues allowed to recover from the Na-rich state in this medium.

(5) A large, rapid increase in membrane potential was observed when Na-rich tissue were exposed to 46 mM Cs-Krebs solution. After 30 min. exposure to 46 mM Cs-Krebs solution Na-rich tissues were depolarised to about 20 mV. The depolarisation was not increased or reversed by the removal of Cs at this time. A more rapid depolarisation occurred when Na-rich tissues were exposed to 46 mM K-Krebs solution.

Removal of K at this time produced an increase in membrane potential,
the increase in membrane potential was reduced by the presence of
ouabain during the removal of K.

IIIB (e) Modification of recovery medium.

(iv) Changes in Sodium concentration

Replacement of total Na in normal Krebs medium with sucrose
caused a fall in resting potential in 3 fresh tissues from 49.3 ± 0.5
(23 penetrations) to 31.1 ± 0.6 (23 penetrations) within 30 min.
Fig.22A shows a control contraction recorded in normal Krebs medium
and, B a typical penetration of about 30 mV after 25 min. in Na-free
Krebs solution. Fig.22 also shows the effect of addition of Na-free
Krebs solution (sucrose substituted) containing 5.7 mM K (as $KHCO_3$)
to a Na-rich tissue. In 3 different tissues the membrane potential
of Na-rich tissues increased to 70.6 ± 0.5 (30 penetrations) in
response to the addition of Na-free Krebs solution. Fig.22D shows
the increase in membrane potential and Fig.22E and F the decrease
in membrane potential to about 30 mV after 30 min. in Na-free Krebs
solution. The elevated K concentration (i.e. 5.7 mM as opposed to
4.6 mM) in the sucrose medium was probably not responsible for the
depolarisation because Na-rich tissues allowed to recover in normal
Krebs solution containing 9.6 mM K had not depolarised 60 min. or
more after the addition of this concentration of K to the recovering
tissue. Fig.23 shows graphically the recovery of the membrane
potential of 2 Na-rich tissues after changing the bathing medium from
K-free Krebs solution, to Na-free (sucrose substituted) Krebs solution
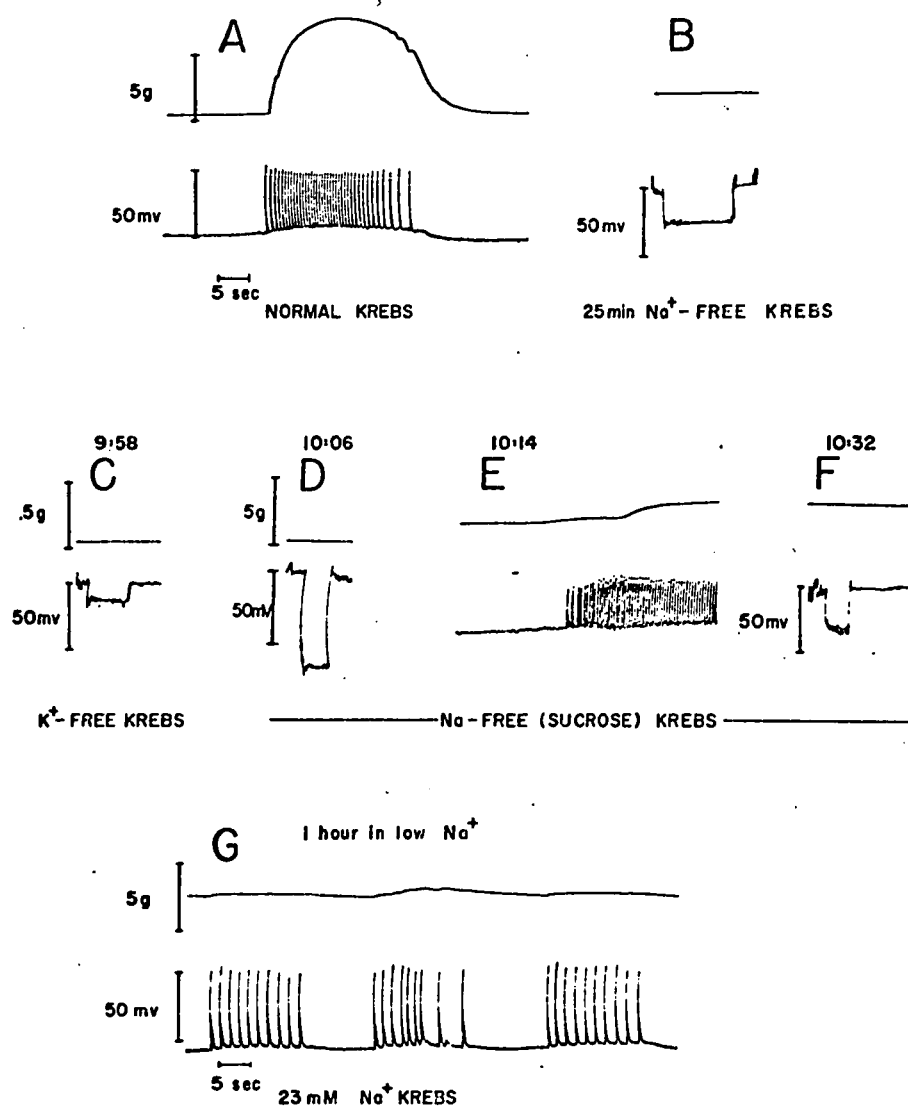
Figure 22. The effect of low Na solutions on the mechanical and electrical activity of fresh and Na-rich rat uterus. Records A and B, show the effect of Na-free solution (sucrose-substituted) on the membrane potential of fresh tissues. B, was taken 25 min. after changing the bathing medium to Na-free solution; C, shows the membrane potential of a Na-rich tissue in K-free Krebs solution; D, shows the hyperpolarisation in response to the addition of Na-free solution (5.7 mM K) to a Na-rich tissue. E and F, show the subsequent decrease in membrane potential in Na-free solutions; G, shows the membrane potential 1 hour after the addition of 23 mM Na-Krebs solution to a fresh tissue.
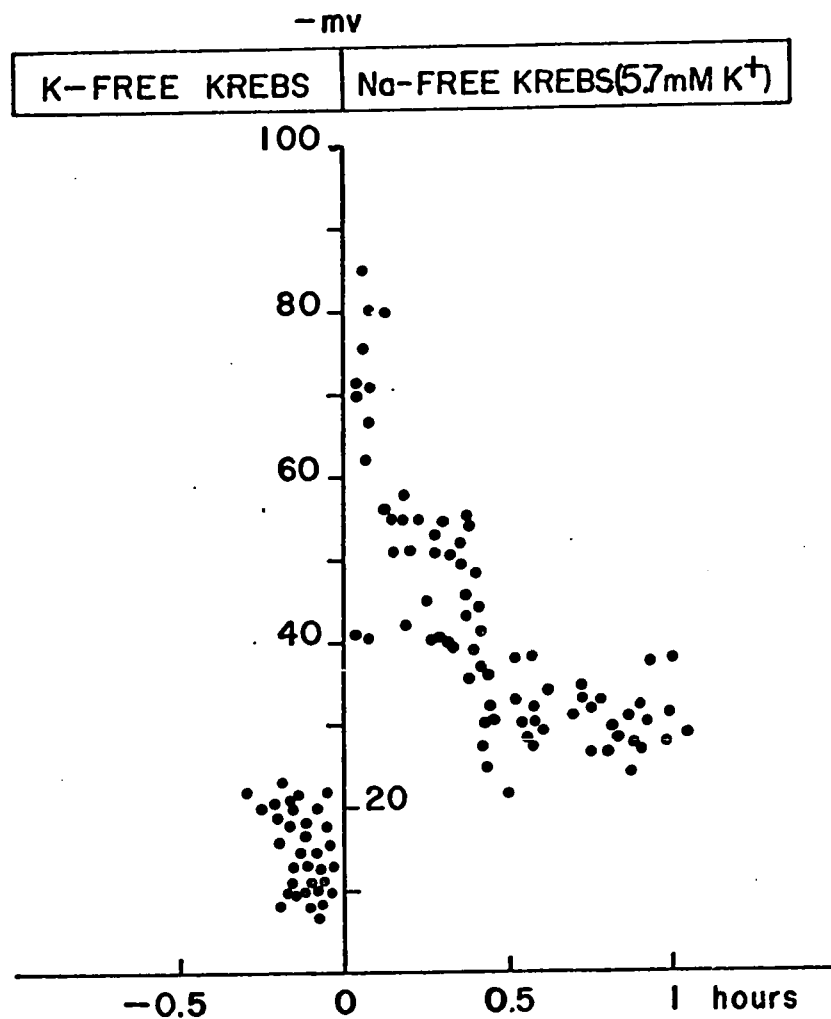
Figure 23. The recovery of the membrane potential of Na-rich rat uterus in Na-free Krebs solution (sucrose-substituted). The membrane potential of a Na-rich tissue is shown to the left of the ordinate. Na-free Krebs solution (containing 5.7 mM K) was added to the tissue at zero time and the membrane potential is shown to the right of the ordinate. Each point represents 1 penetration. Ordinate, membrane potential (-mV). Abscissa - time in hours.

containing 5.7 mM K.   In contrast to the fall in membrane potential

produced in Na-free media, Na-rich tissues allowed to recover in

solutions containing a small amount of Na (23 mM Na, as $NaHCO_3$ + NaH_2PO_4)

did not show a depolarisation after 60 min. in this medium as shown in

Fig.22G.

Incubation of fresh tissues in solutions containing Li as

a substitute for Na demonstrated that Li was unable to support spontaneous

mechanical and electrical activity for longer than 60 min.   The fall

in membrane potential produced by Li-Krebs solution in fresh tissues

is shown in Fig.24.   After one hour in Li-Krebs solution the membrane

potential had fallen to 27.6 $\pm$ 0.9 (20 penetrations in 3 tissues) as

compared to 49.4 $\pm$ 0.5 mV (24 penetrations) in 3 control tissues

incubated for the same time in normal Krebs solution.   Upon the

removal of Li-Krebs solution and addition of normal Krebs solution

the tissue relaxed and showed an increase in membrane potential as

shown in Fig.25 (middle panel).   Thereafter, the tissue showed a

gradual decrease of membrane potential leading to action potentials

and relatively normal contractions as shown in the lower portion of

Fig. 24.

Na-rich tissues placed in Na-free, Li-Krebs solution showed

a rapid increase in membrane potential as shown in Fig.25.   In 2

tissues the membrane potential was 74.4 $\pm$ 1.2 mV (18 penetrations).

A comparison of the effect of Na-free Li-Krebs solution with normal

Krebs solution is shown graphically in the upper part of Fig.25.

The hyperpolarisation produced in Li-Krebs solution declined over the

next 80 min. and small spikes were visible at a membrane potential of
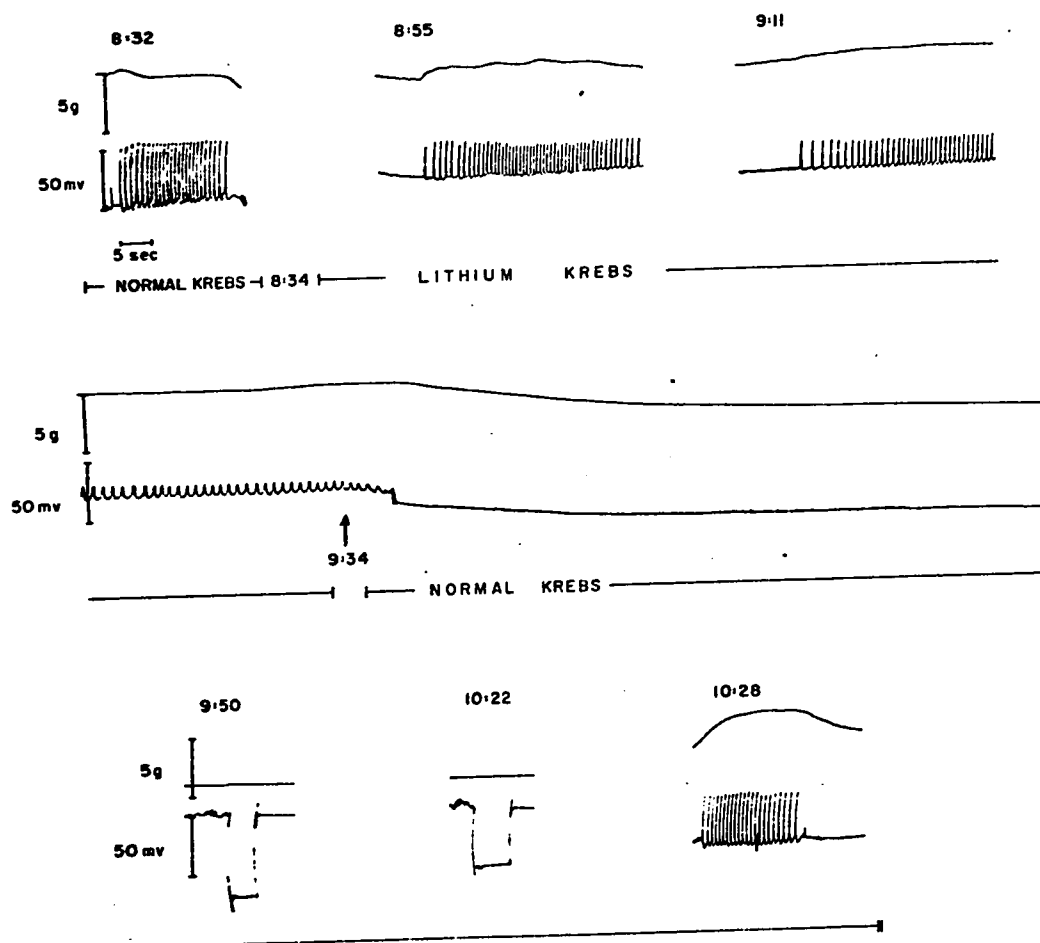
Figure 24. The effect of Li-Krebs solution (Na-free) on the electrical and mechanical activity of fresh tissues. The upper record shows the effect of Li-Krebs solution (Na-free) on the membrane potential and mechanical activity of a fresh tissue after 21, and 37 min. in Li-Krebs solution. The middle record shows the effect of the addition of normal Krebs solution (at 9:34 a.m.) to a tissue depolarised after 60 min. incubation in Li-Krebs solution. The lower record shows the subsequent recovery of spontaneous activity (at 10:28 a.m.) in the same tissue in normal Krebs solution.

Figure 25. The effect of Li-Krebs solution (Na-free) on the recovery of mechanical and electrical activity of Na-rich rat uterus. The two graphs show the change in membrane potential produced by the addition of either normal Krebs solution (left hand graph) or Li-Krebs solution (right hand graph) to Na-rich tissues. The horizontal lines to the left of each ordinate represent the mean membrane potential (± SE) before the addition of either normal Krebs solution or Li-Krebs solution. The lower recordings show the membrane potential changes from a typical experiment during which Li-Krebs solution was added to a Na-rich tissue at 9:07 a.m. At 9:30 a.m. the bathing medium was changed to normal Krebs solution and the penetration made at 9:33 a.m. shows the increase in membrane potential observed. The lower record was taken from the same tissue after 40 min. in normal Krebs solution.

about 20 mV. As described above for fresh tissue, the introduction of

normal Krebs solution to the tissue at this time produced an increase

in membrane potential; however, the magnitude of this potential

observed in 4 experiments was never greater than 60 mV. Fig.25 shows

a recording taken at 9:33 a.m., 3 min. after the introduction of normal

Krebs solution to a Li-depolarised tissue. About 40 min. later

spontaneous mechanical and electrical activity had resumed as shown in

the lower half of Fig.25.

Further experiments were carried out in which Li was

substituted for K in a normal Krebs solution. In these experiments

4.6 mM Li in a K-free Krebs solution (containing normal quantities of

Na) was not able to restore the membrane potential of a Na-rich tissue.

However, these tissues subsequently recovered a normal membrane

potential after initial hyperpolarisation when 4.6 mM K was introduced

to the organ bath. Higher concentrations of Li in the absence of K

were not investigated.

Incubation of fresh tissues in a Na-free Li-substituted

solution containing no K at $4^{\circ}C$ for 18 hours produced a tissue

containing little K and large quantities of Li (Daniel 1965).

Tissues prepared in this way will be referred to as "Li-rich" in an

analogous manner to Na-rich tissues.

The membrane potential recorded from 3 Li-rich tissues was

7.1 $\pm$ 1.8 (52 penetrations). After 40 min. in Li-Krebs containing

K (4.6 mM) the membrane potential was 7.0 $\pm$ 0.4 (30 penetrations in

3 tissues); furthermore, 60 min. after the removal of Li and addition

of normal Krebs solution to these tissues no increase in membrane

potential was observed. Pieces of tissue taken from the same animal in each of 3 experiments above, that were made Na-rich in K-free solution in the usual way and then placed in normal Krebs solution, recovered their membrane potential with hyperpolarisation in a normal fashion. Elevation of the K concentration to 46 mM in Li recovery media bathing Li-rich tissues did not increase the membrane potential above that recorded in the Li-rich state.

The experiments involving substitution of either sucrose or Li for Na may be summarised as follows:-

(1) Fresh tissues incubated in sucrose substituted Na-free Krebs solution showed a decrease in membrane potential after 30 min. in this medium.

(2) If a small quantity of Na (23 mM) was present in the bathing medium surrounding Na-rich tissue, a decrease in membrane potential had not occurred after 60 min.

(3) Fresh tissues placed in Li-containing Na-free solutions showed a reversible fall in membrane potential and decline in spontaneous contractility after 60 min. Na-rich tissues recovered in Li-substituted Na-free solutions showed hyperpolarisation followed by a more marked decline in membrane potential than in Na-containing solutions. The marked fall in membrane potential after exposure to Li-Krebs could be reversed by the addition of normal Krebs solution, although the magnitude of the initial increase in membrane potential

recorded was less than the hyperpolarisation produced by adding

Li-Krebs containing 4.6 mM K to a Na-rich tissue.

(4) Li (4.6 mM) could not substitute for K in restoration

of the membrane potential of Na-rich tissues.

(5) Li-rich tissues showed membrane potentials of about

7 mV.   No increase in membrane potential was observed in these

tissues when either 4.6 mM K, Li-Krebs, normal Krebs, or 46 mM K Li-Krebs

was introduced into the organ bath.

IIIB (e) <u>Modification of the recovery medium.</u>

(v) Replacement of Chloride

The results of the previous section involving sucrose substitute

Na-free solutions generally indicated a deterioration in the condition

of fresh or Na-rich tissues after prolonged exposure to this medium.

Substitution of sucrose for Na in these experiments involved the

replacement of Na and chloride with sucrose.   The experiments of

the following section were carried out in order to investigate the

effects of removal of chloride upon the recovery of Na-rich tissues

by the replacement of NaCl with sodium methyl sulphate $(NaCH_3SO_4)$.

The recovery of the membrane potential of Na-rich tissues

in $NaCH_3SO_4$ - Krebs solution is shown in the potential profile graph

of Fig.26, plotted along with the membrane potential changes

recorded in 2 tissues from the same uterus allowed to recover in

normal Krebs solution.   The membrane potential was significantly

larger (79.9 $\pm$ 0.6 mV 19 penetrations) when $NaCH_3SO_4$-Krebs solution
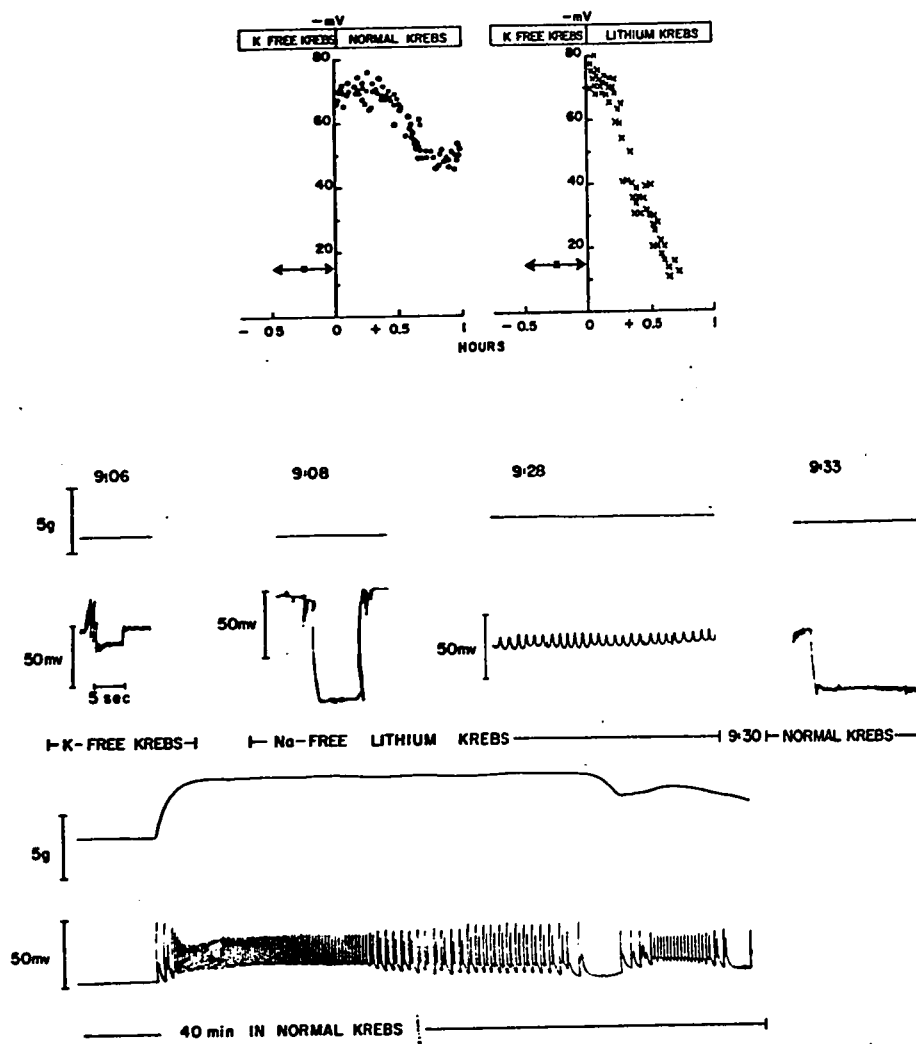
was added to Na-rich tissues as compared to that observed
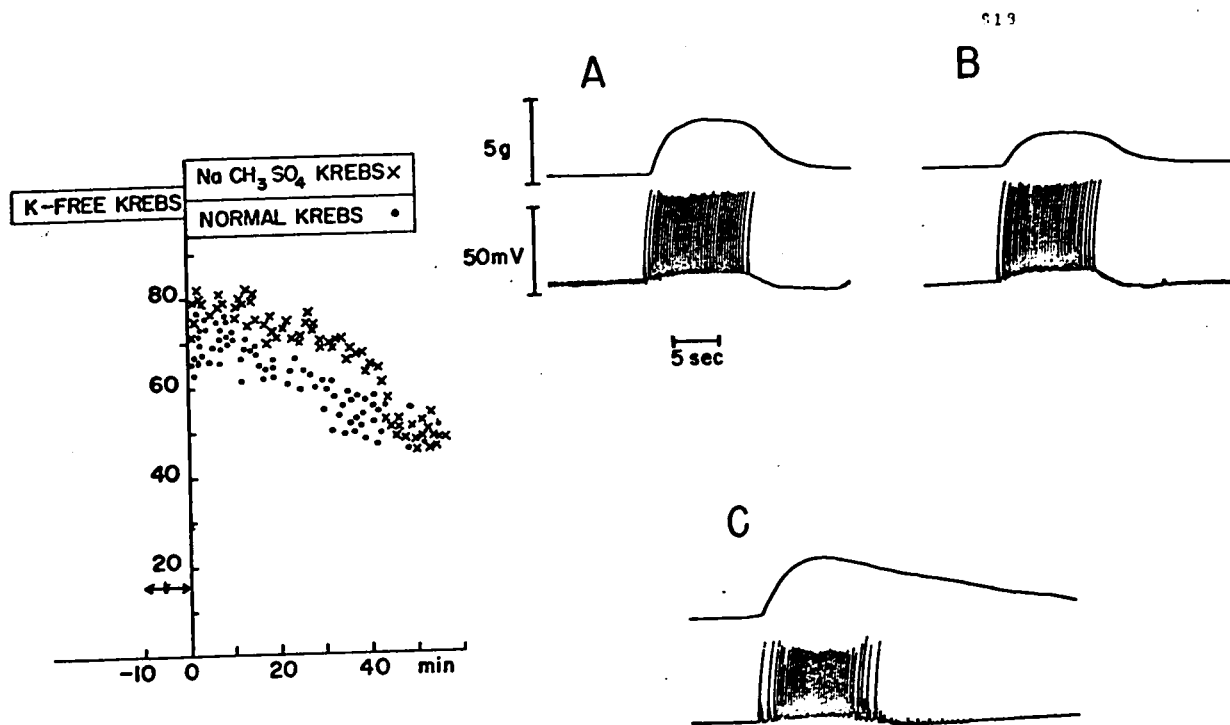
Figure 26. The effect of NaCH₃SO₄-Krebs solution on the recovery of mechanical and electrical activity of Na-rich rat uterus. The graph shows the change in membrane potential produced by changing the bathing medium of a Na tissue to either NaCH₃SO₄-Krebs solution (each x represents 1 penetration) or normal Krebs solution (each ● represents 1 penetration). Recordings A and C show spontaneous activity recorded in an initially Na-rich tissue after 40 min. (A) and 60 min. (c) incubation in NaCH₃SO₄-Krebs solution. For comparison, record B was taken from another tissue and shows typical spontaneous activity after 40 min. in normal Krebs solution.

(70.2 $\pm$ 0.7 mV, 25 penetrations) when normal Krebs solution was added to a Na-rich tissue. Both tissues recovered spontaneous contractions after about 40 min. in their respective media. Fig.26A shows a contraction recorded after 40 min. in $NaCH_3SO_4$-Krebs solution and Fig.26C a similar contraction recorded from tissues recovered in normal Krebs solution for 40 min. Frame B of this figure was recorded 20 min. after record A in $NaCH_3SO_4$-Krebs solution.

The results of the experiments described above using recovery solutions containing low concentrations of chloride may be summarised as follows:-

(1) Na-rich tissues hyperpolarised when placed in media containing K. The observed hyperpolarisation was significantly greater in tissues allowed to recover in the presence of low concentrations (less than 10 mM) of chloride.

IIIB (f) <u>The effect of catecholamines on the recovery of the membrane potential in Na-rich tissues.</u>

In this section attempts have been made to differentiate α and β effects of catecholamines on the membrane potential of partially recovered Na-rich tissues by the use of Noradrenaline with Propranolol (to observe the effect of α-receptor stimulation) and Isopropyl noradrenaline (to observe the effects of β-receptor stimulation).

In the present series of experiments the figures shown represent the effects of catecholamines whilst the microelectrode

remained within the same cell. All the observed effects were repeated with the same results in at least 5 different tissues. No statistical analysis has been attempted as numerous penetrations were not made before and after the addition of each drug.

In the presence of propranolol (1 μg/ml)noradrenaline did not modify the hyperpolarisation caused by the addition of normal Krebs solution to a Na-rich tissue as shown in Fig.27A. Record B was taken from the same cell about 5 min. after the addition of noradrenaline. The decrease in membrane potential caused by removal of K from Na-rich tissues is shown in frames C and D of Fig.27; the effect of norepinephrine (1 μg/ml) on the depolarisation is shown in Fig.27E. Frame F of Fig.27 shows the membrane potential about 2 min. after the addition of norepinephrine. Norepinephrine did not modify the initial hyperpolarising response or the depolarisation produced upon the removal of K within 2 min. of application of this drug. 7 similar experiments were carried out using propranolol and norepinephrine as described above and showed no detectable change in membrane potential upon the addition of norepinephrine to partially recovered Na-rich tissues.

Fig.28A shows the inhibition of spontaneous activity of a fresh tissue produced by the addition of 1 μg/ml isoproplynorepinephrine (INE) to the bath. Spontaneous contractions ceased and, although no detectable change in resting membrane potential was observed in the same cell for 4 min. (Fig.28B), action potentials were inhibited by this catecholamine. Frames C and D of Fig.28 show the lack of effect of INE on the membrane potential when added to a quiescent fresh

A 2:03 p.m.

NORMAL KREBS

5g

NE 1 μg/ml

B 2:08

50mV

5 sec

C 2:14

NORMAL KREBS

K FREE KREBS

D 2:16

K FREE
WASH

E 2:17

K FREE KREBS

NE 1μg/ml

F 2:19
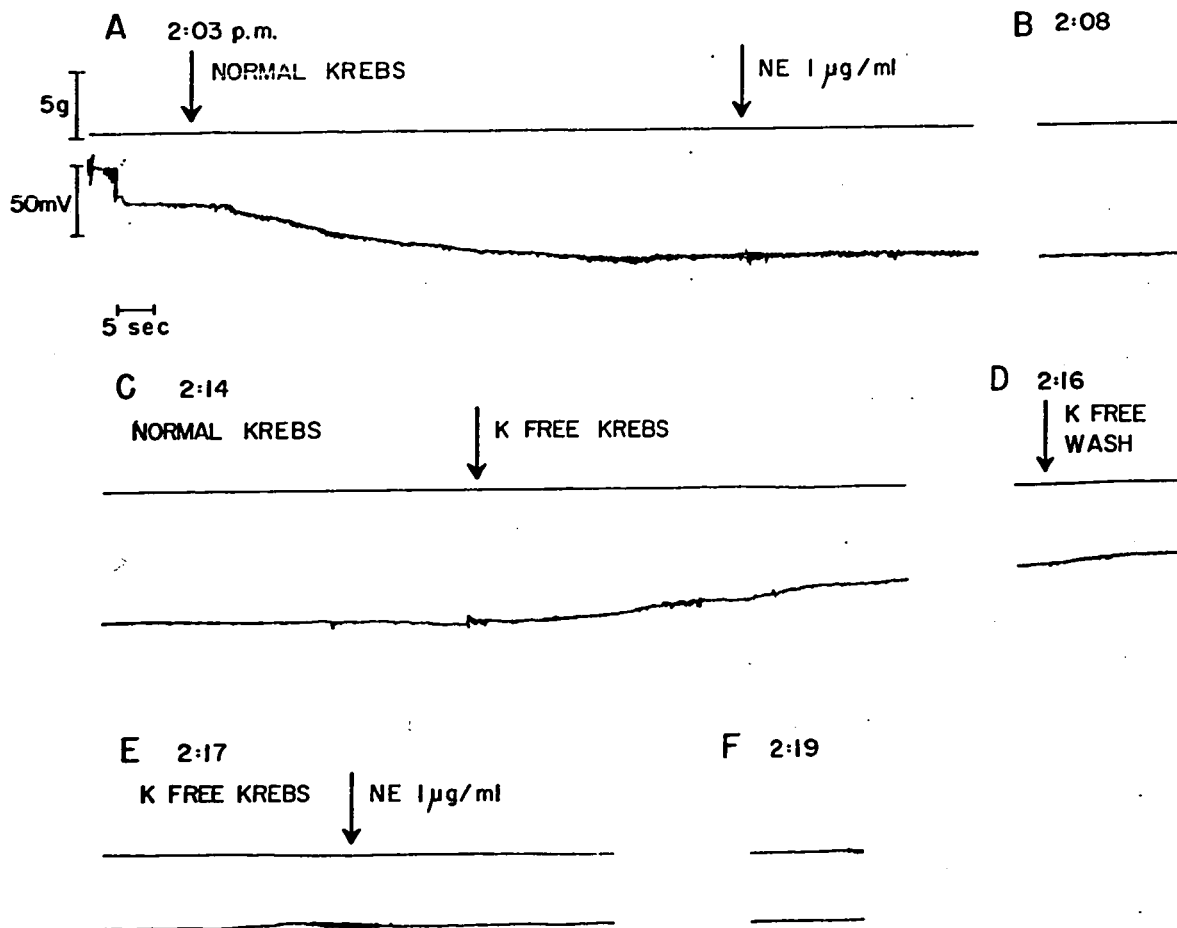
Figure 27. The effect of Norepinephrine (NE) on the recovery of the membrane potential of Na-rich rat uterus (propranolol 1 μg/ml present throughout). A, membrane potential change upon the introduction of normal Krebs solution to a Na-rich tissue and the effect of NE on the induced hyperpolarisation; B, 5 min. after introduction of NE recorded from the same cell as A; C, decrease in membrane potential upon changing bathing medium from normal Krebs solution to K-free Krebs solution; D, further reduction of membrane potential in the same cell as C after washing with K-free Krebs solution; E, effect of NE on membrane potential after reduction of membrane potential by removal of external K; F, 2 min. later, same cell as E.
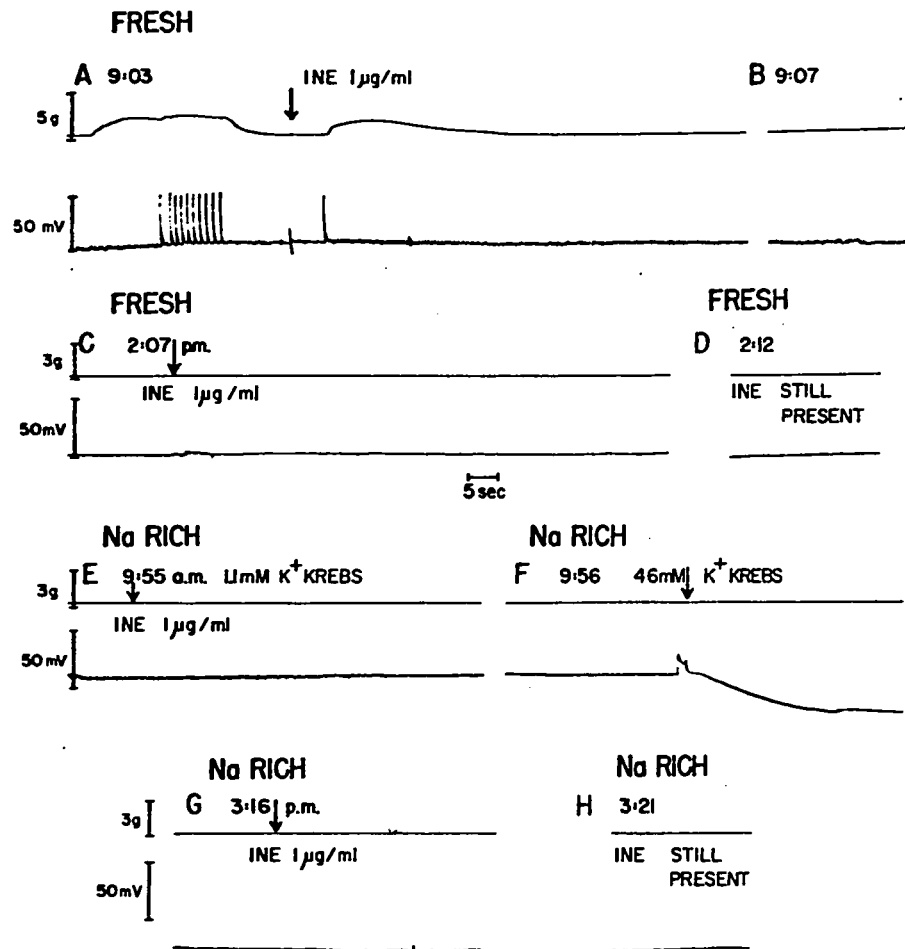
Figure 28. The effect of isoproterenol (INE) on the membrane potential
and contractility of Na-rich and fresh pregnant rat uterus.
A, effect of INE on membrane potential and contractility of
fresh spontaneously active rat uterus;   B, same cell as A
4 min. later in the presence of INE;   C, effect of INE on
membrane potential of fresh quiescent rat uterus;   D, same
cell as C 5 min. later;   E, membrane potential of Na-rich
uterus after 25 min. in 1.1 mM K-Krebs solution;   F, membrane
potential change induced by changing bathing medium to
46 mM K-Krebs solution recorded from the same cell as E;
G, membrane potential 3 min. after changing bathing medium of
Na-rich tissue to normal Krebs solution and effect of INE upon
hyperpolarisation after 5 min;   H, recorded from the same cell
as G.

preparation.   25 min. after exposure of a Na-rich tissue to a Krebs

solution containing 1.1 mM K, INE had no detectable effect on the

membrane potential as shown in Fig.28E.   The addition of 46 mM K to

the same tissue caused an immediate hyperpolarisation as shown in

frame F of Fig.28.   (electrode in the same cell as frame E).   The

effect of INE on the membrane potential during hyperpolarisation

induced in a Na-rich tissue 3 min. after addition of normal Krebs

solution is shown in Fig.28G.   Record H was taken from the same cell

5 min. later.

The effect of INE was also investigated in Na-rich tissues

allowed to regain their membrane potential in 9.2 mM Cs-Krebs.

Fig.29A shows the typical reduction in membrane potential induced by

withdrawal of Cs and the lack of effect of INE on this depolarisation

is shown in Record B along with the ability of 9.2 mM Cs to restore

the membrane potential in the same cell.   Records C and D of Fig.29

were taken from another experiment in which the effect of INE was

observed upon the spontaneous contractions that occurred after

prolonged exposure to 9.2 mM Cs-Krebs.   Although a small relaxation

of contractile activity occurred there was no change in the membrane

potential or spike activity after 12 min. exposure to INE.

In summary, the effects of $\propto$ and β stimulation upon

recovering Na-rich tissues may be stated as follows:-

(1)   Stimulation of $\propto$ receptors with noradrenaline in the

presence of propranolol did not modify the hyperpolarisation after

restoration of normal Krebs to a Na-rich tissue.   Similarly

$\propto$ receptor stimulation did not modify the depolarisation produced
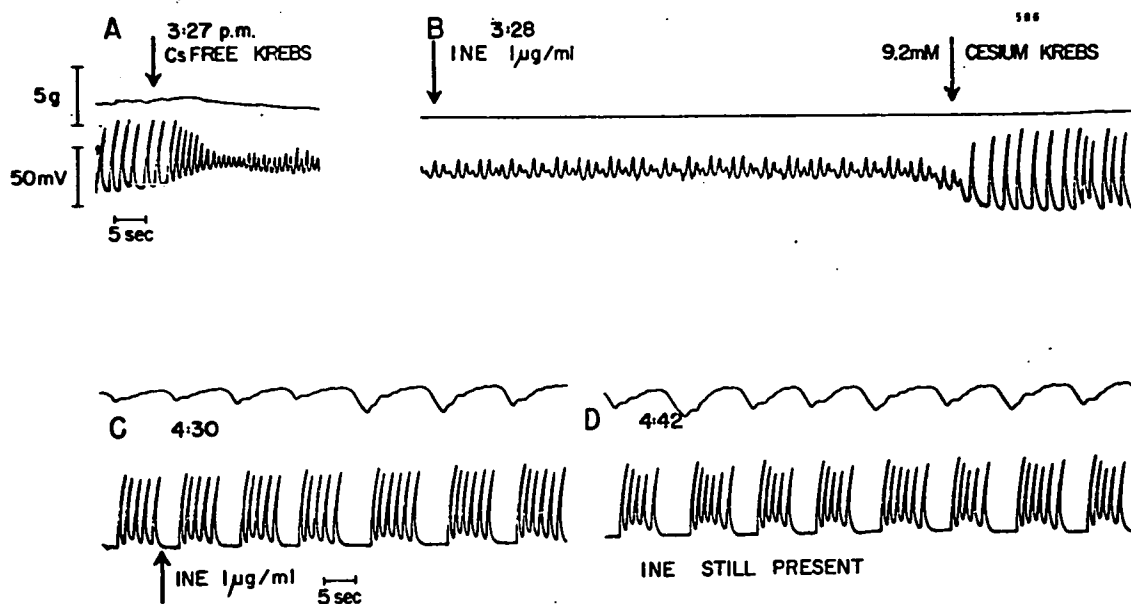
Figure 29. The effect of isoproterenol (INE) on the membrane potential of Na-rich tissues after 70 min. in 9.2 mM Cs-Krebs solution. A, effect of removal of Cs (replacement with Cs-free Krebs solution) upon the membrane potential of Na-rich tissue after 70 min. in 9.2 mM Cs-Krebs solution; B, lack of effect of INE on low membrane potential in absence of Cs, and the effect of reintroduction of 9.2 mM Cs-Krebs solution; C, lack of effect of INE on spontaneous activity in another tissue after prolonged exposure to 9.2 mM Cs-Krebs solution; D, same cell as C after 12 min. exposure to INE.

by removal of K during the early phase of the recovery of Na-rich tissues.

(2) Stimulation of β receptors with isopropylnorepinephrine caused an inhibition of spike activity and relaxation of fresh tissues without immediate change in membrane potential. Na-rich tissues recovered in low concentrations of K did not show any increase in membrane potential upon the addition of INE.

(3) Na-rich tissues recorded in 9.2 mM Cs-Krebs and then depolarised by withdrawal of Cs did not show any change in membrane potential upon the addition of INE. After prolonged exposure to 9.2 mM Cs no change in membrane potential was detected after 12 min. exposure to INE.

IIIB (g)   Extracellular space measurement in rat uterus.

An estimate of the extracellular space was made using ^{14}C-inulin as described in Methods (see Methods IId) and the results are shown, expressed in ml $H_2O/100$ gm. tissue, for two different experimental conditions in Fig.30. There was no significant difference between tissues prepared as described above for each of the uptake periods. A least squares best fit line between uptake periods 120 min. and 240 min. provided an intercept of 42.6 ml/100 g tissue. Using the last three points, viz 60, 120, 240 min. a least squares plot gave an intercept of 35.2 ml/100 g. The same data was applied to a method described by Goodford (1968) for approximate determination of the extracellular space and gave a value of 37.0 ml/100 g.
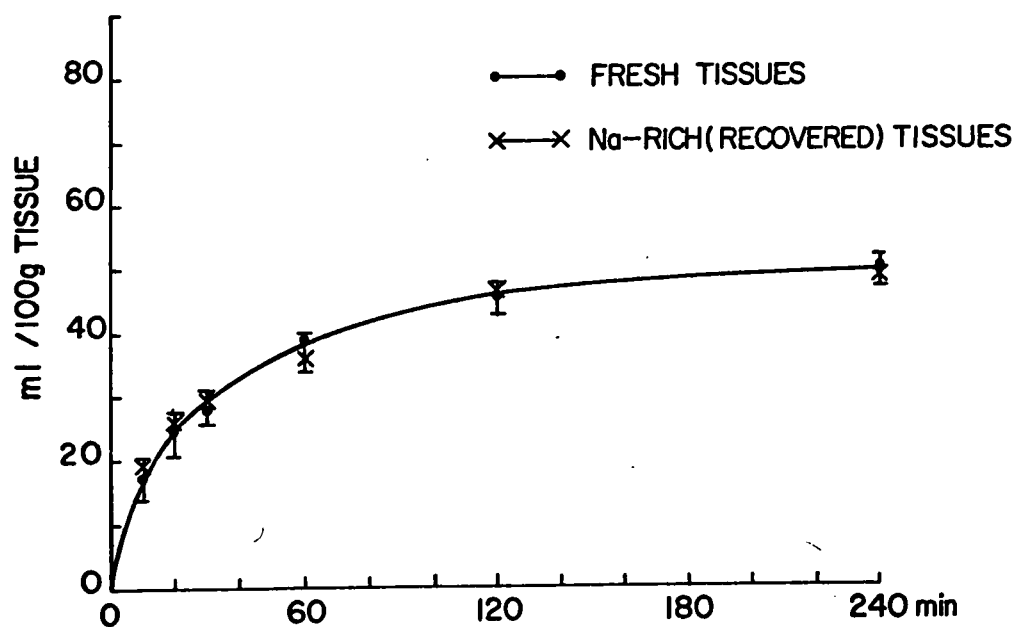
Figure 30. The uptake of ^{14}C-inulin in fresh rat uterus and Na-rich
rat uterus after recovery of membrane potential and
spontaneous contractility in normal Krebs solution. The
uptake of ^{14}C-inulin is shown as ml/100g tissue (see
Methods Ii and Results IIIB(g)) against time. Each
point represents mean $\pm$ SE for 7 tissues.

The latter method assumes that the uptake of tracer is represented by a double exponential function of the form.

$$F_{(t)} = A (1 - e^{-k_1 t}) + B (1 - e^{-k_2 t}) \quad .. \quad .. \quad .. \quad (5)$$

which may be approximated after the first few min. of uptake to

$$R = A + B (1 - e^{-k_2 t}) \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (6)$$

or

$$t \, k_2 = \log_e B - \log_e (1 - R) \quad .. \quad .. \quad .. \quad .. \quad .. \quad (7)$$

Where, R is the uptake of tracer at time t expressed as a fraction of the saturation level of uptake (R = 1). The first term contains the rate constant (k_1) for the initial uptake of inulin, which will be very large in comparison with k_2, therefore $e^{-k_1 t}$ may be taken as zero. (A + B) is the asymptotic limit of F(t), A and B are the amounts of ^{14}C-inulin present in compartments A and B expressed as a fraction of R.

A graph of $\log_e (1 - R)$ vs t from equation (7) provides a straight line with a slope of k_2 and an ordinate intercept of B, from which A the fraction of ^{14}C-inulin may be deduced since A + B = 1.

It is likely that after 4 hours the marker employed for the extracellular space, in experiments such as these, will occupy a greater volume than that regarded as the extracellular space. Preliminary studies in this laboratory by Dr.F.H. Osman have shown that the efflux of substances often used as extracellular markers (e.g. ^{14}C-inulin and ^{14}C-mannitol) do not obey the kinetics of a 2-compartment system after a 2 hour preincubation period (see Discussion).

For the purposes of the present study and until more information is available on this complex problem the value of 37.0 ml H_2O/100 g tissue determined in the present study by the method of Goodford (1968) has been chosen to represent the extracellular space. This value is in reasonable agreement with the value of 35.2 ml/100 g reported above on the basis of a least squares best fit line of the last three points of the saturation curve of ^{14}C-inulin. Casteels and Kuriyama (1965) reported a value of 376 ml/kg wet weight for the extracellular space of pregnant rat myometrium after 90 min. exposure to non-radioactive inulin.

IIIB (h) The uptake of Cesium and Potassium into Na-rich tissues.

In view of the differences noted above in the recovery of the membrane potential and subsequent action potentials of Na-rich tissues in either Cs or K-containing solutions, a series of preliminary experiments were conducted to determine the uptake of K and Cs by Na-rich tissues. In such experiments the amounts of Cs or K and Na present in the inulin space have been subtracted from the total tissue ion contents of Na, K and Cs. The extracellular spaces were not determined in Cs-containing solutions in these preliminary experiments and the value of 370ml/kg obtained in normal Krebs solution has been used for estimating the ionic contribution of the extracellular space to the total uptake. Thus the uptake data (see Figs.31,32 and 33) represents the tissue content of Na, Cs or K after subtraction of approximate extracellular ion content from total ion content.
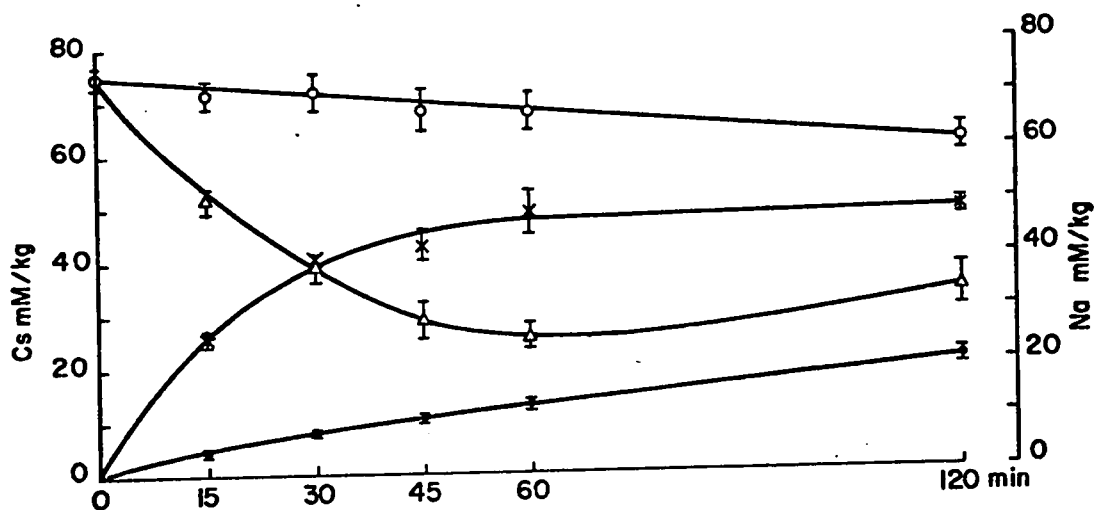
Figure 31. The uptake of Cs (as measured with ^{137}Cs-see Methods IIe) and loss of Na in Na-rich rat uterus at 37°C. Uptake of Cs into Na-rich tissues bathed in 4.6 mM (●—●) and 46 mM (x—x) Cs-Krebs solution; concomitant loss of Na from Na-rich tissues bathed in 4.6 mM (o—o) or 46 mM (△—△) Cs-Krebs. Each point is the mean ± SE of 8 determinations. The amounts of Cs or Na present in the extracellular space has been subtracted in each case (see text).

The initial uptake of Cs into Na-rich tissues from solutions containing 4.6 mM Cs was slow and after 120 min. in this solution had attained only about 22 mM/kg and had not yet equilibrated (Fig.31). By comparison the uptake of Cs from solutions containing 46 mM Cs was rapid and attained a higher concentration of tissue Cs. The tissue contents of Na of tissues exposed to Cs, 4.6 or 46 mM, are also shown in Fig.31. It can be seen that Na was extruded, approximately equal quantities of Na and Cs being moved during the 120 min. period. The uptake of Cs in solutions containing 4.6 mM Cs was quite a slow (20 mM/kg after 120 min.) and was accompanied by a slow extrusion of simular amounts of Na.

The tissue content of K of Na-rich tissues exposed to Krebs solution containing K (4.6 mM) for 40 min. (after subtraction of extracellular contribution) was about 28 mM/kg (see Table V),whereas the corresponding tissue content of Cs after 40 min. was only about 10 mM/kg. Similarly Na extrusion from a tissue incubated in 4.6 mM Cs-Krebs solution was less than the amount extruded from tissues incubated in 4.6 mM K-containing Krebs solution over the same time period. Table IX shows the approximate values for the net uptake of Cs and net extrusion of Na during incubation in 4.6 mM Cs-Krebs solution compared with the net uptake of K and net extrusion of Na for tissues incubated for 40 min. in 4.6 mM K-Krebs solution.

Na-rich tissues incubated in 46 mM Cs showed a rapid loss of Na and gain of Cs, as shown in Figs.31 and 32. Changes in Na and Cs content are also shown in the Table IX after incubation of Na-rich tissues in solutions containing 46 mM Cs or 46 mM K for 40 min.
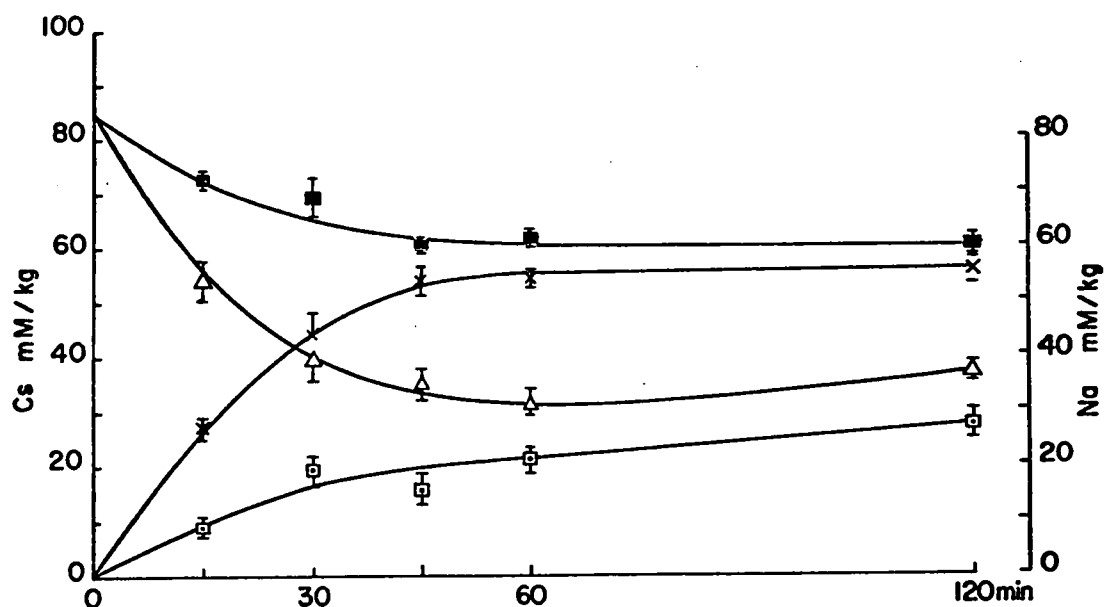
Figure 32. The uptake of Cs (as measured with ^{137}Cs - see Methods IIe) and loss of Na in Na-rich rat uterus. Uptake of Cs from a solution containing 46 mM Cs in Na-rich tissues in the presence of 10^{-3}M ouabain (▣—▣). Control uptake of Cs from Krebs solution containing 46 mM Cs (✕—✕). Concomitant loss of Na in Krebs solution containing 46 mM Cs (△-△); and 46 mM Cs-Krebs solution containing ouabain 10^{-3}M (■—■). The amount of Cs or Na, in the extracellular space, has been subtracted for each observation. Each point represents the mean ± SE of 8 determinations.

TABLE IX

4.6 mM Cs-Krebs Solution		4.6 mM K-Krebs Solution	
+ △ Cs	-△Na	+ △ K	- △ Na
9	6	14	17
46 mM Cs-Krebs Solution		**46 mM K-Krebs Solution**	
+ △ Cs	-△Na	+ △ K	- △ Na
51	50	55	50

Approximate uptake of Cs (+ △Cs) and loss of Na (-△ Na) from tissues incubated in solutions containing 4.6 mM Cs or 4.6 mM K-Krebs solution, and 46 mM Cs or 46 mM K-Krebs solution for 40 min.   Values are in mM/kg tissue after subtraction of approximate quantities of Cs or K in the extracellular space.   Data taken from Table V and Figs.31,32 and 33.

After incubation in elevated K or Cs solutions (46 mM) the quantities of Cs or K accumulated were approximately the same, this was in marked contrast to the lower uptake of Cs as compared with K, when 4.6 mM of Cs or K were used in the uptake media.

The effects of ouabain 10^{-3}M on the uptake of Cs and K from solutions containing either 46 mM Cs or K is shown in Figs. 32 and 33. After 120 min. in 46 mM Cs-Krebs solution the inhibition of uptake of Cs (38%) was greater than the inhibition of uptake of K (29%) caused by ouabain in solutions containing 46 mM K.   During the early part of the uptake of Cs or K from solutions containing 46 mM Cs or K, the uptake
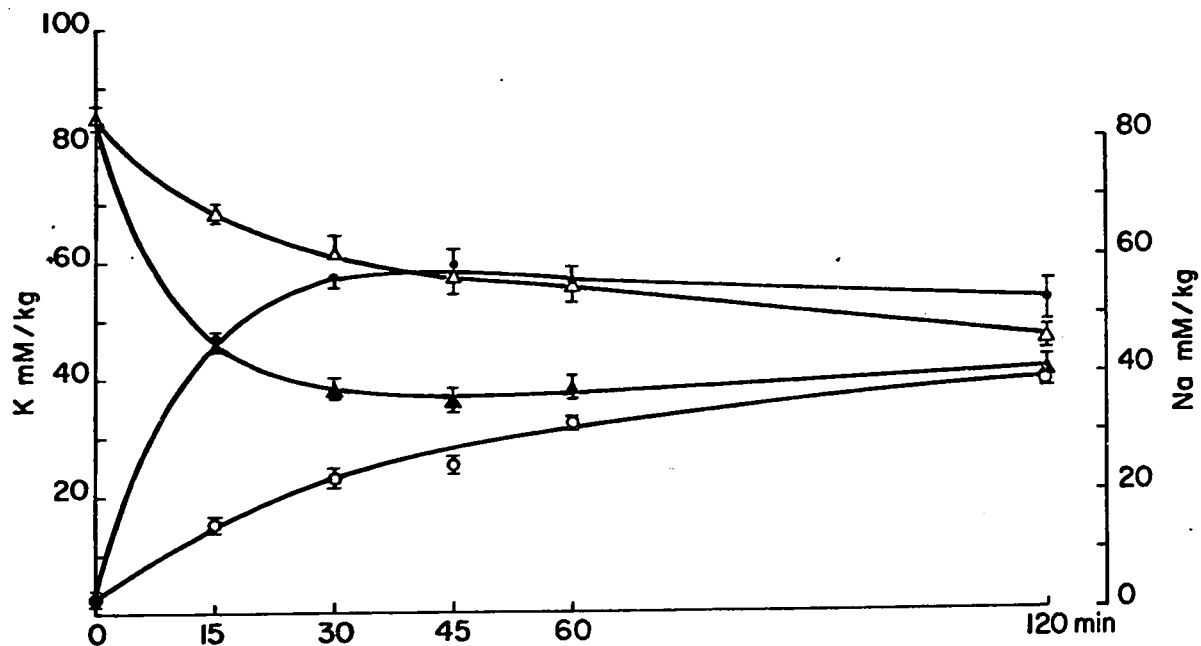
Figure 33. Uptake of K and loss of Na in Na-rich tissues incubated in 46 mM K-Krebs solution in the presence and absence of ouabain 10^{-3}M. Uptake of K from 46 mM K-Krebs solution in the absence of ouabain (•—•). Uptake of K from 46 mM K-Krebs solution in the presence of ouabain 10^{-3}M (o—o). Concomitant loss of tissue Na in the presence (△—△) and absence (▲—▲) of 10^{-3}M ouabain. Each point is the mean ± SE of 8 values. The amounts of Na or K in the extracellular space have been subtracted in each case (see text).

was similar as shown in Table X.

TABLE X

% Inhibition of Cs or K uptake in the presence of 10^{-3}M Ouabain.

TIME	% Inhibition of Cs Uptake	% Inhibition of K Uptake
15	66	71
30	62	64
45	63	60
120	39	28

Data are taken from Figs.32 and 33 and expressed as % inhibition of

control ion content in 46 mM Cs or 46 mM K-Krebs solution in the

absence of ouabain.

In the case of uptake of K the small quantity of K initially present

in Na-rich tissues has been subtracted before the percent data was

calculated.

Membrane potential measurements discussed above showed that

after 120 min. in either 46 mM Cs or 46 mM K-Krebs solution were

depolarised.   The extrusion of Na in the presence of either 46 mM K

or 46 mM Cs was inhibited by the presence of ouabain.   Figs. 32 and 33

show that Na-extrusion was inhibited less in Krebs solution containing

46 mM K than in Krebs solution containing 46 mM Cs after 120 min.

In summary the preliminary experiments on the uptake of Cs and K at different concentrations showed that:-

(1) Na-rich tissues incubated in Krebs solution containing 4.6 mM Cs accumulated less Cs than tissues incubated in solutions containing 46 mM Cs. Na extrusion during Cs uptake was dependent upon the Cs concentration in the bathing medium. Approximately equal quantities of Na and Cs were exchanged during uptake in either 4.6 mM Cs or 46 mM Cs-Krebs solution.

(2) Cs uptake from solutions containing 4.6 mM Cs was less than uptake of K from solutions containing 4.6 mM K over a 40 min. incubation period. The uptake of Cs or K from solutions containing 46 mM Cs or 46 mM K showed approximately equal quantities of Na and Cs or Na and K were exchanged after a 2 hour incubation period.

(3) Both Cs and K movements into Na-rich tissues were inhibited by 60%-70% after 60 min. in the presence of 10^{-3}M ouabain. After 120 min. the inhibition of uptake of both ions was decreased; inhibition of Cs uptake from 46 mM Cs-Krebs solution was greater than the inhibition of K uptake from 46 mM K-Krebs solution.

IIIB (i) <u>Efflux of Cesium (^{137}Cs) and Potassium (^{42}K) from</u>

<u>preincubated Na-rich tissues.</u>

Some preliminary experiments were conducted to determine the effect of modified Krebs solution on the efflux of Cs (using ^{137}Cs) or K (using ^{42}K). Na-rich tissues were loaded with tracer for 90 min. as described in Methods (see Methods IIf) and the efflux of tracer
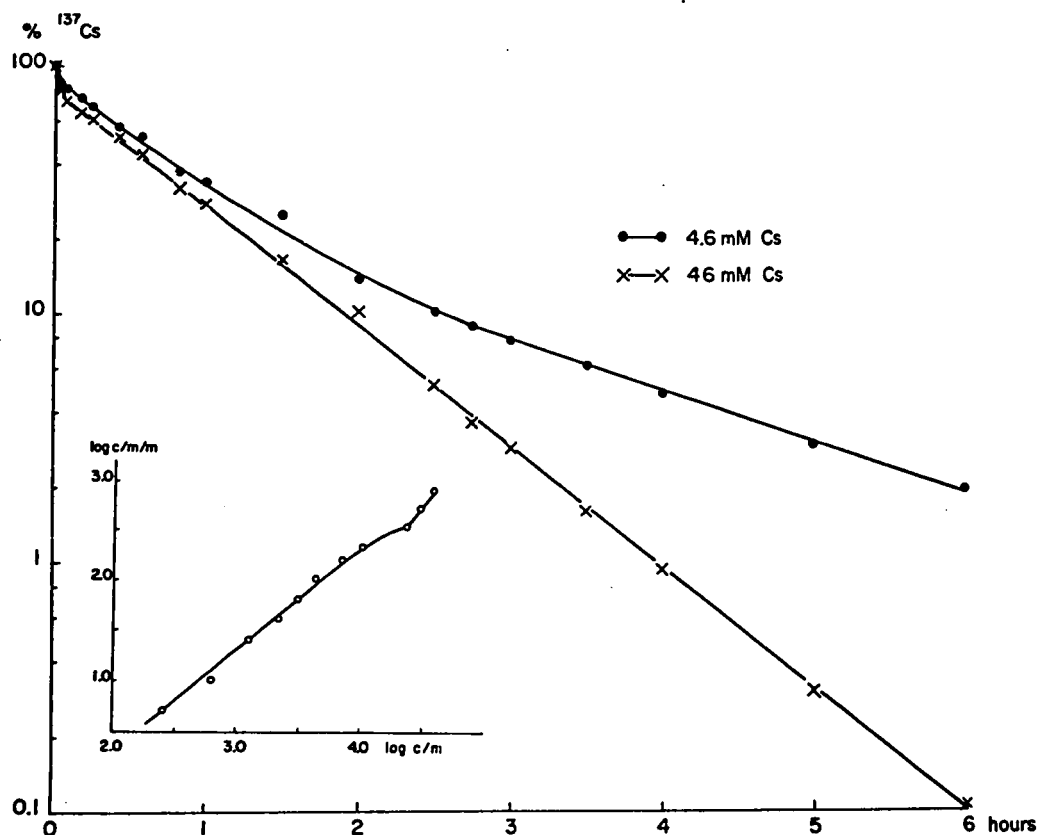
Figure 34. Typical Data for the efflux of ^{137}Cs from rat uterus after preincubation of Na-rich tissues in 4.6 mM Cs or 46 mM Cs-Krebs solution containing ^{137}Cs for 90 min. Upper curve; Efflux of ^{137}Cs from a tissue bathed in 4.6 mM Cs-Krebs solution after preincubation in 4.6 mM Cs-Krebs solution containing ^{137}Cs for 90 min. Lower curve; Efflux of ^{137}Cs from a tissue bathed in 46 mM Cs-Krebs solution after preincubation in 46 mM Cs Krebs solution containing ^{137}Cs for 90 min. Inset graph shows a log c/m/m against log/cm plot for the last 5 hours of efflux for the tissue effluxing into 4.6 mM Cs-Krebs solution. Slope of this line = 1.08.

followed for 360 min. in the appropriate tracer free solution.   Fig.34

shows typical efflux data from two experiments. · The efflux of ^{137}Cs

is shown plotted as two desaturation curves of % log c/m against time

for tissues loaded with ^{137}Cs in 4.6 mM Cs and effluxed into 4.6 mM Cs

and for tissues loaded with ^{137}Cs in 46 mM Cs and effluxed into 46 mM Cs-Krebs

solution.   The inset curve shows a log c/m against log c/m/m plot for

the last 11 points o.f the efflux for the tissue effluxed into 4.6 mM

Cs-Krebs solution.   The last 8 points of the double log plot represent

the efflux from 120-360 min. after transfer of the tissue to tracer free

solution.   This analysis indicates efflux from a single compartment

over the last 120-360 min. of efflux (see Methods IId).   The effects

of modified solutions on efflux was determined after 120 min. of efflux

over a 90 min. test period.   Changes in efflux produced by modified

solutions are expressed in terms of rate coefficients (see Methods IIf).

The efflux of Cs from one control tissue allowed to efflux into

4.6 mM Cs-Krebs solution is shown in Fig.35 along with the effect of

removal of Cs for a 90 min. test period in 3 tissues.   It can be seen

that the removal of Cs did not significantly change the efflux of

Cs over the test period.   The effect of the addition of ouabain $10^{-3}M$

to tissue effluxing into 4.6 mM Cs-Krebs solution is shown in Fig.36.

An increase in efflux was produced by ouabain when added after 120 min.

The increase produced by ouabain was sustained beyond the point at

which ouabain was removed from the solution.   A similar increase in

efflux was also observed in response to ouabain ($10^{-3}M$) in Na-rich

tissues loaded with ^{42}K in normal Krebs solution, and then effluxed
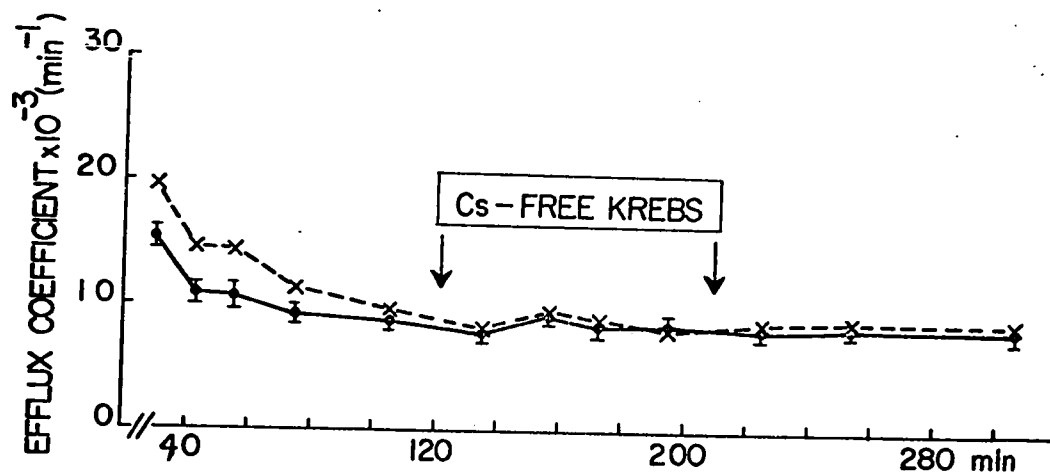
Figure 35. Effect of Cs-free Krebs solution on the efflux of ^{137}Cs from rat uterus. Na-rich tissues were incubated for 90 min. in ^{137}Cs-containing 4.6 mM Cs-Krebs solution. The efflux of ^{137}Cs into 4.6 mM Cs-Krebs solution is shown for a control tissue (x—x) and 3 tissues (●—●) exposed to Cs-free Krebs solution for 90 min. (between vertical arrows) after 120 min. of efflux. Ordinate - efflux coefficient x 10^{-3} (min^{-1}). Abscissa time of efflux (min.) vertical bars represent mean ± SE.
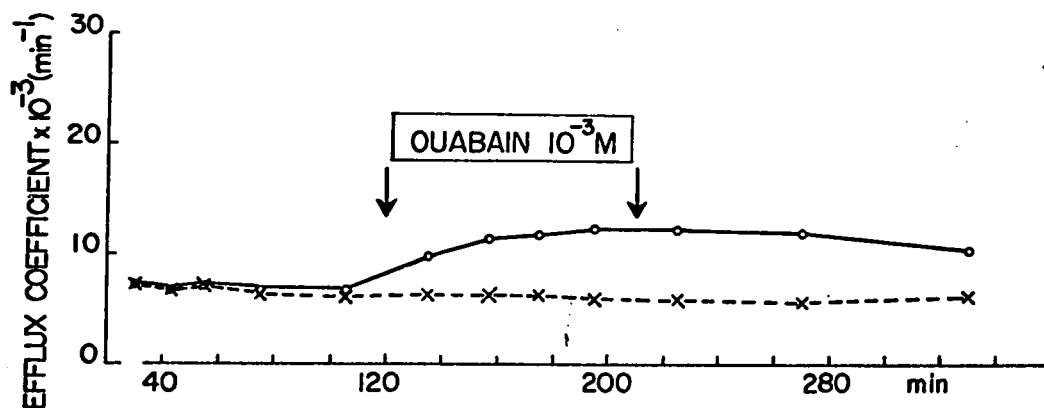
Figure 36. The effect of ouabain (10^{-3}M) on the efflux of ^{137}Cs
from rat uterus. Na-rich tissues were incubated for
90 min. in ^{137}Cs-containing 4.6 mM Cs-Krebs solution
Typical data for the efflux of ^{137}Cs into 4.6 mM Cs-Krebs
solution is shown for a control tissue (x——x) and for a
tissue exposed to ouabain (10^{-3}M) for 90 min. (between
vertical arrows) after 120 min. of efflux. Ordinate:
efflux coefficient x 10^{-3}(min^{-1}). Abscissa; time of
efflux (min).

into tracer free normal Krebs solution. Substitution of K (4.6 mM) for Cs during efflux into 4.6 mM Cs-Krebs solution produced no change in efflux coefficient in 3 experiments as shown in Fig. 37.

Further experiments were conducted in tissues loaded with ^{137}Cs by allowing them to recover from the Na-rich state in a solution containing ^{137}Cs labelled, 46 mM Cs-Krebs solution. Efflux coefficients were then determined during efflux into 46 mM Cs-Krebs solution. Ouabain (10^{-3}M) did not increase efflux of Cs when added to tissues allowed to efflux in 46 mM Cs in 2 tissues. Similar results were obtained when a Na-rich tissue was loaded with ^{42}K in 46 mM K-Krebs solution for 90 min. and then allowed to efflux into 46 mM K-Krebs solution containing ouabain 10^{-3}M for a test period of 90 min.. The efflux of ^{42}K was unaltered by the presence of 10^{-3}M ouabain in 46 mM K.

In summary, preliminary experiments on ^{137}Cs efflux indicated that:-

(1) ^{137}Cs efflux from Na-rich tissues allowed to equilibrate for 90 min. in 4.6 mM Cs-Krebs solution showed no change in efflux after the removal of Cs from, or addition of K (4.6 mM) to the bathing medium. An increase in ^{137}Cs efflux was observed upon the addition of ouabain (10^{-3}M) to the tissue. A similar increase in ^{42}K efflux was the observed response to 10^{-3}M ouabain in a ^{42}K-loaded tissue during efflux into a medium containing 4.6 mM K.

(2) Na-rich tissues loaded with ^{137}Cs from media containing 46 mM Cs-Krebs solution did not show any increase in efflux when ouabain 10^{-3}M was added to the bathing medium.
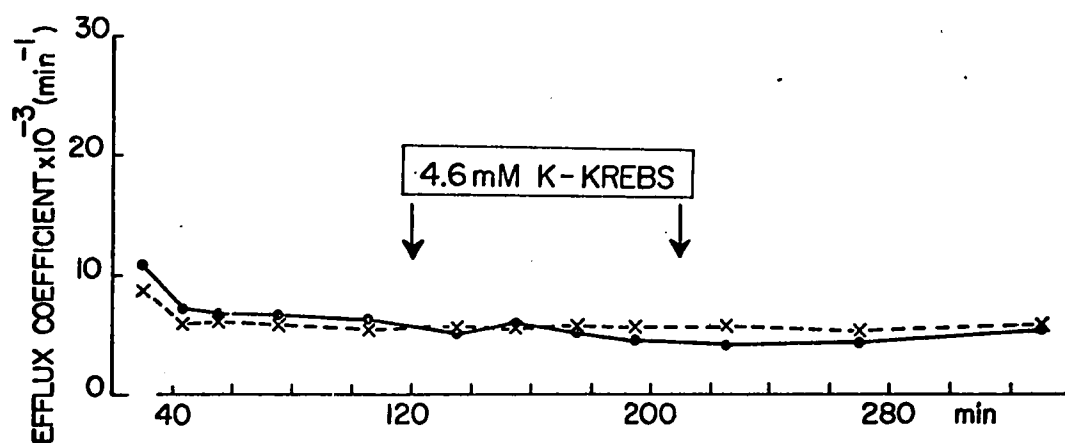
Figure 37. The effect of 4.6 mM K-Krebs solution on the efflux of
^{137}Cs from rat uterus. Na-rich tissues were incubated
for 90 min. in ^{137}Cs containing 4.6 mM Cs-Krebs solution.
Typical data for the efflux of ^{137}Cs into 4.6 mM Cs-Krebs
solution is shown for a control tissue (x——x) and for a
tissue (•——•) exposed to 4.6 mM K-Krebs solution (between
vertical arrows) after 120 min. of efflux. Ordinate:
efflux coefficient x 10^{-3}(min^{-1}). Abscissa: time of
efflux (min).

IV DISCUSSION

IV DISCUSSION

There is now increasing evidence for the existence of
electrogenic Na-pumps in a variety of tissues (see Introduction
iiB). In many of these studies, electrogenic Na-pump activity
has been detected during conditions of net Na-extrusion from
tissues containing an elevated intracellular concentration of
this ion such as existed in the Na-rich tissues used in the
present series of experiments.

The main finding in these studies was that a marked
rapid hyperpolarisation occurred in response to the addition of
K (4.6 mM - 120 mM) to a Na-rich tissue, the observed membrane
potential (V_m) exceeding the calculated K-equilibrium potential
(V_K) during the marked hyperpolarisation. The observed
hyperpolarisation subsequently decreased in K-containing solutions
and spontaneous mechanical and electrical activity was recorded.

It is important at this point to consider the mechanisms
whereby the observed hyperpolarisation of the cell membrane
occurred. In the resting cell hyperpolarisation would be
expected from an increased permeability to K, a decreased
permeability to Na or chloride, or the increased activity of an
electrogenic Na-pump or of an inward chloride pump. In a Na-rich
tissue where the internal concentration of K is very low the
addition of high concentrations of K outside makes V_K positive;
hence the hyperpolarisation observed in the present experiment

cannot be due to an increase in K-permeability. In both Na-rich and fresh tissues a decrease in Na-permeability can cause V_m to increase and approach, but not exceed, V_K. However in the present studies the observed hyperpolarisation exceeded V_K and cannot have resulted from a decrease in Na-permeability. A decrease in chloride permeability would also lead to an increase in membrane potential in a fresh tissue depending upon the value of V_K and V_{Na}, which in Na-rich tissues are less than the observed hyperpolarisation. Hence passive changes in chloride permeability cannot account for the observed hyperpolarisation. There is also the possibility that an inward chloride pump was responsible for the hyperpolarisation observed in response to the addition of K (4.6 mM or greater) to a Na-rich tissue. The results of the experiments in which a Na-rich tissue was incubated in low chloride (less than 10 mM chloride) solutions showed that the initial hyperpolarisation was in fact greater in low chloride solutions than in normal Krebs solution. Under these "low chloride" conditions an inward chloride pump seems unlikely as the cause of the initial hyperpolarisation. However, the activity of an electrogenic Na-pump stimulated by the presence of external K would give rise to a hyperpolarising response as was observed in the above experiments on Na-rich pregnant rat uterus.

One may apply certain criteria in order to distinguish between the operation of a neutral or electrogenic Na-pump: If V_m exceeds V_K during active Na-extrusion then electrogenic Na-pumping is contributing

to the membrane potential (Straub 1967). It is, however, important
at this stage to discuss some of the problems involved in the calculation
of V_K as the accurate determination of this parameter is essential for
fulfillment of the above criterion.

The first problem concerns the determination of the extracelular
space which has to be used to partition total tissue ion content into
intracellular and extracellular components. Extracellular space
measurements are assumed to measure the volume of a homogenous
compartment containing ions with the same activity coefficient as those
present in the intracellular space and external medium. Goodford (1968)
has recently extensively discussed the problems associated with
obtaining an accurate estimate of the extracellular space. Goodford (1968)
has also shown that different extracellular markers equilibrate with
spaces of different volumes; for different markers the extracellular
space does not behave uniformly since in general the size of the marker
is inversely related to the size of the space with which the marker
equilibrates. Preliminary studies in this laboratory by Dr. F.H. Osman
have shown that after 2 hours preincubation in ^{14}C-inulin the efflux of
this molecule from the detrusor muscle of rabbit bladder was occurring
from at least three compartments (Osman and Paton 1969). The half-time
of efflux from the third compartment, the slowest, was greater than
100 min. The actual location of this and the other compartments is at
present under investigation by means of autoradiographic techniques.
It is clear therefore that the determination of the size of the
extracelluler space is subject to many problems as an ideal marker for
the extracellular space is not yet available (Goodford 1968).

In the present study the extracellular space was estimated to be 370 ml/kg using ^{14}C-inulin and calculated using the method of Goodford (1966); this value was in reasonable agreement with the value of 365 ml/kg reported by Casteels and Kuriyama (1965) for myometrium. These authors (Casteels and Kuriyama 1965) also showed that the extracellular space measured by 35S-labelled ethanesulphonate was 570 ml/kg. In the present study, during the marked hyperpolarisation observed in response to the addition of 4.6 mM K-Krebs solution to a Na-rich tissue, V_m exceeded V_K when V_K was calculated using an extracellular space of 370 ml/kg. Only if one assumes that the extracellular space is 600 ml/kg do the values of V_m and V_K become approximately equal. It seems unlikely that the extracellular space could be as high as this value since the total water content was only about 840 ml/kg and the internal K concentration of fresh tissues would then be 334 mEq/l which would seem to be extremely unlikely. When 46 mM K was added to a Na-rich tissue, V_m was -77 mV and V_K calculated using an extracellular space of 370 ml/kg was + 9.1 mV. In this situation even using a larger extracellular space V_K could not equal V_m. A second problem concerns the concentration of K immediately outside the cell membrane. As pointed out by Adrian and Slayman (1966) if the concentration of K immediately outside the cell membrane is less than in the bathing medium, the value for V_K calculated by using the concentration of K in the external medium would underestimate the actual value of V_K. Hyperpolarisation was observed in response to the addition of 46 mM K or 120 mM K to Na-rich tissues; it seems highly improbable that depletion of K in the immediate vicinity of the

outside of the cell membrane could account for the hyperpolarisation in terms of V_K, in view of the large inward gradient for K present in these experiments.

A third problem in calculating V_K arises from the possibility that the internal concentration of K immediately inside the cell membrane is higher than within the remainder of the intracellular fluid. However, hyperpolarisation was observed after the addition of 46 mM or 120 mM K, V_m being 77 and 79 mV respectively. Clearly under these circumstances for V_K to equal V_m the K concentration within such a compartment or area close to the membrane would have to be extremely large.

A fourth problem which may complicate the determination of an accurate value for V_K is the presence of bound electrolytes within the cell membrane. There is now increasing evidence for the presence of bound electrolytes in a variety of tissues including skeletal muscle (McLaughlin and Hinke 1966), non-pregnant rabbit uterus (Kao and Nishiyama 1964; Daniel and Daniel 1957) and non-pregnant rat uterus (Daniel 1963). The presence of bound K in the present experiments could not account for the observed discrepancy between V_m and V_K since the presence of bound K would decrease the intracellular activity of K and lead to a smaller, and not larger, value for the calculated V_K and could not explain the initial hyperpolarisation observed in Na-rich tissues.

A fifth problem concerns the possibility that some of the intracellular water may be bound or sequestered. This would result in

an increase in the intracellular activity of K and hence V_K would be greater than if all the intracellular water was free. However for V_K to equal V_m when 46 mM K was added to Na-rich tissues at least 80% of the total intracellular water would have to be bound.

Thus, in spite of the problems associated with the accurate determination of V_K, it can be concluded that the marked hyperpolarisation observed in Na-rich tissues on the addition of K cannot be accounted for in terms of V_K and thus most probably reflects the activity of an electrogenic Na-pump. The important question then arises as to the nature of the electrogenic Na-pump present in this tissue.

Since the work of Skou (1957) several workers have shown a close relationship between the properties of active transport of Na and the properties of the (Na + K) activated ATPase (hereafter referred to as transport ATPase) (Post et al 1960; Dunham and Glynn 1961; Wheeler and Whittam 1961; Skou 1962). Both the transport ATPase and the active transport of Na possess the following properties (Skou 1964, 1965; Whittam 1964; Baker 1966): Both

    (1)   require metabolic energy in the form of ATP,

    (2)   are inhibited in the presence of ouabain, the effects of ouabain being overcome by increasing the external K concentration,

    (3)   require Na on the inside of the membrane along with magnesium,

    (4)   require K on the outside of the membrane,

(5) require external K but K can be substituted
by other monovalent cations; e.g. Rb, Cs,
NH_4; however, the Na-requirement cannot be
substituted.

(6) are located in the cell membrane.

The above similarities between the properties of the
transport ATPase and active cation transport strongly suggest that
transport ATPase activity is closely related to the active movement
of ions which in turn control the value of the resting membrane
potential. For non-pregnant rat uterus there is now evidence
(Daniel et al 1969a) for a (Na + K)-activated transport ATPase which
is inhibited by ouabain. Similar studies on pregnant rat myometrium
are not yet available, although at this time there is no reason to
suppose that the properties of this enzyme system in pregnant rat
myometrium should be different. It appeared possible that the
electrogenic Na-pumping observed in this study might be related to
the activity of this transport ATPase. Thus subsequent experiments
were designed to examine the effect of various ions, drugs and
procedures (all of which were known to have characteristic effects
on the transport ATPase) on the hyperpolarising response of Na-rich
tissue to the addition of external K. In the Introduction the
properties of other electrogenic Na-pumps have been summarised.
However, these properties are not specific for electrogenic Na-pumps

and in fact are closely similar to the general properties of transport ATPase. Thus they show that the membrane potential changes observed during the various procedures represent ion pump activity and not merely passive permeability changes.

The sensitivity of the membrane ATPase to ouabain is well known (see Skou 1964, 1965 for references). In the present series of experiments the addition of ouabain to a Na-rich tissue abolished reversibly the initial hyperpolarisation. A high concentration of ouabain (10^{-3}M) was used in these studies because of the known insensitivity of rat tissues to cardiac glycosides (Allen and Schwartz 1969). Thomas (1969) and Rang and Ritchie (1968) have also demonstrated that ouabain abolished membrane hyperpolarisation associated with Na-extrusion in snail neurones and mammalian non-medullated nerve fibres respectively.

The hyperpolarisation in the present experiments in response to 4.6 mM K was sensitive to changes in temperature as would be expected if a membrane ATPase controlled the movements of ions across the membrane during recovery from a Na-rich condition. The hyperpolarisation reported in the present study in Na-rich tissues at 25°C in the presence of K is in contrast to the recent findings of Kao and Nishiyama (1969). These authors did not find any hyperpolarisation of the membrane potential during recovery of Na-enriched segments of rabbit myometrium at 25°C. The observations of Kao and Nishiyama were made after overnight immersion at $0-2^{\circ}$C of rabbit myometrium in normal Krebs solution and not K-free Krebs solution; following this treatment their tissues contained 31 mEq/kg K (wet weight) and the observed

V_m was 35 mV. These tissues were less Na-rich than the tissues used in the present study and consequently V_m may have been less influenced by electrogenic Na-pumping. The differences observed in the present study from that of Kao and Nishiyama may also reflect species and other experimental variations and the exact explanation requires further experimentation. The sensitivity of the Na-pump induced hyperpolarising response to temperature has been reported for a variety of other tissues (Kerkut and Thomas 1965; Senft 1967; Carpenter and Alving 1968), a reduction in temperature decreasing the magnitude or duration or both of the electrogenic Na-pump induced hyperpolarisation.

Using several different concentrations of K in the bathing medium the present results show that the initial hyperpolarising response failed to occur in the absence of K and was reversed upon the withdrawal of K from the organ bath. Transport ATPase activity also demonstrates an absolute requirement for the presence of external K. Studies on electrogenic Na-pumps in other tissues have shown that in the absence of external K electrogenic pumping of Na does not take place. In an elegant experimental design in which Na ions are injected into snail neurones by a current pulse Thomas (1969) has demonstrated that extrusion of injected Na does not occur in K-free solutions and that upon the reintroduction of K to the bathing medium the injected Na ions are then extruded giving rise to hyperpolarisation. Rang and Ritchie (1968a,b) have shown that the post-tetanic hyperpolarising response in mammalian non-myelinated nerve fibres was due to the operation of a K-dependent electrogenic Na-pump. Similar K-dependent

electrogenic Na-pump activity has been demonstrated in squid axons

(Hodgkin and Keynes 1956) and in frog muscle (Keynes and Swan 1959).

Before considering the effects of various ions substituted

for K (e.g. Rb, Cs) on the recovery of the membrane potential of Na-rich

tissues it is important to compare the properties of the membrane

potential during recovery from the Na-rich state with the properties of

the membrane potential in fresh tissues, or tissues that had recovered

spontaneous mechanical and electrical activity after initial Na-enrichment.

In fresh tissues, or Na-rich tissues that had recovered spontaneous

activity in normal Krebs solution, the removal of K did not cause any

significant change in membrane potential after 3 hours even though there

must have been considerable loss of internal K. The removal of external

K from such tissue would however be expected to result in an increase in

V_m. The results obtained may indicate therefore that either permeability

to K was reduced and/or electrogenic Na-pumping, maintained by a small

leak of K, kept V_m at a nearly constant level. However flux studies on

non-pregnant myometrium have shown no change in ^{42}K-efflux upon

withdrawal of K (Daniel et al 1969a). Studies on the efflux of ^{22}Na

from non-pregnant rat myometrium at 25°C showed that K-free solutions

increased Na influx in fresh and Na-rich tissue and that Na efflux was

not decreased in Na-rich tissues unless a sufficient time period was

allowed in 4.6 mM K, before beginning efflux, to activate the Na-pump;

Na efflux was not decreased in fresh tissues unless a longer time

period was allowed to bring about some K-depletion and depolarisation

(Daniel 1969 - unpublished observations). This study showed that the

active transport of Na could be maintained in the absence of external K,

presumably as a result of a small leak of K. These results therefore

suggest that V_m in the present study may have been maintained upon

withdrawal of K, as a result of electrogenic Na-pumping. The addition

of ouabain caused a slight initial depolarisation of fresh tissues

followed by a decrease in membrane potential of about 17 mV after 1 hour.

This finding may indicate therefore that V_m was kept at a nearly constant

level as a consequence of an increase of K permeability and/or that

electrogenic Na-pumping was only slowly inhibited by ouabain. Flux data

obtained using ^{42}K showed that ouabain increased K efflux. Furthermore,

ion flux studies on fresh non-pregnant myometrium showed that the

addition of ouabain decreased the efflux of Na and increased the

influx of Na and efflux of K provided that the tissues were exposed to

ouabain for a sufficient time period to inhibit the pump before beginning

efflux and suggesting that turning off the pump increased the passive ion

permeabilities (Daniel 1969 - unpublished observations). The addition

of K to a Na-rich tissue caused a rapid hyperpolarisation which could be

initially reversed by the removal of K. At this stage, as has been

discussed, V_m is maintained by electrogenic Na-pumping.

In contrast to fresh tissue, which depolarised within 3 min.

of the addition of 46 mM K to the bathing medium, Na-rich tissues showed

a marked hyperpolarisation in this medium and depolarised slowly over

the next 40 min. to about 24 mV. The removal of K from the depolarised

Na-rich tissue caused the membrane potential to increase to 49 mV. In

the presence of ouabain the increase in membrane potential caused by the

removal of K from a high K depolarised Na-rich tissue was reduced,

being increased only by 17 mV to 41 mV. The removal of K from

tissues depolarised with high concentrations of K would be expected

on the basis of the Nernst equation to increase the membrane

potential as K outside was decreased and K moved out of the cells.

If the outward movement of K into K-free solutions provided sufficient

K outside the membrane to stimulate an electrogenic Na-pump then the

membrane potential would be a function not only of the passive K efflux

but also of electrogenic Na extrusion. The presence of ouabain may

have inhibited the electrogenic Na-pump component of the membrane

potential such that the membrane potential became only a function of

the K gradient in K-free solutions.

Marshall and Miller (1964) studied the effects of iodoacetic

acid (IAA), 2×10^{-4}M, on the V_m of pregnant rat myometrium; they

found that IAA abolished contractility within 40 minutes but did not

alter V_m until after 180 minutes when 10 mV depolarisation had occurred.

The eventual decrease in membrane potential may have been a consequence

of downhill ion movements. The finding that V_m was unchanged after

40 min. could indicate that electrogenic Na-pumping does not normally

maintain V_m in fresh tissues or that metabolic inhibition resulted in

an increase in K-permeability or that metabolic inhibition was not

complete at this time. In non-pregnant rat uterus IAA $(10^{-3}$M$)$ has

been shown to increase the rate of ^{42}K efflux (Daniel et al 1969a).

From this analysis of the effects of procedures which

inhibit the transport ATPase e.g. ouabain, withdrawal of K, and

metabolic inhibition it is clear that the maintenance of the membrane

potential in fresh tissues is complex. Most procedures which

completely inhibit the Na-pump also have been shown to cause an

increase in K efflux (Daniel et al 1969a) in non-pregnant rat

uterus. This effect would tend to oppose the effect on V_m

of inhibition of electrogenic Na-pumping.

This study has also raised the problem of the relationship of

the Na-pump to K permeability. Flux data using ^{42}K and ^{137}Cs showed

that ouabain ($10^{-3}M$) increased K efflux; it has also been shown that

most procedures which inhibit the Na-pump in non-pregnant rat uterus also

increase K efflux (Daniel et al 1969a). The exposure of Na-rich tissues

to 46 mM K caused an initial hyperpolarisation followed only later by

depolarisation. In contrast fresh tissues were rapidly depolarised by

46 mM K. This suggests that during the early hyperpolarising response

of Na-rich tissues to 46 mM K the membrane permeability to K was considerably

less than in fresh tissues. Furthermore it was found that the maximum

hyperpolarisation developed following the addition of K 4.6 to 120 mM

ranged from 69 mV to 79 mV. If the Na-pump was working maximally at these

concentrations and if K permeability remained constant, it would be

anticipated that the hyperpolarisation developed would have been

greatest at 4.6 mM K and least at 120 mM K, since the greater the external

K concentration the greater would be the inward driving force on K

created by the electrogenic Na-pump. This analysis suggests therefore

that during hyperpolarisation either the permeability to K was extremely low and/or that electrogenic Na-pumping increased as external K was raised and that the rate of Na-pumping in some way regulated the permeability to K such that the greater the rate of pumping, the smaller the permeability to K. In contrast the addition of high concentrations of K (46 mM or 120 mM) to fresh tissues produced an immediate depolarisation. Such tissues have much greater internal concentration of K and a much lower concentration of Na than Na-rich tissues and thus Na-pumping is unlikely to be as large as it is in Na-rich tissues. The depolarisation observed after the exposure of Na-rich tissues to high concentrations of K (46 mM,120 mM) for some time may similarly result from an increase in internal K and a decrease in internal Na thus reducing Na-pump activity. Thus these studies have provided some evidence that the activity of the Na-pump may in some way regulate K permeability; inhibition of the pump resulting in an increase, and stimulation of the pump a decrease in K permeability.

The substitution of various external ions in place of K including Rb and Cs has provided information on the type of pump activity present in various tissues (Adrian and Slayman 1966; Sjodin and Beaugé 1967, 1968). For activation of both Na efflux and the stimulated hydrolysis of ATP by (Na + K)-ATPase the K requirement can also be substituted by other monovalent cation such as Rb, Cs, NH_4. In the present studies both Rb and Cs were able to promote the recovery of the membrane potential of Na-rich rat uterus. Experiments with Rb (4.6mM) in place of K (4.6 mM) showed an initial hyperpolarising response which

was sensitive to the presence of Rb in the bathing medium. Higher

concentrations of Rb induced a more rapid recovery of contractility

after initial hyperpolarisation of the membrane potential. For the

most part the changes in membrane potential and onset of spontaneous

contractility induced by Rb closely resembled the recovery profile

associated with Na-rich tissues placed in media containing similar

amounts of K. The removal of Rb or K from Na-rich tissues,

hyperpolarised by the addition of either one of these ions, caused

an immediate fall in membrane potential. The similar behavior

of Rb and K has been noted in many studies and use has been made

of this similarity in flux studies in which the isotope ^{86}Rb

has been utilised instead of the short-lived ^{42}K (Burgen and

Spero 1968; van Zwieten 1968a, b). Muller (1965) however has pointed

out that the use of ^{86}Rb for determining K fluxes in isolated cardiac

Purkinje fibres is not a safe practice. Adrian (1964) reported that

the permeability of skeletal muscle membranes to Rb was less than the

permeability to K. Subsequently Adrian and Slayman measured the

membrane potential during recovery of Na-rich skeletal muscle in the

presence of Rb and found a greater hyperpolarisation when Rb replaced

K in the recovery medium. However, these authors concluded than, in

spite of the additional inward driving force created by the operation

of an electrogenic Na-pump, 90% of the inward movement of Rb was

coupled to the efflux of Na. Little is known about the relative

permeabilities of K and Rb in rat uterus; however, unlike the study

of Adrian and Slayman (1966) the maximum hyperpolarisation produced in the present study by K and Rb was similar in magnitude in Na-rich tissues. Further experiments on the influx and efflux of Rb would provide more information on this point.

Fresh tissues incubated in solutions containing 4.6 mM Cs in place of K did not show any significant change in membrane potential after several hours in Cs-substituted Krebs solution. On the other hand the recovery of the membrane potential of Na-rich tissues in 4.6 mM Cs-Krebs solution was delayed in comparison to tissues bathed in normal Krebs solution (4.6 mM K); the initial rapid hyperpolarisation recorded in solutions containing K or Rb was not observed with 4.6 mM Cs-Krebs solution, instead a slow increase in membrane potential occurred accompanied by irregular spike activity but without hyperpolarisation relative to the membrane potential of fresh tissues in normal Krebs solution. However, assuming that the extracellular space in Cs is similar to that in normal Krebs solution the value of the Cs-equilibrium potential (V_{Cs}) can be calculated from the uptake data in which Na-rich tissues were incubated in 4.6 mM Cs for up to 2 hours. After 1 hour in 4.6 mM Cs-Krebs solution the membrane potential of Na-rich tissues was about 47 mV, the corresponding V_{Cs} calculated at this time was about 25 mV. Thus the observed membrane potential of Na-rich tissues incubated for 1 hour in 4.6 mM Cs-Krebs solution exceeded the calculated V_{Cs}.

If the concentration of Cs in the recovery medium of Na-rich tissues was increased, a more rapid increase in membrane potential occurred. The increase in membrane potential which occurred when

9.2 mM Cs-Krebs solution was added to Na-rich tissues could be reversed by the removal of Cs even after 2 hours incubation in this solution. An increase or decrease in membrane potential could be induced respectively by the addition or removal of Cs when 18.4 mM Cs was added to a Na-rich tissue that had recovered spontaneous activity. The results using Cs in the recovery fluid were in contrast to those reported above in which Na-rich tissues were allowed to recover in a medium containing K. The removal of K from fresh tissues or recovered Na-rich tissues, after 2 hours incubation in normal Krebs solution, produced no detectable change in membrane potential. The ease with which the membrane potential could be reduced in Cs-free solutions after prolonged exposure to Cs suggests that the existing gradient of Cs was insufficient for the development of a resting potential of greater than about 20 mV. The calculation of V_{Cs} mentioned above would tend to confirm this possibility. This would in turn imply an extremely low permeability to Cs. If a low permeability to Cs was present throughout the two hour exposure of Na-rich tissue to 9.2 mM Cs-Krebs solution then some other mechanism must be invoked to explain the presence of a membrane potential in this medium. One possible explanation for the effects of Cs is that an electrogenic Na-pump is responsible for the membrane potential during the whole of the recovery period even after 2 hours in 9.2 mM Cs-Krebs solution. If activity of the pump requires the presence of external Cs in an analogous manner to that described above for K and Rb then removal of Cs would be expected to cause a depolarisation by inhibition of the Na-pump.

Elevation of the concentration of Cs in the recovery medium, bathing Na-rich tissues, to 46 mM caused a rapid hyperpolarisation (relative to the membrane potential in fresh tissues) when first applied to the tissue. The hyperpolarisation observed under these circumstances was comparable to the large increase in membrane potential caused by lower quantities (4.6 mM) of either K or Rb in Na-rich tissues. It is interesting to note that Beaugé and Sjodin (1968) have shown that 25 mM Cs and 5 mM K had similar effects on Na extrusion in Na-rich frog skeletal muscle.

Studies on the uptake of ^{137}Cs into recovering Na-rich tissues bathed in 4.6 mM Cs showed a very small uptake of Cs and a correspondingly low extrusion of Na. The total tissue uptake of K from a solution containing 4.6 mM K was 30 mM/kg after 40 min. whereas the corresponding uptake of ^{137}Cs from a solution containing 4.6 mM Cs was only about 10 mM/kg. These findings could be interpreted to reflect a low permeability or low rate of coupled influx to Cs as well as the relative inability of Cs to promote Na extrusion through an electrogenic Na-pump. If a coupled pump was present such that Na and Cs were exchanged in a 1:1 manner then removal of Cs would be expected to increase the membrane potential provided the membrane were permeable to Cs and the Cs equilibrium potential was larger than the resting membrane potential.

The experiments on the uptake of ^{137}Cs from solutions containing either 4.6 mM or 46 mM Cs showed that during recovery of Na-rich tissues approximately equal quantities of Na and Cs were

exchanged after a 2 hour incubation period. After 30 min. exposure

of Na-rich tissues to 46 mM Cs-Krebs solution the membrane potential

had fallen to about 20 mV. The uptake studies using ^{137}Cs showed

that after 30 min. in 46 mM Cs-Krebs solution a substantial quantity

of Cs had moved into the tissue during Na extrusion. The removal

of Cs, however, did not increase the membrane potential as was

demonstrated in similar experiments upon the removal of K from Na-rich

tissues depolarised after 40 min. exposure to 46 mM K-Krebs solution.

It is possible that a large fraction of the tissue Cs was in some way

bound and not able to contribute to the resting membrane potential

when the Cs concentration of the bathing medium was reduced to zero.

The lack of increase of the membrane potential when the Cs

concentration of the bathing medium was reduced to zero may also be

due to the low permeability of the larger Cs ion and its inability

to readily move outwards and activate the Na-pump, thereby producing

an increase in membrane potential. Yet another possible explanation

for the lack of increase of the membrane potential in Cs-free

solutions could be that an increase in internal Cs concentration in

some way inhibited the activity of the Na-pump and that even though a

small amount of Cs leaks out of the tissue this is not sufficient to

activate Na extrusion in the presence of large intracellular

concentrations of Cs.

Comparatively little is known concerning Cs movements in

smooth muscle. Beaugé and Sjodin (1968) have studied Cs movements

in frog skeletal muscle and shown that Cs entry into sartorii was

reduced in the presence of strophanthidin by about 80% over a 20 hour period. These authors also showed that the uptake of Cs was dependent upon the intracellular Na concentration and that 25 mM Cs and 5 mM K were roughly equipotent in stimulating Na extrusion. In the present studies in comparison with 4.6 mM K, the uptake of 4.6 mM Cs was significantly less and when Na-rich tissues were incubated in the presence of 4.6 mM Cs the small uptake of Cs was accompanied by a similar small extrusion of Na. The reduced Cs uptake as compared to K may reflect two different processes. If the potential gradient generated by the Na-pump was small in the presence of Cs, because of incomplete activation of the Na-pump, then the inward electrical driving force on Cs would be small and very little would enter, perhaps a small quantity would enter coupled in some way to the extrusion of Na. Secondly if the permeability of the membrane to Cs was less than K, then a smaller quantity of Cs would move passively inward in response to a given potential gradient generated by the Na-pump.

In the presence of Ouabain the uptake of Cs from solutions containing 46 mM Cs was significantly reduced. The inhibition of uptake suggests that part at least of the uptake of Cs is mediated by an active process which was coupled in some way to the extrusion of Na. The more rapid uptake of K from solutions containing 46 mM K as compared with the slower uptake of Cs from solutions containing 46 mM Cs may result from at least two phenomena. The larger size of the Cs ion may well contribute to the slower inward movement of this

ion when exposed to a similar potential gradient (both 46 mM Cs and 46 mM K solutions caused an immediate hyperpolarisation in Na-rich tissues). If the entry of Cs is by means of a coupled pump that extrudes Na then the slower nature of the uptake process may reflect a lesser binding of Cs, as compared to K to the transport sites responsible for the inward movement of these two ions.

At the present time the preliminary data concerning the uptake of Cs have not provided evidence of independent movements of Na and K or Cs. The results are useful in that they suggest different approaches which could be taken to elucidate the problem further. For example, experiments in chloride-free conditions would provide a more reliable estimate of the relationship between calculated equilibrium potentials at any time during uptake and the observed response. If Cs and K are moving inwards by means of a common carrier then a competition between the two ions for uptake might be investigated as has been done by Beaugé and Sjodin (1968) for Cs uptake in skeletal muscle. In addition the actions of Rb and Cs on the transport ATPase of rat uterus requires investigation in view of their apparent different ability to stimulate Na-pumping.

Several workers have shown that many smooth muscles can maintain normal electrical and mechanical activity for prolonged periods when the concentration of Na in the bathing medium is reduced by replacement with either sucrose, lithium chloride, choline chloride or TRIS chloride.

Marshall (1963) showed that a reduction in the external Na concentration from 143 mM to 7.1 mM had no effect on the resting membrane potential after 4 hours. Daniel and Singh (1958) reported that a stepwise reduction in Na to one-ninth of normal did not affect conduction velocity or action potentials of cat uterus. Holman (1968) has pointed out the difficulties of a suitable choice of substance for replacing Na when studying the effects of Na-free solutions: large quantities of choline chloride applied to uterine smooth muscle induce contractions which are not readily blocked by atropine; substitution of Na also presents problems because of the effect of low ionic strength solutions in causing depolarisation of smooth muscle (Holman 1968). In the present studies the effects of Na-free solutions were determined when Na was replaced by either sucrose or lithium. Total replacement of Na with either of these two substances produced a fall in membrane potential and reduction in contractility of fresh tissues. However, if a small quantity of Na (23 mM) remained in the sucrose-substituted Krebs spontaneous mechanical and electrical activity persisted for several hours.

The hyperpolarisation observed when Na-rich tissues were transferred to solutions containing either K or Rb or large quantities of Cs, was also observed when Na-rich tissues were placed in sucrose substituted Na-free solutions containing 5.7 mM K. Although the membrane potential of fresh tissues exposed to Li-substituted Na-free Krebs solution decreased to about 30 mV in 30 min., hyperpolarisation of the membrane potential was observed in response to Li-substituted

Na-free solutions containing 5.7 mM K when applied to Na-rich tissues. The hyperpolarisation decreased rapidly to 25 mV in Li-Krebs solution within 30 min., but could be reversed to about 50 mV at this time by the removal of Li and the addition of normal Krebs solution. An increase in membrane potential in fresh tissues depolarised by exposure to Li-Krebs solution, could be brought about by the removal of Li and addition of normal Krebs solution to these tissues. Fresh tissues rapidly accumulate Li in exchange for both Na and K (Daniel unpublished observations) and it is likely that in both fresh tissues and in Na-rich tissues the intracellular concentrations of both Na and K were considerably reduced after 30 min. in the presence of external Li. The depolarisation of fresh and Na-rich tissues produced by prolonged exposure to Li-Krebs solution could be explained if depletion of intracellular Na had occurred and if internal Li was unable to substitute for Na in activating the Na-pump; this would result in an inhibition of Na-pumping and a fall in membrane potential. The entry of Li and loss of both Na and K in a fresh tissue would also, by removal of internal K, cause a reduction in the value of the K-equilibrium potential and the membrane potential and make V_m depend more closely on the rate of pumping. The restoration of normal Krebs solution to a fresh tissue, depolarised in Li-Krebs, produced an increase in membrane potential which may reflect an increased activity of an electrogenic Na-pump that now extrudes Na ions that have leaked into the cells after the replacement of Li-Krebs solution with normal Krebs solution. A similar type of reasoning can be applied to Na-rich

tissues. The depolarisation which occurs when Na-rich tissues are placed in Li-Krebs solution could be attributed to the loss of internal Na and the inability of the Na-pump to extrude the accumulated Li. However, when Li-Krebs solution was removed and normal Krebs solution added to the tissue the membrane potential subsequently recovered. The recovery of the membrane potential in normal Krebs solution after removal of Li could be explained by the inward leak of Na and the subsequent electrogenic extrusion of Na to initially restore the membrane potential.

Hyperpolarisation was also induced in Na-rich tissues allowed to recover in sucrose-substituted Na-free solutions. After the initial hyperpolarisation the membrane potential of Na-rich tissues declined to about 30 mV after 35 min. exposure to Na-free sucrose-substituted solutions. The fall in membrane potential under these circumstances may reflect the reduced activity of an electrogenic Na-pump due to the greatly reduced internal Na resulting from the outward movement of Na in Na-free solutions.

The initial purpose of the experiments discussed above was to investigate the effects of removal of Na on the hyperpolarisation induced when K was added to Na-rich tissues. The substitution of Li or sucrose for Na in these studies did not affect the hyperpolarisation induced by K in Na-rich tissues. If the hyperpolarisation reflects activity of an electrogenic Na-pump, as was proposed above, then it appears that the activity of the pump is independent of external Na but cannot maintain the membrane potential when intracellular Na is

depleted or replaced by Li.   These characteristics of Na-pumping in this tissue are similar to the properties of other Na transport systems involving transport ATPase (see Skou 1964, 1965; Whittam 1964).

The replacement of NaCl with sodium methyl sulphate $(NaCH_3SO_4)$ in the bathing medium surrounding Na-rich tissues did not prevent the initial hyperpolarising response to K.   Furthermore, fresh tissues placed in solutions containing less than 10 mM Cl showed no significant change in membrane potential or spontaneous mechanical activity after 40 min. in this solution.   Kuriyama (1963) reported that replacement of Cl with SO_4 caused a transient depolarisation in guinea-pig taenia-coli followed by stabilization of the membrane potential at a value about 5 mV less than the resting potential. The hyperpolarisation produced by K containing $NaCH_3SO_4$-Krebs solution was significantly greater than the hyperpolarisation recorded in another segment of the same uterus allowed to recover in normal Krebs solution.   In this context Rang and Ritchie (1968a,b) have shown that the post-tetanic hyperpolarisation in mammalian non-myelinated nerves which normally was less than 5 mV was increased to between 20-35 mV more negative when chloride was replaced by either SO_4 or isethionate. The explanation proposed by these authors for this phenomenon is that small anions such as Cl, Br, NO_2 can short-circuit the potential generated by an electrogenic Na-pump.   Replacement of chloride with SO_4 would be expected to allow a greater potential to be generated across the membrane by electrogenic Na-extrusion. A similar explanation could apply to the prolonged hyperpolarisation observed when Na-rich rat uteri are exposed to solutions in which NaCl has

been replaced by $NaCH_3SO_4$. A firm conclusion as to the lack of short circuiting in the absence of chloride would require the measurement of membrane conductance under conditions of Na-extrusion in the presence of solutions containing NaCl or $NaCH_3SO_4$.

At this point it is important to summarise the characteristics of the electrogenic Na-pump responsible for the initial hyperpolarisation in Na-rich tissues. This study has shown that the pump:-

(1) requires external K, Rb or Cs but not Li being able to substitute for K,

(2) requires internal Na, Li being unable to substitute for Na,

(3) is inhibited by ouabain,

(4) is temperature dependent.

It can be seen that these characteristics are similar to the properties associated with ion transport and transport ATPase in other tissues as discussed earlier. Further study of the transport ATPase in pregnant rat uterus is required to determine more fully the characteristics of this enzyme system, and thus its relationship to the activity of the Na-pump in Na-rich tissues.

The preliminary experiments on the efflux of ^{137}Cs and ^{42}K into solutions containing either 4.6 mM Cs or 4.6 mM K showed that ouabain, in concentrations which would depolarise either fresh or Na-rich tissues, caused an increase in efflux of either ^{137}Cs or ^{42}K. Daniel et al (1969a) demonstrated similar effects on ^{42}K efflux in non-pregnant myometrium and also showed that most of the procedures

that inhibited Na-pump also caused an increase in ^{42}K efflux. The efflux of ^{137}Cs into solutions containing 4.6 mM Cs, was not however increased by removal of Cs from the bathing medium. Similarly withdrawal of K did not increase ^{42}K efflux (Daniel et al 1969a). As discussed earlier external Cs is required to maintain Na pumping and the membrane potential even after 2 hours in 4.6 mM or 9.2 mM Cs-Krebs solution; at this time removal of Cs caused depolarisation. The effects of removal of Cs on the membrane potential were not however monitored, after incubation of Na-rich tissues in 4.6 mM Cs-Krebs solution for 210 min. i.e. the time when the effects on Cs efflux were studied. It is possible however that at this time the removal of Cs also caused a decrease in membrane potential as this was observed up to 120 min. in 4.6 mM or 9.2 mM Cs-Krebs solution. Ouabain also caused depolarisation of tissues in Cs-Krebs solution at a similar time.

The effect of ouabain on efflux cannot be readily attributed to inhibition of Na-pumping since Cs withdrawal did not increase Cs efflux and yet apparently inhibited Na-extrusion. Withdrawal of K similarly did not increase ^{42}K efflux in non-pregnant myometrium (Daniel et al 1969a)but by contrast did not alter the membrane potential of fresh tissues in the present study; as discussed earlier the lack of effect of K-free solution may be due to a leak of K maintaining Na-pumping and thus the membrane potential. A similar explanation cannot be advanced for the effects of Cs-free solutions since the permeability to Cs appears to be small. One possibility is that ouabain produced an increase in Cs and K efflux by an action unrelated to its inhibition of the transport ATPase. This problem

could be further investigated by determining the effect of ouabain on the efflux of ^{137}Cs in tissues depolarised by the withdrawal of Cs. However, in tissues loaded with either ^{137}Cs or ^{42}K and depolarised in 46 mM Cs or 46 mM K-Krebs solution, ouabain did not produce any significant change in Cs-efflux. These high concentrations of K and Cs would tend however to oppose the inhibitory action of ouabain on the Na-pump. In addition, the efflux of ^{137}Cs or ^{42}K from tissues in 46 mM Cs or K was more rapid than in 4.6 mM Cs or K and thus the effect of ouabain on efflux may have been less easy to detect. Clearly further studies on the properties of the efflux of Cs are required.

No attempt was made in the present studies to provide quantitative data concerning the properties of the action potentials recorded in various bathing solutions. However, within the limits of the pen recording system certain characteristics may be discussed. The contractile response, of Na-rich tissues allowed to recover in solutions containing 18.4 mM Rb, was accompanied by a sustained depolarisation showing small superimposed spikes. In view of the lower permeability of Rb as compared to K demonstrated in skeletal muscle (Adrian and Slayman 1966) it is possible that the delayed repolarisation during spike activity in high Rb solutions represents a decreased permeability to Rb. If this is the case then one might argue that the hyperpolarisation produced in response to Rb should have been greater than the hyperpolarisation produced in response to K during the early recovery of Na-rich tissues. In fact the hyperpolarisation induced with Rb (4.6 mM) was not significantly

greater than that induced with a similar concentration of K. However, as discussed earlier the activity of the Na-pump may have reduced the permeability to both K and Rb thus making any differences in hyperpolarisation hard to detect assuming that K and Rb activate the pump to approximately the same extent. Although different mechanisms are probably responsible for the repolarisation phase of the action potential and the movements of Rb in response to electrogenic Na-pumping, the delayed repolarisation of the action potential may reflect a decreased permeability to Rb during spike activity. Another explanation for the sustained depolarisation could be that the electrode had slipped out of the cell. This possibility cannot be completely ruled out, but the frequency with which sustained depolarisation was observed in high Rb solutions, using different electrodes and different uterine segments, makes this explanation unlikely.

According to the ionic theory of the nerve impulse (Hodgkin and Katz 1949; Hodgkin, Huxley and Katz 1952; Hodgkin and Huxley 1952a,b,c,d) the action potential is due to a specific increase in conductance for Na such that the membrane potential approaches the Na-equilibrium potential, followed by inactivation of the Na conductance and a delayed increase in K conductance which returns the membrane potential to its original value. The effect of Cs on the shape of the action potential in Na-rich tissues allowed to recovery in Cs-containing solutions was twofold. The initial action potentials recorded in 4.6mM Cs during recovery of Na-rich tissues had an overshoot of approximately 20-30 mV

whereas the overshoot recorded in fresh tissues was about 6-12 mV.

It appeared that in some way the presence of Cs caused both a large

overshoot, despite the probable presence of high internal Na, and the

generation of plateau-type action potentials. Although there is

still some controversy in the literature concerning the ion or ions

responsible for the rising phase of the action potential in smooth

muscle (see Kuriyama 1968), Anderson (1969) has recently concluded,

from voltage-clamp studies, that in rat uterus the regenerative

excitation mechanism is Na-dependent. The possibility that calcium

is involved in the rising phase of the action potential was not

investigated in the study mentioned above (Anderson 1969), however,

in the absence of Na the membrane potential and the spike amplitude in

taenia-coli have been shown to be a function of the external calcium

concentration (Bülbring and Kuriyama 1963a,b). Baker et al (1962)

have shown that in squid axons the presence of Cs produces a delay

in the Na inactivation process of the action potential. The

increased overshoot recorded in the presence of Cs in the present

study may be a consequence of a similar phenomenon, although proof

that Cs delays Na inactivation in uterine smooth muscle is not

available. In cardiac muscle Niedergerke and Orkand (1966) have

suggested that the upstroke of the action potential involves an inward

Ca current as well as an inward Na current. These workers also proposed

that the inward Ca current contributed to the overshoot of the action

potential of frog's heart. The large overshoot in Cs-Krebs solution

may also be a function of the external calcium concentration in Na-rich

tissues as the internal Na concentration would tend to oppose the

generation of a large spike.   The relationship between calcium

concentration and overshoot produced in the presence of Cs in

recovery Na-rich rat uterus was not investigated in the present study.

The second modification of the shape of the action potential

in Na-rich tissues recovering in 4.6 mM Cs-Krebs solution was the

presence of an extremely large plateau phase during the repolarisation.

The plateau-type action potential is a common observation characteristic

of the ureter of many species (see Burnstock et al 1963 for references).

The plateau phase can be accounted for in terms of a delay in the

increase in permeability to K or a delay in Na inactivation.   A

similar explanation has been advanced to explain the plateau-type

action potentials recorded in cardiac muscle;   Langer (1968) has

recently pointed out that the stabilisation of the membrane potential

during the plateau phase could be the result of a balance between the

prolonged increase in Na-current (inwards) and a delayed increase in

K-current (outwards) in cardiac muscle.   The long plateaus recorded

in the present study in the presence of 4.6 mM Cs may be explained by

a delay in conductance increase for Cs, accompanied by an prolongation

of the inward Na current as described above for cardiac muscle.

Na-rich tissues contain small quantities of K and the plateau phase

may also be explained by an inhibition of K conductance caused by

external Cs, during the repolarisation of the action potential.   If

Cs is responsible for carrying the current during repolarisation the

delay in repolarisation may be caused by the larger size of this ion

as compared to K (Mullins 1961).

It has been postulated that certain catecholamines may

cause hyperpolarisation of smooth muscles by stimulating an electrogenic

extrusion of Na (Burnstock 1958; Bülbring 1962). More recently,

Somlyo and Somlyo (1969) have suggested that in solutions containing

reduced amounts of K (1.0 mM), the hyperpolarisation caused by

isoproterenol in avian slow muscle is due to stimulation of an

electrogenic Na-pump. These authors also showed that the hyperpolarisation

in response to isoproterenol was converted to a depolarisation when

Na-pump activity was inhibited by ouabain, or the substitution of Li

for Na in the bathing medium. Recent studies by Diamond and Marshall

(1969a, b) have shown that epinephrine, norepinephrine and isoproterenol

all hyperpolarised rat uteri and completely abolished spontaneous

activity.

The present studies showed that the α adrenergic agonist,

noradrenaline (in the presence of propranolol) was unable to modify the

initial hyperpolarisation produced by the addition of normal Krebs

solution to a Na-rich pregnant rat uterus. Similarly in a Na-rich

tissue depolarised by the removal of K during the hyperpolarisation

induced by this ion, noradrenaline, in the presence of propranolol,

was unable to modify Na-pump activity and to increase the membrane

potential in Na-rich rat myometrial cells. The lack of effect of

noradrenaline in K-free solutions must be interpreted with caution

however for the early hyperpolarisation associated with electrogenic Na-pumping has been shown earlier in this study to depend upon the presence of external K.

In a fresh tissue the β receptor agonist, Isoproterenol, did not consistently alter the membrane potential although cessation of spike activity and abolition of spontaneous contractions always occurred in continuous recordings made from the same cell. The present series of experiments were conducted in a Krebs solution containing 4.6 mM K and 49 mM d-glucose. The hyperpolarisation, in response to Isoproterenol observed by Somlyo and Somlyo (1969) in avian slow muscle, and Diamond and Marshall (1969a, b) in rat uterus, was recorded from solutions containing less K and d-glucose than used in the present studies. Somlyo and Somlyo (1969) also showed that the magnitude of the hyperpolarisation observed in response to isoproterenol was greatest at low external K concentrations and decreased as the external K concentration was increased. The elevated glucose concentration used in the present study may also in some way have been responsible for the lack of observed hyperpolarisation in response to isoproterenol on fresh tissues.

Na-rich tissues were exposed to a reduced concentration of K in an attempt to reduce but not abolish Na-pump activity. However, the addition of Isoproterenol to such tissues did not increase the membrane potential. In addition no change in membrane potential occurred when maximal Na-pumping had been

induced by the addition of 46 mM K-Krebs solution to Na-rich tissues.
These studies suggest that under the experimental solutions and conditions
employed that isoproterenol was unable to increase Na-pumping in
Na-rich rat uteri.   In addition the membrane potential of Na-rich
rat uteri allowed to recover in Cs-Krebs solution and subsequently
depolarised, by withdrawal of Cs, was not increased by the addition
of Isoproterenol.   These studies have shown that α and β adrenergic
agonists cannot increase or decrease the activity of the Na-pump,
under the present experimental conditions in Na-rich pregnant rat uteri,
at a time when the membrane potential was controlled by electrogenic
Na-pumping.

V SUMMARY AND CONCLUSIONS

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In the introductory chapter of this thesis certain questions were posed relating to the properties of the changes in membrane potential that accompany Na-extrusion in Na-rich pregnant rat uterus. In conclusion, therefore, it is important to summarise the answers which have been obtained to these questions.

(1) <u>What are the changes in membrane potential accompanying Na-extrusion in Na-rich rat uteri ?</u>

The present studies showed that Na-rich tissues had a membrane potential of 15 mV and were quiescent. The addition of K in a concentration of 4.6 mM resulted in a rapid increase in membrane potential to 70 mV. The hyperpolarisation gradually decreased over 30-40 min. and spontaneous contractility resumed accompanied by action potentials and a membrane potential of about 50 mV.

(2) <u>What factors control the membrane potential of (a) Na-rich tissues during recovery from the Na-rich states and (b) fresh tissues.</u>

The addition of K (4.6 mM or greater) to Na-rich tissues caused a marked hyperpolarisation, the observed membrane potential clearly exceeding the K-equilibrium potential. In addition during this early hyperpolarisation, immediate depolarisation occurred upon the withdrawal of K. Those findings indicate that the membrane potential of tissues recovering from the Na-rich state was largely controlled by electrogenic Na-pumping. In fresh tissues removal of external K did not alter the membrane potential and the addition of ouabain produced a small initial

depolarisation.

(3) <u>If electrogenic Na-pumping occurs during the recovery of Na-rich tissues, what are the properties of this pump ?</u>

The initial hyperpolarisation observed when K was added to Na-rich tissues demonstrated an absolute dependence upon the presence of external K; K could be substituted by Rb and to a lesser extent by Cs but not by Li. The Na-pump was temperature dependent and inhibited reversibly by ouabain. For activity the Na-pump did not require the presence of external chloride or Na, but did require intracellular Na which could not be substituted by Li. These properties of the Na-pump are similar to those of (Na + K) activated ATPases in other tissues and suggest that the process of Na-extrusion from Na-rich tissues may result from the activity of a similar ATPase.

(4) <u>If electrogenic Na-pumping occurs during the recovery of Na-rich tissues can α or β adrenergic agonists influence the activity of the pump ?</u>

Under the present experimental conditions no evidence was obtained that α or β adrenergic agonists could modify Na-pumping in rat myometrium recovering from the Na-rich state.

In addition to answers to the above questions this study also provided additional information on the following points:

(1) Evidence was obtained that K permeability was in · some way related to the activity of the Na-pump.

(2) Cs could partially substitute for K in activating the Na-pump and induced action potentials with larger overshoots and a prolonged repolarisation phase. Furthermore, the permeability to Cs appeared to be smaller than that to K.

Further experiments that are suggested as a result of this study are outlined below.

(1) An investigation of ion movements and electrical activity occurring at lower temperatures (e.g. $4^{\circ}-25^{\circ}C$) in the presence of K in Na-rich tissues.

(2) Characterisation of the transport ATPase activity of pregnant rat myometrium in the presence of K, Rb, Li, or Cs.

(3) An electrophysiological investigation of the effects of catecholamines on fresh and Na-rich pregnant myometrium in the presence of varying concentrations of K and glucose.

(4) The properties of the uptake and efflux of Cs during the recovery of Na-rich tissues before and after inhibition of Na-pump activity by metabolic inhibition, cold or ouabain.

(5) A study of the Cs-induced plateau-phase and abnormal spike activity of recovering Na-rich tissues and the relationship of these to external calcium concentration.

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