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The Hormonal Control of Subcellular Calcium and the possible Effects on Contractility in the Rabbit Uterus

by

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ABSTRACT

The Hormonal Control of Subcellular Calcium and the possible Effects on Contractility in the Rabbit Uterus

by

Michael Lewis John Lockey

In striated and smooth muscle, it has been well established that the intracellular level of free ionic calcium regulates contraction: removal of this divalent cation from the sarcoplasm is accompanied by relaxation. Both contraction and relaxation require energy derived from ATP, and in smooth muscle there is evidence that during relaxation calcium ions are metabolically sequestered in the microsomal compartment, which functions as a calcium sink. Progesterone promotes the relaxation of uterine muscle, the inhibition of spontaneous contraction and the maintenance of pregnancy, which could be by a mechanism which modulates microsomal calcium sequestration. It was therefore decided to look for changes that progesterone might produce at this level.

Initially, uterine microsomal fragments were prepared from immature New Zealand White Rabbits which had been pretreated with either estrogen, progesterone, or a combination

of estrogen plus progesterone. The microsomes were then examined by electron microscopy for mitochondrial contamination and this was followed by the measurement of ATP dependent calcium binding. Uterine microsomes were incubated with ^{45}Ca together with ATP in buffer and the incubation was stopped by rapid filtration; the uptake was consistently greater in animals treated with progesterone alone; was maximal in eight minutes; and was saturable and reversible.

Thermodynamic analysis of the binding indicated a greater concentration of binding sites and increased affinity in the progesterone microsomes (n = 9.4 um/g protein and $K_d = 3.1.10^{-7}$ M) compared to the estrogen microsomes (n = 7.2 um/g protein and $K_d = 8.27.10^{-7}$ M). Dose-response and time course of activation and inactivation curves following a single injection of progesterone showed excellent correlation between increases in microsomal calcium binding and inhibition of spontaneous uterine contraction. However, Arrhenius plot analysis showed a single transition temperature in all preparations and suggested that ATPase activity was independent of steroidal hormonal status. This was confirmed by direct Ca-ATPase determination which showed no significant variation following different hormonal pretreatment.

It was concluded that progesterone promotes the metabolic sequestration of Ca by microsomes in amounts which could keep the cellular level below that required for

contraction and that this increase in calcium storage is a result of a greater concentration in uptake sites which bind calcium with increased avidity and not due to increases in available energy from ATP due to changes in enzyme levels.

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This dissertation is dedicated to my wife Elizabeth, who has given me constant encouragement and shown boundless patience during the course of this undertaking.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

INTRODUCTION

In analyzing the hormonal control of uterine muscle, on a species to species basis, progesterone has emerged as the only common factor concerned at some stage or other in the maintenance of pregnancy (Finn and Porter, 1975). This appears to be due to an inhibitory effect of progesterone on uterine contraction, (Schofield, 1957; Csapo, 1961b), but it is apparent that except in the rabbit and possibly the rat and mouse, it does not necessarily involve a direct action on the myometrium. In the cow there is no direct evidence that progesterone acts on myometrial musculature, although circumstantial findings suggest that it may. In sheep and women the picture is so confused that a definite statement on the role of progesterone cannot be made and in the guinea pig, there is reason to believe progesterone does not inhibit the myometrium (Finn and Porter, 1975).

The rabbit, because of its convenient size and availability, has been the most frequently used experimental model in myometrial investigations; thus more is known about the regulation of the uterine muscle in this animal than any other.

It has been established in the rabbit that the action

of progesterone reduces the amplitude of contractions in a dose related manner and that when amounts of progesterone in excess of 5 mg are given, all spontaneous mechanical activity ceases (Csapo and Takeda, 1965). However, when a single injection of progesterone is administered to the rabbit, little change is recorded for 4-6 hours after which a gradual decrease in the amplitude of contractions is observed, until after 10-12 hours activity reaches a minimum. The long interval between injection of progesterone and its effect has never been explained (Csapo and Lloyd-Jacob, 1961; Fuchs, 1964).

It has been a long established notion that, in contrast to progesterone, estrogen has an activating effect on myometrial contraction and this was demonstrated in early work both in vitro (Frank et al., 1925; Newton, 1933; Robson, 1933b) and in vivo (Parkes and Bellerby, 1927; Parkes, 1930; Reynolds, 1933). As better biochemical techniques have developed, it has been shown in rabbit uterus that estrogen maintains actomyosin concentrations, elevates ATP levels, and maximizes muscle tension (Csapo, 1950, 1961a). However, if estrogen is administered to the pregnant doe, it fails to produce uterine contraction (Schofield, 1962) and, indeed, prolongs pregnancy (Allen and Heckel, 1936, 1939; Westman and Jacobsohn, 1937; Robson, 1937, 1939; Keyes and Nalbandov, 1967).

This implies that estrogen and progesterone are not necessarily antagonistic, that estrogen is important in the synthesis and maintenance of the contractile system (Csapo, 1961a) and that progesterone maintains pregnancy by a mechanism which is unknown. In an attempt to explain the above, Csapo (1956, 1963) has formulated "the progesterone block hypothesis," which although controversial, is widely accepted. He has postulated that uterine contraction and, particularly, parturition cannot take place until the effects of progesterone are removed; this is supported by the knowledge that continuous progesterone administration in late pregnancy suppresses labor (Heckel and Allen, 1937). It has been thus concluded that estrogen can influence myometrial activity only after the blocking action of progesterone has been withdrawn (Finn and Porter, 1975). Assay data in the rabbit has shown that parturition is heralded by a fall in plasma progesterone titres (Mikhail, Noall and Allen, 1961; Hilliard, Spies and Sawyer, 1968; Challis, Davies and Ryan, 1973), thus corroborating similar early findings of Knaus (1926) and Schofield (1957, 1960).

The possibility that another myometrial inhibiting factor exists, or that progesterone may induce the production of a secondary inhibiting factor has been considered. When a cross-circulation technique is used to join pregnant rabbits to nonpregnant, ovariectomised rabbits, equipped

with intrauterine balloons, the latter experience a very rapid reduction in uterine activity. The rapidity is such that almost complete inhibition of myometrial activity occurs within one hour of cross circulation; this contrasts sharply with the 10-12 hours latency of progesterone effects (Porter, 1968, 1974).

An intracellular injection of calcium (but not potassium, sodium, or magnesium) into striated muscle elicits a mechanical response even in a calcium free solution (Sandow, 1965) and it has been shown that a minimum intracellular calcium concentration of 0.2 uM is essential for the activation of the contractile proteins (Weber, 1959; Ebashi, 1961a; Portzehl, Caldwell and Ruegg, 1964). It is currently believed that calcium regulates the major contractile proteins actin and myosin through the presence of two other proteins, tropomyosin and troponin. Tropomyosin is thought to normally obstruct the binding of actin and myosin, but in the presence of calcium ions, it is moved out of position by troponin, allowing actin-myosin interaction (Ebashi and Kodama, 1965, 1966). Relaxation of muscle is achieved by the action of a sarcoplasmic reticulum which removes and sequesters calcium from the sarcoplasm, thus restoring tropomyosin to its obstructive position.

The mechanism in smooth muscle is much less clear though considerable evidence suggests that a rise in free

intracellular calcium activates contraction, and a fall leads to relaxation (Bianchi, 1961; Edman, 1963; Daniel, 1964). The lack of a highly developed sarcoplasmic reticulum in smooth muscle has led to the idea that the increase in cytosol calcium concentration essential to activating the contractile system is achieved by transport of calcium into the cell across the plasma membrane (Ebashi and Endo, 1968). An alternate theory is that the endoplasmic reticulum plays a role in smooth muscle similar to that of the sarcoplasmic reticulum in striated muscle, namely the uptake, storage, and release of calcium (Devine et al., 1972) and Gabella (1971) has suggested that pinocytotic vesicles observed in smooth muscle represent a structure analagous to the T system in striated muscles, which is responsible for triggering calcium release from the sarcoplasmic reticulum. Both Devine (1972) and Carsten (1973a) have reported that human uterine and bovine myometrial cells have a well developed sarcoplasmic reticulum during pregnancy and evidence supporting this concept is derived from data which shows that smooth muscle rich in sarcoplasmic reticulum is better able to maintain its mechanical activity in a calcium free medium than smooth muscle with a paucity of sarcoplasmic reticulum.

The manner in which progesterone may affect the sequestration, storage, and release of calcium by the uterine

endoplasmic reticulum and its effect on uterine contraction is the subject of this dissertation.

HISTORICAL ANALYSIS

Hormonal control of myometrial activity

Regnier de Graaf first published a description of the bovine corpus luteum in 1672 but it was more than two centuries later before Prenant (1898) suggested that it might be an endocrine organ. Shortly thereafter, the classic experiments of Fraenkel (1903) emphasized that, in the rabbit, corpora lutea were essential for the maintenance of pregnancy; this was followed by a series of papers (Athias, 1919; Blair, 1922; Keye, 1923; Corner, 1923; Frank, Bonham and Gustavson, 1925) suggesting that the ovary was important in the regulation of myometrial activity. In 1926 Knaus used posterior pituitary extract in an attempt to induce abortion at various stages of pregnancy in the rabbit, but found that the uterus was refractory to this extract and concluded that this was due to the presence of corpora lutea. In 1929 Allen and Corner demonstrated that abortion could be prevented by the administration of luteal extract, but a more precise association became possible when progesterone was identified and isolated from the corpus luteum of pregnant sows (Wintersteiner and Allen, 1934). Subsequently, it was found that pure progesterone alone was

capable of inhibiting abortion (Portman, 1934; Courrier and Kehl, 1938; Allen and Heckel, 1939; Wu and Allen, 1939).

These findings were amplified by both *in vitro* studies, which tended to indicate the inhibitory effect of progesterone on the oxytocin response (Makepiece, Corner and Allen, 1935; Robson, 1935a,b), and *in vivo* investigations, which revealed the inhibitory effect of progesterone on spontaneous uterine activity (Reynolds, 1935). Prior to 1930 the *in vitro* studies of the myometrium invariably used an isotonic method, but in that year, Reynolds developed a technique for establishing a uterine fistula in the rabbit into which a balloon could be inserted to record activity in the conscious animal. It was mainly based on this work and the ability of progesterone to replace corpora lutea, that the concept of progesterone as the "defence hormone of pregnancy" was developed.

Meanwhile, in other endocrine research the role of estrogen in pregnancy was being investigated. Despite a considerable divergence of results, a series of *in vitro* and *in vivo* experiments (Frank *et al.*, Parkes and Bellerby, 1927; Parkes, 1930; Newton, 1933; Robson, 1933; Reynolds, 1933) had suggested that estrogen facilitated uterine contraction. This led to the concept of the estrogen/progesterone ratio mechanism of pregnancy maintenance. This idea postulated that the behaviour of the myometrium was determined

not by the asbolute quantities of either estrogen or progesterone circulating in the blood, but by their ratio; thus, if the effective amount of progesterone in the plasma exceeded the effective amount of estrogen, the uterus was quiescent, whereas if estrogen were in excess the uterus was active. This formed an explanation for the onset of labor, and had numerous proponents (Robson, 1933, 1935a; Reynolds, 1935; Cohen, Marrion and Watson, 1935; Courrier and Kehl, 1938).

However, evidence against the ratio theory gradually accumulated over several decades and it was superceded by the "progesterone withdrawal" and "progesterone block" theories. Schofield (1962) administered 25-30 ug/day of estrogen subcutaneously to the pregnant doe and found that it did not interrupt pregnancy; indeed, it confirmed earlier reports that such treatment prolonged pregnancy presumably due to a luteotrophic effect (Allen and Heckel, 1936; Westman and Jacobsohn, 1937; Robson, 1937, 1939; Heckel and Allen, 1938, 1939). Csapo and Lloyd-Jacob (1961) carried out placental dislocation in the rabbit and measured the interval until abortion occurred. They then combined this procedure with ovariectomy and estrogen treatment; the interval until abortion took place was no shorter than when placental dislocation alone was performed (Porter, Becker and Csapo, 1968), thus showing that the administration of

estrogen to an animal with waning progesterone titres consequent to ovariectomy did not hasten ensuing abortion. It was established by several investigators that the administration of estradiol to rabbits, even in doses as high as 50 ug/day did not increase the dose of progesterone required to produce myometrial refractoriness (Csapo and Corner, 1952; Schofield, 1954; Coutinho and DeMattos, 1968), and studies of estrogen treatment in other animal systems failed to produce evidence that an increase in estrogen was abortifacient or that the ratio theory was tenable (Greene, Burrill and Ivy, 1940; Dewar, 1968).

The concepts of "progesterone withdrawal" being crucial to the preparatory events of parturition was advanced as evidence against the ratio theory mounted (Csapo, 1956, 1963; Mikhail, Noall and Allen, 1961) and support of the withdrawal theory was provided by Hilliard, Spies and Sawyer (1968) with chemical assay data which showed that at parturition in the rabbit there was a dramatic fall in plasma progesterone titres. These findings corroborated the earlier discovery of Heckel and Allen (1937, 1939), that the continuous administration of progesterone in late pregnancy suppressed labor and subsequently, on the basis of more quantitative data, Csapo formulated the "progesterone block hypothesis".

Csapo's hypothesis was based on observations of

myometrial electrical activity; he had observed in the uterus that progesterone reduced the synchrony of electrical phenomena and that a 5 mg dose almost abolished electrical activity completely. Csapo thus argued that progesterone impaired the electrocoupling of the myometrial cells, reducing or blocking contraction. This action persisted until the hormone was removed.

In 1973, the first systematic study was made of estrogen and progesterone concentrations in peripheral rabbit blood, collected throughout the course of pregnancy. Using the radioimmunoassay technique, Challis, Davies and Ryan determined daily the amounts of estrone, estradiol - 17 beta and progesterone in the arterial plasma of pregnant rabbits. They found that the concentration of progesterone gradually increased during gestation until around mid-pregnancy when the highest concentrations were observed; this was followed by a gradual decline until term. The values of estrone in the plasma did not vary over a range that was statistically significant and although the estradiol - 17 beta showed greater variability, the most dramatic change occurred in the mean ratio of progesterone to estradiol - 17 beta. This ratio increased during the first part of pregnancy, remained constant during the mid and latter part and then declined precipitously on the last day before birth. This finding prompted Challis to revive the concept that the estrogen-

progesterone ratio was the important parameter in the termination of pregnancy and he argued that although in the rat the fall of progesterone on the last day of pregnancy was precipitous (Csapo, 1956, 1969) and contrasted with the gradual decline observed in the rabbit, in both animals a spectacular increase in the estrogen-progesterone ratio occurred prepartum.

One important point that remained unexplained by Challis related to the rate of recovery of the uterus following progesterone treatment in non-pregnant rabbits. It had been observed to be characterized by a gradual resumption of mechanical activity over approximately a sixty hour period. This point also remained unexplained by Csapo's progesterone block hypothesis since during the period of declining progesterone titres in late pregnancy in the rabbit there had been virtually no increase in uterine mechanical activity.

In 1968 Porter raised the idea that an alternate component, capable of inhibiting myometrial activity, might be present in plasma, and, in 1974, the same investigator using a cross circulation technique demonstrated the presence of a frequency modulating factor in the plasma of pregnant rabbits which produced rapid changes in myometrial activity compared with the sluggish response observed after the administration of progesterone alone. When non-pregnant rabbits were cross circulated with progesterone treated does

alone, no effect was seen and the existence of a secondary myometrial inhibitory factor acting in conjunction with progesterone in maintaining pregnancy was postulated (Porter, 1974). Such a factor explained both the lag periods unaccounted for by Challis and Csapo and, in addition, the 3-5 day lag periods observed in procedures designed to induce premature delivery by the administration of agents, such as dexamethasone, which reduced plasma progesterone and, presumably, myometrial progesterone (Challis, 1974). It was reported that during this lag period of lowered progesterone concentration (Porter and Challis, 1975), there was no increase in uterine activity indicating that in the rabbit progesterone withdrawal alone was inadequate to precipitate parturition immediately.

The chemical nature of the putative second substance remains unknown although a relaxin-like compound has been predicted (Finn and Porter, 1975) similar to a myometrial inhibitory factor found in the guinea pig (Porter, 1972). It is true that high titres of relaxin have been measured in late pregnancy in the rabbit (Zarrow and Rosenberg, 1953) but attempts to demonstrate myometrial relaxing action of this substance have been unsuccessful (Finn and Porter, 1975).

It has also been postulated that the time lag in recovery from progesterone effects relates to uterine

prostaglandins biosynthesis since in the rat there is good evidence that the stimulatory effect of oxytocin on the uterus is mediated by prostaglandins. Csapo (1972) has advanced the concept that prostaglandins are "the intrinsic myometrial stimulants" and that prostaglandins expression follows removal of progesterone block. This concept is supported by the finding that administration of a prostaglandin synthetase inhibitor to pregnant rabbits prolonged gestation (Challis, 1975).

Studies on the fate of hormone molecules have recently given some insight into the biochemical action of progesterone at the molecular level. Two models have been found suitable for investigation: the uterine deciduoma reaction in rats and the induction of avidin synthesis in the chick oviduct.

The former has been used to study the implantation of the blastocyst into the 'receptive' uterine endometrium (DeFeo, 1967). It has been found that this response is facilitated by progesterone alone and that this hormone exerts a profound stimulatory effect on decidual RNA synthesis. Actinomycin D blocks the reaction suggesting the response is dependent on new RNA synthesis. So far no systems are available to investigate specific progesterone mediated changes in nucleic acid and protein metabolism in the endometrial cell transformation; however, in the chick

oviduct model it has been possible to study the induction of a single new specific protein, avidin.

Studies of this system have indicated that the oviduct epithelium contains specific receptors with a high affinity for progesterone. Interaction of these cells involves the association of hormone with extranuclear receptor protein followed by the translocation of the resulting complex to the nucleus where the synthesis of new sequences of oviduct nuclear RNA is induced. This nuclear RNA is subsequently transported into the cytoplasm where the synthesis of avidin occurs. The molecular mechanism by which progesterone produces uterine quiessence is still completely unknown. However, from the evidence reviewed thus far one may speculate that progesterone in itself does not maintain pregnancy nor cause uterine inhibition, but that it may induce the synthesis of a "second messenger" which is capable of modifying smooth muscle activity. This idea gains some support from time course observations, cross circulation experiments and the avidin model (O'Malley, 1969).

Similarly, estrogen by itself appears not to give rise to uterine contraction, but in a mechanism that may be similar to that of progesterone, it gives rise to an increase in ATP and muscle protein synthesis. Additionally, estrogen appears to sensitize these contractile elements so that stimulation by prostaglandins, oxytocin or catecholamines

more readily produces contraction (Csapo and Wiest, 1969).

A major challenge of molecular endocrinology in this field will be elucidation of these mechanisms.

Control of muscle contraction by alpha and beta adrenergic receptors.

In 1906 A. R. Cushny and Sir Henry Dale, working independently, observed that in the cat, stimulation of the hypogastric nerves gave rise to contraction of the uterus in the pregnant animal and to relaxation in the non-pregnant animal. Dale termed this phenomenon as "pregnancy reversal" and found that it was also mimicked by epinephrine stimulation. In 1913, he made a similar discovery whilst monitoring changes in blood pressure in the carotid artery of the pithed cat. He found that 'adrenine' (a crude adrenal extract), when injected into the femoral vein, normally produced a marked rise in blood pressure. If however the same animal had been pretreated with ergotoxine, the effects allowed to subside, and then injected with 'adrenine', a dramatic fall in arterial pressure was recorded. In other words, a relaxing action of 'adrenine' had been unmasked when its constrictor activity had been blocked with ergotoxine.

The implications of these rudimentary observations did not reveal themselves until almost twenty-five years later

when Rudolph and Ivy (1930) who were concerned with the clinical problem of uterine inertia observed that Dale's "pregnancy reversal" in the rabbit was exactly opposite from that in the cat.

A host of theories were immediately forthcoming to explain "pregnancy reversal", but the most widely accepted was that the hypogastric nerves contained both excitatory and inhibitory fibers and that during pregnancy there was a relative change in the dominance of the two systems (Gruber, 1933).

However, Langley (1901) with amazing perspicacity had previously suggested that effector cells might contain both excitatory and inhibitory substances and that myometrial response depended on the relative proportion of the two substances released during stimulation. In 1926 Gaddum, studying the effects of epinephrine on the ergotamine pretreated, isolated rabbit uterus, had advanced the idea that epinephrine (believed at that time to be synonymous with adrenergic nerve stimulation) acted on specific areas of the myometrial cell, and that ergotamine blocked a fraction of this area so that the concentration of epinephrine had to be increased to overcome the block: Gaddum's ideas had focused on the effector cell rather than the nerve fibers, but unfortunately the idea that changes took place

within the nerve fibers during pregnancy remained in vogue despite demonstration by Loewi (1921) showing chemical transmission, and the work of Cannon and associates on adrenergic transmission (1937).

Today, we know that stimulation of the hypogastric nerves releases both epinephrine together with norepinephrine irrespective of the hormonal or gravid state of the animal (Mann and West, 1951; Vogt, 1965), and that the ability to respond to stimulation resides in the effector cell. Whether the response is excitatory or inhibitory is determined by the reaction of the neurotransmitter with specific molecular sites on or within the effector cell; these sites have been named "adrenoceptors" (Dale, 1954), however we are still largely ignorant as to their precise chemical and morphological nature.

In 1948 Ahlquist classified "adrenoceptors" into two categories: he designated the nonspecific terms alpha and beta to delineate the receptors subserving the two sets of responses and formulated the classification of alpha into excitatory responses, including vasoconstriction, contraction of the uterus and ureter; and beta into those responses associated with inhibition including vasodilation and relaxation of uterine and bronchial musculature. In addition he maintained that neither were alpha receptors wholly excitatory nor were beta receptors entirely inhibitory. The

response which was observed depended on the extent to which one or the other dominated. Using the Ahlquist classification, all uteri examined thus far have been found to contain both alpha and beta adrenergic receptors (Miller, 1967) and the relative dominance of either type of receptor is apparently under hormonal control. Pretreatment of immature rabbits with the gonadal steroids estrogen and progesterone has been found to alter the response to catecholamines. Specifically, uterine contraction, mediated by alpha adrenergic receptors, is enhanced in uteri from estrogen pretreated animals, whilst relaxation, the beta adrenergic response, predominates after progesterone treatment.

The response of the uteri from several species has been observed to convert from contraction to relaxation depending on the concentration of the steroids. This conversion from alpha to beta response with hormonal environment has prompted the hypothesis that alpha and beta adrenergic receptors may be structurally related and interconvertable. This general relationship has not been demonstrated experimentally.

However, some generalizations concerning alpha and beta receptor mechanisms have been established using pharmacological agents classified as agonists and antagonists. Agonists activate receptors and, whilst antagonists produce no observable effect by themselves, they reduce the effect of agonists. A careful and systematic analysis of the

actions of these agents, together with the use of radioligands has so far characterized the rate at which receptors react, their affinities, and the efficacy of drugs on these receptors.

An important breakthrough in the study of adrenoceptors was achieved by Sutherland and Rall (1958) who discovered adenylate cyclase, a membrane bound enzyme, stimulated by catecholamines and peptide hormones but not by steroids. From their finding grew the concept that cellular receptors and effectors were physically distinct when it was observed that activation of adenylate cyclase accelerated the intracellular formation of 'a second messenger', cyclic AMP, which regulated various metabolic processes. It was subsequently found that cyclic AMP occurred in all animal species, including bacteria and unicellular organisms.

The existence was then shown of a second natural universally occurring cyclic nucleotide, cyclic GMP; the cellular levels of this chemical were elevated by biological agents that produced physiological responses exactly opposite to those when tissue cyclic AMP concentrations were elevated (George et al., 1970; Goldberg, 1973). These observations led Goldberg (1973) to propose the dualistic or yin-yang hypothesis of biological control which stated that systems stimulated by an increase in cyclic AMP were suppressed by an increase in cellular cyclic GMP concentration. In other

words, in systems where an increase in cyclic AMP had been observed to be inhibitory, (the beta response), an increase in cyclic GMP concentration was thought to be stimulatory (the alpha response).

The discovery of the ubiquitous nature of cyclic AMP and cyclic GMP has initiated an explosive research effort producing a considerable number of publications supporting the idea that beta adrenergic effects are mediated by cyclic AMP (Andersson and Mohme-Lundholm, 1969; Kawasaki et al., 1969; Walaas and Walaas, 1970; Skelton et al., 1970; Kukovetz and Poch, 1970; Shinebourne and White, 1970; Krause et al., 1970; Bowman and Hall, 1970) and that alpha adrenergic responses are associated with the intracellular accumulation of cyclic GMP (Ball, 1972; George, 1972). However, despite these encouraging findings the yin-yang theory cannot yet be considered proven and its establishment or rejection must await the outcome of future work.

Calcium and cyclic nucleotides as interrelated 'second messengers'.

In 1970, two investigators working independently (Rasmussen, 1970; Rubin, 1970) suggested that calcium and cyclic AMP were connected sub-cellular regulators. The available information on the interactions between these substances is still very fragmentary but cyclic AMP has been

shown to activate cellular mechanisms for the removal of calcium by sarcoplasmic reticulum (Entman, Levey and Epstein, 1969) and aortic microsomes (Baudouin-Legros and Meyer, 1973).

Bohr (1973) reported that calcium was essential for the alpha adrenergic response, prompting Williams and Lefkowitz (1976) to set out to determine if this calcium dependence was at the alpha adrenergic receptor site. They found that neither the addition nor the removal of calcium affected the number of alpha sites or the affinity of adrenergic agonists. From this it was concluded that the effect of calcium was not at the receptor but at some physically distal step in the alpha response. This conclusion agreed with one previously published by Steer, Atlas and Levitski (1975) who in an examination of receptor sites, adenyl cyclase and calcium, had proposed that, in the beta adrenergic response, the cellular binding sites for calcium were distinct from the receptor sites for hormone.

Much more work needs to be done on the relationships between calcium and cyclic nucleotides but the evidence accumulated so far indicates that they play a vital role in regulating cell activity. The available information suggests that in a wide range of systems, but not all, cyclic nucleotides modulate the levels of intracellular calcium; and it would appear that the primary intracellular second messenger

is calcium and that cyclic nucleotides play a secondary role in either dampening or enhancing the calcium signal (Rasmussen and Goodman, 1975).

Role of calcium in muscle contraction

The recognition by Galvani (1791) that a piece of metal induced the contraction of muscle was largely ignored and it wasn't until the classical studies of Ringer (1882, 1883a, 1883b) with physiological salt solutions that the way was paved for subsequent investigation of cell function and electrolyte environment. In 1907, working with mammalian cardiac muscle, Locke and Rosenheim discovered that calcium was in some way related to the coupling of chemical energy with mechanical work and shortly thereafter, Straub (1912) recorded that the removal of calcium from a solution bathing a frog's heart reduced the initial heartbeat. One year later Mines (1913) demonstrated that if calcium was excluded from Ringer's solution there occurred in heart muscle an uncoupling of action potential from contraction and he showed conclusively that although the excitatory process could proceed in the absence of calcium, under these conditions the muscle was unable to contract.

Thus, calcium was established at an early date as an integral part of the muscle contraction process but it was almost thirty years before a relationship between

calcium and muscle protein was proposed by Bailey (1942). In the literature prior to 1942 the term myosin usually referred to a crude extract of protein first obtained and named by Kuhne almost a century previously. This extract consisted of a complex of myosin, as it is understood today, and another protein, actin, discovered by Straub (1942). Nevertheless, the ATPase activity of myosin had been unveiled by Englehardt and Ljubimova (1939) and myosin catalyzed dephosphorylation of ATP had provided an early suggestion that this was the energy source for muscle contraction. Bailey looked at numerous metallic ions and their ability to activate myosin ATPase: he tentatively suggested that free ionic calcium regulated myosin ATPase activity which in turn controlled muscle contraction.

At this time the molecular biology of muscle was very much in its infancy and Szent-Gyorgyi (1945, 1948) who was trying to clarify the relationship between the recently discovered actin, myosin and ATP, believed that the potassium ion was primarily responsible for the regulation of muscle contraction. However, supporting evidence for Bailey came in 1947 from Heilbrunn and Wiercinski who used a microdissection technique to inject solutions of various salts into the interior of isolated muscle fibers of the frog. The effect of the solutions was measured under the microscope and it was observed that the calcium ion, even

when highly diluted, gave rise to an immediate and pronounced shortening; this effect was not seen when any other ion normally present in muscle was used.

In the years that followed, great progress in elucidating the basic molecular features of muscle contraction was made; most of the work was done in striated muscle and in 1953 Hanson and Huxley put forward the interdigitating filament model which is still currently accepted. According to this model, the contractile material consists of a long series of partially overlapping arrays of thin actin filaments and thick myosin filaments and the space between them, the sarcoplasm, is a dilute aqueous solution of salts and other proteins. When the muscle contracts or relaxes, the model supposes that the length of the filaments remain relatively constant but that the overlapping arrays slide past each other (Huxley H.E., 1954; Huxley, A.F., 1954). The sliding force is developed as a consequence of direct physical contact, termed cross bridges, between the myosin and actin filaments.

The molecular role of calcium in the interdigitating model was completely unknown in 1953 but it was noted by Perry and Grey (1956) that the ATPase activity of extensively purified actomyosin was refractory to the absence of calcium; unlike the activity of unpurified systems it continued high when calcium was withdrawn. The significance

of this was not understood until 1965 when Ebashi dramatically showed that calcium sensitivity could be restored to such purified systems by adding back a certain protein fraction. This fraction was subsequently shown to contain two principal proteins, tropomyosin B (Bailey, 1948) and a new protein troponin (Ebashi, 1965). Ebashi (1967) then ingeniously showed that ionic calcium acted directly on the troponin moiety rather than on the actomyosin and that it also did so in sufficient amounts to activate ATPase, something which actomyosin could not do alone (Weber, 1963). It had been suspected for several years that tropomyosin was present as part of the thin actin filaments (Huxley, 1957, 1960; Perry, 1958; Hanson, 1963), and it was confirmed by Pepe (1966) using a florescent antibody technique and by Endo (1966), who also demonstrated that the troponin molecule occupied part of the actin filament. Ebashi (1965) demonstrated that troponin combined with the tropomyosin complex but not with myosin (Ebashi, 1967b; Kominz, 1967). Thus at the end of the sixties a very compelling molecular picture was emerging that troponin acted as a safety catch, functioning as an allosteric regulatory unit, preventing the activation of myosin ATPase by actin when calcium was absent, but allowing activation to occur as soon as calcium was bound to troponin.

Thus the chemical trigger setting off a contraction was

seen to be the release of calcium into the sarcoplasm, which in turn stimulated myosin ATPase giving rise to the breakdown of ATP and the formation of cross bridges. However, one important question remained unanswered: how did the muscle relax again? The first natural factor thought to effect relaxation was the socalled Marsh factor (Marsh, 1951, 1952). Marsh discovered that a specific extract of muscle prevented the in vitro syneresis of myofibril bundles in the presence of ATP and that this inhibition could be overcome by the addition of small amounts of calcium. Later, Ebashi and Lipmann (1962) confirmed that the relaxation of muscle fibers could be achieved by decreasing the concentrations of calcium ions in the intracellular fluid. Marsh's factor had been prepared by extracting striated muscle homogenates at low ionic strength; the preparations had been crude and contaminated and it was only when high speed centrifugation was readily available that the factor was purified and identified with the microsomal fragment.

In 1957 Porter and Pallade published a detailed study of the relaxing factor which they described as a system of membrane limited vesicles, tubules and cysternae associated within a continuous reticular structure forming lace-like sleeves around the myofibrils. This structure is now universally known as the sarcoplasmic reticulum. However, even after the sarcoplasmic reticulum had been morphologically

portrayed, its calcium sequestering activity was not recognized for several years and had to await the advent of radio-active calcium. Meanwhile, Fleckenstein (1954) and Mommaerts (1955) had observed that intramuscular levels of ATP were about the same during contraction and relaxation and surprisingly both processes required the presence of ATP. Thus, it was logical that shortly thereafter a theory that the concentration of ionized calcium determined whether ATP gave rise to contraction or relaxation was advanced and was supported by kinetic data on myofibrillar ATPase activity (Weber, 1959).

Ebashi (1960, 1961a, 1961b, 1962) as well as Hasselbach and Makinose (1961) discovered that fragments of the sarcoplasmic reticulum bound calcium in the presence of ATP and magnesium. Ebashi considered this binding of calcium by the reticulum as the basis of its relaxing effect (1961a, 1961b) thus concurring with a previous suggestion of Weber (1959) that the relaxation of muscle was effected by the sequestration of calcium by the sarcoplasmic reticulum. Furthermore Ebashi demonstrated that the reticulum reduced the amounts of calcium bound to actomyosin (1961a) and more detailed quantitative studies (Weber, 1963) showed that the reticulum caused maximum inhibition of syneresis and ATPase activity of myofibrils after reducing their calcium content from 2 to 1 uM/g protein. Time course experiments indicated

calcium removal was strictly parallel to increasing restraint of ATPase activity and reversal of syneresis.

A number of ancillary investigations showed that when calcium accumulation by the sarcoplasmic reticulum was inhibited by salyrgran (Hasselbach, 1961, 1964b), oleinate (Hasselbach, 1962, 1964b), cetyltrimethylammonium chloride (Hasselbach, 1964b), phospholipase (Ebashi, 1958; Martonosi, 1963), amytal (Muscatello, 1962; Carsten, 1964), ADP (Ebashi, 1962; Hasselbach, 1962) and caffeine (Nagai, 1962; Hasselbach, 1964b; Herz, 1965), relaxation by the reticulum was also inhibited. Conversely, agents which augmented calcium uptake by the reticulum, such as oxalate (Baird, 1960; Hasselbach, 1961), pyrophosphate (Baird, 1960; Martonosi, 1964a; Hasselbach, 1964b), citrate (Hasselbach 1964b) and inorganic phosphate (Lorand, 1962; Seidel, 1964; Hasselbach, 1964b) also increased the relaxation effect of the medium.

Simultaneously, Hasselbach (1961, 1963) found that the sarcoplasmic reticulum was capable of accumulating up to 8 uM calcium oxalate per mg protein which was deposited in the interior of the vesicles as a calcium oxalate precipitate (Hasselbach, 1964b). The same investigator also established that the calcium was transported against an active gradient. Calcium and oxalate moved from a level of ion activities below the solubility product in the medium to a level above the solubility product in the interior of the vesicles.

Oxalate was not accumulated without calcium but calcium was accumulated without oxalate; it was concluded, therefore, that calcium was the actively transported ion.

The development of detailed cohesive theories of muscle contraction based on ion fluxes and the sliding filament theory were possible using information and data derived almost entirely from striated muscle but the numerous studies on smooth muscle have produced some bewildering differences as well as similarities.

Csapo, working in the laboratory of Szent-Gyorgyi applied the latter's extraction technique to smooth muscle and, using uterine muscle from women, rabbits, rats and cows, found that actin and myosin were present in all samples. He also found that the amounts were greater in gravid than in non-gravid uteri, but that the content of smooth muscle was only 10% of skeletal muscle.

There is considerable evidence that in smooth muscle a rise in free intracellular calcium activates contraction and a fall leads to relaxation (Durbin, 1961; Bianchi, 1961, 1968a, 1968b; Edman, 1962; Grossman, 1964; Jobsis, 1964; Weber, 1964; Daniel, 1964, 1965; Sandow, 1965; Nayler, 1966; Schild, 1967; Peachey, 1968; Winegrad, 1968). However, the source of activator calcium, whether extracellular or intracellular and the ultrastructural organization of the myosin molecules in smooth muscle has remained speculative

for several decades.

Structurally, smooth muscle differs strikingly from striated, consisting of small bundles of cells separated by collagen. There are no well defined bands and the various contractile filaments do not lie in an ordered array as in voluntary muscle: both the myosin and actin filaments are irregularly distributed (Bagby, et al., 1974). Isolated muscle studies have suggested that these contractile elements are grouped into fibrils, which lie obliquely to the cell axis.

The regulatory components have not been at all characterized and there is doubt whether specialized regulatory proteins exist. Ebashi, et al., (1966) described a tropomyosin-like compound in chicken gizzard and Carsten (1971) reported the isolation of troponin from the uterus. However, Hartshorne, et al., (1977) have been unable to confirm these reports.

The work of Sobieszek and Bremel (1975), in chicken gizzard, has suggested a calcium-myosin regulating mechanism of muscle contraction, reminiscent of that seen in invertebrates. If this finding is confirmed it would suggest that vertebrate smooth muscle is a primitive type of contractile cell, lacking the specialization of striated muscle.

It has been postulated that activator calcium arises from an increase in the influx of extracellular calcium ions or that it may arise from the translocation of calcium bound

at the surface of the plasma membrane or concentrated at an intracellular calcium sink analagous to the sarcoplasmic reticulum in striated muscle. A large volume of indirect evidence has been presented in the literature for both possibilities.

The first paper dealing with calcium influxes into smooth muscle was published by Schatzmann in 1961, who measured ^{45}Ca uptake. Unfortunately, Schatzmann recorded uptakes only at incubation times of 20 and 50 minutes. However, his work did stimulate subsequent publications in which the experiments were further refined. The data obtained in the years immediately subsequent to Schatzmann's work was explained on the basis of the existence of only one type of calcium binding site (Daniel, 1963, 1964) but in 1965 it became obvious that this model was inadequate (Daniel, 1965).

Frank (1965) and Nayler (1965) had shown in striated and heart muscle sites at which calcium was loosely bound and lost rapidly by diffusion together with a second type of site at which calcium was more tightly bound and not released by depolarization. Dissociation of calcium from these latter sites could be achieved by acetylcholine or caffeine and was therefore termed pharmomechanical coupling (Somlyo, 1968).

The sites were called superficial and sequestering

sites respectively and the general hypothesis arose that calcium bound at superficial membrane sites might be released by depolarization and give rise to the release of calcium from the sequestering sites. Release of calcium from these latter sites, it was suggested, might be sufficient to initiate contraction.

The hypothesis was not universally accepted (Daniel, 1965) since it was believed by many that sarcoplasmic reticulum was absent or extremely scanty in smooth muscle and that the amounts of calcium bound at these sites would be extremely minute and very slow to exchange.

It wasn't until 1971, using electron miscroscopy, that an extensive calcium sequestering sarcoplasmic reticulum was seen in smooth muscle from the guinea pig ileum and human uterus (Gabella, 1971; Somlyo, 1971). However, these studies also revealed mitochondrial fractions with similar calcium sequestering and releasing properties. Since that time there has been lingering speculation in the literature regarding the calcium binding roles of both the smooth muscle sarcoplasmic reticulum and the mitochondria (Carafoli, 1973; Wikstron, 1974).

Mitochondria isolated from several sources have been intensively studied but quantitative questions have been hampered by difficulties which have arisen in determining the calcium binding capacity of muscle mitochondria and

whether this capacity is sufficient to be a sink for activator calcium (Ford, 1976).

Non-mitochondrial reticular preparations have also been obtained from a variety of smooth muscles but the methods of preparation and the properties of these fractions have shown a great deal of variation: it is certain that in many instances they have been a heterogeneous mixture of sarcoplasmic reticulum and plasma membrane. Despite the uncertainties as to the nature of these non-mitochondrial preparations it has been reported that cyclic AMP or dibutyryl cyclic AMP increased calcium uptake in preparations from guinea pig ileum (Anderrson, 1972) and rabbit aorta (Baudouin Legros, 1973) but that there was no effect in rat uterus (Batra, 1971). Baudouin Legros (1973) reported that epinephrine increased calcium uptake in rabbit aorta microsomes and Batra (1971) obtained an increased uptake in rat uterus. Isoproterenol produced a similar increase in guinea pig ileum (Anderrson, 1972). Epinephrine has been reported to decrease net calcium uptake in other guinea pig ileum preparations (Godfraind, 1973) and in rabbit aorta microsomes angiotensin II decreased uptake of calcium. In the same preparation norepinephrine also decreased the calcium uptake (Baudouin Legros, 1973) and dibutyryl cyclic AMP had the opposite effect.

Although the results both from mitochondrial and non-

mitochondrial in vitro experiments have been seen as a promising approach to a better understanding of the roles of various smooth muscle subcellular elements, general interpretations so far have not been possible. Recently, Ford (1976) has reviewed the evidence and concluded that in smooth muscle the classical excitation-contraction couple, in which rapid membrane depolarization precedes muscle tension development, depends mainly on extracellular sources for activator calcium (Waugh, 1962; Northover, 1968; Somlyo, 1968; Somlyo, 1968; Godfriand, 1972; van Breeman, 1972). However, he is quick to point out that precise storage locations for this extracellular calcium are still unknown (Somlyo, 1968; van Breeman, 1973). He then postulates that the link between membrane stimulation and tension development is cytoplasmic calcium and that the latter event relies upon intracellular sources for activator calcium.

The morphological location of intracellular calcium sinks has recently been identified positively in a refined series of electron microscope experiments by the Somlyos (1976). These studies have again confirmed two subcellular structures capable of sequestering calcium and other divalent cations, mitochondria, in close apposition to the surface vesicles, and sarcoplasmic reticulum. The sarcoplasmic reticulum has been found in all smooth muscle examined and consists of a system of tubules which have been classified

into smooth walled and rough walled varieties.

In influx experiments extracellular markers, including ferritin, colloidal lanthanum and horseradish peroxidase, have been observed not to enter the sarcoplasmic reticulum but only into the surface vesicles. The volume of the sarcoplasmic reticulum has been estimated and appears to vary in different types of muscle; its extent within a muscle correlates well with the ability of a given smooth muscle to contract in the absence of extracellular calcium.

The outer tubules and the laminae of the sarcoplasmic reticulum approximate closely to the plasma membrane of the smooth muscle cell and Somlyo (1971) has postulated that in this region action potentials may release calcium thus activating contraction.

The same author (Somlyo, 1971, 1974) incubated strontium (an electron opaque divalent cation capable of substituting for calcium in numerous biological reactions) with vascular smooth muscle prior to electron microscopy. This procedure revealed extensive deposits of strontium in the lumen of the smooth walled, but not the rough walled, reticulum. Somlyo (1976) considered that these findings supported the concept that the sarcoplasmic reticulum in smooth muscle plays a similar role to that in striated muscle as the source of activator calcium.

The majority of the evidence described so far suggests

that there is energy dependent calcium binding to microsomal vesicles. However, there is no direct evidence, like that available for striated muscles, that the calcium found in this microsomal reticulum is released when the muscle contracts (Winegrad, 1965). On the other hand, muscles with the more abundant sarcoplasmic reticulum can still respond to external stimuli in calcium free media, whereas others that have little sarcoplasmic reticulum cannot; this correlation may be coincidental, but does support the views that calcium ions are stored intracellularily and not on surface vesicles.

Steroids and calcium

The first tentative attempt to correlate ovarian hormones, intracellular calcium and uterine contraction was made in 1950 by Walaas who injected estradiol benzoate into female rats and measured the calcium content of the uterus. He was unable to find any significant changes compared to control animals.

In 1956, Csapo undertook a more extensive study of the endocrine regulation of smooth muscle electrolytes, and, using rabbit myometrium, set out to compare the estrogen dominated uterus with that dominated by progesterone. In comparing excised uterine strips under contrasting endocrine conditions, he found exactly opposite behaviour. If rabbit

myometrium was perfused with calcium free Kreb's solution, tension rapidly declined in the estrogen dominated uterus, whereas very little change was observed if the uterus was under the influence of progesterone. Csapo interpreted this to mean that either a strong calcium complex had been formed in which calcium was not freed easily and therefore could not be washed out of the muscle or that the progesterone dominated uterus required less calcium.

In 1959 Goto and Csapo again noticed that progesterone increased the amount of bound calcium in the uterus and two years later Berger and Marshall rediscovered the earlier findings of Csapo; specifically they reported that when myometrium from both estrogen and progesterone treated animals was repeatedly washed with calcium free Kreb's solution, the time to reach complete inhibition of response was 15 minutes in estrogen pretreated muscle but 45 minutes in those pretreated by progesterone. They concluded that calcium was bound more strongly in the latter.

There is a paucity of recent work on the relationship between gonadal steroids and intracellular calcium but in a different approach Carsten (1973) isolated fragmented endoplasmic reticulum from both gravid and non-gravid bovine uteri. The specific calcium binding of both types of uterine reticular fragments were measured and compared. Carsten reported a striking increase in yield of reticulum

from the uteri of pregnant animals which when compared with non-pregnant ones was 50% higher. It was also found that the intrinsic calcium was higher in the pregnant uterus, but that the *in vitro* ATP dependent calcium binding was not statistically different between the pregnant and non-pregnant specimens. However, due to the higher sarcoplasmic reticulum yield, the calcium binding capacity of the pregnant uterus *in vivo* was computed to be significantly higher ($P < 0.05$).

THE NATURE OF THIS STUDY

Intracellular ionic calcium has been shown to be required for all muscle contraction and the alpha adrenergic response. These concepts are compatible but it is not known whether the role of calcium is identical in both mechanisms. However, evidence does suggest that calcium is involved in both processes at intracellular effector sites and not at plasma membrane receptors.

The beta adrenergic response is thought to be associated with an increase in intracellular cyclic AMP and there is evidence that cyclic AMP promotes the lowering of cytoplasmic calcium levels by increasing its removal by sarcoplasmic reticulum and microsomes. In striated and smooth muscle it has been well documented that such removal of calcium is accompanied by muscle relaxation.

Progesterone promotes the beta adrenergic response, the inhibition of spontaneous uterine contraction and the maintenance of pregnancy. Recent findings concerning its molecular mechanism suggest that it enters its target cell directly, forms a cytosol receptor complex and then migrates to the cell nucleus where it reacts with nuclear chromatin

resulting in the synthesis of new cytoplasmic products which modify cell function.

The time course of this mechanism of action is supported by observations that there is an onset period of approximately twelve hours before the effects of progesterone are physiologically manifested and a 60-72 hour period before the effects wear off. Additional support comes from cross-circuit experiments, in which it has been recorded that the blood from an animal with established progesterone effects can transfer these effects to another animal without the twelve hour lag period.

This study examined the manner in which the gonadal steroids estrogen and progesterone modulate subcellular calcium and attempted to correlate this with uterine muscle contractility. Since there is evidence that the microsomal compartment in uterine muscle serves as a sink for the storage and release of activator calcium, it was in order to look for changes that steroids might produce at this level. Initially, uterine microsomal fragments were prepared from rabbits treated with ovarian steroids and the fragments were characterized by electron microscopy. This was followed by the measurement of ATP dependent calcium binding by the microsomes and after standardizing the optimal <u>in vitro</u> conditions for this binding through manipulating the pH, temperature, and concentrations of ATP and magnesium ions

in the incubation medium, thermodynamic determinations allowed the computations of the equilibrium time course, the maximum number of calcium binding sites and the dissociation rate constant under different hormonal dominance.

An investigation was then carried out on the in vitro effects of catecholamines on uterine microsomal calcium binding and an attempt was made to obtain a dose-response relationship. This was followed by a similar study using adrenergic agonists and antagonists.

Next, using data derived thus far, that suggested progesterone promoted a three-fold increase in uterine microsomal calcium binding, it was attempted to correlate this with physiological observations by comparing time course and dose-response curves of progesterone produced uterine inhibition with calcium uptake data obtained under identical conditions.

Additionally, in experiments designed to indicate a possible molecular mechanism for progesterone in this system, the in vitro effects of dibutyrl 3' 5' -cyclic AMP on uterine microsomal calcium binding were assayed.

Finally, in order to indicate if progesterone induces the synthesis of a microsomal bound enzyme capable of providing an increase in available energy from ATP, the activity of microsomal calcium dependent ATPase was measured from each experimental group.

MATERIALS AND METHODS

The investigation was carried out on immature, female, New Zealand White rabbits weighing approximately 5 lb., obtained commercially and maintained on a standard laboratory diet* of pelleted foodstuffs guaranteed by the manufacturer to be free of estrogenic steroid hormones. The animals were allowed water ad libitum and room temperature was $78^{o} \pm 1^{o}F$. Animals were pretreated according to a modified regimen described by Miller and Marshall (1965).

The animals were divided into four groups: the first was estrogen treated with each animal receiving intramuscularly 100 ug estradiol benzoate in sesame oil with propylbaraben 1 mg/cc as preservative every day for four days. On the fifth day the animals were used experimentally. The

*The standard laboratory diet consisted of a mixture of the following: ground yellow corn, dehydrated alfalfa meal, soybean meal, ground oats, wheat middlings, cane molasses, dicalcium phosphate, iodised salt, calcium carbonate, calcium pantothenate, vitamin B_{12} supplement, niacin, pyridoxine hydrochloride, riboflavin supplement, folic acid, D activated animal sterol, vitamin E supplement, vitamin A supplement, choline chloride, methionine hydroxy analogue calcium, cobalt carbonate, iron sulfate, iron carbonate, manganous oxide, copper sulfate, and zinc oxide. The guaranteed analysis was crude protein 16%, crude fat 2.5%, crude fiber 18%, ash 8%, added minerals 2.1%.

second group was estrogen plus progesterone treated; this group received estradiol benzoate injections identical to the first group, but on the fifth day and daily thereafter for four more days received, intramuscularly, 5 mg of progesterone in sesame oil with 20% Benzyl Alcohol as preservative. On the tenth day the animals were used experimentally. The third group was injected intramuscularly with 5 mg progesterone for four days and on the fifth day were used experimentally. The fourth group were control animals and were either untreated or injected with diluents only, according to the same schedule as the three experimental groups.

Preparation of the microsomes

On the day of experimentation each animal was weighed and then sacrificed using intravenous nembutal. As soon as the animal expired (within thirty seconds of the injection) the peritoneal cavity was opened and the uterine horns removed as quickly as possible. The entire procedure took less than four minutes. All adipose and connective tissue was then carefully dissected free from the exterior of the uterus, after which the horns were split open and the interior was scraped so that only the muscle layer remained. This was then weighed and the wet weight recorded.

The uterine muscle was then placed in chilled extraction

buffer consisting of a 0.25 M sucrose and 30 mM histidine hydrochloride, pH 7.2. Care was taken to insure that there was at least 5 ml buffer per gram of tissue.

The entire procedure was then transferred to the cold room at 4°C where the uterus was cut into small portions approximately 2 mm wide using cold scissors. These portions were then made into a crude homogenate using a Virtis rotating blender for 20 seconds, after which the rudimentary preparation was further homogenized using an all glass hand homogenizer. This was then centrifuged at 1000g for ten minutes to remove nuclear material, large proteins and unbroken tissue, and the supernatant was then further centrifuged at 15,000g for twenty minutes to remove mitochondria. Both centrifugations were carried out in a refrigerated Sorvall RC-2 Centrifuge.

The microsomal pellet was sedimented by centrifugation of the remaining supernatant at 105,000g for sixty minutes in a Beckman L5-65 ultracentrifuge.

Electron microscopy

Pellets were fixed in buffered 2% glutaraldehyde, (pH 7.1) post-fixed in 2% osmium tetroxide and dehydrated in methanol. They were then embedded in Epon and sectioned at 700Å using a Sorval MT 2 Ultramicrotome; sections were stained with uranyl acetate and lead citrate and photographed

on a Zeiss EM 10 electron microscope. Several sections from different preparations were examined.

Calcium uptake

Pellets were resuspended in buffer using a teflon glass homogenizer: the final uniform suspensions contained 0.1-0.3 mg protein per ml.

The microsomal suspensions were either used immediately or stored at -80°C. Under this latter condition it was found that the microsomes retained their ability to take up calcium but that this declined at a rate of 12% per 24 hours. This finding approximates that of Batra (1971) who reported that frozen microsomes from rat uterus retained 95% calcium binding activity after 18 hours and 80% activity after 36 hours.

Aliquots of microsomal protein were incubated in 5 ml plastic tubes and the calcium uptake reaction was initiated by the timed simultaneous addition of two separate solutions: the first solution contained freshly made ATP, pH 7.2; the second solution consisted of a mixture $CaCl_2$ and ^{45}Ca, pH 7.2. The concentration of these solutions was predetermined by the specific protocol for each experiment.

The reaction was terminated by filtration of 200 ul of the reaction mixture through a millipore filter (HA 0.45 u) which had been prewashed with 5 ml chilled, 0.25 KCl to

decrease non-specific calcium binding to the filter (Palmer, 1970). The filter was then washed rapidly with 3 aliquots of 2 ml ice cold, calcium free, distilled water. This process took less than 5 seconds, after which the filter was removed and dried under a heat lamp. The filter was finally dissolved overnight in 5 ml Bray's solution and counted in a Hewlett Packard scintillation counter.

The ability of the microsomes to bind calcium in the absence of ATP was measured at each determination point and the amount of calcium binding to the millipore filter, in the absence of microsomes, was recorded also: corrections for these amounts were applied to all measurements.

Each determination was performed in triplicate and the microsomal protein concentration was determined by the method of Lowry (1951) using bovine serum albumin as standard.

Temperature dependence of calcium binding activity

Aliquots of microsomal protein in uptake medium were prepared from each experimental group previously described. The calcium binding activity of each of these fractions was measured in constant temperature water baths for ten minutes at 4^{o}, 10^{o}, 20^{o}, 25^{o}, 30^{o}, 37^{o}, and 40^{o}C. Three such experiments were completed and the mean value at each temperature computed.

Standardization of the calcium binding assay

Effect of ATP concentration

Calcium binding by uterine microsomes was measured by the method already described at 37°C in the presence of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 mM freshly made ATP solution (pH 7.2). The uptake was plotted against the ATP concentration and the optimum value determined.

Effect of pH

Calcium binding by uterine microsomes was measured at 37°C at the following pH values: 6.00, 6.25, 6.50, 6.75, 7.00, 7.25, 7.50, 7.75, and 8.00. Calcium binding was then tabulated at each value to determine the optimum range.

Effect of magnesium ions

Calcium binding by uterine microsomes was determined at 37°C in the presence of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 mM magnesium chloride. Calcium uptake was then plotted against magnesium chloride concentration.

Effect of oxalate

Equilibrium time course curves for calcium uptake by uterine microsomes were plotted at 37°C (pH 7.2) in the presence and absence of 5 mM ammonium oxalate. Differences in uptake were determined by visual inspection.

Thermodynamic Studies

The microsomal pellet was resuspended in the following buffer:

0.25 Sucrose

50 mM Histidine Hydrochloride

5 mM Magnesium Acetate

5 mM Potassium Chloride

Calcium Chloride in various concentrations

plus ^{45}Ca in amounts such that the ^{45}Ca to calcium chloride ratio remained constant. The pH was adjusted to 7.2.

The binding of calcium to microsomal protein was allowed to proceed from 1 to 30 minutes at 37°C and measured at 1 minute intervals. These data enabled the time to reach equilibrium to be calculated. Binding curves were then plotted by incubating calcium chloride with ^{45}Ca as the radioactive label for 10 minutes in concentrations ranging from 1 mM to 1 pM, from which the concentration range at which saturation occurred was determined. ATP dependent binding was estimated by subtracting non-specific binding determined in the absence of ATP from total binding, specific and non-specific, in the presence of ATP. The saturation curves were then analyzed by Scatchard Plot using a Hewlett Packard computer which plotted a least squares line with a minimum relative error of less than 1 in 10^{-3}. From

the plot the maximum number of binding sites available and the equilibrium dissociation constant (K_d) was calculated.

Rate of dissociation

When a procedure is used that employs washing the sample to separate free from bound ligand, consideration of the rate of dissociation is required, in order to validate the procedure.

The time course of dissociation of calcium from the microsomal protein was determined by first allowing ^{45}Ca and uterine microsomes to come to equilibrium for ten minutes, at which time dissociation was initiated by either a twenty-five fold dilution of calcium free buffer, or by the addition of excess unlabeled calcium chloride (0.1 mM). The amount of radioactivity remaining bound was measured at ten second intervals for the first three minutes and at one minute intervals thereafter for twelve minutes. From this $t_{\frac{1}{2}}$ was calculated.

Effect of Norepinephrine and Calcium binding

Animals were pretreated and uterine microsomal pellets obtained by the procedures already described.

Pellets were resuspended in a calcium uptake buffer which consisted of 5 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM $CaCl_2$, 40

mM Morpholinopropane Sulfonic Acid, (MOPS), 0.1 M KCl, and ^{45}Ca adjusted to give approximately 10^4 cpm. The pH was 6.8 at 37°C.

Calcium binding was measured by the standard method described, in the presence of norepinephrine in the following concentrations: 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M. The binding was measured both in the presence and absence of ATP and it was attempted to obtain a dose-response relationship.

Effects of adrenergic receptor agonists and antagonists

Microsomes were prepared by exactly the same procedure as that used previously to determine the effects of catecholamines. Calcium binding was measured in the presence and absence of ATP (5 mM) and norepinephrine (1 uM), together with a wide range of concentrations (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M) of the following:

Alpha adrenergic agonist ---- Phenylephrine

Alpha adrenergic antagonist - Phentolamine

Beta adrenergic agonist ----- Isoproterenol

Beta adrenergic antagonist -- Propanolol

It was attempted to obtain a dose-response relationship in each incubation.

Time course of the effects of progesterone on uterine microsomal calcium binding and spontaneous uterine contraction

Calcium binding and spontaneous contraction

Rabbits were injected intramuscularily with 5.0 mg progesterone or the diluent. The animals were sacrificed after 4, 6, 8, 10, 12, 16, 20, 24, 36, 48, 54, 60, or 72 hours and the uterus removed as described previously. The uterus was divided into two parts: one horn was used to prepare a microsomal fraction and ATP dependent calcium binding was assayed by the standard method; the other horn was cut into helical strips approximately 3.0 mm by 2.0 cm and suspended in mammalian Ringer's solution.

The temperature was maintained at 37^{o}C and the solution bathing the muscle strip was aerated continuously with oxygen. A standard testing tension of 1-4 g was applied and isometric contractions were recorded for two hours by means of a linear force transducer in conjunction with a Grass polygraph recorder. Muscle response was expressed as millimeters of pen deflection on the chart recorder.

Dose-response relationship between progesterone, calcium binding, and spontaneous uterine contraction

The animals were divided into two subgroups. The first

group received diluent only and the second group was comprised of animals which received different dose levels of progesterone. Rabbits received a single intramuscular injection and doses of progesterone were 0.01, 0.05, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, and 10.00 mg per animal. Sixteen hours after the administration of the progesterone the animals were sacrificed and the uterus divided and treated in exactly the same manner as previously described in the time course procedures.

Effect of dibutyryl 3' 5'-cyclic AMP on microsomal binding

Animals were hormonally pretreated according to the Marshall and Miller regimen already mentioned and microsomes were prepared from each uterus.

Calcium binding was measured in the presence of the following concentrations of dibutyryl 3' 5'-cyclic AMP: 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M and it was attempted to obtain a dose-response relationship.

Determination of calcium-ATPase activity

Uterine microsomal pellets were prepared from each group as described. Using a teflon glass homogenizer, the pellets were resuspended in 20 mM MOPS, 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM $CaCl_2$ in 1% Triton X100, pH 6.8 to

give a protein concentration of approximately 100 ug/ml.

Ca-ATPase activity was assayed by the one-step method of Lin and Morales (1977) using a mixed reagent of molybdovanadate and sodium dodecyl sulfate for measuring inorganic phosphate in the presence of proteins. To measure Ca-ATPase activity, the reaction was started by adding ATP to the microsomal solution to give a final concentration of 1 mM. At standard time intervals 2 ml aliquots of microsomal suspension were pipetted into an equal volume of prepared reagent and thoroughly mixed. The absorbance of the color complex, formed by the P_i and Molybdovanate was measured at 350 nm in a Zeiss PMQ 11 spectrophotometer using a cell with a one centimeter path. The absorbance of the colored solutions was read against a blank prepared in the same way, but excluding ATP. The P_i concentrations were ascertained by comparison with a calibration curve obtained using K_2HPO_4 solutions of known concentrations as standards.

RESULTS

Electron Microscopy

The representative electron micrographs in Figures 1 and 2 show the morphology of the preparations isolated at 15,000g (Mitochondrial Fraction) and 100,000g (Microsomal Fraction) respectively, 1650 um^2 of each preparation was systematically examined for mitochondria and in the 15,000g fraction which was not used in calcium uptake determinations, the concentration of mitochondria was found to be 8.8/100um. The microsomal fraction contained no intact mitochondria in any of the sections examined and was characterized by vesicular structures between 500 and 2,000 $\mathring{A}$ in diameter, together with large numbers of ribsomes. This latter preparation was used in all calcium binding measurements.

Calcium binding by uterine microsomes

Figure 3 shows the equilibrium time course of calcium binding by uterine microsomes at 4^{o}, 25^{o}, and $37^{o}C$, following pretreatment with estrogen, estrogen plus progesterone, progesterone and diluent only. Equilibrium appears to be reached after 8 - 10 minutes in all reactions and the figure

shows that at 37°C the calcium uptake in the progesterone treated microsomes is approximately twice that from other groups. Table 1 lists the equilibrium calcium binding by uterine microsomes at 37°C after ten minutes incubation. The data shows a significant increase in calcium binding by all groups in the presence of 5 mM ATP ($p < 0.001$) when compared to the absence of ATP, and by all hormonal groups ($p < 0.002$) when compared with diluent controls. The table further indicates a statistically significant increase in calcium binding of progesterone treated microsomes, when compared with other hormonal groups ($p < 0.001$), but there was no apparent difference in calcium uptake between the animals treated with estrogen and those treated with estrogen plus progesterone.

Effect of temperature on calcium binding activity

Uterine microsomal calcium binding activity was assayed at temperatures from 10° to 40°C and the results were analyzed by Arrhenius plot: the log of calcium binding activity was plotted against the reciprocal of the absolute temperature (Figure 4).

It was apparent that uterine microsomal calcium binding is temperature dependent and that it is different below and above 20°C since the linear plot exhibits a break at this temperature. The slopes enabled the energy of activation (E_a)

to be calculated within the experimental temperature ranges and it was found that activation energy below 20°C 17.7 Kcal/mole and 10.9 Kcal/mole at temperatures above 20°C. The activation energy appeared to be the same for all groups.

Microsomal calcium uptake as a function of ATP concentration

As shown in Figure 5, calcium binding by all rabbit uterine fractions increased with increasing ATP concentration up to 3-5 mM: this generally agreed with a previous report by Tomiyama (1974). However, at ATP concentrations above 5 mM there was a conspicuous decline in calcium binding until at 8 mM ATP calcium binding had decreased to the non-ATP level. Clyman (1976) has reported a similar inhibitory effect of ATP on calcium uptake in human umbilical artery microsomes and has postulated that changes in intracellular ATP concentration together with concomitant pH changes may be responsible for physiological chelation and sequestration of intracellular calcium; specifically, in controlling arterial patency and closure with pO_2 changes. However, support for this hypothesis awaits the demonstration of changes in ATP and hydrogen ion concentrations within localized compartments of the cell.

Effect of pH on calcium binding

Calcium binding by the differentially pretreated

uterine microsomes was measured at 37°C following incubation for ten minutes over a pH range 6.0 - 8.0. The results are shown in Table 2 and suggest strongly that calcium uptake is strictly pH limited. There was very low uptake below pH 6.5 or above pH 7.5 with a rapid increase from pH 6.5 to 6.8 and an equally rapid decline from pH 7.2 to 7.5. Maximum uptake was between pH 6.8 and 7.2 in all preparations. This optimal pH plateau has been found to be typical in many biochemical systems, especially those involving enzyme action. Since enzymes are proteins, pH changes profoundly affect the ionic character of the amino and carboxylic group on the protein surface: additionally low or high pH values give rise to considerable denaturation and inactivation of enzyme molecules. Since the uptake of calcium by microsomes requires the presence of ATP it is likely that enzymatic factors are involved and that the control of pH in various parts of the cell is important. A marked shift in enzyme activity results if pH stability is not maintained.

Calcium uptake as a function of magnesium ion concentration

In the presence of 5 mM ATP at 37°C and pH 7.2 magnesium potentiated the maximal uptake of calcium. Increases in magnesium concentration up to 3 mM gave a concomitant increase in calcium binding, but increasing magnesium concentration above this to 10 mM had no further effect

(Figure 6). Similar findings have been reported in calcium binding experiments carried out using the sarcoplasmic reticulum from striated muscle (Ebashi, 1962; Hasselbach, 1964; Carvalho, 1967) and several hypotheses have been put forward to explain this phenomenon. It is possible that increased calcium binding is induced by the formation of a Ca-Mg-ATP complex resulting from membrane conformational changes (Onishi and Abashi, 1964) or that the calcium binding due to active transport and hydrolysis of ATP requires magnesium ions (Hasselbach, 1964).

Effect of oxalate ions

Katz and Repke (1967) have reported that when oxalate penetrates the vesicular arrangement of microsomal membranes, calcium oxalate is precipitated inside the vesicles increasing calcium uptake.

Calcium uptake by uterine microsomes was measured at $37^{o}C$ and followed at one minute intervals for ten minutes. This was done in the presence and absence of 5 mM ammonium oxalate. In all preparations, except blanks, the uptake of calcium was approximately tripled. This is illustrated in Figure 7.

Thermodynamic studies

The time dependence of calcium binding required the

selection of an appropriate time at which to measure the extent of ligand uptake: this was achieved by utilizing the equilibrium time course curves of calcium sequestration previously obtained (Figure 3). Direct plots of bound versus free calcium were obtained over a wide range of calcium concentrations (10^{-3} - 10^{12}M) to establish if calcium binding was saturable and the range over which saturation occurred. Bound was separated from free ligand by filtration and the procedure was validated by determining the rate of dissociation of the bound ligand from the microsomes by both addition of a large excess of unlabeled calcium and by washing with a calcium free medium.

Visual inspection of the direct plots (Figure 8) and the dissociation rate curves (Figure 9) in the estrogen and progesterone pretreated preparations revealed that the calcium binding was both saturable and reversible.

Double reciprocal plots of the saturation curves could be fitted by a straight line, but since this type of plot tended to weigh most heavily the data at low substrate concentrations, giving a high reciprocal, it was not used in the calculation of the equilibrium dissociation constant (K_d) or the maximum number of binding sites (n). Instead a more accurate graphic determination of these parameters was obtained by Scatchard Plot (Figure 10) in which data at high and low concentrations were weighed more equally. The

Scatchard method of analysis (Scatchard, 1949) plots the bound/free ratio (B/F) for the ligand against the concentration of bound ligand (B). The straight line obtained has a slope equal to the negative of equilibrium association constant ($-K_a$) from which the equilibrium dissociation constant can be calculated (K_d). The absicca intercept gives the number of binding sites R_o, in moles/liter which can be converted to moles/gram protein if the protein concentration is known.

A Hewlett-Packard 9821A computer, which plotted a least squares line with a minimum relative error of less than 1 in 10^{-3} was used in the Scatchard analysis: from which the equilibrium dissociation constant and the maximum number of binding sites were calculated.

As shown in Table 3, the K_d for progesterone pretreated microsomes ranged from $2.00.10^{-7}$ to $3.78.10^{-7}$ M^{-1} and for estrogen pretreated microsomes from $7.15.10^{-7}$ to $9.15.10^{-7}$ M^{-1}. The means were compared by unpaired t test and proved to be statistically significant ($p < 0.001$). The maximum number of binding sites per milligram of microsomal protein ranged from 7.65 to 11.65 uM Ca/g protein in the progesterone microsomes and from 6.50 to 7.80 uM Ca/g protein in the estrogen microsomes. These means were also compared by unpaired t test and were again significantly different ($p < 0.005$).

The rate of dissociation for progesterone and estrogen pretreated microsomes after addition of a large excess of calcium is shown in Figure 9 and the $t_{\frac{1}{2}}$ for dissociation of ligand both in calcium excess and calcium absence is displayed in Table 4. The rate of dissociation in the presence of calcium was greater than its absence ($p < 0.05$) suggesting that the affinity of the calcium for its microsomal binding sites was decreased by the addition of further ligand. This could have been due to the process termed negative cooperativity; however it was not suggested by the Scatchard Plots obtained from the thermodynamic determinations, since this type of cooperativity would have yielded a non-linear plot. Alternatively the addition of excess calcium ions may have interfered with equilibrium or binding of small quantities of ^{45}Ca and appears more feasible since the difference in $t_{\frac{1}{2}}$ were 25% or less. This contradiction did not invalidate the filtration technique for separating bound from free in these experiments: the minimum $t_{\frac{1}{2}}$ was 39.66 seconds for the estrogen treated microsomes whereas the time for filtration and washing in the calcium binding assay was approximately 5 seconds.

Effects of Norepinephrine on Calcium binding

The effects of norepinephrine on uterine microsomal calcium binding are indicated in Table 5. Norepinephrine

appeared to have a slight inhibitory effect which was unaffected by hormonal pretreatment: this effect however was statistically significant in only 10% of all the determinations and it was not possible to obtain a dose-response relationship in any of the groups of animals.

Effects of adrenergic agonists and blocking agents

The effects on uterine microsomal calcium binding, produced by Phenylephrine, Phentolamine, Isoproterenol and ± Propanolol both in the absence and presence of 1 uM Norepinephrine, are shown in Tables 6 - 13 and Figures 11 and 12. The data indicates that *in vitro* incubation with these agents produced a reduction of calcium binding in all determinations.

Incubation with alpha adrenergic agonist phenylephrine, both in the presence and absence of norepinephrine, resulted in a marginal inhibition of calcium binding at all concentrations and in all hormonal groups. It was not possible to obtain a dose-response relationship and the data obtained was similar to that using norepinephrine alone (Table 5).

The effects of Phentolamine on calcium binding are shown in Tables 8 and 9: these indicate a substantial inhibition of calcium uptake in all hormonally treated groups and at the majority of concentrations examined. However it was not possible to plot dose-response curves for

the inhibition within any one group and the diluent control group failed to show any significant drop in microsomal calcium binding.

Isoproterenol, both in the presence and absence of norepinephrine, failed to produce a consistent drop in calcium binding in any group of animals except in those treated with progesterone alone: in this group a highly significant inhibition was seen at all concentrations and in each determination. In the estrogen treated animals inconsistent, significant inhibition of calcium binding was seen, but both in this group and in the others a dose-response plot was not possible.

Tables 12 and 13, together with Figures 11 and 12, show the effect of ± Propanolol on uterine microsomal binding. It was possible to plot dose-response curves both in the presence and absence of norepinephrine, but these curves were similar in all hormonal groups. At a concentration of 10^{-3} M ± Propanolol reduced calcium binding to non-ATP levels.

Non-ATP controls

Parallel non-ATP controls were incubated simultaneously in all determinations throughout the above experiments: Phenylephrine, Phentolamine, Isoproterenol and ± Propanolol failed to produce a statistically significant change in

calcium binding in any of the non-ATP determinations ($p > 0.63$). This data is not shown.

Effects of dibutyryl 3' 5'cyclic AMP on calcium binding

Table 14 depicts the effects of a range of concentrations of dibutyryl 3' 5'cyclic AMP on uterine microsomal calcium uptake. At concentrations above 10^{-6} M there was an apparent increase in calcium binding in all animal groups except those treated with progesterone alone. However these increases did not prove to be statistically significant despite a maximal increase in binding of 27.8% obtained with 10^{-3} M dibutyryl 3' 5'-cyclic AMP in the estrogen treated animals.

Dose-response relationship between progesterone, uterine inhibition and calcium binding

The relationship between the dose of progesterone administered and the response observed (specifically, the inhibition of spontaneous uterine contraction and uterine microsomal calcium binding) is shown graphically in Figure 13. It can be seen that a single injection of 10^{-5}g progesterone failed to produce either inhibition of spontaneous uterine contractions or an increase in microsomal calcium binding. However, administration of 5.10^{-5}g progesterone

inhibited uterine contraction and this inhibition increased with increasing doses of progesterone until it became maximal after a single injection of $2.5.10^{-3}$g: after this no increase in inhibition was observed. The dose-response curve for microsomal calcium binding approximated that seen for contraction restraint but did not exhibit the same sensitivity. An increase in microsomal calcium uptake could not be measured until administration of 10^{-4}g progesterone, after which the dose-response curve paralleled that for the inhibition of contraction. The dose required to produce maximal calcium binding was 5.10^{-3}g progesterone.

Time course of activation and inactivation of progesterone

As shown in Figure 14, the time course of activation and inactivation of spontaneous uterine contractions following a single injection of 5 mg progesterone intramuscularly correlates well with its effect on uterine microsomal calcium binding. A decrease in amplitude of uterine contractions was observed approximately six hours after injection, which was followed by a gradually increasing onset until after twelve hours the contraction amplitude had reached a minimum: it remained minimal until approximately forty hours after the injection when the uterus gradually returned to normal. At forty-eight hours, uterine contractions were 80% of normal but full recovery was not complete

until seventy-two hours had elapsed. A gradual increase in calcium binding started eight hours after the administration of progesterone and became maximal after fourteen hours. This binding remained at this level until thirty-six hours had passed, when it started to decline: at fifty-six hours it had returned to pre-injection levels.

Effect of hormonal treatment on microsomal Ca-ATPase

In an attempt to characterize the effect of hormonal treatment on microsomal Ca-ATPase activity, the method of Lin and Morales (1976) was used to measure the levels of enzyme in the pellets obtained from each group. The results which are shown in Table 13 indicate there was no difference in Ca-ATPase activity in the uterine microsomes, resulting from steroid hormone treatment.

Figure 1

Electron micrograph of crude mitochondrial pellet, obtained at 15,000g from progesterone treated rabbit uterus. 700 Å thick section stained with uranyl acetate and lead citrate. X20,000.

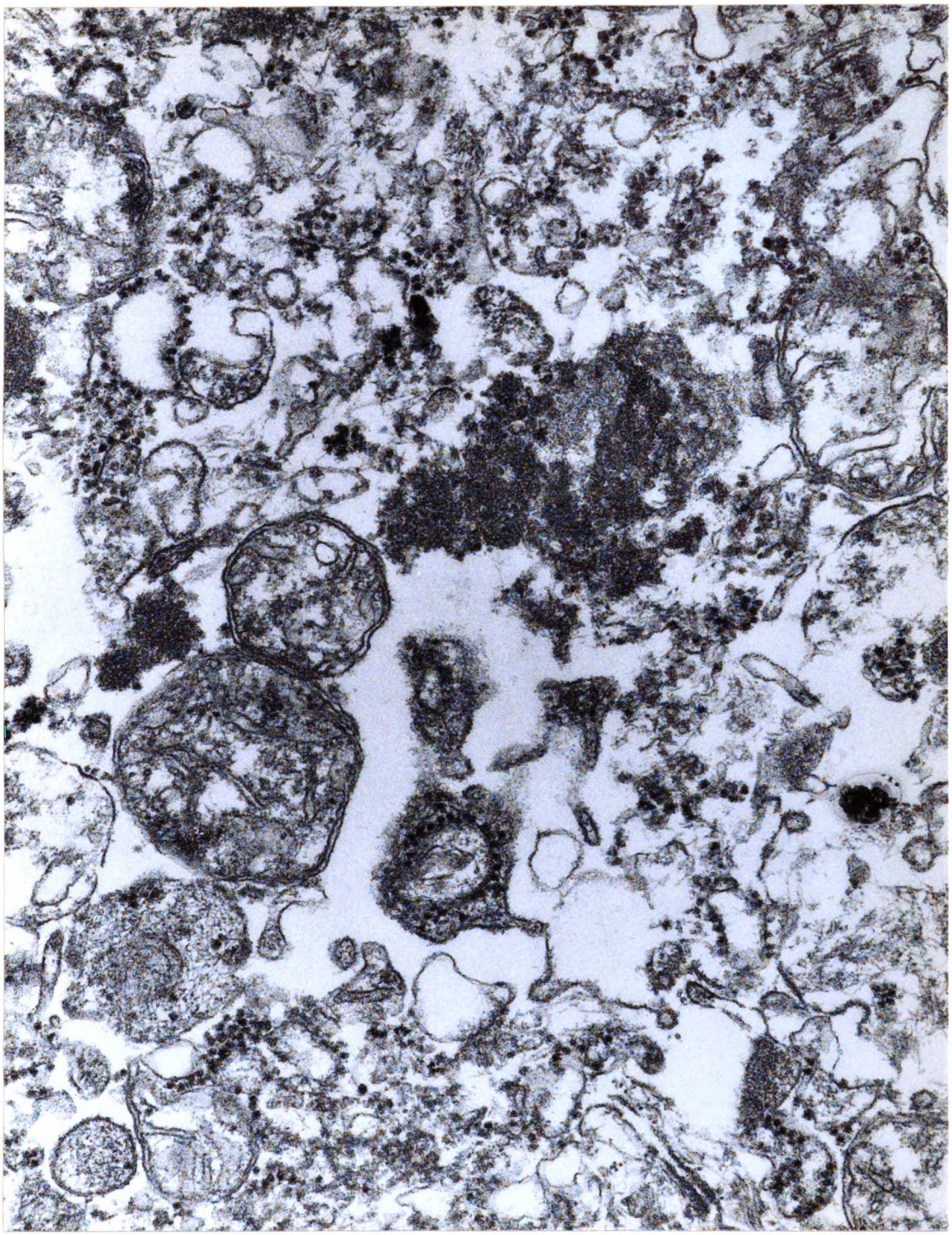
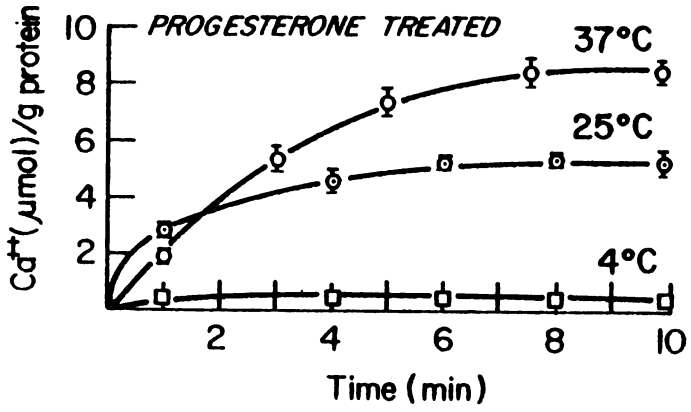


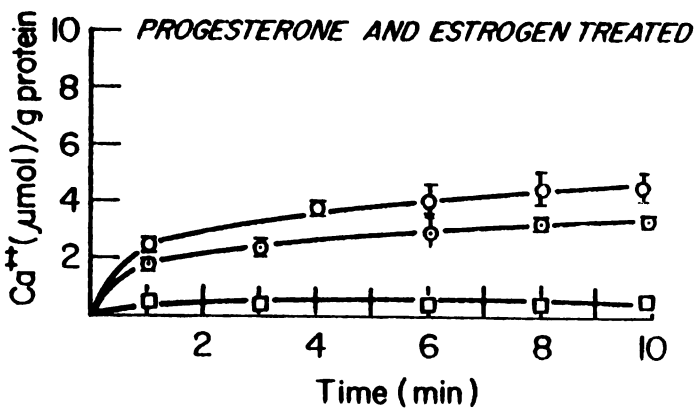
Figure 2

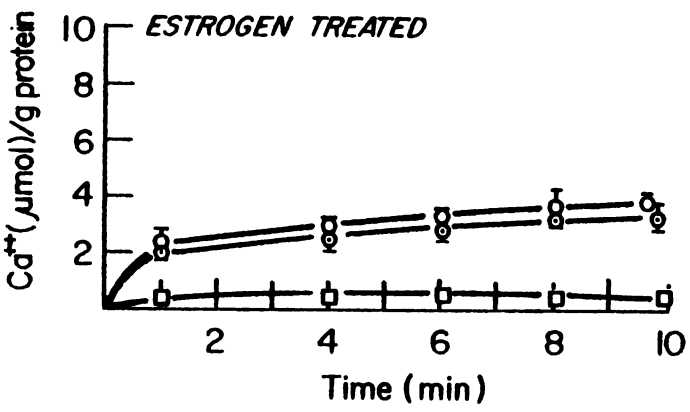
Electron micrograph of microsomal pellet, obtained at 100,000g from progesterone treated rabbit uterus. 700 Å thick section stained with uranyl acetate and lead citrate. X20,000.

Figure 3

The effect of temperature on the equilibrium time course of calcium binding by a microsomal fraction prepared from rabbit uterus pretreated with ovarian hormones. Estrogen treated animals received 100 ug estradiol benzoate daily for four days; estrogen and progesterone animals received estradiol benzoate daily for four days followed by 5 mg progesterone for four days; progesterone treated animals received 5 mg progesterone daily for four days. Control animals received diluent only. Each point represents the mean of six experiments and the solid bars indicate plus and minus one standard error of the mean. The experiments were performed at 37^{o}C, 25^{o}C, and 4^{o}C with a protein concentration of approximately 100 ug/ml.


PROGESTERONE TREATED
37°C
25°C
4°C
Ca++(μmol)/g protein
Time (min)
10
8
6
4
2
2
4
6
8
10


PROGESTERONE AND ESTROGEN TREATED
Ca++(μmol)/g protein
Time (min)
10
8
6
4
2
2
4
6
8
10


ESTROGEN TREATED
Ca++(μmol)/g protein
Time (min)
10
8
6
4
2
2
4
6
8
10

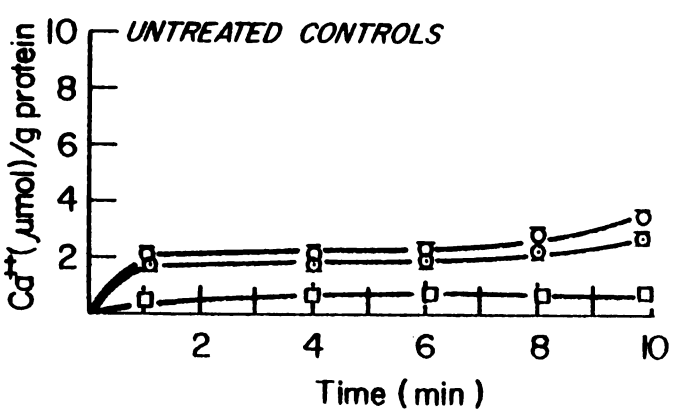

UNTREATED CONTROLS
Ca++(μmol)/g protein
Time (min)
10
8
6
4
2
2
4
6
8
10

Figure 4

Arrhenius Plot

Logarithmic plot of calcium accumulation by rabbit uterine microsomes as a function of reciprocal temperature following hormonal treatment with estrogen, estrogen plus progesterone, progesterone or diluent only. Microsomes were incubated for ten minutes at temperatures from 4 - 40^{o}C. The pH was 7.2.

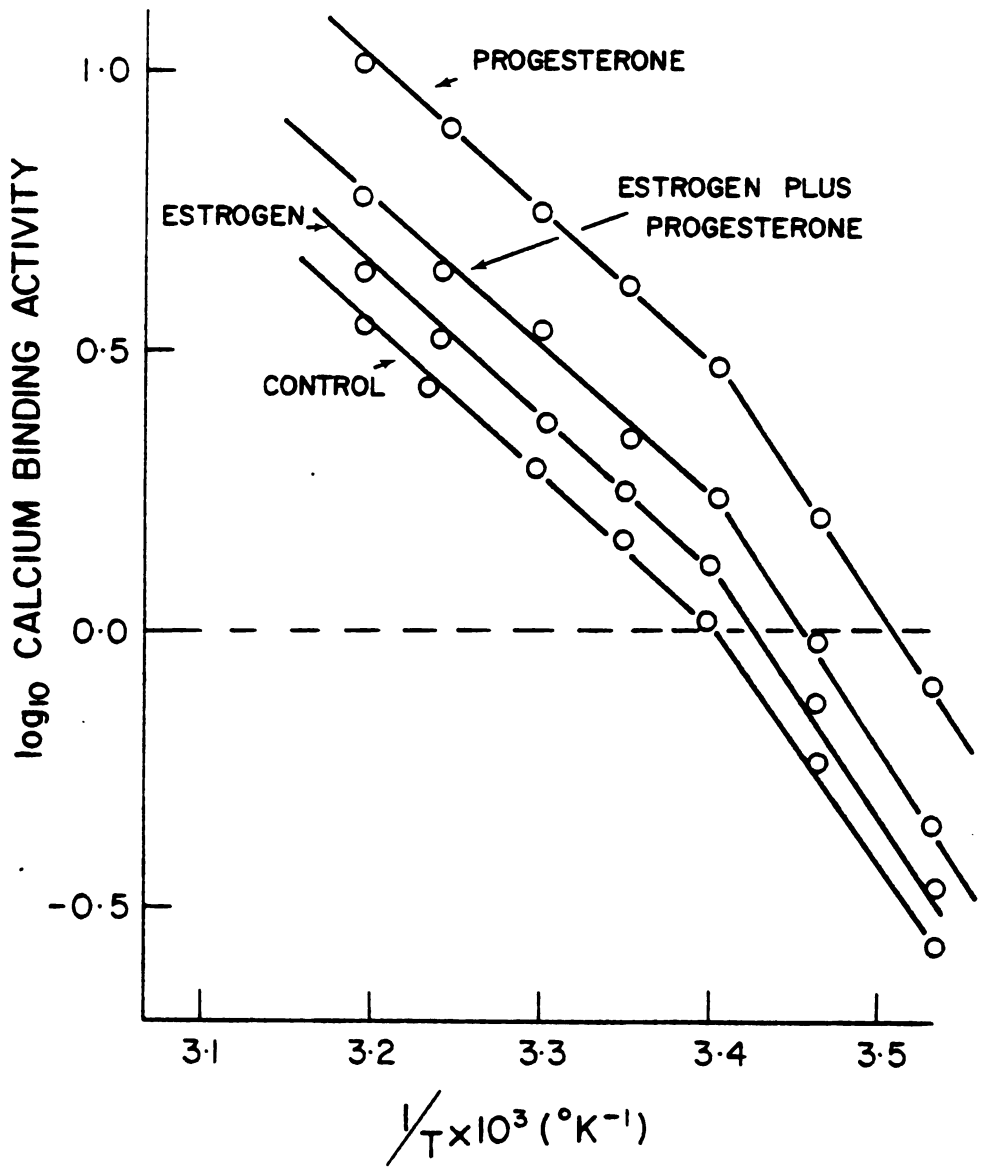

PROGESTERONE
ESTROGEN PLUS
PROGESTERONE
ESTROGEN
CONTROL
1·0
0·5
0·0
-0·5
log10 CALCIUM BINDING ACTIVITY
3·1
3·2
3·3
3·4
3·5
1/T x 10³ (°K⁻¹)

Figure 5

The effect of ATP concentration on calcium binding by rabbit microsomes pretreated with ovarian steroids. Microsomes were incubated for ten minutes at 37°C. Each point is the mean of six experiments and solid bars represent plus and minus one standard error of the mean. The pH was 7.2.

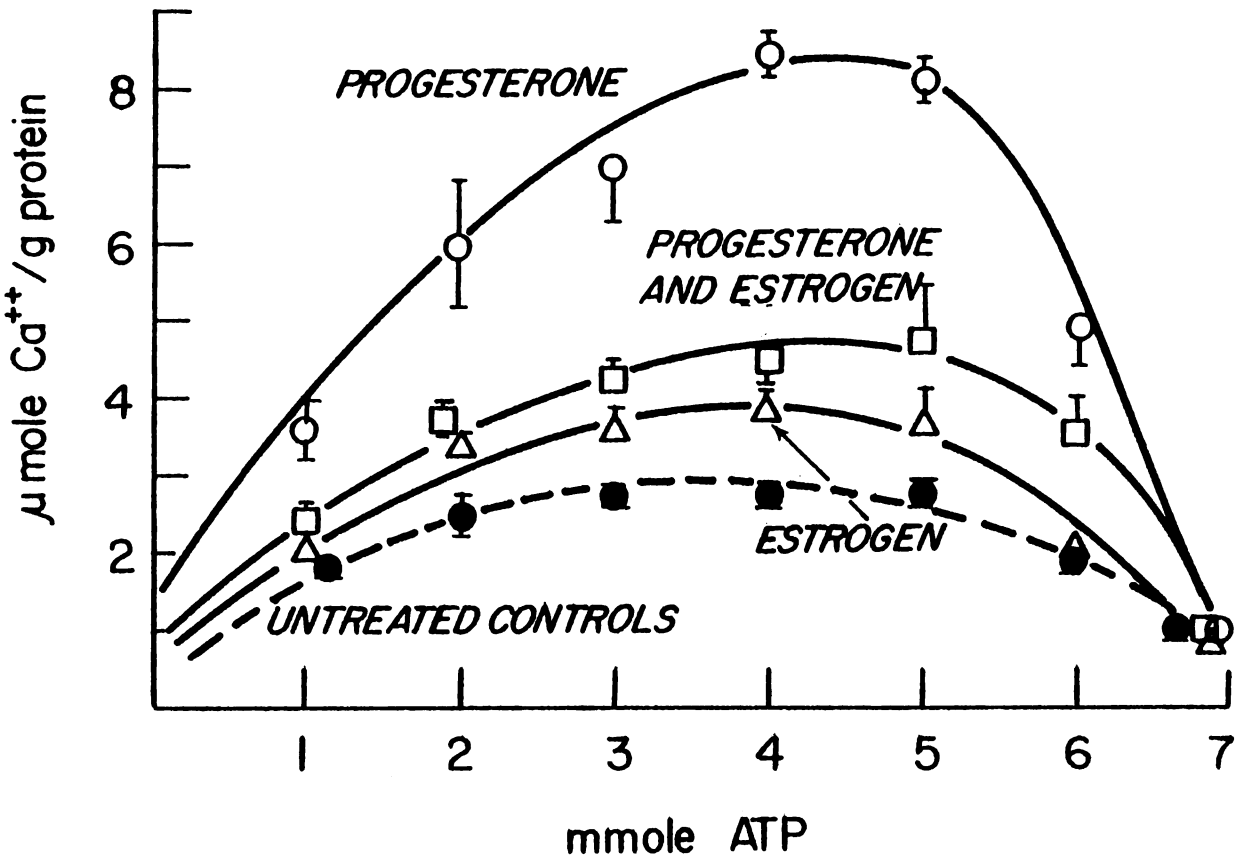

PROGESTERONE
PROGESTERONE AND ESTROGEN
ESTROGEN
UNTREATED CONTROLS
μ mole Ca++ /g protein
8
6
4
2
1
2
3
4
5
6
7
mmole ATP

Figure 6

Influence of magnesium ion concentration on calcium binding by hormonally pre-treated rabbit uterine microsomes. Microsomes were incubated at 37^{o}C for ten minutes. Each point represents the mean of six experiments and solid bars indicate plus and minus one standard error of the mean. The pH was 7.2.

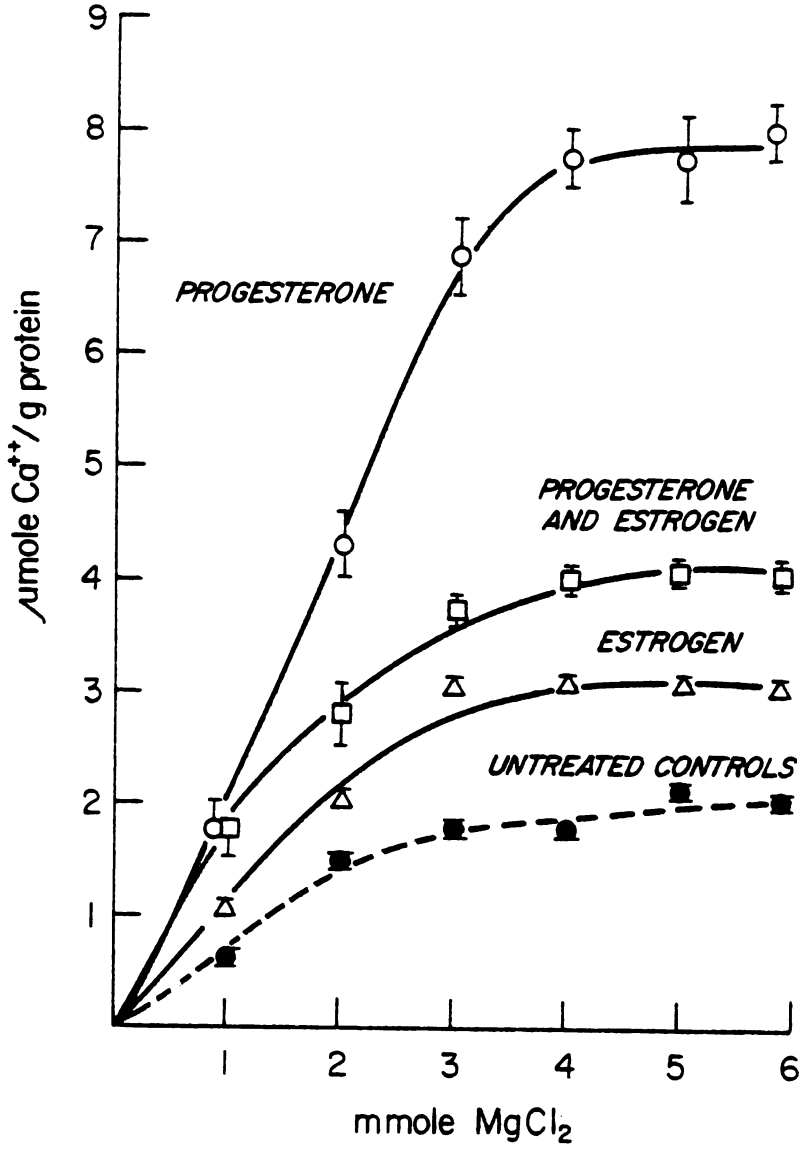

9
8
7
6
5
4
3
2
1
μmole Ca++/g protein
PROGESTERONE
PROGESTERONE AND ESTROGEN
ESTROGEN
UNTREATED CONTROLS
1
2
3
4
5
6
mmole MgCl2

Figure 7

Calcium uptake by pretreated rabbit uterine microsomes at 37°C in the absence (A) and presence (B) of 5 mM ammonium oxalate. Each point represents the mean of six experiments and solid bars indicate plus and minus one standard error of the mean.

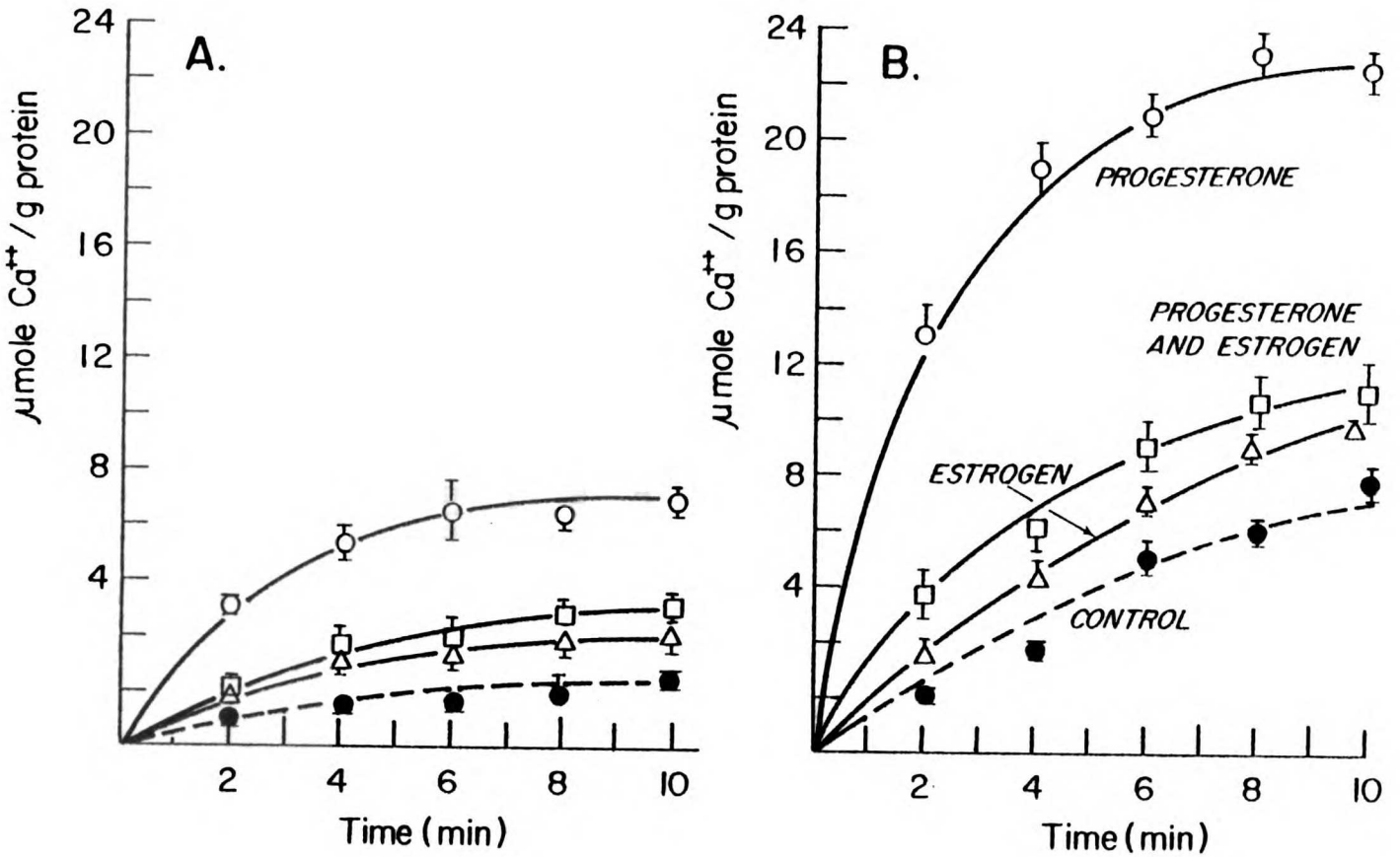

A.
B.
μmole Ca++ /g protein
Time (min)
PROGESTERONE
PROGESTERONE AND ESTROGEN
ESTROGEN
CONTROL

Figure 8

Representative direct plot of the equilibrium binding of Ca^{++} to progesterone pretreated rabbit uterine microsomes measured by filtration. Microsomes were incubated at $37^{o}C$ for ten minutes. The pH was 7.2.

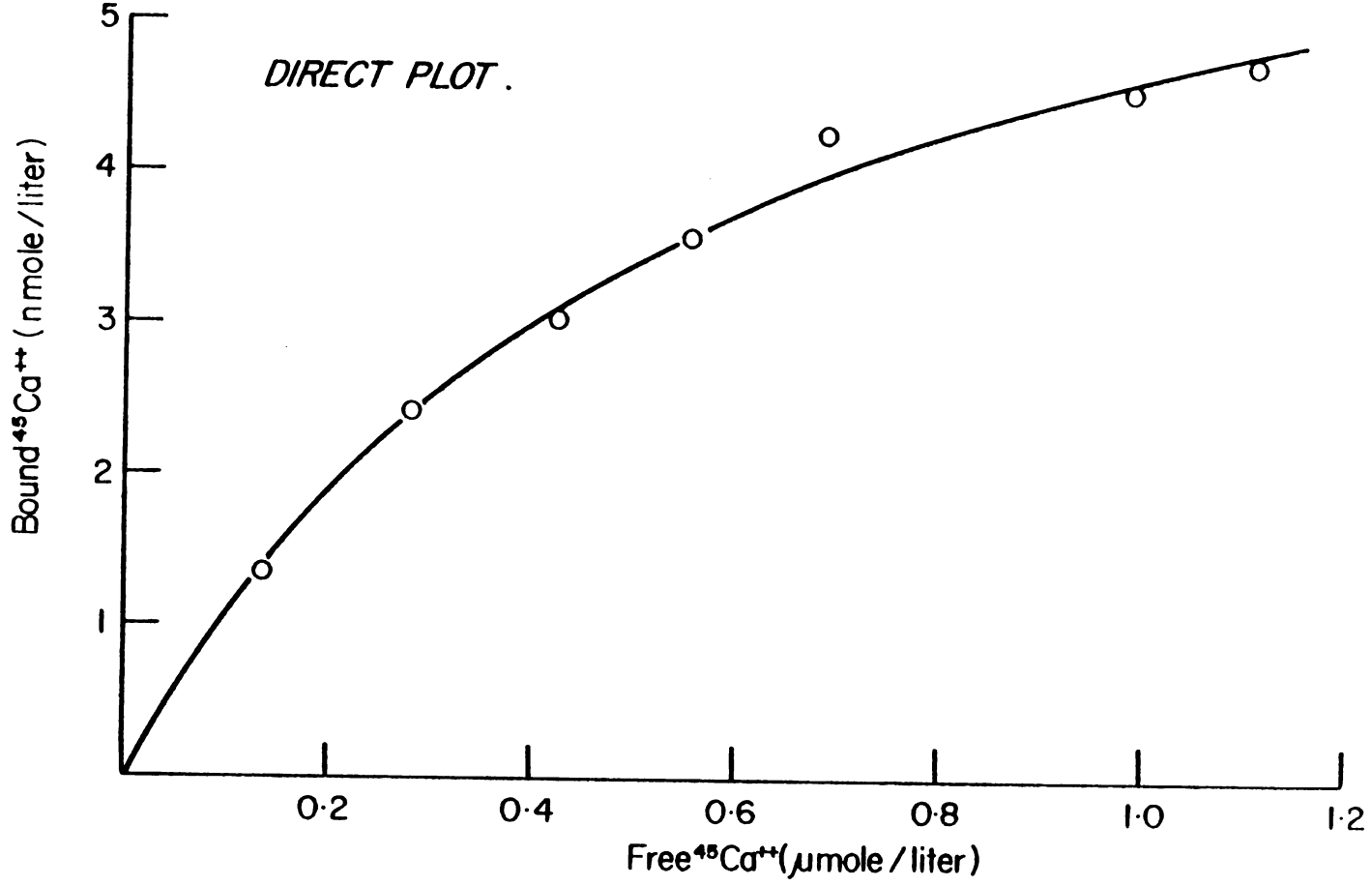
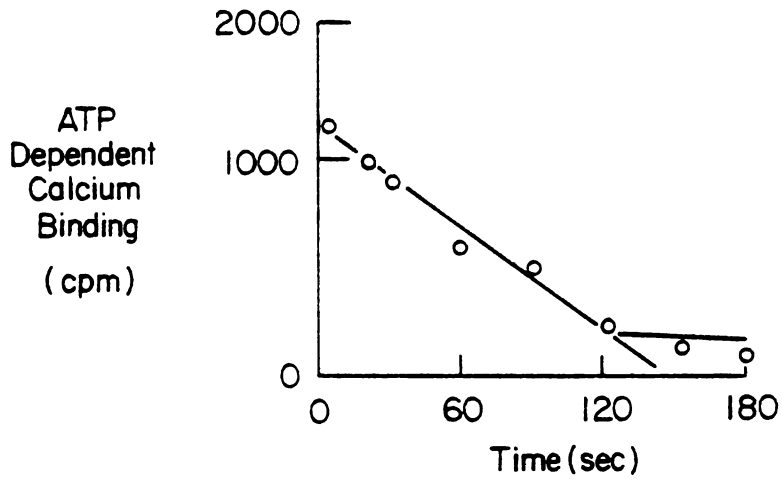

DIRECT PLOT.
Bound $^{45}Ca^{++}$ (n mole / liter)
5
4
3
2
1
0·2
0·4
0·6
0·8
1·0
1·2
Free $^{45}Ca^{++}$ (μmole / liter)

Figure 9

Reversibility of ATP dependent calcium binding examined by the addition of 10^{-5} M $CaCl_2$ to microsomes incubated with ^{45}Ca for ten minutes. The data indicates that in both estrogen and progesterone pre-treated microsomes essentially all the specific binding is reversible. The results also show that the dissociation rate is sufficiently slow to allow separation of free and bound ^{45}Ca by filtration.

PROGESTERONE TREATED MICROSOMES.



ESTROGEN TREATED MICROSOMES

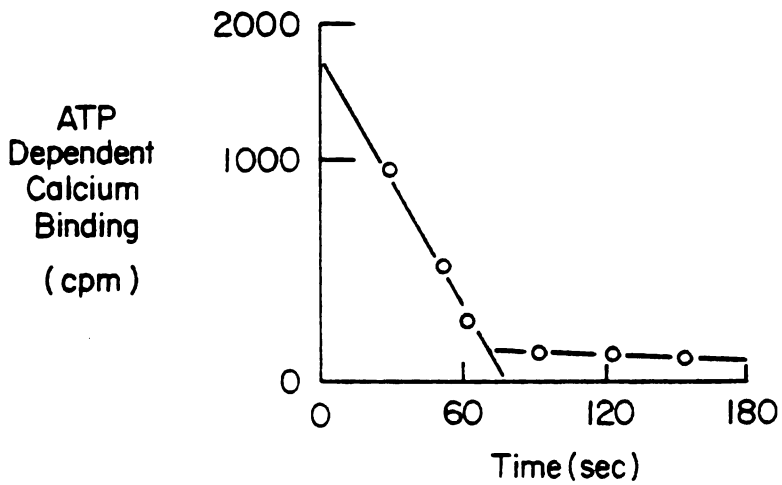


Figure 10

Representative Scatchard plot of equilibrium data shown in Figure 8.

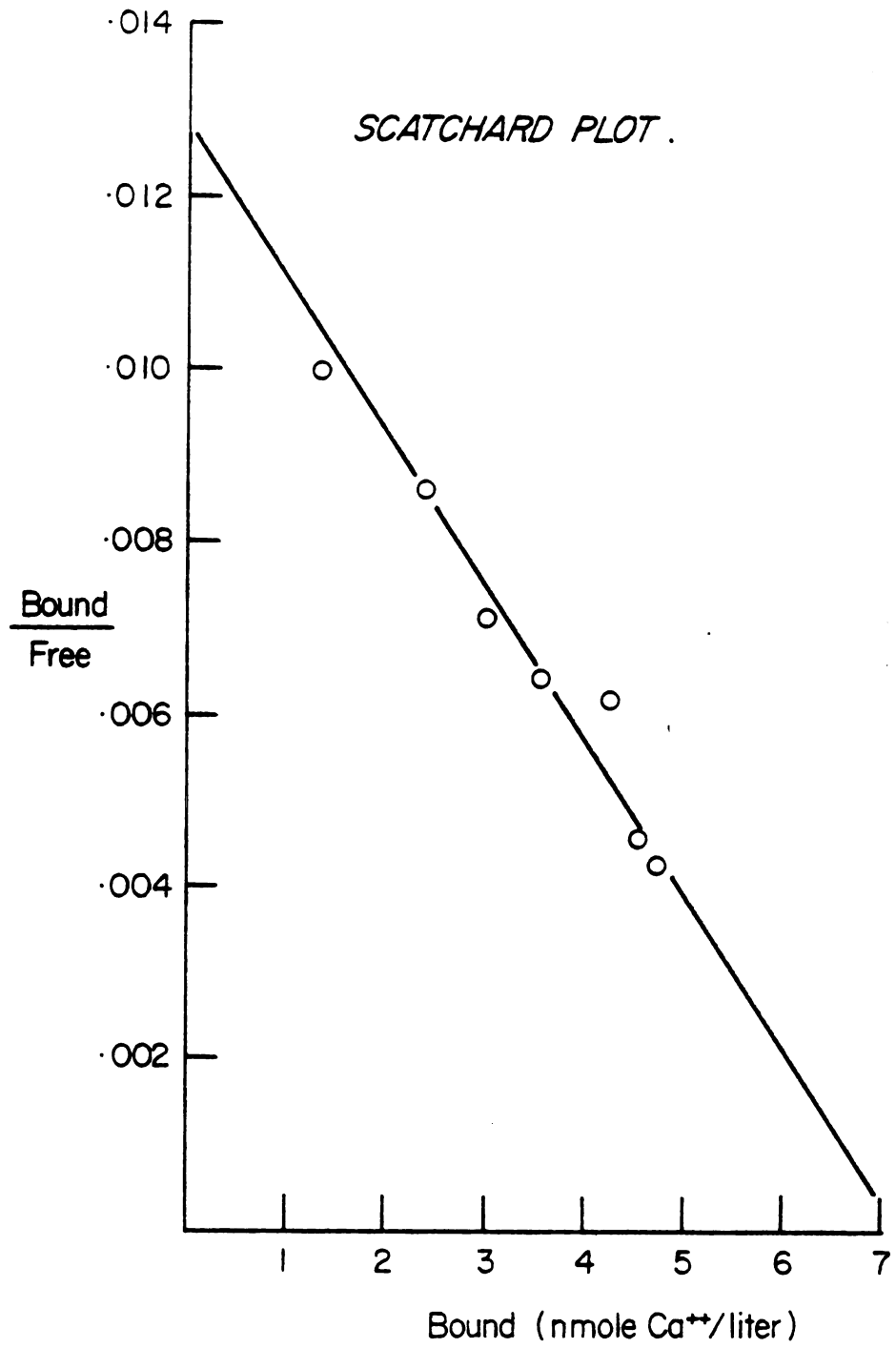

SCATCHARD PLOT.
·014
·012
·010
·008
·006
·004
·002
Bound
Free
1
2
3
4
5
6
7
Bound (nmole Ca++/liter)

Figure 11

Effect of ± Propanolol in the absence of 1 uM Norepinephrine on uterine microsomal calcium binding. Microsomes were incubated for ten minutes at 37°C, pH 7.2. Each point represents the mean of six determinations.

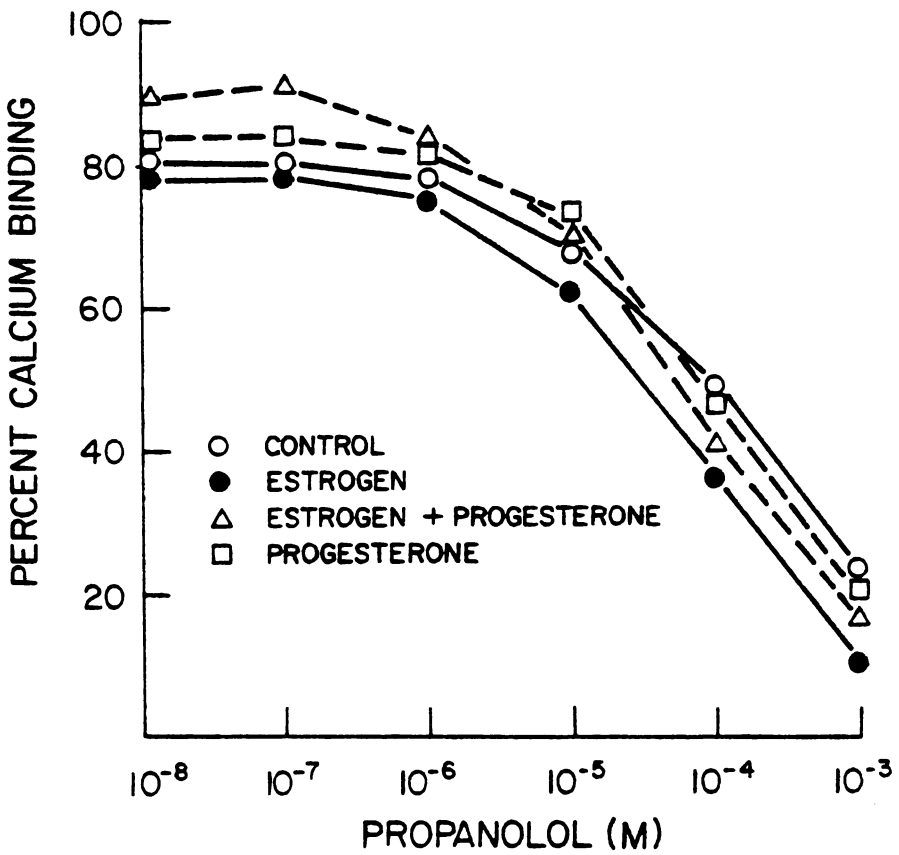

100
80
60
40
20
PERCENT CALCIUM BINDING
CONTROL
ESTROGEN
ESTROGEN + PROGESTERONE
PROGESTERONE
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
PROPANOLOL (M)

Figure 12

Effect of ± Propanolol in the presence of 1 uM Norepinephrine on uterine microsomal calcium binding. Microsomes were incubated for ten minutes at 37°C, pH 7.2. Each point represents the mean of six determinations.

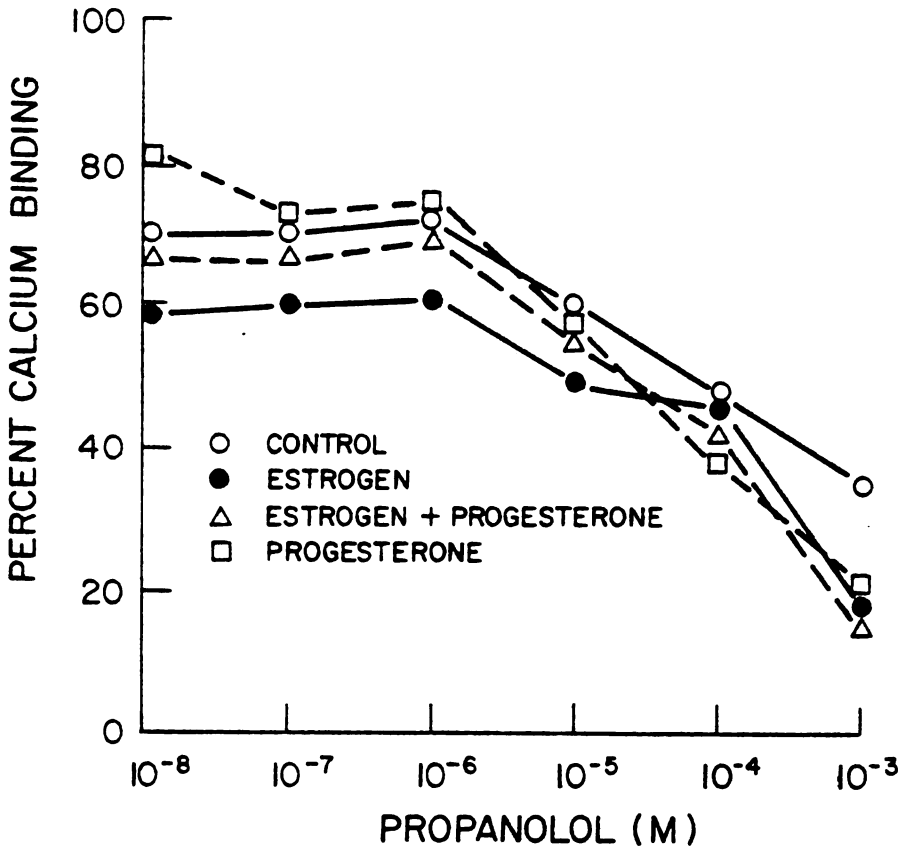

PERCENT CALCIUM BINDING
100
80
60
40
20
0
CONTROL
ESTROGEN
ESTROGEN + PROGESTERONE
PROGESTERONE
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
PROPANOLOL (M)

Figure 13

Effect of a single injection of progesterone on spontaneous uterine contractions and microsomal calcium binding. Rabbits were given a single intramuscular injection of progesterone in doses ranging from 10^{-5} to 10^{-2}g. After 16 hours the spontaneous uterine contraction was measured together with the microsomal calcium binding.

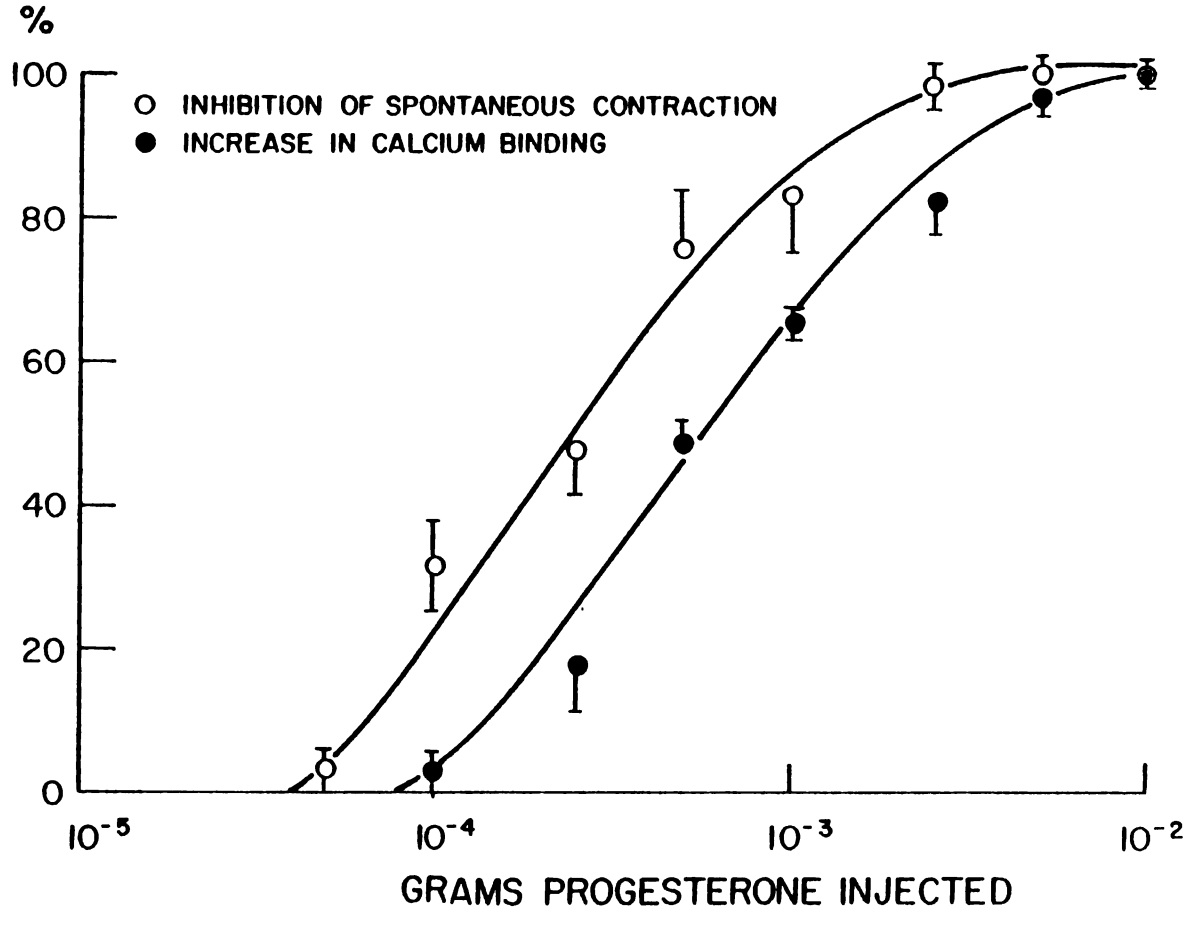

%
100
80
60
40
20
0
INHIBITION OF SPONTANEOUS CONTRACTION
INCREASE IN CALCIUM BINDING
10^{-5}
10^{-4}
10^{-3}
10^{-2}
GRAMS PROGESTERONE INJECTED

Figure 14

Time course of activation and inactivation of inhibition of spontaneous contraction and microsomal calcium binding following a single 5 mg injection of progesterone. Animals were sacrificed at 4, 6, 8, 10, 12, 16, 20, 24, 36, 48, 60 or 72 hours after injection and spontaneous uterine contractions and microsomal calcium binding was measured.

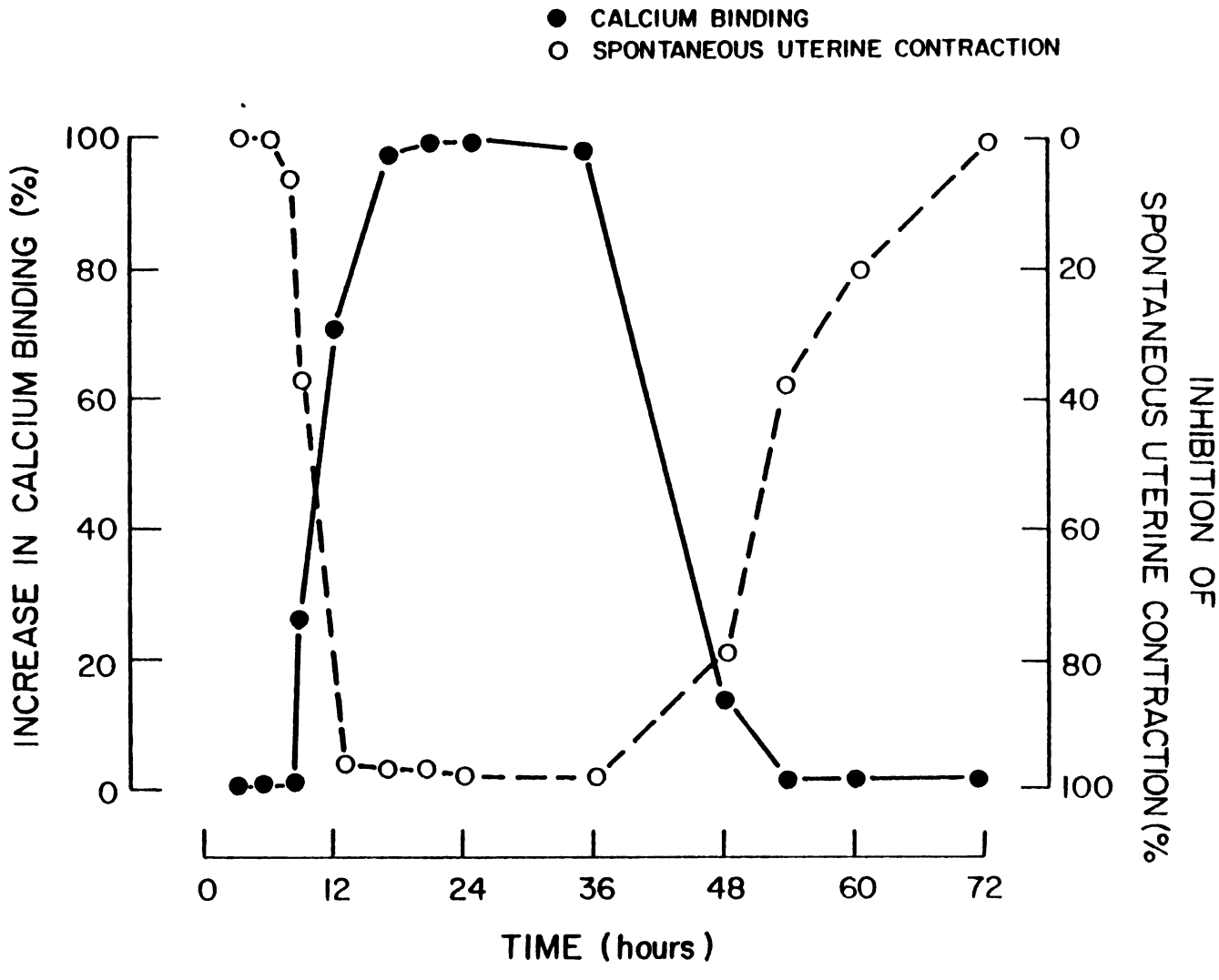

● CALCIUM BINDING
○ SPONTANEOUS UTERINE CONTRACTION
INCREASE IN CALCIUM BINDING (%)
100
80
60
40
20
0
INHIBITION OF SPONTANEOUS UTERINE CONTRACTION (%
0
20
40
60
80
100
0
12
24
36
48
60
72
TIME (hours)

Table 1

Effect of hormonal pretreatment on calcium uptake by rabbit uterine microsomes at 37°C, pH 7.2. Microsomes were incubated for ten minutes and microsomal protein concentration was approximately 100 ug/ml.

TABLE 1

EFFECT OF HORMONAL PRETREATMENT ON CALCIUM UPTAKE BY RABBIT UTERINE MICROSOMES

		HORMONAL PRETREATMENT			
		Control	Estrogen	Estrogen + Progesterone	Progesterone
CALCIUM BINDING (uM Ca/g. Prot.)	Non-ATP Control	1.49 ±0.079 (27)*	1.58 ±0.090 (18)	1.51 ±0.083 (18)	1.49 ±0.099 (27)
	5mM ATP	2.91 ±0.197 (27)	3.88 ±0.199 (18)	4.01 ±0.272 (18)	8.17 ±0.500 (18)
p_1			< 0.001	< 0.002	< 0.001
p_2			< 0.001	< 0.001	
p_3				NS	
p_4		< 0.001	< 0.001	< 0.001	< 0.001

* Figures in parenthesis: Number of observations

p_1 Unpaired t Test: Hormonal groups compared with controls ± SEM included.

p_2 Unpaired t Test: Progesterone group compared with estrogen and estrogen + progesterone group, ± SEM included.

p_3 Unpaired t Test: Estrogen + progesterone compared with estrogen group, ± SEM included.

p_4 Unpaired t Test: ATP binding compared with non-ATP binding, ± SEM included.

Table 2

Effect of pH on calcium accumulation by hormonally pretreated rabbit uterine microsomes. Microsomes were incubated at 37°C for ten minutes. All values represent the mean ± one standard error of the mean of six replicate determinations on different animals.

TABLE 2

EFFECT OF pH ON CALCIUM BINDING BY RABBIT UTERINE MICROSOMES

	Uptake of Calcium (uM/g. protein)			
	Progesterone Treated Microsomes	Estrogen Treated Microsomes	Est.-Prog. Treated Microsomes	Control Microsomes
6.00	0.25 ± 0.014	0.26 ± 0.0129	0.21 ± 0.017	0.27 ± 0.081
6.25	0.20 ± 0.036	0.31 ± 0.030	0.22 ± 0.088	0.27 ± 0.033
6.50	0.56 ± 0.049	0.35 ± 0.028	0.28 ± 0.019	1.11 ± 0.209
6.75	8.24 ± 0.151	3.90 ± 0.130	4.49 ± 0.167	3.59 ± 0.177
7.00	8.81 ± 0.169	4.16 ± 0.196	4.23 ± 0.093	3.86 ± 0.136
7.25	7.82 ± 0.214	3.50 ± 0.074	3.92 ± 0.131	3.71 ± 0.099
7.50	0.16 ± 0.021	0.10 ± 0.011	0.50 ± 0.096	1.60 ± 0.101
8.00	0.15 ± 0.031	0.11 ± 0.024	0.14 ± 0.036	0.20 ± 0.084

Table 3

Equilibrium dissociation constants (K_d) and maximum number of binding sites (n) in hormonally pretreated rabbit uterine microsomes. All values represent the mean $\pm$ one standard error of the mean of six replicate determinations on different animals.

TABLE 3

EQUILIBRIUM DISSOCIATION CONSTANTS AND MAXIMUM NUMBER OF CALCIUM BINDING SITES IN RABBIT UTERINE MICROSOMES

	Progesterone Treated Microsomes	Estrogen Treated Microsomes	Est.-Prog. Treated Microsomes	Diluent Treated Microsomes
$K_D(M^{-1}) \times 10^{-7}$	3.106 ± .279*(18)	8.270 ± .429*(18)	8.390 ± .432*(18)	8.148 ± .450*(18)
n(uM Ca/g. prot.)	9.43 ± .570*(18)	7.20 ± .203*(18)	8.57 ± .659*(18)	7.80 ± .497*(18)

*Standard error of the mean

p_1 Unpaired t Test: Progesterone treated microsomes compared with estrogen treated and controls ± SEM included ($p < 0.0001$).

p_2 Unpaired t Test: Progesterone treated microsomes compared with estrogen + progesterone treated ± SEM included ($p < 0.001$).

p_3 Unpaired t Test: Estrogen treated microsomes compared with estrogen + progesterone treated ± SEM included ($p < 9.844$) NS.

Table 4

Half times of dissociation of rabbit uterine microsomes and ^{45}Ca. Microsomes were incubated with radioactive calcium at 37°C for ten minutes and then either washed with 25 vols. of Ca free medium or allowed to dissociate in the presence of a 0.1 mM $CaCl_2$.

TABLE 4

HALF TIMES OF DISSOCIATION OF RABBIT UTERINE MICROSOMES AND ^{45}CA.

Hormonal Pretreatment	Half Time of Dissociation ($t_{½}$) (seconds) Calcium Excess	Calcium Free
Progesterone	69.16 ± 1.24(6)*	75.16 ± 1.77(6)
Estrogen	39.66 ± 2.10(6)	49.16 ± 2.37(6)

* Number of samples

p_1 Unpaired t Test: Progesterone treated $t_{½}$ Ca excess compared to Ca free ± SEM included ($p < 0.05$).

p_2 Unpaired t Test: Estrogen treated Ca excess compared to Ca free ± SEM included ($p < 0.05$).

p_3 Unpaired t Test: Progesterone treated $t_{½}$ compared to estrogen $t_{½}$ Ca excess ($p < 0.001$).

p_4 Unpaired t Test: Progesterone treated $t_{½}$ compared to estrogen $t_{½}$ Ca free ($p < 0.001$).

Table 5

Effect of Norepinephrine on rabbit uterine microsomal calcium binding: microsomes were incubated for ten minutes at 37^{o}C, pH 7.2 together with 5 mM ATP except where indicated. The means represent six determinations $\pm$ one standard error of the mean. All values were compared with calcium binding in the absence of Norepinephrine by unpaired t test.

TABLE 5

EFFECTS OF NOREPINEPHRINE ON MICROSOMAL CALCIUM BINDING

	HORMONAL PRETREATMENT									
	Diluent Control 0 ATP		Diluent Control 5mM ATP		Estrogen 5mM ATP		Estrogen + Progesterone 5mM ATP		Progesterone 5mM ATP	
		Significance		Significance		Significance		Significance		Significance
0	100 ±9.21		100 ±8.11		100 ±6.33		100 ±5.2		100 ±9.81	
10^{-8}	78.1 ±12.2	NS	90.1 ±9.1	NS	84.2 ±5.1	NS	90.0 ±5.1	NS	89.6 ±3.1	NS
10^{-7}	81.2 ±12.3	NS	84.8 ±4.5	NS	79.1 ±4.2	$p < 0.05$	84.2 ±5.7	NS	90.1 ±5.5	NS
10^{-6}	75.1 ±15.4	NS	87.3 ±5.6	NS	74.2 ±7.1	$p < 0.05$	91.3 ±4.1	NS	79.2 ±8.8	NS
10^{-5}	79.7 ±13.1	NS	86.6 ±3.7	NS	90.8 ±3.9	NS	78.3 ±8.9	NS	89.3 ±10.2	NS
10^{-4}	82.4 ±11.1	NS	86.8 ±5.1	NS	85.1 ±5.5	NS	86.7 ±4.8	NS	81.2 ±8.8	NS
10^{-3}	71.1 ±9.4	NS	74.9 ±6.2	p 0.05	79.2 ±7.7	NS	88.9 ±7.3	NS	95.3 ±6.7	NS

Table 6

Effect of Phenylephrine in the presence of 1 uM Norepinephrine on uterine microsomal calcium binding. Microsomes were incubated for ten minutes at 37°C, pH 7.2. Each point represents the mean of six determinations.

TABLE 6

EFFECTS OF PHENYLEPHRINE TOGETHER WITH 1uM NOREPINEPHRINE ON RABBIT UTERINE MICROSOMAL CALCIUM BINDING

	HORMONAL PRETREATMENT							
	Diluent Control 5mM ATP		Estrogen 5mM ATP		Estrogen + Progesterone 5mM ATP		Progesterone 5mM ATP	
PHENYLEPHRINE (M)		Significance		Significance		Significance		Significance
0	100 ±5.8		100 ±9.3		100 ±8.7		100 ±8.8	
10^{-8}	58.1 ±8.5	$p < 0.01$	91.2 ±9.0	NS	63.7 ±6.1	$p < 0.01$	82.4 ±3.9	NS
10^{-7}	67.7 ±5.4	$p < 0.01$	73.4 ±7.2	$p < 0.05$	81.9 ±4.8	NS	84.3 ±9.9	NS
10^{-6}	68.7 ±6.3	$p < 0.01$	69.5 ±4.3	$p < 0.05$	74.3 ±6.8	$p < 0.05$	72.4 ±9.1	NS
10^{-5}	79.1 ±10.7	NS	77.1 ±9.2	NS	77.7 ±7.3	NS	64.2 ±5.5	$p < 0.01$
10^{-4}	81.7 ±10.2	NS	74.5 ±6.2	$p < 0.05$	87.7 ±4.5	NS	68.1 ±6.8	$p < 0.05$
10^{-3}	67.1 ±7.8	$p < 0.01$	63.4 ±4.4	$p < 0.01$	69.9 ±3.7	$p < 0.01$	83.7 ±5.7	NS

Table 7

Effect of Phenylephrine in the absence of 1 uM Norepinephrine on uterine microsomal calcium binding. Microsomes were incubated for ten minutes at 37°C, pH 7.2. Each point represents the mean of six determinations.

TABLE 7

EFFECTS OF PHENYLEPHRINE ON RABBIT UTERINE MICROSOMAL CALCIUM BINDING

	HORMONAL PRETREATMENT							
	Diluent Control 5mM ATP		Estrogen 5mM ATP		Estrogen + Progesterone 5mM ATP		Progesterone 5mM ATP	
PHENYLEPHRINE (M)		Significance		Significance		Significance		Significance
0	100 ±5.7		100 ±8.3		100 ±6.9		100 ±6.0	
10^{-8}	91.3 ±6.8	NS	83.6 ±9.4	NS	90.4 ±9.7	NS	81.2 ±5.3	$p < 0.05$
10^{-7}	82.9 ±9.1	NS	83.1 ±6.3	NS	91.1 ±4.4	NS	95.3 ±5.3	NS
10^{-6}	81.9 ±6.2	NS	90.6 ±6.1	NS	97.6 ±3.9	NS	96.1 ±10.1	NS
10^{-5}	79.9 ±5.2	$p < 0.05$	75.3 ±8.8	NS	89.8 ±9.3	NS	79.7 ±5.5	$p < 0.05$
10^{-4}	77.9 ±9.4	NS	92.5 ±16.2	NS	82.1 ±5.8	NS	97.1 ±7.7	NS
10^{-3}	72.5 ±2.4	$p < 0.01$	85.9 ±7.0	NS	91.3 ±8.6	NS	90.2 ±8.8	NS

Table 8

Effect of Phentolamine in the absence of 1 uM Norepinephrine on uterine microsomal calcium binding. Microsomes were incubated for ten minutes at 37°C, pH 7.2. Each point represents the mean of six determinations.

TABLE 8

EFFECT OF PHENTOLAMINE ON RABBIT UTERINE MICROSOMAL CALCIUM BINDING

PHENTOLAMINE (M)	HORMONAL PRETREATMENT: Diluent Control 5mM ATP	Significance	Estrogen 5mM ATP	Significance	Estrogen + Progesterone 5mM ATP	Significance	Progesterone 5mM ATP	Significance
0	100 ±3.7		100 ±11.4		100 ±5.1		100 ±7.1	
10^{-8}	97.0 ±5.6	NS	74.8 ±3.1	NS	59.4 ±6.8	$p < 0.001$	51.0 ±6.1	$p < 0.001$
10^{-7}	89.6 ±8.2	NS	72.0 ±4.4	$p < 0.05$	62.8 ±4.2	$p < 0.001$	48.2 ±5.2	$p < 0.001$
10^{-6}	89.8 ±3.5	NS	70.9 ±6.0	$p < 0.05$	63.8 ±6.7	$p < 0.001$	51.2 ±6.3	$p < 0.001$
10^{-5}	84.1 ±9.3	NS	75.0 ±9.1	NS	60.5 ±2.8	$p < 0.001$	52.3 ±7.1	$p < 0.001$
10^{-4}	82.2 ±4.1	$p < 0.01$	70.0 ±12.3	NS	68.2 ±3.1	$p < 0.001$	46.6 ±3.4	$p < 0.001$
10^{-3}	89.1 ±11.5	NS	74.9 ±4.1	NS	74.5 ±9.9	$p < 0.05$	39.9 ±4.0	$p < 0.001$

Table 9

Effect of Phentolamine in the presence of 1 uM Norepinephrine on uterine microsomal calcium binding. Microsomes were incubated for ten minutes at 37°C, pH 7.2. Each point represents the mean of six determinations.

TABLE 9

EFFECT OF PHENTOLAMINE TOGETHER WITH 1uM NOREPINEPHRINE ON RABBIT UTERINE MICROSOMAL CALCIUM BINDING

PHENTOLAMINE (M)	HORMONAL PRETREATMENT							
	Diluent Control 5mM ATP	Significance	Estrogen 5mM ATP	Significance	Estrogen + Progesterone 5mM ATP	Significance	Progesterone 5mM ATP	Significance
0	100 ±3.9		100 ±6.2		100 ±4.1		100 ±5.9	
10^{-8}	99.0 ±6.3	NS	46.6 ±6.9	$p < 0.001$	68.7 ±4.2	$p < 0.001$	71.7 ±7.2	$p < 0.05$
10^{-7}	94.7 ±5.9	NS	64.4 ±7.5	$p < 0.01$	60.9 ±5.5	$p < 0.001$	69.4 ±5.5	$p < 0.01$
10^{-6}	95.9 ±5.0	NS	63.1 ±8.2	$p < 0.01$	66.2 ±8.2	$p < 0.001$	64.6 ±5.7	$p < 0.01$
10^{-5}	91.3 ±3.4	NS	52.7 ±5.2	$p < 0.001$	68.2 ±6.6	$p < 0.01$	41.9 ±6.4	$p < 0.001$
10^{-4}	94.6 ±7.2	NS	62.8 ±4.5	$p < 0.01$	58.7 ±9.2	$p < 0.001$	52.0 ±2.8	$p < 0.0001$
10^{-3}	85.0 ±5.8	NS	79.7 ±6.5	$p < 0.05$	63.6 ±8.8	$p < 0.01$	64.3 ±5.0	$p < 0.01$

Table 10

Effect of Isoproterenol in the absence of 1 uM Norepinephrine on uterine microsomal calcium binding. Microsomes were incubated for ten minutes at 37°C, pH 7.2. Each point represents the mean of six determinations.

TABLE 10

EFFECTS OF ISOPROTERENOL ON RABBIT UTERINE MICROSOMAL CALCIUM BINDING

ISOPROTERENOL (M)	HORMONAL PRETREATMENT: Diluent Control 5mM ATP	Significance	Estrogen 5mM ATP	Significance	Estrogen + Progesterone 5mM ATP	Significance	Progesterone 5mM ATP	Significance
0	100 ±5.1		100 ±4.9		100 ±3.8		100 ±7.6	
10^{-8}	95.1 ±13.7	NS	81.3 ±7.2	NS	79.1 ±5.8	NS	51.2 ±4.2	$p < 0.001$
10^{-7}	98.3 ±8.5	NS	88.1 ±8.1	NS	81.2 ±7.8	NS	50.1 ±5.2	$p < 0.001$
10^{-6}	93.6 ±9.9	NS	73.7 ±5.9	$p < 0.05$	85.1 ±11.1	NS	53.0 ±3.9	$p < 0.001$
10^{-5}	97.1 ±9.4	NS	89.1 ±6.1	NS	79.3 ±5.5	NS	59.1 ±5.1	$p < 0.001$
10^{-4}	94.9 ±6.3	NS	75.1 ±4.1	$p < 0.05$	83.1 ±9.1	NS	61.7 ±3.1	$p < 0.001$
10^{-3}	92.8 ±7.6	NS	74.9 ±3.9	$p < 0.05$	77.7 ±6.2	NS	64.1 ±7.3	$p < 0.005$

Table 11

Effect of Isoproterenol in the presence of 1 uM Norepinephrine on uterine microsomal calcium binding. Microsomes were incubated for ten minutes at 37°C, pH 7.2. Each point represents the mean of six determinations.

TABLE 11

EFFECT OF ISOPROTERENOL TOGETHER WITH 1uM NOREPINEPHRINE ON RABBIT UTERINE MICROSOMAL CALCIUM BINDING

	HORMONAL PRETREATMENT							
	Diluent Control 5mM ATP		Estrogen 5mM ATP		Estrogen + Progesterone 5mM ATP		Progesterone 5mM ATP	
ISOPROTERENOL (M)		Significance		Significance		Significance		Significance
0	100 ±5.2		100 ±8.1		100 ±5.4		100 ±6.1	
10^{-8}	70.1 ±7.8	$p < 0.05$	66.9 ±6.1	$p < 0.001$	79.8 ±7.1	$p < 0.05$	51.0 ±4.4	$p < 0.001$
10^{-7}	89.1 ±9.9	NS	70.8 ±8.1	$p < 0.05$	81.3 ±6.9	NS	49.1 ±3.3	$p < 0.001$
10^{-6}	77.8 ±7.7	NS	81.1 ±7.7	NS	85.1 ±8.9	NS	45.3 ±2.1	$p < 0.001$
10^{-5}	71.6 ±9.6	$p < 0.05$	85.6 ±8.8	NS	83.1 ±10.1	NS	39.1 ±3.7	$p < 0.001$
10^{-4}	81.3 ±5.8	NS	71.8 ±7.8	$p < 0.05$	77.1 ±6.7	$p < 0.05$	50.9 ±4.8	$p < 0.001$
10^{-3}	79.3 ±6.1	NS	69.8 ±5.2	$p < 0.01$	63.1 ±3.8	$p < 0.001$	41.1 ±2.7	$p < 0.001$

Table 12

Effect of ± propanolol in the absence of 1 uM Norepinephrine on uterine microsomal calcium binding. Microsomes were incubated for ten minutes at 37°C, pH 7.2. Each point represents the mean of six determinations.

TABLE 12

EFFECT OF ±PROPANOLOL ON RABBIT UTERINE MICROSOMAL CALCIUM BINDING

	HORMONAL PRETREATMENT							
	Diluent Control 5mM ATP		Estrogen 5mM ATP		Estrogen + Progesterone 5mM ATP		Progesterone 5mM ATP	
PROPANOLOL (M)		Significance		Significance		Significance		Significance
0	100 ±5.5		100 ±6.4		100 ±3.4		100 ±8.3	
10^{-8}	81.8 ±5.6	$p < 0.05$	78.1 ±5.3	$p < 0.05$	88.8 ±8.7	NS	82.9 ±5.1	NS
10^{-7}	79.5 ±3.9	$p < 0.05$	79.9 ±8.5	NS	90.8 ±4.8	NS	81.1 ±6.7	NS
10^{-6}	80.0 ±6.9	$p < 0.05$	77.0 ±9.8	NS	82.1 ±6.2	$p < 0.05$	78.2 ±9.8	NS
10^{-5}	71.2 ±6.1	$p < 0.01$	64.1 ±6.7	$p < 0.01$	69.1 ±5.7	$p < 0.001$	65.0 ±6.2	$p < 0.01$
10^{-4}	52.9 ±4.7	$p < 0.001$	37.2 ±3.5	$p < 0.0001$	41.3 ±3.4	$p < 0.0001$	47.1 ±3.1	$p < 0.0001$
10^{-3}	23.0 ±1.8	$p < 0.001$	15.4 ±2.0	$p < 0.0001$	16.2 ±4.1	$p < 0.0001$	21.1 ±2.0	$p < 0.0001$

Table 13

Effect of ± propanolol in the presence of 1 uM Norepinephrine on uterine microsomal calcium binding. Microsomes were incubated for ten minutes at 37°C, pH 7.2. Each point represents the mean of six determinations.

TABLE 13

EFFECT OF ±PROPANOLOL TOGETHER WITH 1uM NOREPINEPHRINE ON RABBIT UTERINE MICROSOMAL CALCIUM BINDING

	HORMONAL PRETREATMENT							
	Diluent Control 5mM ATP		Estrogen 5mM ATP		Estrogen + Progesterone 5mM ATP		Progesterone 5mM ATP	
PROPANOLOL (M)		Significance		Significance		Significance		Significance
0	100 ±7.3		100 ±5.0		100 ±5.9		100 ±5.8	
10^{-8}	69.4 ±7.1	$p < 0.001$	59.4 ±3.6	$p < 0.001$	74.5 ±5.8	$p < 0.05$	80.1 ±6.5	$p < 0.05$
10^{-7}	68.2 ±6.4	$p < 0.001$	60.1 ±6.7	$p < 0.001$	68.7 ±2.3	$p < 0.001$	69.9 ±7.4	$p < 0.01$
10^{-6}	71.2 ±3.3	$p < 0.005$	61.2 ±4.9	$p < 0.001$	69.7 ±8.3	NS	70.9 ±6.0	$p < 0.01$
10^{-5}	60.9 ±2.6	$p < 0.001$	51.4 ±4.2	$p < 0.001$	55.0 ±4.0	$p < 0.001$	58.5 ±5.5	$p < 0.001$
10^{-4}	48.4 ±4.9	$p < 0.001$	47.3 ±2.8	$p < 0.001$	40.1 ±1.1	$p < 0.001$	38.4 ±3.7	$p < 0.001$
10^{-3}	35.1 ±2.6	$p < 0.001$	18.6 ±1.4	$p < 0.001$	15.4 ±2.5	$p < 0.001$	20.4 ±1.8	$p < 0.001$

Table 14

Effect of Dibutyryl 3' 5'-cyclic AMP in the absence of 1 uM Norepinephrine on uterine microsomal calcium binding. Microsomes were incubated for ten minutes at 37^{o}C, pH 7.2. Each point represents the mean of six determinations.

TABLE 14

EFFECT OF DIBUTYRYL 3' 5'-CYCLIC AMP ON RABBIT UTERINE MICROSOMAL BINDING

DIBUTYRYL 3' 5'-CYCLIC AMP (M)	HORMONAL PRETREATMENT									
	Non-ATP		Diluent Control 5mM ATP		Estrogen 5mM ATP		Estrogen + Progesterone 5mM ATP		Progesterone 5mM ATP	
		Significance		Significance		Significance		Significance		Significance
0	100 ±6.7		100 ±9.7		100 ±8.9		100 ±7.2		100 ±5.4	
10^{-8}	101.4 ±4.3	NS	97.7 ±6.2	NS	102.7 ±7.1	NS	106.3 ±6.2	NS	105.7 ±5.4	NS
10^{-7}	98.1 ±6.2	NS	100.1 ±5.4	NS	106.1 ±6.3	NS	98.9 ±8.4	NS	98.8 ±8.1	NS
10^{-6}	106.7 ±4.1	NS	101.7 ±7.2	NS	97.7 ±7.3	NS	99.8 ±10.5	NS	100.9 ±5.9	NS
10^{-5}	115.0 ±9.8	NS	119.1 ±13.2	NS	106.0 ±7.1	NS	119.0 ±10.2	NS	105.9 ±11.1	NS
10^{-4}	125.6 ±15.3	NS	121.0 ±9.1	NS	125.0 ±10.9	NS	127.1 ±11.1	NS	99.2 ±5.1	NS
10^{-3}	127.6 ±13.1	NS	123.6 ±9.8	NS	127.8 ±8.8	NS	125.3 ±13.4	NS	98.3 ±6.7	NS

Table 15

Effect of hormonal pretreatment of rabbit uterine microsomal Ca-ATPase activity. ATPase activity is expressed in terms of uM inorganic phosphate liberated per second per gram of microsomal protein.

TABLE 15

THE EFFECT OF HORMONAL PRETREATMENT ON RABBIT UTERINE MICROSOMAL Ca ATPase ACTIVITY

Hormonal Pretreatment	Ca ATPase Activity (uM $P_i \cdot g^{-1} \cdot sec^{-1}$)	p_1	p_2	p_3
Diluent Control	0.98 ± 0.14(5)*			
Estrogen	1.06 ± 0.17(5)	NS	NS	NS
Estrogen & Progesterone	0.94 ± 0.13(5)	NS	NS	
Progesterone	1.07 ± 0.14(8)	NS		

* Number of samples

p_1 Unpaired t Test: Hormonal pretreatment compared with controls ± SEM included.

p_2 Unpaired t Test: Progesterone pretreatment compared with estrogen and estrogen + progesterone ± SEM included.

p_3 Unpaired t Test: Estrogen pretreatment compared with estrogen and progesterone ± SEM included.

DISCUSSION

In striated muscle, calcium ions are released from internal storage sites to initiate contraction and sequestered from the sarcoplasm to allow relaxation. In this type of muscle there is general agreement that the sarcoplasmic reticulum is the major regulator of calcium activity (Inesi, 1972) but in smooth muscle there are at least three sites where mobilizable calcium may be sequestered and stored: mitochondria, plasma membranes and the microsomal compartment.

The study isolated a microsomal fraction from rabbit uterine muscle which was characterized by the presence of vesicles and ribosomes and devoid of mitochondria (Figures 1 and 2). This fraction accumulated calcium in a manner which appeared to be ATP driven (Table 1) and, considering previous findings by Fleckestein (1954) and Mommaerts (1955) that muscle relaxation requires ATP derived energy, it is tempting to speculate that uterine microsomal ATP calcium binding may have a physiological significance in regulating the concentration of free cytoplasmic calcium and therefore the contractile state of muscle fibers.

Following treatment with the ovarian hormones, estrogen,

progesterone, or estrogen together with progesterone, it was found that ATP dependent microsomal calcium binding was consistently greater in microsomal fractions treated with progesterone alone (Table 1). Further examination of this binding indicated that in all groups the binding was saturable and reversible but thermodynamic analysis indicated that in progesterone treated microsomes there was a higher concentration of binding sites and a lower equilibrium dissociation rate constant (Table 3).

This increase in calcium binding capacity and avidity suggests that the progesterone dominated uterus may sequester calcium from the sarcoplasm in amounts large enough to keep the intracellular calcium level below that required for muscle contraction: this idea is compatible with the physiological relaxing properties of this hormone. The possibility that progesterone action is mediated through changes it produces in intracellular calcium concentrations should be consonant with the relatively long latent period observed for the onset of its physiological actions, and in order to test this idea the time courses of inhibition of spontaneous uterine contraction, together with that of microsomal calcium binding, were analyzed following a single injection of progesterone. Additionally, to test the hypothesis still further, it was attempted to establish a dose-response relationship between these phenomena. No effect on uterine

contractility or calcium binding was observed before six hours or after 72 hours and the 12-36 hour period of maximum uterine inhibition and calcium binding correlated well. Similarly, the dose-response curves (Figure 13) showed close agreement; however, the onset of effects in both this and the time course experiment did not show such close approximation and it is difficult to speculate on the reasons without further work: it is possible that this discrepancy may be due to other actions of progesterone or to damage to the microsomes incurred during preparation.

If progesterone promotes intracellular sequestration and storage of calcium, the mechanism may involve the induction of a second sub-cellular component: this possibility deserves consideration, especially when the contradictory results from time course and cross circuit experiments are considered together with recent findings concerning the molecular manner in which progesterone acts in other systems (O'Malley, 1969). Since the calcium binding by microsomes appeared to be ATP dependent, it was decided to look for changes in Ca ATPase activity in the microsomal fraction.

Lipid micro-environment has been found critical to ATPase activity (Seelig and Hasselbach, 1971; Martonosi, 1974) and in 1974, Inesi examined this influence on ATPase and calcium binding activity in the sarcoplasmic reticulum of rabbits using temperature dependence studies, followed

by Arrhenius plot analysis; discontinuities in this type of plot are believed to represent lipid phase transitions that in turn modify enzyme activity. Inesi found membrane conformational changes at 19°C affecting Ca ATPase, which he termed the "lipid melt" temperature.

The results of this work show a transition at 20°C in the Arrhenius plot and that hormonal pretreatment with ovarian steroids has no effect on either the transition temperature or the slopes of the graph. This implies the hormonal treatment does not alter drastically the local lipid environment and that characteristics of ATPase in this system are independent of steroid hormonal status. These data agree with other determinations in this study in which Ca ATPase activity in the microsomes was measured directly, subsequent to steroid treatment: it was impossible to reveal significant differences in enzyme activity. Thus it would appear that progesterone does not increase microsomal calcium binding by increasing available energy from ATP by enzyme induction; however, Ostwald and MacLennon (1974) have described the isolation and purification from rabbit sarcoplasmic reticulum of a lipoprotein (Calsequestrin) with high capacity and medium affinity calcium binding properties and in view of the increase in binding sites found in this study in progesterone treated microsomes, one might conject that the hormone could act by inducing the synthesis of a similar

protein.

It has been postulated that estrogen and progesterone alter the sensitivity of alpha and beta receptors to norepinephrine and other experimental evidence has increasingly suggested that calcium ions are often subcellular coupling agents in hormone action (Ebashi and Endo, 1968; Bianchi, 1968; Rubin, 1970; Rasmussen, 1970; Rasmussen et al., 1972). In this investigation various alpha and beta agonists and blockers were used in an attempt to demonstrate a possible relationship between alpha and beta receptors and microsomal calcium binding as an interrelated intracellular messenger system. The results show that propanolol inhibits uterine microsomal calcium uptake and these findings are similar to those in cardiac microsomal preparations (Hess et al., 1968; Scales and McIntosh, 1968; Entman et al., 1969; White and Shinebourne, 1969; Pretorius, 1969). The mechanism by which this block is brought about is controversial. Hess et al. (1968) and White and Shinebourne (1969) have reported that beta adrenergic agonists reversed propanolol inhibitions suggesting that propanolol is mediated in part at least by a beta blocking action. A similar conclusion was reached by Entman et al. (1969) who found that propanolol prevented epinephrine induced cardiac microsomal binding. In the study herein it was found that in rabbit uterine microsomes propanolol inhibited the binding of calcium both in the

presence and absence of norepinephrine. It was also found that norepinephrine had no significant effect on the binding of calcium no matter how the microsomes were pretreated. Baudouin-Legros and Meyer (1973) reported that norepinephrine produced a slight inhibition of calcium uptake in rabbit aortic microsomes and surprisingly that epinephrine had exactly the opposite effect.

In other systems l-propanolol which has beta blocking together with local anaesthetic actions and d-propanolol which is only a local anaesthetic inhibited calcium binding and that this cannot be reversed by isoproterenol (Katz et al. 1974). It has therefore been concluded that inhibition of calcium binding by propanolol cannot be attributed to a beta blocking action on the calcium transport system of the sarcoplasmic reticulum.

In these experiments the beta receptor agonist isoproterenol did not increase the calcium binding in any of the systems examined and in the estrogen, estrogen plus progesterone and controls had little effect. However, in the progesterone pretreated microsomes, isoproterenol, both in the presence and absence of norepinephrine, reduced calcium binding.

Finally, phentolamine, a potent alpha blocking agent, consistently inhibited calcium binding more than any other agent used, suggesting strongly that the agents used in

these investigations must be acting through a mechanism not mediated by alpha or beta receptors.

It is difficult to evaluate the effects of dibutyryl 3' 5'-cyclic AMP on microsomal calcium binding observed in this study since the changes were not statistically significant. However, the suggestion of a dose-response relationship in some of the groups, together with the delicate increase in calcium binding, are compatible with the physiological relaxing properties of the nucleotide (Baudouin-Legros and Meyer, 1973) and do give credence to the idea that cyclic nucleotides play a role in 'fine tuning' intracellular calcium signals (Rasmussen and Goodman, 1975).

In considering the negative results obtained with isoproterenol and 3' 5'-cyclic AMP it is essential to take into consideration the feedback relationships operating between the various subcellular constituents. It is widely believed that in the intact cell, activation of alpha or beta receptors modulates intracellular nucleotide levels, the primary role of which is to stimulate protein kinases which in turn phosphorylate various key proteins; this effect has been particularly well characterized in cardiac muscle where cyclic AMP stimulates a protein kinase which phosphorylates a component of the sarcoplasmic reticulum leading to a stimulation of calcium uptake (Kirchberger et al., 1972; Tada et al., 1974). The kinase does not phosphorylate

Ca-ATPase but a sub-protein of the carrier molecule: this protein, termed phospholamban, is thought to modulate the transport protein (Tada, 1974) and a similar mechanism is thought to exist in smooth muscle where cyclic AMP stimulates calcium uptake into the microsomal compartment. However, the mechanism has not been studied in the same detail as in heart muscle, and in this study the protein kinase or the phosphate acceptor, or both, may have been lost during preparation of the microsomes.

Great care must be taken in the interpretation of these in vitro results. The in vivo physiological significance of the calcium binding remains uncertain because the nature of the intrasarcoplasmic reticulum matrix remains to be determined. Furthermore, the slowness of calcium uptake in vitro indicates that this mechanism alone may not be the in vivo calcium trapping mechanism.

SUMMARY

The administration of various ovarian steroids to immature female rabbits produced changes in the ability of uterine microsomal preparations to bind calcium. The uptake of calcium was consistently greater in animals pretreated with progesterone.

The uterine microsomes were incubated with ^{45}Ca and ATP for ten minutes and the incubation was stopped by rapid filtration. ^{45}Ca uptake was maximal in eight minutes; was saturable and reversible.

An attempt was made to correlate the actions of estrogen and progesterone with changes in the activity of alpha and beta receptors. This was done by incorporating different alpha and beta agonists and blockers into the incubation medium and measuring the effect on microsomal calcium binding. The results were inconclusive but suggested that the pharmacological agents were not acting through the alpha or beta receptor mechanism.

A thermodynamic analysis of calcium binding indicated that in the progesterone pretreated microsomes there was a greater concentration of calcium receptor sites and that binding at these sites displayed greater avidity when

compared to that in estrogen pretreated animals.

It was postulated that progesterone promotes the observed increase in uterine microsomal calcium binding by inducing the synthesis of calcium dependent ATPase in the microsomal compartment: levels of this enzyme were measured in microsomal samples from all groups, however no significant changes were detected.

It was concluded that progesterone promotes the metabolic storage of calcium by uterine microsomal reticulum in amounts large enough to keep the sarcoplasmic calcium level below that required for contraction and that this accounts for observed decreases in contractility.

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