THE KININ SYSTEM AND OVULATION IN MAMMALS

by

CAROLINE MARY SMITH

B.Sc., The University of British Columbia, 1972

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
(Department of Zoology)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 1982

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Zoology

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date Oct 17, 1982

ABSTRACT

In this thesis I investigated the possibility that the kinin system could be involved in the process of ovulation. This study was divided into four parts, these are outlined below.

(1) To determine whether and when the kinin system is activated in relation to ovulation, plasma kininogen levels were estimated in female rats, guinea pigs, and humans at different stages of their estrus or menstrual cycles. Non-ovulating females (women using oral contraceptives, or post-menopausal women) and male guinea pigs served as controls. The ovulating females of all three species showed a marked decline in kininogen levels shortly before ovulation, suggesting that the kinin system was activated at this time. The fall was absent in the non-ovulating controls, with the exception of women using oral contraceptives. In the latter subjects the fall occurred at a similar time in the 'cycle', and was of a similar magnitude as the fall in normal women. These results showed that the fall is a preovulatory change and raised the possibility that a mechanism more fundamental than the events obstructed by the oral contraceptives could be at least partially responsible for the decline.

(2) After establishing the timing of the fall in plasma kininogen levels, an attempt was made to locate the enzymes responsible for the change. The kinin-forming

enzymes of the two locations most likely to be involved in kinin release during ovulation, that is, the plasma and the ovary were examined. The evidence indicated that kinin-forming enzymes were present in both locations and suggested that their concentrations increased as ovulation neared.

(3) In order to examine the possibility that an ovulatory stimulus can activate the kinin system, female rats were treated with an ovulatory dose of luteinizing hormone (LH) or estradiol-17β one day before the anticipated time of ovulation and kininogen level declines. Estimation of plasma kininogen levels revealed marked declines in the LH-treated animals, estradiol-17β had no observable effect. This evidence suggested that LH, but not estradiol-17β could be responsible, at least in part, for the decreased kininogen values just before ovulation.

(4) Lastly, to establish the ability of a kinin to initiate some of the more important events of the ovulatory process, the effects of bradykinin on ovarian smooth muscle contractility and ovarian follicular blood vessel permeability in the rat were examined. Bradykinin stimulated ovarian contractility in <u>in vitro</u> preparations to a significantly greater degree in ovaries isolated during the ovulatory period than at any other stage of the cycle. Also, the degree of movement of the dye Trypan Blue from the general circulation throughout ovarian follicular tissue over a ten minute exposure period was significantly greater

in tissue from animals treated with bradykinin than those that were not. This suggests that bradykinin can increase ovarian follicular blood vessel permeability in the rat. Both of these bradykinin-induced effects were reduced, but not eliminated by indomethacin, suggesting that prostaglandins may be involved.

Results from this study indicate that the kinin system is activated during the preovulatory period, possibly at the level of the ovary, that LH may be partially responsible for this activation, and that kinins may play a role in triggering increases in ovarian contractility and blood vessel permeability both directly and possibly via the release of prostaglandins. More definite proof awaits the development of a satisfactory kinin antagonist.

.....

(Supervisor)

TABLE OF CONTENTS

Page

Page

Page

Page

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

Page

ACKNOWLEDGMENTS

I wish to thank all the people who assisted me throughout this thesis. I am especially grateful to my supervisor, Dr. A.M. Perks, for his critical advice and guidance throughout the course of my studies. I am grateful to Dr. E. Vizsolyi, Dr. W. Hoar, Dr. J. Phillips and Dr. D. Fisher for guidance and advice.

I would also like to acknowledge the aid and assistance of the following people: Dr. J. Ames, Dr. B. Copping, Mrs. E. Fenner and members of the Richmond Biomedical Laboratories for collection of human blood samples; all the willing volunteers who donated blood; Dr. H.Nordan and Mr. A. Tepper for breeding and care of the rats and guinea pigs used in this study; Mr. Fergus O'Hara for mechanical assistance; my parents (David and Carol Smith) and Colin Parkinson for moral support; Mrs. Carol Smith for the typing of this thesis; and finally Dr. J. Hanrahan for encouragement of my research endeavors.

This research was supported by N.R.C. Grant No. 67-2584.

GENERAL INTRODUCTION

THE KININ SYSTEM

1. Background information

Mammalian kinins, a group of short polypeptides, belong to a class of "tissue or local hormones" (Rocha e Silva, 1963); that is, they are generated in the plasma surrounding their target site, rather than in specialized glands.

The kinins have numerous actions that suit them for a role as mediator in a variety of pathological and physiological reactions. Although components of the kinin system are widely distributed throughout the body, their relationship to any strictly physiologic function remains uncertain.

The history of the kinins began in 1909, when Abelous and Bardier found a substance in human urine that caused a prolonged fall in the arterial blood pressure of dogs. A similar factor was found in the pancreas (Kraut, et al., 1930). The substance was named "kallikrein" (Greek for pancreas).

In 1936 Werle discovered that kallikrein can release a very potent substance from plasma which contracts smooth muscle. This substance was later termed "kallidin" and its precursor "kallidinogen".

Rocha e Silva and associates (1949) found that when the

venom of the Brazilian snake Bothrops jararaca was added to blood or serum, it caused release of a factor which had varied, and apparently unrelated effects on living tissues. They named this potent substance "bradykinin" (from the Greek - bradys kinea - meaning slow moving) because of its slow contractile effect on various smooth muscles. The precursor was thus labelled "bradykininogen".

Bradykinin has since been sequenced and synthesized (Boissonnas et al., 1960). It is nona-peptide with the following composition:

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH

The bradykininogen-bradykinin system is now known to be closely related to the kallidinogen-kallidin system since kallidin is, in fact, lysine-bradykinin. There are several other similar peptides all containing the same basic structure, consisting of the nona-peptide bradykinin, with additional amino acids attached to the amino or carboxyl ends. In 1954, the generic term "kinin" was adopted for this whole group of related peptides.

2. Components of the kinin system

The mammalian kinin system is an integrated series of prekallikreins, kallikreins (kininogenases), kininogens, free kinins, and kininases (Schacter, 1969; Eisen, 1970; Pisano, 1975; Erdos, 1976).

A. Prekallikreins to kallikreins

Kallikreins are a group of substrate-specific serine proteases capable of cleaving free kinin from a plasma kininogen molecule. Kallikreins have been found in several tissues, and in plasma (Pisano, 1975; Schacter and Barton, 1979). Plasma kallikreins only attain detectable levels during pathological situations; under normal conditions they circulate in the preactive form (prekallikreins) (Colman et al., 1969; Eisen, 1970; Sampaio et al., 1974; Pisano, 1975; Cochrane and Griffin, 1979; Carretero and Scicli, 1981). Glandular, or tissue, kallikreins have been found in both active and/or preactive form, depending on the tissue (Pisano, 1975; Schacter and Barton, 1979).

A variety of endogenous and exogenous mechanisms are capable of converting prekallikrein to kallikrein. Some of these mechanisms include contact with activated Hageman Factor (clotting factor XII, which also activates the plasmin system, and clotting processes), contact with foreign surfaces (for example, broken blood vessels), contact with tissue proteases (for example, trypsin or cathepsin), dilution of plasma, and changes in temperature, pH, and pO_2 (see Eisen, 1970; Pisano, 1975).

Other serine proteases, with less substrate-specificity, for example, trypsin, cathepsin, and plasmin, can also release kinins from kininogen. These enzymes are usually referred to as "kininogenases" in the current literature.

Often in this thesis there is no justification for distinguishing between kallikreins and kininogenases, and so the more general term "kinin-forming enzymes" will be used.

B. Kininogen to kinin

Kininogens are large protein molecules containing the bradykinin sequence. They are produced in the liver (Bryan et al., 1972) and circulate in the plasma in amounts sufficient to generate a kinin concentration several thousand times higher than biologically effective levels (see Eisen, 1970). Although the complete structure of kininogen is not known, there are believed to be several different forms (in Eisen, 1970). At least one form is resistant to kallikreins, so that under natural physiological or pathological conditions plasma kininogen stores are never totally depleted. However, in strictly artificial situations, such as injection of trypsin into the blood stream, the more resistant forms of kininogen can also release their active peptides.

The kinins, which are rapidly inactivated by kininases in both blood and several tissues (see Eisen, 1970; Pisano, 1975; Erdos, 1976; Leme, 1978; Schacter and Barton, 1979), are transient, rarely reaching detectable levels, except under pathological conditions. For this reason, measurements of kininogen levels are often used as an indication of kinin release (Brocklehurst and Zeitlin, 1967; Habermann, 1970). Once the kinins have been destroyed they are

thought to pass out in the urine (Bumpus et al., 1964).

Figure 1 presents a relatively simple scheme outlining the above processes.

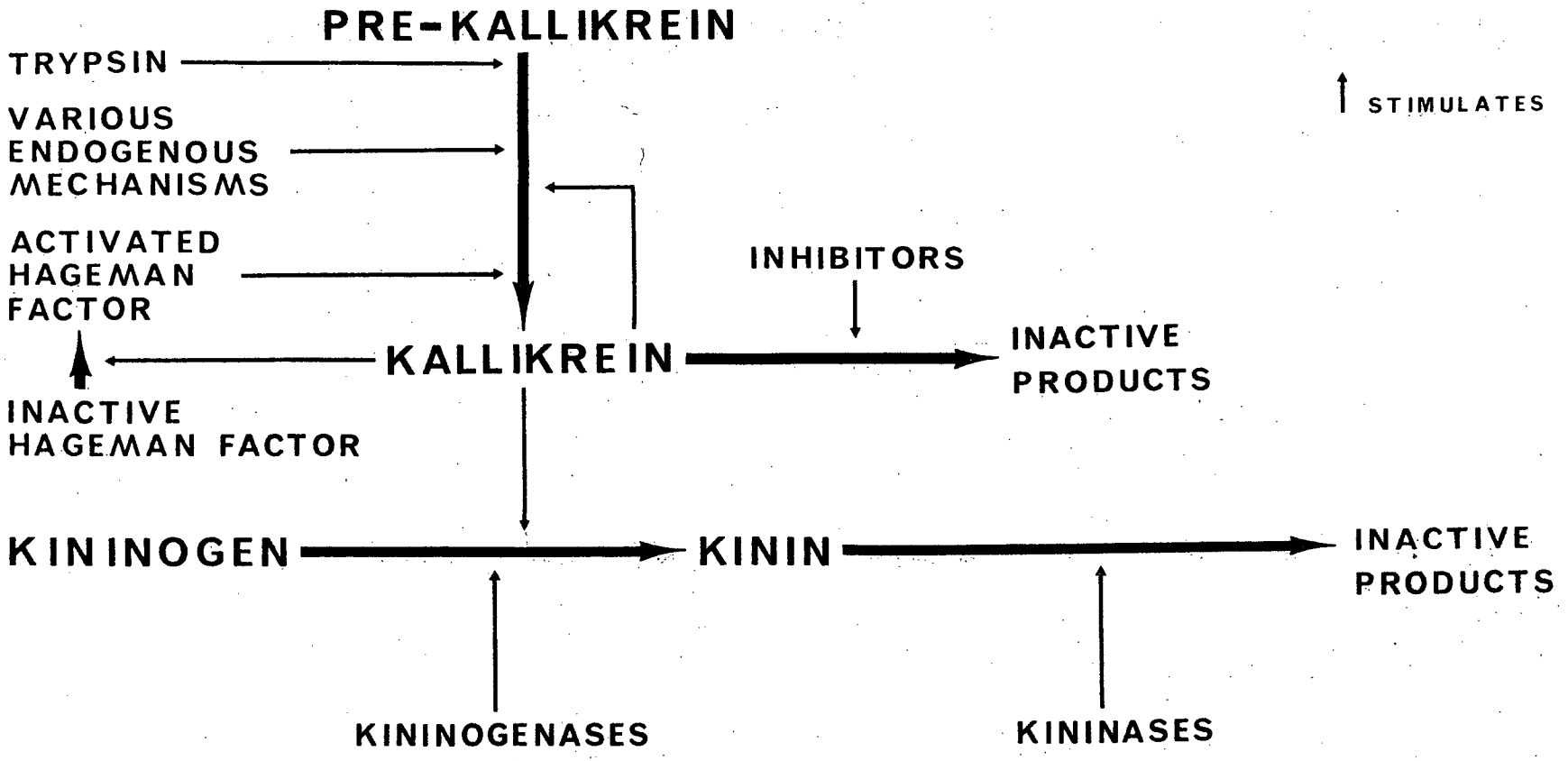
3. Biological actions of the kinins

Bradykinin, the fundamentally active portion of all kinins, is powerful in its ability to stimulate smooth muscles, to increase capillary permeability, and to dilate some and constrict other blood vessels. There is also much interplay between the kinin system and several other systems, including the prostaglandin, plasmin and clotting systems.

A. The effects of bradykinin on smooth muscles

Bradykinin is potent in its ability to contract many and relax a few other smooth muscles (Konzett and Sturmer, 1960; Elliot et al., 1960; Antonio, 1968). The mechanism by which bradykinin acts on smooth muscle is not yet clear, but evidence suggests that it acts directly on specific smooth muscle receptors on the outside of the cell membrane, changing the flux of ions, possibly calcium, across the membrane (Khairallah and Page, 1963; Walaszek, 1970).

FIGURE 1 : A SIMPLIFIED VERSION OF THE MAMMALIAN KININ SYSTEM



THE MAMMALIAN KININ SYSTEM

B. The effects of bradykinin on blood vessels

Bradykinin has potent and numerous effects on the vascular system. When given systemically, this peptide has been shown to cause arteriolar dilation and a decrease in peripheral vascular resistance (Fox et al., 1961; Nakano, 1965; see also Kellermeyer and Graham, 1968). In fact, vasodilation in response to bradykinin occurs so often, and in so many places, some workers believe that bradykinin might play an essential role in this area. Lewis (1963), on the basis of his findings, has suggested that the main function of the kinins is to act as a mediator of functional vasodilation in glands. In addition, several investigators have found that bradykinin constricts some veins in a number of species (Rowley, 1964; Tsuru et al., 1976). Bradykinin can also increase the permeability of vascular walls; whether it achieves this by increasing the number and size of pores or gaps (Majno, 1964; Bignold and Lykke, 1975), or by increasing the size and turnover rate of pinocytotic vesicles (Renkin et al., 1974), or simply by dilating blood vessels, causing the opening of more capillaries (Renkin et al., 1974), remains controversial. Collectively, the evidence indicates that in general, bradykinin increases the blood supplies to tissues at the local level.

C. Interaction of the kinin system with other physiological systems

The kinin system interacts with several other enzyme-hormone systems. Bradykinin possesses the capacity to activate prostaglandin synthesis in several tissues (see Nasjletti and Malik, 1979). Kallikreins can activate Hageman Factor (clotting factor XII) (Spragg, 1974; Cochrane and Griffin, 1979), which in turn, activates the plasmin system and clotting processes (Spragg, 1974). Several positive-feedback loops also exist, for example, activated Hageman Factor, prostaglandins, and plasmin can each activate the kinin system (see Spragg, 1974; Sharma, 1978).

4. Physiological and pathological functions of the kinin system

A. Physiological function

Despite their potent effects, an understanding of the physiological functions of the kinins has been limited by several factors including: (1) the lack of specific inhibitors, (2) the difficulties in measuring kinin concentrations in small samples of biological tissues, (3) their short half-life in the general circulation (t 1/2 = 20 sec) (Arrigoni-Martelli, 1977), (4) the complexity of the system's interactions with other systems, and (5) the ease with which activation of the kinin system is accomplished during experimental or surgical procedures.

The only reasonably well established role for the kinin system lies within the pathological process of inflammation (Lewis, 1970; Arrigoni-Martelli, 1977). To date, any strictly physiological function of the system remains speculative (Schacter, 1979).

B. Pathological function

Much evidence, although circumstantial, has accumulated implicating the involvement of the kinin system in inflammation (Arrigoni-Martelli, 1977). Evidence for this comes from five different directions:

(1) The cardinal signs of inflammation, that is, vasodilation, increased vascular permeability, pain, and accumulation of leucocytes, are all known responses to the kinins (Lewis, 1970).

(2) Due to their short half-life, it is difficult to detect free kinins at an inflammatory site; however, Melmon et al., (1967) have managed to accomplish this.

(3) Increased kinin-forming activity has been observed in lymph collected from injured tissue (Edery and Lewis, 1963).

(4) Many of the necessary stimuli to activate the kinin system are present at the inflammatory site - for example, a foreign surface (eg. broken blood vessel), a change to acid pH, dilution with edema fluid, passage of specific kallikrein activators (eg. trypsin, plasmin), or their generation within the inflammatory site, activation

of Hageman Factor (clotting factor XII), and increased diffusion of kininogen from plasma into the interstitial space, which might result from an increased vascular permeability, perhaps initiated by co-mediators (Lewis, 1970). (5) The kinin system can also interact with several other systems involved in the inflammatory process. For example, (a) bradykinin stimulates increases in cyclic AMP production of fibroblasts (Fahey et al., 1977), which, in turn, initiates prostaglandin synthesis in the fibroblasts (Chandrabose et al., 1978). During inflammation prostaglandins are usually formed subsequent to bradykinin release (Greaves et al., 1976); they serve to maintain the inflammatory symptoms of vasodilation (Greaves et al., 1976; Bonta and Parnham, 1978), increased vascular permeability (Greaves et al., 1976; Vane, 1976; Bonta and Parnham, 1978), and edema (Greaves et al., 1976; Vane, 1976). (b) The processes of coagulation and plasminogen activation are both initiated by Hageman Factor, which can be activated, in turn, by kallikrein, trypsin, and plasmin (see Cochrane and Griffin, 1979). (c) The possibility also exists that bradykinin releases histamine (another mediator of inflammation) from cellular stores, because anti-histamines can reduce bradykinin-induced increases in vascular permeability in several tissues (Becker et al., 1968; Shvarts, 1980). (d) Lastly, collagenolytic activity increases in inflamed tissue (Bertelli et al., 1969; Bonta and

Parnham, 1978), and evidence suggests that plasmin, kallikrein, and other serine proteases associated with the kinin system activate collagenase in inflamed tissue (Eeckhout and Vaes, 1977).

Confirmation of the hypothesis that the kinin system is involved in inflammation, awaits the discovery of an anti-inflammatory agent with specific antagonistic properties toward the kinins (Lewis, 1970).

Therefore, even though the involvement of the kinin system in inflammation is reasonably well established, the exact role(s) of this peptide in an *in vivo* inflammatory response remain unclear.

OVULATION IN MAMMALS

The ovulatory process, as defined in this thesis, includes the events leading to, as well as, the actual rupture of the mature (Graafian) follicle and extrusion of its ovum. Despite extensive research, the exact mechanisms involved in this process remain unclear.

1. The Graafian follicle

The specialized portion of the ovary that contains the mature ovum is termed the Graafian(or mature)follicle. In mammals this follicle consists of a cavity (follicular cavity or antrum) surrounded by a layer of cells termed the

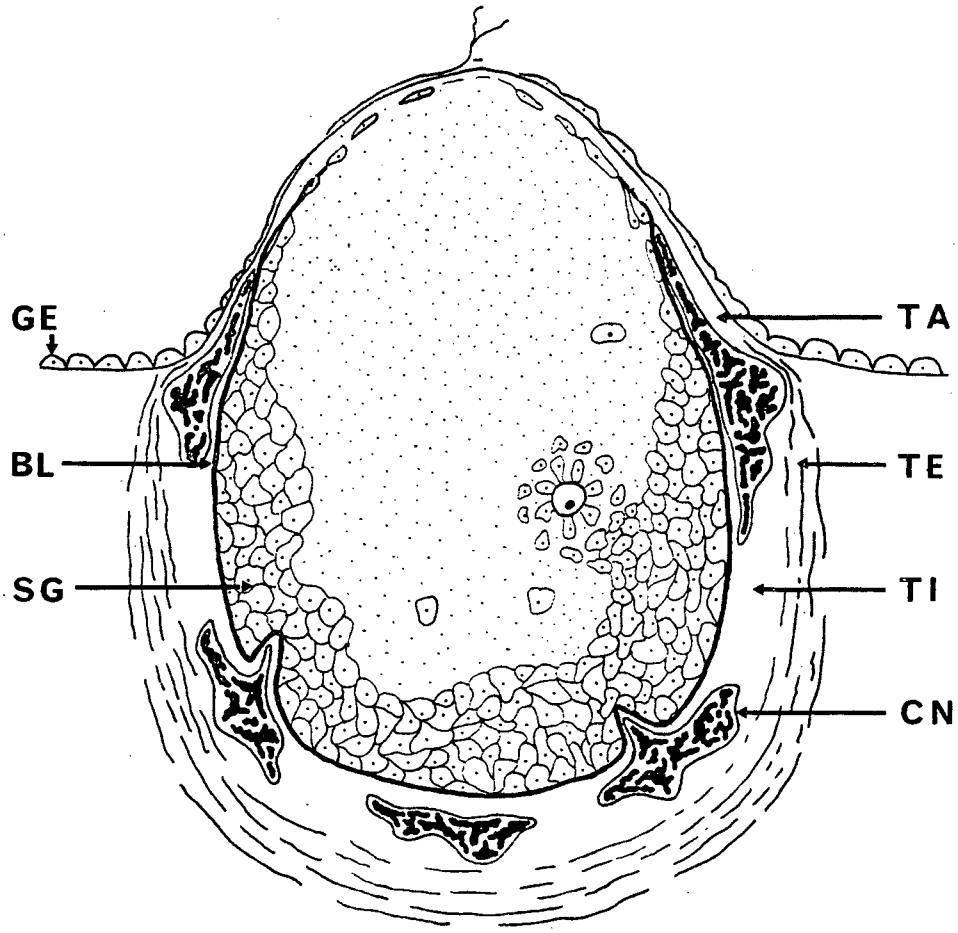
stratum granulosum. The follicular cavity contains follicular fluid, as well as the ovum, which is embedded in a mass of cells, the cumulus oophorus, extending from the granulosa layer into the antrum. A basal lamina surrounds the granulosa layer and separates it from the next layer, the theca. The thecal layer is divided into the innermost theca interna, and the outermost theca externa. The mature follicle bulges out from the ovarian stroma, where it is covered by the ovarian surface, consisting of a tunica albuginea, and a germinal epithelium. Figure 2 illustrated diagramatically, a typical mammalian Graafian follicle.

The granulosa layers of mature follicles are avascular, with the capillary network extending no further than the thecal layers. Hence, products entering the follicular cavity from the general circulation must do so by diffusing across, not only the blood vessel walls, but also the basal lamina and stratum granulosum. As ovulation nears, the blood supply to the maturing follicles increases several fold (Basset, 1943; Burr and Davis, 1951; Szego and Gitin, 1964), and is accompanied by ovarian edema (Szego and Gitin, 1964; Bjersing and Cajander, 1975).

Electron microscopy has shown the presence of smooth-muscle-like cells in the theca externa layer of maturing follicles of several species; these cells are believed not to be part of blood vessel walls (Osvaldo-Decima, 1970;

FIGURE 2: A DIAGRAMATIC REPRESENTATION OF A TYPICAL MAMMALIAN GRAAFIAN FOLLICLE

Two cell layers surround the entire ovary; these are the germinal epithelium (GE) and tunica albuginea (TA). The follicle wall beneath these layers consists of the theca externa (TE), theca interna (TI) and stratum granulosa (SG). The stratum granulosa and theca interna layers are separated by a basal lamina (BL) and a blood capillary network (CN) is found just outside of this membrane (taken from Motta et al., 1971).


GE
TA
BL
TE
SG
TI
CN

O'Shea, 1970b, 1971; Amenta et al., 1979). Also, the technique of fluorescent histochemistry has revealed the presence of elongated cells which contain contractile proteins. These cells form concentric layers in the theca externa of Graafian follicles (Amsterdam et al., 1977; Walles et al., 1978). Numerous branches of adrenergic fibres have been discovered in between, and in close association with these contractile cells. The nerve fibres appear to affect the motor activity of the follicle wall (Owman et al., 1975; Walles et al., 1977a; Walles et al., 1978). In addition, it is known that the ovarian stroma of several species (human, rat, rabbit, cat, mouse) also possesses smooth muscle cells (O'Shea, 1970a; Okamura et al., 1972; Amenta et al., 1979). As ovulation approaches, the spontaneous contractile activity of the follicle wall and ovary appears to increase (Virutamasen et al., 1972a,b; Virutamasen et al., 1973; Sterin-Borda et al., 1976; Virutamasen et al., 1976; Wright et al., 1976).

The apex of the Graafian follicle also undergoes several changes as ovulation nears. First, blood flow to a small area (later to become the rupture site or stigma) decreases (Parr, 1975), and the cells of the underlying germinal epithelium becomes flattened and stretched (Motta et al., 1971). Immediately underlying this area the fibres of the connective tissue become disorganized (Motta

et al., 1971). The granulosa at the apex is frequently reduced to a single layer of cells, or to layers of partially luteinized flat cells, which are loosely connected (Motta et al., 1971). Many dissociated granulosa cells can be found in the follicular fluid at this time (Edwards, 1974), very shortly after, the germinal epithelium overlying the site sloughs off and the tunica albuginea and thecal layers loosen to expose the basal lamina. This membrane bulges, forming a secondary cone and becomes stretched and thinned out; eventually it ruptures and the follicular contents flow out of the follicle (see Blandau, 1966; Parr, 1975).

2. The mechanism of ovulation

Although it is well agreed that a surge in LH initiates the sequence of events ending in follicular rupture and ovum extrusion, the local ovarian events which intervene between the LH surge and ovulation itself remain obscure. Various mechanisms have been proposed (Blandau, 1966). These include: 1) enzymatic digestion of the follicle wall (Espey and Lipner, 1965; Espey and Rondell, 1968; Rondell, 1970), 2) contractions of smooth muscle components of the follicle wall, ovarian stroma, and/or ovarian vasculature (Lipner and Maxwell, 1960; Blandau, 1967; Burden, 1972; Espey, 1978), and 3) increases in follicular volume possibly created by local vascular changes (Boling et al., 1941;

Basset, 1943; Burr and Davis, 1951; Szego and Gitin, 1964; Bjersing and Cajander, 1975). These three possible mechanisms will be discussed in greater detail.

A. Ovulation by enzymatic digestion

Several studies indicate that follicular rupture is caused by proteolytic digestion of the follicle wall. As ovulation nears, the wall becomes more flaccid, and there is a measurable decrease in the tensile strength of this tissue (Rondell, 1970; Espey, 1974). Also, several proteolytic enzymes have been extracted from the follicle, including trypsin and cathepsin, along with a collagenolytic enzyme which increases in activity in follicular fluid as ovulation approaches (Espey, 1974; Espey, 1975; Morales *et al.*, 1978). Several of these enzymes have been found highly effective, *in vitro*, in both decomposing follicle wall tissue, and in reducing its tensile strength (Espey, 1974). Also, Espey and Lipner (1965) injected small amounts of these enzymes directly into the antrum of rabbit Graafian follicles and noted changes similar to normal ovulation.

B. Ovulation by ovarian contractions

The involvement of ovarian contractile activity in the ovulatory process remains controversial. However,

considerable evidence lending support to a causal relationship has accumulated. Both light and electron miscoscopic studies have revealed the presence of non-vascular smooth muscle-like cells and associated autonomic nerve fibres in the vicinity of Graafian follicles (Burden, 1972; Owman et al., 1975; Amenta, 1979). Several investigators, working independently, have demonstrated the ability of ovarian tissue from different species (including cat, rabbit, human, monkey, sheep, cow, guinea pig and rat), to contract spontaneously both in vivo and in vitro (Rocerto et al., 1969; Palti and Freund, 1972; Coutinho and Maia, 1972; Virutamasen et al., 1972a; Diaz-Infante et al., 1974; Gimeno et al., 1974; O'Shea and Phillips, 1974; Walles et al., 1974; Diaz-Infante et al., 1975; Gimeno et al., 1975, 1976; Roca et al., 1976, 1977). Also, neurohormonal agents, prostaglandins, and other substances present in ovulatory tissue have been observed in numerous studies to stimulate ovarian contractility (Rocerto et al., 1969; Virutamasen et al., 1971; Coutinho and Maia, 1972; Virutamasen et al., 1972a,b; Gimeno et al 1973; Diaz-Infante et al., 1974; Gimeno, 1974; O'Shea and Phillips, 1974; Walles et al., 1974b; Diaz-Infante et al., 1975 Gimeno et al., 1975, 1976; Roca et al., 1976; Sterin-Borda et al., 1976; Virutamasen et al., 1976; Roca et al., 1977). Lastly, substances known to inhibit smooth muscle

contractility, for example EDTA, have been found to inhibit ovulation in in vitro perfused preparations (Wallach et al., 1978). When considered together, these data supply convincing support for the contention that ovarian smooth muscle contractions may participate in the process of ovulation.

Whether ovarian contractility and the process of ovulation are causally related remains to be proven. This, however, has not prevented speculation concerning the potential role of ovarian contractions in the ovulatory process. Ovarian mobility could enhance ovulation in several ways. (1) The smooth muscle-like cells in the theca externa of maturing follicles are capable of contracting tonically (Owman et al., 1975; Gimeno et al., 1976; Walles et al., 1976). These tonic contractions could enhance follicular rupture and extrusion of the ova by maintaining intrafollicular pressure until an increasingly distensible follicular wall reaches its breaking point (Rondell, 1964, 1970). (2) The tonic contractions might also be responsible for the vascular bed changes required to produce ischemia at the follicular apex, which appears important to the rupture mechanism (Walles et al., 1977a). (3) The ovarian stroma possesses the ability to contract rhythmically as well as tonically (Walles et al., 1975; Gimeno et al., 1976). Espey (1978) has hypothesized that the rhythmic movements are the result of spasms of the ovarian vasculature, just as are known to occur in the uterine vasculature (Markee, 1932).

These contractions might facilitate, in part, increases in the local circulation. A sustained increase in the local filtration rate could explain the rapid increase in follicular volume that occurs just prior to ovulation (Boling et al., 1941). The tonic contractions of the ovarian stroma might reflect changes in the diameter of blood vessels (Osvaldo-Decima, 1970); if a constrictive mechanism acts on the venous side of the capillary bed concomitantly with periferal vasodilation, much in the same manner as bradykinin often works (see: Kellermeyer and Graham, 1968; Tsuru et al., 1976: Barabe et al., 1979), the filtration rate through capillary walls would be markedly raised (Landis and Pappenheimer, 1963). It is also possible that muscle cells could induce transient openings of certain endothelial junctions (Osvaldo-Decima, 1970).

C. Ovulation through increased follicular volume associated with an increased ovarian blood supply

As ovulation approaches the blood supply to the ovary increases due to both a dilation of the ovarian vasculature (Motta, 1971) and an increase in the permeability of ovarian blood vessel walls (Burr and Davis, 1951). This enhanced blood supply has long been known to be essential for a successful ovulation, since Heape (1905) demonstrated that reduction of the ovarian blood supply inhibits ovulation in

the rabbit. The increased blood supply is believed responsible, at least in part, for the ovarian edema and follicular swelling observed as ovulation nears.

In spite of the large increases in follicular volume as ovulation nears (Boling, 1941; Blandau, 1966; Parr, 1975), there are little or no significant increases in intrafollicular pressure in most species due to the increased distensibility of the walls (Rondell, 1970; Parr, 1975). Rondell (1964) has suggested that the increased follicular volume may "distend a weakened follicle wall to its breaking point"; however, there is some doubt concerning the interpretation which can be made from the methods used.

D. Concluding remarks

Evidence indicates that *in vivo* the events and processes which may lead to follicular rupture and subsequent ovum extrusion are numerous. On this basis, it seem unlikely that any single event could be solely responsible for ovulation. The ovulatory process probably involves (1) release of an ovulatory stimulus (most likely LH), (2) subsequent release of LH mediators at the local ovarian level, (3) proteolytic digestion of the follicular wall, (4) contractions of the follicle wall, and (5) a rapid increase in follicular volume caused, in part, by local vascular changes. The substance(s) which mediate

the effects of LH on the ovary remain poorly understood. However, there is increasing evidence that prostaglandins are involved.

3. Prostaglandins as mediators of LH-induced ovulation

In 1971 Vane made the discovery that prostaglandins are intrinsically involved in the inflammatory response. The inflammatory response is similar in many respects to ovulation (review: Espey, 1980). On the basis of Vane's 1971 study, Tsafriri et al., (1972) administered prostaglandin E_2 to rats in which the LH surge had been blocked by nembutal; ovulation was induced.

There is now considerable evidence suggesting that prostaglandins act as mediators of some of the effects of LH (Clark et al., 1978). The majority of the research has been done on the rabbit and is outlined below.

(1) Levels of prostaglandins of the E and F series increase markedly in the rabbit Graafian follicle as ovulation approaches (Yang et al., 1973; LeMaire and Marsh, 1975). Bauminger and Linder, (1975) found a similar increase in the rat ovary; the prostaglandin concentration had declined to baseline levels the morning after ovulation. Also, the concentrations of prostaglandins of the E and F series increase strikingly in both the rabbit and the rat Graafian follicle in response to administrations of

ovulatory doses of ganadotropins (rabbit: LeMaire et al., 1973; Yang et al., 1973; Bowring, 1975; rat: Bauminger and Lindner, 1975). To be more specific, Challis et al., (1974) cultured rabbit granulosa cells and found that they produce large quantities of prostaglandin F in response to HCG (human chorionic gonadotropin, an LH substitute).

(2) Inhibition of prostaglandin synthesis by the systemic or local administration of indomethacin blocks ovulation in the rat and rabbit (rat: Tsafriri et al., 1973; Osman and Dullaart, 1976; rabbit: O'Grady et al., 1972). This inhibition is thought to be exerted at the ovarian rather than the hypothalamic level for two reasons: First, indomethacin does not block the ovulatory surge of LH (Tsafriri et al., 1973; Osman and Dullaart, 1976). However, it has been noted that in rats, the infusion of E-type prostaglandins into the third ventricle of the brain, or into specific areas of the hypothalamus, caused a marked increase in the release of either LH-RH or LH into the plasma (Harms, 1973; Eskay, 1975; Ojeda et al., 1977). Second, histological examination of follicles from indomethacin treated animals display well developed follicles. However, the ova had either not been shed, or they had been extruded into the ovarian stroma (O'Grady et al., 1972; Tsafriri et al., 1973; Osman and Dullaart, 1976).

How prostaglandins contribute to the rupture of the Graafian follicle is not known. However, it has been

observed that F-type prostaglandins are capable of stimulating ovarian contractions (Virutamasen et al., 1972b; Diaz-Infante Jr. et al., 1974). Prostaglandins can also effect capillary permeability and vasodilation in several tissues (see Zurier, 1974), and indomethacin can reduce prostaglandin-induced increases in ovarian blood flow (Lee and Novy, 1978). Therefore, it is possible that prostaglandins might effect these processes in the ovary.

THE KININ SYSTEM AND OVULATION IN MAMMALS

Several lines of evidence suggest that the kinin system may be involved in the process of ovulation. In 1969, Ramwell et al., indicated the presence of a substance, with the same pharmacological properties as bradykinin, in human, rabbit and bovine follicular fluids. However, these workers hypothesized that this substance might initiate contractions of the oviduct after ovulation, rather than in the actual ovulatory process itself. Later, marked declines in the plasma kininogen levels, around the anticipated time of ovulation, were observed in several species, in two separate studies (humans and rats: McDonald and Perks, 1976; goats: Prasad et al., 1975). Also, several similarities exist between the known actions of the kinins and known events of the ovulatory process. For instance, the kinins are potent in their ability to contract smooth muscle, an

event associated with ovulation. Also, the vascular changes and resultant edema mediated, in part, by bradykinin during inflammation, are comparable to the changes observed in the ovary, as ovulation nears. Even further, the kinins, or other members of the kinin system, can increase the production of several substances believed to be important to ovulation, for example: prostaglandins (Nasjelletti and Malik, 1979). histamine (Becker et al., 1968), plasminogen activator (Spragg, 1974, Movat, 1979), and perhaps even collagenase (Eeckhout and Vaes, 1977) (review: Espey, 1980).

Considering the evidence cumulatively, the suggestion that the kinin system plays an important part in the ovulatory process, was a reasonable possibility.

STATEMENT OF THE PROBLEM

Although the evidence outlined above suggested that a link exists between the kinin system and ovulation, the evidence was scant, and required additional support. Therefore, the present investigation was undertaken to establish with greater certainty that the kinin system and ovulation are interrelated.

To determine whether any substance has a particular mediator function within a physiological process, certain criteria have to be met. Some of the more important criteria include: 1) evidence for the release of the substance near the time of the event (ovulation in this case), or in response to known stimuli (ovulatory stimuli), 2) the presence of the substance or its forming enzyme at its target site (the ovary), 3) the production of the response (ovulation), or some of its events by the proposed mediator, and 4) evidence that specific mediator antagonists can block the physiological response.

With the above criteria in mind, the present investigation was designed to examine four main questions. (1) When, in relation to ovulation, are kinins released? It is usually not possible to detect free kinins in plasma because of their extremely short half-life *in vivo* (Brocklehurst and Zeitlin, 1967; Lewis, 1970). For this reason changes in kininogen levels, and sometimes kinin-

forming enzyme levels were used to indicate kinin formation.
(2) Does the ovary, the proposed target site, possess kinin-forming enzymes?
(3) Can an ovulatory stimulus activate the kinin system?
(4) Is bradykinin capable of initiating some of the events of ovulation, namely ovarian smooth muscle contraction and increases in blood supply to ovarian follicular tissue?

No attempts were made to block ovulation with a kinin antagonist, because little is known of any effective agents at this time. This experiment, was left for future investigation.

GENERAL METHODS

This work involved several general procedures which are detailed below.

1. Siliconing procedure

Contact with glass surfaces decreases kinin activity (probably due to adsorption to negative sites on the glass) and also activates unwanted enzymes (Margolis and Bishop, 1963; Cochrane and Griffin, 1979). Pretreating glass surfaces with silicone prevents these phenomena. Therefore, all glassware was washed, immersed in a 1% solution of Prosil 28 (PCR Research Chemicals Inc., U.S.A.) or Siliclad (Clay-Adams, U.S.A.) for 10 seconds, rinsed in distilled water, and dried for 20 minutes at 100°C.

2. Experimental animals and their treatment

The animals used in this study included rats, guinea pigs, and humans. No animals were induced to ovulate with exogenous hormones but were allowed to ovulate naturally.

A. Rats

The rats were mature females (200-300 g), of an inbred departmental stock (Wistar strain). They were allowed food and water ad libitum, and exposed to light between 06.00 and 18.00 h. Their estrus cycles were followed by

daily vaginal smears (Zarrow et al., 1964; Turner and Bagnara, 1971). Only animals displaying at least three consecutive four-day cycles were used. Ovulation times were estimated from the findings of Everett, 1948; McCormack and Bennin, 1970; McCormack, 1978; McCormack and Sridarin, 1978; Stoklosowa and Szoltys, 1978; Ovulation presumably occurred between 01.00 and 05.00 h on the morning of estrus.

B. Guinea pigs

The guinea pigs were adults, both male and female, of an inbred departmental stock, weighing approximately 500-600 g. They were kept on a 14 hours light: 10 hours dark regime (lights on between 06.00 and 20.00 h). The phases of the estrus cycle in females were followed by daily vaginal smears (Stockard and Papanicolaou, 1917). Only females displaying at least two consecutive, regular 16-day cycles (± 2 days) were used. The ovulation times were estimated from the data of Stockard and Papanicolaou (1917) and Donovon and Lockhart, 1972. Ovulation presumably occurred at the end of the short estrus period, between 24.00 and 12.00 h.

C. Humans

Only healthy, female, human volunteers, ranging in age between 18 and 52 years, were used in this study. The ovulation times were estimated from graphs displaying

the fall and rise in basal body temperature, close to ovulation. Ovulation presumably occurred 24 to 48 hours before the first clear rise in body temperature (Billings et al., 1972; Zuspan and Zuspan, 1979).

3. Estimation of plasma kininogen levels

The method of Brocklehurst and Zeitlin (1967), outlined below, was used to determine the plasma kininogen content.

A. Withdrawal of blood

At the appropriate time, rats and guinea pigs were lightly anaesthetized with ether, and 0.6-0.8 ml of arterial blood were collected by cardiac puncture from the left side of the heart. With human subjects, approximately 0.8 ml of blood were removed from a superficial vein in the forearm, with all neccessary sterile procedures. The blood was withdrawn, without anticoagulant, in a sterile, polypropylene, disposable syringe (Becton-Dickinson Co., U.S.A.).

B. Treatment of the blood samples

With the shortest possible delay, 0.5 ml of the whole blood were inactivated by forcibly ejecting them into 5.0 ml of chilled 95% ethanol, contained in a 50 ml, polyethylene centrifuge tube. Each sample was prepared immediately, or covered with parafilm and stored at -15°C for up to 72 hours;

preliminary experiments showed that this storage did not effect the results. Haematocrit estimations were made on the remaining blood in order to express the results as activity per ml of plasma.

C. Preparation of the samples

Centrifuging the blood/ethanol mixture (2000xg for 30 minutes, at 4°C) separated the kininogen in the precipitate from the free kinin in the supernatant. The precipitate was then suspended in 5.0 ml of 80% ethanol and recentrifuged as before.

To ensure complete denaturation, the precipitate was resuspended in 5.0 ml of 80% ethanol and placed in a boiling water bath for 10 minutes. The mixture was then centrifuged for a third time (4,000xg for 30 minutes, at 4°C) and the supernatant was discarded.

The precipitate was washed twice with 5.0 ml aliquots of distilled water, suspended in 1.5 ml of 2.5 M NaCl, and homogenized in a ground glass blender (Cole Palmer, U.S.A.). 0.2 ml aliquots of the homogenate were incubated at 37°C for 30 minutes with 5.0 ml of a mixture composed of 1.0 mg pure trypsin (two times crystallized, Sigma Chemical Co., U.S.A.), 10.0 ml sodium phosphate buffer (pH 7.35) (see below), and 40.0 ml of distilled water.

The trypsin was then inactivated by heating the incubate in a boiling water bath for 10 minutes. The

samples were stored at -15°C until bioassay (method to follow). All values for bradykininogen are expressed in terms of maximum bradykinin liberated from it by trypsin, the values are given as µg bradykinin-equivalent per ml plasma (µg Bk-equiv./ml plasma).

4. Assessment of kinin activity

A. The rat uterus bioassay

The kinin content of various samples was measured with the rat uterus bioassay, as perfected by Munsick (1960). This bioassay remains the most sensitive and accurate, although sensitive techniques for radioimmunoassay are currently being developed (Odya et al., 1978). This simple assay can measure a concentration of bradykinin as low as 50 pg/ml.

Female virgin rats (Wistar strain) weighing 180-250 g, and in the sensitive proestrus and estrus states (determined by vaginal smear) were used.

The animals were killed rapidly by cervical dislocation. The uterus was exposed by a sagittal incision in the abdominal wall, rapidly dissected free of mensentery and fatty tissue, and placed in a warm (31°C) buffered saline (Munsicks' modification of Van Dyke-Hastings solution, without magnesium present [Munsick, 1960]), with the following composition:

Van Dyke - Hastings Solution

Chemical	(g/l)	Chemical	(ml/l)
NaCl	6.704	$CaCl_2$ (1 M)	0.50
KCl	0.459	*Phosphate buffer	10.0
$NaHCO_3$	2.590		
Glucose	0.50		
Sodium Phenol-sulfonephthalein	0.054		

The ingredients were aerated with 95% O_2 and 5% CO_2 until a pH of 7.4 was reached, as estimated visually by the orange color of the phenol red.

*Phosphate buffer:

Two solutions were prepared as follows:

a) 22.714 g of Na_2HPO_4 were added to 1.0 liter of distilled water.

b) 6.349 g of NaH_2PO_4 (hydrated form) were added to 1.0 liter of distilled water.

The solutions were titrated together until a final pH of 7.4 was maintained.

A single uterine horn was placed in a 5 ml organ bath containing the Van Dyke-Hastings solution with 95% O_2 and 5% CO_2 bubbling through to maintain the pH at 7.4. The organ bath was connected to a two litre reservoir, also aerated with 95% O_2 and 5% CO_2. Both organ bath and reservoir were in a water bath regulated to $\pm$ 0.1°C, and

the assay temperature set between 30-33°C.

The uterine horn was anchored at its posterior end by a glass muscle hook at the bottom of the organ bath, and the ovary was connected to an isotonic muscle transducer (Harvard Tranducers: Harvard, U.S.A.) by silk surgical thread under a tension of 1-2g. The transducer was connected via an amplifier (Harvard, U.S.A.) to a chart recorder (Harvard, U.S.A.).

After the preparation had equilibrated for at least 30 minutes, the solutions to be assayed were added directly to the organ bath with Hamilton glass syringes, and the resulting contractions recorded on the chart recorder. After each response had reached a maximum, the bath was flushed with an excess of buffered saline from the reservoir, and the tissue was left to recover for 5 minutes.

B. Calculations

Estimation of activity was based on four, "four-point" assay groups, each consisting of matched responses to high and low doses of standard and unknown, according to the method of Holten (1948). The standard used in all assays was synthetic bradykinin (BRS 640, Sandoz Co., Switzerland, or later, Bradykinin Triacetate, Sigma Chemical Co., U.S.A.) stored at 0.1 mg/ml and diluted to 50 ng/ml distilled water as needed. The second standard was assayed and checked against the first. Responses were measured as the

contraction height elicited in mm.

The biological activity of each sample was calculated according to the following formula (Holten, 1948):

$$\text{Activity (ng/ml)} = \frac{(S)\ (R)}{\text{volume of U}}$$

where S = the amount of bradykinin on the high dose of standard

R = antilog of M

U = high dose of unknown

M is calculated by the formula:

$$M = \frac{(A+D) - (B+C)}{C+D - (A+B)} (\log c - \log b)$$

where A = the sum of responses to the low dose of standard

B = the sum of responses to the low dose of unknown

C = the sum of responses to the high dose of standard

D = the sum of responses to the high dose of unknown

c = high dose of standard

b = low dose of standard

Confidence limits for the assays were calculated at the 95% level by the method of Holten (1948), and checked by computer.

In the majority of cases in this study the values recorded are averages from groups of samples ± S.E.M. Occasionally only one value was recorded and this is given with the 95% confidence limit of its assay. The results throughout were expressed as µg or ng bradykinin equivalent (Bk-equiv.) per ml plasma or per g tissue (wet weight).

SECTION I

THE CONCENTRATION OF PLASMA AND OVARIAN KININ-FORMING ENZYMES AND/OR OF PLASMA KININOGEN BEFORE AND AFTER OVULATION IN RATS, GUINEA PIGS, AND HUMANS

INTRODUCTION:

McDonald and Perks (1976) noted a marked fall in plasma kininogen around the time of ovulation in rats with five-day estrus cycles, and in the human. Independently, the records of Prasad et al., (1975) showed a similar change in the goat. These findings suggested that the kinin system might be involved in ovulation. However, the work on the rat and human did not show whether the peptide was likely to be concerned in the initiation of ovulation, or in changes which followed from it, because the time course of events was not clear.

The following investigation was undertaken for three reasons:

(1) First, to corroborate the findings of a temporal link between the fall in plasma kininogen levels and ovulation in rats and humans, and to determine whether the fall preceded or followed ovulation.

A detailed analysis of the kininogen levels of rats with four-day estrus cycles through the hours of proestrus and the night of ovulation was performed for two reasons.

First, early work on rats with four-day estrus cycles had failed to record any changes in kininogen throughout the cycle (McCormick and Senior, 1974; Senior and Whalley, 1976). Second, rats were most suitable for further study because the characteristics of their cycle, in particular the timing of ovulation and of the LH surge (Butcher et al., 1974; Fink, 1976), had been well documented.

In women, the timing of ovulation was assessed more carefully than in earlier studies, by use of the basal body temperature method. Also, women using oral contraceptives and postmenopausal women were included as non-ovulating controls.

(2) The second purpose was to provide further evidence for a temporal link by determining whether similar changes in plasma kininogen levels occured in another species (the guinea pig), with an estrus cycle of an entirely different length. Male guinea pigs were included as controls.

(3) The last function was to determine whether other members of the kinin system, namely kinin-forming enzymes, changed over the critical periods of ovulation in the rat. Attempts were made to detect kinin-forming enzymes in the two locations most likely to be involved in kinin release during ovulation, that is the plasma and the proposed target site - the ovary.

MATERIALS and METHODS

1. Experimental animals

A. Rats

Seventy-four mature female rats, maintained as described previously (see General Methods), were divided according to their state of estrus into the following eight groups: 18.00 h diestrus; 12.00, 15.00, 18.00, 21.00 and 24.00 proestrus; 10.00 h estrus; and 18.00 h metestrus. Each animal was sampled only once.

B. Guinea pigs

Eleven adult female guinea pigs were maintained as described previously (see General Methods). One animal was sampled through five cycles, eight animals through three cycles, and two animals through two cycles (total: 33 cycles).

Seven control male guinea pigs were maintained under the same conditions and sampled at the same time as the females. The males had to be dealt with in two groups. Group 1 consisted of two animals from the same stock as the cycling females. Group 2, which consisted of five males, was added later in the experiment, and was of a different stock; for this reason it was dealt with separately.

C. Humans

(a) Women with normal cycles:

This group consisted of eight subjects, 18-35 years of age, with known and regular menstrual cycles. In six subjects, blood samples were taken over two consecutive cycles, and in two subjects over three consecutive cycles (total: 18 menstrual cycles).

(b) Women on oral contraceptive treatment:

Blood samples were obtained from six subjects (18-35 years of age), who took the combined oral contraceptive, norethindrone and mestranol (Orthonovum; Ortho Pharmaceuticals Canada Ltd., Don Mills, Ont.). The daily dose was: norethindrone/mestranol, 1.0/0.05 mg (three subjects); 1.0/0.08 mg (two subjects); 2.0/0.1 mg (one subject). Menstrual flows (four-six days) were induced at the usual times by withdrawal of medication for seven days. Four subjects were followed over two consecutive 'cycles' and two subjects over three consecutive 'cycles' (total: 14 controlled 'cycles').

(c) Postmenopausal women:

Blood samples were taken from two subjects in the early postmenopausal period, when it was possible to estimate the potential time of ovulation from the timing of the last cycle. The subjects were 46 and 52 years of age and had lacked cycles for 18 and 24 months, respectively, before being accepted as postmenopausal for the purposes of this

study. In both subjects, blood samples extended over a three month period (equivalent in total to six potential cycles).

2. Notation of the cycles

Variations in different cycles were coordinated by placing the timing on a proportional basis, with ovulation fixed at 0.5, and the whole cycle designated as 1.0. A similar notation was used for, (1) male quinea pigs, who were assigned an "ovulation" time in accordance with one of the female guinea pigs sampled at the same time, (2) human subjects on oral contraceptives, where the limits of 0.0 and 1.0 were set by the menstrual flows produced by the withdrawal of medication (as based on the timing of untreated cycles), and (3) early postmenopausal subjects, where the absolute times were projected from the last cycle.

3. Collection of tissue

A. Rats

At the appropriate time, each animal was lightly anaesthetized with ether, and two blood samples were rapidly withdrawn from the left side of the heart by cardiac puncture. The first sample consisted of approximately 0.8 ml of blood. 0.5 ml was used for estimating kininogen levels and the residual blood was used for determining the haematocrit (see General Methods section). The second

sample of 2.0 ml blood was withdrawn into a chilled, citrated, polypropylene syringe and used for estimating kinin-forming enzyme levels (see below).

The abdominal cavity was exposed by a sagittal incision in the abdominal wall and the rats were rapidly exsanguinated through the inferior aorta. Finally, both ovaries were removed, dissected free of fat, cleared of blood with 0.32 M sucrose solution, and prepared for estimating the ovarian kinin-forming enzyme levels (see below).

B. Guinea pigs

With each animal, blood samples were taken at intervals of ten days, or more, and between 11.00 and 13.00 h. Each guinea pig was lightly anaesthetized with ether and approximately 0.8 ml of arterial blood was collected from the left side of the heart by cardiac puncture. As before, 0.5 ml were treated for plasma kininogen estimation and the remainder used for determining the haematocrit (see General Methods).

C. Humans

With each subject, blood samples were taken once each week, between 11.00 and 13.00 h. Approximately 0.8 ml of blood were removed from a superficial vein in the forearm, with all necessary sterile procedures. As with the other species, 0.5 ml were treated for estimating kininogen

concentrations and the residual blood was used for determining the haematocrit (see General Methods section).

4. Estimation of plasma kininogen levels

The 0.5 ml of blood obtained from each of the species were immediately and forcefully ejected into 5.0 ml chilled 95% ethanol. This mixture was centrifuged, and the precipitate estimated for kininogen as described by Brocklehurst and Zeitlin (1967). Essentially, the method consisted of incubating the precipitate with an excess of trypsin, which converted kininogen to free kinin. The free kinin was then assayed using the rat uterus bioassay (see General Methods section for details of the incubation and assay procedures). Final kininogen levels were expressed in terms of the maximum kinin liberated from it by trypsin per milliliter of plasma (µg Bk-equiv./ml plasma).

5. Estimation of plasma kinin-forming enzyme levels

The 2.0 ml of blood obtained from the rats by cardiac puncture were immediately ejected into a chilled solution of 1.0 ml 0.32 M sucrose and 0.02 ml of 38% sodium citrate, contained in a 50 ml polypropylene centrifuge tube. The plasma-sucrose supernatant obtained after centrifuging the mixture (2000xg,4°C, for 30 minutes) was treated for kinin-forming enzyme estimation by a modification of the method of Kobayashi et al., (1979). The mixture was adjusted to pH

2.0 with 1 N HCl and incubated at 37°C for 20 minutes; this both activated plasma kinin-forming enzyme and abolished kininase activity.

After neutralizing the incubate to pH 7.0 with 1N NaOH and centrifuging it (700xg, 4°C, for 10 minutes) 1.0 ml of the supernatant was taken for enzyme determination; this involved incubating the supernatant with kininogen substrate (see below), at pH 7.8 (0.2 M tris buffer) for 30 minutes, at 37°C according to Carvalho and Diniz (1966). The reaction was terminated by boiling for 10 minutes and the mixture centrifuged (10,000xg, 4°C, for 30 minutes). In addition, 1.0 ml of the same preparation was incubated without kininogen substrate in order to estimate any detectable background activity, which was subtracted from the final measurement. Before incubating either preparation, 1.0 ml of the kininase inhibitor 8-hydroxquinoline sulphate (2.5 mg/ml in 0.9% saline; Matheson, Coleman, and Bill U.S.A.) was added to both; preliminary studies had shown some kinin loss during incubation without the inhibitor. The samples were stored at - 15°C before assay on the rat uterus bioassay preparation (see General Methods section). Enzyme activities were expressed as nanograms bradykinin-equivalent per milliliter original plasma, per minute of incubation (ng Bk-equiv./ml plasma·min.).

The rat kininogen substrate used above was prepared by the method of Marin-Grez and Carretero (1972), with minor

modifications; the process was repeated every three weeks, since the activity of the product declined after that time. Large male rats were anaesthetized with ether and the abdominal cavity was exposed by a sagittal incision through the abdominal wall. Blood was obtained rapidly from the inferior aorta with a citrated polypropylene syringe. The blood was ejected into a chilled 50 ml polyethylene centrifuge tube containing one volume 38% sodium citrate/99 volumes blood. After centrifuging the mixture (2000xg, 20 min., 4°C) the plasma was incubated at 58-60°C for three hours, to inactivate the kininases, inhibitors, and enzyme precursors. After recentrifugation (2000xg, 20 min., 4°C) the supernatant was fractionated with ammonium sulphate, and the precipitate formed between molarities of 0.97 and 1.87 was dissolved in a minimum of distilled water, and dialyzed overnight according to Zeitlin et al (1976). The kininogen preparation which resulted was flash evaporated at 40°C, and stored in powder form at room temperature; its activity was checked by incubating a small portion of it with excess trypsin, followed by bioassay (see General Methods section), and only preparations yielding 50-70 ng Bk-equiv./mg were used.

6. Estimation of ovarian kinin-forming enzyme levels

The ovaries were weighed, homogenized into cold 0.32 M sucrose at 1.0 g wet tissue per 10.0 ml, and estimated for

kinin-forming enzymes by the same method used for the plasma (after Kobayashi et al., 1979). Enzyme activities were expressed as nanograms bradykinin-equivalent produced per gram wet tissue per minute (ng Bk-equiv./g wet tissue•min).

7. Statistics

All values recorded are averages from groups of animals (± S.E.M.); the numbers in each group are indicated on the graphs. The significance of differences between groups was determined by Student's t-test for two independent samples (Steel and Torrie, 1960).

RESULTS

1. Plasma kininogen levels before and after ovulation

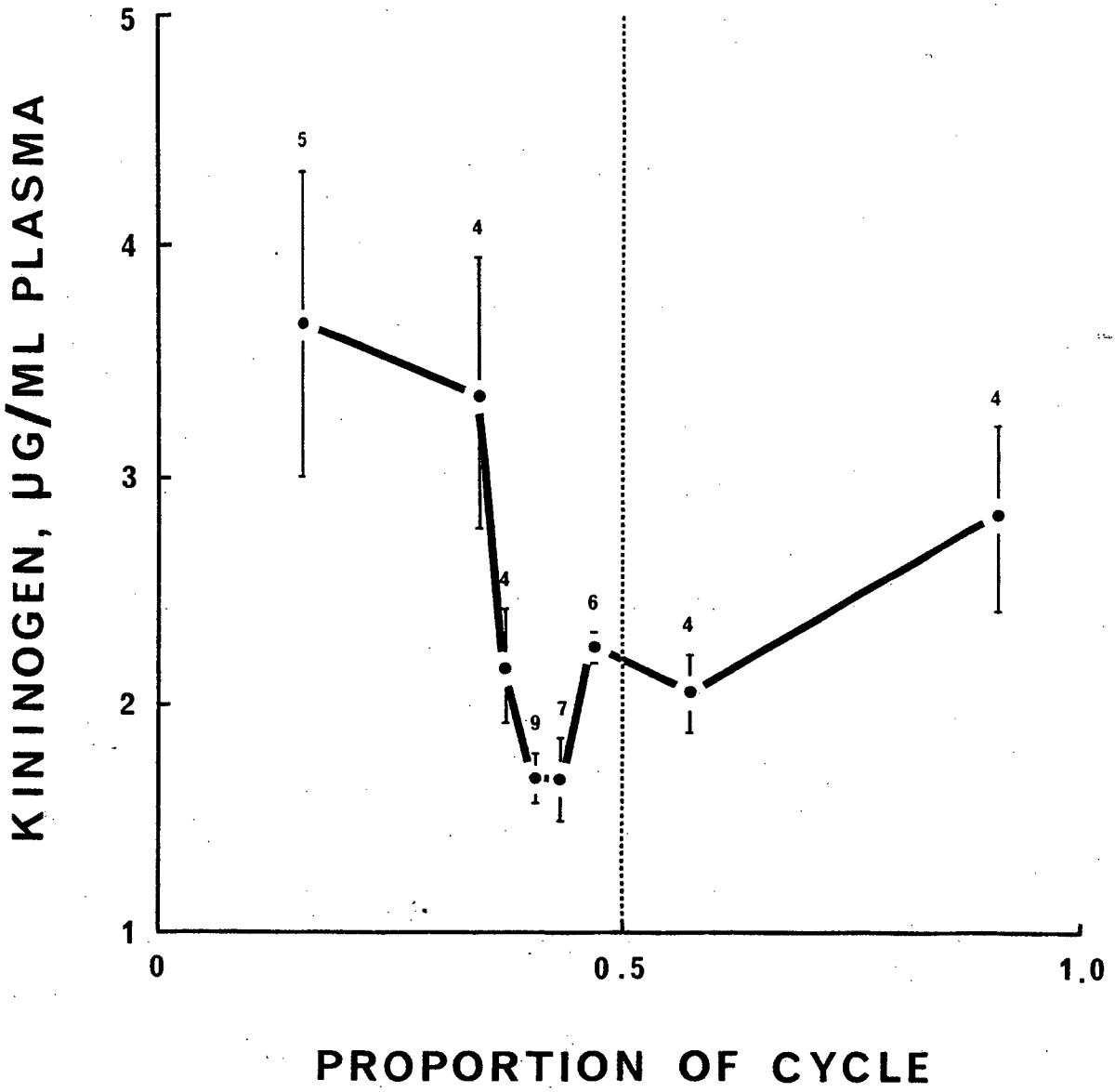
A. Studies in the rat

Data from 44 rats with the short four-day cycle showed, as in the longer cycles, a pronounced fall in plasma kininogen concentrations just before ovulation (Figure 3). The fall, which reached a maximum of 51%, was significant at the $P < 0.01$ level (decline: 3.36 ± 0.59 to 1.65 ± 0.01 µg Bk-equiv./ml plasma). The effect started early in proestrus, between 12.00 and 15.00 h, and reached its maximum at 18.00 h in proestrus. The values remained depressed throughout the period of ovulation, after which they recovered steadily through the rest of the cycle (Figure 3). The period of marked decline corresponded well with the LH surge, as reported for rats with the same four-day cycle by Butcher *et al.*, (1974). The initial decline did not correspond with ovulation itself (range: 01.00 - 05.00 h, in estrus - Everett, 1948; McCormack and Bennin, 1970; McCormack and Sridaran, 1978; McCormack, 1978; Stoklosowa and Szoltys, 1978).

The 51% fall in kininogen levels closely approximated the 59% fall observed earlier by McDonald and Perks (1976). Also, these findings extended the previous results by clearly indicating that the change had begun before ovulation.

FIGURE 3: PLASMA KININOGEN LEVELS DURING THE RAT FOUR-DAY ESTRUS CYCLE

Mean concentrations of plasma kininogen from eight groups of rats at different stages of the four-day estrus cycle. Ordinate: Mean plasma kininogen concentrations (µg-Bk-equiv./ml plasma). Vertical bars represent standard errors of the means and numbers above represent the number of animals in each group. Abscissa: Time expressed as proportion of the cycle (the length of the cycle = 1.0). The midpoint of the time range for the anticipated time of ovulation is fixed at 0.5 and is indicated by vertical broken lines.


KININOGEN, µG/ML PLASMA
5
4
3
2
1
0
0.5
1.0
PROPORTION OF CYCLE
5
4
4
9
7
6
4
4

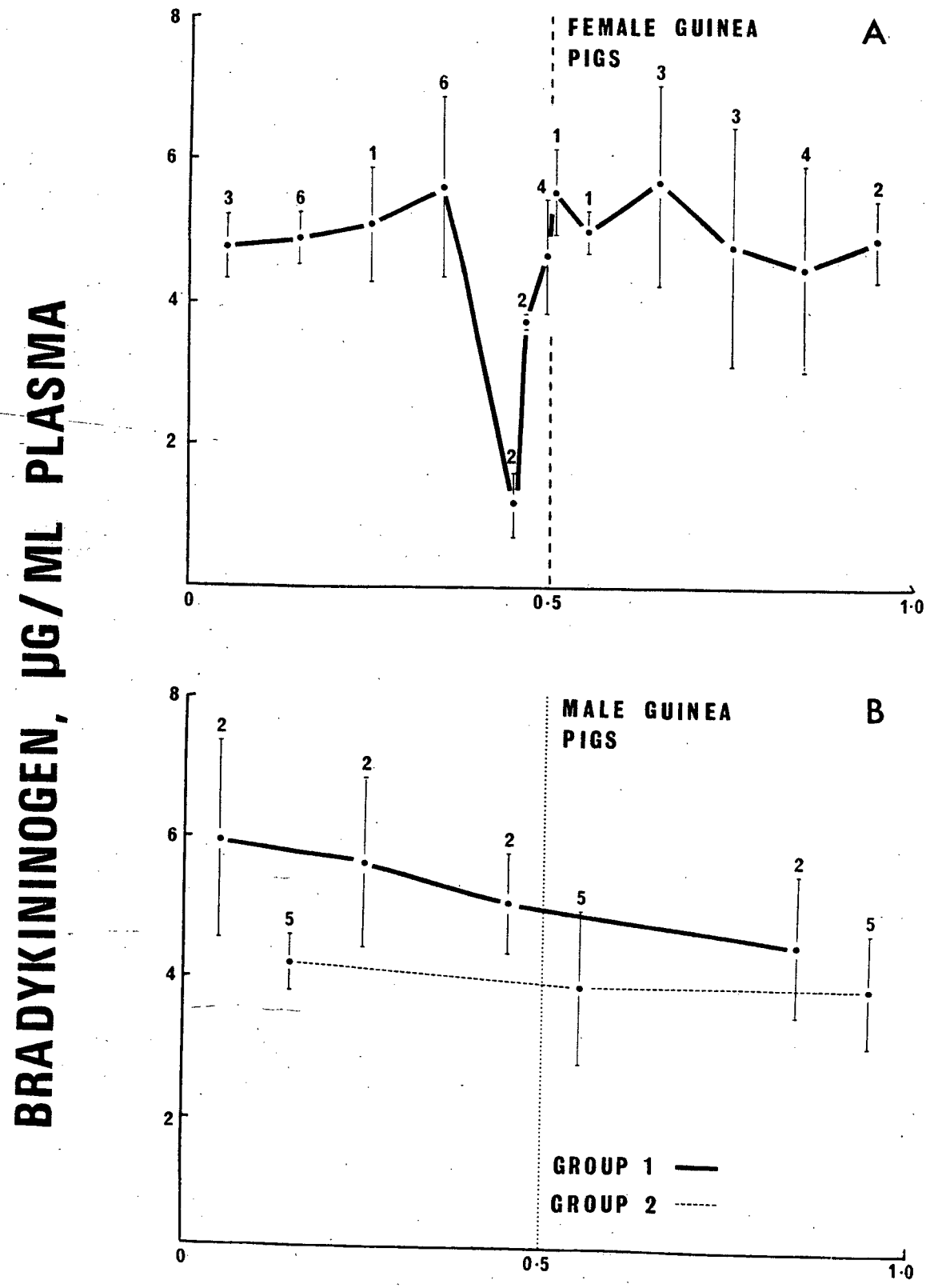
B. Studies in the guinea pig

(a) Plasma kininogen levels in guinea pigs with normal estrus cycles:

Studies of the guinea pig allowed the collection of a number of blood samples within the immediate preovulatory period. In addition, the length of the cycle was long compared with the short period of estrus (14 - 17 days, as compared with 2 - 4 hours), and it seemed unlikely that a relationship between kininogen and ovulation would occur again, if it had been purely accidental in the previous species studied. Figure 4A shows the average values for kininogen assays of 41 blood samples, taken over 33 cycles, from 11 adult female guinea pigs. The kininogen levels changed little over most of the cycle. The average value, excluding the three points in the immediate preovulatory period, was 5.1 $\pm$ 0.3 µg Bk-equiv./ml plasma. However, a 79% fall, from 5.6 $\pm$ 1.2 to 1.2 $\pm$ 0.4 µg Bk-equiv./ml plasma occured 24 hours before the anticipated time of ovulation. The estimated limits of the timing were 18-30 hours. The fall was significant at the $P < 0.05$ level, if the pre-ovulatory values were compared with those outside the immediate preovulatory period (Student's t-test for two independent samples). After the fall in kininogen concentration, the levels recovered slowly to almost normal values by the time of ovulation itself.

FIGURE 4: PLASMA KININOGEN LEVELS DURING THE FEMALE GUINEA PIG ESTRUS CYCLE AND IN MALE CONTROLS

Mean plasma kininogen levels were estimated in groups of guinea pigs at different stages of the estrus cycle and in male controls. (A) Female guinea pigs (based on 33 cycles from 11 animals). (B) Male guinea pig controls (Group 1: data collected from 2 male guinea pigs over the same time period as the females in (A); Group 2: data collected from 5 male guinea pigs of a different stock from the females). Ordinates = plasma kininogen, µg Bk equiv./ml plasma. Mean values are given with vertical bars which represent standard errors of the means; the values above indicate the number of animals in each group. For single observations, the bars give the confidence limits of the assay at the $P = 0.05$ level. Abscissae = time expressed as a proportion of the cycle (the length of the cycle = 1.0). The midpoint of the time range for the anticipated time of ovulation is fixed at 0.5 and is indicated by a vertical line; a broken line represents normal ovulation; a dotted line indicates the equivalent time in the males.


FEMALE GUINEA PIGS
A
MALE GUINEA PIGS
B
GROUP 1
GROUP 2
8
6
4
2
0
0·5
1·0
BRADYKININOGEN, µG/ML PLASMA
PROPORTION OF CYCLE

(b) Plasma kininogen levels in control male guinea pigs:

Figure 4B shows the results for blood samples from two groups of control male guinea pigs. The males were sampled at the same time as the cycling females. Group 1 (two males) was of the same stock as the females. Group 2 (five males) was added later in the experiment, and was from a different stock. Neither group showed any fall in plasma kininogen levels which resembled the preovulatory fall in the females. The mean values obtained from all the control males in Figure 4B were never significantly different; the standard errors always overlapped. The overall average value of 4.7 $\pm$ 0.8 µg Bk-equiv./ml plasma, was not significantly different from the corresponding value in the females (for consistency, the average in the males omitted the value which corresponded to the time before ovulation in the females).

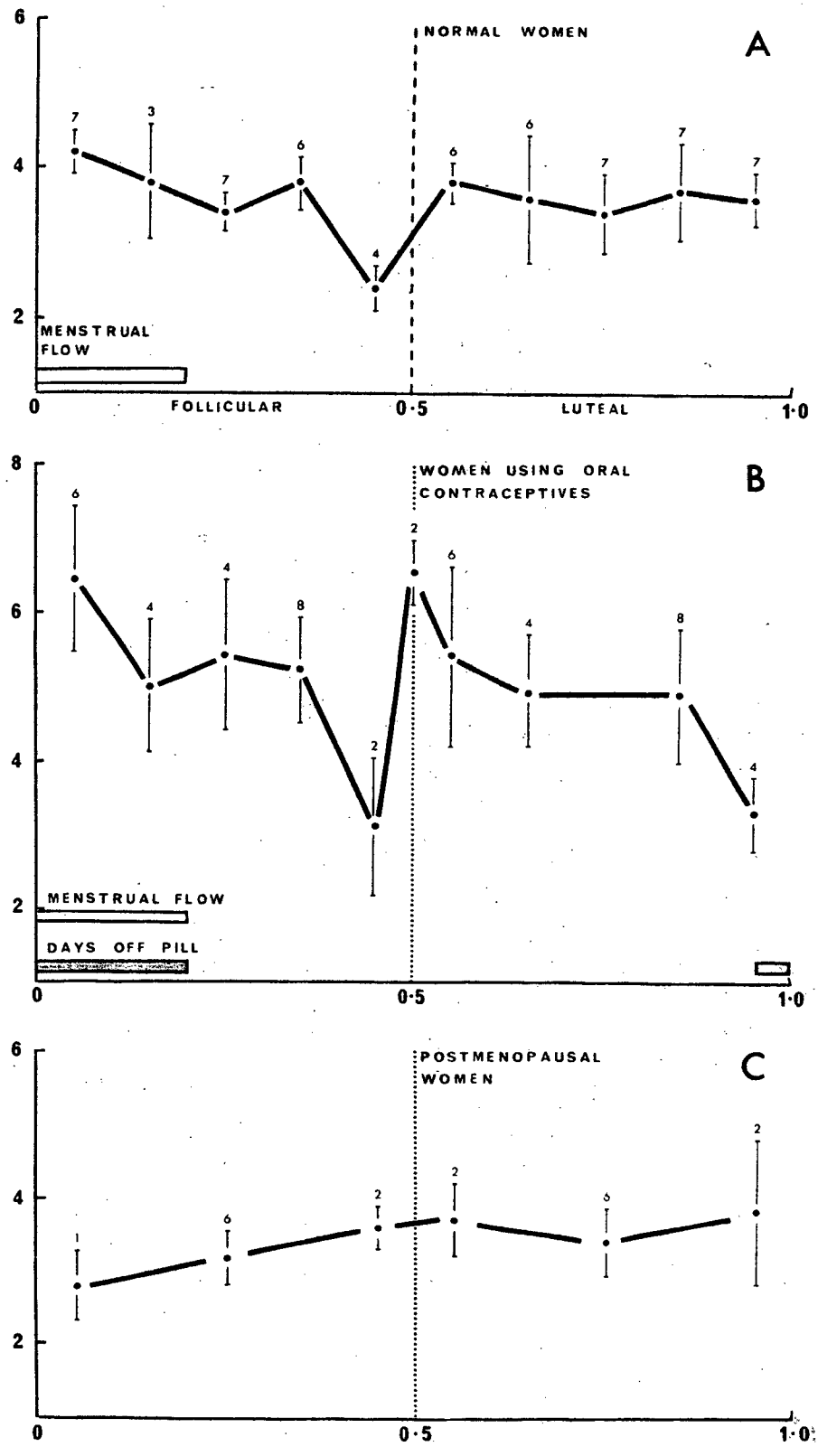
C. Studies in the human

(a) Plasma kininogen levels in women with normal menstrual cycles:

Figure 5A shows the average values for kininogen assays of 60 blood samples, taken over 18 menstrual cycles, from eight healthy women. The subjects monitored their ovulation dates by the basal body temperature method (for details, see the General Methods section). The kininogen

FIGURE 5: PLASMA KININOGEN LEVELS IN WOMEN WITH NORMAL AND MODIFIED MENSTRUAL CYCLES

Mean concentrations of the plasma kininogen levels of groups of women at different stages of the menstrual cycle. (A) Women with normal cycles (based on 18 cycles from 8 subjects). (B) Women on the oral contraceptives, norethindrone and mestranol (1.0/0.05 - 2.0/0.1 mg per day) (based on 14 controlled 'cycles' from 6 subjects). (C) Postmenopausal women (based on the equivalent of 6 cycles in 2 subjects). Ordinates = plasma bradykininogen (μg Bk-equiv./ml plasma). Mean values are given with vertical bars which represent standard errors of the means; the values above indicate the number of subjects in each group. For single observations, the bars give confidence limits of the assay at the $P = 0.05$ level. Abscissae = time, expressed as a proportion of the cycle (the length of the cycle = 1.0). The midpoint of the time range for the anticipated time of ovulation is fixed at 0.5 and is indicated by a vertical line; a broken line represents normal ovulation; a dotted line indicates a potential or calculated ovulation.


NORMAL WOMEN
A
MENSTRUAL FLOW
FOLLICULAR
LUTEAL
WOMEN USING ORAL CONTRACEPTIVES
B
MENSTRUAL FLOW
DAYS OFF PILL
POSTMENOPAUSAL WOMEN
C
BRADYKININOGEN, µG/ML PLASMA
PROPORTION OF CYCLE

levels did not vary significantly throughout most of the cycle. The average value, excluding that immediately before ovulation, was 3.7 ± 0.2 µg Bk-equiv./ml plasma. However, a 41% fall, from 3.8 ± 0.6 to 2.4 ± 0.3 µg Bk-equiv./ml plasma, occured approximately 48 hours before ovulation. The estimated limits of this timing were 36-60 hours. The fall was significant at the $P < 0.05$ level, if the depressed values were compared with those obtained during the rest of the cycle (Student's t-test for two independent samples). The 41% fall in kininogen was closely similar to the 42% fall seen earlier by McDonald and Perks (1976), but it again extended their results by demonstrating that the change had begun before ovulation.

(b) Plasma kininogen levels in women on oral contraceptives:

Because oral contraceptives will inhibit ovulation, it was important to find out whether they would prevent the preovulatory fall in plasma kininogen. Figure 5B shows the average results for kininogen assays of 48 blood samples, taken over 14 artificially regulated cycles, from six women who took the combined oral contraceptives, norethindrone and mestranol (1.0/0.05 to 2.0/0.1 mg/day, respectively). The cycles were divided by the menses, which were produced at the usual times by the withdrawal of medication; the potential time of ovulation was taken as 14 days after the onset of the previous menstrual flow.

A comparison of Figures 5A and 5B demonstrates two points. Firstly, there was an increase in the general level of plasma kininogen throughout most of the cycle, in the women on the oral contraceptives. The average value, excluding that immediately before a potential ovulation, was 5.1 $\pm$ 1.0 µg Bk-equiv./ml plasma. This value was 38% higher than the corresponding value for women with normal cycles, and this increase confirmed the earlier, but single, observation of McDonald and Perks (1976). Individual records showed no relationship between the rise in kininogen, and the dose of oral contraceptives used, within the dose-range tested. Secondly, Figure 5B shows a 39% fall in plasma kininogen, from 5.1 $\pm$ 0.8 to 3.1 $\pm$ 0.9 µg Bk-equiv./ml plasma, 48 hours before the potential time of ovulation. However, the fall was significant at the $P < 0.05$ level, when the reduced values were compared with those obtained during the rest of the 'cycle' (Student's t-test for two independent samples). However, these results must be regarded with caution, since the number of observations was lower than in other studies. Even so, the similarities in both magnitude and timing of the falls in the normal and treated subjects suggests the persistence of the fall despite oral contraceptive treatment. This finding indicates that the fall can not be a consequence of ovulation, because these women, most likely, were not ovulating. (Percival-Smith, 1982).

(c) Plasma kininogen levels in postmenopausal women:

Because the results outlined above suggested that changes in plasma kininogen persisted in subjects on oral contraceptives, it became important to examine early postmenopausal women, where ovulation had stopped from natural causes. Figure 5C shows the average results for kininogen assays of 19 blood samples, taken over 6 'potential cycles', from two early postmenopausal women. Cycles had stopped 18 and 24 months previously, and the time of potential ovulation was projected from the last complete cycle. Figure 5 shows that the general levels of kininogen were not significantly different from those of women with normal cycles: the average value, excluding that immediately before a potential ovulation (omitted for consistency), was 3.4 ± 0.3 µg Bk-equiv./ml plasma. More important, there was no evidence for any fall before the calculated time of 'ovulation'. These results must be taken with some reservation, since the number of experimental subjects was low; nevertheless, they do suggest that the preovulatory fall in plasma kininogen was lost, along with ovulation, at menopause.

2. Plasma kinin-forming enzymes before and after ovulation in the rat

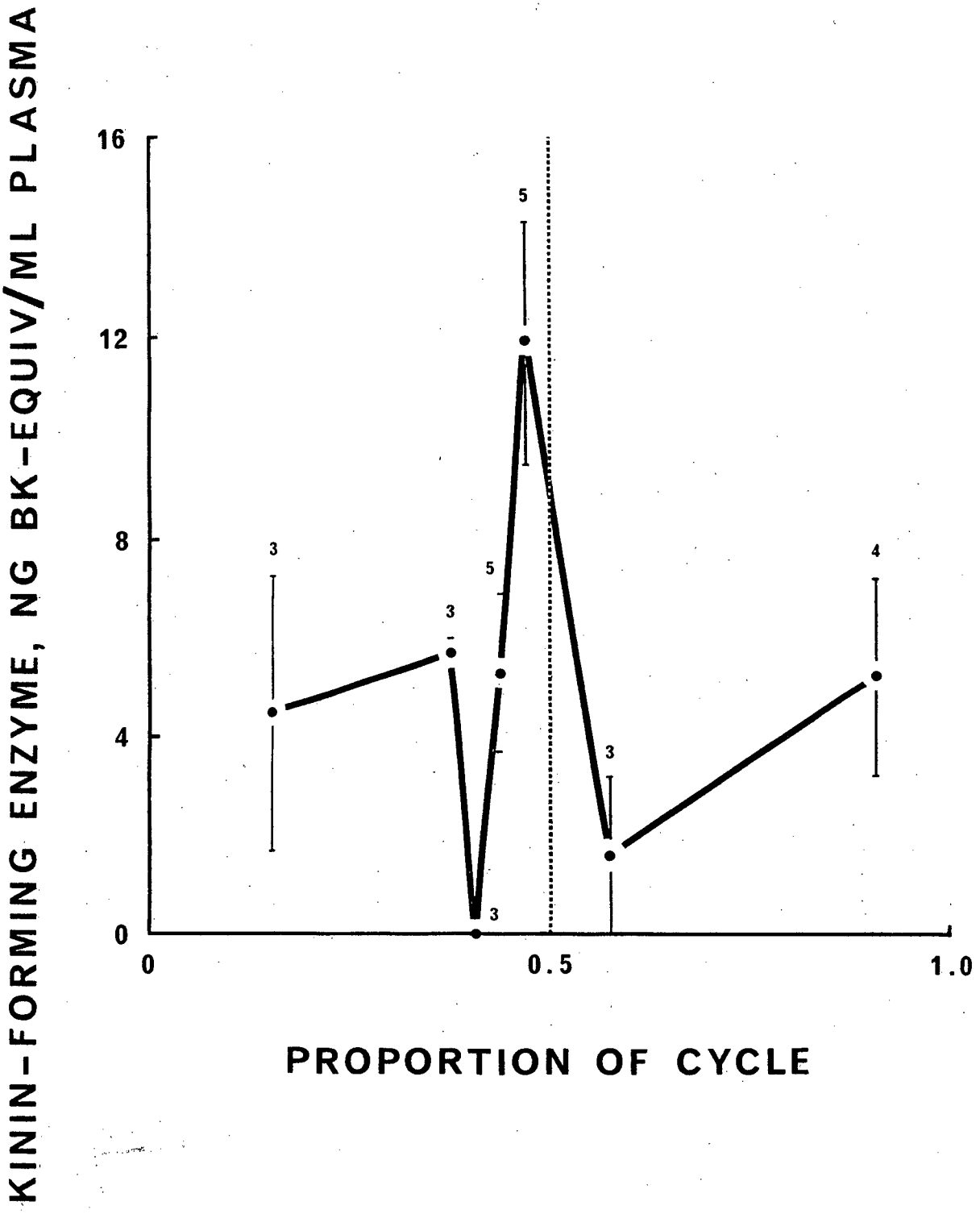
After establishing the timing of the fall in plasma kininogen levels, an attempt was made to find the enzymes

responsible for the change; the first tissue examined was the blood itself. Plasma kinin-forming enzymes circulate in the preactive form, where proteases (such as trypsin and plasmin), usually from inflamed tissue, as well as Hageman Factor (formed in blood during the clotting process), activate them (see Carretero and Scicli, 1981). However, due to their rapid destruction, the active form of the enzyme rarely attains detectable levels in the general circulation (Eisen, 1970; Pisano, 1975). Hence, it was assumed that the enzymes measured in this portion of the study were in the preactive form, and that a marked decline in precursor level would suggest enzyme activation and subsequent destruction.

Figure 6 shows the results from 26 rats. The study showed two significant declines in precursor levels. The first, a 100% fall (from 5.7 $\pm$ 0.3 to 0 ng Bk-equiv./ml plasma·min between 15.00 and 18.00 h proestrus - significant at the $P < 0.01$ level), occured just after the start of the plasma kininogen decline. The second, an 87% decline, occured during the estimated ovulatory period (from 11.0 $\pm$ 2.4 to 1.6 $\pm$1.6 ng Bk-equiv./ml plasma · min between 24.00 h proestrus and 10.00 h estrus - significant at the $P < 0.05$ level). These data suggest that plasma kinin-forming enzymes might have been activated during the preovulatory and, possibly, even the ovulatory periods.

FIGURE 6: PLASMA KININ-FORMING ENZYME LEVELS DURING THE RAT FOUR-DAY ESTRUS CYCLE

Mean concentrations of plasma-kinin-forming enzymes from groups of rats at different stages of the estrus cycle were estimated. Ordinate: Mean plasma kinin-forming enzyme concentrations (ng Bk-equiv./ml plasma). Vertical bars represent standard errors of the means and numbers above represent the number of animals in each group. Abscissae: Time, expressed as a proportion of the cycle (the length of the cycle = 1.0). The anticipated time of ovulation (midpoint of the time range) is fixed at 0.5 and is indicated by a broken vertical line.


KININ-FORMING ENZYME, NG BK-EQUIV/ML PLASMA
16
12
8
4
0
0
0.5
1.0
PROPORTION OF CYCLE
3
3
3
5
5
3
4

3. Ovarian kinin-forming enzymes before and after ovulation in the rat

The above results indicated that the kinin-forming enzymes responsible for initiating the preovulatory plasma kininogen decline must occur in some tissue other than the blood. Because kinins, as local-acting agents, are usually activated at their site of action, and because ovulation seemed to be involved, the studies were extended to the ovaries.

Tissue kinin-forming enzymes have been found in the active and inactive precursor form, depending on the tissue (Pisano, 1975; Schacter and Barton, 1979). Because ovarian tissue had never been examined for its kinin-forming capabilities, it was not known whether the enzymes, if present, would be in the active or preactive form. The assumption was made that ovarian kinin-forming enzymes would be in the active form, at least in the period just before and during ovulation, on the basis of the following circumstantial evidence: (1) kininogen levels fell as the ovarian enzyme levels rose, (2) studies have shown the presence in the ovarian follicles of several proteases possessing the ability to activate but not inactivate tissue kinin-forming enzymes (Vogel and Werle, 1970; Espey, 1974; Strickland and Beers, 1976), and (3) Ramwell (1969) has demonstrated kinin-like activity in follicular fluid. However, confirmation of this assumption awaits further

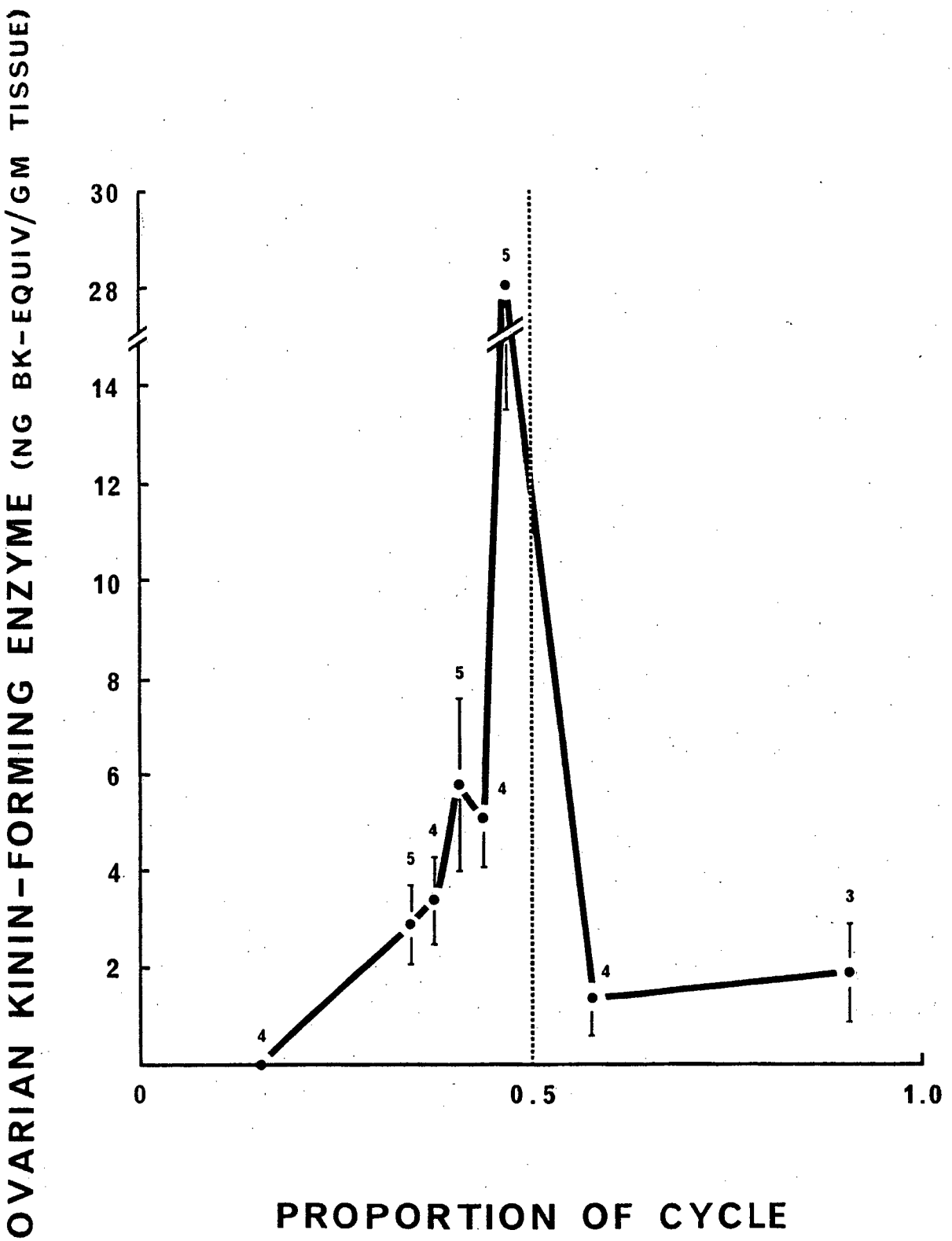
investigation.

Figure 7 shows the results of 70 ovaries from 35 rats. during the quiescent diestrus stage, no kinin-forming enzymes were detected in any ovary. However, by 12.00 h in proestrus all ovaries examined presented strong activity (average: 2.87 ± 0.83 ng Bk-equiv./g wet tissue · min). The activity continued to rise, and increased almost 10-fold by 24.00 h in proestrus (28.14 ± 14.63 ng Bk-equiv./g wet tissue · min; significantly above all levels outside proestrus at $P < 0.01$). However, activity declined rapidly over the ovulatory period, with a 95% fall between the peak in proestrus (28.14 ± 14.63 ng Bk-equiv./g wet tissue · min; significantly above all levels outside proestrus at $P < 0.01$). Some activity persisted into the luteal phase but was lost by diestrus.

Plasma left behind within the ovary could not have been responsible for the activity of the ovary because the ovaries would have had to contain twice their own volume of blood to account for the activities concerned. In addition, residual blood could have had little effect, since diestrus ovaries, similarily contaminated, were without activity. Therefore, these data not only indicate the presence of kinin-forming enzyme activity in the ovary, but also suggest that the activity increased as ovulation neared.

FIGURE 7: OVARIAN KININ-FORMING ENZYME LEVELS DURING THE RAT FOUR-DAY ESTRUS CYCLE

Mean concentrations of ovarian kinin-forming enzymes from groups of animals at different stages of the estrus cycle were estimated. Ordinate: Mean ovarian kinin-forming enzyme concentrations ng Bk-equiv./g tissue (wet weight). Vertical bars represent standard errors of the means and numbers above represent the number of animals in each group. Abscissa: Time, expressed as a proportion of the cycle (the length of the cycle = 0.1). The anticipated time of ovulation (midpoint of the time range) is fixed at 0.5 and is indicated by a broken vertical line.


OVARIAN KININ-FORMING ENZYME (NG BK-EQUIV/GM TISSUE)
30
28
14
12
10
8
6
4
2
0
0.5
1.0
5
5
4
5
4
4
4
3
PROPORTION OF CYCLE

DISCUSSION

The results given here confirm the findings of McDonald and Perks (1976) that plasma kininogen levels fall around the time of ovulation in rats and in humans. Similar findings were observed in yet a third species, the guinea pig. One of the main advances over the original study was the clear demonstration that the fall preceded the anticipated time of ovulation. In addition, kinin-forming enzymes were detected in both the ovary and plasma during this preovulatory period.

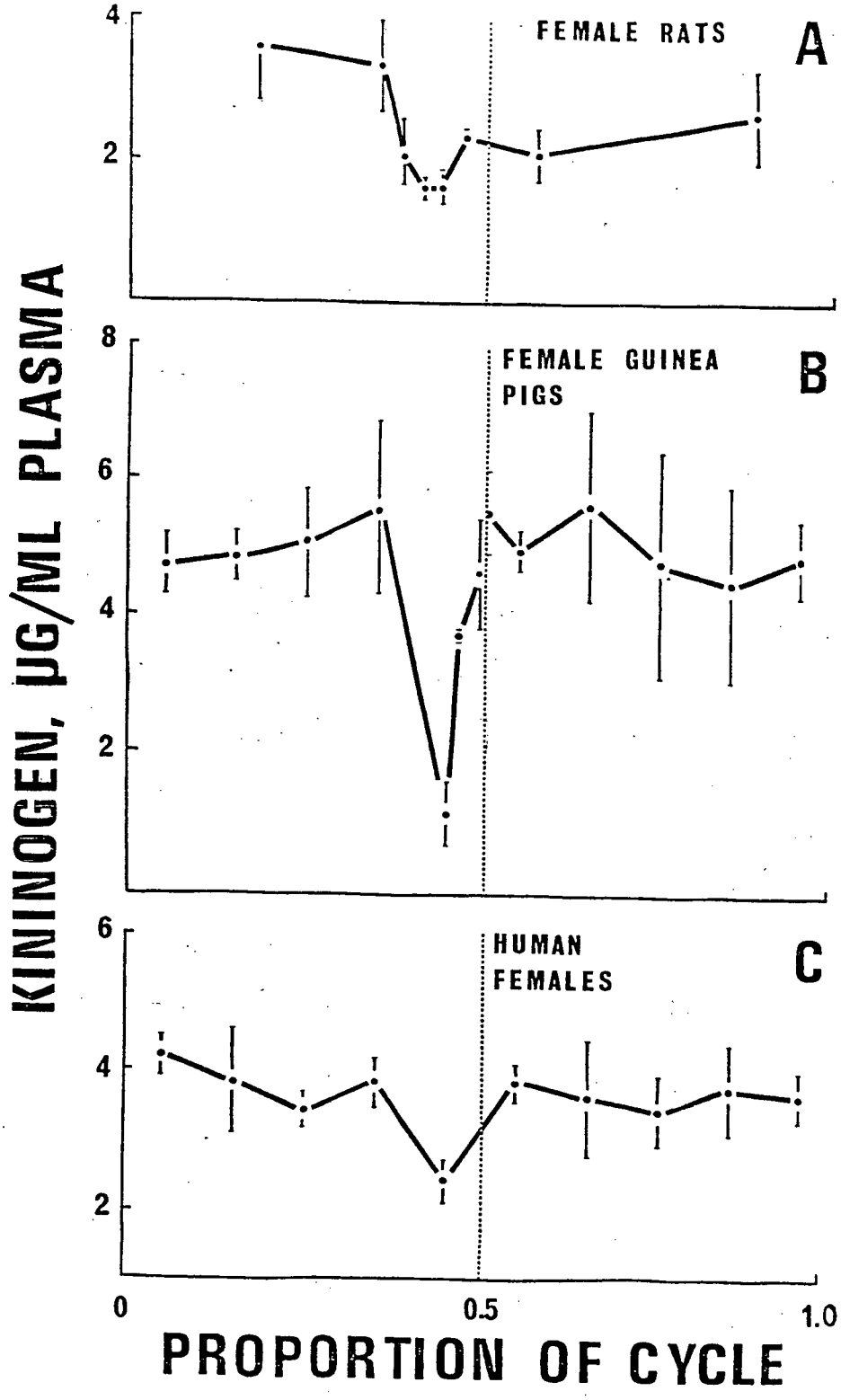
1. Plasma kininogen levels before and after ovulation

Figure 8 integrates the data presented here on the plasma kininogen levels of each of the three species examined. The figure shows a strikingly similar pattern of kininogen changes in the three different species, with cycles that differed in time and length and blood samples as different as arterial blood from the heart and venous blood from a superficial vein in the forearm. Irrespective of their differences, each species had a marked fall in plasma kininogen just before ovulation.

The fall was surprisingly large, averaging 57% over all the species (humans, 41%; guinea pigs, 79%; rats, 51%). These results all agree well with the findings of McDonald and Perks (1976) who noted a decline in plasma kininogen

FIGURE 8: A COMPARISON OF PLASMA KININOGEN LEVELS DURING THE ESTRUS OR MENSTRUAL CYCLES OF RATS, GUINEA PIGS, AND HUMANS

Mean plasma kininogen levels were estimated in groups of animals at different stages of the estrus or menstrusl cycle. (A) Female rats. (B) Female guinea pigs. (C) Human females. Ordinates = plasma kininogen, ug Bk-equiv./ml plasma. Mean values are given with vertical bars which represent standard errors of the means. For single observations, the bars give the confidence limits of the assay at the $P = 0.05$ level. Abscissae = time expressed as a proportion of the cycle (the length of the cycle = 1.0). The midpoint of the time range for the anticipated time of ovulation is fixed at 0.5 and is indicated by a vertical broken line.


FEMALE RATS
A
FEMALE GUINEA PIGS
B
HUMAN FEMALES
C
KININOGEN, µG/ML PLASMA
PROPORTION OF CYCLE
0
0.5
1.0
2
4
6
8

concentrations of 42% in humans and 59% in rats with five-day estrus cycles. These data suggested that about one-half of the total circulating plasma kininogen was lost just before ovulation.

In this study, the timing of the change in kininogen levels was followed more carefully than in previous work The fall occured before the anticipated time of ovulation, by about 48 ± 12 hours in the human, 24 ± 6 hours in the guinea pig and 12 ± 5 hours in the rat. The fall also coincided approximately with the estimated time of the LH surge in each species (rat: Butcher et al., 1974; guinea pig: Donovan and Lockhart, 1972; human: Hilgers et al., 1978). The levels in the guinea pig had almost recovered by the anticipated time of ovulation. In the rat, however, there was only a slight, but not significant recovery, and the levels remained depressed many hours after the anticipated ovulatory period. These results indicate that the initial fall was essentially preovulatory.

The timing of the fall suggested that it could be linked in some way to the subsequent ovulation. Its regularity in three differing species, and its apparent absence in non-ovulating animals, that is, postmenopausal women, and male guinea pigs, left little doubt that it was connected to the reproductive cycle. However, there was one line of evidence against a link to ovulation; this was the surprising persistence of the change in women whose ovulations were presumably inhibited by oral contraceptives.

The evidence for a fall in kininogen in women taking norethindrone and mestranol was not as strong as in the other groups because of the small sample size; however, it was significant at the $P < 0.05$ level (Student's t-test for two independent samples), and in close agreement with the cycling females of all the species shown in Figure 8. At first it was difficult to see how the drop could still be linked to ovulation because these women were almost certainly not ovulating. Clearly, these results implied that the change could not be a consequence of the extrusion of the ovum; this agreed with the time-sequence studies discussed earlier. However, the possibility existed that the change was involved in some early phase of the ovulatory mechanism, one which was more fundamental than the events obstructed by the contraceptives. This hypothesis is reasonable, because evidence of a link between kininogen and ovulation, although circumstantial, is hard to reject, and the fall in kininogen in women on oral contraceptives still retained its usual relationship to the 'potential' time of ovulation. Two possibilities exist: (1) either some aspect of the previous menses (or the change in drugs which produced it) triggered the fall, at long range, or (2) the fall was related to some persistent and fundamental rhythmicity, perhaps of the ovary or hypothalamus. However, due to the small number of samples in the "preovulatory" group, these data should be considered with reservation. Obviously more research is

required in this area.

2. Plasma kinin-forming enzymes of the rat

Only the precursor form of plasma proteases, including kinin-forming enzymes, are normally found in the plasma, because once activated, several circulating proteolytic inhibitors (including kallikrein, plasmin, trypsin, chymotrypsin inhibitors) rapidly inactivate them (see Eisen, 1970; Heimburger et al., 1975; Pisano, 1975). The plasma kallikreins are believed to be activated by: (1) tissue proteases such as trypsin and cathepsin, (2) activated Hageman Factor (clotting factor XII). and (3) enhanced fibrinolytic activity (for review: Eisen, 1970) all presumably associated with the local target site. Therefore, it appears that plasma kinin-forming enzymes circulate in the preactive form until they are activated at the target site and then rapidly inhibited (Heimburger et al., 1975).

Apparently then, the plasma kinin-forming enzymes detected in this study were in the precursor form, and so any sharp drop in their level would suggest precursor activation followed by rapid enzyme destruction, similar to the kininogen-kinin situation.

The first decline in kinin-forming enzyme precursors occurred after the initial decline in kininogen levels (12.00 - 15.00 h proestrus) and at the same time as the

later fall of kininogens to their lowest recorded level (15.00 - 18.00 h proestrus). Clearly, the fall in plasma kinin-forming enzyme precursors could not have initiated the preovulatory fall in plasma kininogen, although it may have contributed to the subsequent additional lowering.

The second decline occurred over the anticipated period of ovulation. If this decline represents a fall in plasma kinin-forming enzyme precursors it may have contributed to the maintenance of the lowered plasma kininogen level (presumably by continuously releasing free kinin).

3. Ovarian kinin-forming enzymes of the rat

Kinin-forming enzymes have been found in the active form, inactive form, or both, depending on the tissue (Eisen, 1970; Pisano, 1975; Schacter and Barton, 1979). The kinin-generating ability of ovarian tissue has not been examined previously. In this initial study, the experiment was designed only to detect the presence and levels of kinin-forming enzymes, and not to determine their state of activity in the natural situation. Circumstantial evidence indicating that the kinin-forming enzymes detected here were in the active form include: (1) concurrent changes in kininogens, (2) the presence of pre-kallikrein activators (trypsin, cathepsin [Espey, 1974] and possibly plasmin [Strickland and Beers, 1976]) in the maturing follicle, (3) the presence of an agent similar to

bradykinin in follicular fluid (Ramwell et al., 1969), and (4) kallikreins, except those of the plasma and pancreas, are resistant to proteolytic degradation (Vogel and Werle, 1970). Also, little appears to be known of the existence of tissue kallikrein inhibitors, except in bovine organs (Schacter, 1980). Evidence from other glands suggests that endogenously-released kallikrein has access to the general circulation, where it might convert kininogen to kinin. In the blood it is inhibited by kallikrein inhibitors (see Nustad et al., 1978). However, the state of activity, as well as the approximate half-life of the ovarian kinin-forming enzymes, is uncertain.

Nevertheless, the results of this study indicate that kinin-forming enzymes, possibly in the active state, arose rapidly in the ovary at the time of both the major fall in plasma kininogen concentration and the anticipated time of the LH surge. Eighteen hours before this stage, no enzyme could be detected. Concurrent decreases in plasma kininogen and increases in ovarian kinin-forming enzymes are highly indicative of kinin release in the ovary at this time. The temporal relationship with the LH surge suggests LH involvement.

Understandably, attention is drawn to the major peak of enzyme activity found in the ovary just before ovulation. This peak might have been responsible for the slowing in recovery of kininogen levels, possibly due to continued

free kinin release. However, one might question why its effect was not greater, because there was no lack of kininogen. It is possible that the remaining kininogens were of a type that is resistant to these enzymes, but not to the exogenous trypsin used to activate them during kininogen estimation (Rocha e Silva, 1974). In fact, in mild inflammatory reactions, the maximum kininogen released rarely exceeds 30-40%. Only in cases of severe shock by proteolytic enzymes such as trypsin, are drastic reductions, sometimes to zero, ever seen (Rocha e Silva, 1974).

Besides the state of activity of the ovarian kinin-forming enzymes, several aspects of this preliminary study remain to be clarified. For example, the exact location of the enzymes within the ovary has not been determined. However, this study supports the possibility that the enzymes came from the Graafian follicle, because the high enzyme levels declined after ovulation.

Also, the substrate-specificity of these enzymes remains to be examined. The developing follicles produce powerful proteases (trypsin, cathepsin, and probably plasmin), capable of digesting the follicle at ovulation, and these are able to act as non-substrate-specific kinin-forming enzymes (reviews: Schachter, 1969; Eisen, 1970; Pisano, 1975). Lastly, the possibility that similar enzymes, arising in other tissues might facilitate the preovulatory kininogen changes requires further investigation. As an example,

Powers et al., (1981) have reported kinin-forming activity in the anterior pituitary of the male rat. A similar study of this activity in the female, at different stages of the estrus cycle, could be interesting.

Despite the uncertainties, the appearance of enzymes in the ovaries seemed particularly significant, because kinins, as 'local hormones', are usually liberated at their site of action (Feldberg, 1955; Rocha e Silva, 1963), and a temporal link between kinins and ovulation had already been established.

4. Concluding remarks

The following general picture summarizes the results presented here. In all three species studied about one-half of the circulating plasma kininogen was lost just before the anticipated time of ovulation. In rats, the kininogen decline and low level over the preovulatory and ovulatory periods coincided with changes in both ovarian and plasma kinin-forming enzymes. Although circumstantial, this evidence suggests that free kinins may be released in ovarian tissue at this time.

The timing suggests that these changes could be linked to preovulatory and perhaps later events in the mechanism of ovulation. The fact that when ovulation did not occur (ie. in postmenopausal women and male guinea pigs) the plasma kininogen change was not present, provides additional

support for a link between the kinin system and ovulation. However, the fact that the plasma kininogen change appeared to persist in women on certain oral contraceptives, raises the possibility that the change is connected to some mechanism, more fundamental than the events obstructed by the drugs.

One observation of particular interest was the apparently close relationship between the timing of the fall in kininogen and the LH surge reported in the four-day cycling rat. This important observation will be dealt with next.

SECTION II

THE EFFECTS OF EXOGENOUS EQUINE LUTEINIZING HORMONE AND OF ESTRADIOL - 17β ON RAT PLASMA KININOGEN LEVELS

INTRODUCTION:

The following study was designed to determine if any of the hormones commonly associated with the induction of ovulation might be responsible for initiating the observed preovulatory changes in the kinin system. Due mainly to the timing of events within the estrus cycle, two reproductive hormones, LH and estradiol - 17β , could be involved.

According to Butcher et al., (1974) plasma LH levels rise on the afternoon of proestrus in the four-day cycling rat. This rise in plasma LH coincided well with the fall in kininogen concentration in rats with the same four-day cycles, as shown in the previous section. In addition, LH has long been implicated in the hyperemia and swelling of the ovary close to ovulation, and these are known effects of kinins particularly associated with inflammation (Lewis, 1970; Arrigoni-Martelli, 1977). In fact, ovulation may prove to be a "controlled inflammation" produced by LH. For these reasons it was felt that LH may be involved in initiating the preovulatory changes in the kinin system.

Serum estradiol - 17β levels of four-day cycling rats, as reported by Butcher et al., (1974), rise gradually after the early morning of proestrus, and their peak coincides well with the preovulatory decline in plasma kininogen levels. This timing of events argues for a possible role of estradiol - 17β in triggering the decline in plasma kininogen levels. However, McCormack and Senior (1971) and Senior and Whalley (1974) reported that relatively long-term treatment of four-day cycling rats with estradiol - 17β (daily subcutaneous injections for five days) elicited no effect after one or two days of treatment, but created a rise in the plasma kininogen concentration after three days. However, these investigators did not examine the short-term effects, that is, over a twelve hour period, presumably because they were interested in simulating the events of pregnancy, not of ovulation.

In this study, an initial attempt was made to advance the preovulatory kininogen changes in rats by administering exogenous LH or estradiol - 17β early in the estrus cycle, before any spontaneous effects could be expected. For comparison purposes, the spontaneous changes in kininogen levels throughout the preovulatory period of the rat were also analyzed, using the same parameters, and compared to the results produced by the two hormones under study.

MATERIALS AND METHODS

1. Experimental animals

Studies were performed on 51 adult female rats, housed and maintained as described in the General Methods section of this thesis. The animals were divided into two groups according to their state of estrus: 30 were in proestrus, for analysis of spontaneous changes in kininogens, (group 1), and 21 were in diestrus (group 2), for hormone or saline treatments.

2. Experimental procedures

With each animal under light ether anaesthesia, one blood sample of approximately 0.8 ml was withdrawn rapidly from the left side of the heart by cardiac puncture. 0.5 ml of blood were treated for kininogen estimation, and the remainder was used for assessing the haematocrit (see General Methods).

3. Treatment groups

A. Group 1

One blood sample was taken from each animal at 12.00, 15.00, 21.00, or 24.00 h proestrus (as outlined above), and used for kininogen assessment.

B. Group 2

An initial blood sample was taken from each animal at 06.00 h diestrus, as described previously, and used for estimating kininogen levels. In order to minimize the number of cardiac punctures, the syringe needle was left *in situ* while the syringe barrels were exchanged and the hormone and/or carrier rapidly injected into the heart. Trauma was minimized by completing the procedure as quickly as possible.

These rats were further divided into four sub-groups according to their treatment regime: a) six animals received 110 IU equine LH (Sigma Chemical Co., U.S.A.) in 0.5 ml 0.9% saline; b) five recieved 0.5 ml 0.9% saline alone; c) six recieved 1.0 ug estradiol - 17β /100g body weight (Sigma Chemical Co., U.S.A.) (20 µl of 50 µg estradiol - 17β per ml 95% ethanol per 100 g body weight, volume adjusted to 0.5 ml with 0.9% saline); and d) four recieved 20 µl 95% ethanol per 100 g body weight, volume adjusted to 0.5 ml with 0.9% saline.

Additional blood samples were taken from each animal at 12.00 and 18.00 h diestrus, or both, and used for measuring kininogen levels (see below). A maximum of three blood samples were taken from each animal, and any animals displaying signs of post-injection distress were eliminated from further study.

4. Measurement of plasma kininogen levels

The 0.5 ml blood obtained above were immediately denatured by ejecting them forcefully into 5.0 ml chilled 95% ethanol. After centrifuging, the precipitate was treated for kininogen estimation as described in the General Methods (Brocklehurst and Zeitlin, 1967). Essentially, the treatment involved incubating the precipitate with excess trypsin to convert the kininogen to kinin. The sample was then assayed using the rat uterus bioassay. Plasma kininogen concentrations were calculated as the maximum kinin liberated from the sample by trypsin in 30 minutes. The responses in group 1 animals were expressed as the percent change in plasma kininogen concentration between 12.00 h proestrus (the approximate starting time of the LH surge - Butcher et al., 1974), and the time the blood sample was collected. The 12.00 h proestrus sample served as the "base-line" value (ie.,0% change). Responses in group 2 animals were expressed as the percent change in plasma kininogen concentration between the time the hormone and/or carrier was injected and the sample collected. The sample taken immediately before treatment served as the "base-line" value in this portion of the study.

5. Statistics

All values recorded are averages from groups of animals (± S.E.M.); the numbers in each group are indicated on the graphs. The significance of differences between groups was determined by Student's t-test for two independent samples (Steel and Torrie, 1960).

RESULTS

1. The correlation between sponteneous preovulatory changes in plasma kininogen, serum LH, and plasma estradiol - 17β levels

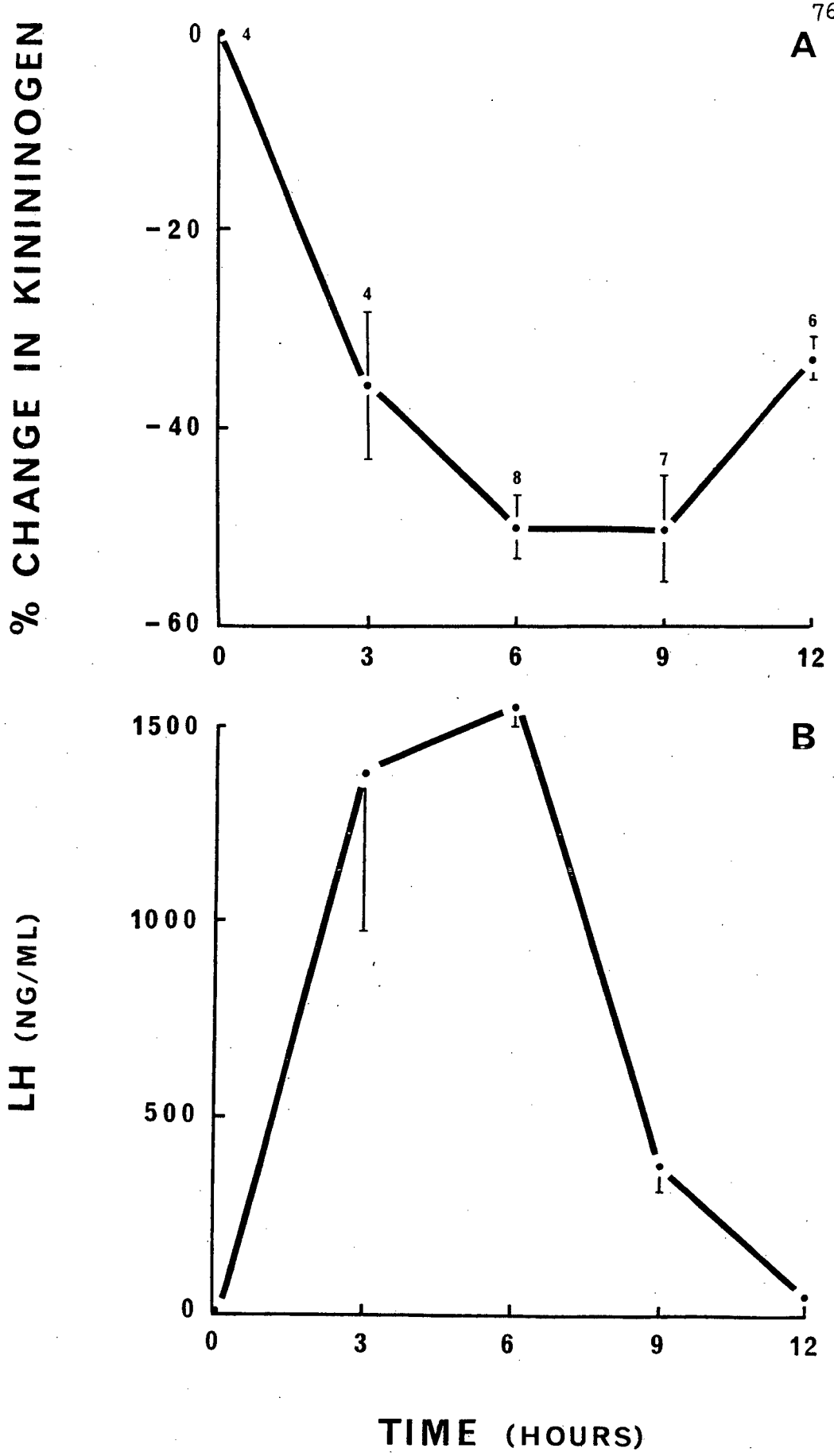
Studies on 30 rats showed a pronounced fall in plasma kininogen levels, which coincided well with the LH surge, and followed closely after the preovulatory rise in serum estradiol, as reported for rats with the same four-day cycle by Butcher et al., (1974). As Figure 9 shows, the decline began during the same 3 hour interval in which the LH surge was thought to start (12.00 to 15.00 h proestrus); the decline reached a maximum of -50.0 $\pm$ 3.0% change 3 hours later, coinciding well with the anticipated time of the LH peak (18.00 h proestrus). The kininogen levels remained depressed at nine and twelve hours; however, there was a slight but significant recovery to -33.0 $\pm$ 2.1% change by twelve hours (significantly different at $P < 0.02$ from the maximum decline value reached at six hours). The start of the kininogen fall trailed the anticipated time of the first significant preovulatory rise in serum estradiol by approximately six to twelve hours, and coincided with its peak.

2. The effect of exogenous LH on plasma kininogen levels

Having established that the plasma kininogen fall coincided well with the natural LH surge, an attempt was

FIGURE 9: A COMPARISON OF THE CHANGES IN PLASMA KININOGEN AND LH LEVELS DURING THE PREOVULATORY PERIOD OF THE RAT FOUR-DAY ESTRUS CYCLE

The values for LH levels during the preovulatory period from 12.00 to 24.00 h proestrus, as reported by Butcher et al., 1974 are shown for comparison purposes. (A) Blood samples were taken for kininogen level estimations at the same time as samples were taken in the LH study. (B) The sample taken at 12.00 h proestrus (approximate time of the start of the LH surge) (time 0) was used as the "baseline" value (0% change) from which the percent change in plasma kininogen at later times was calculated. The values are expressed as the mean ± the standard error of the mean (represented by vertical bars). The numbers above represent the number of animals sampled in each group.
Ordinates: (A) Plasma LH levels in ng/ml plasma, from Butcher et al., 1974, and (B) percent change in plasma kininogen concentration from the base-line value.
Abscissae: Time from the start of the LH surge (12.00 h proestrus) in hours.


A
% CHANGE IN KININOGEN
0
-20
-40
-60
4
4
8
7
6
0
3
6
9
12
B
LH (NG/ML)
1500
1000
500
0
0
3
6
9
12
TIME (HOURS)

made to determine whether exogenous LH could advance this decline. To my knowledge this is the first report of the effect of exogenous LH on plasma kininogen concentrations. Figure 10 shows that treating diestrus rats one day before proestrus (diestrus) with a single injection of equine LH in a saline carrier decreased their plasma kininogen levels. A maximum decline of -30.8 ± 6.7% change was attained by six hours post-injection. This value was significantly different from both pre-injection levels ($P < 0.01$) and the levels of control animals recieving only carrier ($P < 0.01$). Strangely, the controls exhibited a slight, but significant rise in plasma kininogen levels at six hours post-injection, as compared to pre-injection levels (+12.8 ± 4.3% change significant at $P < 0.01$). The levels remained depressed in experimental animals at 12 hours post-injection (-21.3 ± 5.8% change); again this was significantly lower than the pre-injection levels ($P < 0.01$).

3. The effect of exogenous estradiol - 17β on plasma kininogen levels

The start of the natural fall in plasma kininogen appeared to coincide with the peak in serum estradiol - 17β levels, suggesting that estradiol - 17β might also precipitate the plasma kininogen decline. Figure 11 shows that a single relatively high dose of estradiol - 17β (1.0 µg/100 g body weight in 0.5 ml saline/ethanol carrier), injected into

FIGURE 10: THE EFFECT OF EQUINE LH ON PLASMA KININOGEN LEVELS IN RATS

At time 0 each animal recieved a single injection of equine LH (110 IU) in 0.5 ml 0.9% saline carrier (Figure A), or 0.5 ml saline carrier alone (Figure B). A sample taken just prior to injection was used as the "baseline" value (0% change) from which the percentage change in plasma kininogen at various time intervals after was calculated. The values are expressed as the mean ± the standard error of the mean (represented by vertical bars). The numbers above, represent the number of animals sampled in each group.
Ordinates: Percent change in plasma kininogen concentration from the base-line value. Abscissae: Time from injection of LH and/or carrier in hours.

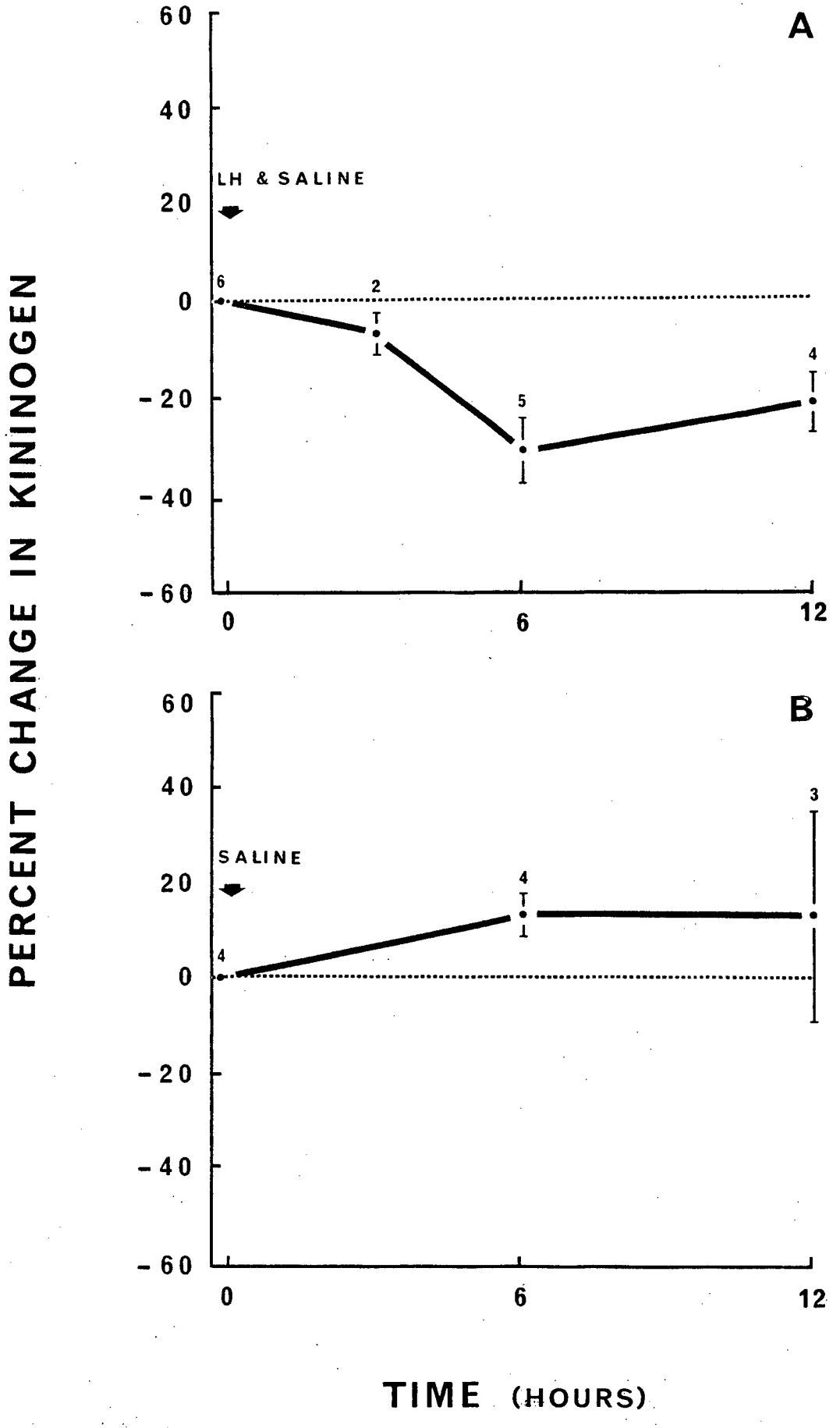
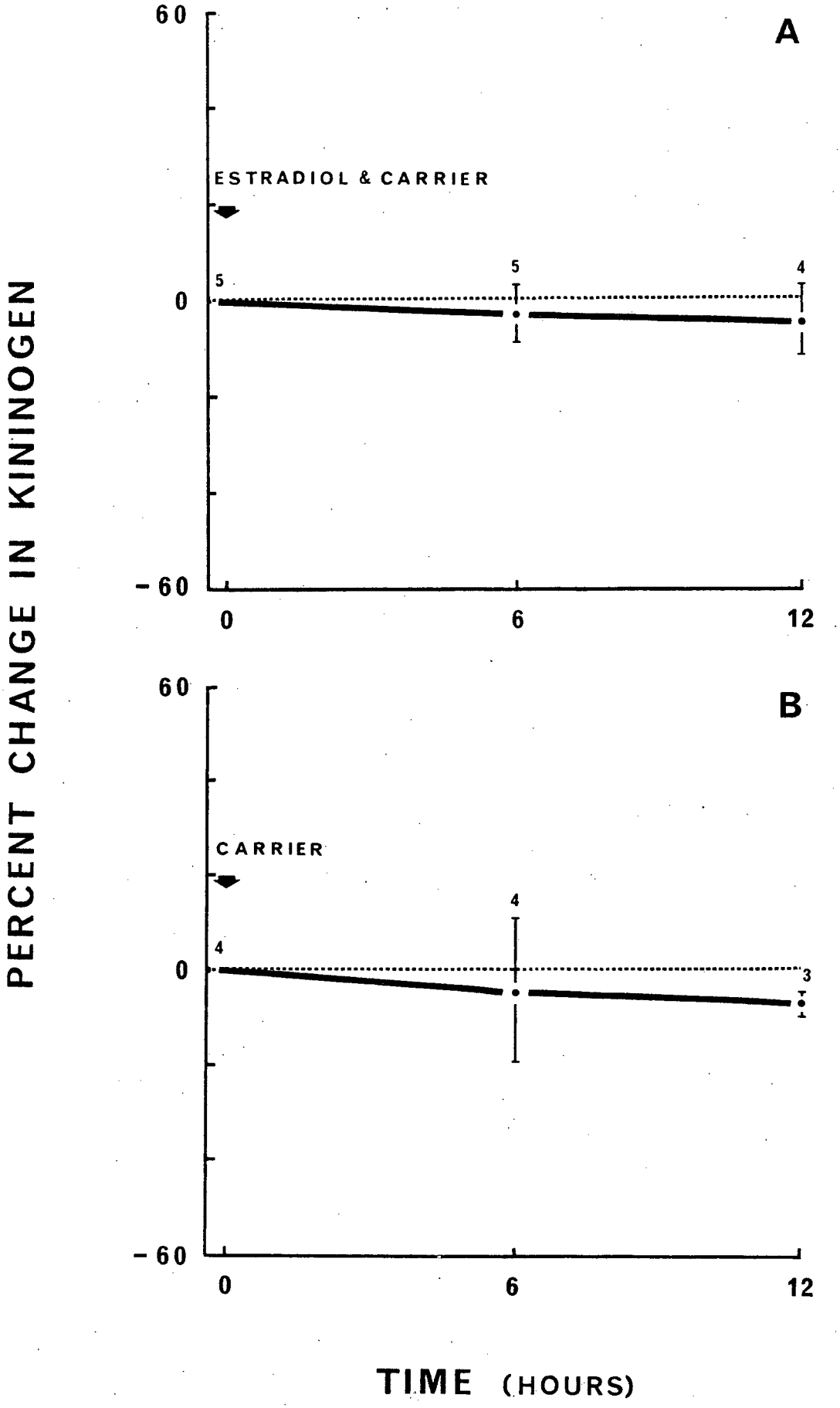

A
60
40
20
0
-20
-40
-60
LH & SALINE
6
2
5
4
0
6
12
B
60
40
20
0
-20
-40
-60
SALINE
4
4
3
0
6
12
PERCENT CHANGE IN KININOGEN
TIME (HOURS)

FIGURE 11: THE EFFECT OF ESTRADIOL - 17β ON PLASMA KININOGEN LEVELS IN RATS

At time 0 each animal recieved estradiol - 17β (1.0 ug/100 g body weight) in 0.5 ml saline/ETOH carrier (Figure A), or carrier alone (Figure B). A sample taken just prior to hormone administration was used as the base-line value (0% change) from which the percentage change in plasma kininogen concentration at various time intervals was calcualted. The values are expressed as the mean $\pm$ the standard error of the mean (represented by vertical bars). The numbers above each bar represent the number of animals sampled in each group. Ordinates: Percent change in plasma kininogen concentration from the base-line value. Abscissae: Time from injection of hormone and/or carrier in hours.


A
60
0
-60
ESTRADIOL & CARRIER
5
5
4
0
6
12
B
60
0
-60
CARRIER
4
4
3
0
6
12
PERCENT CHANGE IN KININOGEN
TIME (HOURS)

the general circulation, failed to elicit any changes in plasma kininogen concentrations throughout the 12 hour observation period. Control experiments (animals received carrier alone) showed the same results. In this case there was a slight reduction in kininogen levels in the control experiments, in contrast to those used for the LH experiments; this may well reflect the difference in the carrier, which contained 95% ethanol in this portion of the study.

DISCUSSION

The data presented in this study demonstrate that, in the animals examined, preovulatory declines in plasma kininogen levels occurred approximately in parallel with the anticipated time of the endogenous LH surge, on the afternoon of proestrus, and also within three to six hours after a single injection of exogenous LH on the day before proestrus. Furthermore, the results showed that the anticipated time of the peak in endogenous estradiol - 17β levels coincided with the start of the kininogen decline. However, exogenous estradiol - 17β failed to advance the decline when administered one day before proestrus.

1. The effect of LH on plasma kininogen levels

Two minor differences between the spontaneous and exogenous LH-induced effects were observed, that is, the effect produced by exogenous LH appeared to have a longer latency period (spontaneous decline: started between 0-3 hours after the anticipated start of LH-surge; exogenously-induced decline started between 3-6 hours after LH administration), and was lesser in magnitude (spontaneous decline: -50.9 $\pm$ 3.0% change; exogenously-induced decline: -30.8 $\pm$ 6.7% change). Several factors could explain these differences, these are outlined below:

(1) Follicular development - the animals recieving exogenous LH were not as close to ovulation as those exposed to endogenous LH. If the follicles were involved in the changes in kininogens, they may have been too immature to function appropiately. This idea is supported by the work of Bauminger et al., (1975) who found that the effect of LH on increasing prostaglandin synthesis in rat follicles increased with the proximity of the follicles to ovulation.

(2) The endocrine environment - many reproductive processes take place under multiple hormonal influences; this hormonal milieu may not have been adequate earlier in the cycle.

(3) The form of LH - the exogenous LH was equine in origin and so may not have been as effective as the animals' own endogenous supply.

(4) The mode of treatment - under normal physiological conditions, the ovary is exposed to LH in a continuous pulsatile fashion over several hours (see Gallo, 1980). However, under the experimental conditions of this study, which tried to avoid the accidental activation of the kinin system by surgical or other factors, the animals were exposed to a single injection of LH, which is probably not as efficient as the natural mode of exposure.

(5) The carrier - in addition, the apparent tendency of the saline carrier to raise kininogen levels might have reduced the magnitude of the kininogen decline.

In any case, the observations that plasma kininogen levels decline in parallel with the anticipated time of the surge of LH, and also following exogenous LH administration, suggest a role for kinins as mediators of this gonadotropin in the ovulatory process.

Previous studies on humans showed that blockage of the LH surge with oral contraceptives failed to alter the "preovulatory" pattern of plasma kininogen changes. These data appear to discredit the concept of kinins functioning as LH intermediaries. Two possibilities could explain this result. First, there could be species variations regarding the substance responsible for initiating the preovulatory decline in plasma kininogen values. In this regard, it would be interesting to determine if blockage of the LH surge, perhaps with nembutal, would inhibit the preovulatory plasma kininogen changes in the rat. Second, the possibility still exists that two separate events are responsible for the plasma kininogen decline, one involving LH-release and the other preceding the release. In this case, if kinins are released before the LH-surge they could assist in initiating it. The finding of kinin-forming activity in the rat anterior pituitary (Powers et al., 1981) supports this hypothesis. However, Steele et al., (1980) could detect no differences in the plasma LH levels of rats receiving third ventricular injections of bradykinin, as compared to the levels of saline-injected control animals.

Their results should be considered with some reservation though, because there was a noticeable rise in LH levels in the bradykinin-treated rats at 15 and 60 minutes post-injection when the levels were compared to pre-injection levels.

Therefore, although still speculative, it is possible that the LH surge could be concerned in producing the pre-ovulatory decline in plasma kininogen levels, as well as being produced by it. Such a positive feedback system, operating at this crucial period, would be considerably important.

2. The effect of estradiol - 17β on plasma kininogen levels

The fact that exogenous estradiol - 17β failed to initiate any declines in the plasma kininogen levels of the rats studied here, suggests that estradiol - 17β is not directly involved in the preovulatory plasma kininogen decline. However, this lack of effect could be due to one or more factors:

(1) Incorrect dosage - the studies of McCormack and Senior (1971) and Senior and Whalley (1974) demonstrate that the dose of estradiol is crucial for producing changes in kininogen levels. However, the same dose was used in both their study and the work reported here; although Senior and his group, being interested in long-term effects, administered the dose daily for five days. This treatment

produced significant increases in plasma kininogen levels, similar to the changes observed during pregnancy. However, in this study of the short-term effects, only one single dose was administered.

(2) The mode of treatment - as in the LH studies the method of hormone administration, that is, a single injection, as opposed to continuous infusion could explain the lack of effect.

(3) Follicular development and hormonal milieu - again, the level of follicular maturation and the hormonal environment may be crucial for an estradiol-induced response.

Regarding the latter two possibilities, it should be remembered that LH was able to elicit an effect, albeit somewhat reduced, under the same treatment conditions. Obviously, these results are only preliminary and further study is required.

3. Concluding remarks

From results reported in this investigation, it appears that LH, but not estradiol - 17β , is responsible, at least in part, for the decreased plasma kininogen values just before ovulation. How LH exerts its effect is open to speculation, but most assuredly, an increase in kinin-forming enzyme activity is involved. The coincident timing of the LH surge and the decrease of plasma kininogen level during the preovulatory period argues for activation

of a pre-formed enzyme, rather than enzyme synthesis, as the mechanism responsible. Evidence obtained in Section I indicates that this increased activity occurs in the ovarian tissue, and/or perhaps in the plasma, but exactly how this effect is accomplished requires further investigation.

SECTION III

THE EFFECTS OF EXOGENOUS BRADYKININ ON OVARIAN CONTRACTILITY AND FOLLICULAR BLOOD VESSEL PERMEABILITY IN THE RAT

INTRODUCTION:

When the known biological actions of the kinins are compared to the major events of the ovulatory process, two outstanding similarities are evident. First, kinins are potent in their ability to contract many different smooth muscles (review: Eisen, 1970), and it has been hypothesized that contraction of ovarian smooth muscle-like cells at ovulation may have an important role in this process (Lipner and Maxwell, 1960; Blandau, 1967; Burden, 1972). Second, evidence has shown that kinins have powerful vasoactive capabilities, causing dilation and increased permeability of many blood vessesl (see Eisen, 1970), and subsequently edema in the surrounding tissue (Arrigoni-Martelli, 1977). It is generally believed that preovulatory ovarian hyperemia and increased blood vessel permeability are at least partially responsible for the rapid increase in follicular volume (Basset, 1943; Burr and Davis, 1951; Szego and Gitin, 1964; Bjersing and Cajander, 1975) which may be vital to follicular rupture and ovum extrusion. However, despite the importance of ovarian contractions and the increased blood supply, the

question of what triggers these events remains unanswered.

The similarity in timing between the preovulatory kinin system changes and several ovarian changes including: (1) swelling of the ovarian follicle (Boling et al., 1941), (2) ovarian hyperemia (Basset, 1943), (3) increased ovarian weight (Osman and Lieuwma-Noordanus, 1980), and (4) increased ovarian contractility noted in a few species (Virutamasen et al., 1972a; Gimeno et al., 1975), suggested that kinins could be involved in promoting these events during the ovulatory process.

In addition, because prostaglandins appear to be involved with the ovulatory process (see Clark et al., 1978), and because they often act as mediators of kinins in other tissues (see Nasjeletti and Malik, 1979), the possibility that prostaglandins might mediate a bradykinin-induced ovarian response was also examined.

The effects of bradykinin (with or without prostaglandin inhibition) on the contractile activity of ovaries isolated from rats at different stages of the estrus cycle were examined in the first part of this study. In the second part, the effects of the same peptide, and prostaglandin inhibitor on the follicular blood vessel permeability of ovaries from similar animals was explored.

A. THE EFFECTS OF BRADYKININ ON CONTRACTILE ACTIVITY OF RAT OVARIES ISOLATED AT DIFFERENT STAGES OF THE ESTRUS CYCLE:

Introduction:

The present study was designed to fulfill four objectives. First, to corroborate the finding that isolated rat ovaries can contract spontaneously. Second, to determine whether bradykinin can stimulate contractile activity in the rat ovary. If so, this evidence would support the hypothesis that bradykinin is a factor involved in regulating ovarian contractility and so, perhaps, ovulation. Third, to examine whether the state of estrus has any effect on the response of the tissue to bradykinin. Fourth, to explore the possibility of prostaglandin involvement in any bradykinin-induced contractile responses.

Spontaneous contractile activities of isolated whole ovary preparations taken from rats at different stages of the estrus cycle were examined. The ovaries were then treated with various doses of bradykinin, with or without indomethacin (a prostaglandin synthesis inhibitor) and the effect noted.

Materials and methods:

(a) Experimental animals:

Studies were performed on 43 ovaries, from 28 adult female rats, maintained as described in the General Methods section. The animals were divided into five groups according to their state of estrus: three were in diestrus, ten in proestrus, five in early estrus, six in mid estrus, and four in metestrus. The sampling times were chosen to coincide with particular events of the ovulatory process. Thus, the early estrus samples, taken between 00.00 and 04.00 h estrus, coincided approximately with the anticipated time of ovulation; the proestrus samples (12.00 to 16.00 h proestrus) were taken at about the same time as the estimated time of the LH surge and initial changes in the kinin system. The remaining samples were taken between 12.00 and 16.00 h on the days of diestrus, estrus, and metestrus.

(b) Experimental procedure:

At the appropriate time, each animal was killed by cervical dislocation. Both ovaries were removed as rapidly as possible, and placed in Tyrode's solution, kept at room temperature, and composed as follows:

Tyrode's Solution

Chemical	g/l	Chemical	g/l
NaCl	6.00	$NaH_2PO_4 \cdot H_2O$	0.0583
KCl	0.60	$NaHCO_3$	0.3333
$CaCl_2$	0.60	Glucose	1.00
$MgCl_2 \cdot 6H_2O$	0.65		

The ingredients were aerated with 95% O_2 and 5% CO_2 until a pH of 7.4 was reached.

Sometimes both ovaries were tested at the same time, but usually one of the ovaries was refrigerated (4°C) in Tyrode's solution until tested never more than four hours later. Just before the experiment, each ovary was quickly trimmed of extraneous tissue and both poles were sutured with silk thread (3-0 gauge). Thread from one pole was attached to a stainless steel hook and promptly immersed and anchored in an organ bath filled with 20 ml Tyrode's solution, also aerated with 95% O_2 and 5% CO_2. The temperature was maintained at 37°C by means of a circulating water bath surrounding the organ bath. Thread from the opposite ovarian pole was connected to a Harvard isometric force transducer (Model 363 - Harvand Apparatus, U.S.A.). The output of the transducer was amplified and coupled to an ink-writing recorder (Fisher Recordall Series 5000 - Fisher Scientific Ltd., U.S.A.).

The apparatus was calibrated to give a positive deflection of 3.94 inches on the recording paper for every 100 mg tension applied to the transducer. A basal resting tension of between 125-200 mg was applied to the isolated preparations by means of a micrometric device (Narishge, Japan).

After a 30 minute equilibration period and a further 10 minute control period, the preparations were exposed to various doses of bradykinin triacetate (0.1 mg/ml - Sigma Chemical Co., U.S.A.) at final concentrations in the bathing fluid of 3.75, 7.50, 15.00, and 25.00 ng/ml. The various doses of bradykinin were diluted in Tyrode's solution and added forcefully, directly into the bottom of the organ bath in amounts of 0.15 - 1.00 ml. Each dose was left in the organ bath for 10 minutes; the preparation was then washed out with fresh Tyrode's solution pumped through the bottom of the organ bath. After 15 minutes or longer another dose was given. Each ovary was treated only once with each of the four doses, which were given in random order. Preliminary tests suggested that the responses were not influenced by refractory effects when these procedures were used. At the end of the experiment the initial dose was usually repeated to determine whether the responses had remained constant. Testing was carried out for up to four hours. The other ovary was mounted in the same way, as soon as the preceding

experiment was finished.

Some of the stored ovaries were mounted after 24 hours refrigeration. It was found that these preparations were particularly sensitive to bradykinin, regardless of their state of estrus, and so they were not included with the "normal" ovaries of this study.

To test the involvement of prostaglandins in the bradykinin-induced responses, indomethacin, a known prostaglandin synthesis inhibitor (Vane, 1971) (Sigma Chemical Co., U.S.A.) was introduced into the organ bath of three preparations in the final concentration of 312.5 ng or 187.5 ng/ml. bathing solution. The indomethacin was made up in a 95% ETOH carrier to the concentration of 50 ug/ml and 0.125 or 0.075 ml were added forcefully, directly to the organ bath of four preparations.

All concentrations of bradykinin and indomethacin in the results are expressed as the final concentration in the organ bath in ng/ml. Responses were evaluated by measuring the area under the tracing corresponding to the response to bradykinin and subtracting from it the area under the tracing corresponding to the control (before bradykinin) period. Both areas, each corresponding to a ten minute recording, were measured by a planimeter (La Sico, model L30M - Los Angelos Scientific Instrument Co., Inc., U.S.A.). The difference of areas is expressed in milligrams x minutes (mg · min), as calculated from the area

in inches^2 (Height: 26.1 mg/inch and length: 5 min./inch). Changes in (1) the frequency of contractions, (2) the average amplitude of contractions (mg), and (3) the basal tone (mg) between the ten minute experimental and ten minute control periods were also estimated.

Results:

(a) Spontaneous contractile activity of the in vitro rat ovary:

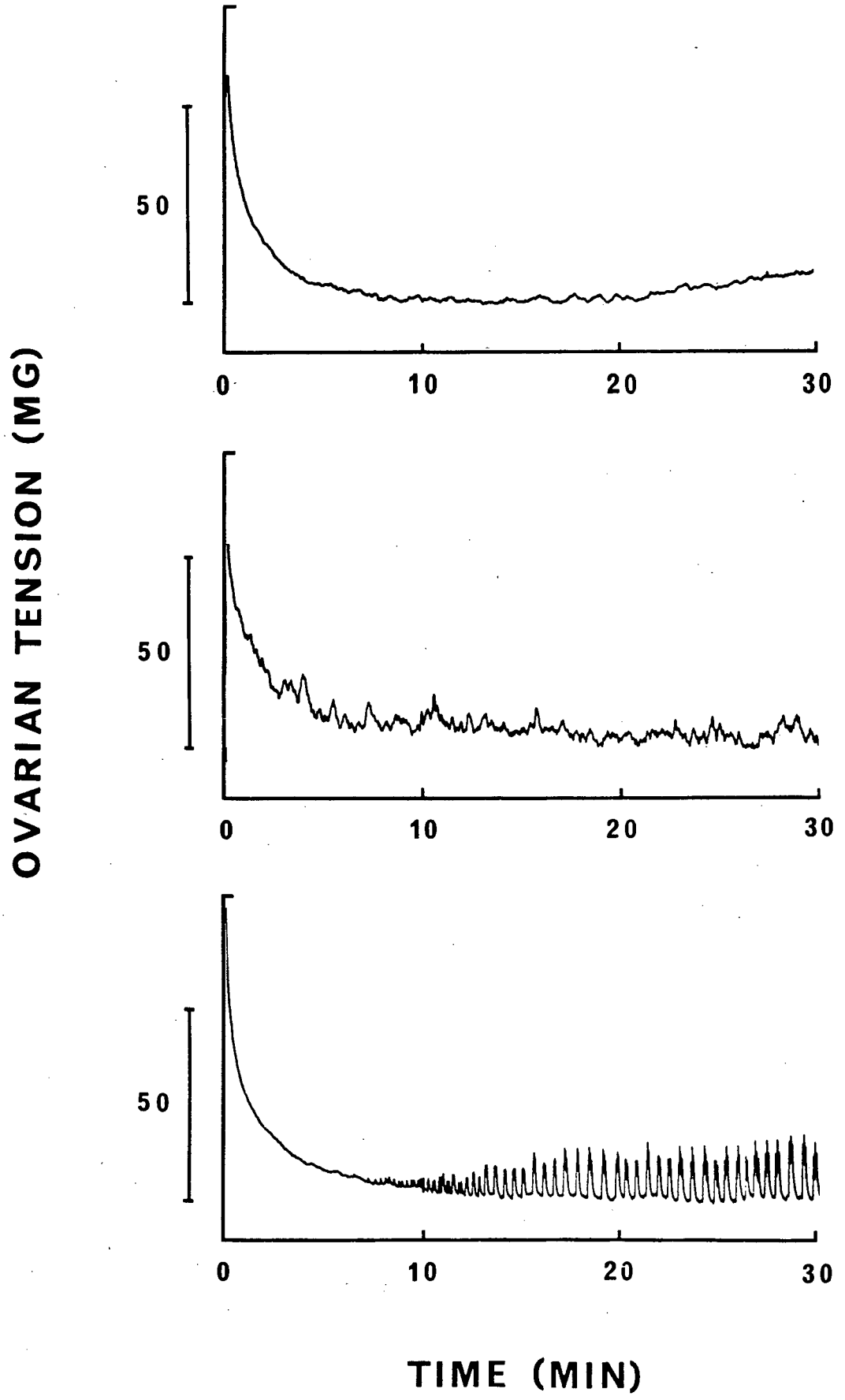
Spontaneous ovarian contractility was recorded during the 30 minute equilibration period. Detectable spontaneous contractions occured in slightly less than half of the preparations (18 out of 43). The contractile patterns varied from one experiment to the other and showed no correlation to the state of estrus. Spontaneous increases in tonic tension were detected in only 3 of the 43 preparations explored. Figure 12 shows some of the more typical contractile patterns.

(b) The effect of bradykinin on the contractile activity of the rat ovary isolated at different stages of the estrus cycle:

Bradykinin, in doses as low as 3.75 ng/ml, stimulated ovarian contractility to a significantly greater degree during the early estrus phase of the cycle than at any other time ($P < 0.01$ with 3.75 ng/ml, 7.50 ng/ml, 15.00 ng/ml, and 25.00 ng/ml when the value observed at early estrus was

FIGURE 12: TYPICAL PATTERNS OF OVARIAN SPONTANEOUS CONTRACTILE ACTIVITY *IN VITRO*

Three original tracings of ovarian contractile activity during the 30 minute equilibration period are shown. Ordinates: Ovarian tension (mg). Abscissae: Time from the start of the experiment (minutes).


OVARIAN TENSION (MG)
50
0
10
20
30
50
0
10
20
30
50
0
10
20
30
TIME (MIN)

compared to those observed at the other stages). Figure 13 shows the difference between the area under the response curve as compared to the area under the control curve with the different doses of bradykinin, at different stages of the estrus cycle. Clearly, there was an increase in response with the dose of bradykinin in early estrus. Analysis suggested that the $\log_{10}$ - dose / response relationship showed a reasonable agreement with an exponential curve ($r^2 = 0.80$) (see Figure 14). However, it seems possible that the curve represents the lower sections of an S - shaped curve, commonly found for log dose/response relationships to biologically active peptides and other agents. Higher doses might have revealed the top of the curve, but it is the lower doses which are important in this study.

Also, a comparison of the number of ovaries responding at each stage of the estrus cycle demonstrated that all (100%) of the early estrus ovaries responded to all of the doses of bradykinin, except the lowest dose, where 5 of the 6 ovaries (83%) responded. The percentage of responses was lower during the other stages of the cycle (except with the highest dose of bradykinin on diestrus ovaries). The results, according to the number of responding ovaries at each stage, are listed in Table 1. These data indicate that the ovaries were sensitive to bradykinin and that the level of sensitivity reached a peak during the

TABLE I

OVARIAN CONTRACTILE RESPONSES TO BRADYKININ

Cycle phase	Dose bradykinin (ng/ml bathing solution)	No. of ovaries	No. of responses	(%)
Diestrus	3.75	4	2	(50)
	7.50	4	1	(25)
	15.00	4	2	(50)
	25.00	4	4	(100)
Proestrus	3.75	11	2	(18)
	7.50	11	1	(9)
	15.00	13	5	(38)
	25.00	14	3	(21)
Early estrus	3.75	6	5	(83)
	7.50	7	7	(100)
	15.00	6	6	(100)
	25.00	7	7	(100)
Estrus	3.75	12	5	(42)
	7.50	10	3	(30)
	15.00	10	5	(50)
	25.00	12	6	(50)
Metestrus	3.75	5	0	(0)
	7.50	4	0	(0)
	15.00	5	0	(0)
	25.00	6	1	(17)

FIGURE 13: THE EFFECTS OF VARIOUS DOSES OF BRADYKININ ON CONTRACTILITY OF RAT OVARIES ISOLATED IN DIFFERENT STAGES OF THE ESTRUS CYCLE

Ovarian contractile responses to bradykinin were evaluated by measuring the area under the tracing corresponding to the response to bradykinin, and subtracting from it the area under the tracing representing the control (before bradykinin) period. The values are expressed as the mean + the standard error of the mean (represented by the dots). Numbers above each bar represent the number of samples in each group. All concentrations of bradykinin are expressed as the final concentration in the organ bath in ng/ml. Ordinate: The difference of areas expressed in mg • min Abscissae: The phases of the estrus cycle.

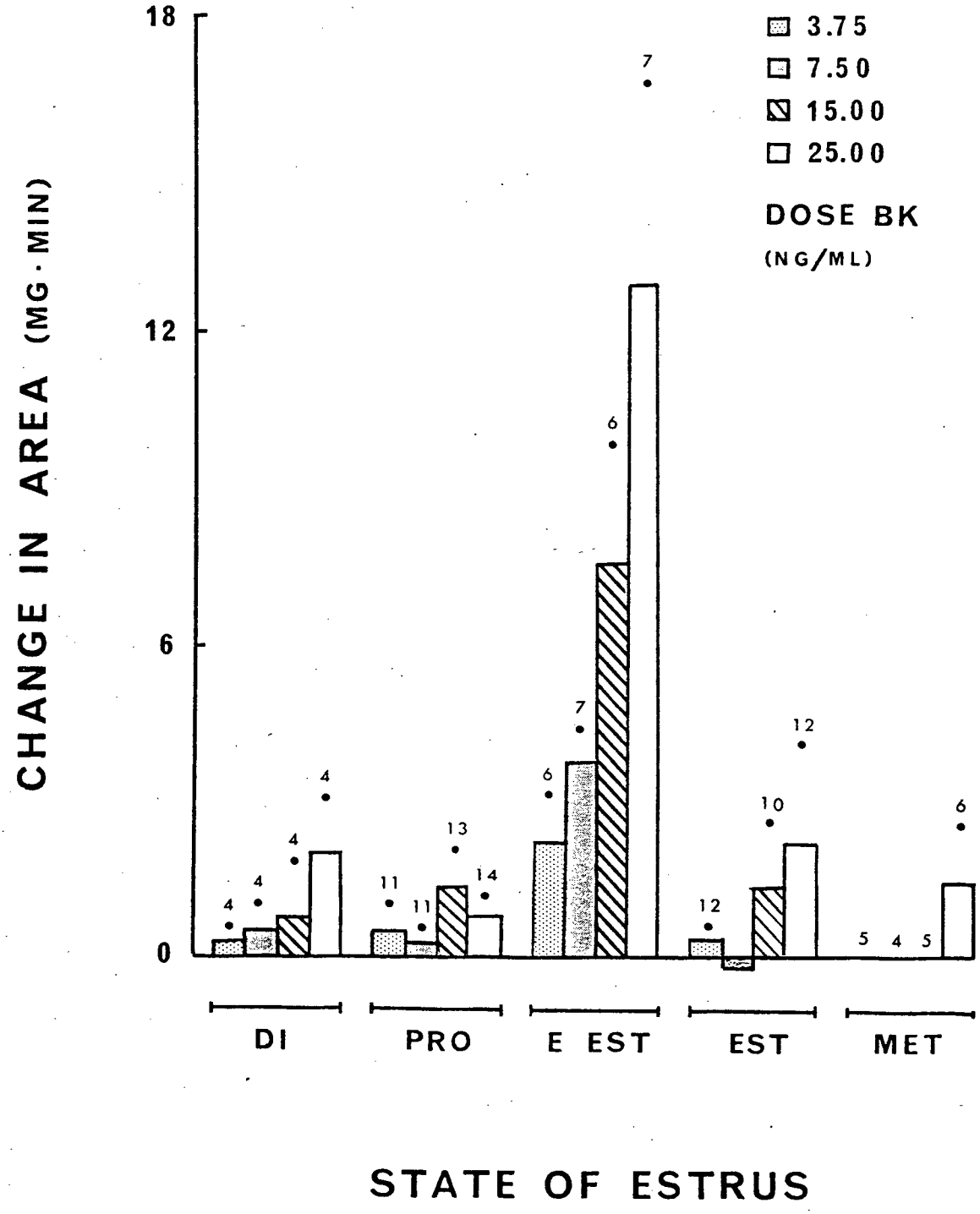
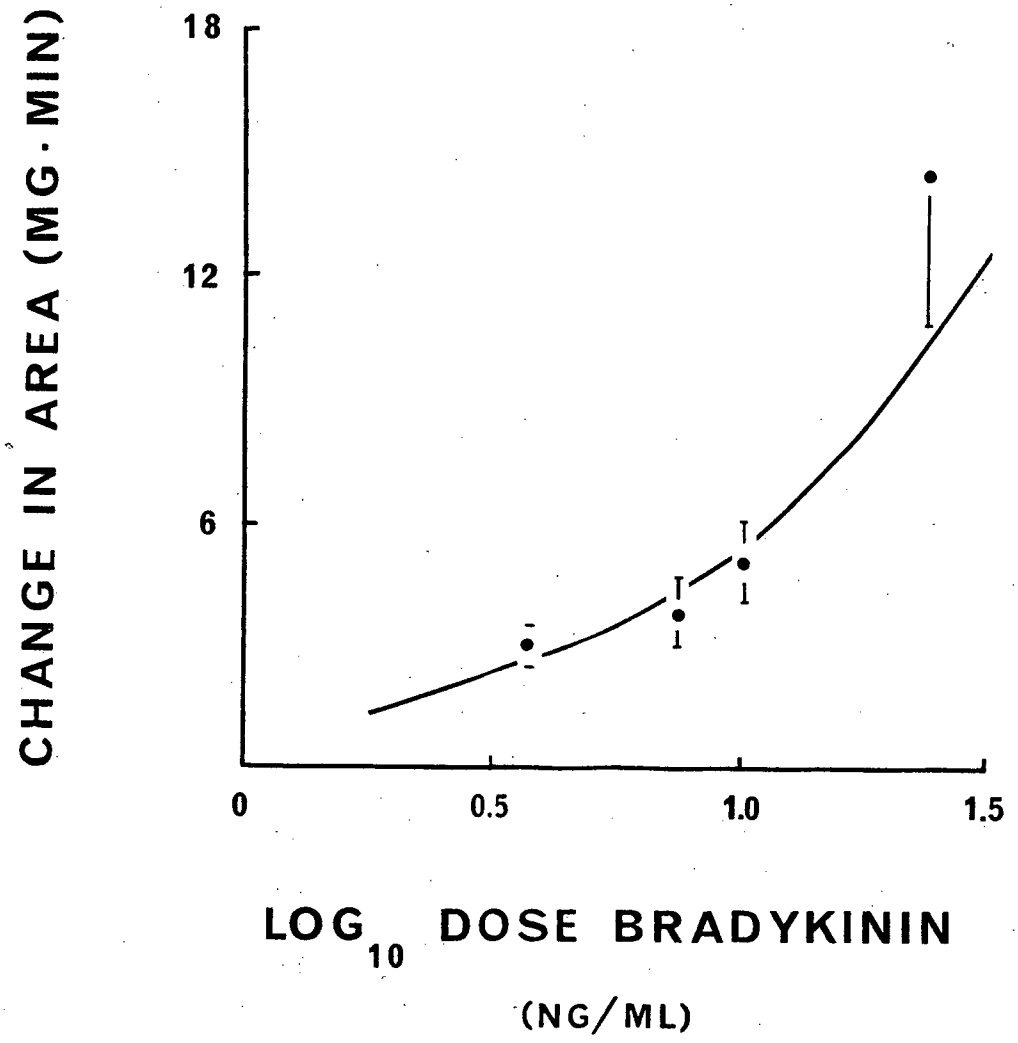

18
12
6
0
CHANGE IN AREA (MG·MIN)
3.75
7.50
15.00
25.00
DOSE BK
(NG/ML)
4
4
4
4
11
11
13
14
6
7
6
7
12
10
12
5
4
5
6
DI
PRO
E EST
EST
MET
STATE OF ESTRUS

FIGURE 14: LOG-DOSE RESPONSE CURVE FOR BRADYKININ-INDUCED CONTRACTILE ACTIVITY IN RAT OVARIES EXCISED AT EARLY ESTRUS

Each point represents the mean value of bradykinin-induced contractile activity from a group of early estrus ovaries recieving the same dose of bradykinin (dose range: 3.75 - 25.00 ng/ml bathing fluid). The vertical bars represent the standard errors of the means and the numbers above represent the number of ovaries in each group. Ordinate: The difference in area under the tracing between the bradykinin-induced response and the control period (mg • min). Abscissae: Log_{10}-dose bradykinin (ng/ml).


CHANGE IN AREA (MG·MIN)
18
12
6
0
0.5
1.0
1.5
LOG10 DOSE BRADYKININ
(NG/ML)

anticipated time of ovulation.

In most cases the response induced by bradykinin consisted solely of an increase in ovarian tone. However, bradykinin initiated phasic contractions, as well as increases in tone, in a few initially quiescent preparations. The latency period varied from almost instantaneous to two minutes. Figure 15 illustrates several types of ovarian, bradykinin-induced responses.

(c) The influence of indomethacin on bradykinin-induced ovarian contractility:

Indomethacin, at a final concentration in the organ bath of 312.5 or 187.5 ng/ml reduced, markedly in most cases, bradykinin-induced ovarian contractile activity when administered along with the bradykinin, or 10 minutes after bradykinin had been administered to the preparation. These preliminary findings suggested prostaglandin involvement in the bradykinin-induced contractility. However, these findings should be considered with reservation, due to the small number of trials (four) and the small sample size (three). Figure 16 shows the original tracings from each of these experiments.

Discussion:

The results given here indicate that bradykinin can enhance contractility of both the quiescent and spontaneously contracting isolated rat ovary. The state of estrus

FIGURE 15: EXAMPLES OF THE EFFECTS OF BRADYKININ ON OVARIAN CONTRACTILE ACTIVITY IN VITRO

Four original tracings of ovarian contractile activity during the 10 minute control and 10 minute response period are shown. Ordinate: Ovarian tension (mg). Abscissae: Time from the start of the control period (minutes).

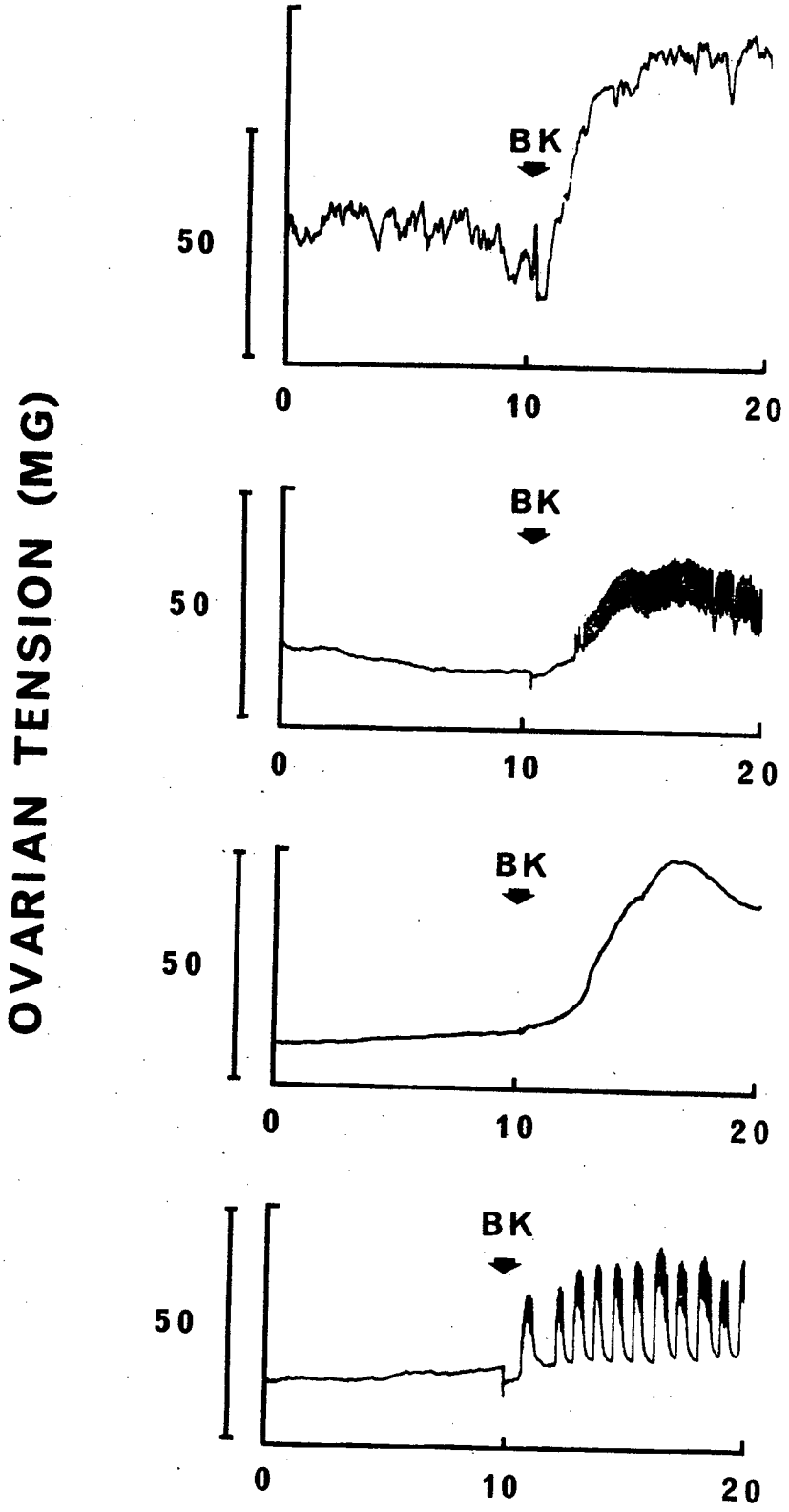
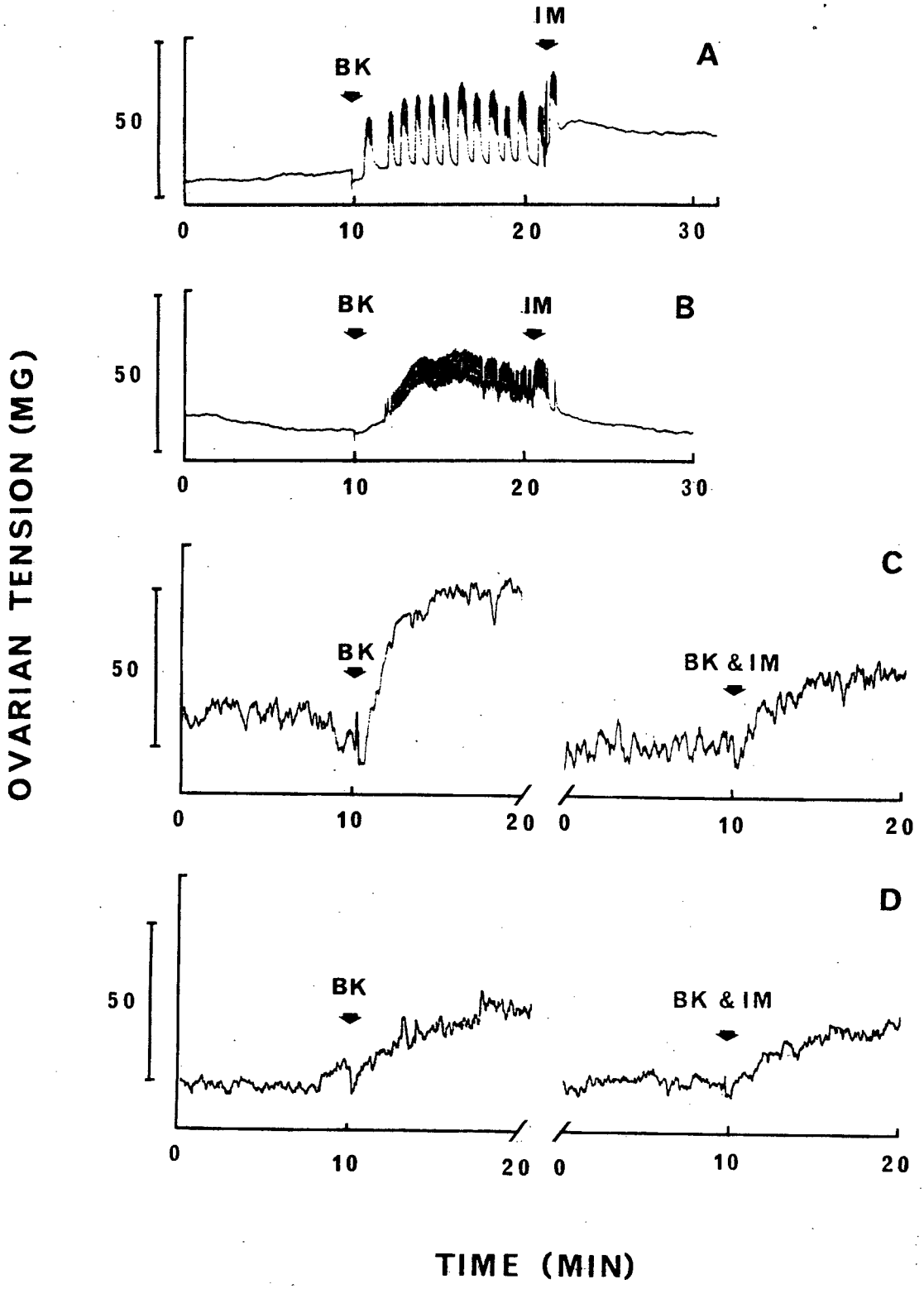

OVARIAN TENSION (MG)
BK
50
0
10
20
BK
50
0
10
20
BK
50
0
10
20
BK
50
0
10
20
TIME (MIN)

FIGURE 16: THE INFLUENCE OF INDOMETHACIN ON BRADYKININ-INDUCED OVARIAN CONTRACTILE ACTIVITY

Original tracings of ovarian contractility: A) and B) during a 10 minute control period, a 10 minute response period to bradykinin, and a 10 minute period following the addition of indomethacin; C) and D) during a 10 minute control period and a 10 minute response period to bradykinin alone, followed by a 10 minute control period and a 10 minute response period to bradykinin and indomethacin.
Ordinate: Ovarian tension (mg)
Abscissae: Time (minutes).


IM
BK
A
50
0
10
20
30
BK
IM
B
50
0
10
20
30
C
BK
BK & IM
50
0
10
20
0
10
20
D
BK
BK & IM
50
0
10
20
0
10
20
OVARIAN TENSION (MG)
TIME (MIN)

appears to influence the sensitivity of this tissue to bradykinin. In addition, preliminary findings suggest that the bradykinin-induced response may be partially mediated by prostaglandins.

(a) Spontaneous contractile activity of the isolated rat ovary:

Previous investigations have demonstrated spontaneous contractile activity in a proportion of, or all of the isolated rat ovaries examined (Gimeno et al., 1973,1974; Sterin-Borda et al., 1976; Roca et al., 1976). In agreement, spontaneous contractions were observed in slightly less than half of the preparations examined in this study, indicating that the techniques and apparatus used in this series of experiments were suitable to detect changes in ovarian contractile patterns.

Increases in ovarian contractility as ovulation approaches have been seen in several species including guinea pig (in vitro) (Gimeno et al., 1975), monkey (both in vivo and in vitro) (Virutamasen et al., 1973), and rabbit (both in vivo and in vitro) (Virutamasen et al., 1972a,b). In the rat, however, the results remain controversial regarding this point. Gimeno et al., (1974) and Sterin-Borda et al., (1976) have reported increases in the proportion of ovaries contracting spontaneously and or in the magnitude of isometrically developed tension of ovaries as ovulation nears. Others (Rocha et al., 1976,1977) have failed to establish

this correlation. In this study there was considerable variation in the patterns of spontaneous contractile activity in all groups studied, but neither the proportion of ovaries, nor the magnitude of isometrically developed tension varied with the state of estrus. Therefore, the relationships between ovarian contractile patterns and the state of estrus in the rat remains uncertain.

(b) The action of bradykinin on contractile activity of rat ovaries isolated at different stages of the estrus cycle:

The *in vitro* observation of spontaneous contractions in rat ovaries is consistent with earlier reports (Gimeno *et al.*, 1973; Roca *et al.*, 1976). Contractions have also been induced in the ovaries of rats by substances such as prostaglandins (Sterin-Borda *et al.*, 1976), and oxytocin (Gimeno *et al.*, 1973,1974; Sterin-Borda, 1976; Roca *et al.*, 1976,1977). However, this study documented, for the first time, the contractile activity of an ovary in response to bradykinin.

The results of this investigation demonstrate that the delivery of bradykinin, in a wide dose range, to the *in vitro* rat ovary enhances ovarian contractility to a greater degree near the anticipated time of ovulation than during any other phase of the estrus cycle. This increased responsiveness has been noted in some studies with prostaglandins (Virutamasen *et al.*, 1972b) and oxytocin (Sterin-

Borda et al., 1976). The proposal of Burden (1972) that the differentation of fibroblasts from the theca externa of ovarian follicles into "muscle-like" fibroblasts may be regulated by gonadotropins or ovarian hormones, could explain the increased sensitivity of the rat ovaries to bradykinin during early estrus, that is, soon after the LH peak. Therefore, it is possible that LH could regulate ovarian contractions by two different mechanisms; it may increase both the synthesis of ovarian contractile agents and the sensitivity of the ovary to them.

In the present investigation bradykinin caused an increase in tonic tension in both quiescent and spontaneously contracting preparations and it occassionally elicited phasic contractions in quiescent preparations. This suggests that bradykinin could be involved in tonic contracture of smooth-muscle-like cells of the follicle wall, ischemia of the follicular apex, and/or tonic changes in diameter of the ovarian vasculature. The bradykinin-induced phasic contractions could be due to rhythmic vascular spasms (see Espey, 1978). The hypothesis that kinins are involved in ovarian vascular changes was tested later (section III, B).

The latency period of bradykinin was relatively short (range: 0-2 minutes) when time for diffusion into the tissue is taken into consideration. This indicates that the peptide acts near and possibly directly on the ovarian

smooth-muscle tissue. Bradykinin may influence ovarian smooth muscle by regulating calcium transport, just as it is thought to do in myometrial tissue of the rat uterus (Khairallah and Page, 1963; Walaszek, 1970) where the latency period is similar (one minute) (Walaszek, 1970).

The physiological concentration of the kinins is difficult to assess because of the difficulty in measuring the peptide directly. However, some idea of the possible levels can be made by considering the values in the literature, and by rough estimates from the changes in kininogen reported here. Values in the literature under normal conditions, range from less than 3 ng/ml plasma (Pisano, 1975) to 5 $\pm$ 2 ng/ml plasma (Spragg, 1974). During anaphylactic shock and under parasitic invasions, levels can reach approximately 20-25 ng/ml plasma (see Eisen, 1970). However, destruction in the general circulation is so efficient, that these levels are probably below physiologically effective levels in the target organs. Melmon et al (1967) measured free kinin levels in inflammatory synovial effusion from arthritides of various etiologies and found levels ranging from 1.6-58.0 ng/ml fluid. From the amount of plasma kininogen lost during the preovulatory decline (section I), it appears that the maximum amount of kinin liberated was approximately 1.7 $\pm$ 0.6 ug/ml plasma. When all of these concentrations are compared to the effective dose range observed in this study, 1.75-25.00 ng/ml bathing solution,

it can be seen that even the highest dose was within possible physiological/pathological levels, suggesting that bradykinin-induced ovarian contractions could occur during, and perhaps aid, the natural *in vivo* ovulatory process.

(c) Effects of indomethacin on the bradykinin-induced contractile activity of isolated rat ovaries:

Indomethacin, a prostaglandin synthesis inhibitor, reduced the response of the ovarian musculature to bradykinin. However, due to the small number of experimental trials (four), this finding should be regarded with caution and taken only as preliminary evidence suggesting that prostaglandins partially mediate the contractile effects of bradykinin on ovarian smooth-muscle tissue. In support of this idea, there is much evidence that prostaglandins mediate some of the ovulatory events (Clark *et al.*, 1978) including ovarian contractions (Virutamasen *et al.*, 1972b; Gimeno *et al.*, 1975; Sterin-Borda *et al.*, 1976). This evidence has been described previously in the General Introduction. Also, bradykinin can increase production of prostaglandins in several other tissues (review: Nasjletti and Malik, 1979). On the basis of these findings it is reasonable to postulate that bradykinin could increase the production of intrafollicular prostaglandins in preovulatory ovaries. The prostaglandins could then act additively

with the exogenously administered bradykinin in eliciting ovarian contractions.

(d) Concluding remarks:

These findings, in a preparation totally isolated from somatic influences, provide supportive evidence that bradykinin may play a role in triggering ovarian contractility at the ovarian level, independent of extra-ovarian, neuronal, and endocrine influences. The heightened ovarian contractility in response to bradykinin observed around the anticipated time of ovulation suggests that prevailing local ovarian conditions, probably induced by the preceding LH-peak, influence the sensitivity of the ovarian smooth musculature to bradykinin. Also, it appears that bradykinin can increase contractile activity both directly, and possibly indirectly, via prostaglandins.

Therefore, the data given here suggest that kinins, and the kinin-system are potential agents for producing ovarian muscle contraction, which could be important in ovulation. Although in vitro work can not prove that this is the case, the results suggest that further work could add a new aspect to the ovulatory process. A second aspect, the possibility that kinins could initiate the increased preovulatory ovarian hyperemia, was examined next.

B. THE EFFECTS OF BRADYKININ ON THE PERMEABILITY OF THE RAT OVARIAN FOLLICULAR VASCULATURE:

Introduction:

The purpose of this study was two-fold. First, to determine whether bradykinin could be involved in increasing blood vessel permeability in the ovary of the rat. If so, this work would support the contention that bradykinin is a factor involved in eliciting hyperemia of the ovary observed prior to ovulation, and so may have a physiological function within the ovulatory process. The second goal was to examine the possibilty of prostaglandin involvement in this response.

Rats were treated with the dye Trypan Blue at various stages of the estrus cycle. After specific lengths of exposure, the animals were killed and their ovaries examined by light microscopy to determine the degree of dye movement through mature follicular tissue (the measure used to estimate blood vessel permeability). Similar animals treated in the same manner, also recieved various doses of bradykinin, with or without indomethacin treatment, and the degree of movement was compared.

Materials and methods:

(a) Experimental animals:

Mature female rats were maintained as described in the General Methods section of this thesis. The animals were divided into three groups. Group 1 was used to establish the normal pattern of vascular changes during the estrus cycle. It consisted of 18 rats in various stages of estrus; four were in diestrus, five in proestrus, three in estrus and six in metestrus. Group 2 animals, consisting of 54 rats in the diestrus stage, were used to examine the effects of bradykinin on vascular leakage. Seventeen of these rats were treated with bradykinin for various exposure times and 14 untreated animals served as controls. The other 23 were exposed to different doses of bradykinin for 10 minutes. Group 3 consisted of 14 rats in the diestrus stage. These were used to examine the effects of indomethacin on the bradykinin-induced response. Six animals were treated with bradykinin alone, four with both bradykinin and indomethacin, and 4 with dye alone.

(b) Experimental procedure:

All experiments were performed between 13.00 and 16.00 h. At the appropriate time, each animal was lightly anaesthetized with ether and injected with Trypan Blue (4% - 0.625 ml/ 100 g body weight - Matheson, Coleman, Bill, U.S.A.) alone, or combined with various doses of bradykinin (2.5 - 26.0 µg/

100 g body weight - bradykinin triacetate - Sigma Chemical Co., U.S.A.). The dye or bradykinin-dye combination were injected into the left ventricle of the heart, thus by-passing the lungs (an organ with particularly high kininase activity) and ensuring direct transport of a portion of the material to the ovary. This method was adopted to avoid anything but the least amount of surgery, in order to minimize artificial activation of the kinin system. The animals treated with indomethacin (10mg) (4ml of 2.5 mg/ml carrier [7.0 mg sodium carbonate /ml 0.9% saline]: Sigma Chemical Co., U.S.A.) were given the inhibitor IP, 30 minutes prior to the injection of bradykinin and or dye (according to Cassin, 1980).

After 5, 10, or 15 minutes of exposure to the peptide and/or dye, the animals were killed rapidly by cervical dislocation. The ovaries were then removed and placed in Sierras' Fixative (composition to follow).

Sierras' Fixative

Chemical	% volume
100% ethanol	60
Formalin	30
Glacial acetic acid	10

After 24 hours of fixation the ovaries were embedded in paraffin wax and sectioned at 40μ. The sections were examined by light microscopy.

(c) Treatment groups:

Group 1 - Each of the 20 animals were exposed to Trypan Blue alone for ten minutes.

Group 2 - Fourteen animals recieved Trypan Blue alone; five were sacrificed after 5 minutes exposure, 4 after 10 minutes, and 5 after 15 minutes. The other eighteen rats in this group recieved Trypan Blue plus bradykinin (26.0 µg/100 g body weight); seven were killed after 5 minutes, 6 after 10 minutes, and 5 after 15 minutes. A further 23 rats recieved Trypan Blue and bradykinin in various doses, four recieved no bradykinin, four recieved 2.5 µg/100g body weight, five recieved 7.0 µg/100 g body weight, four recieved 13.0 µg/100 g body weight and six recieved 26.0 µg/100 g body weight. All were sacrificed 10 minutes later.

Group 3 - Six animals in this group served as controls, and were injected with Trypan Blue and bradykinin (26.0 µg/100 g body weight). The other four were pretreated with indomethacin 30 minutes prior to an injection of Trypan Blue and bradykinin (26.0 µg/100 g body weight). All were sacrificed 10 minutes after the dye/peptide injection.

(d) Assessment of blood vessel permeability:

Increases in the permeability of blood vessels were assessed from the ability of Trypan Blue to accumulate in areas where the capillary permeability had increased (Menkin, 1940). Precise measurement was difficult, but an

attempt was made to quantitate the results on an arbitrary scale of 0-3 (see Table 2).

(e) Statistics:

All values recorded are averages from groups of follicles (± S.E.M.); the number of follicles in each group are indicated on the graphs. Results were compared, using the Student's t-test for two independent samples (Steel and Torrie, 1960).

Results:

(a) Changes of dye movement through maturing ovarian follicles at different stages of the estrus cycle:

Figure 17 shows the relative amount of dye movement (quantified according to the arbritrary scale) in the maturing follicles of rats sacrificed at various stages of the estrus cycle. The graph demonstrates that there was little or no dye in the follicular tissue from animals in the diestrus and metestrus stages. However, at proestrus and estrus (newly ruptured follicles were examined at the estrus stage), the dye had diffused completely throughout the follicle, and accumulated in the antrum. This finding corroborates the evidence of others (Burr and Davis, 1951, Motta, 1971) indicating that blood supply to the ovarian tissues increases as ovulation nears.

TABLE II

THE ARBITRARY SCALE USED TO QUANTIFY DYE DISTRIBUTION THROUGHOUT THE FOLLICULAR TISSUE

SCALE	APPEARANCE OF DYE DISTRIBUTION THROUGHOUT THE FOLLICULAR TISSUE
0	No dye (blue color) visible outside of the blood vessels (Plate 1A).
1	Small quantity of dye leaking from the blood vessels. Dye appears as a narrow "halo" around some blood vessels. No dye has reached the follicular cavity (Plate 1B).
2	Dye has permeated further from the blood vessels and traces are often seen within the follicular cavity. Dye concentrations within the blood vessels are still heaviest (Plate 1C).
3	The dye has completely dispersed throughout the follicle. The dye is quite often no more concentrated in the blood vessels than in the tissue. Dye has accumulated in the follicular cavity (Plate 1D).

FIGURE 17: THE DEGREE OF DYE MOVEMENT THROUGHOUT MATURING OVARIAN FOLLICLES OF RATS AT DIFFERENT STAGES OF THE ESTRUS CYCLE

Groups of rats at four different stages of the estrus cycle (diestrus, proestrus, estrus, and metestrus) were exposed to Trypan Blue for 10 minutes. The amount of dye movement throughout maturing follicles (new corpora lutea at estrus) was examined by light microscopy and according to an arbitrary scale (see Table 2). The values are expressed as the mean + the standard error of the mean (represented by the dots above). Numbers above represent the number of follicles in each group. Ordinate: The degree of dye movement (quantified according to an arbitrary scale). Abscissa: The phases of the estrus cycle.

*During the estrus phase of the cycle tissue from newly ruptured follicles (corpora lutea) were examined.

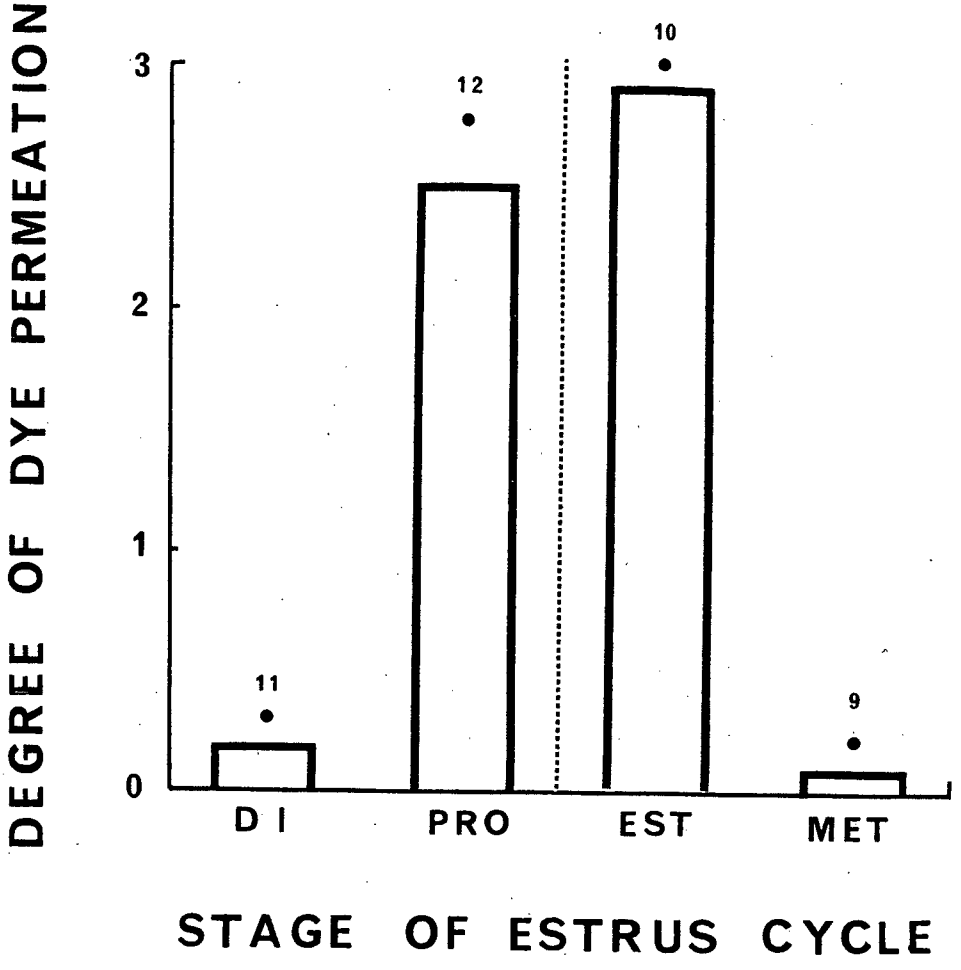
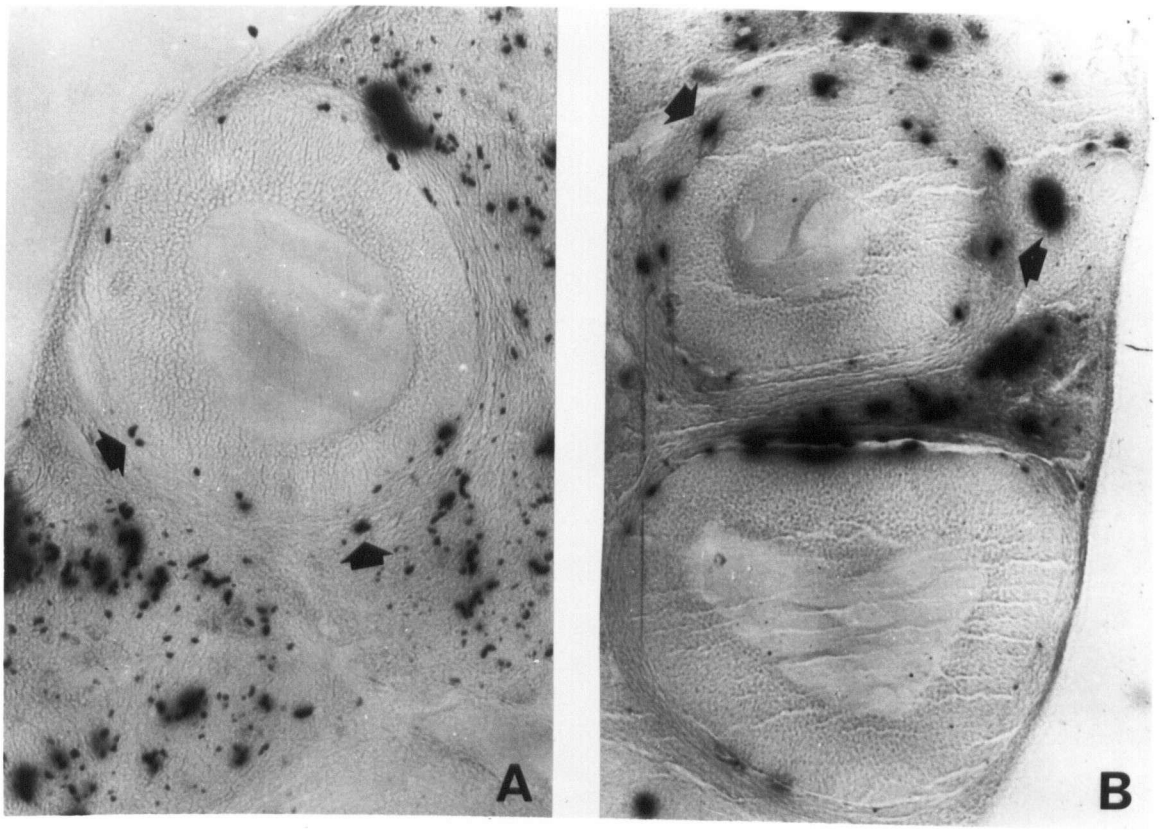
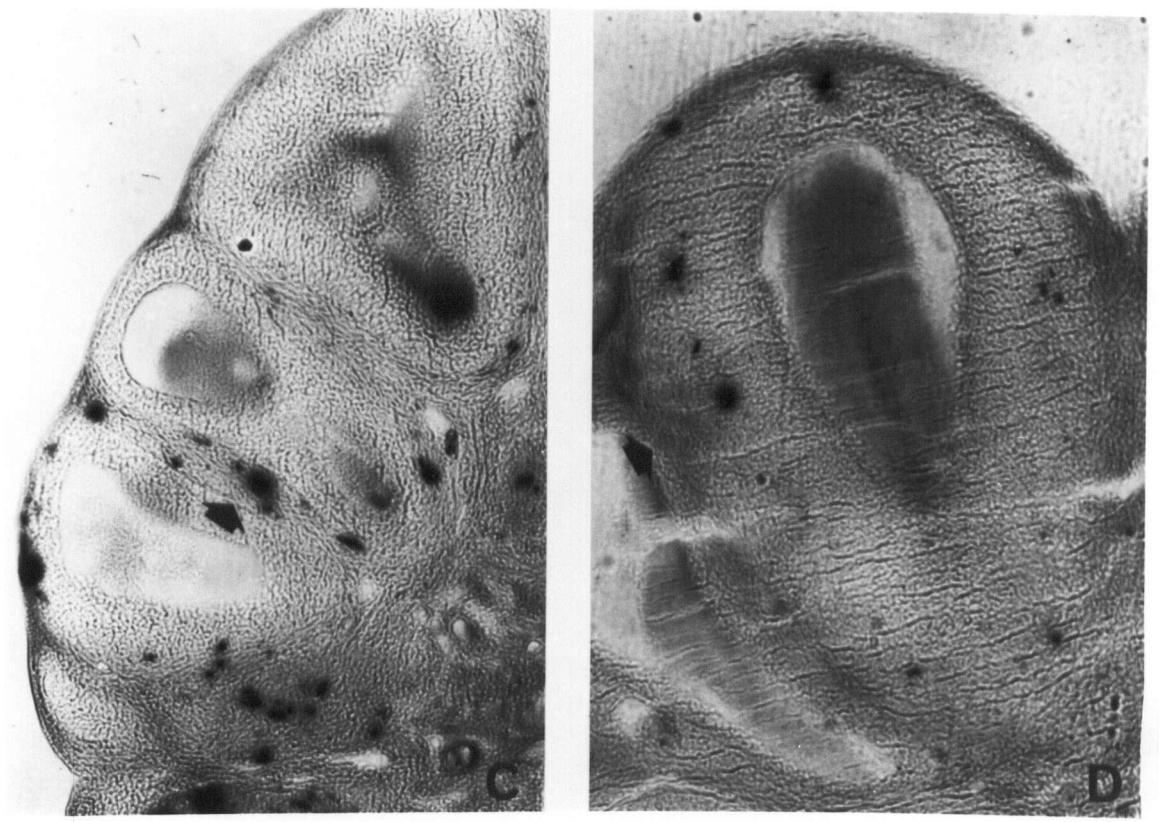

DEGREE OF DYE PERMEATION
3
2
1
0
11
12
10
9
DI
PRO
EST
MET
STAGE OF ESTRUS CYCLE

PLATE I: EXAMPLES OF THE FOUR CLASSIFICATIONS OF DYE DISTRIBUTION THROUGH THE RAT OVARIAN FOLLICULAR TISSUE

Arrows indicate thecal vasculature of the Graafian follicle studied.
Magnified 150X.

A. Graafian follicle representative of "0" on the arbitrary scale.
B. Graafian follicle representative of "1" on the arbitrary scale.
C. Graafian follicle representative of "2" on the arbitrary scale.
D. Graafian follicle representative of "3" on the arbitrary scale.


A
B


C
D

(b) The effect of bradykinin on dye movement in the maturing ovarian follicle:

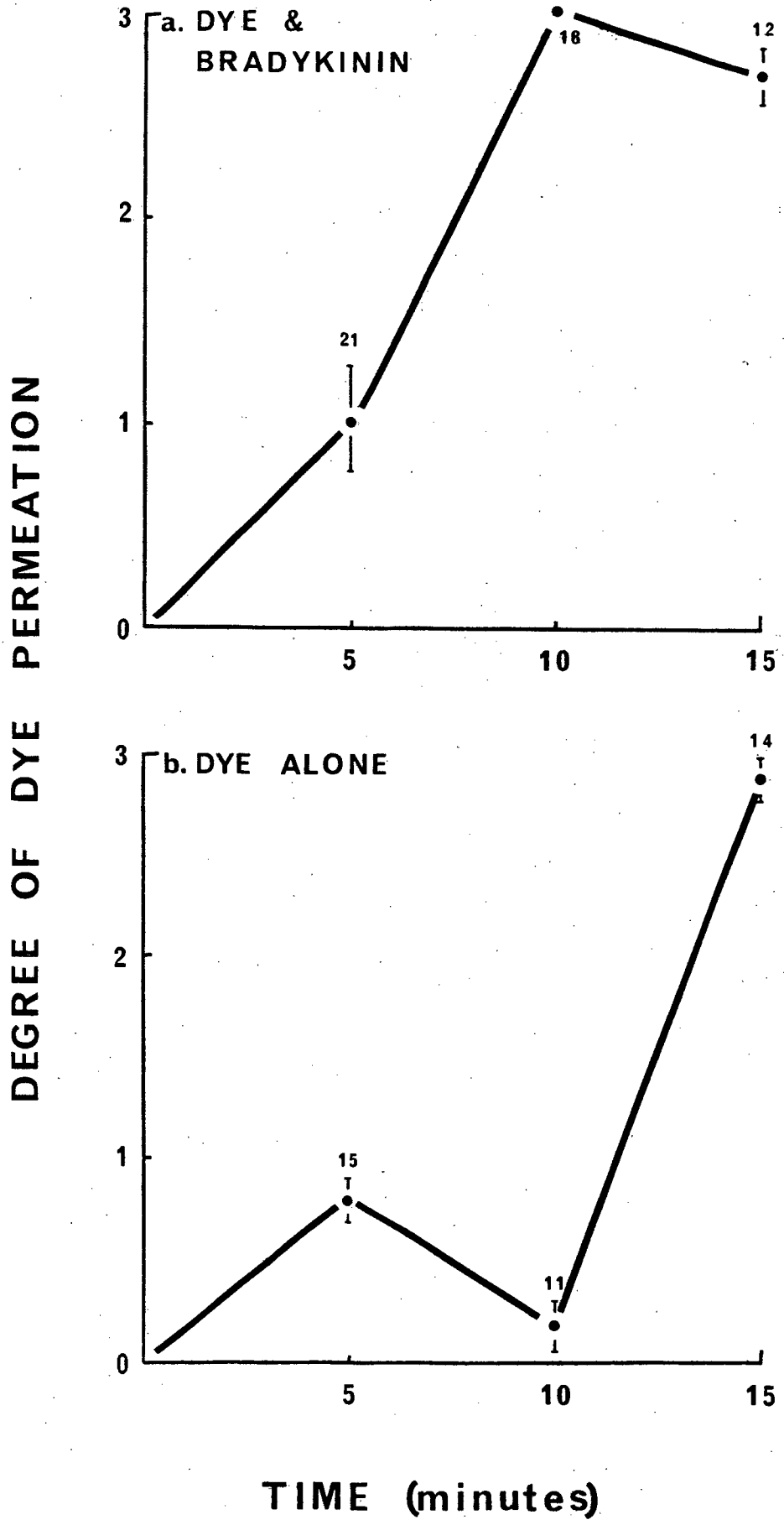
The natural changes in the kinin system studied previously (see section I) appeared to coincide approximately with the natural increases in dye movement noted in this study. This correlation suggested that the kinin system could be involved in producing this phenomenon. To test this hypothesis, diestrus animals were exposed, for various periods, to different doses of bradykinin along with Trypan Blue, or Trypan Blue alone (controls), and their ovaries were compared.

(i) The effect of different exposure times:

In order to test the above hypothesis, as well as to gain some insight regarding the latency period of a possible response, and to establish the optimal exposure period for demonstrating bradykinin-induced responses, diestrus animals were exposed to a relatively high dose of bradykinin (21.0 ug/100 g body weight) and Trypan Blue, or Trypan Blue alone (controls). After various exposure times (5-15 minutes), the ovaries were compared. As Figure 18 demonstrates, after a five minute exposure period there was very little difference in the amount of dye movement throughout follicular tissue from control and experimental animals. However, after a 10 minute exposure period follicular tissue from the experimental animals showed a significantly greater amount of dye movement than similar tissue from the

FIGURE 18: THE EFFECT OF DIFFERENT LENGTHS OF EXPOSURE TO BRADYKININ ON THE DEGREE OF DYE MOVEMENT THROUGHOUT MATURING OVARIAN FOLLICLES OF RATS IN THE DIESTRUS STATE

Groups of rats in the state of diestrus were exposed to (a.) Trypan Blue alone or (b.) in combination with bradykinin (26.0 µg/100 g body weight) for exposure times of 5, 10, or 15 minutes. The amount of dye movement throughout maturing follicles was examined by light microscopy and quantified according to an arbritrary scale (see Table 2). Each point represents the mean value of dye movement from a group of similarily treated follicles. The vertical bars represent the standard errors of the means. Numbers above represent the number of follicles examined in each group.
Ordinate: The degree of dye movement (quantified according to an arbritrary scale).
Abscissae: The length of exposure to the hormone and/or dye (minutes).


a. DYE &
BRADYKININ
b. DYE ALONE
DEGREE OF DYE PERMEATION
TIME (minutes)
0
1
2
3
5
10
15
21
18
12
15
11
14

control animals ($P < 0.01$)groups again. However, with a 15 minute exposure the dye had reached all areas of the follicles, in both groups. These data suggest that (1) bradykinin was capable of increasing dye movement in the diestrus follicular tissue, (2) the latency period of the response was between 5 and 10 minutes, and (3) the 10 minute exposure period was optimal for demonstrating bradykinin-induced responses.

(ii) The effect of different doses of bradykinin:

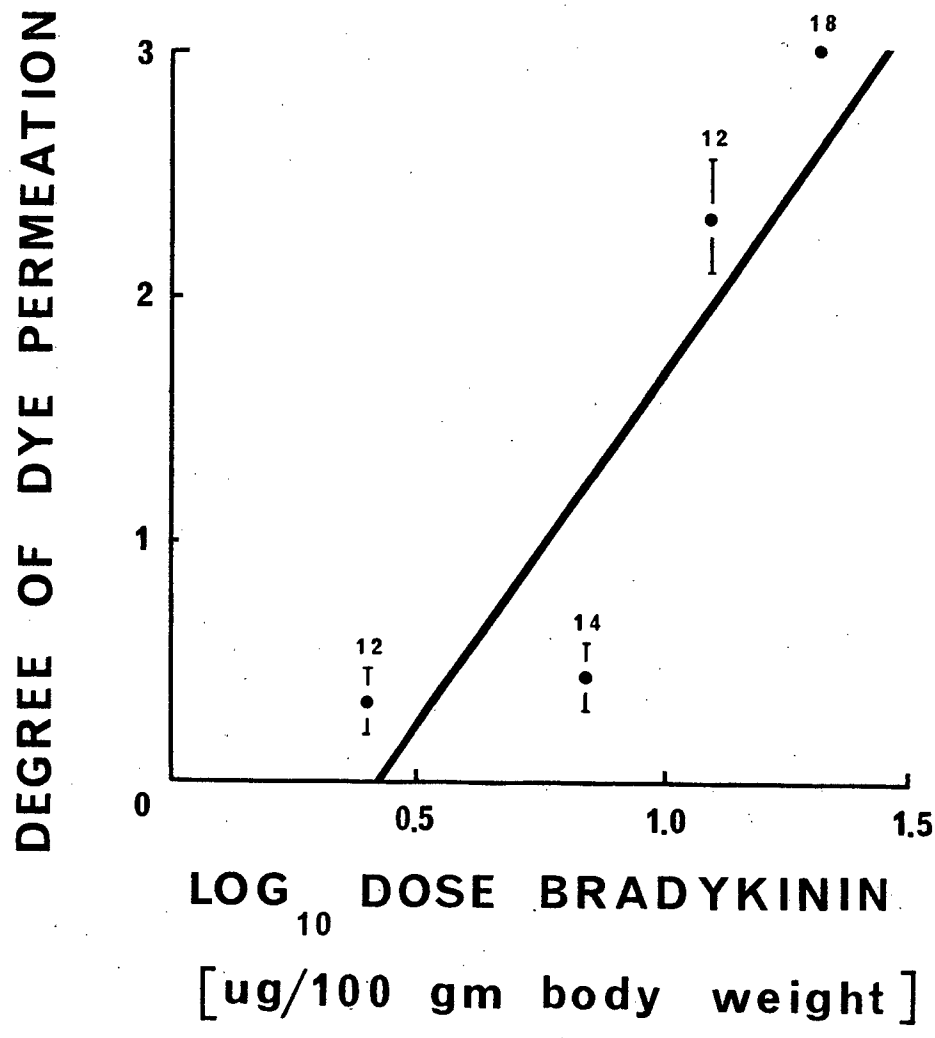
Figure 19 demonstrates that there was an approximately linear $\log_{10}$ - dose/response relationship between bradykinin and dye movement in maturing follicles from diestrus rats exposed for 10 minutes to various doses of bradykinin. The lowest dose capable of causing any additional dye movement into this tissue (threshold dose) within the 10 minute exposure period was 7.0 µg/100 g body weight. Doses as low as 26.0 µg/100 g body weight could cause complete diffusion of dye throughout the tissue with this time interval.

(c) The influence of indomethacin on the bradykinin-induced response:

In order to determine if prostaglandins were mediating the bradykinin-induced response, experimental animals were pretreated with indomethacin (10 mg - IP) 30 minutes prior to a 10 minute exposure to bradykinin (26.0 µg/100 g body weight) and dye. Control animals were divided into two

FIGURE 19: THE EFFECT OF DIFFERENT DOSES OF BRADYKININ ON THE DEGREE OF DYE MOVEMENT THROUGHOUT MATURING OVARIAN FOLLICLES OF RATS IN THE DIESTRUS STATE

Groups of rats in the state of diestrus were exposed to Trypan Blue and various doses of bradykinin for 10 minutes. The amount of dye movement throughout maturing follicles was examined by light microscopy and quantified according to an arbitrary scale (see Table 2). Each point represents the mean value of dye movement from a group of follicles treated with the same dose of bradykinin. The vertical bars represent standard errors of the means. Numbers above the bars represent the number of follicles examined in each group.
Ordinate: The degree of dye movement (quantified according to an arbitrary scale).
Abscissa: Dose of bradykinin (ug/100 g body weight).


DEGREE OF DYE PERMEATION
3
2
1
0
0.5
1.0
1.5
18
12
12
14
LOG 10 DOSE BRADYKININ
[ug/100 gm body weight]

groups. Control group 1 recieved bradykinin and dye in the same dose as the experimental animals (but no indomethacin). Control group 2 recieved only dye. Figure 20. shows that the amount of dye movement was significantly lower in follicles of the experimental animals (those pretreated with indomethacin) than in follicles of control group 1 animals (those receiving bradykinin and dye) ($P < 0.01$). However, the amount of dye movement in experimental follicles was still significantly higher than that in similar tissues from control group 2 animals (those recieving only dye) ($P < 0.01$). These findings suggest that prostaglandins partly mediated this bradykinin-induced response.

Discussion:

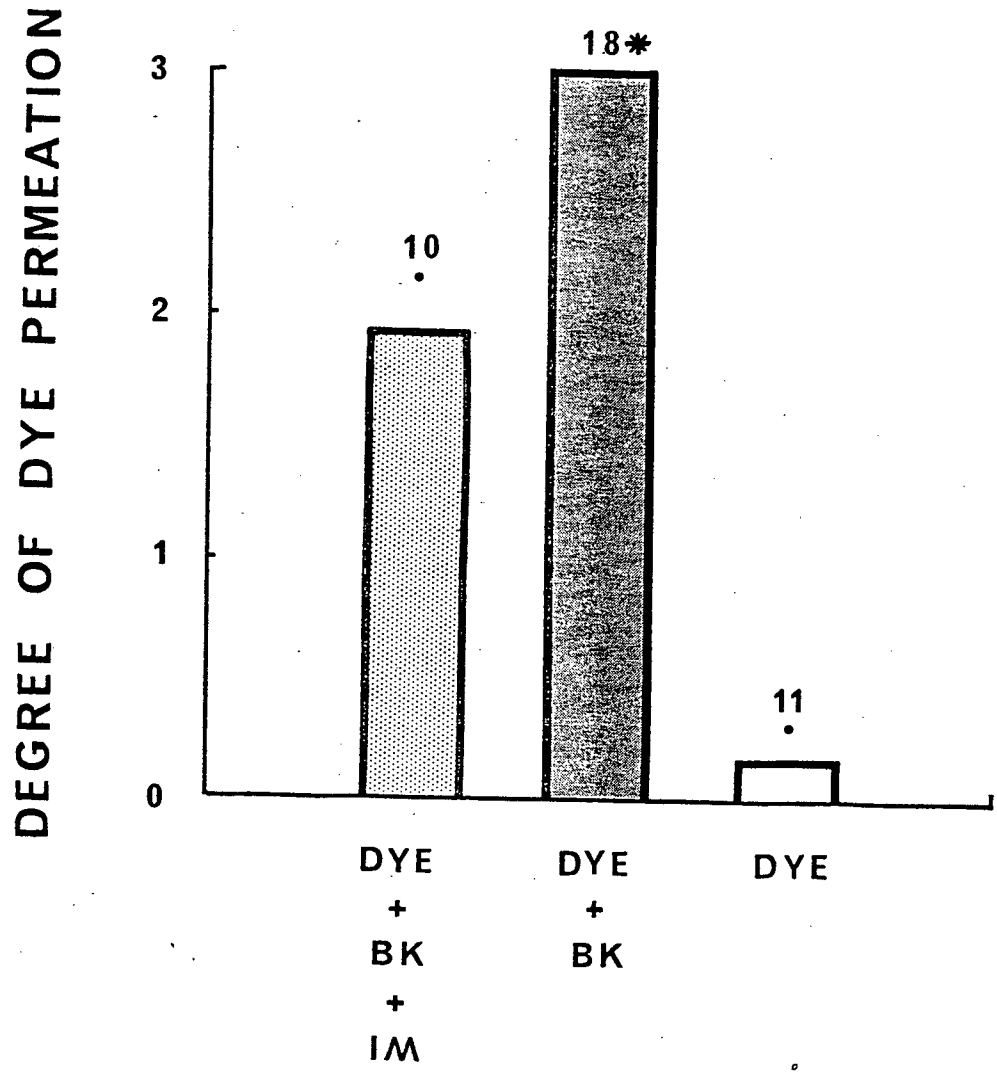
The results given here suggest that bradykinin is capable of modifying the ovarian follicular vasculature of the rat, in a similar manner to the changes that occur as ovulation nears. The preliminary finding that pretreatment with indomethacin can reduce, but not eliminate, this bradykinin-induced response suggests some degree of prostaglandin involvement.

(a) Permeability of the ovarian vasculature before and after ovulation:

In this study, by utilizing the technique of Trypan Blue injection, the degree of follicular blood vessel

FIGURE 20: THE INFLUENCE OF INDOMETHACIN ON BRADYKININ-INDUCED INCREASES IN THE DEGREE OF DYE MOVEMENT THROUGHOUT MATURING OVARIAN FOLLICLES OF RATS IN THE DIESTRUS STATE

Groups of rats in the state of diestrus were exposed for 10 minutes to (1) Trypan Blue alone, (2) Trypan Blue and bradykinin (26.0 ug/100 g body weight), or (3) Trypan Blue and bradykinin (26.0 ug/100 g body weight) as well as indomethacin (10 mg - IP) 30 minutes prior to the hormone - dye injection. The amount of dye movement throughout maturing follicles was examined by light microscopy and quantified according to an arbitrary scale (see Table 2). The values are expressed as the mean value for each group of follicles ± the standard error of the mean (represented by the dots above). Numbers above the bars represent the number of follicles in each group.
Ordinate: The degree of dye movement (quantified according to an arbitrary scale).
Abscissa: The type of treatment recieved.


DEGREE OF DYE PERMEATION
3
2
1
0
10
18*
11
DYE + BK + IM
DYE + BK
DYE

permeability at different stages of the rat estrus cycle was estimated and compared. Near the anticipated time of ovulation (proestrus) there was a marked increase of Trypan Blue diffusion out of the follicular blood vessels and into the surrounding tissue, suggesting increased vascular permeability. This increase persisted in the newly formed corpora lutea, just after ovulation (estrus). These findings were in agreement with those of other workers (rats - Basset, 1943; Parr, 1974; rabbits - Burr and Davis, 1951; Zachariae, 1958), and indicate that this technique is capable of detecting the natural changes in follicular vasculature associated with ovulation.

When administered into the general circulation, Trypan Blue forms a complex with plasma proteins which is small enough to pass through endothelial gaps and the basement membrane, and to enter the surrounding tissue (Majno, 1964). Therefore, Trypan Blue indicates blood vessel permeability not only to fluids, but to plasma proteins as well. Therefore, the enhanced permeability of ovarian blood vessels during the crucial preovulatory period may not only increase follicular volume but may also increase the rate of exchange of proteins and possibly hormones between the ovary and the general circulation.

(b) The effect of bradykinin on permeability of the follicular blood vessels:

In diestrus rats, where the degree of dye movement was normally low, bradykinin significantly increased the degree of dye movement, imitating the natural increase observed near ovulation (proestrus and estrus). Therefore, from this evidence it appears that bradykinin is capable of increasing the permeability of follicular blood vessels to fluids and plasma protein-like molecules in an apparently similar way to that occurring during the ovulatory process.

The exact mechanism(s) by which bradykinin altered blood vessel permeability could not be determined with the techniques used here. From electron microscopic studies of other tissues, it appears that bradykinin might act by: (1) contracting endothelial cells thus increasing the number and size of pores or intercellular gaps (Majno, 1964; Majno et al., 1969; Joris et al., 1972; Bignold and Lykke, 1975), (2) increasing the size and turnover rate of pinocytotic vesicles (Renkin et al., 1974) and/or (3) opening up more capillary beds through its vasodilator abilities (Renkin et al., 1974). Simionescu et al., (1978) noted that bradykinin opened endothelial intercellular junctions of the mouse diaphragm to 30-60 Å.. If bradykinin has the same effect on follicular blood vessels, it could possibly increase their permeability not only to fluid and protein, but also to gonadotropins (Stokes radius -

Bovine LH: 27.6 Å [Riechert et al., 1969]).

One would expect any direct action of exogenous bradykinin to be relatively short-lived because of this hormone's extremely short half-life in the general circulation (Eisen, 1970; Arrigoni-Martelli, 1977).

Although difficult to assess, the effective doses of bradykinin in this study were probably within, or close to physiological range. As mentioned previously, values in the literature range from approximately less than 3 to 25 ng/ml plasma and at least 1.7 ± 0.6 ug Bk-equiv./ml plasma were released just before ovulation in the rat (see section I) (calculated from the amount of plasma kininogen lost during the preovulatory decline). If rats have approximately 2.5 ml plasma/100 g body weight (calculated on the basis of data from Rowett, 1974), then the range from the literature represents roughly 7.5 to 62.5 ng bradykinin/100 g body weight, and the preovulatory kininogen decline represents a release of roughly 3.7 µg bradykinin/100g body weight. However, whilst the spontaneously-released kinin may be released in higher levels within the target site, the amount of injected kinin reaching the ovary is reduced by rapid destruction in the general circulation. Therefore, the noted effective dose range of 7.0 µg - 26.0 µg/100 g body weight could be close to the physiological range; if so, these bradykinin-induced increases in follicular blood vessel permeability could occur during natural

in vivo ovulatory processes.

(c) The effect of indomethacin on the bradykinin-induced ovarian response:

In this study, blocking prostaglandin synthesis diminished, but did not abolish, the increased follicular blood vessel permeability response to bradykinin, in the majority of cases. This finding, although preliminary, due to the small number of samples, suggests that bradykinin might stimulate the synthesis of prostaglandins in the ovary, and that the resultant prostaglandins enhance the effect of bradykinin on the follicular vasculature. Additional, although circumstantial, evidence for this hypothesis comes from other sources. First, there is much evidence suggesting that prostaglandins mediate some of the ovulatory events (Clark *et al.*, 1978), possibly including increases in ovarian hyperemia (Lee and Novy, 1978). Second, prostaglandins often act as mediators and/or modulators of kinins in many tissues (see Nasjletti and Malik, 1979).

(d) Concluding remarks:

In conclusion, the results presented here indicate that physiological doses of bradykinin have the potential to initiate at least one event of the ovulatory process, namely the functional hyperemia necessary for increased follicular volume. Whether bradykinin exerts its effect directly, or through prostaglandin synthesis, remains uncertain, but the preliminary evidence suggests some prostaglandin involvement.

GENERAL DISCUSSION

The experiments described in the preceding sections of this thesis have uncovered four main lines of evidence supporting the contention that the kinin system plays a functional role in the mammalian ovulatory process. First, results described in section I provide evidence for a temporal relationship between kinin system activation, the preovulatory LH surge, and ovulation. There was a marked preovulatory decline in plasma kininogen levels of three different species along with a concomitant change in kinin-forming enzymes in the ovary and plasma of rats around the anticipated time of the LH surge. This suggests that kinins were released just before ovulation. Second, the evidence suggesting the presence of kinin-forming enzymes in ovarian tissue provided support for a spatial relationship between the kinin system and its proposed target site, indicating that the system could be activated locally, at the level of the ovary. Third, in section II it was observed that LH, the only well established ovulatory stimulus, but not estradiol - 17β , was able to activate the kinin system as indicated by the lowered kininogen levels in response to both exogenous and anticipated endogenous LH. Fourth, and last, the evidence presented in section III gives a basis for potential actions of kinins in the ovulatory process.

The results indicate that exogenous bradykinin can initiate two important ovulatory events, namely ovarian contractions, and enhanced follicular blood vessel permeability.

Although further investigations are required, the evidence indicates with added certainty that the kinin system is involved in mammalian ovulation.

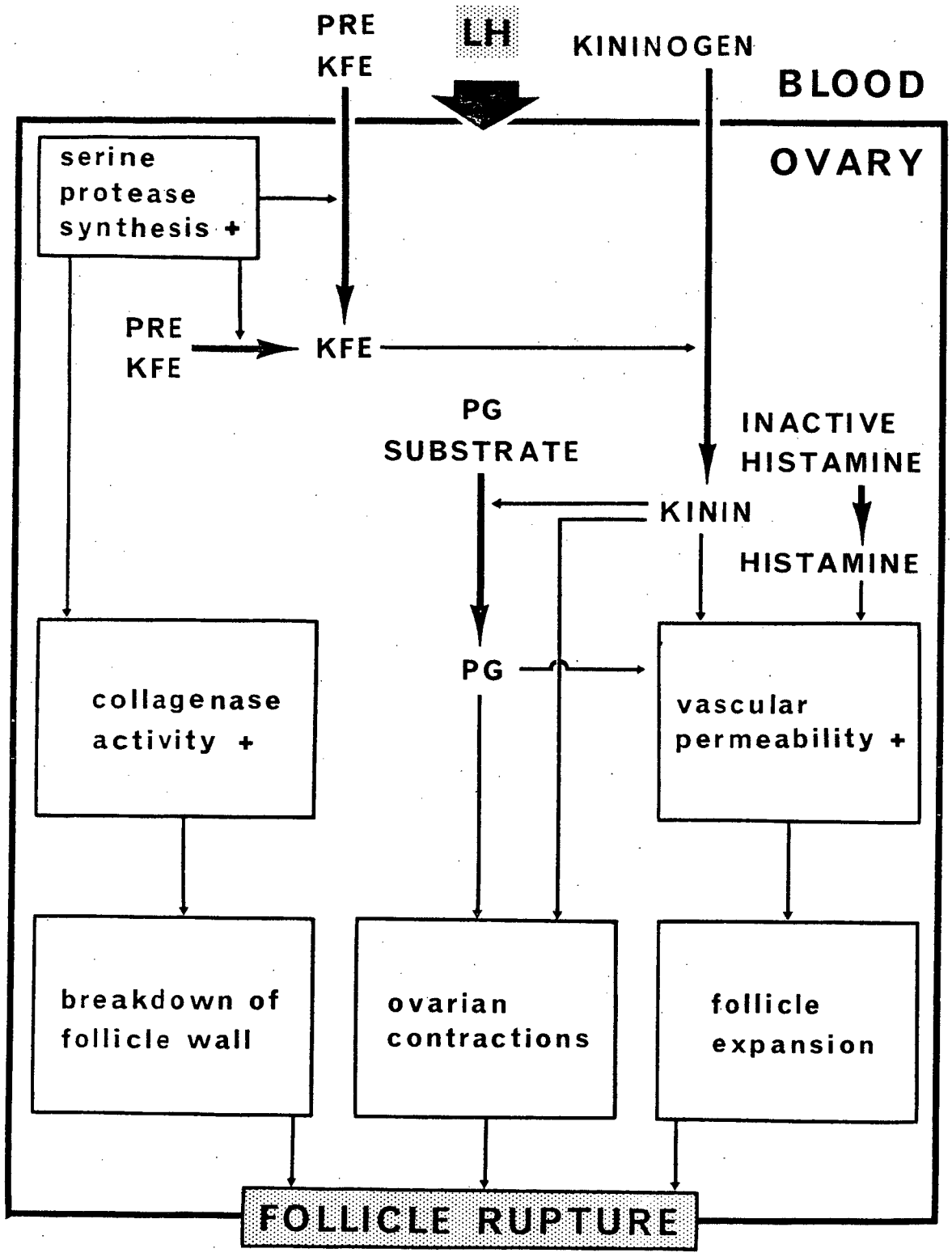
1. A hypothetical model of the involvement of the kinin system in ovulation

The evidence collected in each section of this thesis has been discussed previously with regards to the validity and meaning of the data, as well as its relationship to the work of others. In the remaining section, I have attempted to integrate all of the available data into a hypothetical model of the involvement of the kinin system in mammalian ovulation. Although often speculative, it seems to have potential value in integrating the evidence for the involvement of bradykinin in ovulation, and in suggesting further, more definitive studies.

Figure 21 outlines a hypothetical model of how the kinins might mediate the action of LH in inducing ovulation at the level of the ovary. As illustrated, the ovulating surge of LH, and perhaps some as yet unknown mechanism, probably initiates processes leading to activation of kinin-forming enzymes in the ovary. The kinin-forming enzymes (kallikreins and/or kininogenases) once formed,

FIGURE 21: A HYPOTHETICAL MODEL OF THE KININ SYSTEM INVOLVEMENT IN OVULATION

This model is based on evidence presented in this thesis and in the current literature. KFE = kinin-forming enzyme, PG = prostaglandin, and + = increased.


PRE
KFE
LH
KININOGEN
BLOOD
OVARY
serine
protease
synthesis +
PRE
KFE
KFE
PG
SUBSTRATE
INACTIVE
HISTAMINE
KININ
HISTAMINE
PG
collagenase
activity +
vascular
permeability +
breakdown of
follicle wall
ovarian
contractions
follicle
expansion
FOLLICLE RUPTURE

could perpetuate their own production directly, or through Hageman Factor activation, and would convert plasma kininogen to kinin as it circulated through the ovarian tissue. The kinins could then act directly, and indirectly, through prostaglandins, to enhance ovarian contractions and again, directly or indirectly through prostaglandins and histamine, to increase follicular hyperemia (and hence follicular swelling). These two events, combined with a weakening of the follicle wall, are probably directly responsible for follicular rupture and ovum extrusion.

2. Concluding remarks

In conclusion, the findings to date provide strong support, but not complete proof of the hypothesis that the kinin system mediates, at least in part, the control of ovulation in mammals. A more definite assessment awaits the availability of a satisfactory kinin antagonist. Further investigation in this area will most likely be important to the field of reproductive physiology and perhaps even fertility regulation.

LITERATURE CITED

Abelous, J.and Bardier, E. (1909). Les substances hyptenive de l'urine humaine normale. Compt. Rend. Soc. Biol. 66:511 (quoted from Burr and Davis, 1951).

Amenta, F., Allen, D., Didio, L., and Motta, P. (1979). A transmission electron microscope study of smooth muscle cells in the ovary of rabbits, cats, rats, and mice. J. Submicrosc. Cytol. 11:39.

Amsterdam, A., Linder, H., Groschel-Stewart, U. (1977). Localization of actin and myosin in the rat oocyte and follicular wall by immunofluorescence. Anat. Rec. 187:311.

Antonio, A. (1968). The relaxing effect of bradykinin on intestinal smooth muscle. Br. J. Pharmacol. Chemother. 32:78.

Arrigoni-Martelli, E. (1977). Bradykinin. In: Inflammation and Antiinflammatories. Spectrum Publications, New York. pp 29-41.

Barabe, J., Marceau, F., Theriault, B., Drouin, J., and Regoli, D. (1979). Cardiovascular actions of kinins in the rabbit. Can. J. Physiol. Pharmacol. 57:78.

Basset, D. (1943). The changes in the vascular pattern of the ovary of the albino rat during the estrus cycle. Am. J. Anat. 73:251.

Bauminger, S., Lieberman, M., and Linder, H. (1975). Steroid independent effect of gonadotropins on prostaglandin synthesis in rat Graafian follicles in vitro. Prostaglandins 9:753.

Bauminger, S. and Linder, H. (1975). Periovulatory changes in ovarian prostaglandin formation and their hormonal control in the rat. Prostaglandins 9:737.

Becker, E., Mota, I., and Wong, D. (1968). Inhibition by antihistamines of the vascular permeability increase induced by bradykinin. Br. J. Pharmacol. 34:330.

Bertelli, A., Donati, L., and Marek, J. (1969). Proteases in inflammatory reactions. In: Bertelli, A. and Houck, J. (editors). Inflammation, Biochemistry, and Drug Interaction. Williams and Wilkins Co., Baltimore. pp 66-83.

Bignold, L. and Lykke, A. (1975). Increased vascular permeability in synovialis of the rat by histamine, serotonin, and bradykinin. Experentia 31:671.

Billings, E., Brown, J., Billings, J., and Burger, H. (1972). Symptoms and hormonal changes accompanying ovulation. Lancet I:282.

Bjersing, L. and Cajander, S. (1975). Ovulation and the role of the ovarian surface epithelium. Experentia 31:605.

Blandau, R. (1966). The mechanisms of ovulation. In: Greenblat, R. (editor). Ovulation; Stimulation, Supression, Detection. Lippincott, Philadelphia.

Blandau, R. (1967). Anatomy of ovulation. Clin. Obstet Gynecol. 10:347.

Boissonnas, R., St. Guttman, A., and Jaquenod, P. (1960). Synthesis of L-ARG-L-PRO-L-PRO-L-GLY-L-PHE-L-SER-L-PRO-L-PHE-L-ARG, a nonapeptide with the properties of bradykinin. Helv.Chim. Acta. 43:1349.

Boling, J., Blandau, R., Soderwell, A., and Young, W. (1941). Growth of the Graafian follicle and the time of ovulation in the albino rat. Anat. Rec. 79:313.

Bonta, I. and Parnham, M. (1978). Prostaglandins and chronic inflammation. Biochem. Pharmacol. 27:1611.

Bowring, N., Earthy, M., and Mangan, F. (1975). Changes in prostaglandin F content of the rabbit ovary associated with ovulation. J. Endocrinol. 64:11P.

Brocklehurst, W. and Zeitlin, I. (1967). Determination of plasma kinin levels in man. J. Physiol. 191:417.

Bryan, F., Ryan, J., and Niemeyer, R. (1972). Bradykininogen synthesis by liver. In: Back, N. and Sicuteri, F. (editors). Vasopeptides, Chemistry, Pharmacology, and Pathophysiology. Plenum Press, New York. pp 43.

Bumpus, F., Smeby, R., Page, I., and Khairallah, P. (1964). Distribution and metabolic fate of angiotensin II and various derivatives. Can. Med. Assoc. J. 90:190.

Burden, H. (1972). Ultrastructural observations on ovarian perifollicular smooth muscle in the cat, guinea pig, and rabbit. Am. J. Anat. 133:125.

Burr, J. and Davies, J. (1951). The vascular system of the rabbit ovary and its relationship to ovulation. Anat. Rec. 111:273.

Butcher, R., Collins, W., and Fugo, N. (1974). Plasma concentrations of LH, FSH, prolactin, progesterone, and estradiol-17β throughout the four day estrus cycle of the rat. Endocrinol. 94:1704.

Carretero, O. and Scicli, G. (1981). Possible role of kinins in circulatory homeostasis. Hypertension 3:I-4.

Carvalho, I. and Dinez, C. (1966). Kinin-forming enzyme (kininogenin) in homogenates of rat kidney. Biochem. Biophys. Acta (Amst.) 128:136.

Cassin, S. (1980). Personal communication.

Challis, J., Erickson, G., and Ryan, K. (1974). Prostaglandin F production in vitro by granulosa cells from rabbit preovulatory follicles. Prostaglandins 7:183.

Chandrabose, K., Lapetina, E., Schmitges, C., Siegel, M., and Cuatrecasas, P. (1978). Action of corticosteroids in regulation of prostaglandin biosynthesis in cultured fibroblasts. Proc. Natl. Acad. Sci. USA 75:214.

Clark, M., Triebwasser, W., Marsh, J., and LeMaire, W. (1978). Prostaglandins in ovulation. Ann. Biol. Anim. Biochim. Biophys. 18:427.

Cochrane, C. and Griffin, J. (1979). Molecular assembly in the contact phase of the Hageman factor system. Am. J. Med. 67:657.

Coleman, R., Mattler, L., and Sherry, S. (1969). Studies on the prekallikrein (kallikreininogen)-kallikrein enzyme system of human plasma. J. Clin. Invest. 48:11.

Coutinho, E. and Maia, H. (1972). Effects of gonadotrophins on motility of human ovary. Nature (New Biol.) 235:94.

Diaz-Infante, A., Virutamasen, P., Connaughton, J., Wright, K., and Wallach, E. (1974). In vitro studies of human ovarian contractility. Obstet. Gynecol. 44:830.

Diaz-Infante, A., Wright, K., and Wallach, E. (1975). Influence of estrogen and progesterone treatment on ovarian contractility in the monkey. Fertil. Steril. 26:101.

Diaz-Infante, A., Wright, K., and Wallach, E. (1974). Effects of indomethacin and prostaglandin F_2 on ovulation and ovarian contractility in the rabbit. Prostaglandins 5:567.

Donovan, B. and Lockhart, A. (1972). Light and the timing of ovulation in the guinea-pig. J. Reprod. Fertil. 30:207.

Edwards, R. (1974). Follicular fluid. J. Reprod. Fertil. 37:189.

Eeckhout, V. and Vaes, G. (1977). Further studies on the activation of procollagenase, the latent precursor of bone collagenase. Biochem. J. 166:21.

Eisen, V. (1970). Formation and function of kinins. Rheumatol. 3:103.

Elliot, D., Horton, E., and Lewis, G. (1960). Actions of pure bradykinin. J. Physiol. 153:473.

Erdos, E. (1976). The kinins. Biochem. Pharmacol. 25:1563.

Eskay, R., Warberg, J., Mical, R., and Porter, J. (1975). Prostaglandin E_2-induced release of LHRH into hypophysial portal blood. Endocrinol. 97:816.

Espey, L. (1967). Ultrastructure of the apex of the rabbit Graafian follicle during the ovulatory process. Endocrinol. 81:267.

Espey, L. (1974). Ovarian proteolytic enzymes and ovulation. Biol. Reprod. 10:216.

Espey, L. (1975). Evaluation of proteolytic activity in mammalian ovulation. In: Reich, E., Rifkin, D., and Shaw, E. (editors). Proteases and Biological Control. Cold Spring Harbor Conferences on Cell Proliferation. Vol. 2, pp767-776.

Espey, L. (1978). Ovarian contractility and its relationship to ovulation: A review. Biol. Reprod. 19:540.

Espey, L. (1980). Ovulation as an inflammatory reaction - A hypothesis. Biol. Reprod. 22:73.

Espey, L. and Lipner, H. (1965). Enzyme-induced rupture of rabbit Graafian follicle. Am. J. Physiol. 208:208.

Espey, L. and Rondell, P. (1968). Collagenolytic activity in the rabbit and sow Graafian follicle during ovulation. Am. J. Physiol. 214:326.

Everett, J. (1948). Progesterone and estrogen in the experimental control of ovulation time and other features of the estrous cycle in the rat. Endocrinol. 43:389.

Fahey, J., Ciosek, C., and Newcombe, D. (1977). Human synovial fibroblasts: The relationships between cyclic AMP, bradykinin, and prostaglandins. Agents Actions 7:255.

Feldberg, W. (1955). The physiological significance of local hormones. In: Gaddum, J. (editor). Polypeptides Which Stimulate Plain Muscle. Livingstone, Edinburgh. pp7-11.

Fink, G. (1976). Control of the ovarian cycle in the rat. In: Crosignani, P. and Mishell, D. (editors). Ovulation in the Human. Academic Press, New York. pp95-114.

Fox, R., Goldsmith, R., Kidd, D., and Lewis, G. (1961). Bradykinin as a vasodilator in man. J. Physiol. 157:589.

Gallo, R. (1980). Neuroendocrineregulation of pulsatile luteinizing hormone release in the rat. Neuroendo. 30:122.

Gimeno, M., Borda, L., Rettori, V., Borda, E., and Gimeno, A. (1973). In vitro contractile activity of rat and guinea pig ovaries. Effects of oxytocin and electrical stimulation. Acta Physiol. Latinoam. 23:363.

Gimeno, M., Borda, E., Sterin-Borda, L., Vidal, J., and Gimeno, A. (1976). Pharmacological influences on human ovarian contractions. Obstet. Gynecol. 47:218.

Gimeno, M., Gimeno, A., and Rettori, V. (1975). Physiologic and pharmacologic studies on the motility of isolated guinea pig ovaries. Fertil. Steril. 26:422.

Gimeno, A., Gimeno, M., Sterin de Borda, L., and Borda, E. (1974). Endocrine factors influencing in vitro pharmacological responses of tubal and ovarian smooth muscle. Acta Physiol. Latinoam. 24:164.

Greaves, M., Yamamoto, S., and Mahzoon, B. (1976). The mast cell: Interrelationships between histamine and prostaglandins. Clin. Exp. Dermatol. 1:327.

Haberman, E. (1970). Kininogens. Handbook Exp. Pharmacol. 25:250

Harms, P., Ojeda, S., and McCann, S. (1973). Prostaglandin involvement in hypothalamic control of gonadotropin and prolactin release. Sci. 181:760.

Heape, W. (1905). Ovulation and degeneration of ova in the rabbit. Proc. R. Soc. Lond. Ser B 76:260.

Heimberger, N. (1975). Proteinase inhibitors of human plasma - their properties and control functions. In: Reich, E., Rifkin, D., and Shaw, E. (editors). Proteases and Biological Control. Cold Spring Harbor Conferences on Cell Proliferation. Vol 2. pp367-386.

Hilgers, T., Abraham, G., and Cavanagh, D. (1978). Natural family planning: I. The peak symptom and estimated time of ovulation. Obstet. Gynec. 52:575.

Holten, P. (1948). A modification of the method of Dale and Laidlaw for standardization of posterior pituitary extract. Br. J. Pharmacol. 3:328.

Joris, I., Majno, G., and Ryan, G. (1972). Endothelial contraction in vivo: A study of the rat mesentery. Virchow's Arch. Abt. B. Zellpath. 12:73.

Khairallah, P. and Page, I. (1963). Effects of bradykinin and angiotensin on smooth muscle. Ann. N.Y. Acad. Sci. 104:212.

Kellermeyer, R. and Graham, R. (1968). Kinins - possible physiologic and pathologic roles in man. N. Engl. J. Med. 279:802

Kobayashi, M., Shikimi, T., Miyata, S., and Ohata, K. (1979). Studies on kinin-forming enzyme in rat stomach. Jap. J. Pharmacol. 29:947.

Konzett, H. and Sturmer, E. (1960). Biological activity of synthetic polypeptides with bradykinin-like properties. Br. J. Pharmacol. 15:544.

Kraut, H., Frey, E., and Werle, E. (1930). Der nachivus unes kreislaufhormons in der pankreasdriese. Hoppe-Seyler's Z. Physiol. Chem. 189:53.

Landis, E. and Pappenheimer, J. (1963). Capillary permeability. Handbook of Physiol. 2:961.

Lee, W. and Novy, M. (1978). Effects of luteinizing hormone and indomethacin on blood flow and steroidogenesis in the rabbit ovary. Biol. Reprod. 18:799.

LeMaire, W. and Marsh, J. (1975). Interrelationships between prostaglandins, cyclic AMP, and steroids in ovulation. J. Reprod. Fertil. Suppl. 22:53.

LeMaire,W., Yang, N., Behrman, H., and Marsh, J. (1973). Preovulatory changes in the concentration of prostaglandins in rabbit Graafian follicles. Prostaglandins 3:367.

Leme, J. (1978). Bradykinin-system. In: Vane, J. and Ferreira, S. (editors). Handbook of Experimental Pharmacology. Springer-Verlag, New York, pp 464-522.

Lewis, G. (1963). Pharmacological actions of bradykinin and its role in physiological and pathological reactions. Ann. N.Y. Acad. Sci. 104:236.

Lewis, G. (1970). Kinins in inflammation and tissue injury. In: Erdos, E. (editor). Handbook of Experimental Pharmacology. Vol 25. Springer-Verlag, New York. pp516-529.

Lipner, H. and Maxwell, B. (1960). Hypothesis concerning the role of follicular contractions in ovulation. Sci. 131:1737.

Majno, G. (1964). Mechanisms of abnormal vascular permeability in acute inflammation. In: Thomas, L., Uhr, J., and Grant, L. (editors). Injury, Inflammation, and Immunity. Williams and Wilkins, Baltimore. pp 58-93.

Manjo, G., Shea, S., and Leventhal, M. (1969). Endothelial contraction induced by histamine-type mediators. An electron microscopic study. J. Cell Biol. 42:647.

Margolis, J and Bishop, E. (1963). Studies on plasma kinins. I. Composition of the kininogen complex. Aust. J. Exptl. Biol. Med. Sci. 41:293.

Marin-Grez, M.and Carretero, O. (1972). A method for measurement of urinary kallikrein. J. Appl. Physiol. 32:428.

Markee, J. (1932). Rhythmic vascular uterine changes. Am. J. Physiol. 100:32.

McCormack, C. (1978). Personal communication.

McCormack, C. and Bennin, B. (1970). Delay of ovulation caused by exposure to continuous light in immature rats treated with pregnant mare's serum gonadotrophin. Endocrinol. 86:611.

McCormack, C. and Sridaran,. (1978). Timing of ovulation in rats during exposure to continuous light: Evidence for a circadian rhythm of luteinizing hormone secretion. J. Endocrinol. 76:135.

McCormack, J. and Senior, J. (1971). Effect of sex hormones on the concentration of plasma kininogen in the female rat. Br. J. Pharmacol. 43:421P.

McCormick, J. and Senior, J. (1974). Plasma kinin and kininogen levels in female rat during oestrus cycle, pregnancy, parturition, and puerperium. Br. J. Pharmacol. 50:237.

McDonald, M. and Perks, A. (1976). Plasma bradykininogen and reproductive cycles: Studies during the oestrous cycle and pregnancy in the rat, and in the human menstrual cycle. Can. J. Zool. 54:941.

Melmon, K., Webster, M., Goldfinger, S., and Seegmiller, J. (1967). The presence of a kinin in inflammatory synovial effusion from arthitides of varying etiologies. Arthr. Rheum. 10:13.

Menkin, V. (1940). Dynamics of Inflammation. MacMillan, New York. pp 16-23.

Morales, T., Woessner, J., Howell, D., Marsh, J., and LeMaire, W. (1978). A microassy for the direct demonstration of collagenolytic activity in Graafian follicles of the rat. Biochim. Biophys. Acta. 524:428.

Motta, P., Cherney, D., and Didio, L. (1971). Scanning and transmission electron microscopy of the ovarian surface in mammals with special reference to ovulation. J. Submicr. Cytol. 3:85.

Movat, H. (1979). The kinin system and its relation to other systems. Curr. Top. Pathol. 68:111.

Munsick, R. (1960). Effect of the magnesium ion on the response of the rat uterus to neurohypophyseal hormones and analogues. Endocrinol. 66:451.

Nakano, J. (1965). Effects of synthetic bradykinin on the cardiovascular system. Arch. Int. Pharmacodyn 153:1.

Nasjletti, A. and Malik, K. (1979). Relationships between kallikrein-kinin and prostaglandin systems. Lif. Sci. 25: 99.

Nustad, K., Orstavik, T., Gautvik, K., and Pierce, J. (1978). Glandular kallikreins. Gen. Pharmacol. 9:1.

Odya, C., Goodfriend, T., Stewart, J., and Pena, C. (1978). Aspects of bradykinin radioimmunoassay. J.Immunol. Meth. 19:243.

O'Grady, J., Caldwell, B., Auletta, F., and Speroff, L. (1972). The effects of an inhibitor of prostaglandin synthesis (indomethacin) on ovulation, pregnancy, and pseudopregnancy in the rabbit. Prostaglandins 1:97.

Ojeda, S., Jameson, H., and McCann, S. (1977). Hypothalamic areas involved in prostaglandin (PG)-induced gonadotropin release. I: Effects of PGE_2 and PGF_2 implants on luteinizing hormone release. Endocrinol. 100:1585.

Okamura, H., Virutamasen, P., Wright, K., and Wallach, E. (1972). Ovarian smooth muscle in the human being, rabbit, and cat. Am. J. Obstet. Gynecol. 112;183.

O'Shea, J. (1970a). An electron microscope study of smooth muscle, and its innervation, in the ovary of the rat. J. Anat. 106:196A(Abstr).

O'Shea, J. (1970b). An ultrastructural study of smooth muscle-like cells in the theca externa of ovarian follicles in the rat. Anat. Rec. 167:127.

O'Shea, J. (1971). Smooth muscle-like cells in the theca externa of the ovarian follicles in the sheep. J. Reprod. Fertil. 24:283.

O'Shea, J. and Phillips, R. (1974). Contractility of ovarian follicles from sheep in vitro. J. Reprod. Fertil. 36:457.

Osman, P. and Dullaart, J. (1976). Intra-ovarian release of eggs in the rat after indomethacin treatment at pro-estrus. J. Reprod. Fertil. 47:101.

Osman, P. and Lieuwma-Noordanus, C. (1980). Ovulation from rat and hamster ovaries in vitro. J. Reprod. Fertil. 59:431.

Osvaldo-Decima, L. (1970). Smooth muscle in the ovary of the rat and monkey. J. Ultrastruct. Res. 29:218.

Owman, C., Sjoberg, N., Svensson, K., and Walles, B. (1975). Autonomic nerves mediating contractility in the human Graafian follicle. J. Reprod, Fertil. 45:553.

Palti, Z. and Freund, M. (1972). Spontaneous contraction of the human ovary in vitro. J. Reprod. Fertil. 28:113.

Parr, E. (1975). Rupture of ovarian follicles at ovulation. J. Reprod. fertil. Suppl. 22:1.

Parr, E. (1974). Histological examination of the rat ovarian follicle wall prior to ovulation. Biol. Reprod. 11:483.

Percival-Smith (1982). Personal communication.

Pisano, J. (1975). Chemistry and biology of the kallikrein-kinin system. In: Reich, E., Rifkin, D., and Shaw, E. (editors). Proteases and Biological Control. Cold Spring Harbor Conferences on Cell Proliferation. Vol. 2.

Powers, K., Malik, K., and Nasjletti, A. (1981). Kinin-forming activity in the anterior pituitary of the rat. Fed. Proc. 40:401. (Abstr).

Prasad, S., Raviprakash, V., Sabir, M., and Bhattacharyya, N. (1975). Changes in blood histamine and bradykininogen levels during different periods of the oestrous cycle in goats. J. Reprod. Fertil. 42:229.

Ramwell, P., Shaw, J., and Jessup,S. (1969). follicular fluid kinin and its action on fallopian tube. Endocrinol. 84:931.

Reichert, L., Rasco, M., Ward, D., Niswender, G., and Midgley, A. (1969). Isolation and properties of sub-units of bovine pituitary luteinizing hormones. J. Biol. Chem. 244:5110.

Renkin, E., Carter, R., and Joyner, W. (1974). Mechanism of the sustained action of histamine and bradykinin on transport of large molecules across capillary wall in the dog paw. Microvasc. Res. 7:49.

Roca, R., Garofalo, E., Piriz, H., Martino, I., and Rieppi, G. (1977). Influence of the estrous cycle on the action of oxytocin on rat ovarian contractility in vivo. Fertil. Steril. 28:205.

Roca, R, Garofalo, E., Piriz, H., Martino, I., Rieppi, G. and Sala, M. (1976). Effects of oxytocin on in vitro ovarian contractility during the estrus cycle of the rat. Biol. Reprod, 15:464.

Rocerto, T., Jacobowitz, D., and Wallach, E. (1969). Observations of spontaneous contractions of the cat ovary in vitro. Endocrinol. 84:1336.

Rocha e Silva, M. (1963). The physiological significance of bradykinin. Ann. N.Y.Acad. Sci. 104:190.

Rocha e Silva, M. (1974). Present trends of kinin research. Lif. Sci. 15:7.

Rocha e Silva, M., Beraldo, W., and Rosenfeld, G. (1949). Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulins by snake venoms and by trypsin. Am. J. Physiol. 156: 261.

Rondell, P. (1964). Follicular pressure and distensibility in ovulation. Am. J. Physiol. 207:590.

Rondell, P. (1970). Biophysical aspects of ovulation. Biol. Reprod, 2, Suppl. 2:64.

Rowett, H. (1974). The Rat as a Small Mammal. J. Murray Ltd., London. pp 40.

Rowley, D. (1964). Venous constriction as cause of increased vascular permeability produced by 5-hydroxytryptamine, histamine, bradykinin, and 48/80 in rat. Br. J. Exper. Pathol. 45:56.

Sampaio, C., Wong, S., and Shaw, E. (1974). Human plasma kallikrein - purification and preliminary charactarization. Arch. Biochem. Biophys. 165:133.

Schacter, M. (1969). Kallikreins and kinins. Physiol. Rev. 49:509.

Schacter, M. (1980). Kallikreins (kininogenases) - a group of serine proteases with bioregulatory actions. Pharmacol. Rev. 31:1.

Schacter, M. and Barton, S. (1979). Kallikreins (kininogenases) and kinins. In: DeGroot, L., Cahill, G., Martini, L., Potts, J., Nelson, D., Steinberger, E., and Winegrad, A. (editors). Endocrinology. Grune and Stratton, New York. pp 151-152.

Senior, J. and Whalley, E. (1974). Variation in the oestrogen-progesterone ratio and its effect on plasma kininogen levels in the rat. J. Reprod. Fertil. 41:425.

Senior, J. and Whalley, E. (1976). Plasma kininogen during the oestrous cycle, early pregnancy, and pseudopregnancy in the rat. J. Physiol. 256:167.

Sharma, J. (1978). Effect of prostaglandins (PGE_2 and PGF_2) on plasma kininogen levels in rats. Biomed. Expr. 29:292.

Shvarts, G. (1980). Effects of antihistamines on the action of bradykinin. Bu-l. Exp. Biol. Med. 88:1135.

Simionescu, N., Simionescu, M., and Palade, G. (1978). Structural basis of permeability in sequential segments of the microvasculature of the diaphragm. Microvasc. Res. 15:17.

Spragg, J. (1974). The plasma kinin-forming system. In: Weissmann, G. (editor). Mediators of Inflammation. Plenum Press. New York. pp 85-111.

Steel, R. and Torrie, J. (1960). Principles and Procedures of Statistics. McGraw-Hill Co. Inc. New York. pp481.

Steele, M., Negro-Vilar, A., and McCann, S. (1980). Effect of central injection of bradykinin and bradykinin potentiating factor upon release of anterior pituitary hormones in ovarectomized female rats. Peptides 1:201.

Sterin-Borda, L., Borda, E., Gimeno, M., and Gimeno, A. (1976). Spontaneous and prostaglandin- or oxytocin-induced motility of rat ovaries isolated during different stages of the estrous cycle: Effects of norepinephrine. Fertil. Steril. 27:319.

Stockard, C. and Papanicolaou, G. (1917). The existence of a typical oestrous cycle in the guinea pig with a study of its histological and physiological changes. Am. J. Anat. 22:225.

Stoklosowa, S. and Szoltys, M. (1978). Hormonal dynamics in the preovulatory ovarian follicle and the oocyte in rat during proestrus. Ann. Biol. Anim. Biochim. Biophys. 18:503.

Strickland, S. and Beers, W. (1976). Studies of the role of plasminogen activator in ovulation. J. Biol. Chem. 251:5694.

Szego, C. and Gitin, E. (1964). Ovarian histamine depletion during acute hyperaemic response to luteinizing hormone. Nature 201:682.

Tsafriri, A., Koch, Y., and Lindner, H. (1973). Ovulation rate and serum LH levels in rats treated with indomethacin or prostaglandin E_2. Prostaglandins 3:461.

Tsafriri, A., Lindner, H., Zor, U., and Lamprecht, S. (1972). Physiological role of prostaglandins in the induction of ovulation. Prostaglandins 2:1.

Tsuru, H., Ishikawa, N., and Shigei, T. (1976). Responsiveness of isolated dog veins to bradyinin and other bioactive peptides: Distribution of sensitivity to bradykinin and possible correlation with genesis of the venous system. Blood Vessels 13:238.

Turner, C. and Bagnara, J. (editors). (1971). General Endocrinology. (5th edition). W.B. Saunders Co., Philadelphia. pp516.

Vane, J. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature New Biol. 231:232.

Vane, J. (1976). Prostaglandins as mediators of inflammation. Adv. Prosta. Throm. Res. 2:791.

Virutamasen, P., Hickok, R., and Wallach, E. (1971). Local ovarian effects of catecholamines on human chorionic gonadotropin-induced ovulation in the rabbit. Fertil. steril. 22:235.

Virutamasen, P., Smitasiri, Y., and Fuchs, A. (1976). Intraovarin pressure changes during ovulation in rabbits. Fertil. Steril. 27:188.

Virutamasen, P., Wright, K., and Wallach, E. (1972a). Effects of catecholamines on ovarian contractility in the rabbit. Obstet. Gynecol. 39:225.

Virutamasen, P., Wright, K., and Wallach, E. (1972b). Effects of prostaglandins E_2 and F_2 on ovarian contractility in the rabbit. Fertil. Steril. 23:675.

Virutamasen, P., Wright, K., and Wallach, E. (1973). Ovarian contractility - its relationship to ovulation. Fertil. Steril. 24:763;

Vogel, R. and Werle, E. (1970). Kallikrein inhibitors. In: Erdos, E. and Wilde, A. (editors). Handbook of Experimental Pharmacology. Vol. 25. Springer-Verlag, New York. pp 213.

Walaszek, E. (1970). The effect of bradykinin and kallidin on smooth muscle, In: Erdos, E. and Wilde, A. (editors). Handbook of Experimental Pharmacology. Vol. 25. Springer-Verlag, New York. pp 421-429.

Wallach, E., Wright, K., and Hamada, Y. (1978). Investigation of mammalian ovulation with an in vitro perfused rabbit ovary preparation. Am. J. Obstet. Gynecol. 132:728.

Walles, B., Edvinsson, L., Nybell, G., Owman, C., and Sjoberg, N. (1974). Amine-induced influence on spontaneous ovarian contractility in the guinea pig and the cat. Fertil. Steril. 25:602.

Walles, B., Edvinsson, L., Owman, C., Sjoberg, N., and Sporrone, B. (1976). Cholinergic nerves and receptors mediating contraction of the Graafian follicle. Biol. Reprod. 15:565.

Walles, B., Edvinsson, L., Owman, C., Sjoberg, N., Sporrong, B., and Stefenson, A. (1977a). Influence of sympathetic nerves, amine receptors and anti-adrenergic drugs on follicular contractility and ovulation. Acta. Phys. Scand. Suppl. 452:113.

Walles, B., Edvinsson, L., Owman, C., Sjoberg, N., and Svensson, K. (1975). Mechanical response in the wall of ovarian follicles mediated by adrenergic receptors. J. Pharmacol. Exp. Therap. 193:460.

Walles, B., Falck, B., Owmann, C., and Sjoberg, N. (1977). Charactarization of autonomic receptors in the smooth musculature of human Graafian follicle. Biol. Reprod. 17: 423.

Walles, B., Groschel-Stewart, U., Owman, C., Sjoberg, N., and Unsicker, K. (1978). Fluorescence histochemical demonstration of a relationship between adrenergic nerves and cells containing actin and myosin in the rat ovary, with special reference to the follicle wall. J. Reprod. Fertil. 52:175.

Werle, E. (1936). Uber kallikrein aus blut. Biochem. Z. 287:235. (quoted from Schacter, 1969).

Wright, K., Wallach, E., Fromm, E., and Jeutter, D. (1976). Studies of rabbit ovarian contractility using chronically implanted transducers. Fertil. Steril. 27:310.

Yang, N., Marsh, J., and LeMaire, W. (1973). Prostaglandin changes induced by ovulatory stimuli in rabbit Graafian follicles. The effect of indomethacin, Prostaglandins 4:395.

Zachariae, F. (1958). Studies on the mechanism of ovulation. Acta Endocrinol. 27:339.

Zarrow, M., Yochim, J., and McCarthy, J. (editors) (1964). Experimental Endocrinology. A Scourcebook of Basic Techniques. Academic Press, New York, pp 24.

Zeitlin, I., Singh, Y., Lembeck, F., and Theiler, M. (1976). The molecular weights of plasma and intestinal kallikreins in rats. Naunyn-Schmiedeberg's Arch. Pharmacol. 293:159.

Zurier, R. (1974). Prostaglandins. In: Weissmann, G. (editor). Mediators of Inflammation. Plenum Press, New York. pp 163-180.

Zuspan, K. and Zuspan, F. (1979). Basal body temperature. In: Hafez, E. (editor). Human Ovulation. Elsier/North-Holland Publishing Co., Amsterdam. pp 291-298.