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Localization of relaxin in the corpus luteum of the pregnant rat

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LOCALIZATION OF RELAXIN IN THE CORPUS LUTEUM OF THE PREGNANT RAT

by

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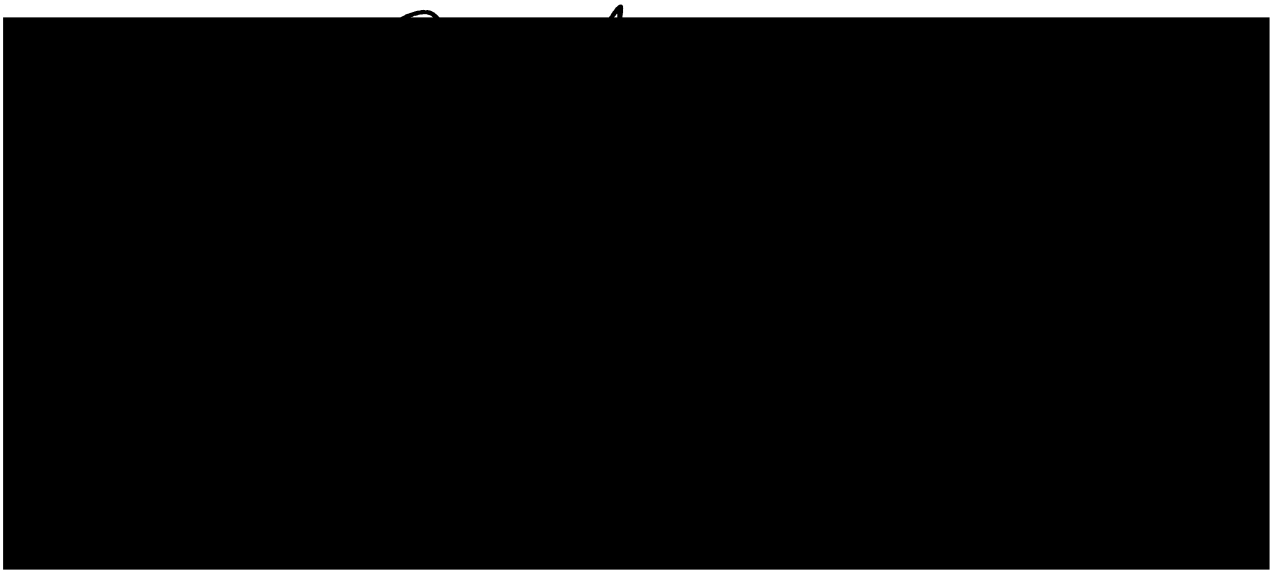

Localization of Relaxin in the Corpus Luteum of the Pregnant Rat.
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The site(s) of relaxin storage in the pregnant rat - ovary vs. uterine metrial gland - has been in dispute. Three different approaches were employed to determine the site(s) of relaxin storage. By means of the fluorescent antibody technique and bioassay of extracts from various rat tissues it can be concluded that relaxin is stored in luteal cells of the ovary in the pregnant rat. Furthermore, the fluorescent antibody method demonstrated that relaxin was localized in the paranuclear area of the cell, presumably in the Golgi region, indicative of a secretory process taking place in the cell. Cell fractions prepared from luteal cells further demonstrated, by means of bioassay of the fractions and electron microscopic study of the fractions, that relaxin was confined either to granules or mitochondria. I feel that one can safely rule out mitochondria as the source of relaxin since this organelle has never been known to be involved in synthesis or storage of a protein hormone destined for secretion. The granule population is, therefore, responsible for the storage of relaxin. Furthermore, this and other work strongly suggests that a specific granule population, not lysosomal in nature, is the specific site of localization of relaxin. This, however, cannot be demonstrated until the intracellular localization of relaxin at the ultrastructural level is performed using peroxidase labelled antibody to relaxin.

The role of granular cells of the uterine metrial gland as a source of relaxin in the pregnant rat is still not settled. Bioassay of tissue extracts from various rat tissues revealed that relaxin activity in the metrial gland was not detectable. By means of the indirect fluorescent antibody method only one experiment out of

six demonstrated the presence of relaxin in the granular cells of the metrial gland. However, other investigators claim to have localized relaxin in these granular cells by the indirect fluorescent antibody technique. Failure to obtain such consistent results in my studies may have been a result of low concentrations of relaxin in the granular cells and/or the presence of a different molecular species of the relaxin molecule. Either of these possibilities might require longer incubation times with the anti-relaxin antiserum and fluorescein labelled anti-rabbit gamma globulin to demonstrate the presence of relaxin than were utilized in my investigations.

Therefore, it can be concluded that relaxin is present in luteal cells of the pregnant rat, and it is strongly suggested that a specific granule population is the storage site of relaxin. The granular cells of the metrial gland as a source of relaxin is still questionable. This problem may not be resolved until further information concerning the nature of the relaxin molecule(s) is obtained.

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TABLE OF CONTENTS

LIST OF TABLES

I. INTRODUCTION

The role of relaxin in reproductive physiology has been a topic of considerable discussion and investigation since 1926 when Hisaw first named and described an ovarian hormone which relaxed the pelvic ligaments of the guinea pig in preparation for delivery of its young. Since that time relaxin has been demonstrated to be present in a variety of animals. Furthermore, species variations are evident in regard to the actions of relaxin and the tissue localization of the hormone. Therefore, it appears that relaxin is adapted to the particular requirements of a species for parturition.

There has been controversy as to whether the hormone is stored in the ovary or the uterine metrial gland in the pregnant rat. There is evidence in the literature which supports both views. The actions of relaxin in the pregnant rat are, nonetheless, well known. The hormone causes softening and dilation of the cervix, inhibits spontaneous motility of the uterus, alters the uterine composition by increasing the content of water, glycogen, and alkaline phosphatase, aids in the lobuloaveolar duct growth in the mammary gland, and, along with estrogen and progesterone, stimulates uterine growth as indicated by an increase in nitrogen concentration and an increase in dry weight.

Approach to the Problem

The aim of this investigation is to clarify the controversy surrounding the storage site(s) of relaxin in the pregnant rat. Long (1973) demonstrated the presence of a distinct granule population, not lysosomal in nature, in luteal cells

of the pregnant rat. This granule population first appears at day 14 of gestation, migrates to the luteal cell surface by day 20, and is absent after parturition. This parallels the increase and decrease of relaxin content in ovaries of the pregnant rat (L.L. Anderson, 1971). Thus, it was suspected that these granules may be a source of relaxin in this species.

Three different approaches were taken to investigate the sites of relaxin storage in the pregnant rat.

(1) To determine the sites of localization of relaxin the indirect fluorescent antibody technique was applied to various tissues of the pregnant rat. These included the ovary, metrial gland, nonplacental uterus, and adrenal gland.

(2) Tissue extracts were prepared from various tissues of the pregnant rat including luteal cells, other ovarian tissue, metrial glands, and nonplacental uteri. These extracts were subsequently bioassayed for relaxin activity.

(3) Utilizing continuous sucrose gradient centrifugation and differential centrifugation, cell fractionation studies of luteal cells from pregnant rats were undertaken to isolate a fraction which, upon bioassay, would reveal high relaxin activity. This information would then be correlated with the morphology of the fraction to determine the subcellular component which stores relaxin.

II. LITERATURE REVIEW

Isolation, Purification, and Physicochemical Properties of Relaxin

Progress in the investigation of relaxin has been hampered by the lack of a national or international standard. The main reason for this is the lack of a relaxin preparation which has been isolated as a chemical entity. To date no relaxin preparation demonstrates homogeneity as revealed by electrophoretic and chromatographic studies; rather, all preparations reveal several components. Therefore, the manner in which the potency of relaxin is measured is based upon a bioassay method in which the increased distensibility of the pelvic girdles of guinea pigs is a function of the amount of relaxin administered to the animals (Abramowitz et al., 1944). These investigators defined one guinea pig unit (GPU or U) as the amount of relaxin which, when injected into ovariectomized guinea pigs 24 hours after the last of four daily injections of estradiol, produces six hours later unmistakable mobility in two-thirds of a group of twelve animals. This method was later replaced by a 'degree or proportion response' (Frieden and Hisaw, 1950, 1953). Each guinea pig was rated on a scale of 0 to 4 depending on the degree of mobility achieved. The 'total response' of a group of guinea pigs was calculated as:

$$\% \text{ positive responses} \times \frac{\text{sum of individual responses in group}}{\text{number of animals}}$$

One GPU was then defined as that amount of relaxin which results in a total response of 100. This was later modified to 120 (Noall and Frieden, 1956).

Therefore, in reviewing the literature one must keep in mind the different definitions of one GPU.

Even though there is no relaxin standard or pure preparation of relaxin, it has been possible to isolate preparations with high relaxin activity which lend themselves to evaluation. Thus, considerable information regarding the physicochemical nature of relaxin has accumulated.

Isolation and Purification of Relaxin.--Attempts have been made to obtain relaxin in its pure form since it was first discovered in 1926. This hormone has been found in the blood, placenta, ovaries, and/or uteri of many species (Hall, 1960). Progress has been made in the isolation of relaxin but a pure preparation as demonstrated by chromatographic and electrophoretic studies has not been obtained. Originally, pregnant rabbit serum was thought to be the best source of relaxin (Hisaw, 1926, 1929; Fevold et al., 1930a,b,c). However, Albert and Money (1946) found that the original methods of extraction resulted in a 30 to 50% loss of the hormone. They devised a method of extracting relaxin from pregnant rabbit serum by means of precipitation with ethanol and acetone in which complete recovery was achieved.

It was soon discovered that there was a better starting material for the isolation of relaxin. Preparations of dried corpora lutea from sows were employed by Hisaw et al., (1930) and Fevold et al., (1930a,b,c). However, fresh whole ovaries of sows were shown to be an even better source of relaxin from which to isolate relaxin. It was found that the whole ovaries of pregnant sows contain between 3,000 and 9,600 GPU/gram of tissue (Albert et al., 1947). At the present

time this is still the best starting material for the isolation of relaxin since it contains the greatest amount of relaxin per unit wet weight tissue (Hall, 1960).

Various attempts have been made to purify relaxin from these crude ovarian extracts, but a purified preparation of relaxin has not been obtained. Griss and associates (1967) claim to have isolated a purified form of relaxin that does exhibit homogeneity when subjected to paper electrophoresis, acetylcellulose membrane electrophoresis, starch-gel electrophoresis, and paper chromatography by the descending method. The pH at which the electrophoretic studies were performed ranged from 1.4 to 11.0. Relaxin was prepared by a procedure in which the inactive contaminants were denatured by heat; this was followed by fractionation with trichloroacetic acid, gel filtration on Sephadex, and chromatography on a column of DEAE Sephadex. However, Oliver and Larkin (1973) in examining different relaxin preparations by electrophoresis at pH 8.65 found that relaxin prepared according to the method of Griss et al., (1967) had three cathodal components as did an NIH preparation with an activity of 460 GPU/mg and Warner Lambert Reference Relaxin preparation (W1165-A, Lot 8) with an activity of 150 GPU/mg.

Cohen and Kleinberg (1960) extracted relaxin from sow ovaries by means of hot glacial acetic acid, followed by oxycellulose adsorption and elution. The potency of this preparation was 150 GPU/mg. Cohen (1963a) further purified his preparation by means of pH fractionation, fractionation with trichloroacetic acid, and column chromatography using DEAE Sephadex. Upon electrophoresis at pH 8.6 this preparation possessed two cathodal components - one major component followed by a weaker one. It was felt that the weaker component might

be a deamination product of the stronger one. Protein and biological activity were fully recovered by this method of purification. The potency of this preparation was 1,800 GPU/mg.

More recently Sherwood and associates (1973) have described a method for the purification of relaxin and have further characterized its structure. Relaxin was extracted with HCl and 70% acetone from pregnant sow ovaries. The active fraction was then precipitated by increasing the acetone concentration to 95%. For further purification the crude extract was subjected to gel filtration on Sephadex G-50. It was then adsorbed on a column of carboxymethyl cellulose and eluted with a linear gradient of NaCl. Upon elution three distinct relaxin components were collected, one of which was distinct from the other two as demonstrated by acrylamide disc gel electrophoresis at pH 8.9. The mean relative potency of the three relaxin preparations ranged from 2,500 GPU/mg to 3,000 GPU/mg. This is by far the most potent preparation to date.

Physicochemical Properties of Relaxin.--The properties of relaxin were originally investigated by Fevold et al (1930a, b, c). The first crude relaxin preparations of Hisaw and Fevold revealed that relaxin had an isoelectric point of 5.4 to 5.5, was stable in acid solution, and was insoluble in nonpolar solvents. Alkali, oxidizing agents, and proteolytic enzymes destroyed the activity of the hormone. Nitrogen content of the crude preparations was 11% and it was suggested that relaxin may have a peptide-like structure.

Later an extensive biochemical investigation of relaxin was undertaken by Frieden and Hisaw (1953). The most potent preparation which they studied had an activity of 450 GPU/mg. The hormone was characterized as being a water

soluble, neutral peptide or protein with a molecular weight between 10,000 and 12,000. The nitrogen content was approximately 14%. Sugars (hexose and hexosamine) were present but were lost as purification proceeded. They demonstrated that activity was lost by subjecting the preparation to hot alkali or proteolytic enzymes. Activity was also lost by the reduction of disulphide bonds by cysteine, and by methylation of carboxyl groups. However, loss of amino groups by either deamination or acetylation did not significantly impair relaxin activity. The relaxin preparation was stable at elevated temperatures (100^{o}C for 10 to 15 minutes). The absence of arginine was noted and the isoelectric point was found to be pH 7.0. However, they determined that the isoelectric point was a function of the purity of the preparation. They felt that relaxin was probably bound to other proteins in cruder preparations, but could not exclude the presence of more than one molecular species of relaxin.

Frieden (1963) further investigated this problem, but only found a number of substances with different electrophoretic mobilities at pH 8.2 with the bulk of the relaxin activity remaining at the origin. The potency of this preparation was 1000 GPU/mg.

Cohen (1963a) determined the molecular weight of his relaxin preparation, which had a potency of 1,800 GPU/mg, to be between 8,000 and 10,000 and the nitrogen content to be 15.1%. Upon electrophoresis at pH 8.6 the active components in the relaxin preparation migrated toward the cathode, indicative of the basic nature of the preparation. This is not in agreement with the work of Frieden and Hisaw (1953) who characterized relaxin as a neutral peptide or protein. Amino acid analysis of Cohen's relaxin preparation revealed a preponderance of

basic amino acids, including arginine. Carbohydrate was absent, which is in keeping with the observation of Frieden and Hisaw (1953) that more purified preparations lost their carbohydrate moieties.

Griss et al. (1967) found that their relaxin preparation was also a strongly basic polypeptide as demonstrated by electrophoretic studies and amino acid analysis, and had a molecular weight of 5,000 to 10,000.

Recent information on the properties of relaxin has been furnished by Sherwood and associates (1973). Their relaxin preparation, which assayed 2,500 to 3,000 GPU/mg, revealed the presence of three components, one of which was electrophoretically distinct from the other two at pH 8.9. The average molecular weight of these components was approximately 6,300 and the isoelectric point was between pH 10 and pH 11. Gel filtration of reduced and carboxymethylated purified relaxin indicated that relaxin contained two subunits linked by disulphide bond(s). The β subunit had a molecular weight of 3,400 and the α subunit had a molecular weight of 2,900. Amino acid analysis of the subunits revealed the absence of histidine, proline, and tyrosine. All of these amino acids had earlier been reported to be present.

Summary of the Biochemistry of Relaxin.--It can be concluded that relaxin is a water soluble, basic polypeptide which is sensitive to reducing agents and proteolytic enzymes. It is heat stable and stable in acid solution. It's molecular weight is between 5,000 and 10,000. The isoelectric point appears to go to a higher pH as the purification is improved. Carbohydrate moieties appear to be present initially but are lost upon purification. The nitrogen content ranges from

11% in the earlier crude preparations to 15% in one of the more potent purer preparations. It appears that the best relaxin preparations to date are still composed of 2 to 3 components. There are three possible explanations for the heterogeneity of these preparations. It is possible that (1) some of the relaxin is attached to other proteins, (2) some deamination could have taken place, or (3) more than one molecular species of the relaxin molecule exists.

Uterine Relaxing Factor vs. Relaxin.--Since relaxin has not been isolated as a chemical entity, there has been some controversy over the possibility that different relaxin molecules are responsible for different physiologic actions of the hormone (Frieden and Hisaw, 1953). Those who believe this to be the situation use the term 'relaxin' for the component which causes relaxation of the pelvic ligaments and term the component responsible for the inhibition of uterine contractions the 'uterine relaxing factor' (URF). Since the controversy cannot be solved until relaxin is isolated as a chemical entity or the existence of different molecular species is established, this presentation will use the term 'relaxin' throughout and will make no distinction based upon the physiologic actions of the hormone.

Physiologic Actions of Relaxin

The physiologic actions of relaxin have evolved along different lines in different species. However, two generalizations regarding the actions of this hormone can be made: (1) relaxin seems to be adapted to the particular requirements of a species for parturition and (2) relaxin elicits maximal effects on tissues which have been pretreated or primed with estrogen.

Relaxation of Pelvic Ligaments.--Separation of the pubic symphysis elicited by relaxin has been described in the guinea pig, mouse, and deermouse (See Hall, 1960 for references). In all instances estrogen priming is a necessary prerequisite for the action of relaxin.

(a) Guinea pig: The identity of the ovarian hormones responsible for the relaxation of the pubic symphysis in the guinea pig was a point of controversy for quite some time. Zarrow (1946, 1948) investigated the effects of the ovarian hormones on the pubic symphysis in the guinea pig and was able to demonstrate that estradiol, progesterone, and relaxin could separately produce relaxation. However, based upon the time required for each hormone to elicit its effect, it was shown that relaxin was the main hormone involved. Zarrow concluded from his investigations that the separation of the pubic symphysis may be produced by either relaxin or prolonged treatment with estrogen, and that the action of progesterone was an indirect one which caused the release of relaxin from the uterus. It is now well established that, after the injection of relaxin into an estrogen primed guinea pig, symphyseal separation occurs within 4 to 6 hours.

Histological changes in the pubic symphysis of the guinea pig support the observations of hormonal effects on the mobility of pubic symphysis as determined by manual palpation. Relaxation produced after prolonged estrogen treatment resulted from the growth and proliferation of symphyseal tissue and resorption of bone (Talmage, 1947a,b; Heringa and Van der Meer, 1948). Relaxin causes a breakdown and dissolution of the symphyseal cartilage and collagenous connective tissue fibers (Talmage, 1947b). Histological changes following treatment with progesterone were identical to those seen following injection of relaxin (Talmage,

1947b). This supports the conclusion of Zarrow (1946, 1948) that progesterone causes the release of relaxin in the guinea pig.

(b) Mouse: Normal formation of the interpubic ligament in the pregnant mouse and its experimental reproduction by relaxin in ovariectomized, estrogen primed mice has been described by Hall and Newton (1946, 1947) and Hall (1947, 1948). Interpubic separation can be produced by estrogen alone, but it requires much more time (Hall and Newton, 1947). Ovariectomy at the end of the second week of gestation prevents the separation of the pubis. It appears that progesterone is not involved in pelvic relaxation in mice and does not cause the secretion of endogenous relaxin as it does in the guinea pig. In fact, progesterone appears to have an inhibitory effect on interpubic ligament formation (Hall, 1949, 1956a,b).

The histological changes which take place in the mouse interpubic ligament in the latter days of gestation involve the resorption of the pubic bone and the lengthening of the interpubic ligament by the formation of new cartilage and its conversion to a primitive type of collagenous connective tissue (Hall, 1947). Similar histological changes were observed in the formation of the interpubic ligament in estrogen primed mice which had been injected with relaxin (Hall, 1947). This increase in collagenous fibers caused by relaxin in the mouse is unlike the situation in the guinea pig in which breakdown of collagenous fibers occurs.

Symphyseal relaxation does not take place in the rabbit, ewe, rat, or pig (Hall, 1960).

Inhibition of Spontaneous Myometrial Contractions.--Inhibition of spontaneous uterine activity by relaxin preparations has been described in the guinea pig,

rat, and mouse (Krantz et al., 1950; Sawyer et al., 1953; Wiqvist, 1959a; Porter, 1971). The uterus, however, must be pretreated with estrogen before relaxin can elicit its maximal effect. Wiqvist (1959b) demonstrated that uterine motility of spayed mice and rats was inhibited by relaxin, and the effective doses of relaxin were 3 to 7 times larger if the animal had not been pretreated with estrogen. The effects of relaxin on the uterus are thought not to be due to adrenaline or histamine, and uteri which had been exposed to relaxin still responded to oxytocin and acetylcholine (Sawyer et al., 1953).

Little is known about the mechanism of action of relaxin on uterine smooth muscle, but the hormone is specific since it does not inhibit the spontaneous motility of ileal smooth muscle (Sawyer et al., 1953). Miller and Murray (1959) suggested that, in the rat, relaxin acted on the uterus by an adrenergic mechanism since adrenaline shows the same properties as relaxin with regard to the inhibition of uterine motility. However, Paterson (1965) demonstrated that in the presence of adrenergic blocking agents relaxin continued to cause inhibition of uterine contractions, while adrenaline did not. Furthermore, since relaxin does not inhibit the activity of the smooth musculature of the ileum it would not seem to operate by the release of adrenaline since adrenaline will inhibit the motility of the ileum.

Effects on the Uterine Cervix.--During the estrous cycle and pregnancy, histological and physiological changes occur in the uterine cervix. Furthermore, during pregnancy there is a softening and dilation of the cervix in preparation for delivery of the young; this phenomenon has been observed in the cow, rat, pig, mouse, monkey, and human being (Graham and Dracy, 1953; Hisaw and

Zarrow, 1948; Zarrow et al., 1956; Steinetz et al., 1959; Zarrow and Yochim, 1961; Hisaw, 1959; Birnberg and Abitbol, 1958; and Eichner et al., 1956). In these estrogen-primed experimental animals, relaxin has been shown to be responsible for this effect on the cervix.

Yochim and Zarrow (1959) measured dilatability and tensile strength of the rat cervix and found that the former was increased and the latter significantly decreased by relaxin. Kroc and associates (1959) investigated the factors responsible for cervical dilatability in the rat. Estrogen was observed to cause an increase in weight by causing cell growth, progesterone had no effect on cervical dilation, and relaxin in unprimed and primed rats produced a dose-proportional increase in dilatability without an increase in cervical weight. The mechanism of action involved here is not known although investigations have been made in this area (Zarrow and Brennan, 1957; Harkness and Harkness, 1956).

Effects on Uterine Composition.--Zarrow and Brennan (1959) first demonstrated an effect of relaxin on the water content of the uterus in weanling rats. The increase in water content was proportional to the dose of relaxin, and the maximum effect was observed within six hours after administration of the hormone. True growth of the uterus, as demonstrated by an increase in nitrogen concentration and dry weight, is generally preceded by an increase in water content. When investigating the factors which influence the growth and composition of the uterus in the rat, Kroc et al. (1959) observed that estrogen caused only a transient increase in uterine water content even with continued estrogen therapy, whereas relaxin maintained high uterine water levels as long as therapy was continued.

True growth of the uterus (increase in nitrogen content and dry weight) was studied in spayed rats (Kroc et al., 1959). Estrogen caused uterine growth, whereas high doses of relaxin or progesterone produced only slight or no growth effects. When progesterone and relaxin were given to rats which were estrogen primed, uterine weight was significantly greater than that observed in the factorial controls. Uterine composition under these conditions was observed to be similar to that of rats in late pregnancy. This strongly suggests a synergistic action of the three ovarian hormones on the growth of the uterus. Wada and Turner (1961) and Brody and Wiqvist (1961) confirmed that all three hormones were required for complete uterine growth.

Relaxin also synergizes with estrogen in the rat to induce other biochemical changes in the uterus including increases in total glycogen content and alkaline phosphatase activity (Kroc et al., 1959; Steinetz et al., 1957).

In the mouse Hall (1960) described a synergism between estrogen and relaxin in stimulating myometrial glycogenesis and alkaline phosphatase activity, and edematous transformation of the endometrial stroma.

In the monkey Hisaw (1959) reported that there was a marked increase in the thickness of the endometrial stroma and dilation of endometrial capillaries and venules with hypertrophy and hyperplasia of their endothelium after the administration of relaxin.

Other Roles of Relaxin in Gestation and Parturition.--Besides relaxation of the pelvic girdle, and cervical softening and dilation in preparation for delivery of the young, relaxin is believed to play other roles in the maintenance of gestation and parturition.

In ovariectomized mice less progesterone was needed to maintain pregnancy if relaxin were also given. Relaxin also facilitated the punctual delivery of live

young mice (Hall, 1957). Relaxin, but not estrogen, increased the delivery response to oxytocin in spayed mice maintained on progesterone (Steinetz et al., 1957).

In the rat, however, the additional role of relaxin in the maintenance of gestation and parturition is not clear. The effect of relaxin on gestation appears to be a function of the relative quantities of estrogen and progesterone (Yochim and Zarrow, 1961). At low estrogen:progesterone ratios, relaxin may increase fetal survival or have no effect. At high estrogen:progesterone ratios relaxin inhibits the maintenance of gestation.

Wiqvist (1959a) investigated the effects of prolonged relaxin administration on nonpregnant uteri of mice and rats. Uterine sensitivity to oxytocin in the rat was not influenced by prolonged exposure to relaxin, while uterine distensibility was markedly enhanced. Whether this is the situation in the pregnant rat remains to be demonstrated since the role of the other ovarian hormones would have to be considered.

The action of relaxin in the maintenance of gestation and parturition in the rat is in need of much more investigation.

Effects on the Mammary Gland.--A synergism between relaxin, estradiol, and progesterone on lobuloalveolar growth in the rat has been described by Hamolsky and Sparrow (1945) and Smith (1954). This effect has been confirmed in the rabbit and guinea pig (Garrett and Talmage, 1952).

Summary of Relaxin Effects in the Rat.--The effects of relaxin in the rat after estrogen priming include cervical softening and dilation, inhibition of uterine

contractions, increased uterine water content, increased uterine glycogen content, and increased uterine alkaline phosphatase activity. Relaxin acts synergistically with estrogen and progesterone to cause maximum uterine growth, (increased uterine nitrogen content and dry weight) and to promote lobuloaveolar growth in the mammary gland. Relaxin does not cause pubic symphysis relaxation in this species.

Methods of Measuring Relaxin Activity

The measurement of relaxin activity in the blood and tissues of different animals has been based upon physiologic actions of the hormone. The methods most commonly used are based upon the relaxation of the guinea pig pubic symphysis, relaxation of the mouse pubic symphysis, and the inhibition of spontaneous uterine contractions in the mouse and the rat. These methods are reviewed by Hall (1960) and Steinetz et al., (1962).

Guinea Pig Pubic Symphysis Method.--A relaxin bioassay using ovariectomized, estrogen-primed guinea pigs was established by Abramowitz and associates (1944). Relaxation was determined by manual palpation and was recorded as an all or none response. Frieden and Hisaw (1950) introduced a grading system for the responses. The bioassay method is now performed by injecting a standard relaxin preparation into intact, estrogen-primed virgin female guinea pigs and subsequently palpating the pubic symphysis for mobility 6 and 12 hours later. Those which respond positively to this treatment are used as assay animals. One week following the preliminary screening, the guinea pigs are again primed with estrogen and injected five days later with the standard relaxin preparation or the unknown preparation to be tested. The pubic symphysis is palpated 6 hours after

the injection and the responses are graded on a scale of 0 to 6 (4-6 is considered a positive response). This is the bioassay method by which the activity of relaxin is most commonly measured - guinea pig units (see Isolation, Purification, and Physicochemical Properties of Relaxin).

Mouse Interpubic Ligament Method.--Measurement of the separation of the pubic symphysis in the estrogen-primed mouse in response to relaxin was first performed by X-ray visualization of the interpubic distance (Hall and Newton, 1946; Kliman et al., 1953). An important contribution was made by Kliman and Greep (1958) when they reported that relaxin activity was markedly enhanced by the use of a repository vehicle for injection of the hormone. Thus, relaxin injected in beeswax-oil was 65 times more potent than an identical preparation injected in saline solution.

Rather than visualizing the interpubic separation with X-rays, this was later done by direct measurement using a calibrated microscope eyepiece and trans-illumination of the exposed pubic symphysis (Kroc et al., 1959; Steinetz, et al., 1960). This is the procedure now employed when using this method of assay. Virgin female mice are estrogen-primed by a single injection of estradiol in oil. One week later the animals are injected with the relaxin standard or the preparation to be tested. Autopsies are performed 36 hours after the injection of the test material, the pubic ligament is exposed and measured with a binocular microscope using a calibrated eyepiece and transillumination of the exposed area.

Inhibition of Spontaneous Uterine Contractions.--Relaxin specifically inhibits the motility of the estrogen-primed uterus in-vivo and in-vitro. Krantz et al. (1950) described an in vivo method for the study of uterine motility in the guinea

pig. More recently Porter (1971) has described the action of relaxin on the myometrial activity of the guinea pig uterus in-vivo. However, these methods have not been utilized as a bioassay to any great extent.

Inhibition of spontaneous uterine motility of the estrous rat or mouse has also been employed as a bioassay method for relaxin (Sawyer et al., 1953; Wiqvist, 1959; Frieden and Hisaw, 1953; Steinetz et al., 1959). This method of assay, whether employing the estrogen-primed mouse or rat, involves the suspension of the uterine horn in an aerated organ bath containing either Locke's or Ringer's solution. The horn is anchored in the bath and the other end is attached to an isotonic lever which records the uterine contractions. The relaxin standard or preparation to be tested is injected into the organ bath after regular contractions have been established. A grading system of 1 to 4 for responses has been suggested by Wiqvist and Paul (1958).

Comparison of Relaxin Bioassay Methods.--The mouse interpubic ligament method of assay appears to be the best method to employ for bioassay of relaxin since it has an objective end point which can be quickly and accurately measured. The method is also easily learned. The guinea pig method of measuring the effect of relaxin on the pubic symphysis motility is subjective and requires training to make the judgments of how to grade a response. Furthermore, a large guinea pig colony must be maintained. The uterine motility inhibition methods are tedious and time consuming. Grading partial responses is extremely dangerous as was pointed out by Wiqvist and Paul (1958).

Radioimmunoassay of Relaxin.--Recently Bryant (1972) has developed a radioimmunoassay technique for measuring relaxin in plasma. Current bioassay methods are impractical for measuring relaxin in multiple samples and bioassay data is always subject to animal variations. Radioimmunoassay is quite sensitive and may open a door to more accurate and easier ways of measuring relaxin activity in plasma and tissues.

Sites of Relaxin Storage

Relaxin has been found in the blood and reproductive tracts of many pregnant animals, including the pig, rabbit, rat, mouse, guinea pig, dog, cat, horse, sheep, goat, cow, whale, and human being (Hisaw and Zarrow, 1950; Steinetz et al., 1959). The hormone increases from a low level in early gestation to a peak in mid or late gestation and decreases after parturition. The ovary appears to be the primary source of relaxin. This appears to be the case for the sow (Hisaw and Zarrow, 1953) and the mouse (Steinetz et al., 1959). Besides the ovary the placenta and uterine endometrium in the rabbit and guinea pig are also sources of relaxin (Zarrow and Rosenberg, 1953; Zarrow, 1947). Small amounts of relaxin are also found in the endometrium and placenta of the whale as well as the ovary (Steinetz et al., 1959). Bryant (1972) and Bryant et al. (1973) have demonstrated a uterine source as well as an ovarian source of relaxin in the sow and sheep by means of radioimmunoassay. Relaxin was found to be present in the blood of pregnant women, but was absent after delivery (Zarrow et al., 1955). Furthermore, by means of fluorescent antibody studies it has been shown that relaxin is present in the placenta, decidua, and endometrium in the human female (Dallenbach and Dallenbach-Hellweg, 1964). Bryant (1972) also demonstrated

that there was a uterine source of relaxin in the human. A new cell type in the endometrium of the rhesus monkey has been described which is believed to be responsible for the secretion of relaxin (Cardell et al., 1969). As was pointed out by Hall (1960) it appears that the ovaries are the principal source of relaxin in animals which rely on the ovary for gestational hormones (rat, mouse, and sow) and that the placenta and uterus may produce relaxin in those species in which maintenance of gestation is not dependent upon the ovaries (human, rabbit). However, certain tissues contain relaxin-like activity for which a physiologic explanation is not available. It has been shown to be present in the testes of the rooster (Steinetz et al., 1959) and of the armadillo (Steinetz et al., 1964). Bryant (1972) demonstrated the presence of relaxin in the plasma of the ram, boar, and human male by radioimmunoassay.

Metrial Gland versus Ovary in the Rat as a Source of Relaxin.--In the pregnant rat the site of relaxin storage is still in dispute. The uterine metrial gland is considered by some investigators to be the source of relaxin. In the pregnant rat, the metrial gland is located on the mesometrial border of the uterus immediately below the placenta. The most characteristic components of the gland are large, heavily granulated cells called metrial gland cells, and which are considered to be the source of relaxin in the pregnant rat (Dallenbach-Hellweg et al., 1965). Two other cell types are present in the gland - lipid bearing cells and fibroblast-like stromal cells which are present throughout the life span of the gland. Metrial gland cells are present from day 9 of gestation through the 22nd day of lactation (Baker, 1948). These cells reach a maximum in number at day 14 of gestation and then start to undergo necrosis (Selye and McKeown, 1935; Baker,

1948; Larkin and Schultz, 1968). Cells that are morphologically identical to these granular cells have been identified in the pregnant human uterus (Dallenbach-Hellweg and Nette, 1964) and the monkey uterus (Dallenbach-Hellweg et al., 1966; Cardell et al., 1969).

Electron microscopic studies reveal that the mature granular cell possesses an active Golgi complex and appears capable of producing a proteinaceous secretion (Larkin and Flickinger, 1969). The granules resemble secondary lysosomes. They are membrane limited, contain small vesicles and multilaminated myelin figures as well as a dense central core (Peach and Bulmer, 1965; Larkin and Flickinger, 1969). These granular cells also contain lysosomal enzymes which are localized within the granules (Bulmer, 1968).

In a study of granule release from metrial gland cells Larkin (1972) observed that granules were released into the tissue spaces upon the death of the cells. It was suggested that once the granules are free, their limiting membranes rupture and the contents diffuse throughout the tissue. Furthermore some of the granules were observed to have been engulfed by phagocytic cells. It should be noted that this is not the usual method of granule release in other cell types. Larkin (1972) suggested that these granules could be a source of relaxin.

Using the fluorescent antibody method, Dallenbach-Hellweg and associates (1965) demonstrated that metrial gland cells of the rat contain relaxin. Furthermore, they found fluorescence over the blood vessels within the metrial gland as early as day 15 of gestation. This coincides with the evidence of granule release by day 15 of pregnancy (Larkin, 1972). In their investigation of relaxin storage sites, Dallenbach-Hellweg et al. (1965) found no specific fluorescence due to the

presence of relaxin in the ovary.

Other investigators have presented evidence in favor of the ovary as the source of relaxin in the rat. Bloom and associates (1958) prepared tissue extracts from various tissues of the pregnant rat, including the ovary, metrial gland, and nonplacental uterus. These tissue extracts were bioassayed and relaxin activity was found to be present only in the ovary. The extract of the metrial gland did not possess any relaxin activity.

Zarrow and McClintock (1966) found by injecting I^{131} labelled antibody to relaxin into pregnant rats that the labelled antibody localized in the ovary to a great extent but not in the uterus. However, they also localized relaxin in the placenta, cervix, and mammary gland of the rat by this method. The latter two organs are known to be target organs of the hormone.

Battista and Lisk (1963) claim to have localized relaxin in corpora lutea of pregnant rat ovaries by the fluorescent antibody technique. However, this information was presented at a regional meeting of the American Society of Zoologists and was never published.

Long (1973) in his ultrastructural study of the corpus luteum of pregnancy in the rat observed the presence of two distinct granule populations. The larger, less electron dense granules, which were irregular in size were termed type I granules. The smaller, more electron dense granules which were more uniform in size were called type II granules. The type II granules appear at day 14 of gestation and increase in numbers until day 20 when they are observed at the periphery of the cells. After parturition the granules are absent from luteal cells. Type I granules are present throughout gestation and were shown to be

lysosomal in nature by their content of acid phosphatase and aryl sulfatase. The type II granules did not display this property. Long (1973) suggested that the type II granules may be a source of relaxin in the ovary of the pregnant rat.

L. L. Anderson (1971) measured relaxin activity in ovarian tissue during different reproductive stages in the rat and found that relaxin activity occurred only during the latter half of gestation. Relaxin activity was low from day 1 through day 13 of gestation, and began to increase at day 14 and day 17. By day 20 the hormone activity had reached its maximum. It then declined markedly on day 21 and remained low after parturition. These data are in excellent agreement with the appearance and disappearance of the type II granules in luteal cells (Long, 1973).

Electron microscopic studies of luteal cells in other species have revealed the presence of distinct granule populations. Belt et al. (1971) investigated the ultrastructure of the luteal cell at various stages of gestation in the sow. These investigators observed the appearance and accumulation of dense granules in early and mid gestation and the disappearance of the granules in late gestation. Upon determining the levels of relaxin activity in the ovaries of the pregnant sow at various days of gestation, it was observed that the rise and fall of relaxin activity in the corpora lutea closely parallels the appearance and disappearance of the granules.

In an ultrastructural study of the human luteal cell, Crisp et al. (1970) observed the presence of a distinct granule population. Since the luteal cells appeared to be actively engaged in both steroid and protein synthesis, it was suggested that the granules may be a source of relaxin in the human. However,

neither Belt et al. (1971) nor Crisp et al. (1970) utilized histochemistry to rule out the possibility that the granules could be lysosomes.

III. MATERIALS AND METHODS

Animals

Young albino male New Zealand rabbits weighing approximately 2.5 kilograms initially and male guinea pigs of mixed breed weighing approximately 500 grams were used for the production of antisera to porcine relaxin. Virgin female rats of the Long-Evans strain weighing approximately 200 to 250 grams were used in the bioassay procedure for relaxin. For cell fractionation, tissue extraction investigations, and fluorescent antibody studies day 17 pregnant rats of the Long-Evans strain were used with day 0 designated as the day that sperm were found. Day 17 of gestation was chosen for these studies since the luteal cells have attained their maximum size at this time and contain an abundance of type II granules which are thought to be a possible source of relaxin (Long, 1973). Furthermore, the metrial gland at day 17 of gestation is well formed and contains numerous granulated cells which are also believed to be a source of relaxin in the rat (Dallenbach-Hellweg et al., 1965).

Antiserum Production

Three male rabbits were injected with 2.0 mg each of porcine relaxin (NIH #0148) in Freund's complete adjuvant (2.0 mg relaxin/0.2 ml saline + 0.4 ml Freund's). Each rabbit was given 0.2 ml intraperitoneally and 0.2 ml was given subcutaneously into each thigh. Thereafter, two additional injections of 1.0 mg relaxin in Freund's complete adjuvant (1.0 mg relaxin/0.1 ml saline + 0.2 ml Freund's) was given to each rabbit at 7 to 9 day intervals. Half of this dose was given subcutaneously into each thigh. Three weeks after the last injection the

rabbits were given another 1.0 mg of relaxin in Freund's in the manner previously described. Three weeks after the end of this immunization schedule 50 to 70 ml of blood were taken from each rabbit by an ear vein. The antisera were tested by the agar diffusion method of Ouchterlony (1953) and the hemagglutination titer method of Stavitsky (1954). The rabbits were given a booster injection of 1.0 mg relaxin in complete Freund's in the manner described above at 3 to 6 week intervals and were bled two weeks after each booster injection. The antisera at each bleeding were tested by agar diffusion plates and by the hemagglutination titer test until maximum responses were achieved.

Each of three male guinea pigs were immunized initially with 1.0 mg of porcine relaxin (NIH #0148) in complete Freund's adjuvant (1.0 mg relaxin/0.1 ml saline + 0.2 ml Freund's). Half was given intraperitoneally and the remaining half was given subcutaneously into the back of the neck. Thereafter injections were given at two week intervals (0.5 mg relaxin/0.05 ml saline + 0.1 ml complete Freund's) subcutaneously into the back of the neck for a period of four weeks. Three weeks after the last injection the animals were given another 0.5 mg of relaxin in Freund's. Two weeks after this immunization schedule was completed the guinea pigs were lightly anesthetized with ether and approximately 3 to 5 ml of blood were taken from each guinea pig by cardiac puncture. The antisera were studied by the agar diffusion method. The guinea pigs were given booster injections at 2 to 4 week intervals with 0.5 mg relaxin in the manner described above and were bled two weeks after each booster injection. The antisera at each bleeding were tested by the agar diffusion method until maximum responses were achieved.

Agar Diffusion Studies

The double diffusion technique of Ouchterlony (1953) was used to determine the specificity of the antisera. Petri dishes which contained between 8 to 9 ml of 1% agar in 0.05 M veronal buffer at pH 8.6 with a final concentration of 0.01% merthiolate were employed. The plates contained a center well with a capacity of 150 ul and either six peripheral wells of 50 µl each or five peripheral wells of 150 µl each. The plates were permitted to remain at room temperature (approximately 24° C) for reaction, and were read and photographed at 24 and 48 hour intervals.

Hemagglutination Titer Test

Using the method of Stavitsky (1954) the hemagglutination titer test was employed to determine the antibody levels of the antisera. This is a very sensitive test in which the antigen is adsorbed on tannic-acid coated sheep red blood cells; these cells are then added to serial dilutions of the antisera. The antibodies in the antiserum agglutinate with the antigen on the red cells and create a lattice or sheet of red cells in the bottom of the tube. Serum which lacks the antigen will not agglutinate the coated red cells and, therefore, the red cells will form a round button instead of a sheet. A control, in which tanned red cells uncoated with antigen were added to the diluted antiserum, was utilized to test for any nonspecific reaction of the tanned cells with the antiserum. The only deviations from the published method were the use of tannic acid at a dilution of 1:40,000 instead of 1:20,000 and the use of another control. This additional control, in which the antiserum was omitted, was employed to test

for any nonspecific interaction between normal rabbit serum being used as serum diluent and the antigen coated red blood cells. One milligram of porcine relaxin (NIH #0148) was used for coating the red cells in the procedure. The antisera used were those prepared as described above.

Fluorescent Antibody Studies

The indirect fluorescent antibody method was used as described by Glass (1971) to locate the sites of relaxin storage in tissues of pregnant rats. Ovaries, uterine metrial glands, nonplacental uteri, and adrenal glands were taken from day 17 pregnant rats, fixed in Carnoy's fixative, dehydrated, cleared, and embedded in paraffin. Sections 7 um in thickness were cut and mounted on acid cleaned slides which had been coated with albumin. Paraffin was removed from the sections and the sections were hydrated to phosphate buffered saline (PBS) with a pH of 6.8. A few drops of undiluted anti-relaxin antiserum were added to the sections and they were incubated for 10 minutes in a moist chamber at 37^{o} C. The sections were washed for 15 minutes in PBS and then exposed to anti-rabbit gamma globulin, produced in goats and labelled with fluorescein isothiocyanate (Antibodies Inc., Davis, California) for a period of 10 minutes in a moist chamber at 37^{o}C. The slides were washed again for 15 minutes in PBS, a cover slip was applied using 50% glycerol as a mounting medium and rimmed with paraffin to prevent evaporation.

Frozen sections of ovary and metrial gland were cut at 12 um. The sections were air dried and fixed in 95% ethanol for 10 minutes according to the technique employed by Dallenbach-Hellweg et al. (1965). The sections were hydrated to PBS and stained in the same way as the paraffin sections. After air drying,

frozen sections were also fixed for 30 minutes in 95% ethanol at 37°C and were permitted to air dry again for 30 minutes at 37°C before staining. (Zarrow and O'Connor, 1966).

Several controls were employed to test the specificity of the immunofluorescent staining reaction. The saline controls were sections which were simply hydrated to observe the presence of any autofluorescence and to check for possible contamination of the tissues by fluorescent compounds during all previous processing of the tissues. A second control used only the fluorescein labelled anti-rabbit gamma globulin to check for any nonspecific reaction between the rat tissues and the fluorescein labelled compound. The third control consisted of incubating the sections using normal rabbit serum (NRS) instead of the antiserum to check for nonspecific reactions between NRS proteins and rat tissues. The last control involved the incubation of anti-relaxin antiserum with porcine relaxin (NIH #0147) for 60 minutes at 37°C before using the antiserum for staining in order to demonstrate the specificity of the antiserum for relaxin. Since the antiserum was diluted by this procedure the exposure time of the relaxin-absorbed antiserum was increased to 20 minutes. Antiserum which was not absorbed by relaxin was diluted with PBS to the same volume as the relaxin-absorbed antiserum and was also exposed to the sections for 20 minutes to determine if the dilution would effect the reaction. In addition, all rabbit antisera were absorbed by a mixed homogenate of liver, spleen, and kidney from a male rat to further control any nonspecific reactions between the antisera and the rat tissues.

Photomicrographs were taken using Anscochrome 500 film and Tri-X Pan film.

Bioassay of Relaxin

The bioassay method selected for use in this investigation is based upon the ability of relaxin to cause inhibition of uterine contractions in the estrous rat. Since the study was concerned with a hormone from rat tissues, a bioassay which utilizes the rat was thought to be desireable. The in-vitro methodology used in this research was based on previous work by Griss et al. (1967), Wiqvist and Paul (1958), Sawyer et al. (1953), and Staff of the Department of Pharmacology, Edinburgh University (1968).

The day before the assay was to be performed, virgin estrous rats were injected subcutaneously with 10 to 20 μg estradiol in corn oil. Rats were also ovariectomized and given estrogen therapy as described by Wiqvist and Paul (1958). I found that there was no noticeable difference with respect to the regularity of the amplitude and frequency of uterine contractions between the two methods of preparing assay animals. Therefore, intact female rats in estrous were injected with 10 to 20 μg estradiol one day prior to the assay and utilized for assaying relaxin activity.

On the day of bioassay the rats were killed by decapitation immediately before use. The uterine horns were dissected free, being careful not to stretch the muscle or to handle it more than necessary. The uterine horns were separated, and each horn was cut in half transversely yielding a total of four muscle segments from each animal. The uterine segments were stored in Locke's solution aerated with 1% CO_2 in oxygen at 37^{o}C until used. The

uterine segments for bioassay were suspended in a 10 ml glass chamber containing Locke's solution maintained at 37°C (Figure 32). The chamber was aerated with 1% CO_2 in oxygen at a bubble rate of approximately 120 bubbles/minute. The pH was maintained at 7.6 by this method. The uterine muscle segments were attached to a heart lever under 1.0 gram of tension and contractions were recorded on an ink writing kymograph. The uterine muscle was permitted a period of 10 to 15 minutes to establish regular contractions. If, in this time interval, such conditions were not established, the uterine segment was discarded. After regular contractions were established, the sample to be investigated was injected into the chamber in a volume of 0.1 to 0.4 ml. All dilutions of samples were made in Locke's solution or normal saline. The sample was allowed 5 to 7 minutes to elicit its effect, if any, on the uterine muscle. After this period of time, if there had been partial or total inhibition of contractions, the chamber was rinsed with 75 to 125 ml of Locke's solution at 37°C, which had been aerated to establish a pH of 7.6. This was performed in a manner that caused no stretching of the muscle. Ideally, the uterine segment should recover its previous amplitude and frequency of contraction. This would demonstrate that the response was due to the material added to the bath, and was not due to fatigue of the muscle. It was noted that very high concentrations of relaxin caused permanent inhibition of rhythmic activity. The end point in the bioassay was total inhibition of uterine contractions since partial responses were quite variable from animal to animal and even from uterine segment to uterine segment within the same animals. Partial responses are defined as reduction in amplitude and/or frequency of contractions. A fresh uterine muscle segment was used for each bioassay.

Controls utilized to determine if uterine contraction inhibition was specifically due to the presence of relaxin were the same as those employed by Sawyer et al. (1958). Strips of ileum from estrogen primed rats were suspended in the organ bath under the same conditions used for rat uterine muscle segments, except for the ileal muscle being under 0.5 g tension instead of 1.0 g. Relaxin is specific in its effect on uterine smooth muscle and should not affect other smooth musculature such as that in the ileum. A contaminant such as adrenaline would affect smooth muscle in general and would cause inhibition in both instances. Samples showing relaxin activity were boiled in 1 N NaOH for 10 minutes and readjusted to a pH of 7.6. Relaxin survives such treatment while many other substances, such as histamine, would not. Therefore, this treatment of samples containing relaxin should not interfere with their ability to cause inhibition of uterine muscle contractions.

Relaxin Extraction

Corpora lutea, remaining ovarian tissue, metrial glands, and nonplacental uteri were collected from day 17 pregnant rats. Each group of tissues was pooled and treated as described by Frieden and Hisaw (1950) and Bloom et al. (1958). The whole homogenized tissues were extracted with a 5% salt solution. The salt extracts were added to 95% ethanol which precipitates some of the extraneous material. The supernatants were brought to pH 4.3 with acetic acid which results in the precipitation of another inactive fraction. The supernatants were then dialyzed against absolute alcohol. The precipitates formed during dialysis were discarded and the relaxin-containing supernatants were

precipitated with acetone. The active precipitates were dissolved in water and these final products were freed of salt by dialysis and were then lyophilized. Deviations from the original method (Frieden and Hisaw, 1950) included omission of the alkali after dialysis against alcohol and the removal of acetone by dialysis was carried out for only 4 hours with two water changes.

Cell Fractionation of Luteal Cells

Since the hypothesis to be tested is that luteal cells in the pregnant rat are sites of storage of relaxin, fractionation of luteal cells from day 17 pregnant rats was undertaken to isolate a fraction which possesses a high concentration of relaxin activity. Morphological studies of fractions were also performed in an attempt to identify the subcellular component which contains relaxin.

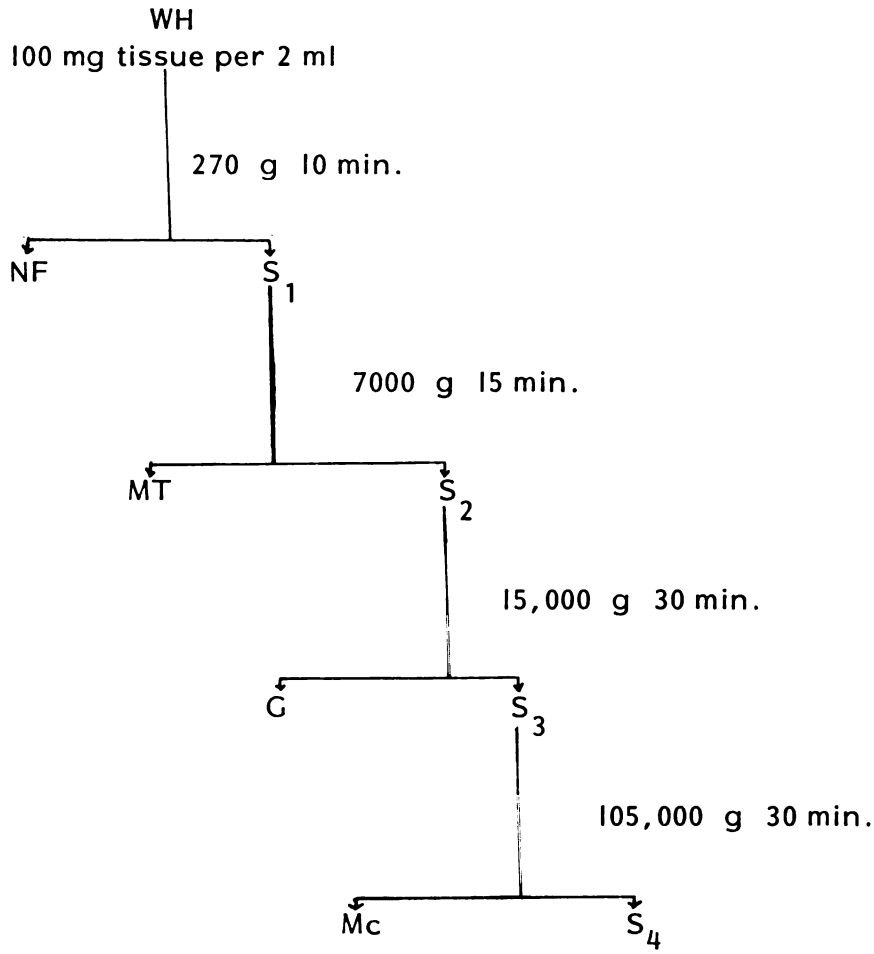
Continuous Density Gradient Centrifugation.--Corpora lutea from day 17 pregnant rats were dissected free of other ovarian tissues. A 5% homogenate (10 corpora lutea or approximately 50 mg/ml) was prepared in 0.25 M sucrose containing $5 \cdot 10^{-4}$M EDTA using a smooth glass tissue grinder with a teflon pestle. The pH of the solution of sucrose and EDTA was adjusted to 7.6 since that was the pH of the bioassay organ bath. The whole homogenate was centrifuged at 270 g for 10 minutes in a Sorvall RC2-B centrifuge to remove the nuclear fraction. The remaining homogenate was carefully layered on a continuous sucrose gradient, which was prepared using a Hoefer gradient maker. The concentrations of the sucrose solutions used for making the gradient were 10% and 55%, whose pH's were adjusted to 7.6. The tubes were placed in a swinging bucket rotor (SW41) and centrifuged for 30 minutes at 90,000 g in a Beckman L3-50 ultracentrifuge. Centrifugation beyond 30 minutes did not alter the number,

distribution, or location of the bands. Fractions of 0.7 ml were collected from the bottom of the tube using a Hoefer fraction collector. The fractions were either frozen for bioassay or were fixed for morphological studies.

Differential Centrifugation.--Corpora lutea were dissected free from other ovarian tissue at day 17 of gestation. A 5% homogenate was prepared by gently homogenizing 20 corpora lutea (100 mg/2ml) in 0.25 M sucrose containing $5 \cdot 10^{-4}$M EDTA. The homogenization was done by hand using a smooth glass tissue grinder with a teflon pestle (6 strokes). The whole homogenate was centrifuged at 270 g for 15 minutes in a Sorvall RC2-B centrifuge to remove the nuclear fraction (Text Figure 1). The supernatant fluid was transferred to another tube and diluted to a volume of approximately 7.7 ml with the sucrose-EDTA solution. The tube was placed in a swinging bucket rotor (SW-41) and centrifuged for 15 minutes at 7,000 g in a Beckman L3-50 ultracentrifuge. The resulting pellet was called the mitochondrial (Mt) fraction. The Mt fraction was washed twice in the sucrose-EDTA solution (7,000 g, 15 minutes). The next fraction, which was brought down at 15,000 g for 30 minutes, was termed the granular (G) fraction. The two washes from the Mt fraction were added to the G fraction and centrifuged at 15,000 g for 30 minutes. The remaining supernatant was then centrifuged at 105,000 g for 30 minutes to collect a fraction which was called the microsomal (Mc) fraction. Again the two washes which were carried over from the Mt fraction were added to the Mc fraction and centrifuged at 105,000 g for 30 minutes. All fractions were resuspended in saline and aliquots of each were made, which were either frozen for bioassay or were fixed for morphological studies.

TEXT FIGURE 1.

Procedure for Fractionation of Homogenates of Corpora Lutea from Day 17 Pregnant Rats by Differential Centrifugation



WH Whole homogenate
NF Nuclear fraction
MT Mitochondrial fraction
G Granular fraction
Mc Microsomal fraction
S_1 Supernatant 275 g.
S_2 Supernatant 7000 g.
S_3 Supernatant 15,000 g.
S_4 Supernatant 105,000 g.

Protein Determinations

The amount of protein present in the fractions obtained by differential centrifugation was determined by the method of Lowry et al. (1951). In reporting these protein values I am actually reporting bovine serum albumin equivalents. These values were used to determine the specific activity of relaxin in fractions (μg of protein/ml which causes total inhibition of uterine contractions).

Morphological Studies

Electron microscopy was employed to examine the subcellular components present in the fractions collected from both continuous density gradient centrifugation and differential centrifugation. All fractions were fixed overnight in an equal volume of unbuffered 4% osmium tetroxide. The fractions were pelleted using a Beckman microfuge, dehydrated in acetone, and embedded in Epon. Sections approximately 1 μm thick were cut from the center of the pellet and stained with toluidine blue. The sections were examined by light microscopy to determine if all areas of the pellet were the same or if there had been a formation of layers in the pellet. Thin sections (silver) of representative areas were cut on a Porter-Blum MT-2 ultramicrotome, collected on parlodion coated specimen grids, and stained with 5% uranyl acetate (30 minutes) and 0.4% lead citrate (7 to 10 minutes). The sections were then examined and photographed using an Hitachi HS-8 electron microscope.

IV. RESULTS

Agar Diffusion Studies

The double diffusion method of Ouchterlony (1953) was used to demonstrate the antigenicity of porcine relaxin and to determine at each bleeding if the antibody response of the host had improved from the previous bleeding. Undiluted rabbit or guinea pig antiserum (150 µl) was placed in the center well and allowed to react with various concentrations of porcine relaxin in the peripheral wells (50 µl each). Precipitin responses were judged to be best at the well where the precipitin line was the sharpest and most intense. This is then the optimal proportion between the antibody and the antigen molecules for reaction.

The antiserum of rabbit 1 (R-1) after 8 weeks of immunization displayed a fair precipitin reaction consisting of a single line, which was sharp but not very intense. The best response was observed at a concentration of 2.5 µg relaxin. The sera of the other two rabbits were negative.

The result with the antiserum at the second bleeding of R-1 (6 to 8 weeks after the first bleeding and 2 weeks after the last booster injection of relaxin) was essentially the same as before except that the line formed against relaxin was sharper and more intense (Figure 1). Again the best response was at a concentration of 2.5 µg relaxin. At this time the antiserum of another rabbit (R-2) also formed a line against porcine relaxin. However, the best response was at a concentration of 20 µg relaxin (Figure 4).

The plates set up to study the sera from the third bleeding revealed that a much different response to relaxin had been achieved. The antiserum from R-1 now displayed the formation of three precipitin lines against porcine relaxin

(Figure 2). The inner diffuse line was best at 1.25 μg, the middle at 2.5 μg, and the outer at 5.0 μg relaxin. The normal pig serum (NPS) did not react with the antiserum thus demonstrating the absence of any antibodies which may have formed to nonspecific porcine proteins present in the relaxin preparation. The antiserum from R-2 at the third bleeding formed two precipitin lines against porcine relaxin (Figure 5). The inner line is diffuse but is best at 2.5 μg relaxin while the outer line shows the best response at 10.0 μg relaxin. Again the serum of the third rabbit (R-3) proved to be negative.

At the final bleeding the antiserum from R-1 again formed three precipitin lines against porcine relaxin but the lines were not as sharp or intense as those formed by the antiserum at the previous bleeding (Figure 3). For some unknown reason rabbits will fail to continue to respond to an antigen which they have received for a period of time. The antiserum from R-2 now formed three precipitin lines against relaxin (Figure 6). The inner two lines are diffuse and tend to run together making it difficult to determine the concentration of relaxin which gives the best response for these two lines. The outer line is best at a concentration of 10.0 μg relaxin. NPS again did not form any lines when tested against the antiserum. R-3 displayed a weak precipitin response to relaxin as indicated by the presence of a light line at 2.5 μg and 5.0 μg relaxin (Figure 7). There is also an inner diffuse line at all concentrations of relaxin.

Agar plates were set up to determine if R-1 and R-2 were forming antibodies to the same components in the porcine relaxin. For this study plates with five peripheral wells containing 150 μl each were utilized (Figures 8 and 9). The single line produced against relaxin by the antiserum from R-2 (bleeding 2) was

shown to be two lines on these plates. One possible explanation for this phenomenon is that the antigen underwent some breakdown upon storage in the refrigerator. The breakdown product, however, still contained active sites to interact with the antibody and due to its smaller size diffused faster in the agar, thus forming a second line. The inner line is identical for all antisera shown; that is, the lines join. Therefore, the animals produced antibodies to similar or identical structures of the hormone. Identity of the middle and outer lines for R-1 and R-2 were difficult to determine since the lines were so close to the peripheral wells, but it is possible that if lower antigen concentrations had been used the lines may have joined and shown identity. The lowest antigen concentration used was 5.0 µg relaxin, which appeared the same as when 10.0 ug or 20.0 ug of relaxin were used.

Agar diffusion studies were also performed on the guinea pig antisera according to the same procedure employed for the rabbit antisera. The guinea pig responses to the antigen were not as complicated as those seen in the rabbits. All three guinea pigs responded to only one component in the relaxin preparation as demonstrated by the formation of a single intense line between the antiserum and porcine relaxin (Figure 10 and 11). The best responses were at 40 µg relaxin. Therefore, the guinea pig antisera were not as sensitive as the rabbit antisera in detecting minute amounts of relaxin; that is, smaller amounts of the antigen are required to cause a precipitin reaction with the rabbit antisera than with the guinea pig antisera. There were no detectable precipitin reactions between the antiserum of GP-1 and NPS. It appears that one or more components

in the antiserum is non-specifically precipitating to form a ring that goes all the way around its well. Another plate was set up to determine if the antibodies formed by all three guinea pigs were against the same component in the relaxin preparation (Figure 12). It was shown that this was the case, since the precipitin lines formed between all three guinea pig antisera and relaxin exhibited identity.

Finally a plate was set up to determine if the guinea pigs had produced antibodies to one of the same relaxin components as the rabbits (Figure 13). This was not the case since the line formed between the guinea pig antisera and porcine relaxin did not show identity with any of the lines formed between the rabbit antisera and porcine relaxin.

Hemagglutination Titer Test

The hemagglutination titer test was employed to determine the antibody level

The results from both the hemagglutination titer test and the agar diffusion plates on the antisera of R-1 were in excellent agreement. The highest titered antiserum produced by R-1 was at the third bleeding. The agar plate demonstrated that three precipitin lines were formed between the antiserum and porcine relaxin (Figure 2), while the hemagglutination titer test gave a positive reaction up to a dilution of 1:4000 of the antiserum.

Table I.

Results of Hemagglutination Titer Test on

Rabbit Antisera to Porcine Relaxin

	Rabbit 1		Rabbit 2		Rabbit 3	
Bleeding	#lines	Titer	#lines	Titer	#lines	Titer
1	1	1:500 3+ 1:1000 1+	0	1:500 -	0	1:500 -
2	2	1:500 3+ 1:1000 1+	1	1:500 1+ 1:1000 1+	0	1:500 2+
3	*3	1:500 4+ 1:1000 3+ 1:2000 1+ 1:4000 1+	*2	1:500 2+ 1:1000 2+ 1:2000 1+	0	1:500 2+ 1:1000 1+
4	3	no results	*3	1:500 1+ 1:1000 1+	1	1:500 $\pm$

*Antisera used for the fluorescent antibody studies.

4+ Compact granular agglutinate; smooth, even sheet of cells.
3+ Smooth mat on bottom of the tube with folded edges.
2+ Smooth mat on bottom of the tube, edges somewhat ragged.
1+ Narrow ring around edge of smooth mat.
$\pm$ Smaller area of tube covered than 1+, and heavier ring around edge.
- Discrete red button in center of bottom of tube.

Results from both tests performed on the antiserum from R-2 were also in good agreement. The best antiserum produced by this rabbit was also at bleeding 3. The agar plate demonstrated the formation of two precipitin lines between the antiserum and porcine relaxin (Figure 5), while the hemagglutination titer test gave a positive reaction up to a dilution of 1:2000 of the antiserum. At

bleeding 4, however, the titer had decreased but three lines were observed on the agar plate between the antiserum of R-2 and porcine relaxin. However, the lines were more diffuse than at the previous bleeding. The rabbit for some unknown reason was failing to respond to the antigen as R-1 did.

The results obtained from both tests on the antisera of R-3 were not in very good agreement. No detectable lines were formed on the agar plates between the antisera and porcine relaxin at bleedings 2 and 3, while the hemagglutination test demonstrated the presence of antibodies to relaxin. This may be due, at least in part, to the greater sensitivity of the latter test. The agar plate set up using the serum from R-3 at the last bleeding demonstrated the presence of a weak precipitin line, while the hemagglutination test did not reveal the presence of any antibodies to relaxin. No good explanation can be offered for this observation. None of the sera from this animal were used for the fluorescent antibody studies.

Indirect Fluorescent Antibody Studies

The antisera selected for use in these studies were R-1 (3), R-2 (3), and R-2 (4). These antisera were absorbed with a mixed homogenate of liver, kidney, and spleen from a male rat to eliminate any proteins in rabbit serum which may have a nonspecific affinity for rat tissues. The guinea pig antisera were shown to be specific in the localization of relaxin but these antisera when used in the staining procedure did not produce as good a reaction as when the rabbit anti-relaxin antisera were employed. Therefore, rabbit antisera were used for all results reported here.

Ovary.--At day 17 of gestation the ovary contains numerous large corpora lutea and follicles at various stages of development (Figure 14). The corpora lutea are composed of large polygonal cells arranged in cords or clumps around the capillaries (Figure 15).

Fluorescent antibody studies of paraffin sections of ovaries from day 17 pregnant rats demonstrated that relaxin was present only in the luteal cells as demonstrated by the specific yellow-green fluorescence (Figures 19 and 20). Maximum fluorescent emission of fluorescein isothiocyanate is at 520 mμ. Furthermore, the specific yellow-green fluorescence did not involve the whole cytoplasm of the cell, but was paranuclear in location, presumably over the Golgi complex (Figure 21). Approximately 30% of the luteal cells did not possess the fluorescent label, but this is easily explained. Since the luteal cells are quite large the section did not pass through some of the cells in the area of the Golgi complex. All other areas of the ovary, follicles and interstitial tissue, did not display any specific yellow-green fluorescence for the localization of relaxin (Figure 19). Interstitial cells displayed an orange autofluorescence (Figure 19), similar to that seen in the saline controls. This autofluorescence is due to the accumulation of lipofuchsin pigment in the interstitial cells (Reichel, 1968). However, this investigator considered these cells to be tissue macrophages. In the mouse it has been demonstrated that these pigmented cells are interstitial cells (Deane and Fawcett, 1952).

The results of the control sections were negative. The saline control displayed a general very dull background with only the orange autofluorescence of the interstitial cells (Figure 16). Sections which were incubated with only

fluorescein labelled anti-rabbit gamma globulin (no anti-relaxin antiserum) resembled the saline control, displaying no specific fluorescence; rather, a dull background was observed in corpora lutea. Autofluorescence of interstitial cells was prominent (Figure 17). With the substitution of normal rabbit serum (NRS) for the anti-relaxin antiserum in the staining procedure, the sections of ovary were seen to have a low background, with no specific fluorescence being observed in the luteal cells (Figure 18). Control tissues, adrenal gland and nonplacental uterus, demonstrated no localization of relaxin when stained with the antiserum and the fluorescein labelled anti-rabbit gamma globulin.

The same staining procedure was used on frozen sections of ovaries from day 17 pregnant rats which had been prepared according to the method of Dallenbach-Hellweg et al. (1965) (sections were air dried and fixed in 95% ethanol for 10 minutes). After staining these sections according to the same procedure as used for paraffin sections, luteal cells did not display any specific fluorescence for the presence of relaxin (Figure 22). All that could be seen in these sections was the autofluorescence of interstitial cells. The saline control for this series displayed a similar lack of specific fluorescence.

Frozen sections from ovaries of day 17 pregnant rats were also fixed according to the method used by Zarrow and O'Connor (1966) in their study of the localization of relaxin in luteal cells of pregnant rabbits. After air drying, the sections were fixed in 95% ethanol for 30 minutes at 37^{o}C. When these sections were stained according to the same procedure as was used for the paraffin sections, specific localization of the fluorescent label was observed in corpora

lutea (Figure 23). However, the stain appeared to be intercellular and was not as intense as in the paraffin sections. The controls revealed that the stain present in the corpora lutea was specific for relaxin. There was no fluorescence observed in the sections incubated with only fluorescein labelled anti-rabbit gamma globulin or with NRS and fluorescein labelled anti-rabbit gamma

In order to further demonstrate the specificity of the antisera for relaxin in luteal cells, the antiserum from R-1 (3) was incubated with NIH porcine relaxin (#0147). In this way the antigen molecules would occupy all the reaction sites on the antibody molecules. Upon staining the antibody molecules would not be free to react with the antigen in the tissue, and no specific yellow-green fluorescence for the presence of relaxin would be demonstrated. In paraffin sections of ovary after staining with the relaxin-absorbed antiserum, luteal cells, as expected, were devoid of the fluorescent label (Figure 24). The control antiserum, which was diluted with phosphate buffered saline so the dilution factor of the antiserum would be the same as for the relaxin-absorbed antiserum, did display the specific fluorescence due to the presence of relaxin (Figure 25).

Metrial gland.--At day 17 of gestation granulated cells accumulate in large numbers around blood vessels in the metrial gland. Many of the endothelial cells of blood vessels in this area are cuboidal or columnar rather than the more usual squamous shape (Figures 26 and 27).

Paraffin sections of uterine metrial glands from day 17 pregnant rats were stained according to the same procedure used for the ovarian sections to determine if relaxin is present in these structures; in particular, to determine if

relaxin is present in the granulated cells as was stated by Dallenbach-Hellweg et al. (1965). No specific fluorescence for relaxin was seen in the granulated cells (Figure 30). However, hypertrophied endothelial cells of the blood vessels appeared to have a slight fluorescence as did the red blood cells in the lumina. This was also cence for relaxin demonstrated to be present in the granulated cells (Figure 31). This was an exception since all previous and following investigations did not reveal any such fluorescence for the presence of relaxin. In the one study in which positive staining of the granulated cells was demonstrated, it is possible that relaxin levels were more concentrated than usual and permitted the reaction to occur. The control section for this series which was incubated with NRS and fluorescein labelled anti-rabbit gamma globulin did not display any specific fluorescence for the presence of relaxin; however, slight fluorescence of the hypertrophied endothelial cells of the blood vessels was again observed (Figure 29).

Frozen sections of the metrial gland from day 17 pregnant rats, which were fixed in 95% ethanol for 10 minutes according to the procedure of Dallenbach-Hellweg et al. (1965), did not demonstrate any specific fluorescence for the localization of relaxin. Again as in the case of the paraffin sections, all frozen sections, including the saline control, revealed a slight fluorescence of hypertrophied endothelial cells. Furthermore, autofluorescence was observed to be present in a few scattered cells of the metrial gland.

Validation of the Bioassay Procedure

The sensitivity of the bioassay was established by determining the smallest quantity of NIH porcine relaxin (#0147) which would produce total inhibition of uterine contractions. Total inhibition of uterine contractions was obtained at a chamber concentration of 0.027 GPU/ml or 0.06 μg/ml of the NIH relaxin preparation (Figure 33a). At this level there was good recovery of the uterine smooth muscle when the chamber was rinsed with Locke's solution. It was observed that at higher concentrations of relaxin, recovery of rhythmic contractions was poor after the chamber was rinsed; if the concentration of relaxin were high enough, there was no recovery. Furthermore, 0.027 GPU/ml was demonstrated to be very close to the level required for total inhibition since 0.020 GPU/ml did not result in total inhibition (Figure 33b). Furthermore, the quantity of relaxin necessary for total inhibition was reproducible.

To determine if the uterine smooth muscle fatigues and changes its pattern of contractions over the time required for bioassay, several muscle segments were permitted to contract for about an hour. The longest bioassay time was approximately 35 minutes. No change in frequency or amplitude of the uterine contractions was seen in one hour (Figure 33c). Furthermore, it was demonstrated that rinsing the chamber did not influence the pattern of uterine contractions.

Bioassay of Tissue Extracts from Day 17 Pregnant Rat Tissues

The information concerning the tissue extracts from day 17 pregnant rats is presented in Table II.

Table II.

Tissue Extraction of Pregnant Tissues

Rat Tissue	No. Rats	Pooled Tissue (wet weight)	Yield of Preparation (dry weight)	Tissue Extract Required for Total Inhibition (dry weight)
corpora lutea (experiment 1)	15	961.8 mg	5.0 mg	~2.0 ug/ml
corpora lutea (experiment 2)	15	818.2 mg	8.2 mg	~2.0 ug/ml
remaining ovarian tissue (experiment 1)	15	544.2 mg	1.9 mg	>24 ug/ml
remaining ovarian tissue (experiment 2)	15	399.8 mg	4.2 mg	>7.0 ug/ml
metrial gland	10	9.3 g	10.0 mg	>47.0 ug/ml
nonplacental uterus	10	5.5 g	8.1 mg	>47.5 ug/ml

Upon bioassay the following results were obtained. The material extracted from the corpora lutea (experiment 1) gave total inhibition at a chamber concentration of 3.2 μg/ml (Figure 34b). However, the uterine muscle did not recover from this concentration of relaxin. At a chamber concentration of 1.6 μg/ml inhibition was not total, but displayed good recovery (Figure 34a). Therefore, the dose level for total inhibition is between 1.6 μg/ml and 3.2 μg/ml. The corpora lutea extract from a second extraction experiment gave similar results.

Bioassay of the remaining ovarian tissue demonstrated that some relaxin activity was present. Partial inhibition was observed at a chamber concentration of 24.0 μg/ml (experiment 1) and at a chamber concentration of 7.0 μg/ml

(experiment 2) (Figure 34 c,d). However, the amount of material necessary to produce these responses is far in excess of the amount needed when the extract is from luteal tissue. The material from the metrial glands and nonplacental uteri upon bioassay gave negative results at chamber concentrations of 47.0 µg/ml and 47.5 µg/ml respectively (Figure 34e,f).

Investigation of Cell Fractions Acquired by Continuous Sucrose Density Gradient Centrifugation

After centrifugation of an homogenate of luteal cells (minus nuclear fraction) on a continuous sucrose gradient at 90,000 g for 30 minutes, two bands were visible (Figure 35). Fractions of 0.7 ml were collected from the bottom of the tube. Fractions 3 through 5 correspond to band 2; fractions 9 through 11 correspond to band 1. All fractions were assayed in the form in which they were collected and were not diluted or concentrated. Bioassay of 0.4 ml of 55% sucrose resulted in a slight reduction in the frequency of uterine contractions; therefore, effects of sucrose in the fractions can be eliminated as a possible cause of the inhibition of uterine contractions.

Bioassay of the fractions demonstrated that the highest level of relaxin activity was present in the region of the lower band, band 2 (fractions 3 through 5). Total inhibition was achieved by 0.2 ml of a fraction from band 2 (Figure 36a). It was also demonstrated that this effect on the uterine smooth muscle was not due to contaminants such as adrenaline or histamine. Adrenaline would cause inhibition of rhythmic contractions of smooth muscle in the ileum as well as

smooth muscle of the uterus. 0.2 ml of fractions from band 2 had no effect on ileal smooth muscle. NIH porcine relaxin standard also had no effect on the smooth muscle of the ileum. A portion of a fraction corresponding to band 2 was also boiled in 1N NaOH for 10 minutes and the pH readjusted to 7.6. Histamine and other contaminants would be inactivated by this treatment but relaxin is not (Sawyer, et al., 1953). This preparation did not lose its effect upon uterine smooth muscle, that is, motility was inhibited. Results were similar to those shown in Figure 36a. Electron micrographs of band 2 demonstrated the presence of mitochondria and a heterogeneous population of granules (Figure 37), which are similar in appearance to the type I and type II granules of the intact luteal cell.

Relaxin activity was also demonstrated to be present in the region of the upper band, band 1 (Figure 35). However, lower concentrations of relaxin were present in this region since only partial inhibition of uterine contractions was obtained using 0.2 ml of a fraction corresponding to the upper part of the tube (Figure 36c). Electron micrographs of this region demonstrated the presence of membranes, lipid droplets, and clusters of mitochondria and granules (Figure 39).

In the region between the two bands relaxin activity, as demonstrated by bioassay, was essentially nonexistent. Bioassay of 0.2 ml of a fraction from this region had slight or no effects upon uterine contractions (Figure 36b). Electron micrographs of this intermediate area demonstrated the presence of membranes (Figure 38). However, infrequent granules and mitochondria were seen.

Investigation of Cell Fractions Collected by Differential Centrifugation

Protein determinations were performed on the cell fractions collected by differential centrifugation in order to determine the specific activity of relaxin in the fractions. These values are given in Table III. Since sucrose and EDTA, the solution used for fractionation, interferes with the color reaction in the Lowry method of protein determination, each fraction was resuspended in saline for these determinations. The whole homogenate was centrifuged at 105,000 g for 30 minutes (speed at which the microsomal fraction was pelleted). The pellet collected from the whole homogenate was then resuspended in saline for protein determination. Therefore, solubilized protein in the supernatant was not measured.

Table III.

Protein Determinations (bovine serum albumin equivalents) on Cell Fractions (mg)

Experiment No.	I	II	III	IV	V
Whole Homogenate	-	8.4	9.2	6.2	8.0
Nuclear Fraction	-	3.5	4.5	4.3	4.8
Mitochondrial Fraction	0.4	0.7	0.5	0.4	0.5
Granular Fraction	0.8	1.6	1.0	0.6	1.1
Microsomal Fraction	0.3	0.8	0.7	0.6	1.0
Total of Fractions	-	6.5	6.7	5.9	7.4
Protein Loss	-	1.8	2.5	0.3	0.6

The specific activity of these cell fractions was determined by bioassay - the amount of protein necessary for total inhibition. The results are seen in Table IV.

Table IV.

Specific Activity of Cell Fractions

(Amount of protein necessary to cause total inhibition of uterine contractions) µg/ml - chamber concentration

Experiment No.	I	II	III	IV	V
Whole Homogenate	-	7.0	12.8	22.5	20.8
Nuclear Fraction	-	8.0	14.6	28.82	28.8
Mitochondrial Fraction	4.0	4.2	5.5	17.6	9.0
Granular Fraction	1.5	1.0	2.6	7.8	6.0
Microsomal Fraction	3.5	7.2-15.0	10.4	24.4	19.6

The granular fraction was demonstrated to have the highest specific activity; that is, less protein was required to produce total inhibition than was observed for any of the other cell fractions. The mitochondrial fraction had the next highest specific activity, while the microsomal fraction had a low specific activity; that is, large amounts of microsomal protein were necessary to produce an effect on the uterine muscle in the bioassay. In fact, in none of the experiments was the dose level ever found which caused total inhibition of uterine contractions by the microsomal fraction. In all cases the specific activity of the whole

homogenate was higher than the nuclear fraction as would be expected.

The animals used for experiments IV and V appeared to be day 15 or day 16 of gestation. Therefore, less relaxin would be present in the initial homogenate used for the cell fractionation studies in these two experiments. The bioassay results of experiment II are seen in Figure 40.

Electron micrographs of the granular fraction demonstrated the presence of mitochondria and a heterogeneous population of granules (Figure 41). Both type I and type II granules as described by Long (1973) appeared to be present (Figure 42). The mitochondrial fraction contained mitochondria as well as granules (Figure 43). However, systematic photographing of the upper right corner and lower left corner of each space on the grid occupied by a section demonstrated that a higher concentration of granules was present in the granular fraction than in the mitochondrial fraction. The microsomal fraction as seen in electron micrographs was a very clean fraction consisting of smooth and rough surfaced vesicles (Figure 44). Granules were seen only infrequently.

V. DISCUSSION

This investigation was undertaken to help clarify the site(s) of storage of relaxin in the pregnant rat. Day 17 of gestation was selected for investigation since the luteal cells have attained their maximum size at this time and contain an abundance of the type II granules, which are thought to be a possible source of relaxin in the pregnant rat (Long, 1973). Furthermore, the metrial gland at day 17 of gestation is well formed and contains numerous granulated cells, which are also believed to be a source of relaxin in the rat (Dallenbach-Hellweg et al., 1965). The granulated cells at this time are undergoing necrosis, and the granules are being released into the tissue spaces upon the death of the cells (Larkin, 1972).

Production of Antiserum to Relaxin

Relaxin, because of its low molecular weight of 5-10,000, is considered to be a weak antigen. However, several investigators (Cohen, 1963b; Steinetz et al., 1964; McClintock and Zarrow, 1966; Zarrow and O'Connor, 1966) have been successful in the production of antisera to porcine relaxin in rabbits. Furthermore, it appears that there is a lack of immunochemical species specificity of the relaxin molecule(s) as demonstrated by the double diffusion agar method of Ouchterlony (1953). For example, Steinetz and associates (1964) employing a rabbit antiserum to porcine relaxin demonstrated that there was a precipitin reaction to whale corpus luteum, an extract of rooster testes, acetone dried armadillo testes, and acetone dried human corpus luteum.

Cohen (1963b) demonstrated that the antiserum which he produced in rabbits to porcine relaxin caused total inhibition of the physiologic effect of relaxin upon the pubic symphysis of the mouse when 0.2 ml of undiluted antiserum was injected 30 minutes before the injection of approximately 3 GPU of relaxin.

Steinetz and his associates (1964) also employed the mouse interpubic ligament method to test the inhibition of the physiologic action of the hormone by their antiserum, which was prepared in rabbits against porcine relaxin. Total inhibition of 1 GPU was achieved by 0.2 ml of 1:10 dilution of the antiserum. This is more or less equivalent to the response which Cohen (1963b) achieved with his antiserum. Steinetz et al. (1964) also studied their antiserum by the technique of Ouchterlony (1953). On the agar plates it was observed that the antiserum to porcine relaxin produced several precipitin lines when tested against the homologous relaxin. This was also observed by McClintock and Zarrow (1966) who noted the presence of three lines. However, these latter investigators stated that relaxin preparations of higher purity (1000 GPU/mg) formed only one line against the antiserum. By means of the hemagglutination titer test their antisera had titers of 1:1000 to 1:4000.

Zarrow and O'Connor (1966) produced an antiserum to porcine relaxin in rabbits which, by the hemagglutination titer test, gave a 2+ reading at a 1:32 dilution of the antiserum. The interpubic ligament method of measuring relaxin activity in the mouse resulted in only 34% inhibition of 1 GPU of relaxin by 0.2 ml of undiluted antiserum. This is a much weaker response than the responses produced by the antisera of the other investigators. Zarrow and O'Connor (1966) were aware of the weakness of their antiserum; however,

by means of the fluorescent antibody method they did localize relaxin in the corpus luteum of the pregnant rabbit using this antiserum. They were not able to demonstrate the presence of relaxin in the ovary of the pregnant mouse by this method; the reasons presented for this failure were that the mouse ovary contained low levels of relaxin, and that the antiserum was too weak.

By means of the technique of Ouchterlony (1953) I found that rabbits produced antibodies to three components in the NIH porcine relaxin preparation. This is in agreement with the observations of McClintock and Zarrow (1966) and Steinetz et al. (1964). I showed that none of the three components were present in normal pig serum, since no detectable precipitin lines were formed between normal pig serum and the antiserum. The initial response of the rabbits was to a single component in the relaxin preparation as suggested by a single precipitin line on the agar plate, but as the booster injections continued the rabbits produced antibodies to two more components. This is not surprising since Oliver and Larkin (1973) demonstrated the presence of three cathodal components in an NIH porcine relaxin preparation using gel electrophoresis. The results of the hemagglutination titer test support the findings obtained by the agar diffusion method with the exception of one rabbit which did not respond well to the antigen. The best antiserum produced during this investigation formed three precipitin lines against porcine relaxin on the agar plates, and gave a positive reaction in the hemagglutination titer test up to a dilution of 1:4000 of the antiserum. This titer is equivalent to that of the antiserum produced to porcine relaxin in rabbits by McClintock and Zarrow (1966).

It is difficult to compare the potency and specificity of the antiserum produced in this investigation with those produced by other investigators due to the varying

methods of examining the antisera and lack of certain data. In most cases inhibition of a physiologic action was the main criterion used to characterize the antiserum, but this procedure does not give information about the immunological properties of the antiserum; that is, are nonspecific antibodies present? Therefore, the ultimate test of an antiserum is its specificity for the antigen when employing the fluorescent antibody technique.

The precipitin reaction on agar plates between the guinea pig antisera and relaxin was markedly different from that demonstrated between the rabbit antisera and relaxin. Each guinea pig antiserum produced a single, strong line, much stronger than any of the lines formed by rabbit antisera. The best response was at a concentration of 40 μg relaxin. This is a higher concentration of relaxin than was necessary when testing the rabbit antiserum. The best responses for the rabbit antiserum were 1.25 μg, 2.5 μg, and 5.0 μg relaxin depending on which of the lines is being described. This demonstrated that the sensitivity of the rabbit antisera is greater than that of the guinea pig antisera in reacting with minute amounts of the antigen; that is, smaller quantities of relaxin are detected by the antisera of the rabbits than by the antisera of the guinea pigs. The guinea pigs were not on such an extended immunization schedule as the rabbits, so it cannot be determined from this study if the guinea pigs would have produced antibodies to more than one component in the NIH relaxin preparation, or that the optimal concentration of antigen and antibody for the precipitin reaction would have changed. However, it appears unlikely that the sensitivity of the antiserum for the antigen would have changed since the results of the agar diffusion studies of the antisera obtained after the last

two booster injections of relaxin demonstrated that there was no further improvement.

In comparing the guinea pig antisera to the rabbit antisera on the agar plates it appears that there is no identity of the single line produced by the guinea pig antisera to relaxin with any of the three precipitin lines formed by the rabbit antisera against the same relaxin preparation. It is possible that the difference in sensitivity, that is the affinity for the antigen, of the two antisera is great enough to make detection of any identity between the two impossible on the agar plates, or the guinea pigs could be reacting against still another component in the relaxin preparation.

Formation of precipitin lines was not observed between corpora lutea extracts of the pregnant rat and the relaxin antiserum on agar plates. This is in agreement with the previous results of Steinetz and associates (1964). However, these investigators felt that the lack of a reaction was due to the low solubility of the corpora lutea extract. I hardly think that this is the reason since my extracts were dissolved under the same conditions as for bioassay. Total inhibition of uterine contractions was achieved at approximately 2 μg/ml. I can offer no explanation for failure of development of the precipitin reaction between the relaxin antiserum and the rat corpora lutea extract except that there may not have been enough relaxin present in the extract.

Localization of Relaxin by the Indirect Fluorescent Antibody Technique

The results of the fluorescent antibody studies are only as good as the antiserum used. The rabbit antiserum to relaxin used for many of these studies

gave a positive reaction at a 1:4000 dilution of the antiserum when tested by the hemagglutination titer test, and formed three precipitin lines against relaxin. The best responses were at 1.25 μg, 2.5 μg, and 5.0 μg of relaxin depending on the line being described.

Guinea pig antiserum to relaxin was also specific for the localization of relaxin but the sensitivity of the antigen was not as strong as was demonstrated by the agar diffusion studies and by some initial fluorescent antibody studies of the antisera from the two species. Therefore, rabbit antisera rather than those from guinea pigs were utilized for these studies.

Using paraffin sections of the rat ovary, the indirect fluorescent antibody method clearly demonstrated the presence of relaxin in the paranuclear area of luteal cells. This would suggest that the stain is localized over the Golgi complex since the Golgi complex is enlarged at this time and is paranuclear in location. However, not all luteal cells demonstrated this localization of relaxin. One possible explanation for the lack of stain in these luteal cells would be that the section is at a level in these cells which did not include the nuclear or paranuclear areas. Another possible explanation could be that not all luteal cells are engaged in the formation of relaxin. Follicles and interstitial tissue did not display any localization of the antigen. The controls for any nonspecific staining were all negative. The reduction in nonspecific staining was due in part to the absorption of the antisera by a mixed homogenate of liver, kidney, and spleen from a male rat. This removed any serum proteins which may have a nonspecific affinity for rat tissue proteins. The control tissues-adrenal gland and nonplacental uterus - in which relaxin is known not to be present in the rat were also negative. The specificity of the stain was further demonstrated by the staining of

sections of ovary with relaxin antiserum that had previously been absorbed with relaxin. No fluorescence was observed since binding sites on the antibody molecules were occupied by the antigen and were therefore not free to react with antigen present in luteal cells. To determine if the dilution of the antiserum by the addition of relaxin or the incubation would effect the staining reaction, antiserum was diluted with phosphate buffered saline to the same volume as with the relaxin and incubated under the same conditions. Upon staining, the localization of relaxin in luteal cells was demonstrated to be present in the paranuclear area of the cells. Therefore, the dilution and incubation of the antiserum had no effect upon the staining reaction.

Dallenbach-Hellweg et al. (1965) were not able to demonstrate the presence of relaxin in the corpus luteum of day 19 and day 20 pregnant rats by means of the indirect fluorescent antibody technique. Frozen sections were utilized and the relaxin antiserum was acquired from Dr. B.G. Steinetz. Data concerning the antiserum are lacking. Therefore, one must assume that the antiserum used was as good as that previously produced by Steinetz et al. (1964).

I employed frozen sections of ovaries from day 17 pregnant rats and fixed them in 95% ethanol according to the procedure of Dallenbach-Hellweg et al. (1965). Using this procedure I was also unable to demonstrate localization of relaxin in the corpus luteum of the pregnant rat. There are two reasons which could explain the loss of relaxin from the corpus luteum in this procedure. Relaxin is a water soluble hormone which could diffuse out of the sections while air drying. Also, fixation may not have been adequate.

Zarrow and O'Connor (1966) used frozen sections to demonstrate the presence of relaxin in the corpus luteum of the pregnant rabbit by means of the fluorescent antibody technique. Their frozen sections were also permitted to air dry but were fixed in 95% ethanol for 30 minutes at 37°C, and were then permitted to air dry again for 30 minutes at 37°C before staining. In applying this methodology to frozen sections of the pregnant rat ovary, I was able to demonstrate the localization of relaxin in the corpus luteum. However, the stain appeared to be localized mainly in the intercellular spaces of the luteal cells. This is most likely a result of diffusion of the hormone out of the cells during the initial air drying of the sections. Controls were all negative.

Since the localization of relaxin could be demonstrated in frozen sections of the ovary after they were fixed according to the method of Zarrow and O'Connor (1966), it appears that Dallenbach-Hellweg et al. (1965) lost relaxin from frozen sections of the ovary of the pregnant rat due to inadequate fixation of the hormone in situ.

Using the indirect fluorescent antibody method, Dallenbach-Hellweg and associates (1965) claim to have localized relaxin in the granular cells of the metrial gland. They also observed specific fluorescence in the lumina of nearby blood vessels which they interpreted as evidence of secretion of relaxin from the granulated cells. I repeated this work and found no such localization in the day 17 pregnant rat, a day of gestation which was studied by Dallenbach-Hellweg et al. (1965). However, I would assume that if relaxin is lost from the ovary of the pregnant rat as a result of the procedure they used for the indirect fluorescent antibody method, the same loss would occur in the metrial gland. However, Dallenbach-Hellweg et al. (1965) felt that relaxin may be bound more

tightly in the metrial gland than in the ovary and this would explain why they did not localize relaxin in the ovary.

I studied paraffin sections of metrial glands which were processed and stained in the same manner as the ovarian sections. I could not clearly demonstrate the presence of relaxin in granular cells of the metrial gland. If relaxin is present in the metrial gland, I could have not detected it for several reasons. Dallenbach-Hellweg et al. (1965) used prolonged incubation times of the relaxin antiserum (30 minutes to 6 hours) and of the fluorescein-labelled anti-rabbit gamma globulin (15 minutes to 1 hour). I used 10 minute incubations for each component. If relaxin is present in low concentrations, then prolonged staining times may be necessary to demonstrate its presence. Again, there may be different molecular species of the relaxin molecule which require different staining times. I did not employ prolonged staining times so these questions will have to remain unanswered for the time being. Furthermore, I could not compare the potency and specificity of my antisera with the antiserum which Dallenbach-Hellweg et al. (1965) used since they did not provide the pertinent information.

It is not unlikely that relaxin could be synthesized in both the corpus luteum and the uterine metrial gland. The steroid hormones are produced by both the ovary and the placenta in many species. Furthermore, in the rabbit and guinea pig there is both a uterine and an ovarian source of relaxin (Hisaw et al., 1942, 1944). Bryant (1972) has demonstrated that there is both an ovarian and a uterine source of relaxin in the pregnant sow and sheep. There are granular cells in the endometrium of the human female which have been demonstrated to be a source of relaxin (Dallenbach-Hellweg and Nette, 1964; Dallenbach and

Dallenbach-Hellweg, 1964) that are similar in structure to metrial gland granular cells in the rat (Larkin and Flickinger, 1969).

Localization of Relaxin by Bioassay of Tissue Extracts

Bloom and his associates (1958) prepared tissue extracts from day 19 pregnant rats according to the relaxin purification method of Frieden and Hisaw (1950). These tissues included the ovary, embryo, amniotic fluid, blood, endometrial decidua, uterus, and fetal membranes. Bioassay of these tissues (in-vitro inhibition of spontaneous uterine contractions of the rat), revealed relaxin activity to be confined to the ovarian extract.

I repeated this work preparing extracts of corpora lutea, remaining ovarian tissue, metrial glands, and nonplacental uteri from day 17 pregnant rats. I wanted to determine if relaxin activity were confined to the corpus luteum rather than the remaining ovarian tissue and to confirm the negative findings of the bioassay of the metrial gland extract.

Upon bioassay (in-vitro inhibition of spontaneous uterine contractions of the rat) of these tissue extracts I found that the metrial gland and nonplacental uterus extracts did not contain any relaxin activity at a chamber concentration of 47.0 µg/ml and 47.5 µg/ml respectively. This is approximately the same chamber concentration tested by Bloom et al. (1958). However, if there are different molecular species of relaxin which have different physiologic actions, then the method of bioassay may not have detected a different relaxin in the metrial gland.

I found the bulk of the uterine inhibiting capacity to be in the extract prepared from the corpora lutea rather than the remaining ovarian tissue.

Bloom's ovarian extract gave total inhibition at a chamber concentration of 21 µg/ml. Two different corpora lutea extracts that I prepared demonstrated total inhibition of uterine contractions at a chamber concentration of approximately 2 µg/ml. Total inhibition was never achieved with the extract prepared from the remaining ovarian tissue. The material from this extract was always limited in quantity so that the end points were never determined. One extract of the remaining ovarian tissue gave partial inhibition at 24 µg/ml, while a second experiment yielded an extract which produced partial inhibition at 7 µg/ml. Activity present in the remaining ovarian tissue can be explained by the fact that small corpora lutea, mistaken for large follicles upon gross dissection, were left with the remaining ovarian tissue. Fluorescent antibody studies demonstrated that these small corpora lutea were also a very rich source of relaxin.

This section of the study clearly implicates the corpus luteum as a source of relaxin and not the metrial gland in the pregnant rat.

Subcellular Localization of Relaxin

Since the luteal cells in the pregnant rat have been shown to be a source of relaxin, the next area of investigation was to determine the subcellular component in the luteal cells which stores relaxin.

Long (1973) made an ultrastructural study of luteal cells in the rat throughout gestation. The luteal cell on day 17 of gestation presents the appearance of both an active steroid secreting cell and an active protein secreting cell. The luteal cells contain an abundance of agranular endoplasmic reticulum. This is considered to be a consistent feature of steroid producing cells (See review by Christensen and Gillim, 1969). There is also an enlarged Golgi complex which

is also characteristic of steroid secreting cells. However, the significance of this is not understood since the two main functions of the Golgi complex are the condensation of secretory products and the addition of terminal carbohydrate groups to the secretion. In addition, the luteal cells of the pregnant rat possess numerous stacks of granular endoplasmic reticulum and two distinct granule populations (Long, 1973). The type I granule population is lysosomal in nature as demonstrated by their content of acid phosphatase and aryl sulfatase. These hydrolytic enzymes are not present in the second population of granules, or type II granules, which are more electron dense and smaller than the type I granules. The type I granules are present throughout gestation while the type II granules appear at day 14 of gestation and are absent after parturition. In general, cells involved in the secretion of a protein hormone possess a well developed granular endoplasmic reticulum and characteristic secretory granules (Porter, 1961). Thus, it appears that these luteal cells are engaged in the synthesis of a protein destined for secretion.

The mitochondria have undergone a change in structure from the earlier stages of gestation. Early in gestation they were spherical and contained many short tubular cristae. At later stages (day 17) the mitochondria are more elongated and contain lamellar cristae. This change in structure may take place in order to accomodate the cholesterol side chain cleavage enzymes (Christensen and Gillim, 1969).

L. L. Anderson (1971) demonstrated that relaxin activity in the ovaries of the pregnant rat is low between days 1 and 13 of gestation. Relaxin activity in the ovaries then increases and reaches a maximum at day 20. After parturition

the levels decline dramatically. This parallels the appearance and disappearance of the type II granules in luteal cells.

A similar comparison of a granule population in luteal cells and relaxin activity in the plasma of pregnant sows has been made (Belt et al., 1971). It is a well known fact that the pregnant sow ovary is the richest source of relaxin. Belt et al. (1971) observed the appearance of granules in the luteal cells at the fourth week of gestation in the sow. The number of granules continued to increase in numbers through gestation and reached a maximum between days 105 and 110. The level of relaxin in the ovarian vein at this time was low. Peak levels of the hormone in plasma occurred between 16 and 44 hours before parturition and by 1 to 16 hours before delivery the plasma levels of relaxin were extremely low. After parturition the luteal cells were observed to be nearly free of granules.

Crisp et al. (1970) in a study of the corpus luteum of pregnancy in the human observed that the luteal cells appeared to be actively engaged in steroid and protein synthesis. He suggested that the granule population observed in these luteal cells may contain relaxin.

It is strongly suggested that the type II granules observed by Long (1973) in the luteal cells of the pregnant rat are the storage sites of relaxin. Cell fractionation studies were undertaken to isolate a fraction which possesses a high degree of relaxin activity. The morphology of the fraction was also examined in the electron microscope. Initial cell fractionation studies of luteal cells were performed on a continuous sucrose density gradient. The fractions which corresponded to the lower band in the centrifuge tube upon bioassay contained

the greatest amount of relaxin activity. The activity was demonstrated not to be histamine or adrenaline. Electron micrographs of the lower band revealed the presence of only granules and mitochondria. Fractions from the intermediate area between the bands did not possess any relaxin activity. No granules or infrequent granules were present in these fractions as seen in the electron micrographs. The top fractions possessed some relaxin activity, but not to the extent as the lower fractions. The electron micrographs of the top fractions revealed mainly membranes with lipid, and there were areas of mitochondria and granules which must have been trapped at the interface of the gradient and the homogenate. Relaxin activity in the top fractions, therefore, can be explained by the presence of some granules and the solubilization of the relaxin during the homogenization of the luteal cells.

In an attempt to get better separation of the cellular components, differential centrifugation was employed. Furthermore, in order to better quantitate relaxin activity, protein determinations on the cell fractions were performed so relaxin activity could be based on the amount of protein/ml which is required for total inhibition of uterine contractions. de Duve (1967) in reference to enzyme localization in cell fraction studies considered quantitative recovery of activities of cell fractions to be quite essential, and the results to be significant only if the sum of the activities of the fractions corresponds to the activity of the original homogenate. However, the necessity for a balance sheet of enzyme activities is disputed by other workers. In my cell fractionation studies there were protein losses of between 4% and 29%. Some of these losses can be explained by the solubilization of proteins during homogenization and washing of the

fractions. No protein determinations were performed on supernatant fluids because sucrose interfered with the Lowry reaction. Losses were also incurred by the adherence of some material to dispo-pipettes during the washings and the transferring of aliquots of each fraction to tubes for storage or fixation. Even though the protein of the fractions does not add up to the protein content of the whole homogenate, I feel that the distribution of relaxin activities is meaningful as demonstrated by bioassay results and morphological studies of the fractions.

By the method of cell fractionation used in this study, the granular fraction had the highest degree of relaxin activity upon bioassay. The electron micrographs of this fraction revealed the presence of granules and mitochondria. The mitochondrial fraction demonstrated one-fourth to one-third of the specific relaxin activity of the granular fraction. The electron micrographs of the mitochondrial fraction demonstrated only granules and mitochondria. Moreover, systematic photographing of sections from these pellets revealed that more granules were present in the granular fraction than in the mitochondrial fraction. The microsomal fraction had very little relaxin activity and total inhibition was not achieved. The electron micrographs revealed that the microsomal fraction was extremely clean, being composed of smooth surfaced and ribosome-studded membraneous vesicles. Granules were only infrequently seen.

Based upon the bioassay and the morphological study of these cellular fractions, it can be concluded that the subcellular component which stores relaxin is either the granules or the mitochondria. Mitochondria are not known to be sites of storage for hormones destined for secretion. Furthermore, the

mitochondria are present throughout gestation. It appears, therefore, that the granules are the storage sites of relaxin. Since lysosomes or type I granules were not separated from the type II granules, no distinction can be made based on this investigation as to which population of granules possesses relaxin activity. However, since the appearance and disappearance of type II granules parallels relaxin levels in the ovaries of the pregnant rat, it is strongly suggested that type II granules are the storage sites for relaxin.

VI. CONCLUSIONS

The site(s) of relaxin storage in the pregnant rat - ovary vs. uterine metrial gland - has been in dispute. Three different approaches were employed to determine the site(s) of relaxin storage. By means of the fluorescent antibody technique and bioassay of extracts from various rat tissues it can be concluded that relaxin is stored in luteal cells of the ovary in the pregnant rat. Furthermore, the fluorescent antibody method demonstrated that relaxin was localized in the paranuclear area of the cell, presumably in the Golgi region, indicative of a secretory process taking place in the cell. Cell fractions prepared from luteal cells further demonstrated, by means of bioassay of the fractions and electron microscopic study of the fractions, that relaxin was confined either to granules or mitochondria. I feel that one can safely rule out mitochondria as the source of relaxin since this organelle has never been known to be involved in synthesis or storage of a protein hormone destined for secretion. The granule population is, therefore, responsible for the storage of relaxin. Furthermore, this and other work strongly suggests that type II granules which were described by Long (1973) are the specific sites of localization of relaxin. This, however, cannot be demonstrated until the intracellular localization of relaxin at the ultrastructural level is performed using peroxidase labelled antibody to relaxin.

The role of granular cells of the uterine metrial gland as a source of relaxin in the pregnant rat is still not settled. Bioassay of tissue extracts from various rat tissues revealed that relaxin activity in the metrial gland was not detectable. By means of the indirect fluorescent antibody method only one experiment out of

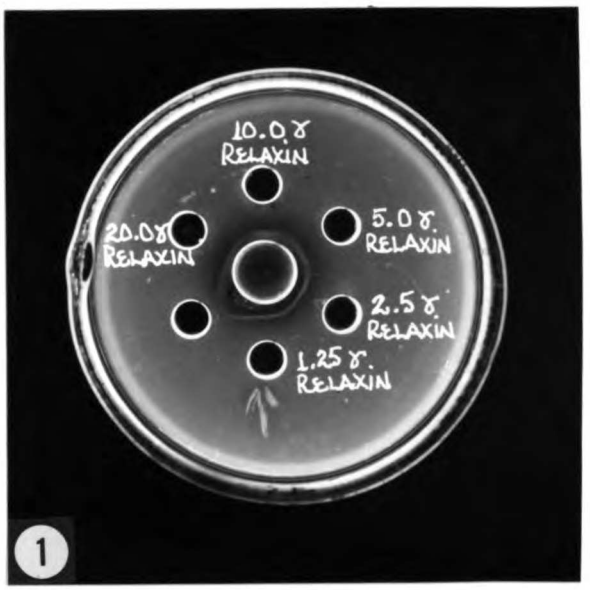
six demonstrated the presence of relaxin in the granular cells of the metrial gland. However, other investigators claim to have localized relaxin in these granular cells by the indirect fluorescent antibody technique. Failure to obtain such consistent results in my studies may have been a result of low concentrations of relaxin in the granular cells and/or the presence of a different molecular species of the relaxin molecule. Either of these possibilities might require longer incubation times with the anti-relaxin antiserum and fluorescein labelled anti-rabbit gamma globulin to demonstrate the presence of relaxin than were utilized in my investigations.

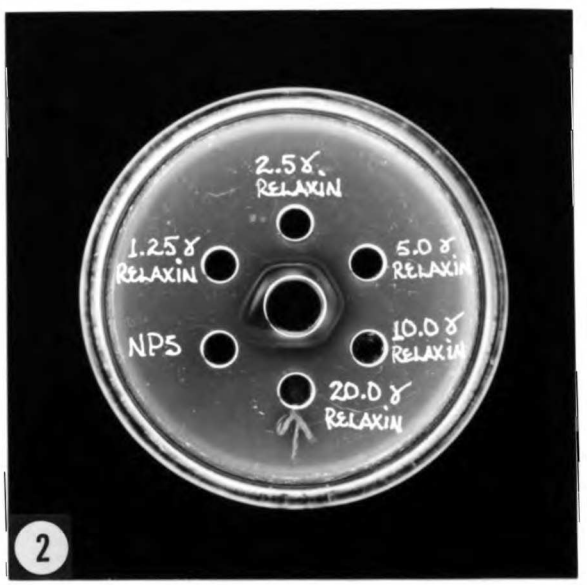
Therefore, it can be concluded that relaxin is present in luteal cells of the pregnant rat and it is strongly suggested that the type II granules are the storage sites of relaxin. The granular cells of the metrial gland as a source of relaxin is still questionable. This problem may not be resolved until further information concerning the nature of the relaxin molecule(s) is obtained.

Figure 1. Ouchterlony plate showing the results of precipitin reactions between the antiserum (rabbit 1, bleeding 2) to porcine relaxin and the homologous relaxin. Antiserum of R-1 (150 µl) was placed in the center well and 50 µl of varying concentrations of porcine relaxin were placed in the peripheral wells. Note that the best response is at a concentration of 2.5 µg relaxin. Photographed at 24 hours. On all figures γ is used as an abbreviation for microgram (µg).

Figure 2. This plate demonstrates the precipitin reactions between the antiserum (rabbit 1, bleeding 3) to porcine relaxin and the homologous relaxin. Antiserum of R-1 (150 µl) was placed in the center well and 50 µl of varying concentrations of porcine relaxin were placed in the peripheral wells. Note the formation of three precipitin lines between the antiserum and porcine relaxin. The inner diffuse line is best at 1.25 µg relaxin, the middle line is sharpest at 2.5 µg relaxin, and the outer line is the sharpest at 5.0 µg relaxin. NPS denotes normal pig serum diluted 1:10 with saline. Note the absence of a detectable reaction with the pig serum proteins. Photographed at 24 hours.

Figure 3. This plate demonstrates the precipitin reactions between the antiserum (rabbit 1, bleeding 4) to porcine relaxin and the homologous relaxin. Antiserum of R-1 (150 µl) was placed in the center well and 50 µl of varying concentrations of porcine relaxin were placed in the peripheral wells. Note that the precipitin lines are not as strong or sharp as at the previous bleeding (Figure 2). The inner diffuse line is best at 1.25 µg relaxin, the middle line is best at 2.5 µg relaxin, and the outer line is barely detectable at 5.0 µg relaxin. NPS denotes normal pig serum diluted 1:10 with saline. Photographed at 24 hours.


10.0 γ
RELAXIN
20.0 γ
RELAXIN
5.0 γ
RELAXIN
2.5 γ
RELAXIN
1.25 γ
RELAXIN
1

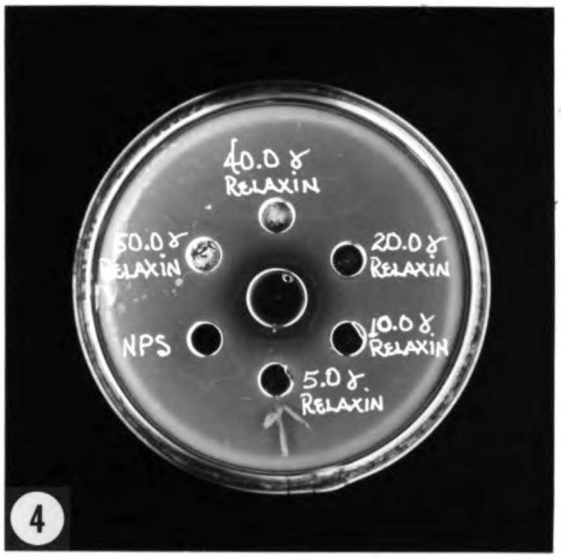

2.5 γ
RELAXIN
1.25 γ
RELAXIN
5.0 γ
RELAXIN
NPS
10.0 γ
RELAXIN
20.0 γ
RELAXIN
2

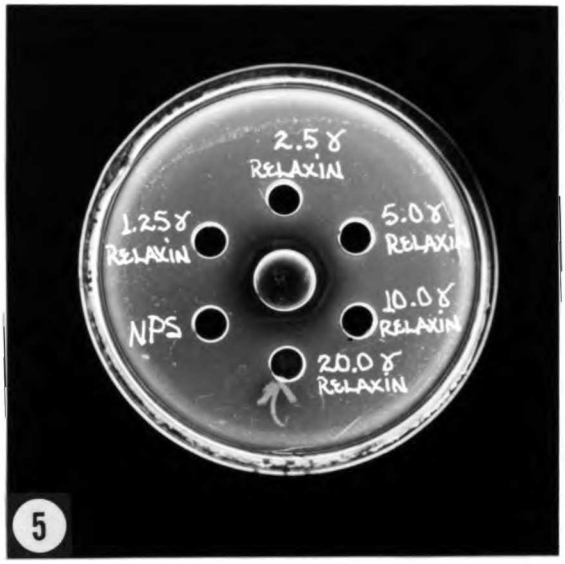

2.5 γ
RELAXIN
1.25 γ
RELAXIN
5.0 γ
RELAXIN
NPS
10.0 γ
RELAXIN
20.0 γ
RELAXIN
3

Figure 4. Ouchterlony plate showing the results of precipitin reactions between the antiserum (rabbit 2, bleeding 2) to porcine relaxin and the homologous relaxin. Antiserum of R-2 (150 µl) was placed in the center well and 50 µl of varying concentrations of porcine relaxin were placed in the peripheral wells. Note that the best response is at a concentration of 20.0 µg relaxin. NPS denotes normal pig serum diluted 1: 10 with saline. Note the absence of a detectable reaction with the pig serum proteins. Photographed at 24 hours.

Figure 5. This plate demonstrates the precipitin reactions between the antiserum (rabbit 2, bleeding 3) to porcine relaxin and the homologous relaxin. Antiserum of R-2 (150 µl) was placed in the center well and 50 µl of varying concentrations of porcine relaxin were placed in the peripheral wells. Note the formation of two precipitin lines between the antiserum and porcine relaxin. The inner diffuse line is best at 2.5 µg relaxin and the outer line is best at 10 µg relaxin. NPS denotes normal pig serum diluted 1: 10 with saline. Note the absence of a detectable reaction with the pig serum proteins. Photographed at 48 hours.

Figure 6. This plate demonstrates the precipitin reactions between the antiserum (rabbit 2, bleeding 4) to porcine relaxin and the homologous relaxin. Antiserum of R-2 (150 µl) was placed in the center well and 50 µl of varying concentrations of porcine relaxin were placed in the peripheral wells. Note the formation of three precipitin lines. The inner diffuse line is best at 1.25 µg relaxin. The middle line is best at 5.0 µg to 10.0 µg relaxin and the outer line is best at 10.0 µg relaxin. NPS denotes normal pig serum diluted 1: 10 with saline. Note the absence of a detectable reaction with pig serum proteins. Photographed at 48 hours.


40.0 γ
RELAXIN
50.0 γ
RELAXIN
20.0 γ
RELAXIN
NPS
10.0 γ
RELAXIN
5.0 γ
RELAXIN
4


2.5 γ
RELAXIN
1.25 γ
RELAXIN
5.0 γ
RELAXIN
NPS
10.0 γ
RELAXIN
20.0 γ
RELAXIN
5

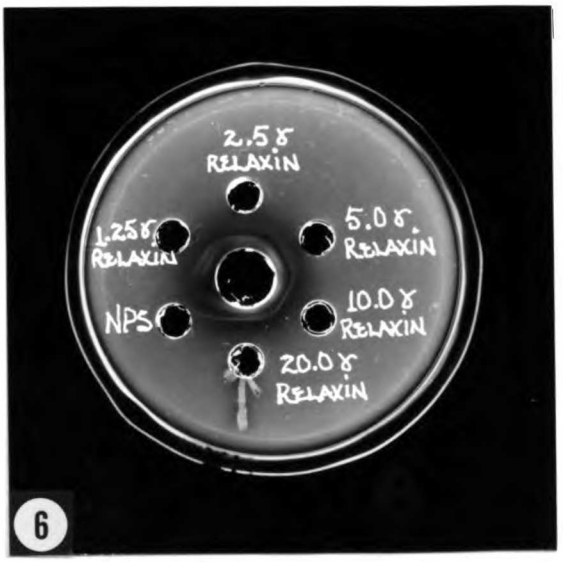
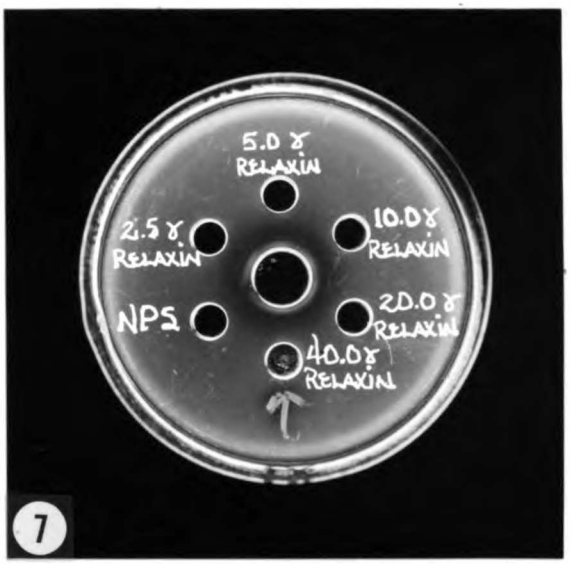
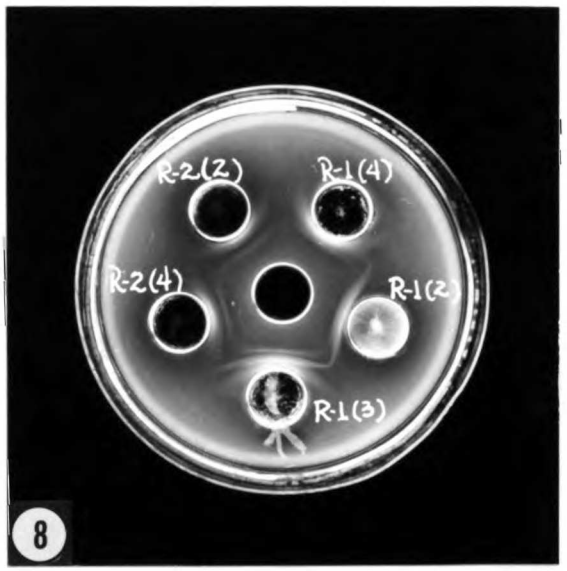

2.5 γ
RELAXIN
1.25 γ
RELAXIN
5.0 γ
RELAXIN
NPS
10.0 γ
RELAXIN
20.0 γ
RELAXIN
6

Figure 7. Ouchterlony plate showing the results of precipitin reactions between the antiserum (rabbit 3, bleeding 4) to porcine relaxin and the homologous relaxin. Antiserum of R-3 (150 µl) was placed in the center well and 50 µl of varying concentrations of porcine relaxin were placed in the peripheral wells. There is an inner diffuse line at all concentrations of relaxin. Note the formation of a weak line at 2.5 µg and 5.0 µg relaxin. NPS denotes normal pig serum diluted 1:10 with saline. Photographed at 48 hours.

Figure 8. This Ouchterlony plate demonstrates the identity of the inner precipitin lines produced by the antisera of rabbits 1 and 2 at different bleedings. The outer two lines are too close to the peripheral wells to demonstrate identity of these lines between the antisera of R-1 and R-2. It does appear that at a lower antigen concentration that the lines may join. The center well contains 10.0 µg relaxin in a volume of 150 µl. The peripheral wells contain 150 µl of each of antiserum. Photographed at 48 hours.

Figure 9. This plate demonstrates the identity of the inner precipitin lines produced by the antisera of rabbits 1 and 2 at different bleedings. The outer two lines are too close to the peripheral wells to demonstrate any identity between the antisera of R-1 and R-2. It does appear that at a lower antigen concentration that the lines may join. The center well contains 20 µg relaxin in a volume of 150 µl. The peripheral wells contain 150 µl each of the antiserum. Photographed at 48 hours.


5.0 γ
RELAXIN
2.5 γ
RELAXIN
10.0 γ
RELAXIN
NPS
20.0 γ
RELAXIN
40.0 γ
RELAXIN
7


R-2(2)
R-1(4)
R-2(4)
R-1(2)
R-1(3)
8

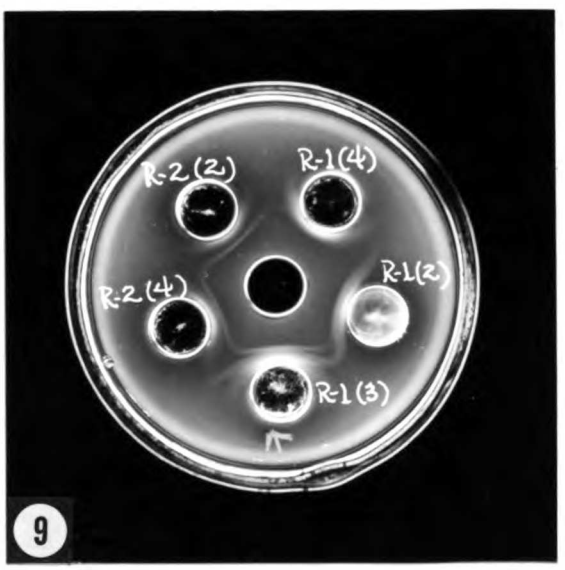
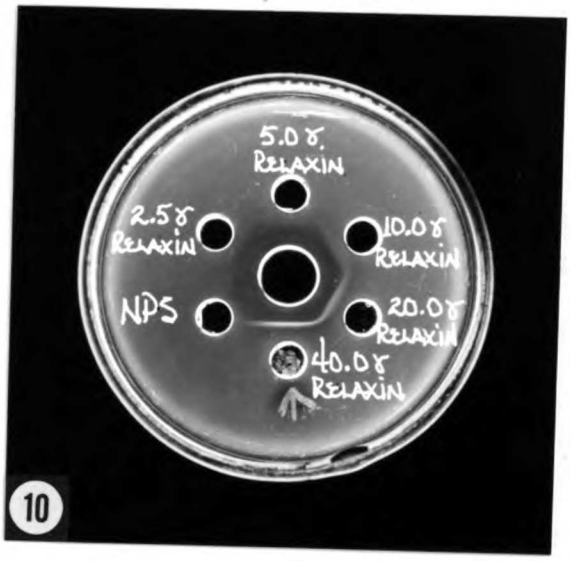

R-2(2)
R-1(4)
R-2(4)
R-1(2)
R-1(3)
9

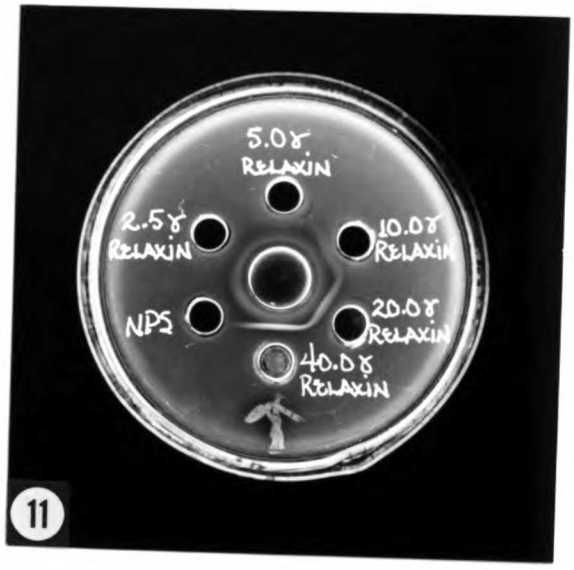
Figure 10. Ouchterlony plate demonstrating the precipitin reactions between antiserum (guinea pig 1, bleeding 3) to porcine relaxin and the homologous relaxin. Antiserum of GP-1 (150 µl) was placed in the center well and 50 µl of varying concentrations of porcine relaxin were placed in the peripheral wells. Note the formation of a single, heavy line. The best response is at a concentration of 40.0 µg relaxin. NPS denotes normal pig serum diluted 1:10 with saline. Note the absence of a detectable reaction with the pig serum proteins. Photographed at 48 hours.

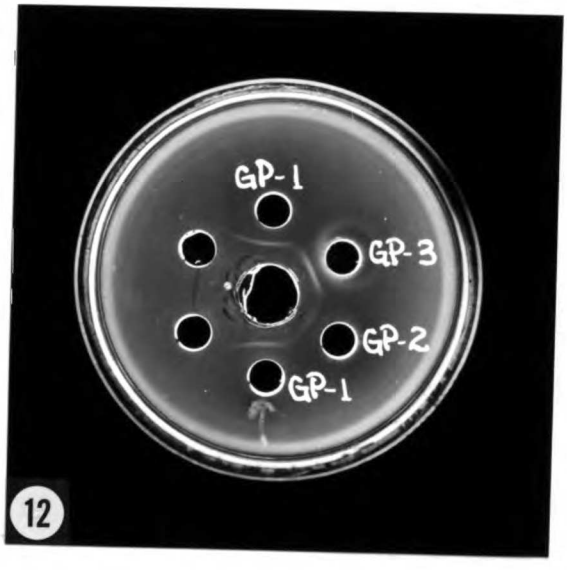
Figure 11. This plate demonstrates the precipitin reactions between the antiserum (guinea pig 3, bleeding 2) to porcine relaxin and the homologous relaxin. Antiserum of GP-3 (150 µl) was placed in the center well and 50 µl of varying concentrations of porcine relaxin were placed in the peripheral wells. Note the formation of a single heavy line. The best response is at a concentration of 40.0 µg relaxin. NPS denotes normal pig serum diluted 1:10 with saline. Note the inner diffuse line which is also present at the NPS well probably indicative of a non-specific precipitation of some component in the antiserum. Photographed at 24 hours.

Figure 12. Ouchterlony plate demonstrating the identity of the single lines produced by the guinea pig antisera to porcine relaxin in response to the homologous relaxin. The center well contains 20.0 µg relaxin in a volume of 150 µl. The peripheral wells possess 50 µl of the guinea pig antiserum. Photographed at 48 hours.

Figure 13. This Ouchterlony plate demonstrates the lack of identity of the precipitin lines formed by the rabbit antisera with precipitin lines produced by the guinea pig antisera. The center well contains 20.0 µg relaxin in a volume of 150 µl. The peripheral wells contain 150 µl each of the antiserum. Photographed at 48 hours.


5.0 γ
RELAXIN
2.5 γ
RELAXIN
10.0 γ
RELAXIN
NPS
20.0 γ
RELAXIN
40.0 γ
RELAXIN
10


5.0 γ
RELAXIN
2.5 γ
RELAXIN
10.0 γ
RELAXIN
NPS
20.0 γ
RELAXIN
40.0 γ
RELAXIN
11


GP-1
GP-3
GP-2
GP-1
12

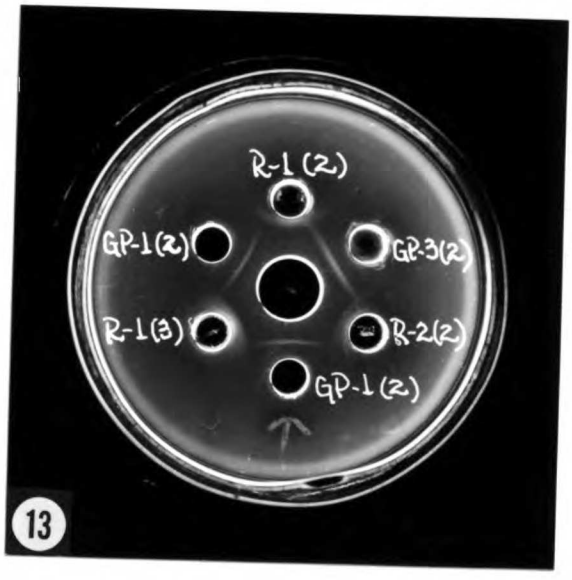
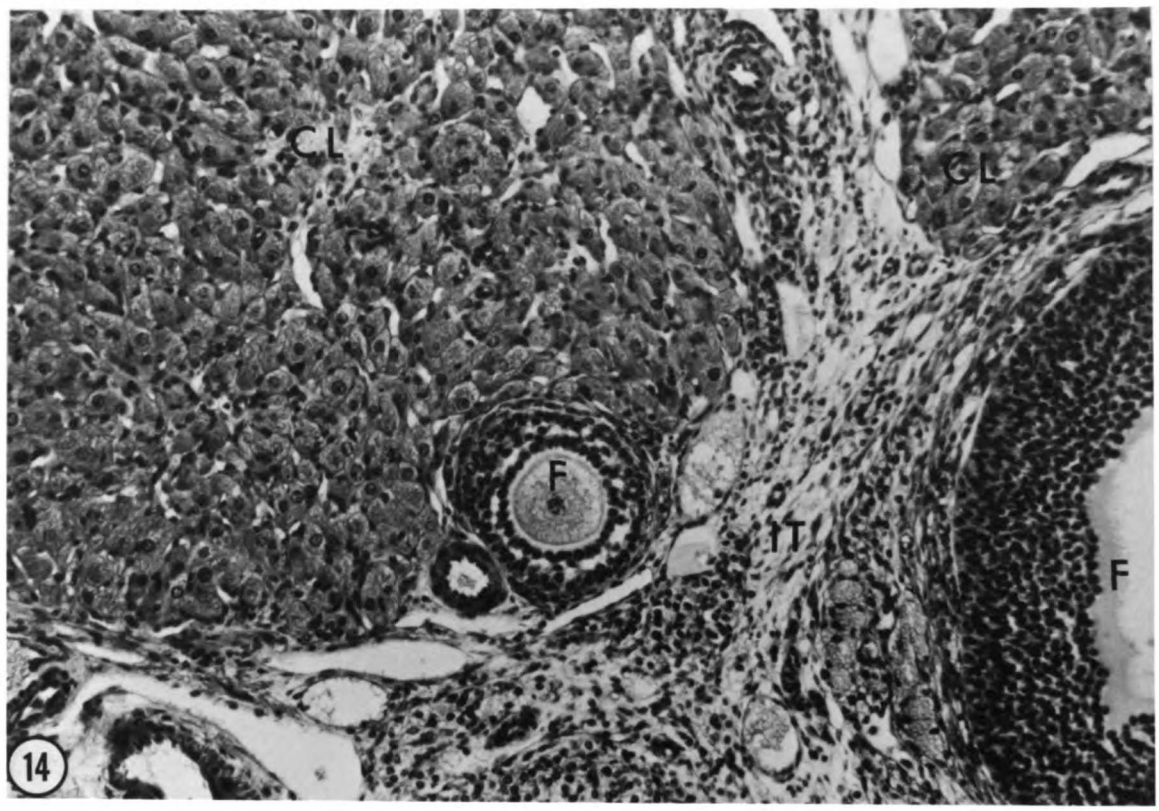

R-1 (2)
GP-1 (2)
GP-3 (2)
R-1 (3)
R-2 (2)
GP-1 (2)
13

Figure 14. Portion of an ovary of a day 17 pregnant rat. The corpora lutea (CL) are quite large. Note the presence of follicles (F) at various stages of development and the interstitial tissue (IT). H. and E. stain; X190.

Figure 15. A higher magnification view of a small area of a corpus luteum showing polygonal luteal cells. H. and E. stain; X500.



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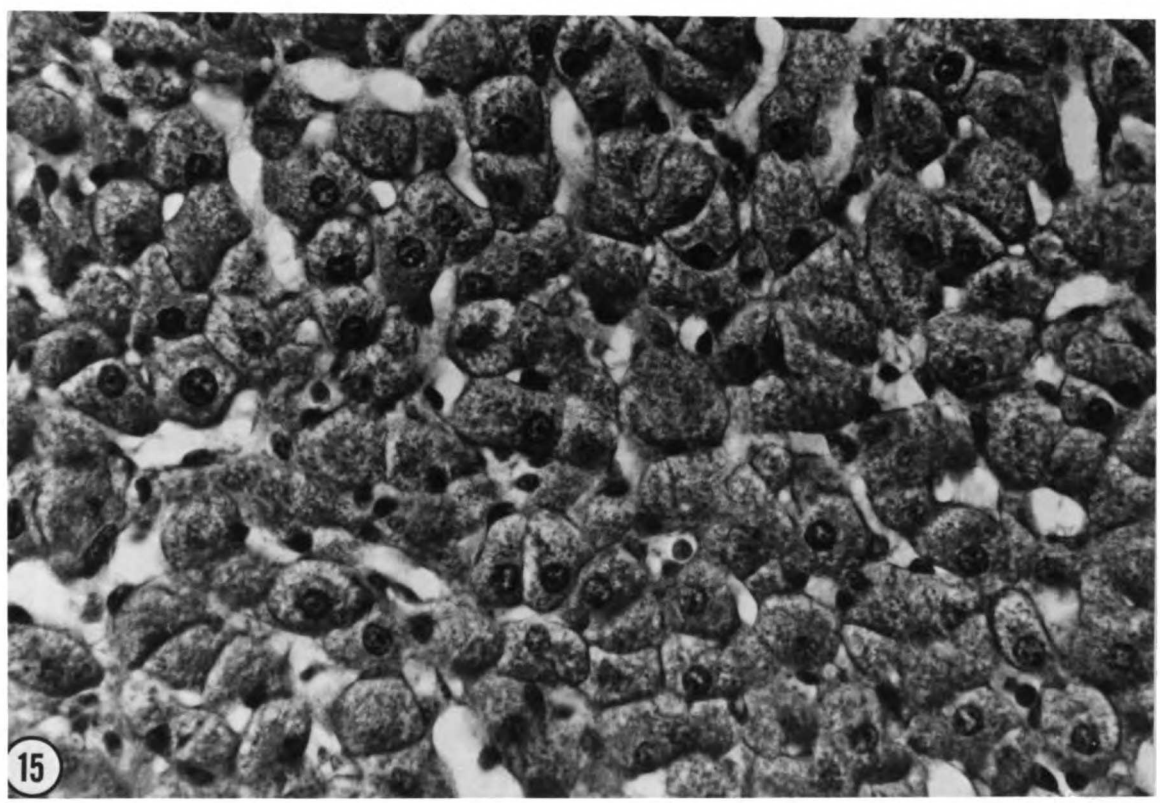
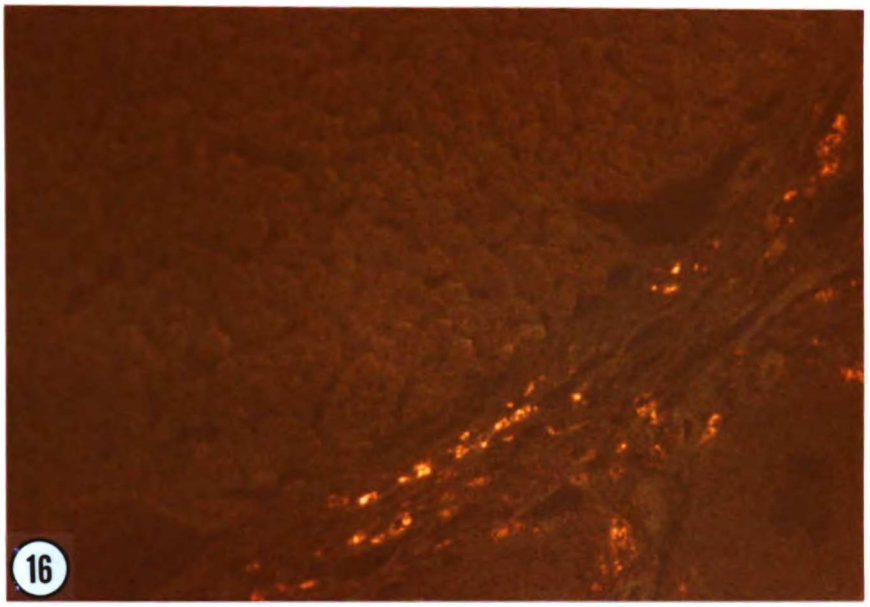


15

Figure 16. Fluorescent photomicrograph. Saline control. Paraffin section of an ovary at day 17 of gestation. There is a general low background in the luteal cells of the corpus luteum (upper left), and in the follicle (lower right). Note the orange autofluorescence of the interstitial cells. X140.

Figure 17. Fluorescent photomicrograph. Incubated with fluorescein labelled anti-rabbit gamma globulin only. Paraffin section of anyovary at day 17 of gestation. There is a general low background in the luteal cells of the corpus luteum (upper half). Note the orange autofluorescence of the interstitial cells (lower half). X140.

Figure 18. Fluorescent photomicrograph. Incubated with normal rabbit serum and fluorescein labelled anti-rabbit gamma globulin. Paraffin section of an ovary at day 17 of gestation. Only luteal cells are present in this photomicrograph. There is a low general background which resembles the saline control (Figure 16). X140.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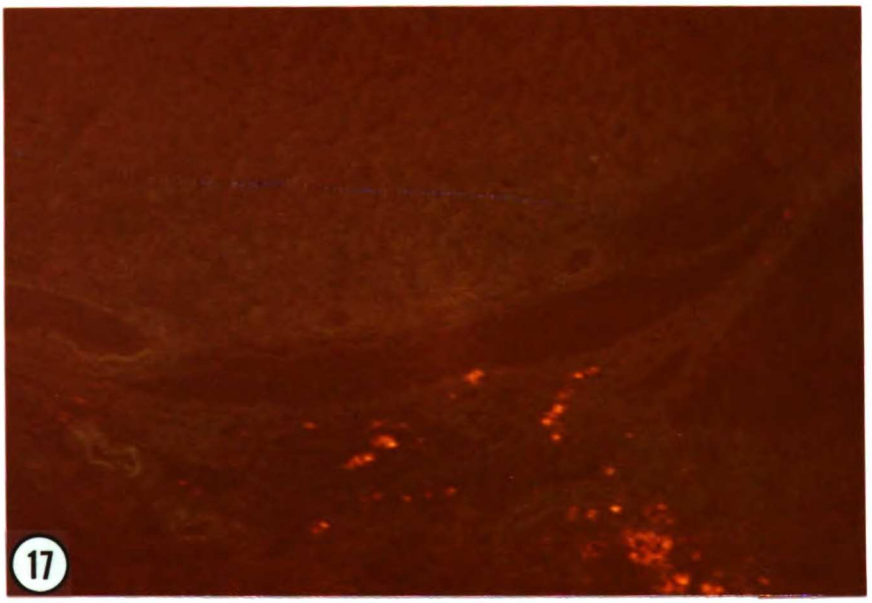
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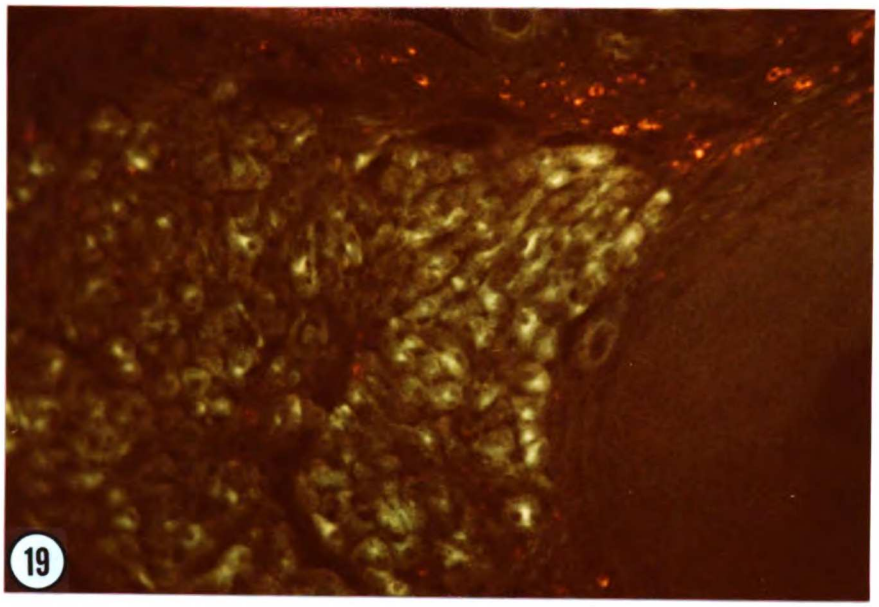
18

Figure 19. Fluorescent photomicrograph. Stained with relaxin antiserum (Rabbit 1, bleeding 3) and fluorescein labelled anti-rabbit gamma globulin. Paraffin section of an ovary at day 17 of gestation. Observe the presence of the fluorescein label in the luteal cells of the corpus luteum (lower left). There is no label present in the follicle (lower right) or the interstitial area (top). Note that the autofluorescence of the interstitial cells is the same as is seen in the saline control (Figure 16). X140

Figure 20. Fluorescent photomicrograph. Stained with relaxin antiserum (Rabbit 2, bleeding 3) and fluorescein labelled anti-rabbit gamma globulin. Paraffin section of an ovary at day 17 of gestation. Only luteal cells are present in this photomicrograph. Observe the presence of the fluorescent label in the luteal cells. However, not all cells appear to possess relaxin. Most likely explanation is that the section does not pass through all the cells in the paranuclear region. X140.

Figure 21. Fluorescent photomicrograph. Stained with relaxin antiserum (Rabbit 2, bleeding 4) and fluorescein labelled anti-rabbit gamma globulin. Paraffin section of an ovary at day 17 of gestation. Only luteal cells are present in this photomicrograph. Observe the paranuclear location of the fluorescent label for relaxin in the luteal cells. X375.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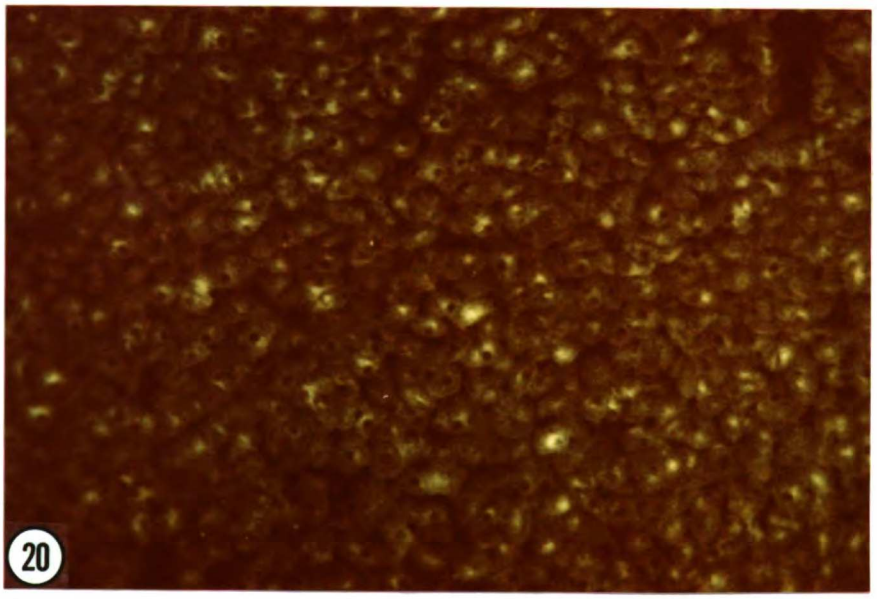
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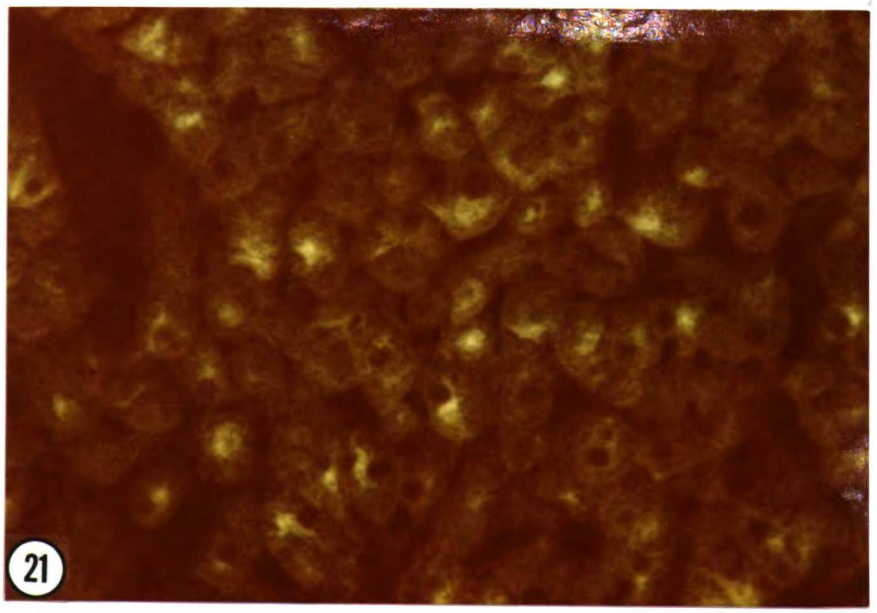
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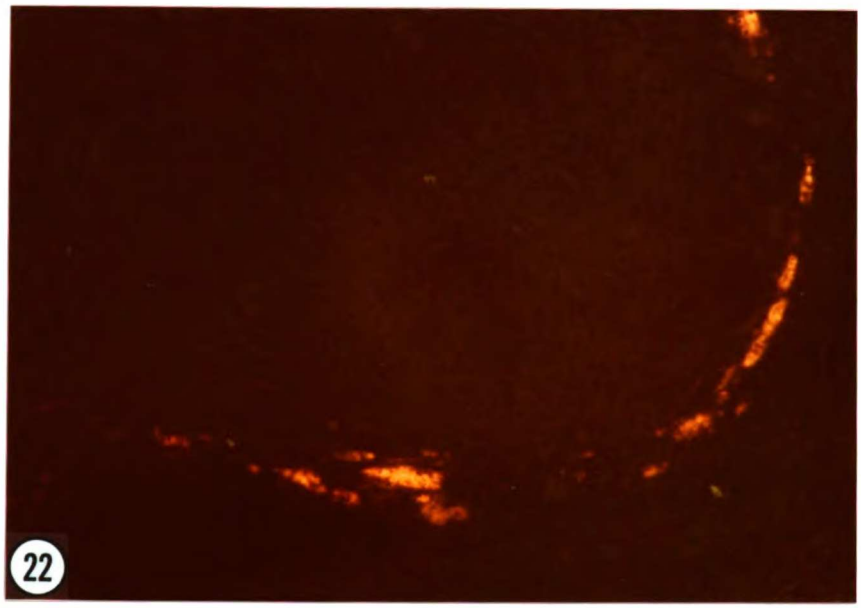
Figgure 22. Fluorescent photomicrograph. Stained with relaxin antiserum (Rabbit 2, bleeding 3) and fluorescein labelled anti-rabbit gamma globulin. Frozen section of an ovary at day 17 of gestation. After air drying, this frozen section was fixed for 10 minutes in 95% ethanol. There is a dark background present in the corpus luteum (upper left) with no specific stain for the localization of relaxin. Observe the orange autofluorescence of the interstitial cells. X140.

Figure 23. Fluorescent photomicrograph. Stained with relaxin antiserum (Rabbit 2, bleeding 3) and fluorescein labelled anti-rabbit gamma globulin. Frozen section of an ovary at day 17 of gestation. After air drying,this section was fixed for 30 minutes in 95% ethanol at 37^{o}C and air dried again for 30 minutes at 37^{o}C. There is a specific localization of the fluorescent label in this photomicrograph of luteal cells. However, the relaxin appears to be intercellular in location. X140.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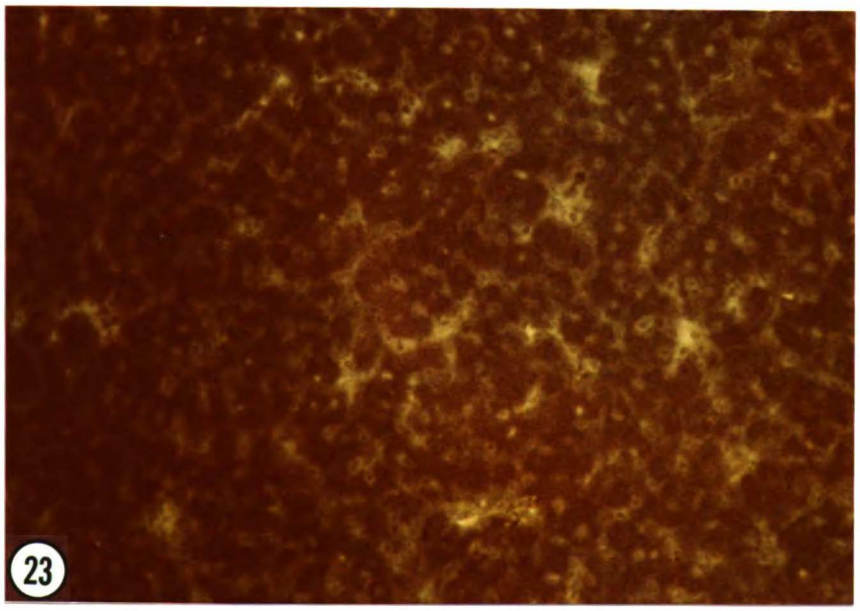
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Figure 24. Fluorescent photomicrograph. Incubated with relaxin absorbed antiserum (Rabbit 2, bleeding 4) and fluorescein labelled anti-rabbit gamma globulin. Paraffin section of an ovary at day 17 of gestation. Luteal cells are seen in this photomicrograph. The antiserum was absorbed by porcine relaxin for 60 minutes at 37^{o}C before staining the section. There is no localization of the fluorescent label in the luteal cells; rather an even dull background is present. X375.

Figure 25. Fluorescent photomicrograph. Stained with relaxin antiserum (Rabbit 2, bleeding 4) and fluorescein labelled anti-rabbit gamma globulin. The antiserum was diluted with phosphate buffered saline so the dilution of the antiserum would be the same as for the relaxin absorbed antiserum (Figure 24). Note the localization of the fluorescent label in the para-nuclear region of the luteal cells. X375.

24

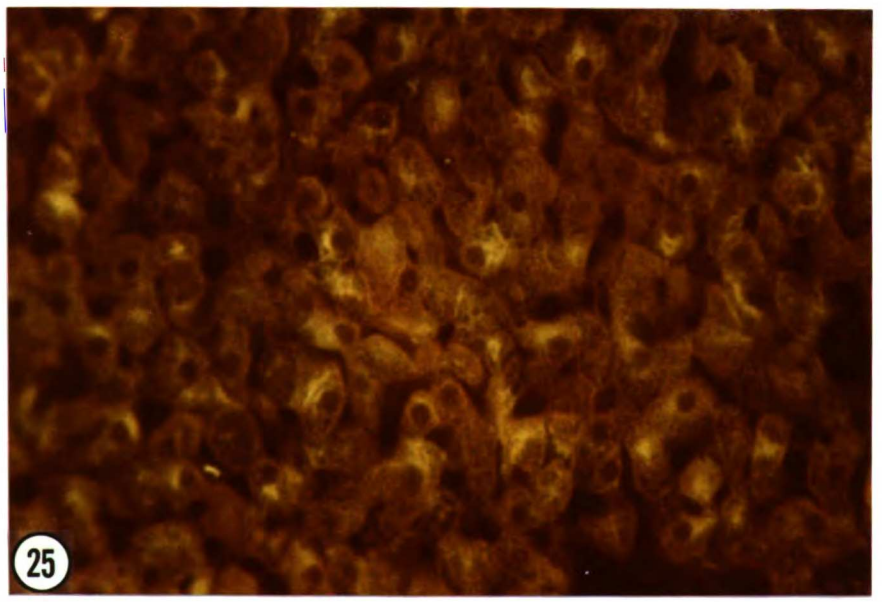

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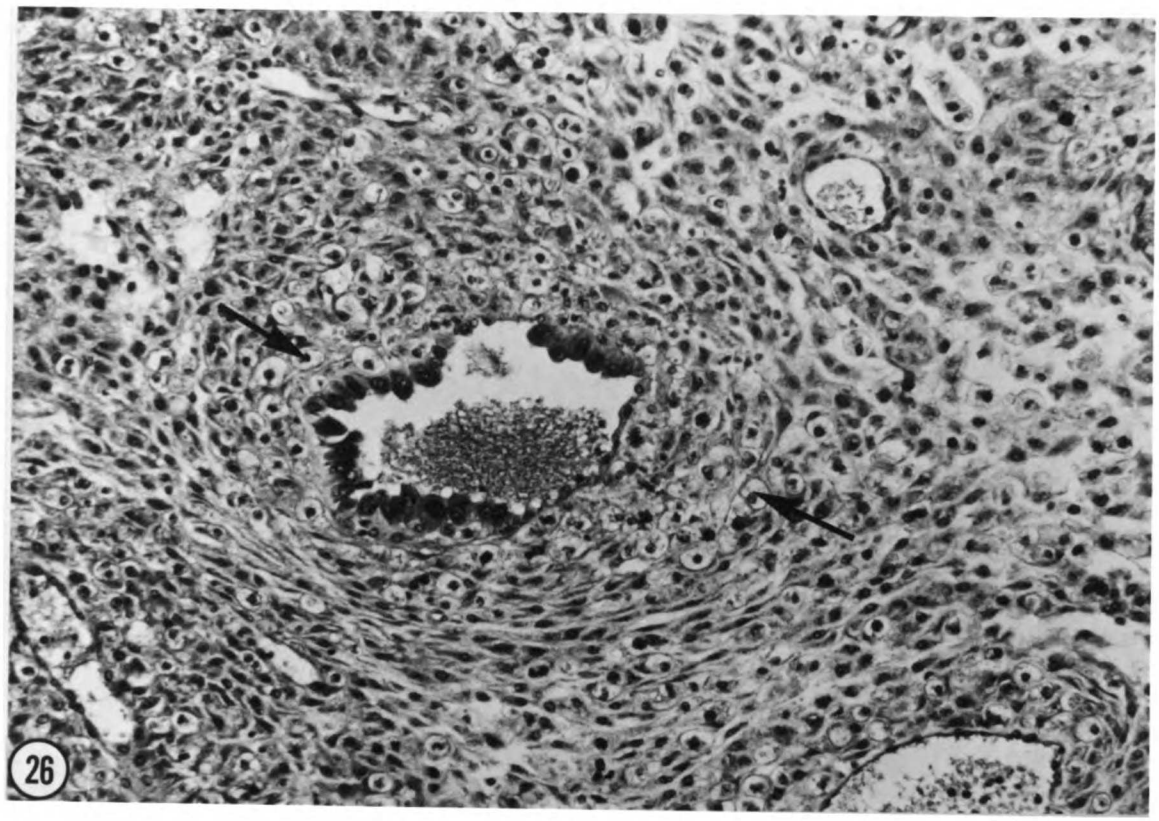
Figure 26. Small area of a metrial gland from a day 17 pregnant rat. The granular metrial gland cells (arrows) accumulate in large numbers around blood vessels. Note the hypertrophy of endothelial cells lining the blood vessel. H. and E. Stain; X200.

Figure 27. A higher magnification of the metrial gland showing the granular metrial gland cells (arrows) and the hypertrophied endothelial cells of the blood vessel. H. and E. Stain; X580.



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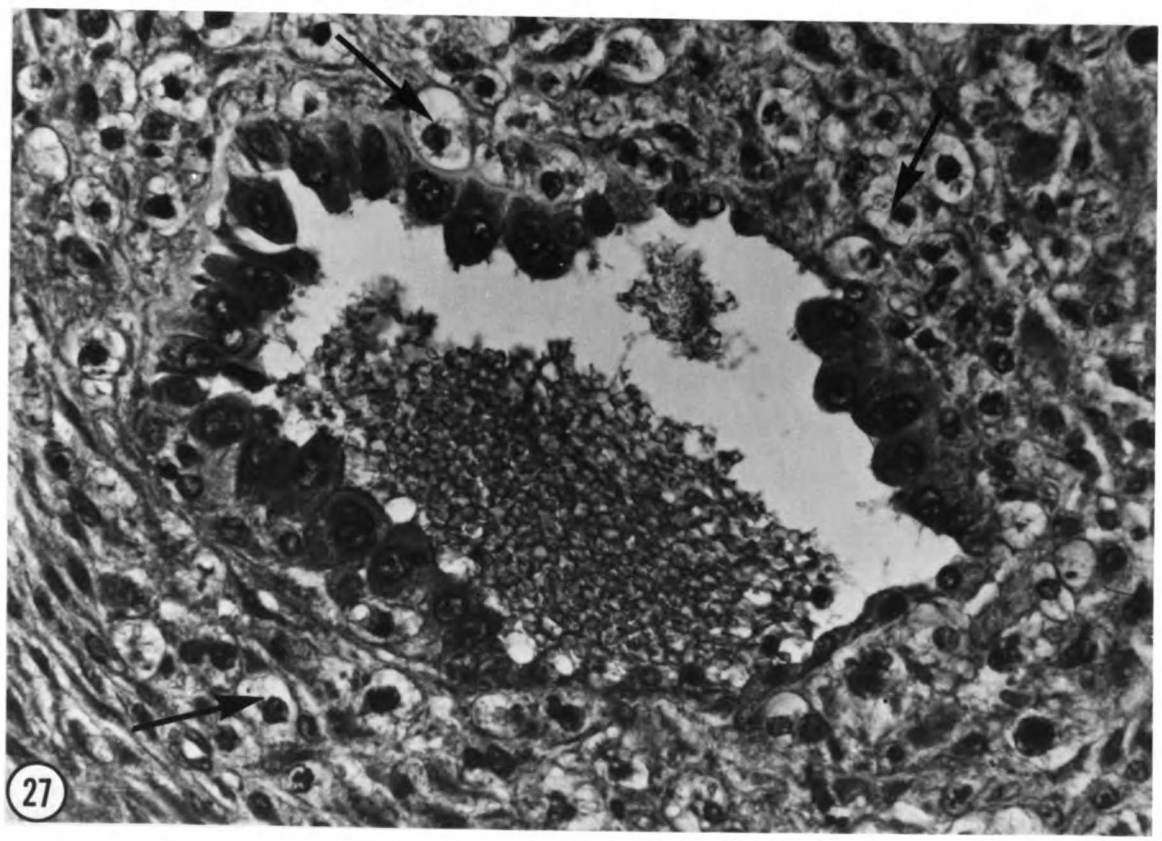

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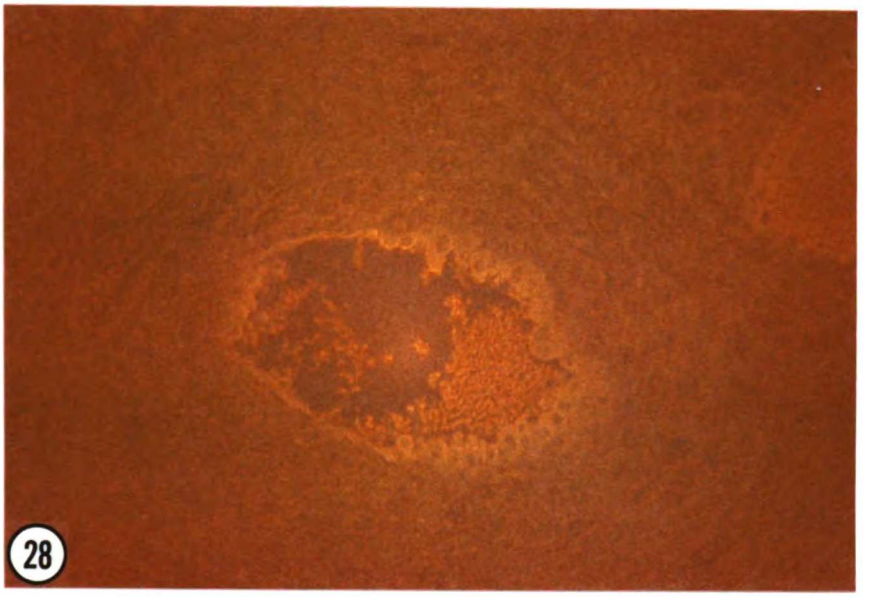
Figure 28. Fluorescent photomicrograph. Saline control. Paraffin section of a uterine metrial gland at day 17 of gestation. Note the autofluorescence of the hypertrophied endothelial cells of the blood vessel and the red blood cells in the lumen. The tissue surrounding the vessel shows a low intensity non-specific fluorescence with no autofluorescence observed in this area. X150.

Figure 29. Fluorescent photomicrograph. Incubated with normal rabbit serum and fluorescein labelled anti-rabbit gamma globulin. Paraffin section of a uterine metrial gland at day 17 of gestation. Note the fluorescence of the hypertrophied endothelial cells similar to that present in the saline control (Figure 28). The surrounding tissue is rather dull and even in appearance. X150.



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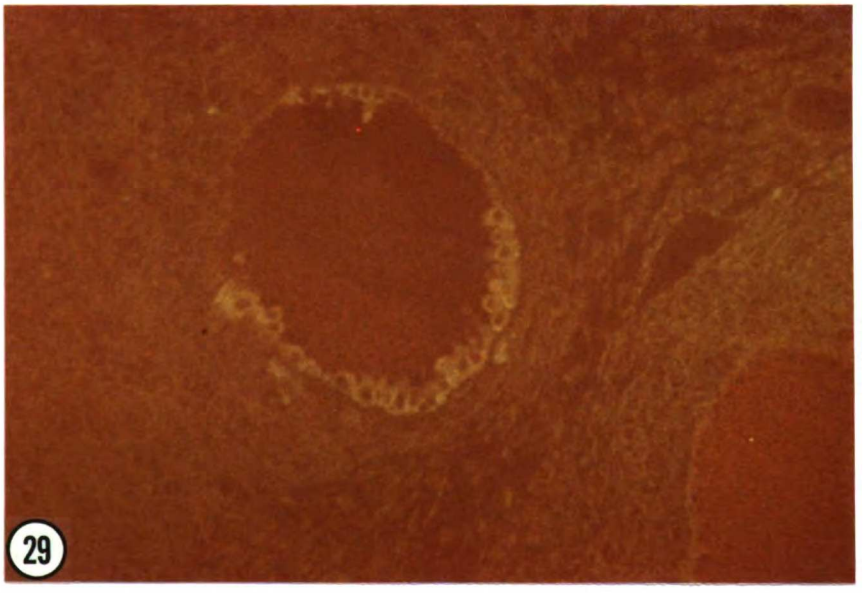

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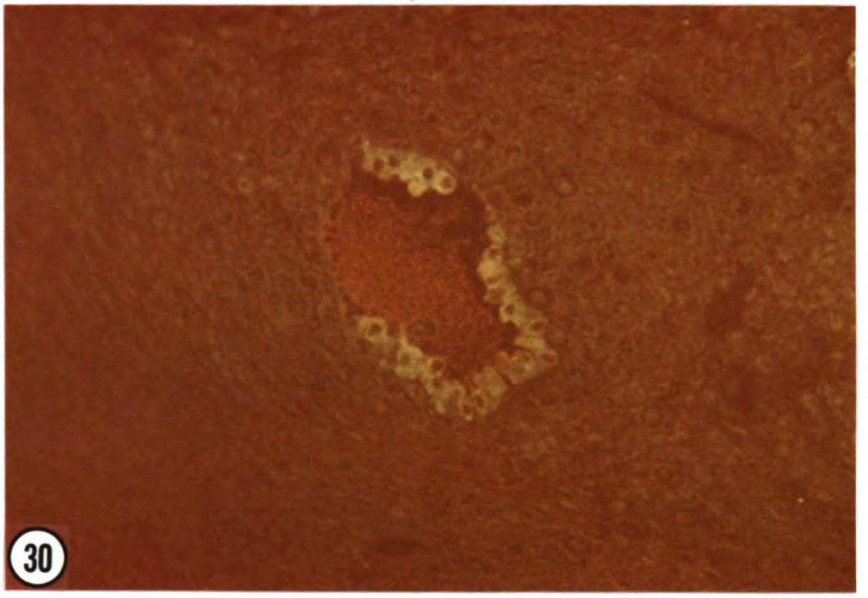
Figure 30. Fluorescent photomicrograph. Stained with relaxin antiserum (Rabbit 2, bleeding 4) and fluorescein labelled anti-rabbit gamma globulin. Paraffin section of a uterine metrial gland at day 17 of gestation. Note the fluorescence of the hypertrophied endothelial cells of the blood vessel and red blood cells which were also observed in the saline control (Figure 28). There is no localization of the fluorescent label in the area surrounding the blood vessel, and granular cells are not stained. X150.

Figure 31. Fluorescent photomicrograph. Stained with relaxin antiserum (Rabbit 2, bleeding 4) and fluorescein labelled anti-rabbit gamma globulin. Paraffin section of a uterine metrial gland at day 17 of gestation. Note the autofluorescence of the red blood cells in the lumen of the blood vessel. Also note the localization of the fluorescent label in the granular cells surrounding the blood vessel. This localization of relaxin was an exception, since all sections from previous and later studies appeared the same as illustrated in Figure 30. X150.



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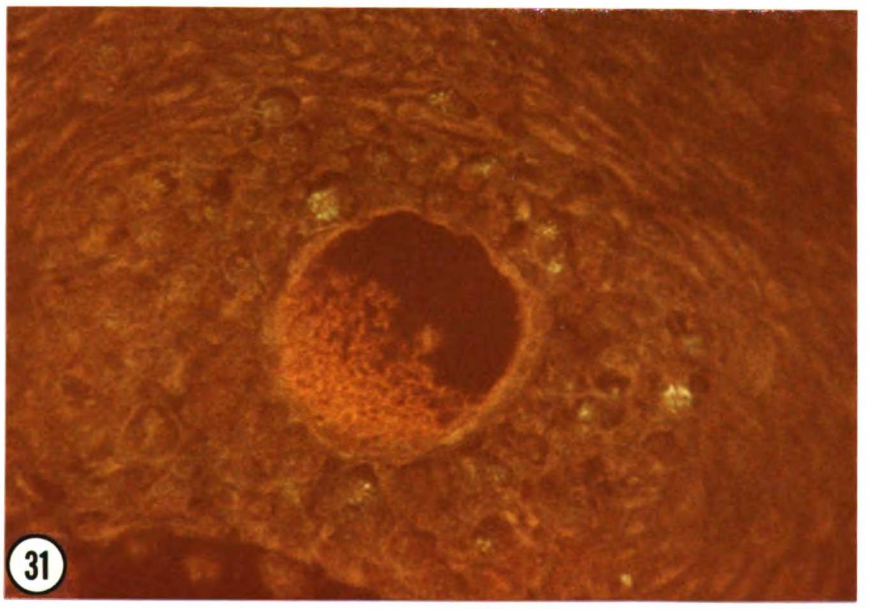

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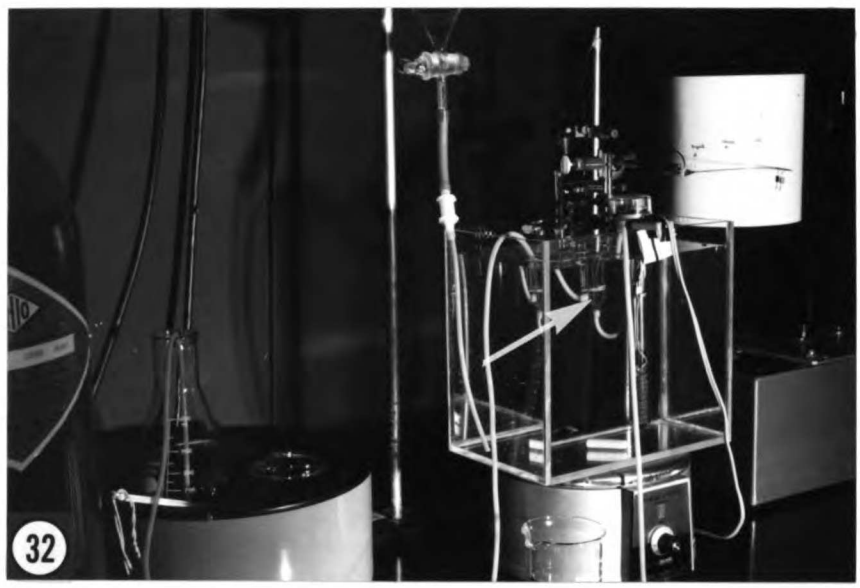
Figure 32. Photograph of the bioassay apparatus. The uterine segment was suspended in Locke's solution in the organ bath chamber (arrow). The water bath was thermostatically controlled to maintain the temperature at 37.5°C. The chamber was aerated with 1% CO_2 in oxygen. The uterine muscle was attached to a muscle level and uterine contractions were recorded on an ink writing kymograph.

Figure 33. Sensitivity of the assay. The scale at the bottom of the page: 1 large division = 100 seconds.

a) This tracing of uterine contractions demonstrates the sensitivity of the bioassay method. Total inhibition was achieved by 0.027 GPU/ml (0.06 µg/ml) of NIH porcine relaxin #0147 (downward arrow). Washing of the chamber (upward arrow) restored uterine contractions.

b) This tracing of uterine contractions demonstrates that 0.027 GPU/ml is just in the range for total inhibition since 0.020 GPU/ml of NIH porcine relaxin (downward arrow) did not cause total inhibition.

c) This uterine tracing demonstrates the effect of time upon the muscle. After 58 minutes the amplitude or frequency has not changed. Even draining and filling the chamber (upward arrow) does not influence the pattern of uterine contractions.



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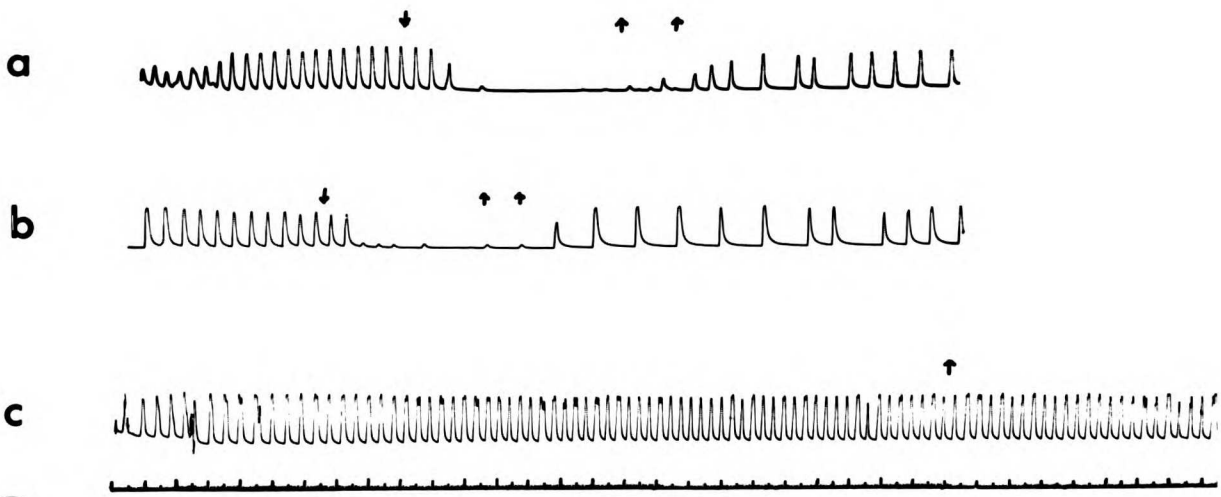

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Figure 34. Bioassay results of the extractions prepared from tissues of the pregnant rat at day 17 of gestation. Downward pointing arrows indicate the time of addition of the tissue extract. Upward pointing arrows indicate the time of rinsing the chamber with Locke's solution. Scale at bottom of page: 1 large division = 100 seconds.

a) Relaxin extracted from day 17 corpora lutea. 16 μg added to the chamber for a chamber concentration of 1.6 μg/ml. Inhibition was not quite complete.

b) Relaxin extracted from day 17 corpora lutea. 32 μg added to the chamber for a chamber concentration of 3.2 μg/ml. Inhibition was complete. There was no recovery upon rinsing the chamber.

c) Extract prepared from day 17 ovarian tissue minus corpora lutea (experiment 1) 120 μg extract added at first arrow. No inhibition is seen. However, the amount of material necessary to produce this response is far in excess of the amount needed when the extract is from luteal tissue. At the second arrow another 120 μg was added to give a total of 240 μg or 24.0 μg/ml (chamber concentration). This resulted in partial inhibition.

d) Extract prepared from day 17 ovarian tissue minus corpora lutea (experiment 2). 70 μg added at the arrow for a chamber concentration of 7 μg/ml. This resulted in partial inhibition as demonstrated by reduced frequency. This dose is far in excess of the amount of luteal extract needed to produce this effect.

e) Extract prepared from day 17 metrial gland tissue. 470 μg extract added to the chamber for concentration or 47.0 μg/ml. No inhibition is observed.

f) Extract prepared from day 17 nonplacental uteri. 475 μg extract added to the chamber for a concentration of 47.5 μg/ml. No inhibition is observed.

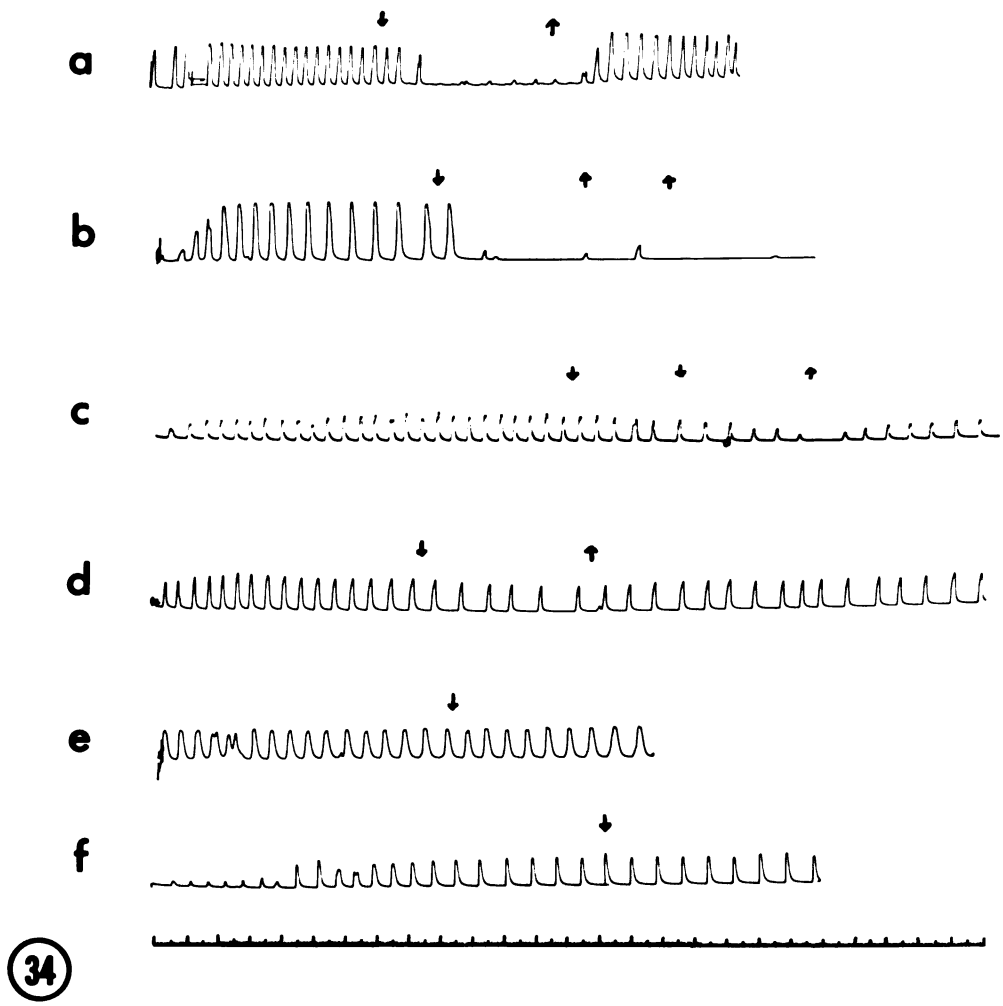

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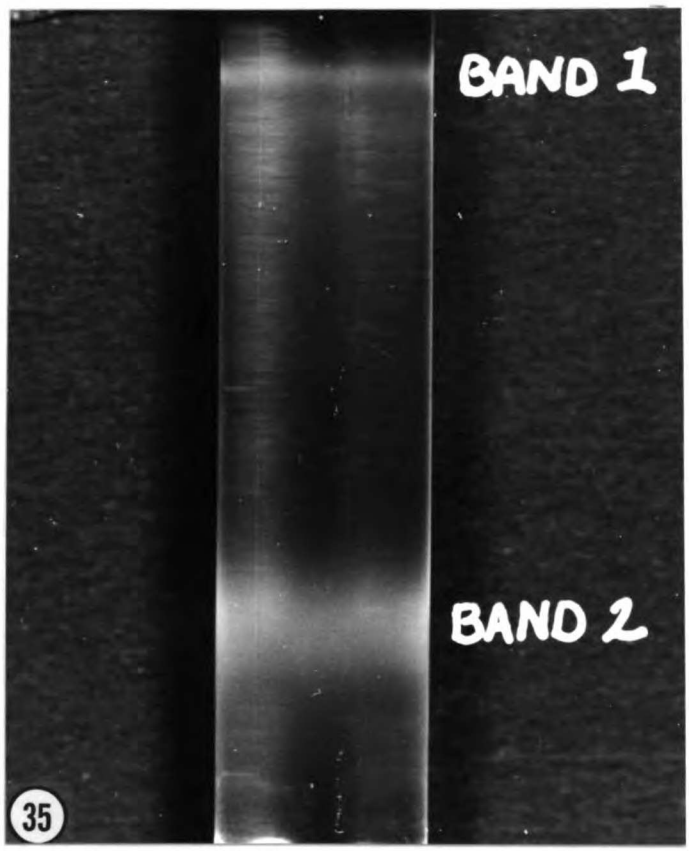
Figure 35. Photograph of a centrifuge tube showing the distribution of bands when 1.0 ml of luteal homogenate (minus the nuclear fraction) is placed on a 10-55% sucrose ($5x10^{-4}$ M EDTA) gradient and centrifuged at 90,000 g for 30 minutes. Fractions of 0.7 ml were collected from the bottom of the tube. Therefore, band 2 corresponds to fractions 3 through 5, and band 1 corresponds to fractions 9 through 11. Band 2 contains mitochondria and a heterogeneous collection of granules. It is in this band that large quantities of relaxin can be found. An electron micrograph of band 2 is seen in Figure 37. An electron micrograph of band 1 is seen in Figure 39.

Figure 36. Bioassay results of cell fractions obtained by continuous density gradient centrifugation. Downward pointing arrows indicate time of addition of 0.2 ml of the fraction. Upward pointing arrows indicate time of rinsing the chamber with Locke's solution. Scale at bottom of page:
1 large division = 100 seconds.

a) 0.2 ml of fraction 3 (band 2) added to the chamber. Complete inhibition indicating concentrated relaxin activity.

b) 0.2 ml of fraction 8 (intermediate region of tube) added. Essentially no inhibition.

c) 0.2 ml of fraction 11 (band 2) added. Partial inhibition.


BAND 1
BAND 2
35

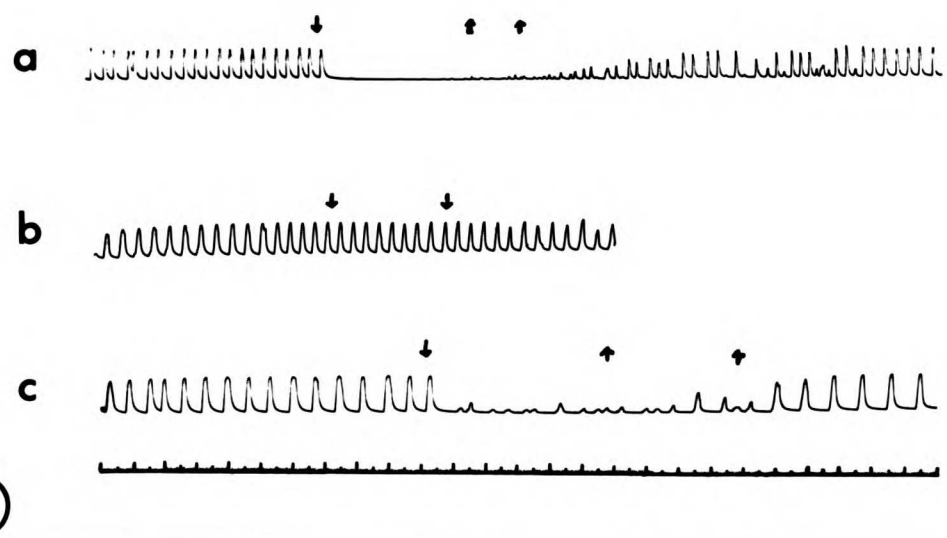
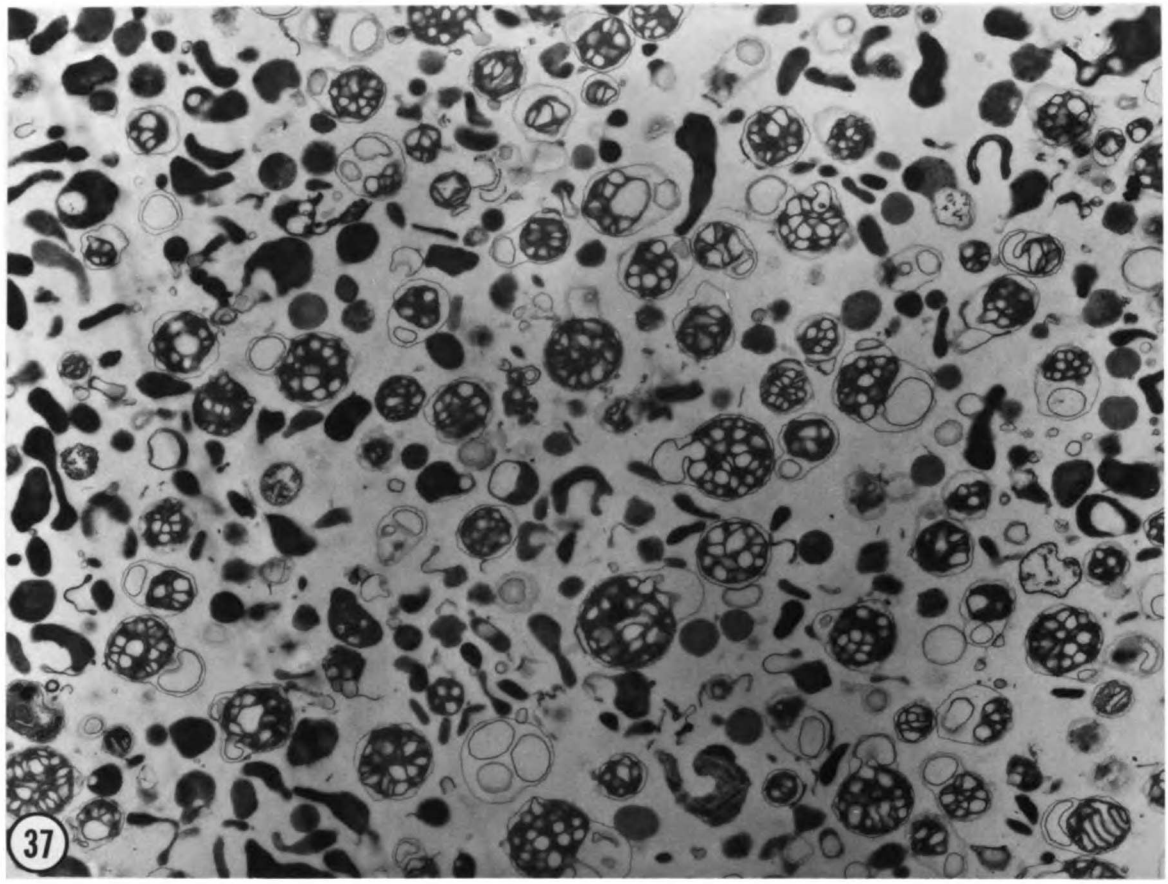

a
b
c
36

Figure 37. An electron micrograph of a thin section through a pellet from band 2 (fraction 5) (continuous density gradient centrifugation). Note the abundance of mitochondria and the heterogeneous granule population X12,250.

Figure 38. An electron micrograph of a thin section through a pellet from the intermediate region between bands 1 and 2 (continuous density gradient centrifugation). Note the abundance of membranes and the lack of granules. X12,250.



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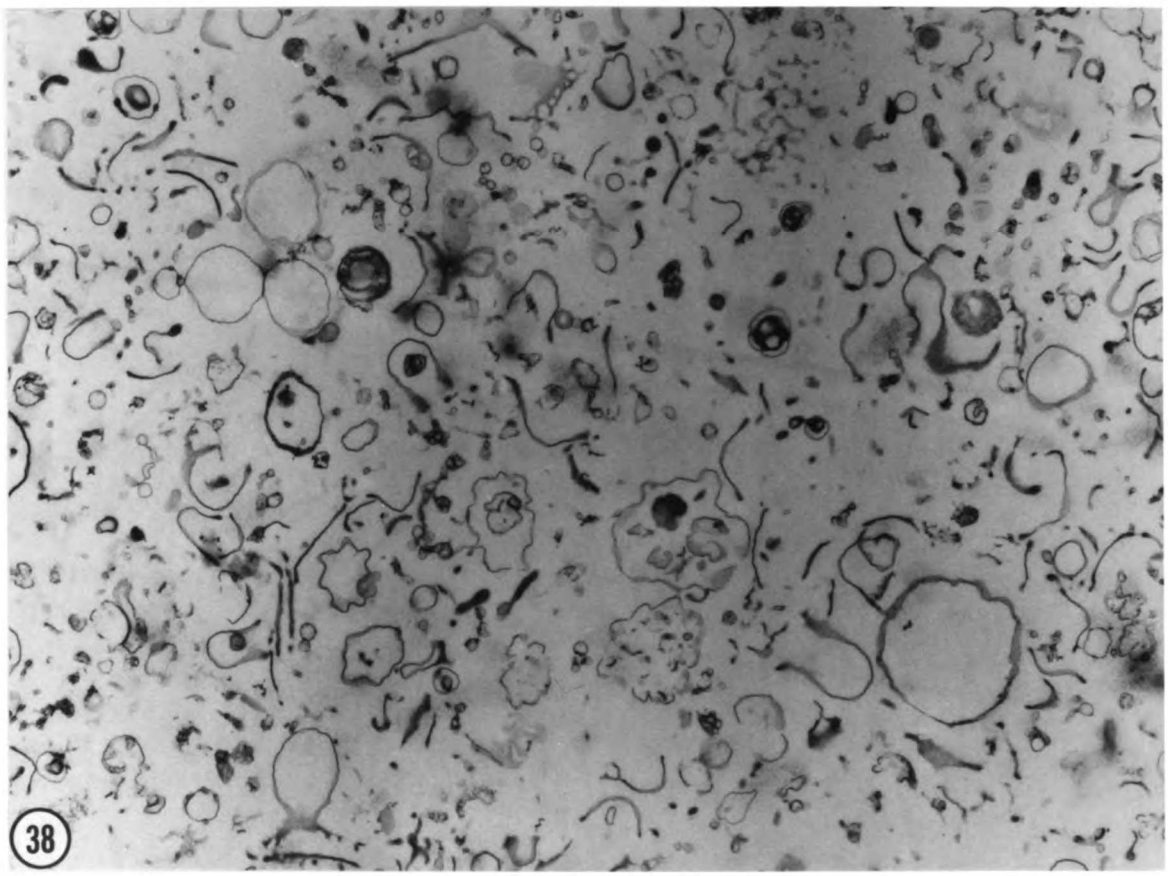

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Figure 39. An electron micrograph of a thin section through a pellet from the region of band 1 (continuous density gradient centrifugation). A heterogeneous population of organelles is present - lipid droplets, mitochondria, granules, and membranes. X12,250.

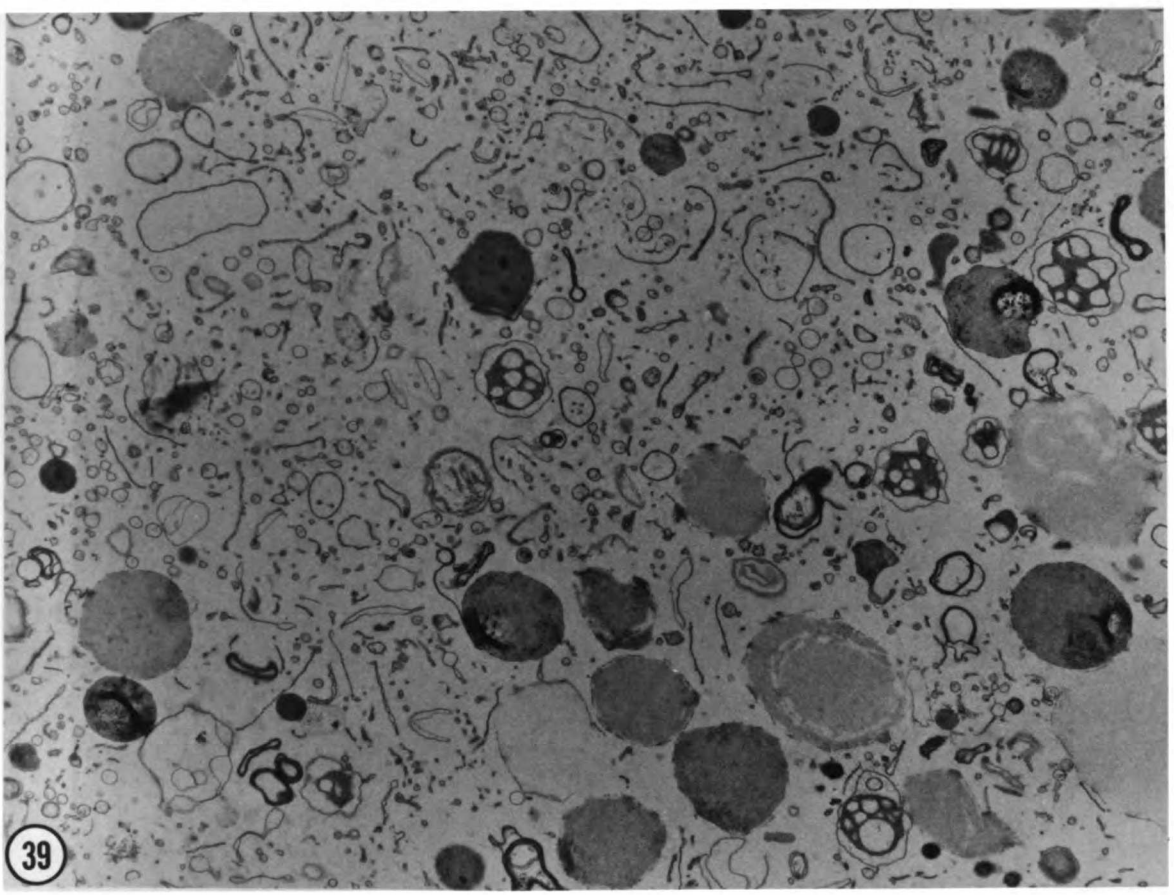
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Figure 40. Bioassay results of cell fractions obtained by differential centrifugation. Downward pointing arrows indicate time of addition of fraction. Upward pointing arrows indicate time of rinsing the chamber with Locke's solution. Scale at bottom of page: 1 large division = 100 seconds.

a) Total inhibition by whole homogenate. 70 µg of protein from the whole homogenate added to chamber for a chamber concentration of 7 µg/ml.

b) Total inhibition by nuclear fraction. 80 µg of protein from the nuclear fraction added to chamber for a chamber concentration of 8 µg/ml.

c) Total inhibition by mitochondrial fraction. 42 µg of protein of the mitochondrial fraction added to chamber for a chamber concentration of 4.2 µg/ml.

d) Total inhibition by granular fraction. 10 µg of protein from the granular fraction added to chamber for a chamber concentration of 1.0 µg/ml. At a chamber concentration of 0.75 µg/ml there was not total inhibition.

e) Inhibition by microsomal fraction. 72 µg of protein of microsomal fraction added to the chamber for a chamber concentration of 7.2 µg/ml. Total inhibition was never obtained with this fraction.

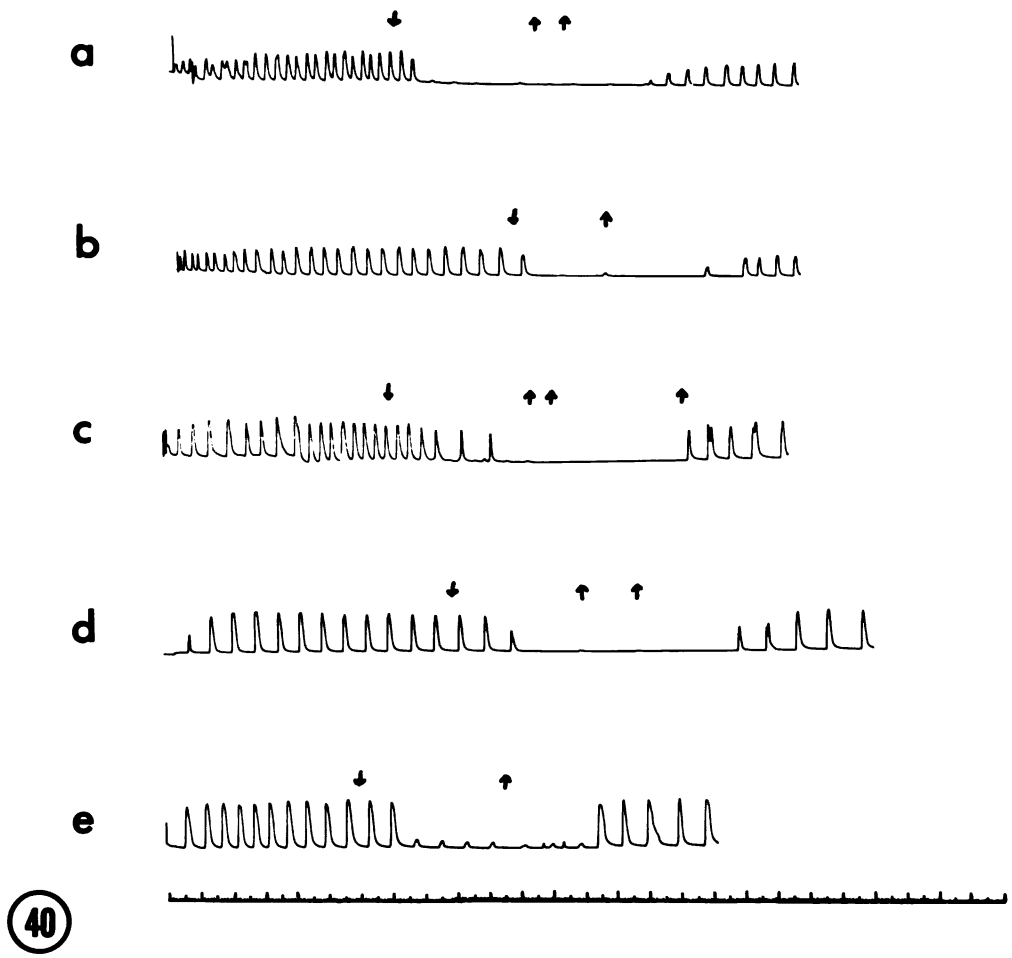
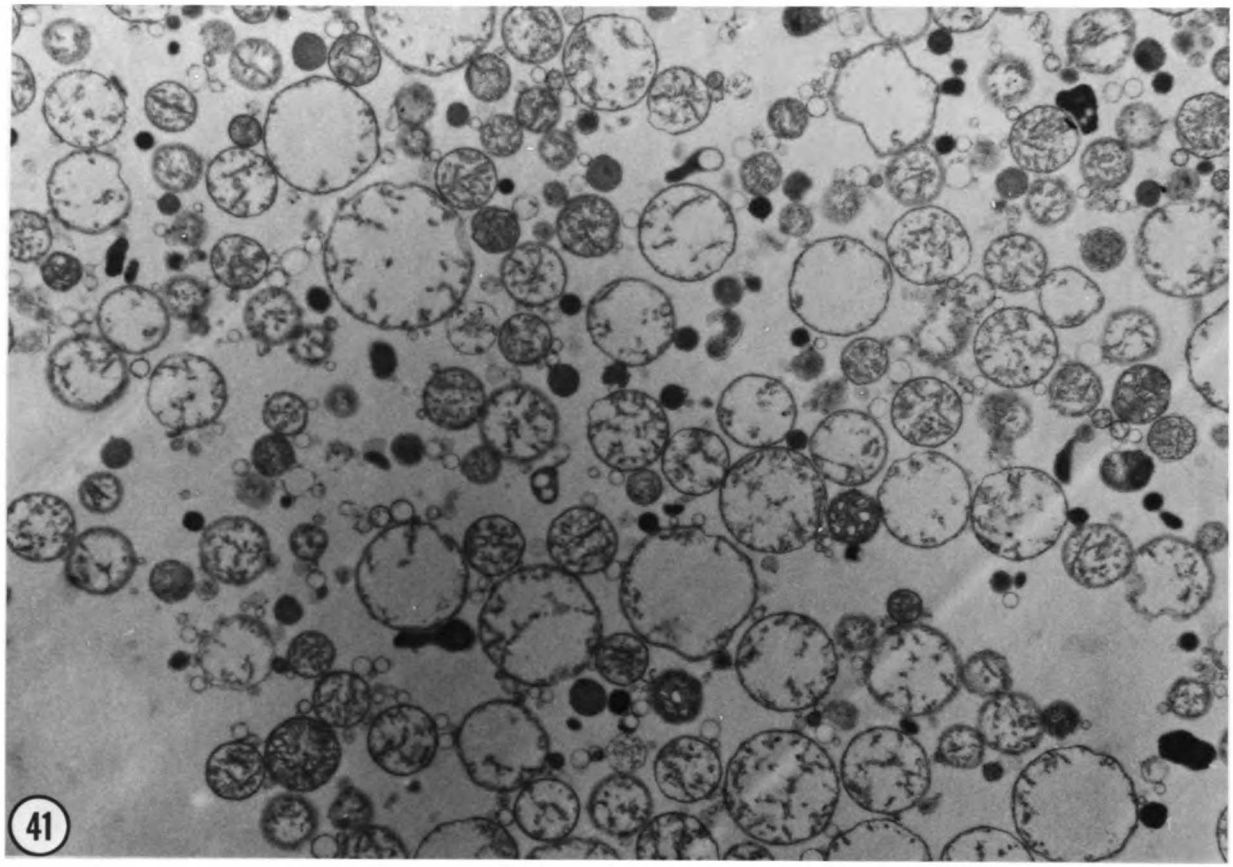

a
b
c
d
e
40

Figure 41. An electron micrograph of a thin section through the granular fraction (differential centrifugation). Note the abundance of mitochondria and granules. X12,250.

Figure 42. A higher magnification of the granular fraction demonstrating the presence of type I (1) and type II (2) granules. The type II granules are smaller, more regular in size, and more electron dense than the type I granules. X33,000.



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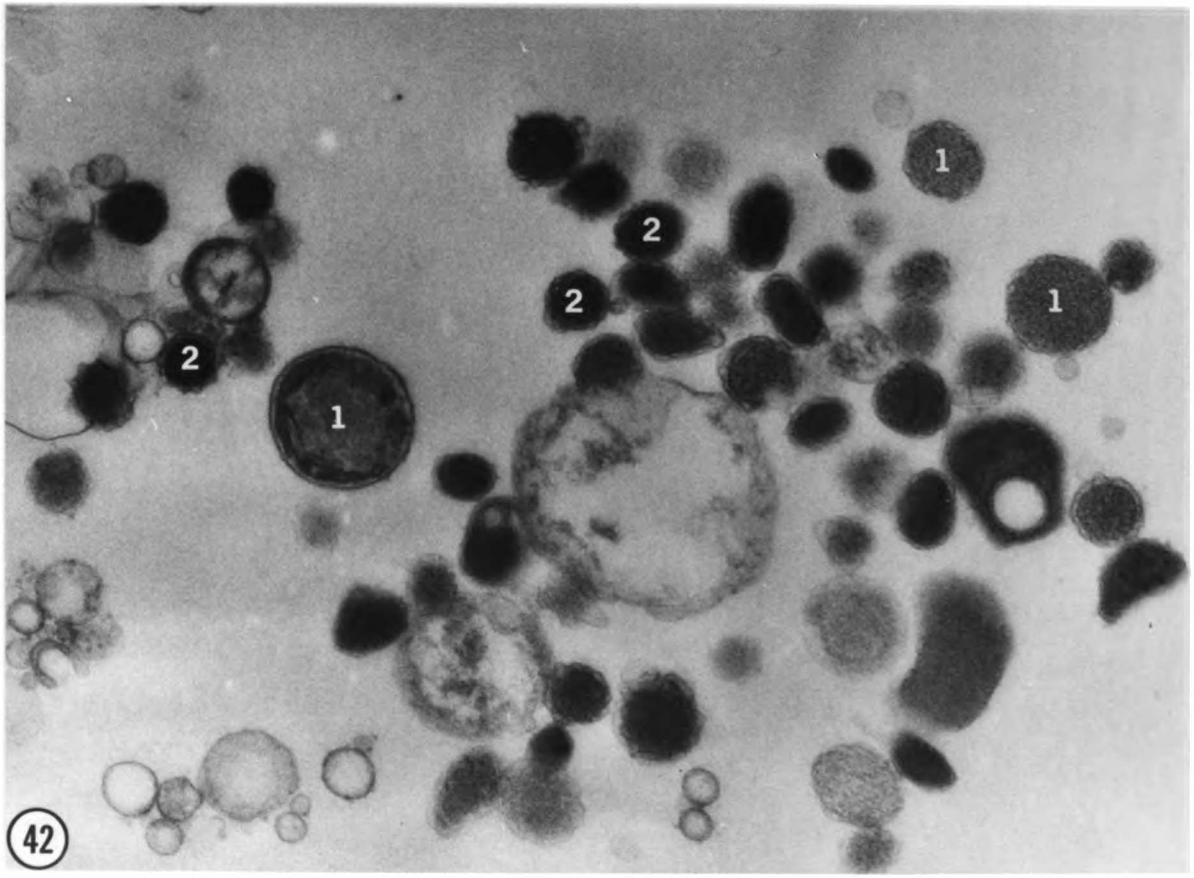

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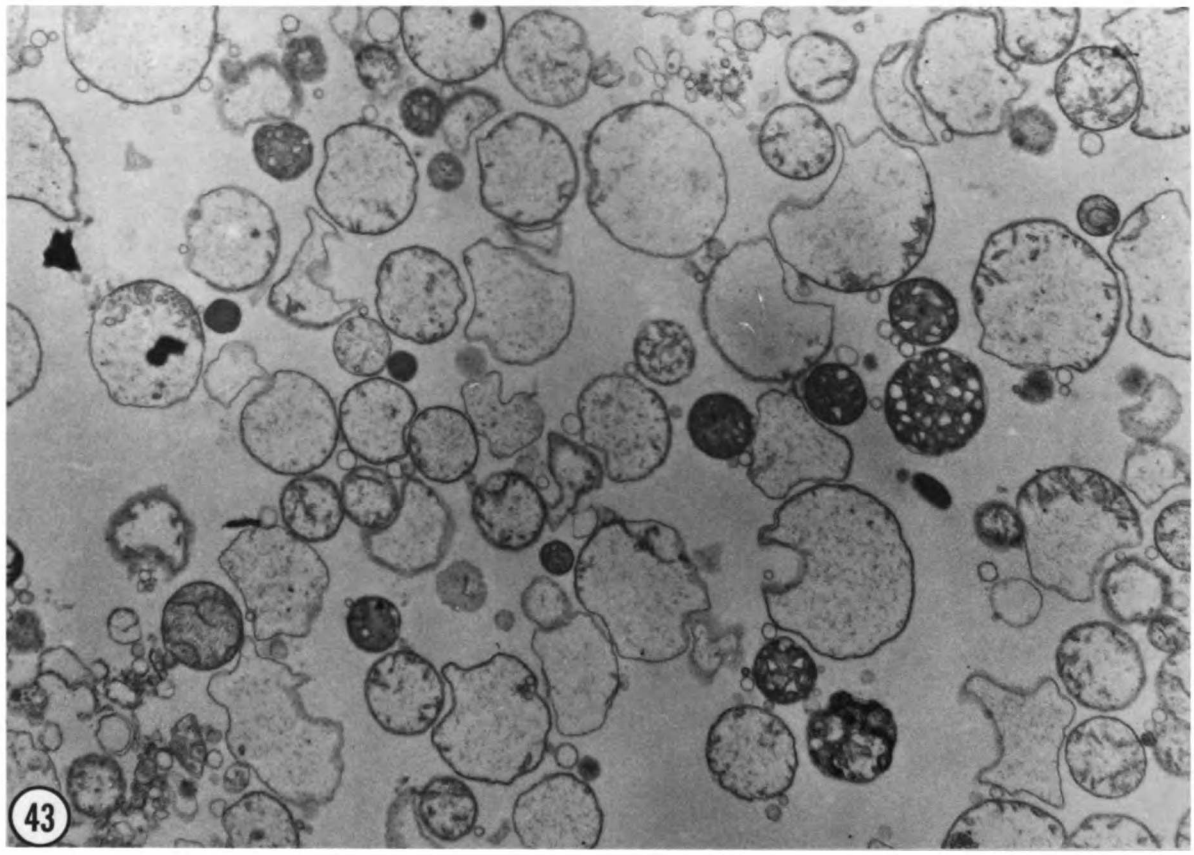
Figure 43. An electron micrograph of a representative area of the mitochondrial fraction (differential centrifugation). Note the abundance of mitochondria and the presence of some granules. X12,250.

Figure 44. An electron micrograph of a representative area of the microsomal fraction. Note the abundance of smooth and rough surfaced vesicles, and the absence of granules. X12,250.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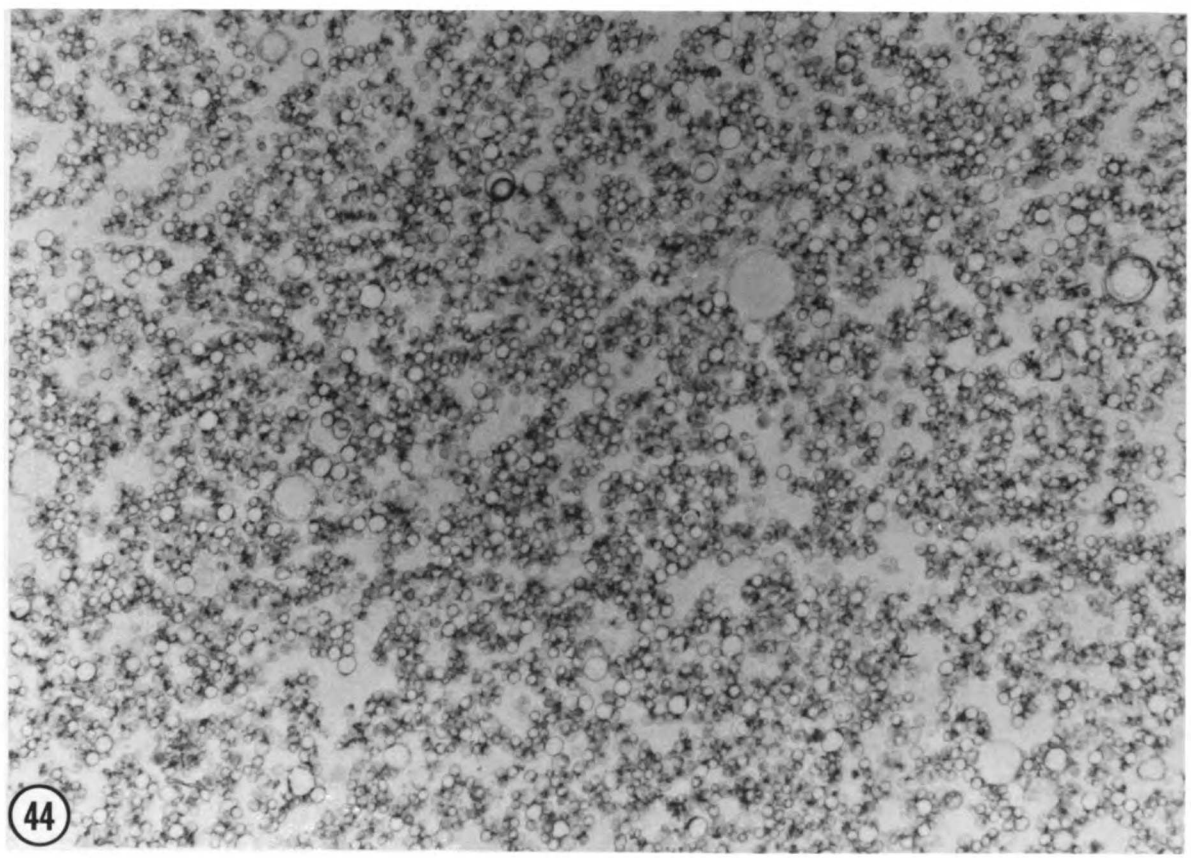
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